Pediatric Autism Petition
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Delaware's Medical Marijuana Program
Dover, De

I, Janie F. Maedler, am petitioning to add Autism as a Qualifying Condition to Delaware's Pediatric Medical Marijuana Law. **Autism Spectrum Disorder, ASD,** is a severe neurodevelopmental disorder that affects brain development. As a "spectrum,' ASD affects individuals in varying degrees, but generally a person with autism will suffer from these core symptoms:

1. Impaired communication

2. Impaired reciprocal social interaction

3. Restricted, repetitive and stereotyped patterns of behaviors or interests

Additional features can include sensory processing dysfunction, poor eye contact, and an insistence on sameness. Patients often have several physical health issues such as sleep and gastrointestinal disturbances. These challenges range from mild to severe, and patients on the more severe end of the spectrum may also exhibit aggressive and/or self-injurious behaviors. Currently there is no psychopharmacological treatment or cure for the core symptoms of ASD.

With the publication of the Diagnostic and Statistical Manual of Mental Disorders in May 2013, all autism disorders were merged under one umbrella diagnosis of ASD. This diagnosis now includes the previously distinct subtypes of autistic disorder, childhood integrative disorder, pervasive developmental disorder-not other specified and Asperger Syndrome. The diagnostic code for ASD is ICD-10-CM, F84.0.

Autism Spectrum Disorder is a condition that emerges early in childhood. Symptoms of autism are most pronounced during childhood and are estimated to affect 1 in every 42 boys and 1 in every 189 girls in the United States according to the statistics from the U.S. Centers for Disease Control and Prevention. Approximately 1 in 68 American children now has ASD- a ten fold increase in prevalence over a 40 year period. Options for the treatment of Autism are extremely limited, and cannabis is emerging as a treatment of choice.

Conditions associated with ASD:

Conditions associated with ASD are multiple. If identified and managed, will result in an improved sense of well being, more effective participation in educational and therapeutic programs, and improved quality of life for the patient and their family. A wide arrange of medical disorders can negatively impact the health and behavior of each autistic individual.

Aggression and self injurious behaviors- It is very common for ASD patients to exhibit aggression towards family members, caregivers, teachers and even themselves over time despite being administered medications aimed at calming them. Recent research findings suggest nearly 28% of 8 year old children with ASD behave in a way that can lead to self injury. (Soke, Rosenberg, Hamman, Fingerline, Carpenter, Giarellie, Lee, et al., 2016) According to the CDC these new findings suggest that self injurious behaviors are now common among autism. Kanne and Mazurka showed that in a population of 1,380 children, ages 4-17, with ASD that 68% showed aggressive behaviors towards caregivers and 49% towards non-caregivers as compared to 7-11% of children with intellectual disability but not autism.

Social Anxiety- It is common for those with autism to have difficulty regulating emotions. This can be displayed in immature behavior, crying, outbursts, tantrums, inappropriate behaviors, disruptive behavior and aggressive behavior. The tendency to be overwhelmed or frustrated in social situations can result in "stemming", self-injurious behaviors such as head banging, hair pulling or self biting. According to a study conducted in 2016 (Wei et al), FAAH is a novel therapeutic target for the social impairment of ASD which can be treated with cannabis. Chakarabarti et al. conducted a clinical study that showed that the human cannabinoid receptor CNR1 modulates our ability to recognize facial expressions. They noted that the "CNR1 is the best characterized molecule of the endocannabinoid system, involved in the processing rewards…four single nucleotide polymorphisms in the CNR1 locus modulate differential stratal response to happy but not to disgust faces. This suggests a role for the variations of the CNR1 gene in underlying social reward responsively…This has implications for medical conditions involving hypo-responsivity to emotional and social stimuli, such as autism."

Delayed and disordered language- Failure to be able to communicate leads to increased frustration and anxiety. A potential link has been found between autism and the CB2 receptors within the endocannabinoid system. The Rowdy, Malenka & Sudhof, 2013 study found that the cell mutations in the brain that have been previously associated with the autism block the action of the molecules that act on the CB2 receptors. A similar study also determined that that mice with autistic like behavioral issues possessed unregulated CB2 receptors (Onaivi, et al, 2011). Another discovered the same in the up regulations of CB2 receptors but in human subjects (Siniscalco, et al, 2013). This suggests that autism could be caused by a disruption of the brain's ability to send clear signals and in turn raises the possibility that using cannabinoids to restore communication and contribute to the treatment of autism thru proper cell function.

Gastrointestinal Disturbances- GI distress affects up to 85% of children with ASD are impacted from GI issues. These conditions range in severity from chronic constipation or diarrhea to inflammatory bowel disease. Pain from GI issues can prompt behavioral changes such as increased self soothing (such as stemming, rocking or head banging) or outbursts of aggression and/or self-injury. The GI tract accommodates and expresses all the components of the ECS. (Hasenoehrl, Taschler, Storr, & Schicho,2016)

Seizure Disorder-seizure disorders, including epilepsy, occur in as many as 39% of those with ASD. Seizures associated with ASD tend to start in either early childhood or adolescence but may occur at any time. Depending on the severity of the autism and due to the lack of communication, some children can go years or a lifetime with seizures without caregivers knowing they are having

seizures. All types of seizures can be found in association with ASD, including major motor, myoclonic, and febrile seizures, but complex partial seizures are the most frequently reported. 66.7% of seizures that occur in ASD become apparent after 12 years of age with 30% occurring by 20 years of age.

Sleep Disorders- Sleep problems are highly prevalent in ASD children and rank as one of the most common concurrent clinical disorders. Prevalence rates vary widely, ranging from 40%-80%, as compared with that of typically developing children in which prevalence rates are approximately 30%. Sleep disorders are reported to more severe in ASD. Other types of sleep disturbances have also been reported such as parasomnias, sleep related breathing disorders, sleep related movement disorders and circadian rhythm sleep disorders.

<u>Current Approved Medications for ASD:</u>

It is important to note that an expanding body of research indicates that the underpinnings of ASD are medically based- as with any medical condition the longer it goes untreated, incorrectly treated or unmanned the more it will likely deteriorate over time. In order to halt the progression of the disorder and address these underlying medical issues, we must ensure that effective treatments are available.

Currently, there are two medications that are FDA approved to treat the irritability associated with ASD (yet does not treat all 3 symptoms of social difficulty, communication difficulties and self injurious behavior) : Risperidone and Aripiprazole. Both of these medications are anti psychotics intended to treat the behavioral symptoms of ASD including aggression, self injurious behavior and severe tantrums. These are two significant issues with these medications as described below:

1. A growing body of medical literature and research points to medical underpinnings for ASD which cannot be effectively treated with antipsychotic medications.

2. These medications often come with severe side effects that are similar or identical to the issues we are trying to solve.

Common Side Effects of Risperidone/Risperdal- extrapyramidal effects (sudden, often jerky, involuntary motions), dizziness, tiredness, fatigue, fever, weight gain, headache, dry mouth, feeling hot or cold, increased appetite, anxiety, insomnia, restlessness, nausea, vomiting, stomach pain, constipation, cough, sore throat, runny or stuffy nose, skin rash, aggressive behavior, agitation, tachycardia

Common Side Effects of Aripiprazole/Abilify- dizziness, lightheadedness, drowsiness, weakness, nausea, vomiting, tiredness, excess saliva or drooling, choking or trouble swallowing, blurred vision, headache, anxiety, weight gain, insomnia, constipation, suicidal thoughts, involuntary repetitive movements, neuroleptic malignant syndrome, tremors, muscle spasms, fainting, restlessness, seizures, distress, self-injury

Additionally, off label drugs including additional antipsychotics, antidepressants, stimulants and mood stabilizers are also often prescribed to address symptoms. ASD patients often suffer from chronic inflammation, particularly the brain and in the digestive tract. Antipsychotics and the off-

label medications used to treat ASD typically do not address inflammation in the human body (yet cannabis does). Antipsychotics such as Risperdal and Ability are Selective Serotonin Reuptake Inhibitors. SSRIs are designed to ease depression by increasing levels of serotonin in the brain, which is believed to contribute to the feeling of well-being. This may mask the underlying medical conditions which must be treated to improve the quality of these patients' lives.

According to a 2013 report published by the National Institute of Health, there is no evidence to support the use of SSRI's to treat ASD in children. Not only do SSRI's lack long term effectiveness in treating the symptoms of ASD but they may cause side effects that may cause the patient to further deteriorate over time.

Other off label drugs include stimulant medications like Adderall or Ritalin and Naltrexone (FDA approved for alcohol and opioid addiction). It is important to note that there have been no significant clinical trials to demonstrate risk or efficacy of these drugs used off label in children with autism. Predicting which drug and what dosage may be effective has proven problematic.

Benefits of Cannabis for ASD Pediatric Patients:

I've included various research articles in support of why cannabis would be effective therapy for ASD. Here are few I'd like to highlight are below.

In the 2013 Journal of Autism and Developmental Disorders study "Cannabinoid Receptor Type 2, but not Type 1, is Up-Regulated in Peripheral Blood Mononuclear Cell of Children Affected by Autistic Disorders" a link between the endocannabinoid system and immune cells in children with autism was revealed. Immune dysfunction is a factor the contributes to autism, the condition is believed to be linked to higher levels of CB2 receptors in cells. CB2 receptors play a significant role in regulation of the immune system. The authors concluded that the study demonstrated "an endocannabinoid-CB2 signaling dysregulation in autism, implying the endocannabinoid system may represent a new treatment opportunity for autism pharmacotherapy."

In 2013, Journal Neuron revealed in a study that autism related mutations in mice resulted in "deficits in endocannabinoid signaling." The study concluded that "alterations in endocannabinoid signaling may contribute to autism pathophysiology."

The study, showed a link between endocannabinoids in it's relation to Fragile X syndrome, the most common known genetic cause of autism. The research showed that "a distinct type of metabotropic glutamate receptor-5-dependent long-term depression at excitatory synapses of the ventral striatum and prefrontal cortex, which is mediated by the endocannabinoid 2-arachidonoyl-sn-glycerol, is absent in fragile X mental retardation protein-null mice......These changes are accompanied by impaired endocannabinoid-dependent long-term depression. Pharmacological enhancement of 2-arachidonoyl-sn-glycerol signalling normalizes this synaptic defect and corrects behavioural abnormalities in fragile X mental retardation protein-deficient mice. The results identify the endocannabinoid signalosome as a molecular substrate for fragile X syndrome, which might be targeted by therapy."

Autism being a neurodevelopmental disorder, Govt Patent 6630507 of cannabis which is patented as a neuroprotectant would be a safer treatment then many of the FDA approved and off label drugs being used to try to treat the symptoms of autism. Abilify and Risperadol are not neuroprotectants

plus they have significant side effects. In the 2016 Study, Role of Endocannabinoids on Neuroinflammation in Autism Spectrum Disorder Prevention, research is done to investigate the role of the endocannabinoid system in the pathogenesis of ASD and it's potential as a preventative treatment of ASD.

I ask that you to consider adding Autism as a qualifying condition to our Pediatric Medical Marijuana Program. I am currently a consultant for families in other legal MMJ states with pediatric autism being a qualifying condition and have over 400 families that I consult with. The results are more then positively life changing for autism families. I have listened to parents ready to commit their tiny children and then a few weeks later their children are doing so much better on cannabis that they are able to take them out in public and enjoy family life. These parents have tried numerous therapies, pharmaceuticals, vitamins, diets, and supplements...you name it and cannabis has been the first thing to bring their child quality of life. With the large population of autistic children that we have here in Delaware our children here deserve this relief legally. This should not be held out of reach in a such a cruel disorder with no specific treatment options. Neurologists who have been able to qualify autistic children thru a dual diagnosis of seizures have expressed to me that they have seen remarkable results for the autism behaviors as well. As far as worrying about the future of autistic children's developing brains....I've included supporting letters on this subject but also, look at them now without cannabis....beating themselves, their parents, their siblings and caregivers. Fracturing their skulls, biting the flesh off of their arms, having seizures daily, having such GI distress that they do nothing but rock themselves all day, going days with no sleep, not allowing anyone to touch them because it's just to painful to add one more thing to their already stimulated body, having a diminished quality of life as well as their family. I have heard from parents who's child has spoken 6 months after starting cannabis for the first time in 7 years, I have heard from parents who's child was in diapers as a teen and now is potty trained in one month, I have heard from parents who's child only slept one night a week but is now sleeping each night and is more content and not self mutilating. The first hand stories are endless. We could have these kinds of successes as well here in Delaware with our pediatric autistic population. Please approve autism as a pediatric qualifying condition. Let parents/guardians and their child's specialists who know that child decide if this is a treatment they need to use.

I thank you for your time in reviewing this petition.

All the best,

Janie F. Maedler

My son Matthew Monge - now 10 years old - was diagnosed with Autism at age 3. He also has ADHD, and Intellectual Disability. I knew he had this disease at 15 months old as he stopped meeting his milestones. He started screaming sometimes continuously on and off for days intermittently as if in pain, and beating on the right side of his head under the ear. Although Matthew is verbal, his cognition is delayed to the level of a 3-4 year old. Matthew can obey one step commands but no more.

Self-injurious behavior and aggression are the biggest concerns although there are many. Matthew has adverse reactions to medications, limiting him to certain ones. One medication required me to have him transported by ambulance to the hospital for a very long admission. Matthew has signs and symptoms of sub-clinical seizures, although numerous EMG's have detected nothing. I have recorded these episodes and others have admitted these episodes are possibly absent seizures.

Matthew has had very thorough ABA therapy and treatment along with placement at a behavior facility in Wisconsin for one year. These approaches have not been effective. Redirection has only been successful 22% of the time. ABA has never been successful and has resulted in discharge of the ABA facility. Matthew has head pain in the right back side the head in which he beats under the ear at full force – his hair has ceased to regrow in the area due to consistency of striking and digging in that area.

Matthew is afraid of children thinking they will hurt him which makes Matthew aggressive. We cannot go to waiting areas of clinics. We must be immediately directed to a quiet room or he will attack others due to noises. Matthew needs to be reduced in his medications. Risperdone causes obesity and trying other medications causes adverse reactions. Depakote was used and continues to be used for aggression although recently Matthew had a high toxicity and was admitted to the hospital. His head pain continues and there is no medication to calm and treat. Nothing works and Benzodiazepines cause adverse reactions.

I discharged Matthew recently due to neglect at a Crisis home as another child abused him. I believe this could have been avoided if put on the program and Matthews cognition and verbalisation would have cleared a lot more up. I believe I have a right to this natural medication after multiple have failed and we have nowhere to turn.

My son also vocally ticks in the morning and randomly throughout the day. He has resorted to biting his forearms as his way of crying out in pain. Do I think he is in pain? Your doggone right I do. When a child tells you that, you don't consider them a liar.

These strong medications need to be reduced and I believe Medical Cannabis administration is the key to keep Matthew healthy. Please make Autism a qualifying condition to help my child - I feel I have tried everything and I don't want to lose my child. I believe after reading about the endocannabinoid system and the benefits of getting Matthew certified by the Minnesota Medical Cannabis Program that we could save Matthew's life and save lots of time and money spent. The expense of multiple admits, inpatient facilities, safety of others and safety of a suffering child, far out way the benefits of the medication itself.

Thank you for understanding my concern.

Nadine Monge

Please see photos on following page.

My son Kyle was diagnosed with Autism at age 3..He didn't speak or respond to his name and was constantly flapping his arms. He would shriek uncontrollably and bang his head forcefully on the floor, walls, and doors. He was unconsolable. He was constantly moving and showed no interest in what others were doing or saying. He had unusual obsessions (the washer, the microwave, the ceiling, holes.) He has cycled through countless repetitive behaviors. Stomping, spitting, clearing his throat, jumping,pushing his younger brothers, pinching, spinning, and most recently echoing the same word or phrase. His aggression and destructive behaviors only got more intense with age. Punching himself in the face, biting his legs and arms, throwing his whole body on the floor, scratching until he bleeds, punching holes in walls, throwing objects. He has multiple scars and has given me 3 black eyes

He had no interest in his brothers and was unable to make eye contact, use gestures, or communicate in any way. He has been nonverbal for most of his life. Lack of communication led to much of the aggressive behaviors, but at times it was random and seemed like he was in pain..

He had Speech Therapy, but was unable to focus or follow simple directions. He did Occupation Therapy but was discharged due to "unknown underlying medical conditions." Kindergarten was a nightmare. He was left out of special events and spent most of his day in a room with only two other students. He was not allowed to join the mainstream classroom or participate in Kindergarten graduation because they did not want to upset him by changing his routine He started ABA (Applied Behavior Analysis) Therapy in August of 2015. The behaviors continued and we felt there was no choice but to try medication, in retrospect this was a mistake. Within a year my 7 year old son was on Buspirone,Tenex, Prozac, Klonopin, Hydroxyzine and Abilify. These medications had zero effect. put him to sleep, or put him into a violent rage. The risks outweighed the benefits.Risperdal was prescribed as a last resort, but thankfully he was re-evaluated and given a diagnosis of Tourette's Syndrome.

Kyle was certified through the Medical Cannabis Program last November. While it has only been a short period of time, his quality of life has dramatically improved.He is able to communicate by speaking simple words and using his AAC (augmentative and alternative communication) device. He is generally in a very good mood and smiling. Eye contact has improved and he plays with his brothers.He can wave at us, label objects, and says mom and dad. He is of all other medications and is much easier to redirect and calm down. Of course we still have off times, but they are rare. I don't have to worry he will have an allergic reaction or hurt himself or others. Medical cannabis has given my family hope.

Kassidy Kay Schmatz

My 11 year old son is not a medical card holder so we wish him to remain anonymous. My son was diagnosed with Autism when he was 4 years old. He had aggression, lack of communication skills, perseverance, side by side play, lack of empathy, anxiety, tantrums, social anxiety, OCD, insomnia, restless leg syndrome, IBS, no eye contact and many repetitive behaviors. He became a student of the Consortium in Lewes, Delaware with receiving therapies year round however went to regular peer classes due to being considered extremely high functioning. During his school day he had a therapist and a detailed IEP of his goals he needed to meet. Life was challenging for me, his mom mostly, as I was pregnant and had to wrestle him when he would tantrum and keep him from hurting his sibling. This started with him before Kindergarten. In 3rd grade, we accidentally discovered the benefits of cannabis when he picked up a MMJ patient's (family member) medication and ate it. It is common for ASD kids to eat things they should not, by the way. His therapist remarked to us that it was the most attentive and appropriate he had been. I had been taking classes and doing tons of research on medical cannabis and knew it was possibly a treatment for autism but seeing this on my own was an eye opener. With using small amounts of THCA our son's behaviors almost did a 180. We soon were in an IEP meeting taking him out of the consortium because he had meet all of his IEP goals. For the following year we agreed with the staff to put him in with a dual certified special ed teacher so that they could keep an eye on him in case he began to regress. Before the 4th grade he had struggled with his grades but suddenly he started getting straight A's and excelling in certain subjects, he starting making bonds with peers which we never thought was possible, he had tons of empathy and would tell us how much he loved us thru out the day. His sleep has improved and not only has his restless leg syndrome but his GI distress has greatly improved. Only 2 1/2 years ago he had a complete work up to have a Nissan Surgery which would lift his stomach for his severe GERD and GI issues. The surgery thankfully made me so nervous that I kept putting it off and now he no longer needs it. He used to get pneumonia regularly from his severe GERD and have to be hospitalized…this has not happened in over 2 years. Now if you were to meet him you would never think he was diagnosed ASD. He still has some small behaviors of ASD but they are ones that will never hold him back in his quality of life or his future endeavors. We still get the occasional doctor who hands us a script for Abilify because my son is socially awkward at times and some of the behaviors come out when he feels anxious but we just let them know we are fine without the scripts. My son is currently taking THCA and occasionally a micro dose of THC if needed for social anxiety. I recently received a phone call from his retired childhood pediatrician and she asked me about the kids…I told her about his progress and what we were doing with cannabis. She was so happy and remembered how he was. I can not wait for her to meet him as a preteen because she will be just as amazed as I am at his recovery and how this plant has given him quality of life. It's given us hope for not only him but others on the spectrum.

Anonymous from Rehoboth, Delaware
August 14, 2017

To whom it concerns,

My name is Katie Kennedy, I am the very proud mom of Tyler Kennedy.

Tyler is 11 yo and has severe autism, as well as Lowe Syndrome (brain/eye and kidney disease) and Tourettes. He has been certified to use Medical Marijuana in the State of MN for his Tourettes condition since September of 2016, nearly one year.

In his one year since beginning Medical Marijuana- Tyler has potty trained daytime hours, he has learned to dress himself with 1:1 step direction, he is able to transition 80% better, his anxiety levels have been decreased significantly, he has returned to fulltime school and is doing well, he is learning new tasks all the time, he is playing appropriately with toys, he is interested in other children and joining in on their play, he is willing to eat new foods and eats them regularly (cucumbers, raw apples, carrots, almonds, walnuts) he is able to receive a haircut with electric clippers without any behavior. His kidney function has increased, he was functioning at 66%, last check he was functioning at 80%- recheck due in August.

Tyler has been weaned off Abilify- please see side effects of this med. He has also weaned ½ off Risperidone, we had to stop weaning as the withdrawal was horrific- he remains at 0.5mg day, we are working up the courage to try again...the withdrawal was heartbreaking for all.

We are very fortunate to have a Tourettes diagnosis as it gives us legal access to Medical Marijuana, I come forward to petition with others again this year as I believe in this medicine, I believe in it- I'm living the difference and feel every person with Autism should have the same freedoms my son does.

Medical Marijuana has been life changing for my son and our entire family.

My son has been using medical marijuana for a year now. We have seen so many positive results. He is now off of one and a half of his medications needed to control range and the use of Ativan as a PRN has gone down from daily to way less than a small dose only as needed once in a while. The rage I am talking about is putting his head through the walls, not touching the walls but putting his head THROUGH the walls and also bashing his teeth into the solid wood trim and biting himself and trying to bite others severely. The amount of physical damage this young man has sustained is real. By using medical marijuana we have seen the uncontrollable rage turn into more of a communication anger because I believe, with my son, it was a learned behavior of banging his head. However, we do see little by little how he is using this to communicate his needs. He clearly can say I want by tapping his chest from the use of medical marijuana. This young man, With the mentality of a two-year-old, is happier and not drugged up. We still have rage at times. Not like before medical marijuana; however, when he is treated with medical marijuana the happy smiling young man returns and continues happily with his day, not laying in bed like a zombie.

Thank you for your time to read this.

Sincerely,

Jeanne Lugaro

I wish to remain anonymous. My 17 year old son has been diagnosed with ASD, ADHD and Depression with Severe Anxiety. Over the years, his aggression (rage attacks) and anxiety have gotten worse. During a rage attack, he punches holes in the wall, smashes windows, bangs his head and breaks items by kicking or throwing stuff. Walking in our house resembles a battle zone. His siblings often lock their bedroom doors, barricaded inside, to avoid him and his behaviors. His anxiety has gotten so bad that he is unable to order food for himself at Mcdonalds or any sit down restaurant. He doesn't leave my side when we're in public as he's afraid of being abducted. He's tried Abilify and Risperdal for ASD, Adderall, Ritalin and Concerta for ADHD, Seraquil, Zoloft, Paxil and Hydroxizine for Anxiety. None seems to help. His current prescriptions are Adderal, Abilfy, and trazadone to help with sleep. My son hates taking these meds and often refuses as they make him feel "out of it", sick to his stomach with no appetite, and his body aches all the time, not to mention what these meds do to his sleep cycle. During his rage attacks he also bites himself, punches us (his parents), and cuts himself. He usually ends up restrained and the police are called to calm him down.

Recently, my son has been self-medicating with marijuana. We as parents disapproved strongly at first. However, since he started using, we've noticed a huge difference in his demeanor. He is calm, rational, happy and we no longer see any rage attacks. He's nice to his siblings and to us, his parents! I don't see him constantly worrying, he has an appetite and he is able to sleep without medications. Please consider adding ASD to the list of approved conditions for medical marijuana use. My son's happiness\life depends on it.

My name is Victoria Grancarich. I'm the mother of a 14 year-old boy named Julian. Julian has severe Autism and a seizure disorder. Julian began having seizures in June of 2011. We have tried many medications over the last 6 years, however seizures and motor tics continued to be an issue.

Julian had always been a kind and affectionate boy in his younger years. When Julian turned 13, the onset of puberty brought new challenges. In February of 2016 Julian became extremely violent toward both family and school staff. He began raging daily and would physically attack us. It got to the point where we as his family needed to wear protective clothing to avoid being bitten, having our hair pulled, and being kicked and punched. His younger sister could not be in the same room and she had to spend all of her time at home locked in her room for her own protection.

In August 2016, Julian began to turn the violence on to himself. He began punching himself in the head full force thousands of times per day. He would use his knee to injure his teeth. He would bang his head into walls. We were powerless to stop him. We were trying to protect him using helmets, arm immobilizers and at times we had to physically restrain him for hours at a time to keep him from harming himself. We believed our son was in terrible pain and was suffering from debilitating headaches. We saw this once vibrant boy lose his will to live. He seemed determined to end his life and came close several times. Between October 2016 and January 2017, Julian was hospitalized 3 times. He suffered self-inflicted skull fractures and massive tissue damage. He had black eyes and giant hematomas on his skull regularly. The hospital staff offered psychiatric medications as well as gabapentin but nothing could stop the daily rages that lasted every moment that he was awake. I felt certain that if we could not get Julian cannabis that he would find a way to end his life.

Julian qualified for cannabis through the state program because of his seizure disorder. After being sent home from Children's Hospital in Minneapolis after another life-threatening episode of self-injury with no plan in place to heal our son we felt cannabis was our only hope. The excruciating pain our son was in was getting worse and we knew no pharmaceutical medication could help him. We had tried everything the doctors offered and had absolutely no success.

We enrolled him in the Minnesota Cannabis program in January 2017. Within a week of beginning cannabis therapy Julian was able to go about an hour without harming himself. As the weeks went on and we reached a therapeutic dose, Julian's behaviors began to slowly melt away. By early March he was smiling again. Within 6 weeks of beginning cannabis Julian was no longer injuring himself or others. He began to take an interest in his life again. He returned to school full time. We were able to remove his helmets and protective gear. By mid-March we were getting smiles and hugs. Julian began to go outdoors again by mid-April. By May Julian began to show interest in using augmentative communication for the first time in his life.

It is now late June. We have not seen 1 episode of self-injury since early March. Julian has not struck me since February. Julian is enrolled in a day camp for children with special needs where he spends 8 hours a day. He is exploring outside and making friends. He is happy and smiling. Julian and his sister have a relationship once again. Julian spends hours in our backyard enjoying bouncing on his trampoline, moving his body and taking in fresh air. He lives safely and happily in our home. He is free from pain. His seizures and motor tics are well managed to the point they are not interfering in his daily life.

Cannabis gave us our beautiful son back. Julian is alive and well today because of this miracle medication.

On the following page are photos of my son before cannabis therapy and after.

June 15, 2017

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Cannabis & Adolescents – Physician Support

This brief summary is meant as additional supportive evidence for Delaware lawmakers to justify debilitating conditions in adolescents for the state's respective medical marijuana program.

Recommendation

Dear Colleagues,

I am the father of a 12 year old son who has autism. I have found great interest in pursuing possible etiologies in the development of the condition and explore safe alternatives to conventional pharmaceutical intervention. I am a speaker at multiple national conferences, specifically focused on autism and cannabis. I have provided testimony in several other states about this same issue at hand. As you know, several states have autism as qualifying diagnosis and more and more are to follow in the coming years.

I appreciate the opportunity to elaborate a little further into pediatric usage of cannabis and its potential benefits.

Since I am sure you are aware, cannabis is non-lethal to humans. There are no reported deaths in recorded history that are attributed to cannabis as the cause of death.

The only question that really should be asked is if chronic cannabis exposure to the developing brain can be harmful. I share the same passion to protect our children. I am a father of four. Recommending cannabis to this sub-population, adolescents with a debilitating and/or devastating condition, comes with a great deal of research.

I am giving my full support for giving parents the choice to choose cannabis as treatment modality for these debilitating diseases in the adolescent population.

  - I have seen countless parents success stories with this approach,

  - I have seen parents with their treated children for several years without adverse effects,

  - I have researched this thoroughly. Cannabis is not only safe, but therapeutic (evidence below),

  - this is not meant to change novel treatment recommendations for any conditions in any way. This is meant simply to be a legal choice for those parents that desire to choose cannabis for their child. It offers them legal protection from prosecution,

  - many do it without having cannabis-cards anyways. This causes additional stress for already struggling families.
I have personally consulted with Professor Grinspoon, who is a renowned Harvard Psychiatrist for 40 years and was reassured that cannabis is non toxic in the developing brain. I have also consulted Professor Mechoulam, who discovered the THC molecule. In fact, he is still active as professor in Israel and is involved in the first human trial with cannabis in children affected with autism.
If you see the powerful transformation yourself, when you listen to powerful parent testimony, it is our duty to protect these parents from state or federal interference. We must protect these very brave, yet vulnerable parents.
Thank you for your time to hear these parents and physicians and thank you for your time researching this subject as thoroughly as I have.

Scientific evidence

Highlights

- "These alterations in endocannabinoid signaling may contribute to autism pathophysiology (Földy 2013, Krueger 2013, Onaivi 2011, Siniscalco 2013)."
- "Endocannabinoids regulate stress responses, in part via the modulation of the 5-HT system (Haj-Dahmane 2011)."
- " Neurogenesis (Galve-Roperh 2007, Jiang 2005, Avraham 2014, Campos 2013)"
- " Neuroprotection (Hampson 2003, Lara-Celador 2013, Sanchez 2012)"
- " Antioxidants (Borges 2013, Pertwee 2010, Hampson 1998, Hampson 2003)"
- " Neuromodulation (Davis 2007, Lara-Celador 2013, Pertwee 2010, Youssef 2012)"
- " Anti-inflammatory (Pertwee 2010, Izzo 2009, Nagarkatti 2009, Klein 2005)"

Direct Links

- NL3 mutations inhibit tonic secretions of endocannabinoids
- ECS is suggested target for fragile X treatment
- CB2 upregulated and is suggested target for ASD treatment
- PPAR alpha/gamma and GPR55 downregulated
- CB1 is key element of perception of basic emotions (like happy faces)

Correlations

- Modulation of GABA efflux via CB1 and CB2
- ECS and 5-HT system closely interrelated
    - eCBs via CB1 modulate 5-HT release
    - 5-HT regulates the release of eCBs via 5-HT2a
    - AEA reduces 5-HT binding
    - THC, THCA, CBD, CBDA are all 5-HT1a agonists
    - THC increases 5-HT1a receptor expression and function
    - Cannabinoid agonists inhibit 5-HT3
    - CBD tryptophan degradation suppressor
- Cannabinoid signaling suppresses cytokine proliferation/release via CB1/CB2 dependent and independent mechanisms
- CB1 regulates synaptic plasticity at synapse onto Purkinje cells
- ECS target for modulating neuronal and glial cell function in epileptogenic developmental pathologies
- Tonic eCBs regulate GI functions (including metabolism)

Phytocannabinoids are compounds that are useful as tissue protectants, such as neuroprotectants. The compounds and compositions may be used, for example, in the treatment of neurological insults due to inflammation, such as autism spectrum disorders.

Cannabinoid receptor type 1 (CB1) receptors are thought to be one of the most widely expressed G protein-coupled receptors in the brain, making cannabinoids an integral part in brain homeostasis. CB2 receptors are mainly expressed on T cells of the immune system, on macrophages and B cells, and in hematopoietic cells, making cannabinoids an integral part in human immune function.

Cannabinoids as antioxidants and neuroprotectants – US Patent 6630507 B1

United States Patent 6630507 by the Department of Health and Human Services, 'Cannabinoids as antioxidants and neuroprotectants'. Cannabinoids have been found to have antioxidant properties, unrelated to NMDA receptor antagonism. This new found property makes cannabinoids useful in the treatment and prophylaxis of wide variety of oxidation associated diseases, such as ischemic, age-related, inflammatory and autoimmune diseases. The cannabinoids are found to have particular application as neuroprotectants.

"It has surprisingly been found that cannabidiol and other cannabinoids can function as neuroprotectants..." "No signs of toxicity or serious side effects have been observed following chronic administration of cannabinoids to volunteers..." "It is an object of this invention to provide a new class of antioxidant drugs..."

The Shafer Commission Report Evidence

The Controlled Substances Act created the Presidential Commission on Marijuana and Drug abuse specifically to advice on the proper scheduling on cannabis. Thus was born a council that would become one of the most legendary fact-finding bodies ever conceived: the Shafer Commission.

In the early 1970s, President Nixon appointed Gov. Raymond P. Shafer of Pennsylvania, a former prosecutor with a "law-and-order" reputation, to run a commission that would demonstrate enough evidence to re-affirm Marijuana to the "most dangerous" list, Schedule I.

The Shafer Commission "recorded thousands of pages of transcripts of formal and informal hearings, solicited all points of view, including those of public officials, community leaders, professional experts and students. They conducted separate surveys of opinion among district attorneys, judges, probation officers, clinicians, university health officials and 'free clinic' personnel. They commissioned more than 50 projects, ranging from a study of the effects of marijuana on man to a field survey of enforcement of the marijuana laws in six metropolitan jurisdictions."

Shafer brought his report to the White House March 21, 1972. It was **1,184 pages** long.

A short summary of the Shafer Commission for pertinent points relating to the Public hearing on Autism as qualified diagnosis for the Texas Medical Marijuana Program in Texas:

"No significant physical, biochemical, or mental abnormalities could be attributed solely to their marihuana smoking... No valid stereotype of a marihuana user or non-user can be drawn... Young people who choose to experiment with marihuana are fundamentally the same people, socially and psychologically, as those who use alcohol and tobacco... No verification is found of a causal relationship

between marihuana use and subsequent heroin use.... Most users, young and old, demonstrate an average or above-average degree of social functioning, academic achievement, and job performance...

"The weight of the evidence is that marihuana does not cause violent or aggressive behavior; if anything marihuana serves to inhibit the expression of such behavior... Marihuana is not generally viewed by participants in the criminal justice community as a major contributing influence in the commission of delinquent or criminal acts... Neither the marihuana user nor the drug itself can be said to constitute a danger to public safety... Research has not yet proven that marihuana use significantly impairs driving ability or performance...

-Shafer Commission report 3/21/1972

Cytokine levels higher in autism

Cytokines are small secreted proteins released by cells that have a specific effect on the interactions and communications between cells. Pro-inflammatory cytokines are involved in the up-regulation of inflammatory reactions. [1] Elevated pro-inflammatory cytokine levels are associated with autism spectrum disorders (ASD) [1]. In ASD, as well as a number of conditions, the expression level of CB2 receptors increases in response to the inflammatory nature of the condition. [2][3] Given that CB2 is up-regulated, and that it's believed to play a neuroprotective role, CB2 is being investigated as a potential target for treatment of ASD. [3] CB1 variations modulate the striatal function that underlies the perception of signals of social reward, such as happy faces. This suggests that CB1 is a key element in the molecular architecture of perception of certain basic emotions. This may have implications for understanding neurodevelopmental conditions marked by atypical eye contact and facial emotion processing, such as ASC. [4] Endocannabinoids are key modulators of synaptic function. [5] Endocannabinoids regulate stress responses, in part via the modulation of the 5-HT system. [6][7] Additional targets of endocannabinoids (and exogenous cannabinoids), PPARα, PPARγ, and GPR55 expression levels have shown reductions in a valproic acid model of autism in rats.[8]

Toxicity

No signs of toxicity or serious side effects have been observed following chronic administration of cannabidiol to healthy volunteers (Cunja et al., Pharmacology 21:175-185,1980), even in large acute doses of 700mg/day (Consroe et al., Pharmacol. Biochem. Behav. 40:701-708,1991) but cannabidiol is inactive at the NMDA receptor [9], indicating that THC is warranted. According to US patent 6630507, safety is demonstrated by stating that in the presence of glutamate alone, and in the presence of glutamate and cannabidiol (CBD) or THC, it was demonstrated that CBD and THC were similarly protective.

United States patents specifically demonstrating evidence of safety:

NMDA receptor antagonism can be achieved with a subset of cannabinoids.
U.S. Pat. No. 5,538,993 (3S,4S-delta-6-tetrahydrocannabinol-7-oic acids),
U.S. Pat. No. 5,521,215 (stereospecific (+) THC enantiomers), and
U.S. Pat. No. 5,284,867 (dimethylheptyl benzopyrans)
have reported that these cannabinoids are effective NMDA receptor blockers.

Terpenes

Phytocannabinoids aid in neuroprotection against oxidative stress in patients affected with neurological diseases. In addition to the cannabinoids, terpens have been found to be helpful in providing CB2 activation. Caryophyllene is the only terpene known to interact with the endocannabinoid system (CB2). β–caryophyllene selectively binds to the CB2 receptor and that it is a functional CB2 agonist. Further, β–caryophyllene was identified as a functional non-psychoactive CB2 receptor ligand in foodstuff and as a macrocyclic anti-inflammatory cannabinoid in cannabis. [16]

Many of the other cannabinoids, terpenoids and flavonoids found in medical marijuana play a role in boosting the therapeutic effect of cannabis. **The FDA and other agencies have generally recognized terpenes as "safe."**

For example, humulene and caryophyllene displayed comparable anti-inflammatory responses to steroid alternatives. [17] Humulene was simultaneously effective in reducing inflammation and offering pain relief. [18] The oral effects of humulene were analyzed and the results suggested that again, this terpene was highly effective at reducing inflammation, proving its usefulness as a topical or oral supplement. [19]

Conclusion

There is evidence that aggressive autism behaviors can be explained by chemical imbalances in the body leading to a multitude of health concerns, including neuro-inflammation. Cannabinoids such as CBD and THC were found to be neuroprotective according to United States Patent #6630507 by the Department of Health and Human Services and many studies as presented above. Rather than causing harm to the developing brain, phytocannabinoids appear to aid in brain neuropsychiatric homeostasis. A strong support towards approval in this case will create relief on many levels.

Please call me directly for questions or concerns or further opportunities to clarify uncertainties,

Sincerely,

Christian Bogner, MD

References

[1] Zhang, Jun-Ming, and Jianxiong An., et al. "Cytokines, Inflammation and Pain." International anesthesiology clinics2 (2007): 27–37. PMC. Web. 11 June 2015.

[2] Fernández-Ruiz, Javier, et al. "Prospects for cannabinoid therapies in basal ganglia disorders." British journal of pharmacology 163.7 (2011): 1365-1378.

[3] Siniscalco, Dario, et al. "Cannabinoid receptor type 2, but not type 1, is upregulated in peripheral blood mononuclear cells of children affected by autistic disorders." Journal of autism and developmental disorders 43.11 (2013): 26862695.

[4] Chakrabarti, Bhismadev, and Simon Baron-Cohen. "Variation in the human Cannabinoid Receptor (CNR1) gene modulates gaze duration for happy faces." Molecular autism 2.1 (2011): 10.

[5] Castillo, Pablo E., et al. "Endocannabinoid signaling and synaptic function." Neuron 76.1 (2012): 70-81.

[6] Di Marzo, V., and F. Piscitelli. "Gut feelings about the endocannabinoid system." Neurogastroenterology & Motility 23.5 (2011): 391-398.

[7] Li, Chen, Peter M. Jones, and Shanta J. Persaud. "Role of the endocannabinoid system in food intake, energy homeostasis and regulation of the endocrine pancreas." Pharmacology & therapeutics 129.3 (2011): 307-320.

[8] Kerr, D. M., et al. "Alterations in the endocannabinoid system in the rat valproic acid model of autism." Behavioural brain research 249 (2013): 124-132.

[9] Cannabinoids as antioxidants and neuroprotectants, US patent 6630507, US dept. of Health

[10] Microbe Inotech test report 03/14/2016, Invoice # GLYPH312

[11] Chemosphere. 2013 Apr;91(4):545-51. doi: 10.1016/j.chemosphere.2012.12.036. Epub 2013 Jan 18.

[12] Toxicology. 2014 Jun 5;320:34-45. doi: 10.1016/j.tox.2014.03.001. Epub 2014 Mar 15.

[13] Great Planes Laboratories, requisition # 467141, 10/18/2016

[14] Neuropsychopharmacology. 2016 Nov 21. doi: 10.1038/npp.2016.260. [Epub ahead of print]

[15] J Pharmacol Exp Ther. 2008 Nov;327(2):538-45. doi: 10.1124/jpet.108.141796. Epub 2008 Aug 12.

[16] Proc Natl Acad Sci U S A. 2008 Jul 1;105(26):9099-104. doi: 10.1073/pnas.0803601105. Epub 2008 Jun 23. Beta-caryophyllene is a dietary cannabinoid.

[17] Eur J Pharmacol. 2007 Aug 27;569(3):228-36. Epub 2007 May 22.Anti-inflammatory effects of compounds alpha-humulene and (-)-trans-caryophyllene isolated from the essential oil of Cordia verbenacea.

[18] Planta Med. 2008 Nov;74(14):1678-83. doi: 10.1055/s-0028-1088307. Epub 2008 Oct 24. Pharmacokinetics and tissue distribution of the sesquiterpene alpha-humulene in mice.

[19] Br J Pharmacol. 2009 Oct; 158(4): 1074–1087. Preventive and therapeutic anti-inflammatory properties of the sesquiterpene α-humulene in experimental airways allergic inflammation

✚AIM
Autism Is Medical

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July 16, 2017

Dear Legislator,

I am writing to you today to ask for your support in making medical cannabis available for pediatric use in patients with autism.

I am a board certified registered nurse and mother to a fourteen-year-old son recovered from autism. I have been a practicing nurse for twenty-three years and have worked in all facets of patient care with most of my experience in large, university setting, acute care hospitals. I also practice as an independent legal nurse consultant, working on litigation for vaccine injury claims filed with the United States Federal Claims Court under the National Vaccine Injury Compensation Program.

I am the co-founder/co-director of AIM, Autism Is Medical, a 501(c)(3) that supports efforts focused on educating parents, practitioners, and school personnel on the underlying medical issues that affect children and adults diagnosed with autism spectrum disorder. It is our strong belief and mission that all patients with autism should have the same access to an appropriate diagnostic investigation of their health problems as all other patient populations. Patient centered focused care is the standard of practice in all settings and we will continue to promote the inclusion of this large group of medically complex individuals. This non-profit was created by myself and two other mothers, who both have children with complex health issues and a diagnosis of autism.

Providing comprehensive medical care is driven by national goals and patient centered care is that model. Providing the patient what they need to regain health and treating them based on their individual health care needs is the standard of practice. In care of our pediatric patient population, the medical home model is that standard and provides the most comprehensive, multidisciplinary medical management of children who have chronic illness, are medically fragile and those that live with illnesses affecting them globally. These children require cutting edge diagnostic and therapeutic treatments provided by highly skilled practitioners. Children affected by autism, are at the pinnacle of this need for the most comprehensive, most cutting edge, and most effective treatments. Autism is a neurobiological disorder and can affect all systems of the body.

Children with autism have a high prevalence of other medical illnesses including gastrointestinal disorders, seizures and epilepsy, anxiety, allergies, recurrent infections and metabolic disorders including mitochondrial disease (Frye, 2015). There are many children with a diagnosis of autism that are considered medically fragile. These children may have severe refractory seizure disorder that does not respond to traditional pharmaceutical management. Some children suffer from upwards of forty to sixty seizures daily. Children with autism suffer from altered pain response and atypical expression of pain including self-injurious behaviors. These can be severe and can cause trauma sometimes requiring emergency medical attention (Courtemanche, Black & Reese 2016). Our medical systems are not well equipped, or adequately trained to serve this ever growing patient population. Our emergency rooms are not able to accommodate the expanding numbers of adult-sized pediatric patients with severe autism that are brought for treatment of escalating behaviors or the resulting injuries. These children need to have available to them, all the possible treatment modalities that may help treat their symptoms and alleviate the devastating effects of these serious medical problems.

The American Academy of Pediatrics policy states, "medical care of infants, children and adolescents ideally should be accessible, continuous, comprehensive, family centered, coordinate, compassionate and culturally effective," and goes on to say that, "physicians should seek to improve the effectiveness and efficiency of health care for all children and strive to attain a medical home for every child in their community" (AAP,2002).

This clearly outlines that pediatric care must be continually improving to provide the most effective treatments to those who are in need. This comprehensive plan of care must include medical cannabis for pediatric use. By not supporting making medical cannabis available to pediatric patients in your state, you will be denying them the opportunity to have access to all of the treatment modalities that are available to remediate some of the devastating effects of autism, seizure disorder and other debilitating illnesses.

My journey began with my now fourteen-year-old son being diagnosed on the autism spectrum before the age of three. He was low verbal at having under ten words, had severe auditory processing disorder preventing him from being able to communicate or express himself. His brain was unable to process speech and he did not recognize language. More difficult to obtain treatment for, where his underlying severe medical issues. Those included small bowel disease, abnormal EEG, failure to thrive, lymphopenia and multiple allergies. My son had severe bowel disease which prevented him from absorbing any nutrients. At age seven, he weighed thirty-nine pounds and was very sick. After extensive treatment of his underlying medical diagnoses, along with intensive therapies, he has recovered from autism and his bowel disease is in remission. He has been formally evaluated and his diagnosis of autism spectrum disorder removed. He is a happy, healthy and independent teenager who requires no assistance in school, and will go on to attend college and be a successful adult. This was only possible with him receiving patient centered care. His specific needs were met when he received targeted medical diagnostic testing and treatment. This should be available to all children.

Nurses as their primary role, are patient advocates. It is within this framework I ask you to support medical cannabis for the use in the pediatric population. Autism is a neurobiological

disorder with devastating associated serious health issues. Patients and families have a right to choose their treatment based on individual need. Medical cannabis can be used to decrease many of the symptoms and there have been many parent reports that it has substantially decreased seizure activity. Cannabinoids offer neuroprotective and antioxidant effects and can improve behaviors, decrease anxiety, and assist with sleep disturbances. Pharmaceutical options available today are often not metabolized well in children with autism, who have altered mitochondrial and metabolic function. Dangerous side effects and long term consequences of antipsychotic use in children, often the first line of treatment, have been well documented. It is imperative that legislators and health care providers work to ensure that children have every available resource and treatment option to improve the quality of their health and life.

Thank you in advance for your critical attention to this important need to serve the children of your community. Please do not hesitate to contact me for any reason. I look forward to hearing about this important legislative initiative moving forward with your support.

Respectfully,

Jill Rubolino, RN-BC, PCCN, LNC

References

Courtemanche, A. B., Black, W. R., & Reese, R. M. (2016). The relationship between pain, self-injury, and other problem behaviors in young children with autism and other developmental disabilities. *American Journal on Intellectual and Developmental Disabilities, 121*(3), 194-203,276,278. doi:http://dx.doi.org.ezproxy.uta.edu/10.1352/1944-7558-121.3.194

Frye, R.E., (2015) Autism spectrum disorder in the emergency department: Looking beyond behavior. *International Journal of Emergency Mental Health and Human Resilience, 17*(3), 661-663.

American Academy of Pediatrics (2002). The medical home: medical home initiatives for children with special needs project advisory committee. *Pediatrics,* 122(2), 184-186. doi:10.1542/peds.110.1.184, retrieved from http://pediatrics.aappublications.org/content/110/1/184

Audrey Ross Powell, MD
Internal Medicine and Pediatrics
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July 2, 2017

Delaware Legislature
411 Legislative Ave
Dover, DE 19901

To Whom it May Concern:

As we know, CBD formulations are widely available for treatment of many
conditions and has broad therapeutic potential. It has medical uses as an anti
inflammatory, anti epileptic, and as an antiemetic. In addition, it has been
researched worldwide for its properties against neoplasms, psychiatric
disorders, chronic pain and many inflammatory conditions.

There are limitations, however, to CBD only preparations. We must exercise
caution in our pediatric population with autism, immune-related disease or
infectious disease. CBD alone may actually worsen symptoms in these
vulnerable individuals. There is endocannabinoid deficiency and replacing
only CBD as opposed to Whole Plant Cannabis can cause Herxheimer
reactions or a die-off effect. CBD is an efficient antimicrobial agent and
aggressively annihilates pathogens, however, if the detox pathways are not
optimized, the toxin load increases. This can cause a paradoxical effect and
worsening of the condition.

On the other hand, if there is access to Whole Plant Cannabis, the availability
of all of the plant components is advantageous. It will reduce the cannabinoid
deficiency that is common in the pediatric population. This will allow more
efficacy and less propensity for worsening symptoms due to dysfunctions in
the detox pathways. Of course, the treatments must be individualized for the
best outcomes. In addition, studies have shown no effect on brain
development.

Sincerely,

Audrey Ross Powell, M.D.

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July 2, 2017

Janie Maedler
Rylie's Smile Foundation
Rylie's Sunshine LLC

Dear Jae

In response to your request for a letter regarding the use of the cannabinoid Tetrahydrocannabinol (Delta 9-THC) "THC" therapeutically in children and adolescents, we provide our expertise of more than four decades at the National Cancer institute, the pharmaceutical and Biotech industry in the development of drugs and immune therapy for Cancer, HIV/AIDS, Autoimmune and Neuroimmuune diseases. Our basic and translational research changed the paradigm for the treatment of these diseases, which are still the standard of care more than a quarter century since our discoveries in the early 1990s

Only since 1994 has the scientific community discovered the Endogenous Cannabinoid System (ECS) in the development of Brain and Immune systems; that the brain and the immune system are inextricably linked from conception until death; and that the immune system changes throughout life.

Thus, the cannabinoids including the phyto-cannabinoid, THC, are critical to health and the maturation of not only hematopoietic stem cells (HSC) but Mesenchymal Stem Cells and Embryonic Stem cells. A 2013 publication by Galve-Roperh et. al. details the effects of cannabinoid on stem cells throughout development. Key to these pioneering and paradigm shifting discoveries is the direct effect of THC on the production of TGF-Beta from HSC. I am a co-discoverer of the regulatory effects of transforming growth factor-beta (TGF-beta) on hematopoietic stem cells. I was awarded the NIH Distinguished Service Award "in recognition of fundamental co-discoveries of Interleukin-2, the first human leukemia virus, and for the discovery of hematopoietic regulatory activities of transforming growth factor beta". Billions of dollars have been spent by NIH and the pharmaceutical industry developing therapeutics for targeting TGF-beta which THC does. It should be approved for every indication whether infants, adolescents or the elderly. Medicine should not be denied to anyone without basis in science. The scientific/medical community has learned this as laws and medicine were applied discriminating against women, children and the elderly in the past.

Our families' health depends on the application of knowledge and discoveries to ALL regardless of age, gender or cultural bias. Please extend the use of THC in all clinical indications.

Sincerely,

Francis W. Ruscetti, PhD and Judy A. Mikovits, PhD

Today you must deliberate on a simple question: whether to permit the parents of children with intractable, treatment-resistant or treatment refractory diseases try full cannabis extracts, including Tetrahydrocannabinol, to see if their children respond favorably/positively in an effort to mitigate or reduce their child's signs and symptoms and slow or halt the progression of the disease to catastrophic complications or death.

This question is actually much easier to answer than you might think, but the right answer requires a little bit of humanity, a little bit of honesty, and a little bit of humility, with a dash of logic thrown in.

The argument against permitting this is that since "we" (who?) don't know what the long-term effects of cannabis oil is on the pediatric/adolescent brain, we should **err** (emphasis on "err") on the side of caution and let children die of cancer, become brain damaged from continuous, uncontrolled seizures, go blind from retinal disease, lest we harm their developing brains somewhere in the future; however, isn't it clear that in doing nothing, that neglect (and I do mean neglect) does the most harm, because the prognosis of each disease is clear--the outcome unacceptable to every parent, any parent.

In fact, long-time cannabis users endorse that cannabis consumption over the long-term is safe: no one has died or suffered such severe complications from its use resulting in death, whereas for many severe pediatric diseases, the result is death. Could there be more subtle deficits induced over the long term?

The answer is that no therapeutic is completely benign, completely safe and without negative side effect(s). Anything consumed has toxicity or some side effect. The practice of administering any therapeutic has always been a tradeoff and a practice of formulation so as to minimize side effect(s). You give a therapeutic because the outcome with the therapeutic is perceived to be better than without--be it to increase quality of life, to reduce pain, to palliate, or to treat. The lens of medicine is skewed more to the near-term: for example, the basic metric in cancer care (oncology) is the 5-year survival rate (their lens looks 5 years out). This is because we have to treat today's problem now, not some theoretical problem at some hypothetical future. We have to do the best we can with the knowledge we have now.

Importantly, it is disempowering at a basic human level, at the level of a parent's fundamental right and need to care for their own child, to not permit what is

instinctual: to secure medicine for their child. No regulatory body should stand between a parent and their child, no regulatory body really can. Where there is a will, there is a way. No regulatory body can say they know better than an individual 100% of the time. It is a basic American right to life, liberty, and the pursuit of happiness and part of self-mastery is that through autonomy, self-determinism, and self-sovereignty, one can change one's fate and choose their own destiny. So-called incurable diseases can be controlled through personal ingenuity and determination-- that is what everyone can learn from the history of Lorenzo's Oil.

The pharmaceutical industry was born from the basic hypothesis that an active pharmaceutical ingredient (API) could be isolated from botanical extracts. This is how digoxin/digitalis was discovered from the herb, foxglove to treat congestive heart failure (CHF). Herbalism, an ancient medicinal art with a longer history, is founded on a simple premise that informs its art: herbs and plants are effective therapeutics against human ills and can be administered skillfully by those experienced in its administration.

Cannabis is an herb, one that is psychoactive under certain conditions (THC > 1% by dry weight in the flower and the CBD:THC ration less than 8:1). However, when psychoactivity is present, qHS (nightly) dosing right before falling asleep provides all the benefit of the plant, without any perceived psychoactivity, because the child sleeps through the psychoactive period.

Please be compassionate and vote in favor of permitting the compassionate use of cannabis, including Tetrahydrocannabinol, for pediatric patients. Science and medicine are finding out what the medical cannabis community knows and has known for a long time: cannabis heals. What better service can we render our community, your constituents, than to heal our children. The children themselves will thank you for your service and I thank you for your service.

Sincerely,
Kang H. Hsu, M.A. (Molecular and Cellular Biology, Harvard University)
CEO SO Young Life Sciences, Inc
(909)929-5120

IMPACT
NETWORK

Letter of Support: Michele Ross, PhD

Remove Limits on THC Potency for Pediatric Patients

July 3rd, 2017,

My name is Michele Ross, PhD. I received my doctorate in Neuroscience from the University of Texas Southwestern Medical Center in 2008 and I currently am the Director of Clinical Research at the nationwide 501c3 nonprofit research institute I founded in 2013, IMPACT Network.

I fully support and urge the removal of THC potency limits for pediatric patients that qualify for medical marijuana. After educating legislators in my home state of Colorado, a bill that would have put a limit on THC potency was dropped. I have attached IMPACT Network's Fact Sheet on THC Potency Limits with references; please keep in mind this was developed for a state with both medical and recreational marijuana.

There is a lack of scientific evidence that cannabis products containing more than 7% THC are harmful for the brain and body. High THC potency is not unsafe for children. In fact, products containing more than 7% THC are often necessary for many children with chronic conditions, because their immature endocannabinoid system requires higher dosage than adults. THC potency should be something left to the discretion of medical professionals and their patients.

It is not possible to die from consuming THC at any potency at any age. Limiting THC potency does not make THC any safer, it only means patients or their caregivers have to buy more product to achieve the same dosage they are accustomed to. If the aim of the 7% THC limit is to reduce consumption of THC products, it may actually have the opposite effect.

There is concern that limiting medical marijuana products for pediatric patients to 7% THC and 15% CBD may result in few products available through the dispensary system for these patients. As most products available have a higher THC potency, a child may suffer needlessly if the supply of products meeting this potency limit runs out. Removing the THC potency limit means a child would never be put in this dangerous and potentially life-threatening situation.

As a neuroscientist, I am both an expert in cannabinoid medicine and in neurological conditions, such as autism. I did my Masters exam on a form of autism, performed basic and clinical research at several universities, and provided hundreds of client consultations on cannabis treatment through IMPACT Network. In fact, I am actually an expert on the effects of drugs on neurogenesis (birth of new brain cells), which occurs through the lifespan. My first paper in 2006 was published on the effects of Cannabinoids on Neurogenesis.

In my expert opinion, the benefits of THC consumption outweigh the risks for a pediatric patient whose chronic condition is not managed by traditional medications. In neurological conditions such as epilepsy, autism, or brain cancer, the risk of permanent brain damage, death, or injury due to self-harm or falls is much greater than the potential risk of THC to brain development. In fact, many of the potentials issues raised in regard to THC exposure during childhood or adolescence have only been investigated in terms of recreational use of smoking marijuana.

Studies looking at brain development and cognitive performance in adolescents taking cannabis products consumed orally or in products containing both THC and CBD have not been performed. We do know CBD increases birth of new brain cells, while THC may reduce them. Thus, products with

both THC and CBD may be ideal for limiting potential risks on brain development in pediatric patients.

We lack the scientific data to state what THC potency cutoff provides safety for children, and in fact that decision should be left to healthcare professionals and not legislated. At IMPACT Network we are currently investigating the impact of cannabinoids singly and in combination on brain waves and cognitive performance in health patients, and patients with neurological conditions such as epilepsy, Parkinson's disease, treatment-resistant depression, and autism, but our study is not done yet.

I'd be happy to answer any questions you may have about our research at IMPACT Network or any other topic that might help you better understand the potential harm limiting THC potency in medical marijuana products will have on children. Feel free to contact at your convenience.

Sincerely,

Michele Ross, PhD

Executive Director

IMPACT Network

720-486-9062

Michele@impactcannabis.org

I am not an advocate for drugs, either legal or illicit. I have never smoked and I don't care at all for alcohol. I agree with Oliver Wendell Holmes: "I firmly believe that if the whole materia medica could be sunk to the bottom of the sea it would be all the better for mankind and all the worse for the fishes."

Recently, our newsletter, *Autism Research Review International*, published a letter from a father in New Jersey whose teenage autistic son had become extremely assaultive, sending members of his family to the hospital and requiring police intervention on a number of occasions. Like that New Jersey father, thousands of parents are dealing with autistic children who are so out of control, and so violent to themselves and others, that they can make their own lives and those of their families hellish.

We then heard from a mother in Florida whose very large autistic son had changed from a sweet, loving boy to a teenager who flew into unpredictable rages that "were usually associated with self-injury, aggression and property damage. At times I had to lock myself in the bathroom; otherwise he would attack me. We gave him many medications, but nothing worked."

A friend suggested a solution: a brownie with marijuana baked into it. "Soon after he ate the brownie," she said, "my son's anxiety disappeared, and his sweet, loving behavior returned. He shows no signs of being under the influence of a drug. He now receives one marijuana brownie and several doses of Marinol, which contains the active ingredient in marijuana, each day. This has clearly saved my child's life and my family's life."

Some severe behavioral problems in autistic children have improved remarkably when the child is given a treatment of high-dose vitamin B6 and magnesium, which has been proven to be safe and effective in more than 20 research studies. But in many cases that treatment does not work. Drugs such as risperidone (Risperdal) are often used to control severe behavior problems in autistic individuals, but they have a large range of highly toxic effects. It seems to me that if one is going to need to use drugs because the safe nutritional supplements do not work, one ought to consider a relatively safe drug such as marijuana, if research bears out the good results that a number of parents have reported.

While medical marijuana is not a drug to be administered lightly, compare its side effects to the known effects of Risperdal, which include massive weight gain, a dramatically increased risk of diabetes, an elevated risk of deadly heart problems, and a host of other major and minor problems. Other psychotropic drugs are no safer, causing symptoms ranging from debilitating tardive dyskinesia to life-threatening malignant hyperthermia or sudden cardiac arrest. Of all drugs, the psychotropic drugs are among the least useful and most dangerous; in comparison, the benefit/risk profile of medical marijuana seems fairly benign. Moreover, the reports we are seeing from parents indicate that medical marijuana often works when no other treatments, drug or non-drug, have helped. Among the comments received:

"I know it's not the end-all answer, but it's been the best answer for the longest time for us in [comparison] to all the other medications. I cannot tell you how many months we would go on a medication wondering if it was doing anything, anything at all. Here we can see the difference in 30 to 60 minutes."

"My son (who is almost nine years old) has been on medications to address his severe autistic behaviors. . . . None of the medications has ever made a difference, except for making his behaviors worse. . . . A few months ago we tried the prescription drug Marinol

and noticed a drop in the severe episodes, no fits and little to no aggression toward his teacher and family members on a daily basis. A few weeks ago we started him on cannabis and stopped the Marinol. He has been in a much better mood and is much easier to keep on task in the classroom now. . . . He still has days when he gets angry and moody, but we can adjust the dose to help him through those days. . . . I feel much more comfortable administering cannabis than something like Risperdal."

Medical marijuana is not legal in most states. Information on whether or not medical marijuana can be legally prescribed in your state is available on the Internet at www.mpp.org. Additional information can be found at www.maps.org/mmj, www.NORML.org, and www.druglibrary.org.

It is important to keep in mind the distinction between legalizing marijuana for medical uses, which has been done in some states, and "recreational" drug use, which is illegal throughout the US. Judging from the evidence in hand, I believe legalization of medical use is justified. Legalizing marijuana for nonmedical use, as has been done for tobacco and alcohol, is quite another issue.

Even if medical marijuana can be legally prescribed in your state, doctors are likely to be very reluctant to help you obtain it. You might be able to obtain information or help from local AIDS awareness and advocacy groups, which have been in the forefront of making medical marijuana available to the public.

Again, I stress that I am strongly opposed to drugs in general, and consider them a last resort, to be employed only when safer and more efficacious treatments fail. But while I am not "pro-drug," I am very much "pro–safe and effective treatment," especially in cases where an autistic individual's behaviors are dangerous or destructive. Early evidence suggests that, in such cases, medical marijuana can be a beneficial treatment, as well as being less harmful than the drugs doctors routinely prescribe.

Written By Bernard Rimland, PhD
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The late, Dr. Rimand was the father of an adult autistic son, the founder of the Autism Society of America and the Director of the Autism Research Institute. He was chief consultant on autism for the 1988 film Rain Man.

Medical Comorbidities in Autism: Challenges to Diagnosis and Treatment

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Summary: Ever since its original description by Leo Kanner in 1943, autism has been generally defined by its clinical characteristics and core symptoms that include impaired social skills, isolated areas of interest, and delayed and disordered language. Over time, it has become apparent that autism is a heterogeneous disorder with regard to its clinical presentation, etiology, underlying neurobiology, and degree of severity. As a result, the termed diagnosis of autism spectrum disorders (ASDs) has come into common usage. With advancements in clinical care, there has come the appreciation that many ASD children, adolescents, and adults may have medically relevant disorders that may negatively impact their developmental progress and behavior, but which frequently go undetected. Many of these medical conditions are treatable, often resulting in improved developmental gains and quality of life for the patient and family. In addition, the possibility exists that some of these medical conditions may suggest the presence of important genetic and/or biologic markers, which, if identified, can refine our ability to be more precise in categorizing clinical and genetic subtypes within the autism spectrum. **Key Words:** Autism, medical conditions, atypical behaviors, diagnosis, management.

INTRODUCTION

Infantile autism is a behaviorally defined disorder first described by Leo Kanner[1] in 1943. Although infantile autism is now recognized as being clinically, etiologically, and biologically heterogeneous, it is generally agreed that the core features of the disorder consist of a triad of characteristics, including impaired social interaction, delayed and disordered language, and isolated areas of interest.[2] Variable additional features can include poor eye contact, stereotypic and repetitive behaviors, sensory processing dysfunction, and an insistence on sameness. Symptoms can range from relatively subtle and mild to very severe. Some level of cognitive impairment has been reported. Until recently, it was generally believed that 75% of those affected with autism functioned in the mentally retarded range. However, more current data suggests that fewer than half of those affected have a significant cognitive deficit.[3] Although it was once believed that autistic persons had no impairment of motor skills, a growing body of research sug-

gests that hypotonia is common and may be associated with deficits in both fine and gross motor skills.[4] Individuals with non-syndromic or "essential" autism and with few, if any, dysmorphic features are generally considered to be attractive in appearance. Measurements of head circumference in childhood has suggested that approximately 20% of autistic children show a frank macrocephaly of greater than 98% for age and sex.[5] Numerous epidemiologic studies have provided evidence for a genetic basis for autism. Linkage studies have identified a number of candidate genes of promise; however, despite modern technology, approximately only 6% to 15% of individuals with autism will be found to have an identifiable genetic diagnosis.[6–8] Current studies suggest a multifactorial inheritance, including genetic heterogeneity with multiple major gene effects, possible contributing environmental effects, and physiologically linked processes with multiple genes. Thus, autism is now considered a heterogeneous disorder, clinically, neurobiologically, and etiologically, resulting in the now common usage of the termed diagnosis of autism spectrum disorders (ASDs).

For the most part, autism research has been largely devoted to the assessment of and understanding of many of the clinical characteristic features of the disorder,

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generally aspects of language patterns, cognitive skills, behavioral manifestations, social interaction, and information processing, and how these features may relate to the underlying gross and microscopic anatomy, neurochemistry, connectivity of the brain, and potential genetic factors. Although we still have much to learn, we have expanded our knowledge regarding the underlying neurobiological mechanisms related to the autistic brain during the past 25 years. However, we have acquired very little knowledge of any other organ systems that may be important in this disorder. Much of this relative lack of information may relate to the fact that individuals with ASD, especially children, can be difficult to examine, often due to poor cooperation and difficult office behavior. Other limitations include the fact that many ASD individuals are nonverbal or hypo-verbal, and many of these patients have deficits in sensory processing, which are features that prevent them from reporting pain or accurately localizing discomfort. Clinical observations suggest that many ASD patients may not present with physical symptoms of an illness that most physicians have been taught to recognize in typically developing persons, which further complicates the physicians' diagnostic efforts. In fact, many "autistic behaviors," such as aggression and self-injurious activity, have been attributed to be just "part of the autism." As a result, the ASD patient is frequently referred for behavioral management and/or psychopharmacological intervention because of failure to interpret these behaviors as indicators of pain and discomfort, suggesting an underlying medical condition.

The importance of identifying comorbid, health-related medical conditions associated with ASD are multiple. First, many of these medical conditions are treatable, and if identified and managed, will result in an improved sense of well being, more effective participation in educational and therapeutic programs, and improved quality of life for the patient and his family. Second, identification of medical disorders involving specific organ systems may help us to identify phenotypic and genetic clusters of ASD persons, thus possibly defining meaningful subtypes that may result in a better understanding of subsets of causative and biological mechanisms. However, at this point in time there are few, well-designed population studies that have carefully examined associated medical disorders in autism, and much of the "data" available stems from clinical observations and anecdotal information involving relatively small groups of subjects. In 2003, the Autism Treatment Network (ATN) was established under the guidance of the Northwest Autism Foundation in Portland, Oregon, with the goal of creating a network of academically based multidisciplinary centers that would develop mutually agreed on protocols to begin assessing the prevalence, presenting features, and treatment of comorbid

medical conditions across the autism spectrum. Today, the ATN is comprised of fourteen academic institutions across the United States and Canada. With the involvement of the ATN, and possibly other multi-site consortium groups, it is hoped that we may be able to look more carefully at a number of comorbid medical conditions that may have broad implications if identified and treated, resulting in improved behavior, developmental progress, and quality of life, as well as providing leads into potential subsets of ASD that may guide genetic and biologic research.

Space does not allow for a detailed description of the multiplicity of possible medical conditions that may affect a person with autism. Suffice it to say that ASD individuals can and do have many of the same medical disorders and diseases that affect neurotypical persons, although they may present differently. Only some of the more common disorders will be highlighted within this chapter, including gastrointestinal dysfunction, sleep irregularities, metabolic disorders, and hormonal imbalances. However, every primary care and specialty physician and caregiver involved in the care of ASD persons must be alert to the possibility of a wide range of potential medial disorders that could negatively impact the health and behavior of each autistic individual.

SEIZURE DISORDERS

The prevalence of seizures in adults with autism has been estimated to be between 20% to 35%,[9] and in ASD children the estimate is between 7% to 14%,[10,11] with peak risk periods said to occur during early childhood and adolescence.[12] All types of seizures can be found in association with ASD, including major motor, myoclonic, and febrile seizures, but complex partial seizures are most frequently reported. 66.7% of seizures that occur in ASD become apparent after 12 years of age,[13] with 30% occurring by 20 years of age. A predilection for seizures appears to be associated with the co-appearance of low cognitive ability, dysmorphic features, and motor impairment, and a long list of syndromes, including tuberous sclerosis and Rett's syndrome. It is likely that the co-occurrence of ASD and seizures may reflect a shared underlying neurobiology.

The clinical identification of seizures may be difficult in some cases, especially partial complex seizures, which can be largely complicated by the presence of atypical body movements and behavioral patterns often seen in association with ASD. Not all body movements or mannerisms observed in ASD are seizure related and may be a manifestation of other medical conditions, such as gastroesophageal reflux disease (GERD).[14] Further complicating the diagnosis is a lack of direct correlation between electroencephalographic (EEG) activity and clinical seizures.[9] However, any behavior such as staring

spells, cessation of activity, eye fluttering, or eye deviation to one side, as well as behavioral changes associated with confusion or followed by fatigue or sleep should raise the suspicion of complex partial seizures, and should lead to further investigation. Obtaining a high-quality EEG, especially in toddlers and young children, can be difficult but is achievable. A prolonged overnight EEG can often be helpful, as well as the use of a video-tape review of the events recorded at home and/or at school.

Treatment approaches have expanded substantially over the past several years, and a number medication options are available for intervention. In a small number of cases, seizure control can be difficult to achieve and may require the use of multiple medications and possibly the use a vagal nerve stimulator. For the most part, seizure management can be successful in the majority of ASD children, adolescents, and adults with good medical oversight.

In recent years, there has been a growing interest in the possible role of EEGs in autism, with or without the presence of epilepsy. Rates of these abnormal EEGs have been reported to be as high as 60%, and it has been suggested by some investigators that these abnormalities may play a causal role in autism symptomatology.[15] The significance of epileptiform EEGs in the absence of clinical seizures remains controversial, and as of yet there is no solid data that exists as to their relevance or treatment implications. However, this is an important area for future research. Although these EEG findings may simply be a reflection of the underlying neuropathology responsible for autism, without implication for treatment, it is also possible that individuals with these abnormalities might potentially benefit from intervention. Large population studies correlating EEG abnormalities with autism phenotypes and clinical medication trials with a well-described baseline and outcome data could help to begin to answer these questions.

SLEEP DISORDERS

Sleep problems are highly prevalent in ASD children and rank as one of the most common concurrent clinical disorders.[16] Prevalence rates vary widely, ranging from 40% to 80%,[17,18] as compared with that of typically developing children in which prevalence rates are approximately 30%.[19] In addition, sleep disorders are reported to be more severe in ASD. Among the sleep problems most commonly reported, sleep onset, sleep maintenance, and sleep duration are consistently the most predominant concerns expressed by parents of children with an ASD.[20] However, other types of sleep disturbances have also been reported in ASD children, including parasomnias (nightmares, screaming), sleep-related breathing disorders, sleep-related movement dis-

orders, and circadian rhythm sleep disorders. Although the frequency of night-time awakening is similar to that of typically developing children, ASD children experience more lengthy periods of night waking (i.e., frequently as much as 2 to 3 hours per night). During this time, the child may laugh, talk, or get up and play with toys/objects in the room, which is a pattern that is not typical of other children with developmental disabilities or typically developing children.[21]

It is known that disordered sleep affects daytime health and can result in neurocognitive dysfunction and behavioral disruptions. In typically developing children, sleep disruption may lead to daytime sleepiness and may manifest itself as hyperactivity, inattention, or aggression.[22] Thus, sleep disorders can have wide ranging implications for daytime functioning, developmental progress, and quality of life for both the child and his family. Effective intervention can have a positive impact on educational and developmental progress, as well as community life opportunities.

Although sleep difficulties are common in ASD, it is unclear why this dysfunction is so prevalent in this disorder. A number of hypotheses have been proposed. It has been suggested that sleep problems could be the result of 1) intrinsic biological or genetic abnormalities that alter brain structure and/or chemistry, 2) psychological or behavioral disorders associated with the core features of ASD, or 3) factors in the home or environment that are not conducive to good sleep. Any one or a combination of these factors could contribute to sleep disturbances in ASD.[21]

Biological mechanisms may involve the role of melatonin, a neurohormone produced from serotonin in the pineal gland and secreted during night-time hours. Its major role appears to be to organize circadian rhythms, including sleep-wake and core body temperature cycles. Sleep problems in ASD may be related to circadian rhythm dysregulation, which may cause the nervous system to fluctuate causing fluctuation in faulty transmission of entrainment cues, along with social and environmental factors and possible intrinsic biological abnormalities.[21] This faulty transmission of entrainment cues may be related to an abnormal expression of one or more of 10-clock genes that are believed to form the core of the biological clock, thereby controlling circadian rhythm. Thus, it has been speculated that sleep disorders in ASD may be related to genetic abnormalities associated with melatonin synthesis and its role in modulating synaptic changes. To date, there have been few studies exploring the role of melatonin in sleep pattern abnormalities associated with ASD, but existing data suggests that further investigations are warranted.

Although biological factors may form the basis of many of the sleep disturbances in ASD, co-morbid psychopathology may also be playing a role, at least in some

cases, especially anxiety, depression, stress, and mood disorders. Symptoms of attention deficit hyperactivity disorder may also play a role and contribute to sleeplessness, as well as sleep disordered breathing, obstructive apnea, respiratory compromise secondary to enlarged tonsils and adenoids, parasomnias, and a variety of medications, including corticosteroids, stimulants, some anticonvulsants, and serotonin reuptake inhibitors, or asthma medications.[18]

It has been recommended that all ASD children be screened for potential sleep disturbances, given the high prevalence rate in this population.[18] A number of questionnaires can be used including the Children's Sleep Habits Questionnaire (CSHQ) and the Pediatric Sleep Questionnaire (PSQ). Each of these questionnaires explores possible obstructive sleep apnea, restless leg syndrome, behaviors suggestive of parasomnias (night terrors, sleep walking), or symptoms that might suggest nocturnal seizures. Depending on the responses to the questionnaires, referrals to a sleep specialist or a neurologist may be indicated. Further studies may be needed to clarify the nature and cause of the presenting behaviors, including the use of actigraphy and polysomnography. Gastrointestinal disorders, such as GERD, should also be considered as a possible cause of nighttime awakenings and may warrant a referral to a gastroenterologist.[23]

When considering treatment interventions, most sleep specialists recommend the implementation of establishing a program to improve sleep hygiene. This approach seeks to correct problematic behavior and promote regulated and predictable bedtime routines. Behavioral interventions can often be useful in treating children with ASD and may be effective in dealing with problems associated with sleep onset and frequent awakenings.

Pharmacologic interventions may also be indicated. Currently, there is growing evidence in support of the use of melatonin, which is considered a nutritional supplement, and as such, it can be obtained over-the-counter and is relatively inexpensive. Studies have shown that this product can be efficacious in promoting sleep and appears to have no long-term adverse side effects. However, melatonin has not been rigorously studied with regard to dosage, safety, and efficacy, and further studies are needed.

Similarly, there is little data to support the use of other medications for use in promoting and sustaining sleep in ASD. Clonidine has been found to be effective in reducing problems with sleep onset and night-time awakenings in some studies.[16] Mediations designed to treat anxiety and depression can also be considered, such as mirtazapine and trazodone, fluvoxamine, or clomipramine, have been used in some cases in which obsessional disorders appear to be a factor. Some anticonvulsant medications with sedating properties may also be useful.[18] It is important, however, that whatever medications are implemented, management be carefully monitored by a sleep specialist or psychopharmacologist with expertise in treating ASD children, because dosage and potential side effects merit cautious and careful implementation and supervision.

GASTROINTESTINAL DISORDERS

The evaluation of abdominal pain and discomfort in an individual with impaired communication skills and possible dysfunction in the processing of sensory information can be a challenging task. Although many patients present with the easily recognized symptoms of vomiting, diarrhea, and constipation, a substantial number of individuals do not provide these clues. Currently there are no reliable signs or symptoms that can consistently aide the primary care physician to distinguish between functional and organic disorders. Many ASD individuals may express their physical discomfort by manifesting atypical behaviors, such as putting pressure on their abdomen, tapping on their chest, acting aggressively toward others, or demonstrating self-injurious behavior.[23]

At the present time, the true prevalence of gastrointestinal (GI) disorders in ASD is largely unknown, with estimations ranging from 9% to 70% or higher.[24] Evidence-based guidelines for the evaluation of GI symptoms are not yet available for individuals with ASD. However, recently, 8 experienced gastroenterologists reviewed the current literature and published guidelines regarding GI symptoms that frequently occur in the general pediatric population. Based on this information and their collective expertise, they adopted guidelines for the current best practices related to the diagnosis and treatment for children with ASD. These guidelines deal with chronic constipation, abdominal pain, chronic diarrhea, and symptoms of GERD. In addition, these guidelines also raise awareness of the association of GI disorders with self-injurious behavior and sleep disturbances, and the frequent atypical presentation of GI dysfunction in individuals with ASD.[22,24]

Although some ASD persons present with symptoms that are easily recognized by most healthcare providers, many others may not. As previously noted, atypical behaviors may include chest tapping, facial grimacing, and intermittent gulping, chewing on nonedible objects, including shirts and sleeves, constant eating or drinking, seeking of abdominal pressure, aggressions, and self-injurious behaviors to name just a few.[25] It is well known that developing children may typically present with behavioral disruptions when not feeling well, and there is little reason to believe that ASD children should act differently. It is also well established that if an individual is ill, he or she is less likely to perform well at school, in a job, or in daily life. Thus, the identification and treatment of medical conditions, including GI disorders, may

have a positive impact on the ability of the ASD person to participate in programs and therapies and to make meaningful progress.

A number of GI disorders have been reported in association with ASD, including GERD, gastritis, esophagitis, inflammatory bowel disease, celiac disease, Crohn's disease, and colitis. Evaluation of a possible GI disorder should consist of a thorough medical history and physical examination, including anal inspection and examination and assessment of the back and spine for a dimple or tuft of hair. Studies may include the analysis of a stool specimen for parasites and enteric pathogens, stool guaiac, electrolytes/osmolarity, and might include serum electrolytes, liver function tests, assessment of nutritional status, and an abdominal roentgenogram to assess bowel gas pattern and possible retention of stool.[23] Depending on the results of these assessments and the outcome of attempted medical management and interventions, endoscopy, and/or colonoscopy may be warranted for diagnostic and treatment purposes.

It is not yet known whether GI disorders are more common in the ASD population than in typically developing persons, or whether these disorders should be treated in the same manner in both groups. Similarly, it is not known whether GI dysfunctions are inherent to ASD in some cases or associated phenomena. Well-designed, well-controlled diagnostic and clinical studies in sizeable populations (probably involving different age groups) are needed to establish evidence-based guidelines for identification and management. Recently, Campbell et al.[26] reported that disrupted MET gene signaling may contribute to an increased risk for ASD that includes familial GI dysfunction. A functional variant in the promoter of the gene encoding the MET receptor tyrosine kinase has been associated with ASD, and MET protein expression has been found to be decreased in the temporal lobe cortex in ASD postmortem brain. MET is a pleotrophic receptor that is known to be important for brain development, as well as GI repair and motility. This study suggests that by carefully investigating ASD individuals and their families medically, as well as behaviorally, we may be able to begin to identify genetically important subgroups within the larger ASD population. Thus, the identification of medical conditions in ASD persons, in this case, GI disorders, may not only impact the general health and well-being of the patients, but it may lead to improved and more precise definitions of phenotypic and genetic subtypes within this complex heterogeneous disorder.

METABOLIC DISORDERS

Metabolic disorders are considered to be relatively rarity among individuals with neurodevelopmental disorders. Among this population, the estimated diagnostic

yield has been found to vary between 1% to 2.5%.[27] In a more recent study, Engbers et al.[28] performed repeated metabolic analyses on a cohort of 433 subjects with neurodevelopmental impairments, whose initial evaluations were said to be unremarkable. Of these subjects, 12 (2.8%) were found to have diagnostic disorders, some of which were treatable. Thus, although metabolic disorders may be rare, it is important to pursue carefully planned diagnostic studies so as to identify potentially manageable disorders. The prevalence of metabolic disorder in ASD is largely unknown.

A number of genetic and metabolic disorders have been associated with ASD, often described in a small number of subjects. In the past, phenylketonuria was often reported to be associated with autistic features, but due to newborn screening, it is less commonly observed. Autistic features have also been reported in association other metabolic disorders, including creatine deficiency syndromes,[29] urea cycle disorders,[30] Wilson's disease, and Lesch-Nyhan syndrome.[31] Space does not allow for a full review of many of these conditions. However, there has been an increasing interest in two of these disorders in particular, and these will be highlighted here in more detail.

There is a growing body of suggestive evidence that mitochondrial dysfunction may play a role in at least a subset of individuals with ASD. Mitochondria perform four central functions in the cell that are relative to the pathophysiology of disease: 1) they provide most of the energy to a cell in the form of ATP, 2) they generate and regulate reactive oxygen species, 3) they buffer cytosolic calcium, and 4) they regulate apoptosis through mitochondrial permeability transition pore.[32] Mitochondrial disorders are heterogeneous and are often multi-systemic. Mitochondria provide much of the energy to the cell, and thus preferentially affect tissues with high-energy demands including the brain, the muscles, the heart, and the endocrine system. However, any organ system can be involved. It has been hypothesized that mitochondria may be a mediator of many common metabolic disorders. Mitochondrial disorders can be caused by genetic defects in mtDNA or nuclear DNA gene that encodes a mitochondrial protein or structural RNAs. Mitochondrial disorders are believed to be rare, with those related to mtDNA estimated to have a frequency of 1 in 5000, and known pathogenic mtDNA mutations detected in cord blood in 1 in 200 live births. The frequency of nDNA mutations affecting mitochondrial genes may be more common than mtDNA mutations.[32]

At the present time, the prevalence of mitochondrial disorders in ASD is largely unknown. In 2005, Oliveira et al.[33] published a population-based study, which was a survey of school-aged children with ASD. The investigators found that 7% of their subjects met criteria for a definite mitochondrial respiratory chain disorder, the af-

fected children being clinically indistinguishable from ASD children without a mitochondrial disorder. In a more recent study, Weissman et al.[34] reviewed the medical records of 25 ASD children with muscle biopsy proven enzyme-defined or mutation-defined mitochondrial electron transport chain dysfunction. Clinically, 24 of the 25 subjects demonstrated one or more abnormalities uncommon in ASD including cardiovascular, endocrine, and hematologic findings. There were 19 subjects exhibiting constitutional symptoms, such as low physical endurance, repeated regressions after the age of 3 years of age, delayed gross and fine motor milestones, and the involvement of multiple organ systems. Abnormalities were found in blood lactate, plasma alanine, and serum ALT and AST in 76%, 36%, and 52%, respectively. No differences were noted between males and females. The most common findings were deficiencies in complex I (64%) and complex III (20%). Thus, although all children carried a diagnosis of idiopathic autism, detailed clinical and biochemical evaluation identified features that appeared to differentiate these children from others with essential autism. The authors concluded that with careful clinical and biochemical analysis, ASD children with co-occurrence of mitochondrial dysfunction can be distinguished from those with idiopathic ASD, and that those with mitochondrial autism may comprise a significant subset of individuals with autism. If this hypothesis is true, this subset should be seen as genetically different from ASD children without a mitochondrial dysfunction. Whether the mitochondrial dysfunction in these cases is primary (causative) or secondary (an associated phenomenon) remains to be determined, and also opens this important question to further research.

Smith-Lemli-Opitz syndrome (SLOS) is another metabolic disorder that has been frequently associated with features of ASD.[35] SLOS is known to be an autosomal recessive disorder associated with an inborn error of cholesterol synthesis caused by mutations of the 7-dehydrocholesterol reductase gene (DHCR7) located on chromosome 11q12-13.[36] The syndrome has an estimated incidence of 1 in 20,000 to 1 in 60,000 births and a carrier frequency of 1%. Cholesterol is essential for neuroactive steroid production, growth of myelin membranes, and normal embryonic and fetal development. In addition, it is believed to modulate oxytocin receptors, ligand activity and G-protein coupling of the serotinin-1A receptor.[37] It has been hypothesized that a deficit in cholesterol may disrupt these biological mechanisms, thereby contributing to clinical features of ASD seen in SLOS. It has been estimated that 50% to 75% of individuals with SLOS may meet criteria for ASD.

Clinically, although physical features may not be readily recognizable in mild cases, individuals with SLOS frequently exhibit dysmorphic features associated with ptosis, upturned nares, small chin, bi-temporal narrowing, microcephaly and webbing between the second and third toes. Additional characteristics include developmental delay, hyperactivity, irritability, temper tantrums, aggressions, and self-injury. The diagnosis of SLOS can be confirmed by the presence of an elevated level of plasma 7-dehydrocholesterol relative to the cholesterol level. Identification of this disorder is important, given the fact that it is one cause of ASD that is partially treatable with cholesterol supplementation.

HORMONAL DYSFUNCTION

There has been an unwritten lore in the world of autism that when children with ASD go through adolescence, then their behavior worsens. This change in behavior has usually been attributed to the hormonal changes associated with the teenage years, but no formal studies have been reported to support this hypothesis. Hormonal changes could also be reflected in association with precocious puberty, accelerated, or reduced physical growth, and/or behavioral disruptions associated with menstrual pain or discomfort. An additional disorder that should be considered is one of congenital adrenal hyperplasia that we have diagnosed in three adolescent girls with ASD.[38] Some of these factors have been reviewed in a study published by Carr et al.,[39] in which both physical discomfort and pain associated with the menstrual cycle was cited as a possible cause of problem behaviors in adolescent girls. The authors also raise the possibility that fluctuations in progesterone and estrogen levels during adolescence may be an important variable. Abnormalities in progesterone and/or estrogen levels have been observed in a subset of behaviorally disruptive adolescent girls with ASD in our clinical program. When this hormonal imbalance is treated, the problem behaviors decline significantly and in some cases, disappear.[38]

This theme has been further expanded in a recent study in which adolescent girls with autism, Down syndrome, and cerebral palsy were evaluated retrospectively regarding gynecological complaints. The girls with autism were significantly more likely to present with behavioral issues than those in the two other groups. Successful management included the use of nonsteroidal anti-inflammatory medications, oral contraceptives, and education.[40] Thus, there is some evidence that hormonal dysregulation during adolescence may be an underlying factor for some of the behavioral problem observed in some of the ASD teenagers. At this time, there is very limited data regarding hormonal changes in adolescent ASD girls, and there is almost no similar research in ASD teenage boys. This is a very important but significantly understudied topic that merits increased attention to research.

There are numerous other medical conditions that deserve mentioning. These include the growing concern

regarding the prevalence of osteoporosis among individuals with ASD and the possible role of self-imposed dietary selection, and/or the use of special diets that may restrict calcium and vitamin D as part of treatment programs.[41] Obesity is also becoming a growing concern, not only among ASD individuals but in regard to the whole population within the United States. In ASD, contributing factors may include the use of food re-inforcers as part of a behavioral program, the preference for carbohydrates shown by many ASD persons, the use of some medications (such as risperidone), and the lack of physical exercise and activity.[42] Additional medical conditions that merit scrutiny include recurrent otitis media, hearing impairment, bladder and renal disorders, hypertension, diabetes, allergies, recurrent headaches/migraine, dental pain, immune disorders, and bacterial and viral illnesses, just to name a few. Any or all of these can result in significant behavior alterations. Much research is needed to assess the prevalence of these and other intercurrent illnesses in ASD, their mode of presentation, and whether medical management should be the same or different from that of typically developing individuals. We have much to learn in regard to organ systems in ASD outside of the brain, and how this information may contribute to a better understanding of the biological processes that underlie this disorder throughout a patient's lifespan.

ASD has been traditionally seen as a disorder primarily involving atypical neurodevelopment in the brain. Very little attention has been paid to other organ systems. It is becoming increasingly apparent that many ASD individuals may have associated medical conditions, which up to this point have gone unrecognized, and which are now only just becoming evident. At this point in time, there are very few, if any, large population studies related to the investigation of potential comorbid medical conditions in individuals with autism. Thus, there is little information regarding clinical presentation, which could guide the primary care physician, prevalence rates, effective modes of treatment, or developmental or behavioral outcomes as the result of diagnosis and management. Research designed to begin to address these questions is a major part of the agenda of the ATN and other clinical collaboratives. It is likely that the recognition of many of these medical conditions followed by appropriate treatment may result in better, more effective participation in and benefit from therapies and interventions as the result of improved health, and ultimately improved developmental outcomes. In addition, some of these medical conditions may begin to help us subcategorize specific populations within the larger ASD framework, resulting in more specific clinical and biological phenotyping and genotyping of ASD patients throughout their lifespan. More research is needed involving organ systems outside the brain. The information

to be obtained from these investigations may broaden our understanding of the interplay between these systems, thereby expanding our knowledge of the underling biologic mechanisms involved in the autism spectrum disorders.

CONCLUSIONS

It is becoming increasingly evident that many ASD children, adolescents, and adults can and often do experience a number comorbid medical conditions, the nature and prevalence of which remain as yet to be poorly defined. Many of these disorders have been largely ignored, at least in part, due to the challenges involved in conducting a meaningful medical history and physical examination in a frequently nonverbal patient whose behavior may interfere with a detailed assessment. However, there is a growing importance in identifying these disorders. First, ASD individuals deserve the same high-quality healthcare available to their neurotypical peers. They deserve to have treatable medical conditions identified and appropriately treated, resulting in decreased discomfort and improved overall health, thus allowing them to better participate in their therapeutic, educational, and vocational programs, and to achieve their best potential. Second, by identifying associated medical conditions and including them as part of the clinical phenotype, we may be able to better clinically subtype groups of ASD individuals, thus leading to more accurate genotypic identification. Much progress has been made during the past 5 to 10 years, but much remains to be learned. With advancing technology and improved diagnostic measures, it is anticipated that our understanding of the ASDs will improve and expand, resulting in more targeted and effective interventions in the future.

REFERENCES

1. Kanner L. Autistic disturbances of affective contact. Nervous Child 1943;2:217–50.
2. American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders, 4th edit (DSM-IV). Washington DC: APA, 1994.
3. Newschaffer CJ, Croen LA, Daniels J, et al. The epidemiology of autism spectrum disorders. Annu Rev Public Health 2007;28:235–258.
4. Ming X, Brimacombe M, Wagner GC. Prevalence of motor impairment in autism spectrum disorders. Brain Dev 2007;29:565–570.
5. Lainhart JE, Bigler ED, Bocian M, et al. Head circumference and height in autism: a study by the Collaborative Program of Excellence in Autism. Am J Med Genet A 2006;140:2257–2274.
6. Landa RJ, Garrett-Mayer E. Development of infants with autism spectrum disorders: a prospective study. J Child Psychol Psychiatry 2006;47:629–638.
7. Landa RJ. Diagnosis of autism spectrum disorders in the first 3 years of life. Nat Clin Pract Neurol 2008;4:138–147.
8. Lauritsen MB, Pedersen CB, Mortensen PB. Effects of familial risk factors and place of birth on the risk of autism: a nationwide register-based study. J Child Psychol Psychiatry 2005;46:963–971.
9. Minshew NJ, Sweeney JA, Bauman, ML. Neurologic aspects of autism. In: Cohen DJ, Volkmar FR, eds. Handbook of autism and

pervasive developmental disorders, 2nd ed. New York: Wiley, 1997:344–369.

10. Rapin I. Historical data. In: Rapin I, ed. Preschool children with inadequate communication: developmental language disorder, autism, low IQ. London: MacKeith, 1996:98–122.

11. Tuchman RF, Rapin I, Shinnar S. Autistic and dysphasic children. II: Epilepsy. Pediatrics 1999;88:1219–1225.

12. Volkmar FR, Nelson DS. Seizure disorders in autism. J Amer Acad Child Adolesc Psychiatry 1990;29:127–129.

13. Rossi PG, Parmeggian A, Bach V, Santucci M, Visconti P. EEG features and epilepsy in patients with autism. Brain Dev 1995;17: 169–174.

14. Buie T. Gastrointestinal issues encountered in autism In: Bauman ML, Kemper TL, eds. The neurobiology of autism. Johns Hopkins University Press: Baltimore, 2005:103–117.

15. Spence SJ, Schneider MT. The role of epilepsy and epileptiform EEGs in autism spectrum disorders. Pediatr Res 2009;65:599–606.

16. Ming X, Brimacombe M, Chaaban J, et al. Autism spectrum disorders: concurrent clinical disorders. J Child Neurol 2008; 23:6–13.

17. Richdale AL. Sleep problems in autism: prevalence, cause and intervention. Dev Med Child Neurol 1999;41:60–66.

18. Johnson KP, Giannotti F, Cortesi F. Sleep patterns in autism spectrum disorders. Child Adolesc Psychiatric Clin N Am 2009;18: 917–928.

19. Ferber R. Childhood sleep disorders. Neurol Clinics 1996;14:493–511.

20. Malow BA, Marzec ML, McGrew SG, Wang L, Henderson LM, Stone WI. Characterizing sleep in children with autism spectrum disorders: a multidimensional approach. Sleep 2006;28:1559–1567.

21. Richadale AL, Schreck KA. Sleep problems in autism spectrum disorders: prevalence, nature and possible biopsychosocial aetiologies. Sleep Med Rev 2009;13:403–411.

22. Owens J, Opipari L, Nobile C, Spirito A. Sleep and daytime behavior in children with obstructive apnea and behavioral sleep disorders. Pediatrics 1998;102:1178–1184.

23. Buie T, Fuchs GJ, Furuta GT, et al. Recommendations for evaluation and treatment of common gastrointestinal problems in children with ASDs. Pediatrics 2010;125(suppl):S19–S29.

24. Buie T, Campbell DB, Fuchs GJ, et al. Evaluation, diagnosis and treatment of gastrointestinal disorders in individuals with ASDs: a consensus report. Pediatrics 2010;125(suppl):S1–S18.

25. Buie T. Clinical presentation of gastrointestinal disorders in nonverbal ASD children. Unpublished data, 2009.

26. Campbell DB, Buie TM, Winter H, et al. Distinct genetic risk based on association of MET in families with co-occurring autism and gastrointestinal conditions. Pediatrics 2009;123:1018–1024.

27. Von Karnebeck CD, Jansweijer MC, Leenders AG, et al. Diagnostic investigations in individuals with mental retardation: a systematic literature review of their usefulness. Eur J Hum Genet 2005;13:6–25.

28. Engbers HM, Berger R, van Hasselt P, et al. Yield of additional metabolic studies in neurodevelopmental disorders. Ann Neurol 2008;64:212–217.

29. Nasrallah F, Feki M Kaabachi N. Creatine and creatine deficiency syndromes: biochemical and clinical aspects. Ped Neurol 2010;42: 1–13.

30. Gorker I, Tuzun O. Autistic-like findings associated with a urea cycle disorder I a 4-year old girl. J Psychiatry Neurosci 2005;30: 133–135.

31. Trauner DA. Cognitive and behavioral manifestations of metabolic and chromosomal disorders. In: Frank Y, ed. Pediatric behavioral neurology. New York: CRC Press, 1996:251–268.

32. Wallace DC, Fan W, Procaccio V. Mitochondrial energetics and therapeutics. Annu Rev Pathol Mech 2010;5:297–348.

33. Oliveira G, Diogo L, Grazina M, et al. Mitochondrial dysfunction in autism spectrum disorders: a population-based study. Dev Med Child Neurol 2005;47:185–189.

34. Weissman JR, Kelley RI, Bauman ML, et al. Mitochondrial disease in autism spectrum disorder patients: a cohort analysis. PLos ONE (serial online) 2008;3:e3815. Available at: www.plosone.org. Accessed December 2008.

35. Bukelis I, Porter FD, Zimmerman AW, Tierney E. Smith-Lemli-Opitz syndrome and autism spectrum disorder. Am J Psychiatry 2007;164:1655–1661.

36. Kelley RI, Hannekam RCH, Smith-Lemli-Opitz syndrome. In: Scriver CR, Beaudet AL, Valle D, Sly WS, eds. The metabolic and molecular basis of inherited disease, 8th edit. New York: McGraw Hill, 2001:6183–6201.

37. Aneia A, Tierney E. Autism: the role of cholesterol in treatment. Int Rev Psychiatry 2008;20:165–170.

38. Herzog A. Hormonal abnormalities may be present in a subset of girls with ASD. Unpublished data, 2008.

39. Carr EG, Smith CE, Glacin TA, Whelan BM, Pancari J. Menstrual discomfort as a biological setting event for severe problem behaviors: assessment and intervention. Am J Mental Retard 2003;108: 117–133.

40. Burke LM, Kalpakjian CZ, Smith YR, Quint EH. Gynecological issues of adolescents with Down syndrome, autism, and cerebral palsy. J Pediatr Adolesc Gynecol 2010;23:11–15.

41. Hediger ML, England LJ, Molloy CA, Yu KF, Manning-Courtney P, Mills JL. Reduced bone cortical thickness in boys with autism or autism spectrum disorder. J Aut Dev Disord 2008;38:848–856.

42. Curtin C, Bandini LG, Perrin EC, Tyboi DJ, Must A. Prevalence of overweight in children and adolescents with attention deficit hyperactivity disorder and autism spectrum disorders: a chart review. BMC Pediatr 2005;5:48–58.

The Endocannabinoid System as it Relates to Autism
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The importance of the discovery and continued elucidation of the crucial role that the endocannabinoid system (ECS) plays in human health and disease cannot be understated. Cannabinoid receptors are the most highly expressed of any GPCR. They're the only ones to play a direct role in virtually every aspect of the human body (CNS and immune systems, throughout the periphery, presynaptic, and postsynaptic). (Alger 2013)

The growing body of data in regards to this aspect of physiology continues to lead to the further elucidation of the physiological basis in a growing number of diseases (including psychiatric) (Pacher 2006). One reason that this is important is because one such pathogenesis is for that of autism (ASD). There are a number of direct correlations between ASD and the ECS. Some will be outlined in this paper.

NL3 Mutations Inhibit Tonic Endocannabinoid Secretions

"Rare mutations in neuroligins and nerexins predispose to autism" (Földy 2013). Neuroligin-3 is the only known protein required for tonic secretion of endocannabinoids that include AEA and 2-AG (Földy 2013). Neuroligin-3 mutations have been shown to inhibit tonic endocannabinoid secretion (Földy 2013). These alterations in endocannabinoid signaling may contribute to autism pathophysiology (Földy 2013, Krueger 2013, Onaivi 2011, Siniscalco 2013). These finding have in part prompted researchers to apply to conduct research with nonhuman primates in order to further elucidate this link (Malcher-Lopes 2013).

Endocannabinoid system deficiencies are suggested to be involved in the pathophysiology of a growing number of diseases (Marco 2012, Russo 2003). Pacher and Pertwee both cover the endocannabinoid system in detail (Pacher 2006, Pertwee 2010). The number of functions that endocannabinoid signaling regulate in the human body is extensive and beyond the scope of this paper (Pertwee 2010). For sake of brevity only a few potentially relevant aspects will be listed:

- "Endocannabinoids are key modulators of synaptic function" (Castillo 2012).

- Tonic secretions of endocannabinoids regulate GI functions (including metabolism) (Di Marzo 2011, Li 2011).

- Endocannabinoids (and exogenous cannabinoids) suppress proliferation and cytokine release (Cencioni 2010).

- Endocannabinoids regulate stress responses, in part via the modulation of the 5-HT system (Haj-Dahmane 2011).

- CB2 is expressed in Purkinje cells (Gong 2006). "In the cerebellar cortex, CB1Rs regulate several forms of synaptic plasticity at synapses onto Purkinje cells, including presynaptically expressed short-term plasticity and, somewhat paradoxically, a postsynaptic form of long-term depression (LTD) (Carey 2011)."

- "CB1 variations modulate the striatal function that underlies the perception of signals of social reward, such as happy faces. This suggests that CB1 is a key element in the molecular architecture of perception of certain basic emotions. This may have implications for understanding neurodevelopmental conditions marked by atypical eye contact and facial emotion processing, such as ASC" (Chakrabarti 2011).

- Additional targets of endocannabinoids (and exogenous cannabinoids), PPARα, PPARγ, and GPR55 expression levels have shown reductions in a valproic acid model of autism in rats (Kerr 2013).

- Endocannabinoids and CB1 agonists increase cerebrocortical blood flow (Iring 2013).

- "The expression patterns in malformations of cortical development highlight the role of cannabinoid receptors as mediators of the endocannabinoid signaling and as potential pharmacological targets to modulate neuronal and glial cell function in epileptogenic developmental pathologies" (Zurolo 2010).

- The endocannabinoid signalosome is "a molecular substrate for fragile X syndrome, which might be targeted for therapy" (Jung 2012).

Exogenous cannabinoids from cannabis display similar pharmacological characteristics to that of endogenous cannabinoids (Pertwee 2010). The potential therapeutic value of systemic administration of phytocannabinoids has been suggested in the treatment of a number of diseases with suspected underlying endocannabinoid deficiencies (Russo 2003). Documentation of their safety and clinical efficacy in a variety of treatments continues to grow (Hazekamp 2013). Some similar characteristics include:

- Neurogenesis (Galve-Roperh 2007, Jiang 2005, Avraham 2014, Campos 2013)
- Neuroprotection (Hampson 2003, Lara-Celador 2013, Sanchez 2012)
- Antioxidants (Borges 2013, Pertwee 2010, Hampson 1998, Hampson 2003)
- Neuromodulation (Davis 2007, Lara-Celador 2013, Pertwee 2010, Youssef 2012)
- Anti-inflammatory (Pertwee 2010, Izzo 2009, Nagarkatti 2009, Klein 2005)

Based on their relative safety, the similar pharmacological characteristics to endocannabinoids that are inhibited in ASD, and the significant role those endogenous cannabinoids play in human health, it's possible that cannabinoids from cannabis could prove therapeutic value in treatments.

Increased Expression of CB2 Receptors Associated with ASD

The second direct link, of possibly equal or greater relevance for treatment, is the upregulation of CB2 receptors in the brains of those with ASD (Siniscalco 2013). This is believed to be part of an endogenous neuroprotective role of the endocannabinoid system:

- "CB2 receptors have been identified in the healthy brain, mainly in glial elements and, to a lesser extent, in certain subpopulations of neurons, and that they are dramatically up-regulated in response to damaging stimuli, which supports the idea that the cannabinoid system behaves as an endogenous neuroprotective system. This CB_2 receptor up-regulation has been found in many neurodegenerative disorders including HD and PD, which supports the beneficial effects found for CB_2 receptor agonists in both disorders. In conclusion, the evidence reported so far supports that those cannabinoids having antioxidant properties and/or capability to activate CB_2 receptors may represent promising therapeutic agents" (Fernández 2011).

- CB2 "expression is increased by inflammatory stimuli suggests that they may be involved in the pathogenesis and/or in the endogenous response to injury… receptors may be part of the general neuroprotective action of the ECS by decreasing glial reactivity. Neuropathological findings in human brains suggest that the upregulation of CB_2 receptors is a common pattern of response against different types of chronic injury of the human CNS. In addition, their selective presence in microglial cells is highly suggestive of an important role in disease-associated neuroinflammatory processes. The anti-inflammatory effects triggered by the activation of the CB_2 receptor make it an attractive target for the development of novel anti-inflammatory therapies" (Benito 2008).

Given that CB2 is upregulated, and that it's believed to play a neuroprotective role in the human brain, CB2 activation is believed to be a potential target for treatment of ASD (Siniscalco 2013). Endocannabinoids (AEA, 2-AG) and the most prominent cannabinoids in cannabis (including THC) are CB2 agonists (Izzo 2009).

Elevated Cytokine Levels Associated with ASD

Elevated cytokine levels are associated with ASD (Napolioni 2013). Whether this is a direct result of inhibited tonic secretion of endocannabinoids remains uncertain. However, endocannabinoids (AEA, 2-AG) have been shown to play key roles inhibiting cytokines via CB2 activation (Cencioni 2010, Panikashvili 2006). "Both THC and CBD have been shown to decrease cytokine production" via CB1/CB2 dependent and independent mechanisms (Juknat 2012, Kozela 2010). The majority of cannabinoids are PPAR gamma agonists (Izzo 2009), which have been shown to inhibit cytokine production (Jiang 1998).

Clinically Diagnosing ASD

A team of researchers recently discovered and patented a process that claims that it's possible to clinically diagnose ASD, and susceptibility to it, via observation of the degree of modulation that acetaminophen has on endocannabinoid levels (Schultz 2012).

Botanical Extracts > Dronabinol

Of equal relevance to this issue is the substantial data, including clinical studies, suggesting that the combined administration of CBD along with THC (and possibly other cannabinoids/terpenes present in cannabis) exhibit additive and synergistic effects resulting in greater clinical efficacies when compared to either cannabinoid alone (McPartland 2001, Izzo 2009, Russo 2011). The second most prominent cannabinoid in cannabis is CBD (Gertch 2010). CBD has been shown to inhibit intoxication, sedation, and tachycardia associated with THC (Russo 2006). It's been shown to increase the clinical efficacy of THC, while adding therapeutic value in its own right (Russo 2006).

- "CBD is demonstrated to antagonize some undesirable effects of THC including intoxication, sedation and tachycardia, while contributing analgesic, anti-emetic, and anti-carcinogenic properties in its own right. In modern clinical trials, this has permitted the administration of higher doses of THC, providing evidence for clinical efficacy and safety for cannabis based extracts in treatment of spasticity, central pain and lower urinary tract symptoms in multiple sclerosis, as well as sleep disturbances, peripheral neuropathic pain, brachial plexus avulsion symptoms, rheumatoid arthritis and intractable cancer pain. Prospects for future application of whole cannabis extracts in neuroprotection, drug dependency, and neoplastic disorders are further examined. The hypothesis that the combination of THC and CBD increases clinical efficacy while reducing adverse events is supported" (Russo 2006).

- "Several studies suggest that CBD is non-toxic in non-transformed cells and does not induce changes on food intake, does not induce catalepsy, does not affect physiological parameters (heart rate, blood pressure and body temperature), does not affect gastrointestinal transit and does not alter psychomotor or psychological functions. Also, chronic use and high doses up to 1,500 mg/day of CBD are reportedly well tolerated in humans" (Machado 2011).

An argument could be made that botanical extracts with CBD present offer safer options for patients, with greater clinical efficacy, when compared to THC (Dronabinol) alone (Russo 2006). CBD offers more than simply increasing the safety and efficacy of THC (Izzo 2009).

- "CBD has been shown to have an inhibitory effect on the inactivation of endocannabinoids (i.e. inhibition of FAAH enzyme), thereby enhancing the action

of these endogenous molecules on cannabinoid receptors, which is also noted in certain pathological conditions. CBD acts not only through the endocannabinoid system, but also causes direct or indirect activation of metabotropic receptors for serotonin or adenosine, and can target nuclear receptors of the PPAR family and also ion channels" (Campos 2012).

Here are some of the demonstrated pharmacological characteristics of CBD that may be relevant:

- CB1/CB2 agonist blocker (can inhibit overstimulation of CB1 by THC)
- FAAH inhibition increases endocannabinoid levels (including AEA, 2-AG)
- AEA reuptake inhibitor
- 5-HT1a agonist
- Suppressor of tryptophan degradation
- PPAR alpha and gamma agonist
- Positive allosteric modulator at glycine receptors
- TRPV1 and TRPV2 agonist
- Adenosine uptake competitive inhibitor
- Antagonist at abnormal-CBD receptor
- Regulator of intracellular Ca 2+
- T-type Ca 2+ channel inhibitor   (Izzo 2009)

If we accept that tonic secretions of AEA and 2-AG are inhibited via NL3 mutations in ASD (both of which being CB1 and CB2 agonists), then it might be possible to suppose the potential benefits of low doses of THC in treatments as well. This seems especially true when the striking pharmacological similarities between THC and AEA are reviewed (Pertwee 2010). The majority of the research conducted thus far with ASD and cannabinoids has been with THC alone. Dronobinal has indicated potential in a single adolescent case study of autism (Kurz 2010). This might suggest that THC along with CBD might offer increased clinical efficacy (Russo 2006).

Treating Symptoms Associated with ASD

A considerably greater body of data can be gathered in regards to aspects of the involvement (and targeting for treatment) of the endocannabinoid system in a number of the symptoms, and diseases, associated with ASD (in comparison to the pathophysiology of ASD itself):

- G.I. Disorders (Camilleri 2013, Di Sabatino 2011, Wright 2008,)
- Repetitive Behaviors (Casarotto 2010, Deiana 2012, Gomes 2011, Kinsey 2011)
- Seizures (Jones 2012, Porter 2013, van Rijn 2011)
- Sleep Dysfunction (Murillo-Rodriguez 2011, Ware 2010)
- Self Injurious Behavior and Tantrums (Müller-Vahl 2004, Onaivi 2011, Passie 2012)
- Tuberous Sclerosis (Krueger 2013, Shu, Hai-Feng 2013, Zurolo 2010)

- Cerebral Ischemia (Schmidt 2012, Choi 2013, Murikinati 2010, Garcia-Bonilla 2014)
- Depression/Anxiety (Hill 2009, Almeida 2013, Campos 2013, Schier 2012)
- Cachexia (Engeli 2012, Gamage 2012, Marco 2012)

Conclusion

Given the known role of the endocannabinoid system in ASD it seems entirely possible, if not likely, that cannabinoid rich botanical extracts from cannabis can be utilized as useful agents targeting the pathophysiology of ASD, as well as the many debilitating symptoms and conditions associated with it. We believe that families and physicians should have the legal right to explore these options on an individual basis without fear of prosecution.

Works Cited

1. Alger, Bradley. "Getting High on the Endocannabinoid System." *Cerebrum* (2013).

2. Almeida, Valeria, et al. "Cannabidiol exhibits anxiolytic but not antipsychotic property evaluated in the social interaction test." Progress in Neuro-Psychopharmacology and Biological Psychiatry 41 (2013): 30-35.

3. Aso, Ester, et al. "Lack of CB1 receptor activity impairs serotonergic negative feedback." *Journal of neurochemistry* 109.3 (2009): 935-944.

4. Benito, C., et al. "Cannabinoid CB2 receptors in human brain inflammation." *British journal of pharmacology* 153.2 (2008): 277-285.

5. Best, Aaron R., and Wade G. Regehr. "Serotonin evokes endocannabinoid release and retrogradely suppresses excitatory synapses." *The Journal of Neuroscience* 28.25 (2008): 6508-6515.

6. Bolognini, D., et al. "Cannabidiolic acid prevents vomiting in Suncus murinus and nausea-induced behaviour in rats by enhancing 5-HT1A receptor activation." *British journal of pharmacology* 168.6 (2013): 1456-1470.

7. Booz, George W. "Cannabidiol as an emergent therapeutic strategy for lessening the impact of inflammation on oxidative stress." *Free Radical Biology and Medicine* 51.5 (2011): 1054-1061.

8. Camilleri, Michael, et al. "Cannabinoid receptor 1 gene and irritable bowel syndrome: phenotype and quantitative traits." American Journal of Physiology-Gastrointestinal and Liver Physiology 304.5 (2013): G553-G560.

9. Campos, Alline Cristina, et al. "Multiple mechanisms involved in the large-spectrum therapeutic potential of cannabidiol in psychiatric disorders." *Philosophical Transactions of the Royal Society B: Biological Sciences* 367.1607 (2012): 3364-3378.

10. Carey, Megan R., et al. "Presynaptic CB1 receptors regulate synaptic plasticity at cerebellar parallel fiber synapses." *Journal of neurophysiology* 105.2 (2011): 958.

11. Castillo, Pablo E., et al. "Endocannabinoid signaling and synaptic function." *Neuron* 76.1 (2012): 70-81.

12. Cencioni, Maria Teresa, et al. "Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors." *PLoS One* 5.1 (2010): e8688.

13. Chakrabarti, Bhismadev, and Simon Baron-Cohen. "Variation in the human Cannabinoid Receptor (CNR1) gene modulates gaze duration for happy faces." *Molecular autism* 2.1 (2011): 10.

14. Deiana, Serena, et al. "Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Δ9-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive–compulsive behaviour." Psychopharmacology 219.3 (2012): 859-873.

15. Di Filippo, Clara, et al. "Cannabinoid CB2 receptor activation reduces mouse myocardial ischemia-reperfusion injury: involvement of cytokine/chemokines and PMN." *Journal of leukocyte biology* 75.3 (2004): 453-459.

16. Di Marzo, V., and F. Piscitelli. "Gut feelings about the endocannabinoid system." *Neurogastroenterology & Motility* 23.5 (2011): 391-398.

17. Di Sabatino, A., et al. "The endogenous cannabinoid system in the gut of patients with inflammatory bowel disease." Mucosal immunology 4.5 (2011): 574-583.

18. Engeli, Stefan. "Central and peripheral cannabinoid receptors as therapeutic targets in the control of food intake and body weight." Appetite Control. Springer Berlin Heidelberg, 2012. 357-381.

19. Fernández-Ruiz, Javier, et al. "Prospects for cannabinoid therapies in basal ganglia disorders." *British journal of pharmacology* 163.7 (2011): 1365-1378.

20. Földy, Csaba, Robert C. Malenka, and Thomas C. Südhof. "Autism-associated neuroligin-3 mutations commonly disrupt tonic endocannabinoid signaling." *Neuron* 78.3 (2013): 498-509.

21. Gamage, Thomas F., and Aron H. Lichtman. "The endocannabinoid system: role in energy regulation." Pediatric blood & cancer 58.1 (2012): 144-148.

22. Garcia-Bonilla, Lidia, et al. "Immune mechanisms in cerebral ischemic tolerance." Frontiers in neuroscience 8 (2014).

23. Gertsch, Jürg, Roger G. Pertwee, and Vincenzo Di Marzo. "Phytocannabinoids beyond the Cannabis plant–do they exist?." *British journal of pharmacology* 160.3 (2010): 523-529.

24. Godlewski, Grzegorz, Manfred Göthert, and Barbara Malinowska. "Cannabinoid receptor-independent inhibition by cannabinoid agonists of the peripheral 5-HT3 receptor-mediated von Bezold–Jarisch reflex." *British journal of pharmacology* 138.5 (2003): 767-774.

25. Gomes, Felipe V., et al. "Facilitation of CB1 receptor-mediated neurotransmission decreases marble burying behavior in mice." Progress in Neuro-Psychopharmacology and Biological Psychiatry 35.2 (2011): 434-438.

26. Gong, Jian-Ping, et al. "Cannabinoid CB2 receptors: immunohistochemical localization in rat brain." *Brain research* 1071.1 (2006): 10-23.

27. Haj-Dahmane, Samir, and Roh-Yu Shen. "Modulation of the serotonin system by endocannabinoid signaling." *Neuropharmacology* 61.3 (2011): 414-420.

28. Hazekamp, Arno, and Franjo Grotenhermen. "Clinical Studies With Cannabis and Cannabinoids, 2005-2009." (2013).

29. Siniscalco, Dario, et al. "Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders." *Journal of autism and developmental disorders* 43.11 (2013): 2686-2695.

30. Iring, András, et al. "Role of Endocannabinoids and Cannabinoid-1 Receptors in Cerebrocortical Blood Flow Regulation." *PloS one* 8.1 (2013): e53390.

31. Izzo, Angelo A., et al. "Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb." *Trends in pharmacological sciences* 30.10 (2009): 515-527.

32. Jean-Gilles, Lucie, Bruno Gran, and Cris S. Constantinescu. "Interaction between cytokines, cannabinoids and the nervous system." Immunobiology 215.8 (2010): 606-610.

33. Jiang, Chengyu, Adrian T. Ting, and Brian Seed. "PPAR-γ agonists inhibit production of monocyte inflammatory cytokines." *Nature* 391.6662 (1998): 82-86.

34. Johnson, Jeremy R., et al. "Multicenter, double-blind, randomized, placebo-controlled, parallel-group study of the efficacy, safety, and tolerability of THC: CBD extract and THC extract in patients with intractable cancer-related pain." *Journal of pain and symptom management* 39.2 (2010): 167-179.

35. Jones, Nicholas A., et al. "Cannabidiol exerts anti-convulsant effects in animal models of temporal lobe and partial seizures." Seizure 21.5 (2012): 344-352.

36. Juknat, Ana, et al. "Cannabidiol affects the expression of genes involved in zinc homeostasis in BV-2 microglial cells." *Neurochemistry international* 61.6 (2012): 923-930.

37. Jung, Kwang-Mook, et al. "Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome." *Nature communications* 3 (2012): 1080.

38. Kawamura, Yoshinobu, et al. "The CB1 cannabinoid receptor is the major cannabinoid receptor at excitatory presynaptic sites in the hippocampus and cerebellum." *The Journal of neuroscience* 26.11 (2006): 2991-3001.

39. Kerr, D. M., et al. "Alterations in the endocannabinoid system in the rat valproic acid model of autism." *Behavioural brain research* 249 (2013): 124-132.

40. Kinsey, Steven G., et al. "Inhibition of endocannabinoid catabolic enzymes elicits anxiolytic-like effects in the marble burying assay." *Pharmacology Biochemistry and Behavior* 98.1 (2011): 21-27.

41. Kishimoto, Yasushi, and Masanobu Kano. "Endogenous cannabinoid signaling through the CB1 receptor is essential for cerebellum-dependent discrete motor learning." *The Journal of neuroscience* 26.34 (2006): 8829-8837.

42. Klegeris, Andis, Christopher J. Bissonnette, and Patrick L. McGeer. "Reduction of human monocytic cell neurotoxicity and cytokine secretion by ligands of the cannabinoid-type CB2 receptor." *British journal of pharmacology* 139.4 (2003): 775-786.

43. Kozela, Ewa, et al. "Cannabinoids Δ9-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-β/STAT proinflammatory pathways in BV-2 microglial cells." *Journal of biological chemistry* 285.3 (2010): 1616-1626.

44. Krueger, Dilja D., and Nils Brose. "Evidence for a common endocannabinoid-related pathomechanism in autism spectrum disorders." Neuron 78.3 (2013): 408-410.

45. Kurz, René, and Kurt Blaas. "Use of dronabinol (delta-9-THC) in autism: A prospective single-case-study with an early infantile autistic child." (2010)

46. Machado Bergamaschi, Mateus, et al. "Safety and side effects of cannabidiol, a Cannabis sativa constituent." *Current drug safety* 6.4 (2011): 237-249.

47. Malcher-Lopes, Renato. "Targeting alterations in the endocannabinoid system of rodents and non-human primates for the study of autism." *Qatar Foundation Annual Research Conference*. No. 2013. 2013.

48. Marco, Eva M., et al. "Endocannabinoid system and psychiatry: in search of a neurobiological basis for detrimental and potential therapeutic effects." *Frontiers*

in behavioral neuroscience 5 (2011).

49. Mato, Susana, et al. "CB1 knockout mice display impaired functionality of 5-HT1A and 5-HT2A/C receptors." *Journal of neurochemistry* 103.5 (2007): 2111-2120.

50. McPartland, John M., and Ethan B. Russo. "Cannabis and cannabis extracts: greater than the sum of their parts?." *Journal of Cannabis Therapeutics* 1.3-4 (2001): 103-132.

51. Mikics, Eva, et al. "Interactions between the anxiogenic effects of CB1 gene disruption and 5-HT3 neurotransmission." *Behavioural pharmacology* 20.3 (2009): 265-272.

52. Müller-Vahl, K. R., et al. "Cannabinoids: possible role in patho-physiology and therapy of Gilles de la Tourette syndrome." Acta Psychiatrica Scandinavica 98.6 (1998): 502-506.

53. Murillo-Rodriguez, Eric, et al. "The emerging role of the endocannabinoid system in the sleep-wake cycle modulation." Central Nervous System Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Central Nervous System Agents) 11.3 (2011): 189-196.

54. Murikinati, Sasidhar, et al. "Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment." The FASEB journal 24.3 (2010): 788-798.

55. Napolioni, Valerio, et al. "Plasma cytokine profiling in sibling pairs discordant for autism spectrum disorder." *Journal of neuroinflammation* 10.1 (2013): 38.

56. Onaivi, E. S., et al. "Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders." Current neuropharmacology 9.1 (2011): 209.

57. Pacher, Pál, Sándor Bátkai, and George Kunos. "The endocannabinoid system as an emerging target of pharmacotherapy." *Pharmacological reviews* 58.3 (2006): 389-462.

58. Palazuelos, Javier, et al. "CB2 cannabinoid receptors promote neural progenitor cell proliferation via mTORC1 signaling." *Journal of Biological Chemistry* 287.2 (2012): 1198-1209.

59. Panikashvili, David, et al. "The endocannabinoid 2-AG protects the blood–brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines." *Neurobiology of disease* 22.2 (2006): 257-264.

60. Pertwee, R. G., et al. "International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2." *Pharmacological reviews* 62.4 (2010): 588-631.

61. Porter, Brenda E., and Catherine Jacobson. "Report of a parent survey of cannabidiol-enriched cannabis use in pediatric treatment-resistant epilepsy." *Epilepsy & Behavior* 29.3 (2013): 574-577.

62. Onaivi, E. S., et al. "Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders." Current neuropharmacology 9.1 (2011): 209.

63. Rock, Erin. *Cannabidiol Indirectly Activates 5-HT1A Somatodendritic Autoreceptors to Attenuate Vomiting and Nausea*. Diss. 2011.

64. Roloff, Alan M., et al. "Homer 1a gates the induction mechanism for endocannabinoid-mediated synaptic plasticity." *The Journal of Neuroscience* 30.8 (2010): 3072-3081.

65. Russo, Ethan B. "Clinical endocannabinoid deficiency (CECD): can this concept explain therapeutic benefits of cannabis in migraine, fibromyalgia, irritable bowel syndrome and other treatment-resistant conditions?." *Neuro endocrinology letters* 25.1-2 (2003): 31-39.

66. Russo, Ethan, and Geoffrey W. Guy. "A tale of two cannabinoids: the therapeutic rationale for combining tetrahydrocannabinol and cannabidiol." *Medical hypotheses* 66.2 (2006): 234-246.

67. Russo, Ethan B. "Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects." *British Journal of Pharmacology* 163.7 (2011): 1344-1364.

68. Schier, Alexandre Rafael de Mello, et al. "Cannabidiol, a Cannabis sativa constituent, as an anxiolytic drug." Revista Brasileira de Psiquiatria 34 (2012): 104-110.

69. Stone, Joe, et al. "Cannabinoids, Ketogenic Diets, Holy Basil, and the PPAR Connection." Unpublished (2014) http://www.scribd.com/doc/207827158/Cannabinoids-Ketogenic-Diets-Holy-Basil-and-the-PPAR-Connection

70. Vaney, C., et al. "Efficacy, safety and tolerability of an orally administered cannabis extract in the treatment of spasticity in patients with multiple sclerosis: a randomized, double-blind, placebo-controlled, crossover study." *Multiple Sclerosis* 10.4 (2004): 417-424.

71. van Rijn, Clementina M., et al. "Endocannabinoid system protects against cryptogenic seizures." Pharmacol Rep 63 (2011): 165-168.

72. Ware, Mark A., et al. "The effects of nabilone on sleep in fibromyalgia: results of a randomized controlled trial." Anesthesia & Analgesia 110.2 (2010): 604-610.

73. Wright, K. L., M. Duncan, and K. A. Sharkey. "Cannabinoid CB2 receptors in the gastrointestinal tract: a regulatory system in states of inflammation." British journal of pharmacology 153.2 (2008): 263-270.

74. Youssef, F. F., and A. J. Irving. "From cannabis to the endocannabinoid system: refocussing attention on potential clinical benefits." West Indian Medical Journal 61.3 (2012).

75. Zurolo, E., et al. "CB1 and CB2 cannabinoid receptor expression during development and in epileptogenic developmental pathologies." *Neuroscience* 170.1 (2010): 28-41.

Role of Endocannabinoids on Neuroinflammation in Autism Spectrum Disorder Prevention

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ABSTRACT

Autism Spectrum Disorder (ASD) disease has become a mounting socio-economical alarm around the world. Neuroinflammtion had been shown in postmortem brain specimens from ASD patients. The Endocannabinoids System (ES) consists of a family of locally produced, short-lived, endogenous, phospholipid-derived agonists (endocannabinoids) that control energy balance and body composition. The growing number of medical benefits of ES, such as their ability to regulate processes like neuroinflammation, neurogenesis and memory, raise the question of their potential role as a preventive treatment of ASD.

To test this hypothesis, basic and clinical studies allow us a thorough investigation of the role of ES in the pathogenesis of ASD. This hypothesis will help to understand the mechanism of ES and its role in ASD.

INTRODUCTION

ASD is a severe neurodevelopmental disorder characterized by repetitive, stereotyped behavior, impai-rment in communication and social interaction in children [1].

The number of cases in the Arab world ranges from 1.4 to 29 per 10000 [2]. While these rates are much lower than those found in the literature, it does not mean the condition is less prevalent in the Arab world, because many cases may remain undiagnosed. In fact, evidence does not support differences in ASD prevalence by geographic region nor of a strong impact of ethnic/cultural factors [1]. Factors that might contribute to an apparent lower prevalence of autism in Arab countries include low awareness of ASD in professionals (e.g., paediatricians) and in the general population (e.g., parents, teachers), few child neuro-psychiatrists in general and fewer specialized in ASD.

Although the diagnosis is made during first three years of age, however the onset of disease can be from birth or usually during the second year [3]. The aetiology of ASD is not fully understood but there is consensus that genetics, environmental and autoimmunity factors interact to play a role in its development [4]. Neuroinflammtion in ASD has attracted the attention of research in targeting the neuroprotective mechanisms [5]. The growing number of medical benefits of Endocannabinoid System (ES), such as their ability to regulate neuroinflammation, neurogenesis and memory, raise the question of their potential role as a preventive treatment of ASD. This hypothesis paper highlights the basic and clinical studies that allow us a thorough investigation of the role of ES in the pathogenesis of ASD [6].

Neuroinflammtion in ASD

Neuroinflammtion is an important aspect in ASD and had been shown in postmortem brain specimens from ASD patients of different age (4–45 years of age) groups [5]. Microglia, resident mononuclear cells of the CNS, takes part in immune surveillance and synaptic pruning [7]. Studies have shown prominent microglia activation and increased inflammatory cytokine and chemokine production, including IFN-γ, IL-1β, IL-6, IL-12p40, TNF-α (tumor necrosis factor α) and chemokine CCL-2 in the brain tissue and cerebral spinal fluid [8-10].

Expression profiling analysis of postmortem brain tissues from ASD patients revealed increased levels of mRNA transcripts of several immune-related genes [11]. In addition to neuroinflammation, there are alterations in the systemic immune responses that are associated with disease severity. For example, different proinflammatory cytokines such as, IL-1β, IL-6, IL-8, IL-12p40, and chemokines CCL2, CCL5, are elevated in ASD and are associated with poor communication and social interaction [12-14]. These findings indicate a trend towards neural and systemic proinflammatory status of the immune system in ASD.

Endocannabinoid system as neuro protective mechanisms

Central nervous system (CNS) has inherent mechanisms of neural cells protection. Activation of ES may be one of those mechanisms which are involved in protecting the negative effects of inflammation, because of its anti-inflammatory effects on CNS [15]. The ES is a novel system of intracellular signalling and consists of two main cannabinoid (CB) receptors type 1 and type 2 (CB1 and CB2). Initially identified in mouse spleen cells, CB1 receptors are located in the CNS, peripheral nervous system, and peripheral organs. CB1 receptors are also found in adipocytes, liver, pancreas and skeletal muscle. CB2 receptors are expressed on immune cells such as microglia (resident mononuclear cells of the immune system in CNS) and neurons. In the CNS, CB1 receptors are concentrated in the cerebellum, hippocampus, and the basal ganglia [16], which are areas in the brain implicated as dysfunctional in autism [17,18].

Linking endocannabinoid system, neuroinflammation and neurodegeneration

Although ES and the immune system are autonomous, however both systems communicate with each other anatomically and physiologically through autonomic nervous system via innervations of lymph nodes, spleen, bone marrow, thymus, liver and gastrointestinal tract. The two systems communicate through chemical messengers, which range from smaller molecules, including nitric oxide, to larger proteins, such as cytokines. Inflammation is one of the example which shows an intimate interaction between the two system acting in a complex manner to generate appropriate adaptive cellular responses [19]. Neuronal wiring of the ANS (sympathetic and parasympathetic) to the main sites of immune system such as thymus, liver, spleen and bone marrow, skin and lymph nodes modulates the immune system. Second, small molecules such as

Multisite Impact of the Endocannabinoid System

Site of Action	Mechanism(s)	Effects	Receptors
Hypothalamus Nuc Accumbens	↑ Food Intake	Body Weight Waist Circumference	**CB 1 Receptors** Cortex, Hippocampus, Basal ganglia, Hypothalamus, Cerebellum, Spinal Cord, Dorsal Root Ganglia, Enteric Nervous System, Adipocytes, GIT, Endothelial Cells, Liver
Adipose Tissue	↓ Adiponectin ↑ Lipogenesis	Visceral fat Dyslipidemia Insulin Resistance	
Muscle	↓ Glucose Uptake	Insulin Resistance	
Liver	↑ Lipogenesis	Dyslipidemia Insulin Resistance	
GI Tract	↓ Satiety	Body Weight Waist Circumference	
Immune System	↓ Inflammtion	Neuroprotection	**CB 2 Receptors** Immune Cells & Tissues, T Cells, B Cells, Spleen, Tonsils, Activated microglial cells

* + ➡ +   +?*

Neuroinflammation in Autism

[Table/Fig-1]: Schematic representation of multisite actions of endocannabinoid system.
*Potential therapeutic target to treat neuroinflammation in autism through selective CB2 receptor agonists.

nitric oxide and large like cytokines and growth factors and their respective receptors connect these two systems. Thus, these two important entities of the body are interlinked physiologically and anatomically to mount an effective response when required [6].

Potential pro-inflammatory role of cannabinoids have also been reported in a few studies, indicating involvement of complex mechanisms that need to be unraveled [20,21].

It is well known that pro-inflammatory cytokines such as IL-6, IL-12, IL-1β, TNF-α released by immune cells in the CNS adds to the development of neuroinflammation and neurodegeneration [22]. Activation of ES is one the mechanism in the CNS, which protect from the detrimental effects of these cytokines. During inflammation, endogenous endocannabinoids such as Arachidonyl Ethanolamide (AEA) and 2-arachidonyl glycerol (2-AG) are released from different immune cells and neurons in the CNS. These endocannabinoid ligands (AEA, 2-AG) bind to endocannabinoids receptors and induce neuroprotection through different mechanisms [23].

Thus, it is possible that participation of ES in the regulation of immune responses is associated with therapeutic effects mediated by the down regulation of cytokine expression.

Regardless of the challenges in targeting ES receptors that will potentially disrupt the processes of learning and memory, approaches for neuroprotection have been taken to avoid those side effects by targeting specifically the ES CB2 receptors [Table/Fig-1], by modulation of the degradation pathway of ES, or by using low, non-psychoactive doses of non-selective agonists of CB1/CB2 endocannabinoid receptors [24].

It has been shown that mRNA and protein levels of CB2 receptor were up-regulated in the blood, peripheral blood mononuclear cells, of children with autistic disorder [25], suggesting the involvement of the endocannabonoid system in the development of autism.

The ES plays an important role in the development of the central nervous system and its activation can induce long-lasting functional adaptations [26]. Use of cannabis (an exogenous cannabinoid) in the still-maturing brain may produce persistent alterations in brain structure and cognition [27]. Due to poor efficacy of current treatments and the likely delay to implement future safe and efficacious treatments, there is an opportunity to develop preventive approaches based on currently available knowledge regarding the pathogenesis and risk factors of ASD. It has been found that endocannabinoids have anti-inflammatory and immuno-suppressive effects and can act as potential therapies [28]. Different pharmacological agents providing anti-inflammatory and neuroprotective effects in the diseases of CNS can modulate Endocannabinoid receptors CB1, CB2 and other EC receptors which are not fully defined. For example, in experimental models of AD, stimulation of CB1, CB2 and other non-CB1 and non-CB2 receptors with cannabinoids reduced microglial activation and microglia-mediated neurodegeneration [29]. It has been shown that increase in endogenous levels of endocannabinids has protective effect against β-amyloid-induced neurotoxicity [30]. In experimental models of multiple sclerosis, stimulation of CBI and CB2 receptors have anti-inflammatory effects in response to pharmacological AMT inhibitors. AMT is a purported high-affinity transporter which is responsible for removal of anandamide and CB receptors and AMT inhibitors like AM404, VDM11 and UCM707 block cellular

uptake of anandamide and CB receptors [31,32]. This therapeutic use of drugs targeting EC system in CNS disorders has a great potential and animal experiments have shown encouraging results in reducing clinical symptoms in degenerative and inflammatory disease conditions. For example AMT inhibitors such as AM404 and VDM11 showed anti-cytotoxic effects in experimental model of Parkinson's disease. Similarly administration of AM404 and VD11 also reduced the spasticity in mice suffering from chronic relapsing experimental allergic encephalomyelitis, a model of MS [33].

Testing the hypothesis

Hence, ES modulators can be explored in autism animal models with well-established face and predictive validities which could serve as a translational link between laboratory findings from animal's studies leading to exploratory studies in humans. Thus, we postulate that modulation of the ES in ASD could prove a valuable tool to prevent or delay the progression of disease [Table/Fig-1].

Implications of the hypothesis

The complex nature of ASD advocates a multimodal drug approach that could protect from the various processes underlying neurodegeneration and thus, at minimum, delay the pathological process. The expected benefit from a chronic treatment aimed at stimulating the endocannabinoid system is a delayed progression of ASD: i.e., reduced inflammation, sustained potential for neurogenesis, and delayed memory impairment. Such results could lead to new therapeutic strategies that target the inflammation and the decline in neurogenesis associated ASD.

AUTHORS' CONTRIBUTIONS

SH, MI, KA and SB participated to the conception of the present hypothesis. All authors drafted and approved the final manuscript.

REFERENCES

[1] Elsabbagh M, Divan G, Koh YJ, Kim YS, Kauchali S, Marcin C, et al. Global prevalence of autism and other pervasive developmental disorders. Autism Res. 2012;5(3):160-79.

[2] Al-Salehi SM, Al-Hifthy EH, Ghaziuddin M. Autism in Saudi Arabia: presentation, clinical correlates and comorbidity. Transcult Psychiatry. 2009;46(2):340-47.

[3] Ozonoff S, Iosif AM, Baguio F, Cook IC, Hill MM, Hutman T, et al. A prospective study of the emergence of early behavioral signs of autism. J Am Acad Child Adolesc Psychiatry. 2010;49(3):256-66.e1-2.

[4] Careaga M, Van de Water J, Ashwood P. Immune dysfunction in autism: a pathway to treatment. Neurotherapeutics. 2010;7(3):283-92.

[5] Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA. Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol. 2005;57(1):67-81.

[6] Ziemssen T, Kern S. Psychoneuroimmunology – cross-talk between the immune and nervous systems. J Neurol. 2007;254:8–11.

[7] Bessis A, Béchade C, Bernard D, Roumier A. Microglial control of neuronal death and synaptic properties. Glia. 2007;55(3):233-38.

[8] Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, Li XM, et al. Elevated immune response in the brain of autistic patients. J Neuroimmunol. 2009;207(1-2):111-16.

[9] Morgan JT, Barger N, Amaral DG, Schumann CM. Stereological study of amygdala glial populations in adolescents and adults with autism spectrum disorder. PLoS ONE. 2014;9(10):e110356.

[10] Morgan JT, Chana G, Pardo CA, Achim C, Semendeferi K, Buckwalter J, et al. Microglial activation and increased microglial density observed in the dorsolateral prefrontal cortex in autism. Biol Psychiatry. 2010;68(4):368-76.

[11] Garbett K, Ebert PJ, Mitchell A, Lintas C, Manzi B, Mirnics K, et al. Immune transcriptome alterations in the temporal cortex of subjects with autism. Neurobiol Dis. 2008;30:303–11.

[12] Mead J, Ashwood P. Evidence supporting an altered immune response in ASD. Immunol Lett. 2015;163(1):49-55.

[13] Xu N, Li X, Zhong Y. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. Mediators Inflamm. 2015;2015:531518.

[14] Careaga M, Ashwood P. Autism spectrum disorders: from immunity to behavior. Methods Mol Biol. 2012;934:219-40.

[15] Micale V, Mazzola C, Drago F. Endocannabinoids and neurodegenerative diseases. Pharmacol Res. 2007;56:382–92.

[16] Drysdale AJ, Platt B. Cannabinoids: mechanisms and therapeutic applications in the CNS. Curr Med Chem. 2003;10(24):2719-32.

[17] Bauman ML, Kemper TL. Neuroanatomic observations of the brain in autism: a review and future directions. Int J Dev Neurosci. 2005;23(2-3):183-87.

[18] Courchesne E, Pierce K, Schumann CM, Redcay E, Buckwalter JA, Kennedy DP, et al. Mapping early brain development in autism. Neuron. 2007;56(2):399-413.

[19] Steinman L. Elaborate interactions between the immune and nervous systems. Nat Immunol. 2004;5:575–81.

[20] Killestein J, Hoogervorst EL, Reif M, Blauw B, Smits M, Uitdehaag BM, et al. Immunomodulatory effects of orally dministered cannabinoids in multiple sclerosis. J Neuroimmunol. 2003;137(1-2):140-43.

[21] Maestroni GJ. The endogenous cannabinoid 2-arachidonoyl glycerol as in vivo chemoattractant for dendritic cells and adjuvant for Th1 response to a soluble protein. FASEB J. 2004;18(15):1914-16.

[22] Wong ML, Sternberg EM. Immunological assays for understanding neuroimmune interactions. Arch Neurol. 2000;57:948–52.

[23] Jean-Gilles L, Gran B, Constantinescu CS. Interaction between cytokines, cannabinoids and the nervous system. Immunobiology. 2010;215(8):606-10.

[24] Centonze D, Finazzi-Agrò A, Bernardi G, Maccarrone M.The endocannabinoid system in targeting inflammatory neurodegenerative diseases. Trends Pharmacol Sci. 2007;28(4):180-87.

[25] Siniscalco D, Sapone A, Giordano C, Cirillo A, de Magistris L, Rossi F, et al. Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders. J Autism Dev Disord. 2013;43:2686–95.

[26] Campolongo P, Trezza V, Palmery M, Trabace L, Cuomo V. Developmental exposure to cannabinoids causes subtle and enduring neurofunctional alterations. Int Rev Neurobiol. 2009;85:117-33.

[27] Jager G, Ramsey NF. Long-term consequences of adolescent cannabis exposure on the development of cognition, brain structure and function: an overview of animal and human research. Curr Drug Abuse Rev. 2008;1(2):114-23.

[28] Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. Future Med Chem. 2009;1(7):1333-49.

[29] Ramírez BG, Blázquez C, Gómez del Pulgar T, Guzmán M, de Ceballos ML. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. J Neurosci. 2005;25(8):1904-13.

[30] Van der Stelt M, Mazzola C, Esposito G, Matias I, Petrosino S, De Filippis D, et al. Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. Cell Mol Life Sci. 2006;63(12):1410-24.

[31] Arévalo-Martín A, Vela JM, Molina-Holgado E, Borrell J, Guaza C. Therapeutic action of cannabinoids in a murine model of multiple sclerosis. J Neurosci. 2003;23(7):2511-16.

[32] Eljaschewitsch E, Witting A, Mawrin C, Lee T, Schmidt PM, Wolf S, et al. The endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. Neuron. 2006;49(1):67-79.

[33] Högestätt ED, Jönsson BA, Ermund A, Andersson DA, Björk H, Alexander JP, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005;280(36):31405-12.

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ORIGINAL ARTICLE

Targeting anandamide metabolism rescues core and associated autistic-like symptoms in rats prenatally exposed to valproic acid

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Autism spectrum disorders (ASD) are characterized by altered sociability, compromised communication and stereotyped/repetitive behaviors, for which no specific treatments are currently available. Prenatal exposure to valproic acid (VPA) is a known, although still underestimated, environmental risk factor for ASD. Altered endocannabinoid activity has been observed in autistic patients, and endocannabinoids are known to modulate behavioral traits that are typically affected in ASD. On this basis, we tested the hypothesis that changes in the endocannabinoid tone contribute to the altered phenotype induced by prenatal VPA exposure in rats, with focus on behavioral features that resemble the core and associated symptoms of ASD. In the course of development, VPA-exposed rats showed early deficits in social communication and discrimination, compromised sociability and social play behavior, stereotypies and increased anxiety, thus providing preclinical proof of the long-lasting deleterious effects induced by prenatal VPA exposure. At the neurochemical level, VPA-exposed rats displayed altered phosphorylation of CB1 cannabinoid receptors in different brain areas, associated with changes in anandamide metabolism from infancy to adulthood. Interestingly, enhancing anandamide signaling through inhibition of its degradation rescued the behavioral deficits displayed by VPA-exposed rats at infancy, adolescence and adulthood. This study therefore shows that abnormalities in anandamide activity may underlie the deleterious impact of environmental risk factors on ASD-relevant behaviors and that the endocannabinoid system may represent a therapeutic target for the core and associated symptoms displayed by autistic patients.

INTRODUCTION

Autism spectrum disorders (ASD) are a group of severe developmental psychiatric disorders emerging in the early life, for which no specific treatments are currently available. ASD are characterized by altered social interaction, compromised verbal and nonverbal communication, stereotyped and repetitive behaviors,[1] often associated with comorbid features,[2-4] such as social and generalized anxiety.[5-7]

Both genetic and environmental factors are involved in the etiology of ASD.[8,9] Environmental factors include a broad range of influences, such as prenatal exposure to drugs, environmental chemicals, infectious agents, or maternal stress.[10] One of the best examples of known environmental risk factors for ASD is prenatal exposure to the antiepileptic and mood stabilizer drug valproic acid (VPA). When this drug is taken during pregnancy, it can result in children displaying autistic-like features, such as impaired communication, reduced sociability and stereotyped behaviors.[11,12] Results from animal models support the clinical data: prenatal VPA exposure in rodents induces autistic-like signs in the offspring, and therefore it has been proposed as a preclinical model of ASD with face and construct validity.[13-16] However, the public awareness of the association between prenatal VPA exposure and adverse outcome in the offspring is still limited. Thus, in March 2016, the French Inspection générale des affaires sociales reported at least 450 cases of malformations in children born between 2006 and 2014 from mothers who had taken VPA during pregnancy,[17] calling for additional research on both environmental risk factors for ASD that can become targets for effective public health risk reduction efforts, and potential treatments for VPA-induced neurobehavioral dysfunctions.

The endocannabinoid system is a unique biological system that affects a wide range of biological processes, including brain development and functioning. It consists of cannabinoid receptors (CB1 and CB2, mainly expressed in the brain and periphery, respectively), their endogenous ligands (endocannabinoids, mainly anandamide and 2-arachidonoylglycerol (2-AG)), and the enzymes for ligand synthesis and degradation.[18-21] In the present study, we used the rat model of ASD based on prenatal VPA exposure to test the hypothesis that an altered endocannabinoid tone contributes to the behavioral alterations observed in ASD, for the following reasons: (1) endocannabinoids are key modulators of socio-emotional responses, cognition, seizure susceptibility, nociception and neuronal plasticity,[22-25] all of which are affected in ASD; (2) variations in the CB1 cannabinoid receptor gene modulate social reward responsivity in reward-related forebrain areas,[26,27] suggesting that subtle changes in endocannabinoid affinity at CB1 receptors owing to these polymorphisms may underlie the deficits in social reward processing observed in ASD; (3) altered endocannabinoid activity has been observed both in genetic[28-31] and environmental[32] models of ASD and in ASD patients.[33]

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We first determined whether the autistic-like behavioral changes exhibited through development by rats prenatally exposed to VPA were associated with changes in the activity of CB1 cannabinoid receptors in brain areas involved in socio-emotional functioning. The endocannabinoid anandamide has a positive modulatory role in key behaviors that are altered in ASD, such as social communication, social play and anxiety-like behaviors,[34–39] and it has recently been shown that pharmacological enhancement of anandamide levels corrected the social deficits observed in two genetic models of ASD.[40] Therefore, we investigated whether prenatal VPA exposure induced changes in brain anandamide synthesis and metabolism, and whether pharmacological manipulation of the endocannabinoid system through modulation of anandamide metabolism was able to restore the altered phenotype displayed by VPA-exposed rats from infancy to adulthood, with focus on behavioral features that resemble the core and associated symptoms of ASD.

MATERIALS AND METHODS

Animals

Female Wistar rats (Charles River, Arbresle, France), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day 1. Pregnant rats were singly housed in Macrolon cages (40 (length) × 26 (width) × 20 (height) cm), under controlled conditions (temperature 20–21 °C, 55–65% relative humidity and 12/12 h light cycle with lights on at 0700 h). Food and water were available ad libitum. On gestational day 12.5, females received a single intraperitoneal injection of either sodium valproate (VPA) or saline (SAL). Newborn litters found up to 1700 h were considered to be born on that day (postnatal day (PND) 0). On PND 1, the litters were culled to eight animals (six males and two females), to reduce the litter size-induced variability in the growth and development of pups during the postnatal period. On PND 21, the pups were weaned and housed in groups of three. The experiments were carried out on the male offspring during infancy (PNDs 9 and 13), adolescence (PND 35) and adulthood (PND 90). One pup per litter from different litters per treatment group was used in each experiment. For every experiment, the exact sample size (n) for each experimental group/condition is indicated in the figure legends. The sample size was based on our previous experiments and power analysis.

The experiments were performed in Wistar rats because previous behavioral studies performed with the VPA preclinical model of autism used this rat strain.[15] Furthermore, we focused on behavioral features, resembling the core and associated symptoms of ASD, that are well documented and can be easily studied in the Wistar rat strain, either from an ethological or pharmacological point of view.

The experiments were approved by the Italian Ministry of Health (Rome, Italy) and performed in agreement with the guidelines released by the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU.

Drugs

VPA (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in saline at a concentration of 250 mg ml⁻¹ and administered at a dose (500 mg kg⁻¹) and time (gestational day 12.5) that have been shown to induce autistic-like behavioral changes in the offspring.[41]

The anandamide hydrolysis inhibitor URB597 (Sigma-Aldrich, Milan, Italy) was dissolved in 5% Tween 80/5% polyethylene glycol/saline and administered intraperitoneally either 2 h (for the behavioral tests performed at adolescence and adulthood) or 30 min (for the behavioral tests performed at infancy) before testing. Drug dose and pre-treatment intervals were based on literature,[29,35,37,38,42] and on pilot experiments. The solutions were administered in a volume of 2.5 ml kg⁻¹ at infancy, 2 ml kg⁻¹ at adolescence and 1 ml kg⁻¹ at adulthood.

Western blot analysis of phosphorylated and total CB1 cannabinoid receptor

The rats were rapidly decapitated and their brains removed and cut into coronal slices on a cold plate. The prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus, amygdala and cerebellum were dissected by hand under microscopic control within 2 min. The tissues were stored at − 80 °C until use.

Lysates from the tissues were homogenized in a homogenization buffer (0.01 M Tris-HCl, 0.001 M CaCl₂, 0.15 M NaCl, 0.001 M PMSF (phenylmethyl-sulfonyl fluoride), pH 7.5) w/v 1:10 (ref. 43) by sonication. An aliquot of homogenate was resuspended in sample buffer (0.250 M Tris-HCl—pH 6.8—containing 20% SDS, 0.001 M PMSF). Lysate samples were boiled for 5 min before loading to the SDS-PAGE (polyacrylamide gel electrophoresis).

Proteins (20 µg) from lysate samples were resolved by 7% SDS-PAGE at 30 mA (constant current) for 120 min and blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Immunoblots were incubated with primary antibodies anti P-CB1 and CB1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA, Catalog No. sc-17555 and sc-10068), followed by secondary peroxidase-conjugated antibodies (1:3000; Santa Cruz Biotechnology, Catalog No. sc-2020). Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK). The nitrocellulose membrane was stripped with Restore western blot stripping buffer (Pierce Chemical, Rockford, IL, USA) for 15 min at room temperature and re-probed with anti-tubulin (Sigma, Catalog No. T9026) antibody. Bound antibodies to proteins onto nitrocellulose were visualized by using enhanced chemiluminescence detection (GE Healthcare) and exposure to Amersham Hyperfilm ECL (GE Healthcare). The images were analyzed with ImageJ (National Institutes of Health).

Quantitative PCR analysis of fatty acid amide hydrolase and N-acylphosphatidylethanolamines-phospholipid D expression

Two micrograms of the total RNA extracted from the whole rat brain were reverse-transcribed using an oligo-dT primer to prime the reverse transcription and the SuperScript II Reverse Transcriptase system (Life Technologies). The quantitative PCR (qPCR) of fatty acid amide hydrolase (FAAH) and N-acylphosphatidylethanolamines-phospholipid D (NAPE-PLD) was performed using a Rotor-Gene 6000 thermal cycling system (Corbett Research, Mortlake, NSW, Australia) and the Hydra SYBR qPCR Master Mix (BioLab, Rome, Italy) as detection system. The thermal cycling conditions for all the transcripts analyzed were: 95 °C for 2 min and 40 cycles at 95 °C for 10 s and 60 °C for 30 s. PCR primers were the following: FAAH_FW: 5′-TGCTGAAGCCTCTGTTTCCT-3′; FAAH_RV: 5′-TCTCATGCTGCAGTTTCCAC; NAPE_FW: 5′-TCGCTGGGGATACTGGTTAC-3′; NAPE_RV: 5′-AAGCTCCAATGG GAATAGCC; β-ACTIN_FW: 5′-AGGCCATGTACGTAGCCATCCA-3′; β-ACTIN_RV: 5′-TCTCCGGAGTCCATCACAATG-3′. The fold changes of FAAH and NAPE transcripts expression in VPA- vs SAL-treated samples were calculated using the $2^{-\Delta\Delta CT}$ method.

Reproduction data

Body weights of the dams were taken daily throughout pregnancy and the length of pregnancy was determined. Litter size, male/female ratio, weight gain of pups and postnatal vitality were also measured.

Isolation-induced ultrasonic vocalizations

On PND 9, the pups were individually placed into a Plexiglas arena (30 (length) × 30 (width) × 30 (height) cm), located inside a sound-attenuating and temperature-controlled chamber, with a camera positioned above the arena. The ultrasonic vocalizations (USVs) emitted by the pup were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Glienicke, Germany) sensitive to frequencies between 10 and 200 kHz. Pup axillary temperature was measured before and after the test by a digital thermometer. The USVs were analyzed, both quantitatively and qualitatively, using Avisoft Recorder software (Version 5.1), and classified in six different groups according to number of syllables, frequency modulation and duration:[44–46] flat calls (calls with constant frequency with a maximum variation of ± 5 kHz); up/downward (single syllable calls emitted at ± 5 kHz with a single frequency modulation); flat high frequency calls (calls with similar constant frequency, but emitted at > 75 kHz); syllable calls (calls composed by two or more syllables); complex calls (calls displaying complex frequency modulation); short calls (calls with durations shorter than 5 ms).

Homing behavior

On PND 13, the litter was separated from the dam and kept for 30 min in a temperature-controlled holding cage. Then, each pup was placed into a Plexiglas box whose floor was one-third covered with bedding from the

pup's home cage and two-third with clean bedding. The pup was located at the side of the box covered by clean bedding, and its behavior was videorecorded for 4 min for subsequent analysis. The following parameters were scored using the Observer 3.0 software (Noldus Information Technology, Wageningen, The Netherlands): latency (seconds) to reach the home-cage bedding area; total time (seconds) spent by the pup in the nest bedding area; total number of entries into the nest bedding area.[45]

Three-chamber test

The apparatus was a rectangular three-chamber box, with two lateral chambers (30 (length) × 35 (width) × 35 (height) cm) connected to a central chamber (15 (length) × 35 (width) × 35 (height) cm). Each lateral chamber contained a small Plexiglas cylindrical cage. The test was performed as previously described.[47] Each experimental rat was individually allowed to explore the apparatus for 10 min, and then confined in the central compartment. An unfamiliar stimulus animal was placed into the Plexiglas cage in one chamber of the apparatus, whereas the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to freely explore the apparatus for 10 min. The percent of time spent in social approach (sniffing the stimulus and the cage confining it) was scored using the Observer 3.0 software (Noldus Information Technology).

Social play behavior

Rats were individually habituated to the test cage for 10 min on each of the 2 days before testing, as previously described.[37,42,48] On the test day, the animals were isolated for 3 h before testing.[49–51] The test consisted of placing VPA- or SAL-exposed rats together with an untreated animal for 15 min. The behavior was assessed for each individual animal of a pair separately using the Observer 3.0 software (Noldus Information Technology). The behaviors of the animals were recorded using a camera with zoom lens, video tape recorder and television monitor.

In rats, a bout of social play behavior starts with one rat soliciting ('pouncing') another animal, by attempting to nose or rub the nape of its neck. The animal that is pounced upon can respond in different ways: if the animal fully rotates to its dorsal surface, 'pinning' is the result (one animal lying with its dorsal surface on the floor with the other animal standing over it), which is considered the most characteristic posture on social play behavior in rats.[52]

We determined 'play responsiveness' (that is, the percentage of response to play solicitation) as the probability of an animal of being pinned in response to play solicitation (pouncing) by the stimulus partner.[42]

Elevated plus maze

The apparatus comprised two open and two closed arms (50 (length) × 10 (width) × 40 (height) cm) that extended from a common central platform (10 (length) × 10 (width) cm). The rats were individually placed on the central platform of the maze for 5 min. Each session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis performed using the Observer 3.0 software (Noldus Information Technology). The following parameters were analyzed:[53]

1. % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open+closed arms) × 100;
2. % open-arm entries (% OE): (the number of entries into the open arms of the maze/number of entries into open+closed arms) × 100;
3. Number of closed-arm entries (number of CE).

Hole-board test

The apparatus was a gray square metal table (40 (length) × 40 (width) × 10 (height) cm) with 16 evenly spaced holes (4 cm in diameter), inserted in a Plexiglas arena (40 (length) × 40 (width) × 60 (height) cm). The rats were individually placed in the apparatus and their behavior was observed for 5 min. Dipping behavior was scored by the number of times an animal inserted its head into a hole at least up to the eye level. Each session was recorded with a camera positioned above the apparatus for subsequent behavioral analysis performed using the Observer 3.0 software (Noldus Information Technology).

Statistical analysis

The data are expressed as mean ± s.e.m. To assess the effects of the prenatal treatment (VPA or SAL) on the offspring, data were analyzed with Student's *t*-tests. Two-way analysis of variance (ANOVA) was used to assess the effects of prenatal and postnatal treatments, using prenatal (VPA or SAL) and postnatal (URB597 or vehicle) treatments as between-subjects factor. Two-way ANOVA was followed by Student's–Newman–Keuls *post hoc* test where appropriate.

The western blot and qPCR experiments were performed in duplicate and the results were comparable.

Behavioral experiments were scored and analyzed by a trained observer who was unaware of the treatment conditions (Noldus Information Technology). Similarly, biochemical experiments were performed and analyzed in blinded conditions.

Supplementary Figure 1 shows three different timeline diagrams for: (1) the behavioral characterization of the VPA- and SAL-exposed offspring; (2) the behavioral effects of URB597 in the VPA- and SAL-exposed offspring; (3) the western blot and quantitative PCR analyses of the brain of VPA- and SAL-exposed animals.

RESULTS

Reproduction data

During gestation, no differences in body weight gains were observed between VPA- and SAL-treated dams. Prenatal VPA exposure did not affect pregnancy length, litter size at birth, male/female ratio, pup weight gain and postnatal vitality (Supplementary Table 1).

CB1 cannabinoid receptor phosphorylation and FAAH and NAPE-PLD expression

Phosphorylation may reflect the activation of CB1 cannabinoid receptors.[54] To investigate whether VPA prenatal exposure induced changes in the activity of brain cannabinoid receptors, we measured both phosphorylated and total CB1 receptor protein expression in the prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus, amygdala and cerebellum of the offspring at PNDs 13, 35 and 90. In VPA-exposed rats, the ratio between phosphorylated and total CB1 receptor protein decreased in the amygdala at PND 13 ($t = 2.103$, $P < 0.05$, df = 10; Figure 1a) and 90 ($t = 11.41$, $P < 0.001$, df = 10; Figure 1c) but not at PND 35 ($t = 0.06718$, $P = $NS (not significant), df = 10) and in the hippocampus at PND 35 ($t = 3.439$, $P < 0.01$, df = 10; Figure 1e) and PND 90 ($t = 4.802$, $P < 0.001$, df = 10; Figure 1f), but not at PND 13 ($t = 1.369$, $P = $NS, df = 10). The phosphorylation state of CB1 receptors increased in the dorsal striatum of VPA-exposed rats at all ages (PND 13: $t = 6.056$, $P < 0.001$, df = 10; Figure 1g; PND 35: $t = 4.082$, $P < 0.001$, df = 10; Figure 1h; PND 90: $t = 5.601$, $P < 0.001$, df = 10; Figure 1i). No differences between VPA- and SAL-exposed rats were observed in the cerebellum (PND 13: $t = 0.8856$, $P = $NS, df = 10; PND 35: $t = 0.05827$, $P = $NS, df = 10; PND 90: $t = 0.8579$, $P = $NS, df = 10), prefrontal cortex (PND 13: $t = 1.897$, $P = $NS, df = 10; PND 35: $t = 0.1135$, $P = $NS, df = 10; PND 90: $t = 0.8790$, $P = $NS, df = 10) and nucleus accumbens (PND 13: $t = 0.3687$, $P = $NS, df = 10; PND 35: $t = 0.1578$, $P = $NS, df = 10; PND 90: $t = 1.365$, $P = $NS, df = 10). Significant changes in CB1 total receptor levels were only observed in the striatum of adolescent ($t = 5.88$, $P = $NS, df = 10) and adult ($t = 2.47$, $P = $NS, df = 10) rats and in the hippocampus of adult animals ($t = 4.15$, $P = $NS, df = 10).

We next evaluated the expression of FAAH, which catalyzes anandamide hydrolysis, and NAPE-PLD, which catalyzes the one-step conversion of NAPEs to anandamide, in the brain of the offspring at PNDs 13, 35 and 90. In VPA-exposed animals, FAAH expression was increased at PND 13 ($t = 3.8832$, $P < 0.05$, df = 4) and unchanged at PNDs 35 ($t = 0.6928$, $P = $NS, df = 4) and 90 ($t = 2.6943$, $P = $NS, df = 4; Figure 2a). NAPE-PLD expression was decreased in VPA-exposed animals at all ages (PND 13: $t = 11.2583$,

4

Amygdala

Hippocampus
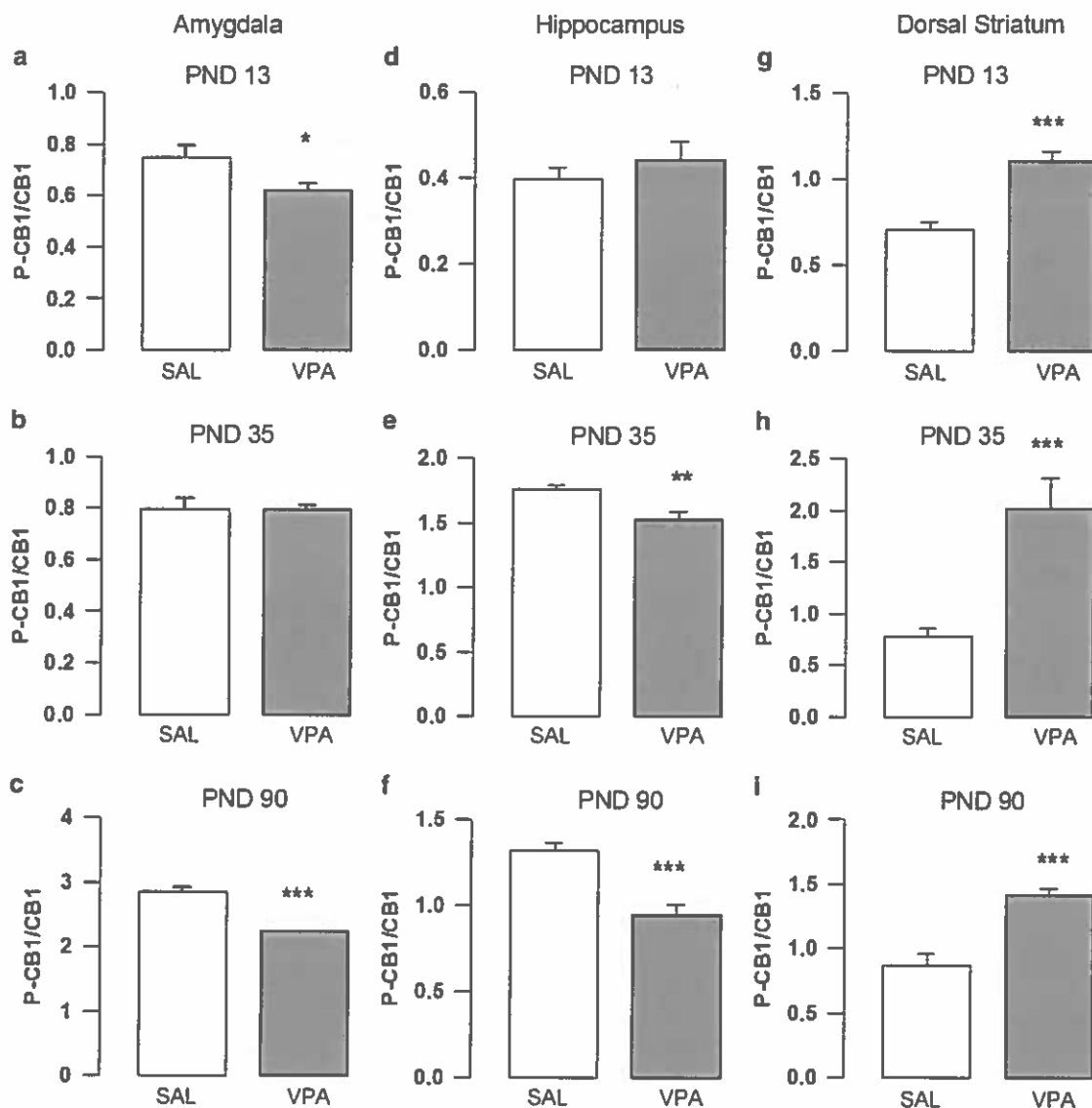
Dorsal Striatum

Figure 1. Activation of CB1 receptors. Ratio between phosphorylated and total CB1 receptor protein in the amygdala (a–c), hippocampus (d–f) and dorsal striatum (g–i) of VPA- and SAL-exposed offspring, evaluated at PNDs 13 (n (SAL) = 6, n (VPA) = 6), 35 (n (SAL) = 6, n (VPA) = 6) and 90 (n (SAL) = 6, n (VPA) = 6). Data represent mean ± s.e.m.; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's t-test). PND, postnatal day; SAL, saline; VPA, valproic acid.

$P < 0.001$, df = 4; PND 35: $t = 18.0133$, $P < 0.001$, df = 4; PND 90: $t = 11.1207$, $P < 0.001$, df = 4; Figure 2b).

Isolation-induced USVs
When separated from the dam and siblings on PND 9, VPA-exposed pups vocalized significantly less compared with SAL-exposed pups ($t = 2.15$, $P < 0.05$, df = 28; Figure 3a). No differences between the two groups were found in the percentage of the different call categories emitted: flat ($t = 0.82$, $P = $ NS, df = 14); flat high frequency ($t = 1.54$, $P = $ NS, df = 14); syllable ($t = 0.69$, $P = $ NS, df = 14); short ($t = 1.08$, $P = $ NS, df = 14); up/downward ($t = 0.67$, $P = $ NS, df = 14); complex ($t = 0.04$, $P = $ NS, df = 14). URB597 reversed the communicative deficits displayed by VPA-exposed animals, without altering the performance of SAL-exposed pups (Figure 3b). A two-way ANOVA analysis performed on the number of total USVs emitted after treatment with URB597 or vehicle gave the following results: total USVs ($F_{(prenatal\ treat.)1,38} = 2.021$, $P = $ NS; $F_{(treat.)1,38} = 2.741$, $P = $ NS; $F_{(prenatal\ treat. \times treat.)1,38} = 4.586$; $P < 0.05$).

Post hoc analysis revealed that VPA-exposed pups emitted less USVs compared with SAL-exposed pups ($P < 0.05$). However, VPA-exposed pups treated with URB597 did not differ from SAL-exposed pups treated with either VEH ($P = $ NS) or URB ($P = $ NS; Figure 3b).

Homing behavior
The VPA-exposed rats showed deficits in the homing test on PND 13: they showed longer latency to reach the home-cage bedding ($t = 2.55$, $P < 0.05$, df = 23; Figure 3c) and spent less time in the nest area ($t = 2.55$, $P < 0.05$, df = 23; not shown) compared with SAL-exposed offspring. The two groups did not differ in locomotor activity (number of crossings: $t = 0.90$, $P = $ NS, df = 23). URB597 reversed the deficit displayed by VPA-exposed pups in this test, without altering the performance of SAL-exposed pups. A two-way ANOVA analysis performed on the parameters measured in the test gave the following results: latency ($F_{(prenatal\ treat.)1,40} = 1.77$, $P = $ NS; $F_{(treat.)1,40} = 5.45$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,40} = 3.53$;

a

b
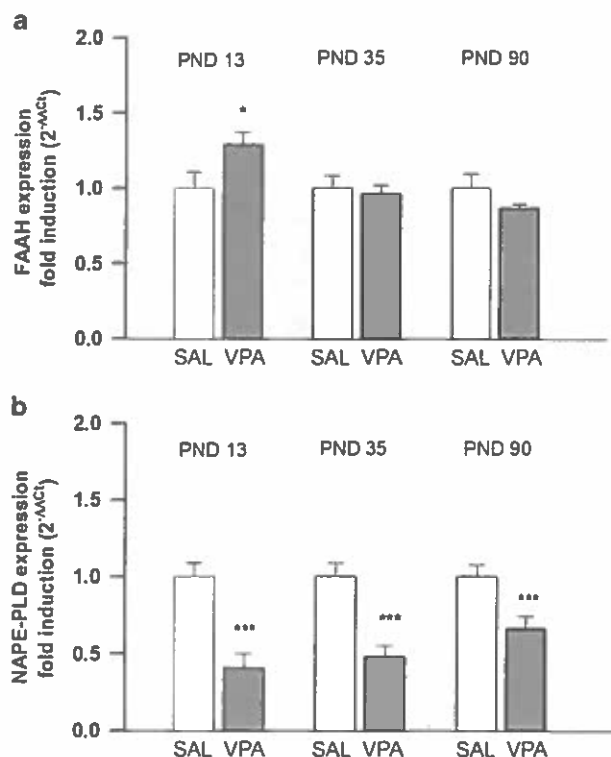
Figure 2. FAAH and NAPE-PLD expression. Fold induction of FAAH (a) and NAPE-PLD (b) expression in the brain of VPA- and SAL-exposed offspring, evaluated at PNDs 13 (n (SAL) = 3, n (VPA) = 3), 35 (n (SAL) = 3, n (VPA) = 3) and 90 (n (SAL) = 3, n (VPA) = 3). Data represent mean ± s.d.; *$P < 0.05$; ***$P < 0.001$ (Student's t-test). FAAH, fatty acid amide hydrolase; NAPE-PLD, N-acylphosphatidy-lethanolamines-phospholipid D; PND, postnatal day; SAL, saline; VPA, valproic acid.

P = NS); nesting time ($F_{(prenatal\ treat.)1,40} = 1.34$, P = NS; $F_{(treat.)1,40} = 6.72$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,40} = 7.11$; $P < 0.05$). *Post hoc* analysis revealed that VPA-exposed pups showed a longer latency to reach the familiar bedding ($P < 0.05$; Figure 3d) and spent less time in the nest area ($P < 0.01$, not shown) compared with SAL-exposed pups. Conversely, no differences were found between VPA-exposed pups treated with URB597 and SAL-exposed pups treated with vehicle.

Social play behavior
The VPA-exposed rats showed reduced play responsiveness compared with SAL-exposed animals ($t = 2.56$, $P < 0.05$, df = 24; Figure 3e). When VPA- and SAL-exposed animals were treated with URB597 or its vehicle, a two-way ANOVA analysis performed on the percentage of response to play solicitation gave the following results: ($F_{(prenatal\ treat.)1,32} = 4.42$, $P < 0.05$; $F_{(treat.)1,32} = 4.52$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,32} = 1.2$; P = NS). *Post hoc* analysis revealed that VPA-exposed rats responded less to social play solicitation ($P < 0.05$; Figure 3f) compared with SAL-exposed rats. Conversely, no differences were found between VPA-exposed rats treated with URB597 and SAL-exposed rats treated with vehicle.

Three-chamber test
Compared with the SAL-exposed animals, the VPA-exposed rats showed decreased sociability in the three-chamber test, as they spent less time sniffing the stimulus animal both at PNDs 35 ($t = 3.14$, $P < 0.01$, df = 15; Figure 4a) and 90 ($t = 3.37$, $P < 0.01$,

df = 26; Figure 4c). No differences between the two groups were found in the total number of entries (PND 35: $t = 0.13$, P = NS, df = 15; PND 90: $t = 1.44$, P = NS, df = 26, not shown). When VPA- and SAL-exposed animals were treated with URB597 or its vehicle, a two-way ANOVA analysis performed on the percentage of time spent sniffing the stimulus gave the following results: PND 35 ($F_{(prenatal\ treat.)1,40} = 6.05$, $P < 0.05$; $F_{(treat.)1,40} = 9.31$, $P < 0.01$; $F_{(prenatal\ treat. \times treat.)1,40} = 2.1$; P = NS); PND 90 ($F_{(prenatal\ treat.)1,35} = 4.13$, $P < 0.05$; $F_{(treat.)1,35} = 1.07$, P = NS; $F_{(prenatal\ treat. \times treat.)1,35} = 1.1$; P = NS). *Post hoc* analysis revealed that VPA-exposed rats spent less time sniffing the stimulus than SAL-exposed rats both at PND 35 ($P < 0.01$; Figure 4b) and PND 90 ($P < 0.05$; Figure 4d). Conversely, no differences were found between VPA-exposed rats treated with URB597 and animals SAL-exposed rats treated with vehicle.

Hole-board test
The VPA-exposed rats showed stereotypic behaviors in the hole-board test, as they made more head dippings at PND 35 ($t = 4.14$, $P < 0.001$, d.f. = 21; Figure 4e) and PND 90 ($t = 2.27$, $P < 0.05$, df = 22; Figure 4g). URB597 reduced the stereotypic behavior found in VPA-exposed rats. A two-way ANOVA analysis performed on the number of head dippings gave the following results: PND 35 ($F_{(prenatal\ treat.)1,74} = 3.93$, P = NS; $F_{(treat.)1,74} = 4.6$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,74} = 10.49$; $P < 0.01$); PND 90 ($F_{(prenatal\ treat.)1,30} = 1.32$, P = NS; $F_{(treat.)1,30} = 10.67$, $P < 0.01$; $F_{(prenatal\ treat. \times treat.)1,30} = 4.82$; $P < 0.05$). *Post hoc* analysis revealed that VPA-exposed adolescent ($P < 0.001$; Figure 4f) and adult ($P < 0.05$; Figure 4h) rats made more head dippings than SAL-exposed rats. Conversely, no differences were found between VPA-exposed rats treated with URB597 and SAL-exposed rats treated with vehicle.

Elevated plus maze test
Both at PNDs 35 and 90, the VPA-exposed rats showed an anxious-like phenotype compared with SAL-exposed animals, as they spent less time in the open arms (PND 35: $t = 3.18$, $P < 0.01$, df = 42; Figure 5a; PND 90: $t = 2.55$, $P < 0.05$, df = 21; Figure 5e) and made less open-arm entries (PND 35: $t = 2.08$, $P < 0.05$, df = 42; Figure 5b; PND 90: $t = 2.86$, $P < 0.01$, df = 21; Figure 5f) compared to SAL-exposed rats. No significant differences have been found in the total number of closed-arm entries either at PND 35 ($t = 1.64$, P = NS, df = 42, data not shown) or 90 ($t = 0.65$, P = NS, df = 21, data not shown). URB597 had anxiolytic-like effects in VPA-exposed rats, without altering the behavior of SAL-exposed animals. A two-way ANOVA analysis performed on the parameters measured in this test gave the following results: PND 35, percentage of time spent in the open arms ($F_{(prenatal\ treat.)1,34} = 6.09$, $P < 0.05$; $F_{(treat.)1,34} = 1.41$, P = NS; $F_{(prenatal\ treat. \times treat.)1,34} = 2.87$, P = NS); percentage of open-arm entries ($F_{(prenatal\ treat.)1,34} = 8.60$, $P < 0.01$; $F_{(treat.)1,34} = 5.93$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,34} = 4.51$, $P < 0.05$); PND 90, percentage of time spent in the open arms ($F_{(prenatal\ treat.)1,54} = 2.12$, P = NS; $F_{(treat.)1,54} = 5.79$, $P < 0.05$; $F_{(prenatal\ treat. \times treat.)1,54} = 8.08$, $P < 0.05$; Figure 5g); percentage of open-arm entries ($F_{(prenatal\ treat.)1,54} = 1.76$, P = NS; $F_{(treat.)1,54} = 1.85$, P = NS; $F_{(prenatal\ treat. \times treat.)1,54} = 5.84$, $P < 0.05$; Figure 5h). *Post hoc* analysis showed that VPA-exposed animals spent less time in the open arms and made less open-arm entries compared with SAL-exposed rats (Figures 5c and d, g and h). Conversely, no differences were found between VPA-exposed animals treated with URB597 and SAL-exposed animals treated either with vehicle or with URB. Last, a two-way ANOVA analysis performed on the total number of closed-arm entries gave the following results: adolescence ($F_{(prenatal\ treat.)1,34} = 1.2$, P = NS; $F_{(treat.)1,34} = 0.45$, P = NS; $F_{(prenatal\ treat. \times treat.)1,34} = 0.06$, P = NS); adulthood ($F_{(prenatal\ treat.)1,54} = 1.1$, P = NS; $F_{(treat.)1,54} = 0.36$, P = NS; $F_{(prenatal\ treat. \times treat.)1,54} = 0.17$, P = NS).

Isolation-induced USV test PND9

Homing test PND13

Social play behavior PND 35
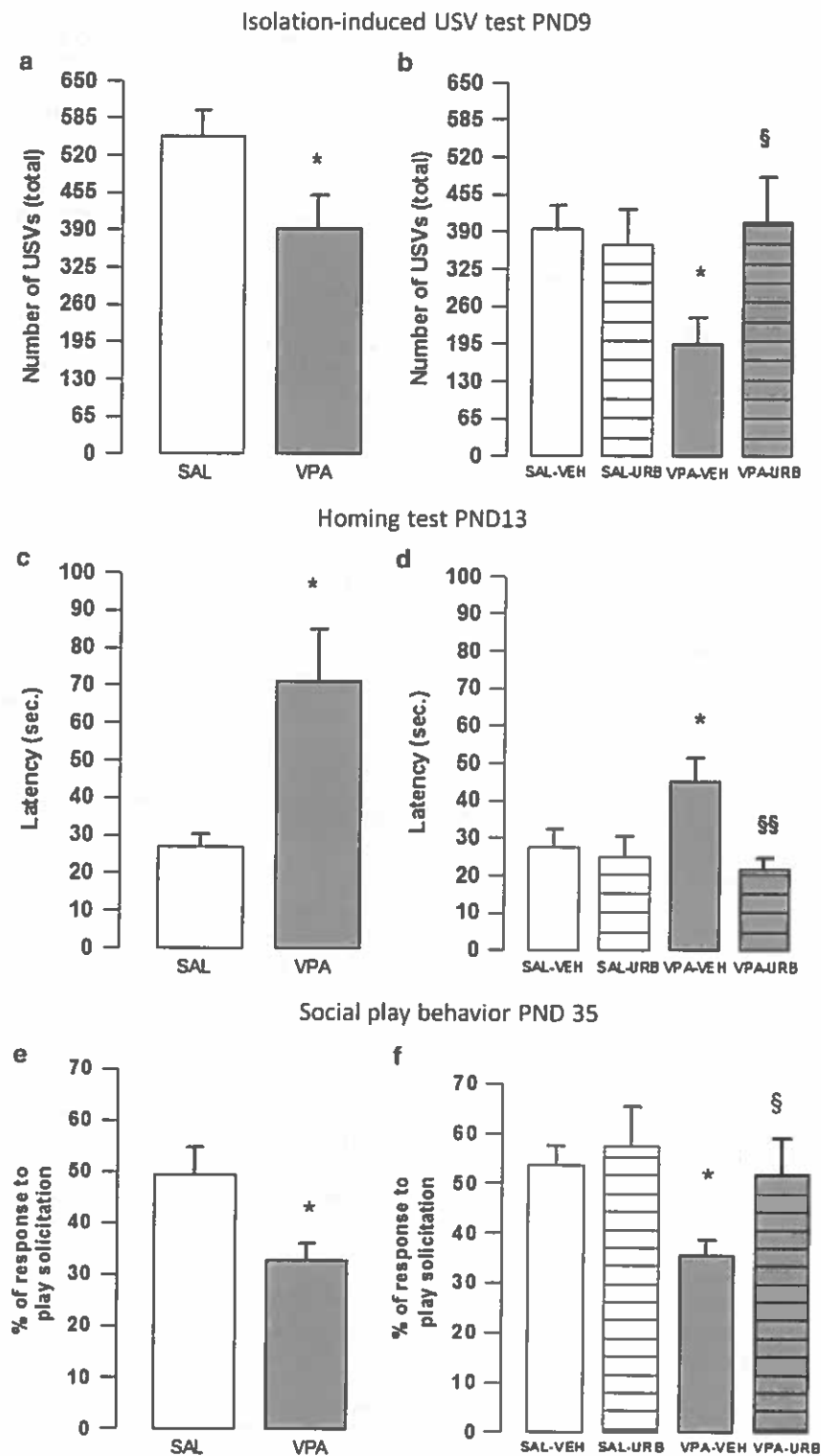
Figure 3. Isolation-induced USV, homing and social play behavior tests. Prenatal VPA exposure reduced isolation-induced USV emission at PND 9 (**a**; n (SAL) = 13, n (VPA) = 17), altered homing behavior at PND 13 (**c**; n (SAL) = 10, n (VPA) = 15) and reduced social play behavior at PND 35 (**e**; n (SAL) = 14, n (VPA) = 12). URB597 reversed the altered behavioral phenotype displayed by VPA-exposed rats in the isolation-induced USV (**b**; n (SAL-VEH) = 12, n (VPA-VEH) = 11, n (SAL-URB) = 10, n (VPA-URB) = 9), homing (**d**; n (SAL-VEH) = 11, n (VPA-VEH) = 11, n (SAL-URB) = 14, n (VPA-URB) = 8) and social play behavior (**f**; n (SAL-VEH) = 9, n (VPA-VEH) = 11, n (SAL-URB) = 8, n (VPA-URB) = 8) tests. Data represent mean ± s.e.m. *$P < 0.05$ vs SAL-VEH group; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs VPA-VEH group (Student's t-test (**a, c, e**); Student's–Newman–Keuls *post hoc* test (**b, d, f**)). PND, postnatal day; SAL, saline; USV, ultrasonic visualization; VEH, vehicle; VPA, valproic acid.

Three-chamber test PND 35

Three-chamber test PND 90

Hole board test PND 35

Hole board test PND 90
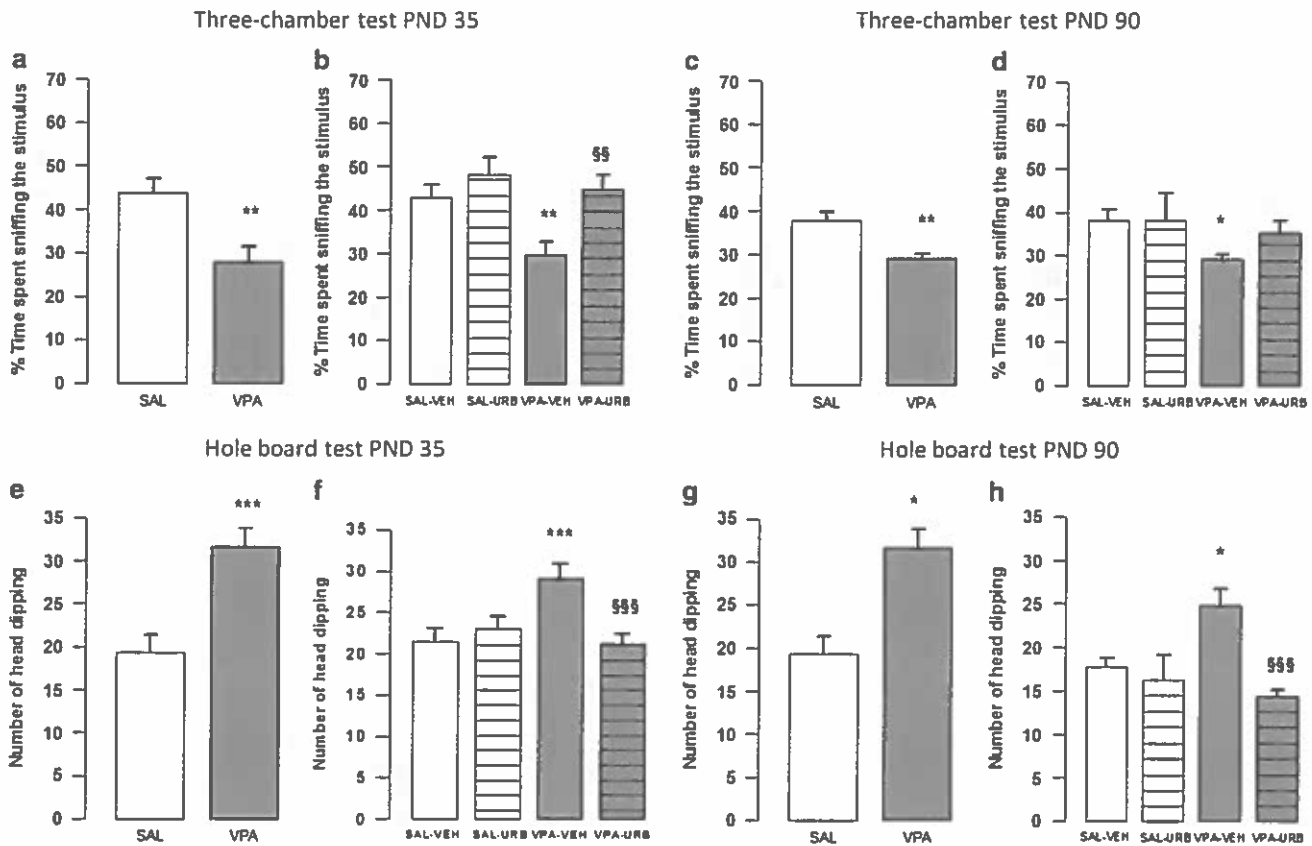
Figure 4. Three-chamber and hole-board tests. Prenatal VPA exposure reduced sociability in the three-chamber test (**a** and **c**) at PND 35 (n (SAL) = 8, n (VPA) = 9) and 90 (n (SAL) = 15, n (VPA) = 13) and induced stereotypic behavior in the hole-board test (**e** and **g**) at PND 35 (n (SAL) = 11, n (VPA) = 12) and 90 (n (SAL) = 12, n (VPA) = 12). URB597 mitigated the altered sociability (**b** and **d**) displayed by VPA-exposed offspring at PND 35 (n (SAL-VEH) = 13, n (VPA-VEH) = 13, n (SAL-URB) = 8, n (VPA-URB) = 10) and 90 (n (SAL-VEH) = 11, n (VPA-VEH) = 12, n (SAL-URB) = 8, n (VPA-URB) = 8). Furthermore, URB597 mitigated the stereotypies (**f** and **h**) displayed by VPA-exposed offspring at PND 35 (n (SAL-VEH) = 20, n (VPA-VEH) = 19, n (SAL-URB) = 20, n (VPA-URB) = 19) and 90 (n (SAL-VEH) = 8, n (VPA-VEH) = 10, n (SAL-URB) = 8, n (VPA-URB) = 8). Data represent mean ± s.e.m. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs SAL-VEH group; $\S\S P < 0.01$, $\S\S\S P < 0.01$ vs VPA-VEH group (Student's t-test (**a**, **c**, **e** and **g**); Student's–Newman–Keuls post hoc test (**b**, **d**, **f** and **h**)). PND, postnatal day; SAL, saline; VEH, vehicle; VPA, valproic acid.

DISCUSSION

Endocannabinoids are known to regulate key brain functions that are altered in ASD. By using a well-validated animal model of ASD based on prenatal VPA exposure in rats,[15,16] we provide new evidence that an altered anandamide tone that manifests already at infancy and persists into adolescence and adulthood may underlie core and associated autistic-like symptoms, thus providing preclinical rationale to a potential role of anandamide signaling as a new therapeutic target for ASD.

At the neurochemical level, we found that rats prenatally exposed to VPA display altered expression of phosphorylated CB1 cannabinoid receptors in the amygdala, hippocampus and dorsal striatum, with no changes in the prefrontal cortex, cerebellum and nucleus accumbens. As phosphorylation may reflect the activation of CB1 cannabinoid receptors,[54] the observed changes may be a compensatory response aimed at normalizing CB1-mediated signaling in VPA-exposed rats as a consequence of a relative imbalance of the system.[55] To support this possibility, we found changes in the expression of the enzymes that catalyze the synthesis and degradation of anandamide in the brain of infant, adolescent and adult rats prenatally exposed to VPA, indicating that prenatal VPA exposure induces long-lasting changes in brain anandamide metabolism. These results extend previous findings showing changes in the expression of mRNA for the enzymes primarily responsible for the synthesis and degradation of 2-AG in the cerebellum and hippocampus of VPA-exposed rats, with no changes in both 2-AG and anandamide content.[32]

At the behavioral level, our results confirm the validity of prenatal exposure to VPA as an animal model of ASD with strong face validity. In particular, our longitudinal study shows that prenatal VPA exposure induces early communicative deficits, altered sociability and social play behavior, enduring stereotypies and increased anxiety in the rat offspring. At infancy, in line with previous studies,[56–59] we found quantitative changes in the USVs emitted by VPA-exposed rats when separated from their mother and siblings compared with control animals. USVs have an essential communicative role in mother–offspring interaction,[60] and therefore the altered USV profile displayed by VPA-exposed pups when separated from the nest may indicate a reduced ability to communicate with their mother. Furthermore, as previously reported,[15,58,61–63] we found that VPA-exposed pups were unable to use olfactory cues to discriminate between a neutral odor and their own home cage odor in the homing behavior test. Olfaction, and in particular the learned association between maternal odors and maternal stimulation, is crucial for the development of social behavior and social recognition.[64,65] Altogether, the altered USV profile and homing behavior displayed by VPA-exposed pups indicate their profound deficits in social communication and social discrimination since the first days of life.

At adolescence and adulthood, the consequences of prenatal VPA exposure on the social behavior of the offspring were analyzed at two different levels: (1) during a free dyadic social encounter with a same-age stimulus animal, to analyze the reciprocal nature of the social interaction and its ethologic

Elevated plus-maze test PND 35

Elevated plus-maze test PND 90
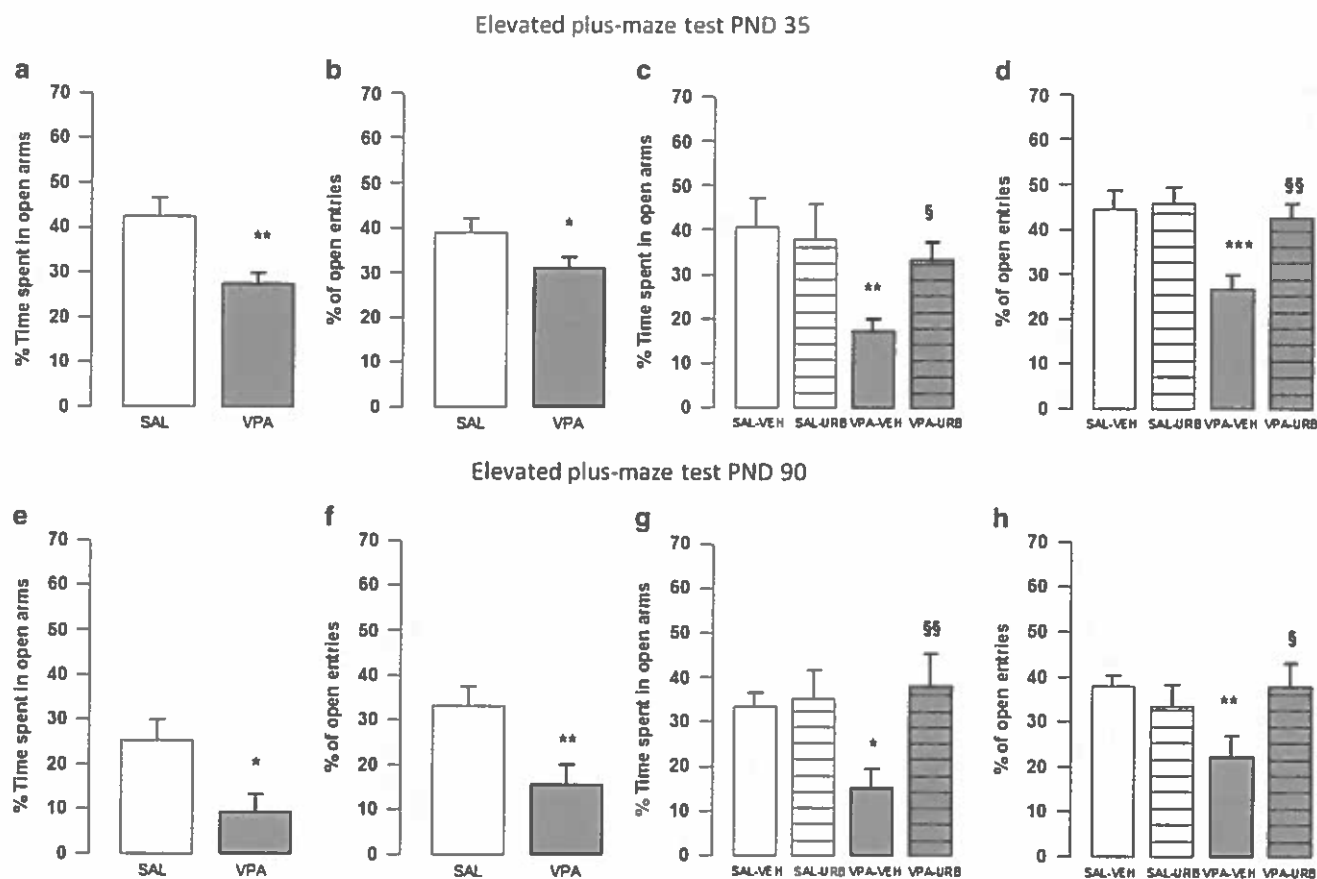
Figure 5. Elevated plus maze test. Prenatal VPA exposure decreased the percentage of time spent by the offspring in the open arms (a and e) and the percentage of open-arm entries (b and f) in the elevated plus maze test at PND 35 (n (SAL) = 23, n (VPA) = 21) and 90 (n (SAL) = 13, n (VPA) = 10). URB597 reversed the anxiety-like behavior displayed by VPA-exposed rats (c, d, g and h) at PND 35 (n (SAL-VEH) = 11, n (VPA-VEH) = 11, n (SAL-URB) = 8, n (VPA-URB) = 8) and 90 (n (SAL-VEH) = 17, n (VPA-VEH) = 15, n (SAL-URB) = 13, n (VPA-URB) = 13). Data represent mean ± s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs SAL-VEH group; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ vs VPA-VEH group (Student's t-test (a-f); Student's–Newman–Keuls post hoc test (c-h)). PND, postnatal day; SAL, saline; VEH, vehicle; VPA, valproic acid.

characteristics; (2) in the three-chamber test that focuses on the social approach of the experimental animal toward a confined stimulus animal, without direct social contact.

In accordance with previous studies,[15,32,41,57,58,61,66-69] the VPA-exposed rats showed deficits in social behavior both at adolescence and adulthood, as they showed decreased responsiveness to play solicitation and reduced sociability in the three-chamber test. The reduced willingness to engage in social interaction or to prolong a playful encounter displayed by VPA-exposed rats are reminiscent of the altered social behavior and deficient social play observed in autistic patients.[1]

The endocannabinoid system modulates different aspects of social behavior. In humans, variations in the CB1 cannabinoid receptor gene predict differences in the striatal response to happy faces, indicating a role of the endocannabinoid system in social reward responsivity.[26,27] In addition, the endocannabinoid system modulates amygdala reactivity to social threat signals.[70] Altered striatal and amygdala activity has been related to the diminished response to social rewards displayed by autistic patients.[71,72] In line with this scenario, we found that VPA-exposed rats show altered phosphorylation of CB1 cannabinoid receptors in the dorsal striatum during all the lifespan, and in the amygdala both at infancy and adulthood.

In rodents, anandamide has a positive modulatory role in several social behavior: it is involved in pup USV production and emotional reactivity,[35,73] it is released during social play in the

amygdala, nucleus accumbens[36] and dorsal striatum,[74] and it is involved in adult forms of social interactions.[34,75] Interestingly, we found that the expression of NAPE-PLD, the main anandamide biosynthetic enzyme, was reduced in VPA-exposed rats from infancy to adulthood; furthermore, VPA-exposed infant rats showed higher expression of FAAH, the main enzyme involved in anandamide catabolism. Thus, a reduced anandamide-mediated signaling may underlie the deficits in the communicative and social domain displayed by VPA-exposed rats through development. In line with this possibility, enhancing anandamide activity through administration of the anandamide hydrolysis inhibitor URB597 normalized the USV profile and the performance of VPA-exposed pups in the homing test, and reversed their social deficits in the three-chamber and social play behavior tests.

Stereotyped behaviors are the second core symptom displayed by ASD patients.[1] In accordance with previous studies,[15,58,66,67,76] we found that both adolescent and adult rats prenatally exposed to VPA showed stereotyped behavior in the hole-board test. It is known that the endocannabinoid system is involved in motor control by modulating dopaminergic neurotransmission in the basal ganglia.[77] In particular, increasing endocannabinoid signaling by inhibiting endocannabinoid uptake or metabolism counteracts the stereotypies induced by systemic or intrastriatal dopaminergic stimulation in rodents,[78-80] whereas antagonism at CB1 cannabinoid receptors increases dopamine receptor-mediated stereotypies.[81] In light of these findings, it is therefore

not surprising that the anandamide hydrolysis inhibitor URB597 counteracted the stereotyped behavior displayed by both adolescent and adult VPA-exposed rats.

Anxiety is a frequent symptom displayed by autistic patients.[2] Accordingly, as previously reported,[41,66,82,83] the VPA-exposed rats showed an anxious phenotype in the elevated plus maze test, both during adolescence and adulthood.

Animal and clinical studies have repeatedly shown that the endocannabinoid system is critically involved in the control of emotionality.[21] In particular, URB597 induces anxiolytic-like effects in rodents,[35,38,39] that become evident particularly when the animals are tested under stressful conditions,[84,85] supporting the idea that endocannabinoids are released 'on demand', in response to challenging situations. Interestingly, we found that URB597, administered at a dose that had no effect in control animals, reduced the anxious phenotype displayed by both adolescent and adult VPA-exposed rats.

Collectively, three main conclusions can be drawn from the present study. First, the altered behavioral profile displayed by VPA-exposed rats from infancy till adolescence and adulthood is a further preclinical proof of the long-lasting deleterious effects induced by prenatal VPA exposure. To date, the potential for some environmental factors to promote ASD is still relatively undetermined, and the recent epidemiological data clearly show that the public health protection strategies adopted so far have been inadequate at best.[17] Thus, targeted research on causative environmental factors for ASD is warranted. Second, we report abnormalities in brain anandamide activity in an environmental animal model of ASD based on prenatal VPA exposure in rats. These findings, that contribute to increase our understanding of the neural underpinnings of ASD, extend previous studies reporting altered endocannabinoid activity in animal models of ASD[28-32] and in ASD patients,[33] and genetic studies in humans showing an important role for cannabinoid receptors in processing appetitive socially relevant stimuli.[26,27] Last, our results prove that pharmacological interference with anandamide metabolism mitigates in rats behavioral features that resemble the core and associated symptoms of ASD. Thus, this study suggests that abnormalities in anandamide activity may underlie the deleterious impact of environmental risk factors on ASD-relevant behaviors, and that the endocannabinoid system may be a therapeutic target for the core and associated symptoms displayed by autistic patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 American Psychiatric Association. *Diagnostic and Statistic Manual of Mental Disorders: DMS-5*, 5th edn. American Psychiatric Publishing: Arlington, VA, 2013.

2 Lai MC, Lombardo MV, Baron-Cohen S. Autism. *Lancet* 2014; **383**: 896–910.

3 Mannion A, Leader G. Comorbidity in autism spectrum disorder: a literature review. *Research in Autism Spectrum Disorders* 2013; 7: 1595–1616.

4 Mazzone L, Ruta L, Reale L. Psychiatric comorbidities in Asperger syndrome and high functioning autism: diagnostic challenges. *Ann Gen Psychiatry* 2012; **11**: 16.

5 de Bruin EI, Ferdinand RF, Meester S, de Nijs PF, Verheij F. High rates of psychiatric co-morbidity in PDD-NOS. *J Autism Dev Disord* 2007; **37**: 877–886.

6 Gillott A, Standen PJ. Levels of anxiety and sources of stress in adults with autism. *J Intellect Disabil* 2007; 11: 359–370.

7 Simonoff E, Pickles A, Charman T, Chandler S, Loucas T, Baird G. Psychiatric disorders in children with autism spectrum disorders: prevalence, comorbidity, and associated factors in a population-derived sample. *J Am Acad Child Adolesc Psychiatry* 2008; **47**: 921–929.

8 Kim YS, Leventhal BL. Genetic epidemiology and insights into interactive genetic and environmental effects in autism spectrum disorders. *Biol Psychiatry* 2015; **77**: 66–74.

9 Tordjman S, Somogyi E, Coulon N, Kermarrec S, Cohen D, Bronsard G *et al.* Gene x Environment interactions in autism spectrum disorders: role of epigenetic mechanisms. *Front Psychiatry* 2014; **5**: 53.

10 Dietert RR, Dietert JM, Dewitt JC. Environmental risk factors for autism. *Emerg Health Threats J* 2011; **4**: 7111.

11 Williams PG, Hersh JH. A male with fetal valproate syndrome and autism. *Dev Med Child Neurol* 1997; **39**: 632–634.

12 Williams G, King J, Cunningham M, Stephan M, Kerr B, Hersh JH. Fetal valproate syndrome and autism: additional evidence of an association. *Dev Med Child Neurol* 2001; **43**: 202–206.

13 Rodier PM, Ingram JL, Tisdale B, Nelson S, Romano J. Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. *J Comp Neurol* 1996; **370**: 247–261.

14 Wagner GC, Reuhl KR, Cheh M, McRae P, Halladay AK. A new neurobehavioral model of autism in mice: pre- and postnatal exposure to sodium valproate. *J Autism Dev Disord* 2006; **36**: 779–793.

15 Schneider T, Przewlocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. *Neuropsychopharmacology* 2005; **30**: 80–89.

16 Servadio M, Vanderschuren LJ, Trezza V. Modeling autism-relevant behavioral phenotypes in rats and mice: do 'autistic' rodents exist? *Behav Pharmacol* 2015; **26**: 522–540.

17 Inspection générale des affaires sociales (IGAS). Enquête relative aux spécialités pharmaceutiques contenant du valproate de sodium. IGAS, France 2016.

18 Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* 2003; **83**: 1017–1066.

19 Piomelli D. The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* 2003; **4**: 873–884.

20 Di Marzo V. A brief history of cannabinoid and endocannabinoid pharmacology as inspired by the work of British scientists. *Trends Pharmacol Sci* 2006; **27**: 134–140.

21 Pacher P, Batkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* 2006; **58**: 389–462.

22 Marsicano G, Lutz B. Neuromodulatory functions of the endocannabinoid system. *J Endocrinol Invest* 2006; **29**: 27–46.

23 Marco EM, Laviola G. The endocannabinoid system in the regulation of emotions throughout lifespan: a discussion on therapeutic perspectives. *J Psychopharmacol* 2012; **26**: 150–163.

24 McLaughlin RJ, Gobbi G. Cannabinoids and emotionality: a neuroanatomical perspective. *Neuroscience* 2012; **204**: 134–144.

25 Trezza V, Campolongo P, Manduca A, Morena M, Palmery M, Vanderschuren LJ *et al.* Altering endocannabinoid neurotransmission at critical developmental ages: impact on rodent emotionality and cognitive performance. *Front Behav Neurosci* 2012; **6**: 2.

26 Chakrabarti B, Kent L, Suckling J, Bullmore E, Baron-Cohen S. Variations in the human cannabinoid receptor (CNR1) gene modulate striatal responses to happy faces. *Eur J Neurosci* 2006; **23**: 1944–1948.

27 Chakrabarti B, Baron-Cohen S. Variation in the human cannabinoid receptor CNR1 gene modulates gaze duration for happy faces. *Mol Autism* 2011; **2**: 10.

28 Maccarrone M, Rossi S, Bari M, De Chiara V, Rapino C, Musella A *et al.* Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. *Neuropsychopharmacology* 2010; **35**: 1500–1509.

29 Jung KM, Sepers M, Henstridge CM, Lassalle O, Neuhofer D, Martin H *et al.* Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. *Nat Commun* 2012; **3**: 1080.

30 Busquets-Garcia A, Gomis-Gonzalez M, Guegan T, Agustin-Pavon C, Pastor A, Mato S *et al.* Targeting the endocannabinoid system in the treatment of fragile X syndrome. *Nat Med* 2013; **19**: 603–607.

31 Liu QR, Pan CH, Hishimoto A, Li CY, Xi ZX, Llorente-Berzal A *et al.* Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Genes Brain Behav* 2009; **8**: 519–530.

32 Kerr DM, Downey L, Conboy M, Finn DP, Roche M. Alterations in the endocannabinoid system in the rat valproic acid model of autism. *Behav Brain Res* 2013; **249**: 124–132.

33 Siniscalco D, Sapone A, Giordano C, Cirillo A, de Magistris L, Rossi F *et al.* Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders. *J Autism Dev Disord* 2013; **43**: 2686–2695.

10

34 Manduca A, Servadio M, Campolongo P, Palmery M, Trabace L, Vanderschuren LJ et al. Strain- and context-dependent effects of the anandamide hydrolysis inhibitor URB597 on social behavior in rats. Eur Neuropsychopharmacol 2014; 24: 1337–1348.

35 Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A et al. Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 2003; 9: 76–81.

36 Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LW, Pasterkamp RJ et al. Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. J Neurosci 2012; 32: 14899–14908.

37 Trezza V, Vanderschuren LJ. Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 2008; 197: 217–227.

38 Patel S, Hillard CJ. Pharmacological evaluation of cannabinoid receptor ligands in a mouse model of anxiety: further evidence for an anxiolytic role for endogenous cannabinoid signaling. J Pharmacol Exp Ther 2006; 318: 304–311.

39 Moreira FA, Kaiser N, Monory K, Lutz B. Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) is mediated by CB1 receptors. Neuropharmacology 2008; 54: 141–150.

40 Wei D, Dinh D, Lee D, Li D, Anguren A, Moreno-Sanz G et al. Enhancement of anandamide-mediated endocannabinoid signaling corrects autism-related social impairment. Cannabis Cannabinoid Res 2016; 1.1: 81–89.

41 Markram K, Rinaldi T, La Mendola D, Sandi C, Markram H. Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology 2008; 33: 901–912.

42 Trezza V, Vanderschuren LJ. Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. Eur Neuropsychopharmacol 2008; 18: 519–530.

43 Segatto M, Manduca A, Lecis C, Rosso P, Jozwiak A, Swiezewska E et al. Simvastatin treatment highlights a new role for the isoprenoid/cholesterol biosynthetic pathway in the modulation of emotional reactivity and cognitive performance in rats. Neuropsychopharmacology 2014; 39: 841–854.

44 Brudzynski SM, Kehoe P, Callahan M. Sonographic structure of isolation-induced ultrasonic calls of rat pups. Dev Psychobiol 1999; 34: 195–204.

45 Scattoni ML, Gandhy SU, Ricceri L, Crawley JN. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. PLoS One 2008; 3: e3067.

46 Gulia KK, Patel N, Radhakrishnan A, Mohan Kumar V. Reduction in ultrasonic vocalizations in pups born to rapid eye movement sleep restricted mothers in rat model. PLoS One 2014; 9: e84948.

47 Moy SS, Nadler JJ, Young NB, Perez A, Holloway LP, Barbaro RP et al. Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. Behav Brain Res 2007; 176: 4–20.

48 Trezza V, Vanderschuren LJ. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. J Pharmacol Exp Ther 2009; 328: 343–350.

49 Vanderschuren LJ, Trezza V, Griffioen-Roose S, Schiepers OJ, Van Leeuwen N, De Vries TJ et al. Methylphenidate disrupts social play behavior in adolescent rats. Neuropsychopharmacology 2008; 33: 2946–2956.

50 Niesink RJ, Van Ree JM. Involvement of opioid and dopaminergic systems in isolation-induced pinning and social grooming of young rats. Neuropharmacology 1989; 28: 411–418.

51 Vanderschuren LJ, Niesink RJ, Spruijt BM, Van Ree JM. Effects of morphine on different aspects of social play in juvenile rats. Psychopharmacology (Berl) 1995; 117: 225–231.

52 Pellis SM, Pellis VC. Play-fighting differs from serious fighting in both target of attack and tactics of fighting in the laboratory rat Rattus norvegicus. Aggress Behav 1987; 13: 227–242.

53 Manduca A, Morena M, Campolongo P, Servadio M, Palmery M, Trabace L et al. Distinct roles of the endocannabinoids anandamide and 2-arachidonoylglycerol in social behavior and emotionality at different developmental ages in rats. Eur Neuropsychopharmacol 2015; 25: 1362–1374.

54 Daigle TL, Kwok ML, Mackie K. Regulation of CB1 cannabinoid receptor internalization by a promiscuous phosphorylation-dependent mechanism. J Neurochem 2008; 106: 70–82.

55 Garcia DE, Brown S, Hille B, Mackie K. Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB1 cannabinoid receptor. J Neurosci 1998; 18: 2834–2841.

56 Gandal MJ, Edgar JC, Ehrlichman RS, Mehta M, Roberts TP, Siegel SJ. Validating gamma oscillations and delayed auditory responses as translational biomarkers of autism. Biol Psychiatry 2010; 68: 1100–1106.

57 Felix-Ortiz AC, Febo M. Gestational valproate alters BOLD activation in response to complex social and primary sensory stimuli. PLoS One 2012; 7: e37313.

58 Moldrich RX, Leanage G, She D, Dolan-Evans E, Nelson M, Reza N et al. Inhibition of histone deacetylase in utero causes sociability deficits in postnatal mice. Behav Brain Res 2013; 257: 253–264.

59 Tyzio R, Nardou R, Ferrari DC, Tsintsadze T, Shahrokhi A, Eftekhari S et al. Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring. Science 2014; 343: 675–679.

60 Wohr M, Schwarting RK. Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 2013; 354: 81–97.

61 Roullet FI, Wollaston L, Decatanzaro D, Foster JA. Behavioral and molecular changes in the mouse in response to prenatal exposure to the anti-epileptic drug valproic acid. Neuroscience 2010; 170: 514–522.

62 Favre MR, Barkat TR, Lamendola D, Khazen G, Markram H, Markram K. General developmental health in the VPA-rat model of autism. Front Behav Neurosci 2013; 7: 88.

63 Degroote S, Hunting D, Sebire G, Takser L. Autistic-like traits in Lewis rats exposed perinatally to a mixture of common endocrine disruptors. Endocr Disruptors 2015; 2: e976123.

64 Melo AI, Lovic V, Gonzalez A, Madden M, Sinopoli K, Fleming AS. Maternal and littermate deprivation disrupts maternal behavior and social-learning of food preference in adulthood: tactile stimulation, nest odor, and social rearing prevent these effects. Dev Psychobiol 2006; 48: 209–219.

65 Terry LM, Johanson IB. Effects of altered olfactory experiences on the development of infant rats' responses to odors. Dev Psychobiol 1996; 29: 353–377.

66 Schneider T, Turczak J, Przewlocki R. Environmental enrichment reverses behavioral alterations in rats prenatally exposed to valproic acid: issues for a therapeutic approach in autism. Neuropsychopharmacology 2006; 31: 36–46.

67 Kim JW, Seung H, Kwon KJ, Ko MJ, Lee EJ, Oh HA et al. Subchronic treatment of donepezil rescues impaired social, hyperactive, and stereotypic behavior in valproic acid-induced animal model of autism. PLoS One 2014; 9: e104927.

68 Baronio D, Castro K, Gonchoroski T, de Melo GM, Nunes GD, Bambini-Junior V et al. Effects of an H3R antagonist on the animal model of autism induced by prenatal exposure to valproic acid. PLoS One 2015; 10: e0116363.

69 Raza S, Himmler BT, Himmler SM, Harker A, Kolb B, Pellis SM et al. Effects of prenatal exposure to valproic acid on the development of juvenile-typical social play in rats. Behav Pharmacol 2015; 26: 707–719.

70 Phan KL, Angstadt M, Golden J, Onyewuenyi I, Popovska A, de Wit H. Cannabinoid modulation of amygdala reactivity to social signals of threat in humans. J Neurosci 2008; 28: 2313–2319.

71 Scott-Van Zeeland AA, Dapretto M, Ghahremani DG, Poldrack RA, Bookheimer SY. Reward processing in autism. Autism Res 2010; 3: 53–67.

72 Baron-Cohen S, Ring HA, Bullmore ET, Wheelwright S, Ashwin C, Williams SC. The amygdala theory of autism. Neurosci Biobehav Rev 2000; 24: 355–364.

73 Bortolato M, Campolongo P, Mangieri RA, Scattoni ML, Frau R, Trezza V et al. Anxiolytic-like properties of the anandamide transport inhibitor AM404. Neuropsychopharmacology 2006; 31: 2652–2659.

74 Marco EM, Rapino C, Caprioli A, Borsini F, Maccarrone M, Laviola G. Social encounter with a novel partner in adolescent rats: activation of the central endocannabinoid system. Behav Brain Res 2011; 220: 140–145.

75 Realini N, Vigano D, Guidali C, Zamberletti E, Rubino T, Parolaro D. Chronic URB597 treatment at adulthood reverted most depressive-like symptoms induced by adolescent exposure to THC in female rats. Neuropharmacology 2011; 60: 235–243.

76 Mehta MV, Gandal MJ, Siegel SJ. mGluR5-antagonist mediated reversal of elevated stereotyped, repetitive behaviors in the VPA model of autism. PLoS One 2011; 6: e26077.

77 Morera-Herreras T, Miguelez C, Aristieta A, Ruiz-Ortega JA, Ugedo L. Endocannabinoid modulation of dopaminergic motor circuits. Front Pharmacol 2012; 3: 110.

78 Martin AB, Fernandez-Espejo E, Ferrer B, Gorriti MA, Bilbao A, Navarro M et al. Expression and function of CB1 receptor in the rat striatum: localization and effects on D1 and D2 dopamine receptor-mediated motor behaviors. Neuropsychopharmacology 2008; 33: 1667–1679.

79 Beltramo M, de Fonseca FR, Navarro M, Calignano A, Gorriti MA, Grammatikopoulos G et al. Reversal of dopamine D(2) receptor responses by an anandamide transport inhibitor. J Neurosci 2000; 20: 3401–3407.

80 Luque-Rojas MJ, Galeano P, Suarez J, Araos P, Santin LJ, de Fonseca FR et al. Hyperactivity induced by the dopamine D2/D3 receptor agonist quinpirole is attenuated by inhibitors of endocannabinoid degradation in mice. Int J Neuropsychopharmacol 2013; 16: 661–676.

81 Ferrer B, Gorriti MA, Palomino A, Gornemann I, de Diego Y, Bermudez-Silva FJ et al. Cannabinoid CB1 receptor antagonism markedly increases dopamine receptor-mediated stereotypies. Eur J Pharmacol 2007; 559: 180–183.

82 Kumar H, Sharma BM, Sharma B. Benefits of agomelatine in behavioral, neurochemical and blood brain barrier alterations in prenatal valproic acid induced autism spectrum disorder. Neurochem Int 2015; 91: 34–45.

83 Schneider T, Roman A, Basta-Kaim A, Kubera M, Budziszewska B, Schneider K et al. Gender-specific behavioral and immunological alterations in an animal model of

autism induced by prenatal exposure to valproic acid. *Psychoneuroendocrinology* 2008; **33**: 728–740.

84 Naidu PS, Varvel SA, Ahn K, Cravatt BF, Martin BR, Lichtman AH. Evaluation of fatty acid amide hydrolase inhibition in murine models of emotionality. *Psychopharmacology (Berl)* 2007; **192**: 61–70.

85 Haller J, Barna I, Barsvari B, Gyimesi Pelczer K, Yasar S, Panlilio LV *et al*. Interactions between environmental aversiveness and the anxiolytic effects of enhanced cannabinoid signaling by FAAH inhibition in rats. *Psychopharmacology (Berl)* 2009; **204**: 607–616.

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

LETTER TO THE EDITOR

Would some cannabinoids ameliorate symptoms of autism?

Rami Bou Khalil

Dear Sir,

There has been a massive growth of public awareness and research funding around autism spectrum disorders (ASD) over the past 10 years. This disorder is one of the groups of neurodevelopmental disorders known as pervasive developmental disorders characterized by three core deficits: impaired communication, impaired reciprocal social interaction and restricted, repetitive and stereotyped patterns of behaviors or interests. At present, there is no psychopharmacologic treatment for the core symptoms of autism.

In addition to their classic physiologic functions in diuresis, parturition and lactation, oxytocin and vasopressin act as important modulators of social cognition and behaviors in a diverse range of species including humans [1]. Some clinical studies have provided a body of evidence that raises optimism for the feasibility of oxytocin-based therapeutics in ASD. For example, administration of oxytocin to patients with ASD has been shown to facilitate processing of social information, improve emotional recognition, strengthen social interactions and increase eye gaze, and even reduce repetitive behaviors [2]. The effects of intranasal oxytocin on the social behavior of 13 patients suffering from high functioning ASD were investigated and compared to the effects of a placebo condition and to the behavior of matched healthy subjects. Oxytocin was shown to enhance visual scanning of faces and, in particular, of the eye region, as compared to a placebo condition. It

enhanced patients' ability to process socially relevant cues and acquire their meaning in an interactive context such as the ball-tossing task [3]. The entangled evolutionary origins of oxytocin and vasopressin in mammals are noteworthy. Although oxytocin and vasopressin have profoundly different hormonal actions in the periphery, a rich body of evidence has shown that both neuropeptides can act on similar substrates in the central nervous system to regulate the related behaviors. These intertwined actions of oxytocin and vasopressin are realized pharmacologically through a phylogenetically related family of four G-protein coupled receptors that include an oxytocin receptor and the three vasopressin receptors (V1a, V2, and V1b). Only modest differences separate the affinity and potency of oxytocin for oxytocin receptor versus its nearest pharmacologic neighbor V1a [4].

Data suggest that the transient receptor potential V2 (TRPV2) protein may play a role in mediating physiological activities associated with oxytocin and vasopressin release, such as parturition, lactation and diuresis [5]. TRPV2 protein, also known as vanilloid receptor-like 1 (VRL-1), is a member of the TRP superfamily of nonselective, ligand-gated cation channels, many of which have been shown to serve as detectors and transducers of thermal sensory stimuli. It has been demonstrated that TRPV2 is activated by moderate thermal stimuli and, in the rat, is expressed in medium to large diameter dorsal root ganglion neurons [6].

Cannabidiol (CBD) is a major nonpsychotropic constituent of cannabis sativa, which unlike the other major constituent delta9-tetrahydrocannabinol (delta9-THC), is virtually inactive at both of its central nervous system receptors. In one study, cell-based calcium mobilization and electrophysiological assays were used to identify and characterize several novel cannabinoid TRPV2 agonists in

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cultured rat dorsal root ganglion neurons. Among these, CBD was found to be the most robust and potent, followed by delta9-THC and cannabinol [7]. Those cannabinoids may, accordingly, possess the ability, due to their action as TRPV2 agonists, to increase the release of both oxytocin and vasopressin enhancing the stimulation of oxytocin receptor and V1a receptors at the same time. CBD displays a plethora of other actions including anticonvulsive, sedative, hypnotic, antipsychotic, anti-inflammatory and neuroprotective properties. CBD and delta9-THC are components of drugs commercialized, in certain countries, as treatments for neuropathic pain, overactive bladder, and spasticity in patients suffering from multiple sclerosis [8]. Thus, despite their action on oxytocin and vasopressin release, CBD and delta9-THC may help in improving symptoms of ASD by their sedative, antipsychotic, anti-convulsant and tranquilizing effects. In addition, the cannabinoid system has already been shown to be implicated in social behavior in rats [9].

The administration of cannabinoids for children and adolescents suffering from ASD is a controversial legal and ethical issue. Instead, those cannabinoids may be tested when administered to animals presenting autistic symptoms. Animal models of autistic symptoms exist especially in rodents that have their oxytocin and/or vasopressin function impaired such as mice or rats lacking the oxytocin or vasopressin gene or one of their receptors [10]. Whenever cannabinoids were found efficient in animal models of autism, the rationale supporting their efficacy may outweigh their legal and ethical adversities, when administered to children in the setting of randomized controlled studies.

References

1. Meyer-Lindenberg A, Domes G, Kirsch P, Heinrichs M (2011) Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. Nat Rev Neurosci 12:524–538
2. Green JJ, Hollander E (2010) Autism and oxytocin: new developments in translational approaches to therapeutics. Neurotherapeutics 7:250–257
3. Andari E, Duhamel JR, Zalla T, Herbrecht E, Leboyer M, Sirigu A (2010) Promoting social behavior with oxytocin in high-functioning autism spectrum disorders. Proc Natl Acad Sci USA 107:4389–4394
4. Sala M, Braida D, Lentini D et al (2011) Pharmacologic rescue of impaired cognitive flexibility, social deficits, increased aggression, and seizure susceptibility in oxytocin receptor null mice: a neurobehavioral model of autism. Biol Psychiatry 69:875–882
5. Wainwright A, Rutter AR, Seabrook GR, Reilly K, Olivier KR (2004) Discrete expression of TRPV2 within the hypothalamo-neurohypophysial system: implications for regulatory activity within the hypothalamic–pituitary–adrenal axis. J Comp Neurol 474:24–42
6. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Peterson-Zeitz KR (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science 288:306–313
7. Qin N, Neeper MP, Liu Y, Hutchinson TL, Lubin ML, Flores CM (2008) TRPV2 is activated by cannabidiol and mediates CGRP release in cultured rat dorsal root ganglion neurons. J Neurosci 28:6231–6238
8. Scuderi C, Filippis DD, Iuvone T, Blasio A, Steardo A, Esposito G (2009) Cannabidiol in medicine: a review of its therapeutic potential in CNS disorders. Phytother Res 23:597–602
9. Onaivi ES, Benno R, Halpern T et al (2011) Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders. Curr Neuropharmacol 9:209–214
10. Belzung C, Leman S, Vourc'h P, Andres C (2005) Rodents model for autism: a critical review. Drug Discov Today Dis Models 2:93–101

ORIGINAL PAPER

Cannabinoid Receptor Type 2, but not Type 1, is Up-Regulated in Peripheral Blood Mononuclear Cells of Children Affected by Autistic Disorders

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Abstract Autistic disorders (ADs) are heterogeneous neurodevelopmental disorders arised by the interaction of genes and environmental factors. Dysfunctions in social interaction and communication skills, repetitive and stereotypic verbal and non-verbal behaviours are common features of ADs. There are no defined mechanisms of pathogenesis, rendering curative therapy very difficult. Indeed, the treatments for autism presently available can be divided into behavioural, nutritional and medical approaches, although no defined standard approach exists. Autistic children display immune system dysregulation and show an altered immune response of peripheral blood mononuclear cells (PBMCs). In this study, we investigated the involvement of cannabinoid system in PBMCs from autistic children compared to age-matched normal healthy developing controls (age ranging 3–9 years; mean age: 6.06 ± 1.52 vs. 6.14 ± 1.39 in autistic children and healthy subjects, respectively). The mRNA level for cannabinoid receptor type 2 (CB2) was significantly increased in AD-PBMCs as compared to healthy subjects (mean $\pm$ SE of arbitrary units: 0.34 ± 0.03 vs. 0.23 ± 0.02 in autistic children and healthy subjects, respectively), whereas CB1 and fatty acid amide hydrolase mRNA levels were unchanged. mRNA levels of N-acylphosphatidylethanolamine-hydrolyzing phospholipase D gene were slightly decreased. Protein levels of CB-2 were also significantly increased in autistic children (mean $\pm$ SE of arbitrary units: 33.5 ± 1.32 vs. 6.70 ± 1.25 in autistic children and healthy subjects, respectively). Our data indicate CB2 receptor as potential therapeutic target for the pharmacological management of the autism care.

Keywords Autistic disorders · Cannabinoid system · Gene expression · PBMCs

Introduction

Autistic disorders (ADs) are variable heterogeneous neurodevelopmental disorders defined by deficits in social

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interaction, adaptive functioning, and communication skills, combined with repetitive and stereotypical behaviours (*Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision* (DSM-IV-TR), American Psychiatric Association 2000; Levy et al. 2009). While autism pathogenesis remains unclear, efforts to define valid treatments for ADs are being pursued. Numerous biochemical and cellular events are associated with ADs (i.e. oxidative stress, mitochondrial dysfunction, intestinal dysbiosis and immune dysregulation) (Ashwood et al. 2006; de Magistris et al. 2010). Among the immunological dysfunctions described in ADs, peripheral blood mononuclear cells (PBMCs) are reported (Enstrom et al. 2010; Siniscalco et al. 2012). AD-PBMCs show increased levels of pro-inflammatory cytokines and interleukins with resultant long-term immune alterations (Molloy et al. 2006; Onore et al. 2009). Recently, it has been demonstrated that AD-PBMCs show altered expression and activation of several caspases (Siniscalco et al. 2012). These caspases are a phylogenetically conserved structurally-related family of aspartate-specific, cysteine-dependent proteases (Lamkanfi et al. 2002). They regulate apoptosis and inflammatory signalling pathways. However, beyond apoptosis, these enzymes show other functions. Caspases are pleiotropic enzymes, functioning in cell differentiation and proliferation, as well as in activation and nuclear reprogramming pathways (Algeciras-Schimnich et al. 2002).

The endocannabinoid system consists of arachidonic acid derived compounds (endocannabinoids), their receptors and the associated enzymes (Li et al. 2011). This represents an intricate network of lipid signalling pathways (Barna and Zelena 2012). Accumulating evidence highlights that the endocannabinoid system is involved in several psychiatric disorders (i.e. autism, anxiety, major depression, bipolar disorder and schizophrenia), as well as developmental disorders (Schneider and Koch 2005; Ishiguro et al. 2010; Robinson et al. 2010; Garcia-Gutierrez and Manzanares 2011; Minocci et al. 2011).

Endocannabinoids, such as N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), are synthesized and released upon demand in a receptor-dependent way (Mouslech and Valla 2009). They exert their effects through the G-protein-coupled cannabinoid receptors CB1 and CB2, which, in turn, are negatively coupled to adenylate cyclase enzyme (Pertwee et al. 2010). After receptor binding, endocannabinoids are transported into cells by a specific uptake system and degraded by the fatty acid amide hydrolase (FAAH).

Recent studies suggested that endocannabinoids exhibit potent anti-inflammatory and immunosuppressive properties. Therefore, this pathway presents therapeutic potential for autoimmune and inflammatory diseases (Klein and Cabral 2006; Nagarkatti et al. 2009).

Schultz hypothesized acetaminophen contributes to the risk of autism via activation of the endocannabinoid system (Schultz 2010). To our knowledge, however, no studies have specifically investigated the endocannabinoid system in the development of autism. Herein we address the issue of whether these disorders are associated with changes in the expression of CB1/2 receptors and endocannabinoid metabolism enzymes, the AEA biosynthetic enzyme N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and the AEA degradative catabolic enzyme FAAH in PBMCs from AD patients.

Materials and Methods

Subjects

Informed consent was obtained from all subjects enrolled in this study in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

We investigated 17 children with autism, and compared them to 22 age and sex matched healthy children used as control group (age ranging 3–9 years; mean age: 6.06 ± 1.52 vs. 6.14 ± 1.39 in autistic and healthy individuals, respectively). The 17 subjects with autism were recruited into the study from the outpatient Centre for Autism of La Forza del Silenzio, Naples-Caserta, Italy. The cohort included 14 boys and 3 girls. Before entering the study, all of the children were administered the Autism Diagnostic Interview-Revised version (Lord et al. 1994), the Childhood Autism Rating Scales (Schopler et al. 1993), and the Autism Diagnostic Observation Schedule-Generic (Lord et al. 2000) to document the diagnosis of autism. All included patients met the Diagnostic and Statistical Manual of Mental Disorders-IV criteria for autism (DSM-IV-TR) (American Psychiatric Association 2000). In addition to meeting the criteria for autistic disorder (AD), subject children were required to score at least 30 points on the CARS scale. Twenty-two healthy children (females 4, males 18) were recruited among staff family members. Potential subjects were excluded if they had any of the following: a neurological or comorbid psychiatric disorder, epilepsy, history of liver, renal or endocrine disorders, current infection of any origin. Mental retardation or behavioural disorders, including Pervasive Developmental Disorder—not otherwise specified (PDD-NOS), inclusion criteria for attention deficit-hyperactivity disorder, were all considered exclusion criteria for control children. Children diagnosed with Asperger's syndrome, fragile X syndrome and tuberous sclerosis were also excluded from the study. IQ test was not performed. Neither AD subjects nor

controls had special diets or other pharmacological interventions. Other exclusion criteria were coeliac disease and/or other major diseases of the intestinal tract, such as inflammatory bowel disease or hepatic disorders.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Fresh peripheral blood samples from AD subjects and control donors were drawn and collected in sterile EDTA tubes (Becton–Dickinson, Franklin Lakes, NJ, USA). Peripheral blood mononuclear cells (PMBCs) were isolated by centrifugation over Histopaque 1,077 density gradient (Sigma Chemical, St Louis, MO, USA). Briefly, blood was diluted 1:1 in phosphate buffer saline (PBS) (Sigma, St. Louis, MO, USA), overlaid onto lymphocyte separation media (Lymphocyte Separation Medium—Lonza, Walkersville, MD, USA), centrifuged at 2,200 rpm for 30 min at room temperature and plasma was removed. Mononuclear cell fraction was harvested and washed twice in PBS. The final pellet was re-suspended in Tri-Reagent solution (Molecular Research Center Inc., Cincinnati, OH, USA) or protein lysis buffer for further molecular analysis.

RNA Extraction and RT-PCR

The RNA was extracted from PBMCs using a RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. The total RNA concentration and integrity were determined by Nanodrop ND-1000 UV spectrophotometer (Nano-Drop® Technologies, Thermo Scientific, Wilmington, DE, USA). The mRNA levels of the endocannabinoid genes under analysis were measured by RT-PCR amplification, as previously reported (Siniscalco et al. 2012). Reverse Transcriptase from Avian Myeloblastosis Virus (AMV-RT; Promega, Madison, WI, USA) was used. For first-strand cDNA synthesis 200 ng total RNA, random examers, dNTPs (Promega, Madison, WI, USA), AMV buffer, AMV-RT and recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) were assembled in diethylpyrocarbonate-treated water to a 20 μl final volume and incubated for 10 min at 65 °C and 1 h at 42 °C. RT minus controls were carried out to check potential genomic DNA contamination. These RT minus controls were performed without using the reverse transcriptase enzyme in the reaction mix. Aliquots of 2 μl cDNA were transferred into a 25 μl PCR reaction mixture containing dNTPs, MgCl₂, reaction buffer, specific primers and GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA), and amplification reactions using specific primers and conditions for human genes under analysis cDNA were carried out. Sequences for the human mRNAs from GeneBank

(DNASTAR INC., Madison, WI, USA) were used to design specific primer pairs for RT-PCRs (OLIGO 4.05 software, National Biosciences Inc., Plymouth, MN, USA) (Table 1). Each RT-PCR was repeated at least three times to achieve the best reproducibility data. The mean of the inter-assay variability of each RT-PCR assay was 0.07. The levels of mRNA measured were normalized with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was chosen as the housekeeping gene. Indeed GAPDH is one of the most stably expressed genes in human peripheral blood (Stamova et al. 2009). To our knowledge, there is no molecular evidence of variation in GAPDH mRNA-levels in autism disorders (Siniscalco et al. 2012). The gene expression values were expressed as arbitrary units ± SEM. Amplification of the genes of interest and GAPDH was performed simultaneously. PCR products were resolved into 2.0 % agarose gel. A semi-quantitative analysis of mRNA levels was carried out by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA, USA).

Protein Extraction and Western Blot Analysis

For protein extraction, PBMCs were suspended in protein lysis buffer [HEPES 25 mM; EDTA 5 mM; SDS 1 %; Triton X-100 1 %; PMSF 1 mM; MgCl₂ 5 mM; Protease Inhibitor Cocktail (Roche, Mannheim, Germany); Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany)]. Protein concentration was determined using the method described by Bradford (1976). Each sample was loaded, electrophoresed in a 15 % SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was blocked in 5 % milk, 1X Tris-buffered saline and 0.05 % Tween-20. Primary antibodies to detect CB2 (Calbiochem-Merck, Darmstadt, Germany) were used according to the manufacturer's instructions at 1:250 dilutions. The rabbit anti-CB2 antibody detects endogenous levels of the human 45 kDa fragment of CB2 receptor protein. The antibody does not cross-react with the CB1 receptor protein and, according to the manufacturer, was validated with a recombinant protein consisting of the first 33 amino acids of human CB2 receptor used as a positive control. Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody and reacted with an ECL system (Amersham Pharmacia, Uppsala, Sweden). To assess equal loading, protein levels were normalized with respect to the signal obtained with Ponceau S staining, as previously reported (Alessio et al. 2010; Romero-Calvo et al. 2010; Zanichelli et al. 2012). We used Ponceau S staining over actin as equal loading control as this method has a better dynamic range and overcomes the possibility that housekeeping proteins could vary in this pathology or be saturated at the levels of

Table 1 Primer sequences, annealing temperatures, and product sizes for RT-PCRs

Gene	Sense primer	Antisense primer	Annealing temperature °C	Product sizes bp
CB1	5'-CAAGGAGAATGAGGAGAACA-3'	5'-CCAGCGTGAGGGACAGGACT-3'	55	318
CB2	5'-TTGGCAGCGTGACTATGACC-3'	5'-AGGAAGGCGATGAACAGGAG-3'	55	274
FAAH	5'-GGCCACACCTTCCTACAGAA-3'	5'-GTTTTGCGGTACACCTCGAT-3'	58	218
NAPE-PLD	5'-GAAGCTGGCTTAAGAGTCAC-3'	5'-CCGCATCTATTGGAGGGAGT-3'	60	178
GAPDH	5'-TCACCAGGGCTGCTTTTAAC-3'	5'-GGACTCCACGACGTACTCAG-3'	55	242

PCR primers were designed by using the computer program OLIGO 4.05 software (National Biosciences Inc., Plymouth, MN, USA) and were purchased from PRIMM (Milan, Italy)

loading (Romero-Calvo et al. 2010). The semi-quantitative analysis of protein levels was carried out by the Chemi-Doc-It 5000, using VisionWorks Life Science Image Acquisition and Analysis software (UVP, Upland, CA, USA).

Immunocytochemistry

For immunocytochemical analysis, PBMCs were extracted and plated as previously reported (Siniscalco et al. 2012). In brief, mononuclear cells were re-suspended at 1×10^6 cell/mL in RPMI 1640 complete medium (Lonza, Verviers, Belgium) containing 10 % fetal bovine serum (FBS) (EuroClone-Celbio, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Lonza), were plated on slides with a 12-well plate and incubated for 4 days at 37 °C with 5 % CO_2. Cells were then fixed with 4 % paraformaldheyde fixative. After washing in PBS, non-specific antibody binding was inhibited by incubation for 30 min in blocking solution (1 % BSA in PBS). Primary antibodies were diluted in PBS blocking buffer and slides were incubated overnight at 4 °C in primary antibodies to human CB1 receptor or to human CB2 receptor (either diluted at 1:200; Calbiochem-Merck, Darmstadt, Germany). Fluorescent-labeled secondary antibodies (1:1,000; Alexa Fluor 488 (for CB1) and 568 (for CB2), Molecular Probe; Invitrogen, Carlsbad, CA, USA) specific to the IgG species used as a primary antibody were used to locate the specific antigens in each slide. Cells were counterstained with bisbenzimide (Hoechst 33258; Hoechst, Frankfurt, Germany) and mounted with mounting medium (90 % glycerol in PBS). Fluorescently labelled slides were viewed with a fluorescence microscope (Leica, Wetzlar, Germany). Immunofluorescence images were analyzed with Leica FW4000 software (Leica, Wetzlar, Germany). Only bis-benzimide counterstained cells were considered as positive profiles so as to avoid overcounting cells.

Statistical Analysis

Biomolecular data are expressed as mean ± SEM ANOVA, followed by Student–Neuman–Keuls post hoc test, was used to determine the statistical significance among groups, without correction for multiple test comparison. $p < 0.05$ was considered statistically significant.

Results

AD- Related Changes in Endocannabinoid System Gene Expressions

We examined endocannabinoid system gene expression mainly by RT-PCR, since this technique is a far more sensitive method for the detection of gene expression than immunocytochemistry (Giordano et al. 2011; Siniscalco et al. 2012). In addition, the genes analysed showed a transcriptional regulative mechanism (Galiègue et al. 1995; Maccarrone et al. 2001; Nong et al. 2002). When compared to controls, the semiquantitative analysis of PBMC-extracted mRNA levels, measured by RT-PCR amplification, showed an increase in the CB2 receptor gene in PBMCs of AD patients (mean ± SE of arbitrary units: 0.34 ± 0.03 vs. 0.23 ± 0.02, $p < 0.05$, in autistic children and healthy subjects, respectively), whereas mRNA levels of NAPE-PLD gene were slightly decreased (mean ± SE of arbitrary units: 0.25 ± 0.04 vs. 0.39 ± 0.03, $p < 0.05$, in autistic children and healthy subjects, respectively); mRNA levels of CB1 receptor (mean ± SE of arbitrary units: 0.51 ± 0.05 vs. 0.69 ± 0.07, $p > 0.05$, in autistic children and healthy subjects, respectively) and FAAH enzyme gene (mean ± SE of arbitrary units: 0.38 ± 0.10 vs. 0.48 ± 0.08, $p > 0.05$, in autistic children and healthy subjects, respectively) were not different (Fig. 1; Table 2).

Fig. 1 Over-expression of CB2 receptor gene, but not of CB1 receptor and FAAH enzyme, and down-expression of NAPE-PLD gene in AD-PBMCs. The measured mRNA levels were normalized with respect to *GAPDH* (housekeeping gene) and gene expression values were expressed as a percentage of arbitrary units ± SEM *open circle* indicates significant difference versus healthy controls. *p* values <0.05 were considered statistically significant. *CTL* healthy control subjects, *AD* autistic patients
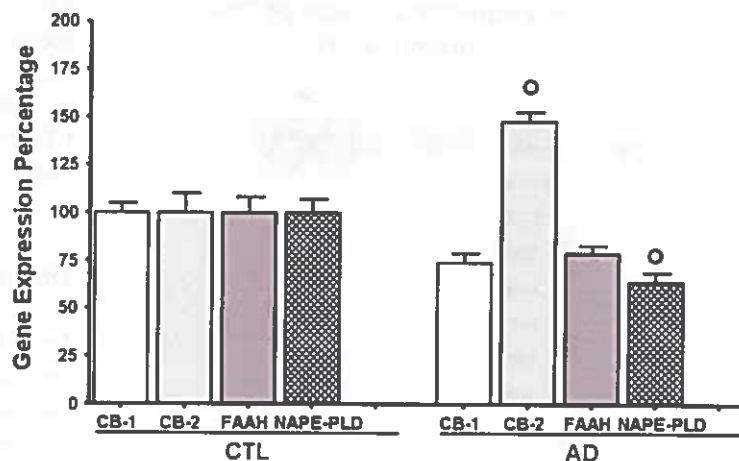
Table 2 The mRNA levels (mean ± SE) of the genes under analysis measured by RT-PCR amplification are reported

Gene	Healthy subjects	PCR coefficient of variation	Autistic patients	PCR coefficient of variation	*p* value	$F_{(1-37)}$
CB-1/GAPDH	0.69 ± 0.07	0.08	0.51 ± 0.05	0.05	0.056	3.91
CB-2/GAPDH	0.23 ± 0.02	0.07	0.34 ± 0.03*	0.05	0.003	9.99
FAAH/GAPDH	0.48 ± 0.08	0.06	0.38 ± 0.10	0.08	0.434	0.63
NAPE-PLD/GAPDH	0.39 ± 0.03	0.05	0.25 ± 0.04*	0.07	0.007	8.17

Each RT-PCR was repeated at least three times. The semi-quantitative analysis of mRNA levels was carried out by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA, USA). The measured mRNA levels were normalized with respect to *GAPDH* (housekeeping gene) and gene expression values were expressed as arbitrary units ± SE. *$p < 0.05$ versus the corresponding healthy controls, as analyzed by four separate ANOVAs, followed by Student–Neuman–Keuls test. The mean of the inter-assay variability of each RT-PCR assay was also reported as PCR coefficient of variation. The values of variance *p* and F rate are also reported (critical alpha is set to 0.05)

CB2 Protein Levels are Increased in AD-PBMCs

As G protein-coupled receptors, the cannabinoid receptors (CBs) also show post-translational regulation (Ardura and Friedman 2011; Peralta et al. 2011). To confirm gene expression change, we therefore determined the protein levels of CB2 receptor by western blot analysis, as well as by fluorescence immunocytochemistry.

To confirm a lack of change in protein levels for CB1 receptor as implied by mRNA levels, we performed immunocytochemical analysis also for this receptor. Western blot analysis showed a remarkable increase in CB2 protein levels in AD children as compared to healthy controls (Fig. 2) (mean ± SE of arbitrary units: 33.5 ± 1.32 vs. 6.70 ± 1.25, *p* < 0.05, in autistic children and healthy subjects, respectively). Protein level analysis in AD patients and control groups was performed simultaneously. CB2 protein level was enhanced in all the AD children evaluated. The control group demonstrated no intragroup variances of significance.

The difference between the increase in CB2 mRNA and in the increase in CB2 protein in AD group is not surprising. Post-translational control of protein function has been described to affect protein levels. Indeed, the CB2 protein, as G protein coupled receptor, show a multilevel

system of regulation, that affects the levels of receptor in the cell (Ardura and Friedman 2011; Peralta et al. 2011; Tománková and Myslivecek 2012).

Moreover, the levels of cellular mRNAs can be regulated by controlling the rate at which the mRNA decays (Wilusz et al. 2001). It is noteworthy to consider that there is not a direct correlation between mRNA transcripts and protein levels. Gene expression is also regulated by the control of mRNA degradation, since the steady-state concentration of mRNA is determined both by its rates of synthesis and decay (Rajagopalan and Malter 1997; Meyer et al. 2004). Changes in mRNA half-life do not reflect changes in transcription (Ross 1996). More importantly, the correlation between mRNA and protein abundances in the cell is insufficient to predict protein expression levels from quantitative mRNA data (Gygi et al. 1999; Maier et al. 2009). Determining a direct relationship between mRNA and protein levels can be problematic (Pascal et al. 2008). However, mRNA expression is informative in the prediction of protein expression (Guo et al. 2008). Increasing in both mRNA and correspondent protein is indicative of a positive correlation between mRNA and protein expression levels (Guo et al. 2008; Yang et al. 2013). Using several and different techniques (i.e. RT-PCR, Western blot, immunocytochemistry), as used here,

Immunoblot analysis of CB2 protein levels
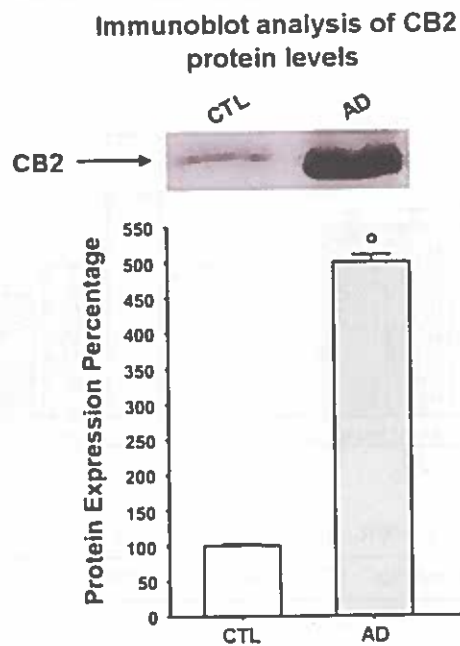
Fig. 2 Representative western blot analysis of CB2 protein levels in the PBMCs obtained from the autistic children and the healthy controls, respectively. *CTL* healthy control subjects, *AD* autistic disorder subjects. The histograms indicate percentage variations in CB2 protein levels in the PBMCs of AD children compared to the healthy controls (CTL). *open circle* indicates significant differences versus healthy subjects. $p < 0.05$ was considered as the level of significance

to study changes in gene expression is a valid tool to assess the correlation between these macromolecules inside the cell (Dong et al. 2012).

Immunofluorescence analysis was carried out using an antibody able to detect endogenous levels of the human CB2 receptor protein, without cross-reacting with the CB1 receptor protein. Immunofluorescence staining confirmed that CB2 was over-expressed in the PBMCs in AD children as compared to healthy controls, while no difference in CB1 receptor related signals were observed in AD children respect to healthy controls (Fig. 3).

Discussion

In this study, we demonstrated for the first time the up-regulation of CB2 receptors in PBMCs from ADs subjects. No differences were observed for CB1 receptor regulation. Alterations in endocannabinoid levels are transient adaptive reactions which attempt to re-establish normal homeostasis disrupted by the disease. However, in some conditions, endocannabinoid systems appear to contribute to a chronic maladaptive disease state (Di Marzo and Petrosino 2007). Emerging studies highlight that endocannabinoid signalling through CB2 receptors could activate a protective system. CB2 receptor activation is known to trigger immune sup-pression (Hegde et al. 2010). After inflammation or tissue injury, there is a rapid increase in local endocannabinoid levels, which appears to mediate immune responses through down-regulation of cytokine expressions (Jean-Gilles et al. 2010; Pacher and Mechoulam 2011). The immunomodu-latory effects of endocannabinoids are mainly mediated by the CB2 receptor expressed on immune cells (Klein et al. 2003; Cencioni et al. 2010). The CB2 gene, which is not expressed in the brain, is principally expressed in immune tissues (Kenny 2011); whereas CB1 is abundant in the central nervous system (Galiègue et al. 1995). It's

Fig. 3 Representative fluorescent photomicrograph of PBMCs showing immunocytochemistry for CB1 receptor (*bottom*) and CB2 receptor (*top*). *Top Arrows* indicate CB2 positive staining (*red fluorescent*). *Bottom Arrows* indicate CB1 positive staining (*green fluorescent*). To correctly identify cells, their nuclei were counterstained with bisbenzimide (*blue fluorescence*), as shown in panel a. a healthy control subjects; b autistic disorder patients. *Scale bars* 15 μm

CB2 receptor positive staining (red fluorescence)

CB1 receptor positive staining (green fluorescence)
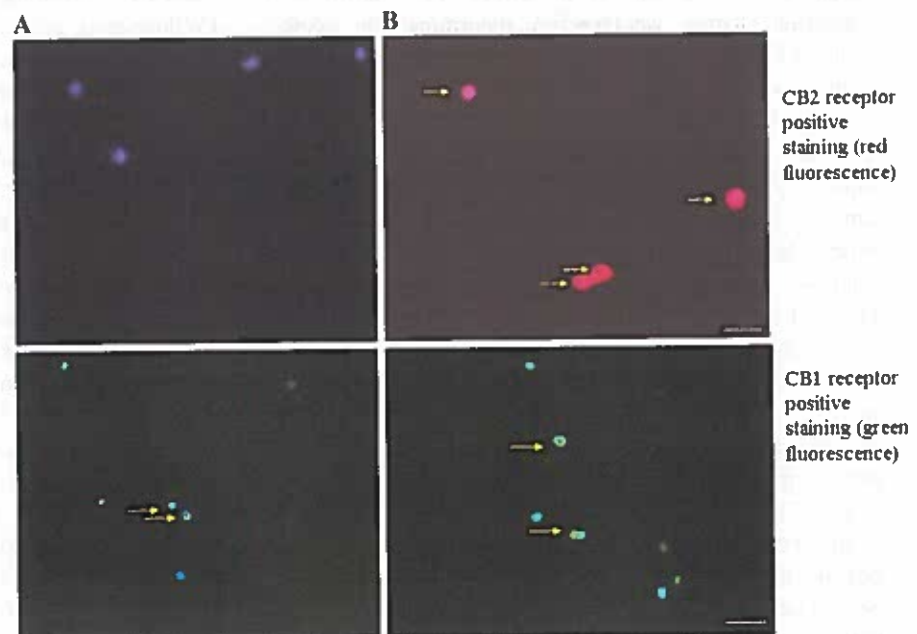
noteworthy that CB2 receptors regulate cannabinoid-induced immune modulation (Tanikawa et al. 2011). Cannabinoids are involved in B cell activation and maturation through the CB2 receptor. Importantly, B lymphocytes express the highest level of CB2 mRNA relative to other immune cells (Agudelo et al. 2008). In addition, CB2 receptor is able to modulate development, migration, proliferation, and effector functions of immune cells (Basu and Dittel 2011). Alterations in immune system in autism pathogenesis have been reported (Gupta et al. 2010; Suzuki et al. 2011). Moreover, AD-PBMCs show increased activation of both Th1- and Th2- mediated immune response, altered cytokine profiles, decreased lymphocyte numbers, imbalance of serum immunoglobulin levels and caspase-mediated immune response changes (Ashwood et al. 2006; Li et al. 2009; Siniscalco et al. 2012). These observations, when combined with the present study data, are suggestive that CB2 receptor up-regulation in PBMCs could be related to AD-immune dysregulation. It is well established that

these cells are key regulators of the immune pathways, and a dysregulation in the PBMC response could result in long-term immune alterations seen in AD (Enstrom et al. 2010). The CB2 receptor alterations we observed in AD-PBMCs indicate the endocannabinoid system may be functionally involved in AD pathogenesis or maintenance. The fact that in PBMCs from autistic children we observed only CB2 receptor changes, but not CB1 and/or the anandamide catabolic enzyme FAAH, could indicate that the main action played by endocannabinoids in these cells is to regulate inflammation and immune responses. CB1 receptors do not seem involved in mediating these events. However, our data cannot exclude CB1 receptor up-regulation in other cell types or within the central nervous system. It is noteworthy that pro-inflammatory stimuli suppress NAPE-PLD expression (Zhu et al. 2011). In fact, the slight down-regulation we observed in mRNA levels for this biosynthetic enzyme could be related to the inflammatory state associated with autism immunopathology.
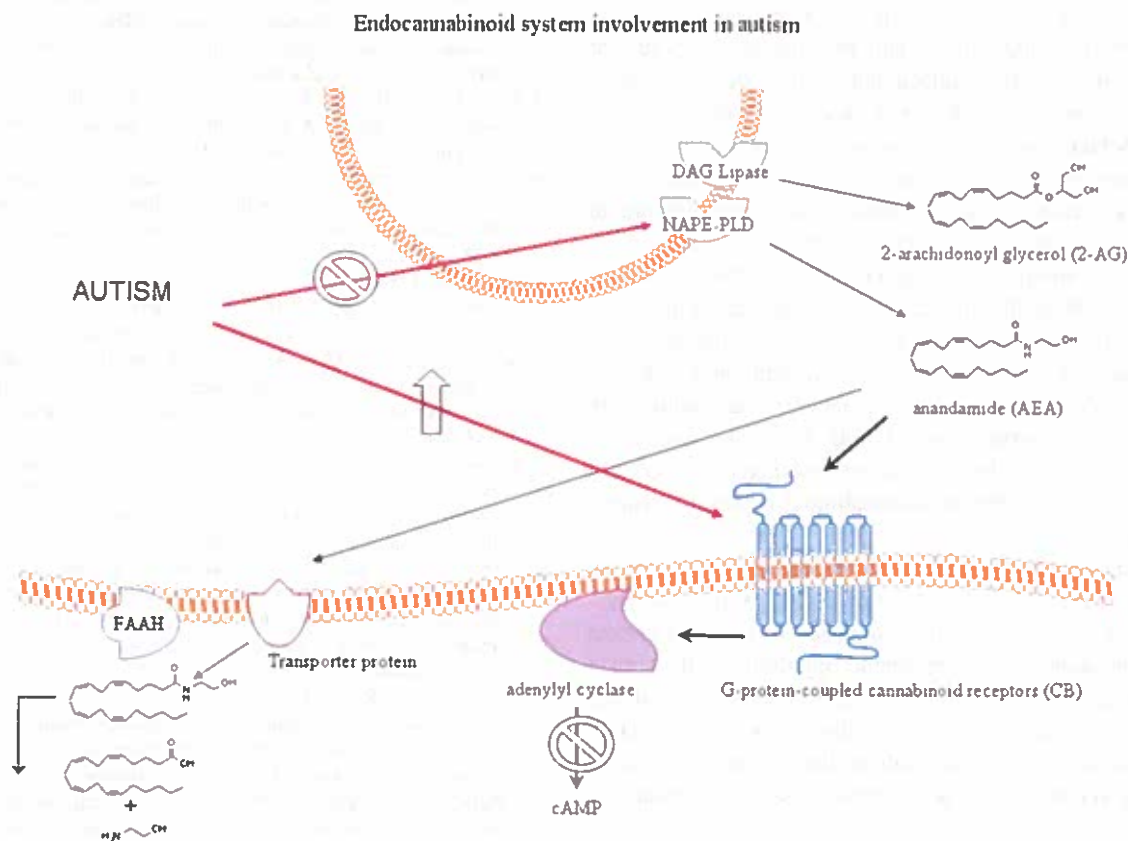
Endocannabinoid system involvement in autism

Fig. 4 Endocannabinoids, such as N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), are synthesized and released upon demand in a receptor-dependent way, through the AEA biosynthetic enzyme N-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and the diacylglycerol (DAG) lipase enzyme, respectively. They exert their effects through the G-protein-coupled cannabinoid receptors CB1 and CB2, which, in turn, are negatively coupled to adenylyl cyclase enzyme. After the specific binding with their receptors, endocannabinoids are transported into cells by a specific uptake system and degraded by the enzymes fatty acid amide hydrolase (FAAH). In peripheral blood mononuclear cells, autistic disorders trigger over-production of CB2 receptor gene expression, as well as protein levels, together with a down-expression of NAPE-PLD

Another hypothesis could be related to a CB2 protective response to AD-mediated inflammatory stimuli derived from the capacity of CB2 to inhibit pro-inflammatory cytokine synthesis and release (Di Filippo et al. 2004). However, it has been demonstrated that pro-inflammatory cytokines are abundantly increased in the plasma of autistic patients (Ashwood et al. 2011). These data, when combined with our study's observations, enhance the hypothesis of a correlation between CB2-mediated immune dysfunction and autism pathophysiology. This further indicates that the endocannabinoid system, through CB2 receptors, could mediate a cross-talk between immune and nervous systems.

As previously mentioned, Schultz reviewed the possible autism activation by endocannabinoid system (Schultz 2010). He reviewed data revealing sulfation deficits in acetaminophen (paracetamol) metabolism with the autism population. Acetaminophen administration in the presence of a sulfation deficiency, creates a metabolic by-product, N-arachidonoylphenolamine (AM404), causing an indirect increase of endocannabinoids levels (Högestätt et al. 2005; Soukupová et al. 2010), which in turn activate CB1/2 receptors triggering autism. This hypothesis invites further consideration of the endocannabinoid system regarding autism pathogenesis However, acetaminophen was not routinely taken by any of the subjects of this investigation, so its involvement with endocannabinoids remains speculative. Nevertheless, the question of the endocannabinoid system involvement in autism pathogenesis remains a potentially important concept deserving further investigation. Apart from the endocannabinoid system, other environmental autism risk factors (i.e. environmental toxics exposure, parental age, low birth weight, and maternal infections during pregnancy) are under consideration. Any of these may further interact with the endocannabinoid system as well. Further experiments are needed in order to better characterize the endocannabinoid system's involvement in AD.

In conclusion, to our knowledge, this is the first study demonstrating an endocannabinoid-CB2 signalling dysregulation in autism, implying the endocannabinoid system may represent a new treatment opportunity for autism pharmacotherapy (Fig. 4). While the therapeutic use of the endocannabinoid systems is inviting, extensive research will be required to further evaluate this complex regulatory pathway and the safety of pharmacological manipulation.

References

Agudelo, M., Newton, C., Widen, R., Sherwood, T., Nong, L., Friedman, H., et al. (2008). Cannabinoid receptor 2 (CB2) mediates immunoglobulin class switching from IgM to IgE in cultures of murine-purified B lymphocytes. *Journal of Neuroimmune Pharmacology, 3*(1), 35–42.

Alessio, N., Squillaro, T., Cipollaro, M., Bagella, L., Giordano, A., & Galderisi, U. (2010). The BRG1 ATPase of chromatin remodeling complexes is involved in modulation of mesenchymal stem cell senescence through RB-P53 pathways. *Oncogene, 29*(40), 5452–5463.

Algeciras-Schimnich, A., Barnhart, B. C., & Peter, M. E. (2002). Apoptosis independent functions of killer caspases. *Current Opinion in Cell Biology, 14*, 721–726.

American Psychiatric Association. (2000). *Diagnostic and statistical manual of mental disorders* (4th ed.). Text Revision. Washington, DC: American Psychiatric Press.

Ardura, J. A., & Friedman, P. A. (2011). Regulation of g protein-coupled receptor function by na +/h + exchange regulatory factors. *Pharmacological Reviews, 63*(4), 882–900.

Ashwood, P., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Pessah, I., & Van de Water, J. (2011). Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. *Brain, Behavior, and Immunity, 25*(1), 40–45.

Ashwood, P., Wills, S., & Van de Water, J. (2006). The immune response in autism: A new frontier for autism research. *Journal of Leukocyte Biology, 80*(1), 1–15.

Barna, I., & Zelena, D. (2012). The biochemical complexity of the endocannabinoid system with some remarks on stress and related disorders: A minireview. *Endocrine Regulations, 46*(2), 107–124.

Basu, S., & Dittel, B. N. (2011). Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. *Immunologic Research, 51*(1), 26–38.

Bradford, M. M. (1976). A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Cencioni, M. T., Chiurchiù, V., Catanzaro, G., Borsellino, G., Bernardi, G., Battistini, L., et al. (2010). Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. *PLoS ONE, 5*(1), e8688.

de Magistris, L., Familiari, V., Pascotto, A., Sapone, A., Frolli, A., Iardino, P., et al. (2010). Alterations of the intestinal barrier in patients with autism spectrum disorders and in their first-degree relatives. *Journal of Pediatric Gastroenterology and Nutrition, 51*(4), 418–424.

Di Filippo, C., Rossi, F., Rossi, S., & D'Amico, M. (2004). Cannabinoid CB2 receptor activation reduces mouse myocardial ischemia-reperfusion injury: Involvement of cytokine/chemokines and PMN. *Journal of Leukocyte Biology, 75*(3), 453–459.

Di Marzo, V., & Petrosino, S. (2007). Endocannabinoids and the regulation of their levels in health and disease. *Current Opinion in Lipidology, 18*(2), 129–140.

Dong, R., Dong, K., Wang, X., Chen, G., Shen, C., & Zheng, S. (2012). Interleukin-33 overexpression is associated with gamma-glutamyl transferase in biliary atresia. *Cytokine, S1043–4666*(12), 00771–00775.

Enstrom, A. M., Onore, C. E., Van de Water, J. A., & Ashwood, P. (2010). Differential monocyte responses to TLR ligands in

children with autism spectrum disorders. *Brain, Behavior, and Immunity, 24*(1), 64–71.

Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, D., Carayon, P., et al. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *European Journal of Biochemistry, 232*(1), 54–61.

Garcia-Gutierrez, M. S., & Manzanares, J. (2011). Overexpression of CB2 cannabinoid receptors decreased vulnerability to anxiety and impaired anxiolytic action of alprazolam in mice. *Journal of Psychopharmacology, 25*(1), 111–120.

Giordano, C., Siniscalco, D., Melisi, D., Luongo, L., Curcio, A., Soukupova, M., et al. (2011). The galactosylation of N(ω)-nitro-L-arginine enhances its anti-nocifensive or anti-allodynic effects by targeting glia in healthy and neuropathic mice. *European Journal of Pharmacology, 656*(1–3), 52–62.

Guo, Y., Xiao, P., Lei, S., Deng, F., Xiao, G. G., Liu, Y., et al. (2008). How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. *Acta Biochimica et Biophysica Sinica (Shanghai), 40*(5), 426–436.

Gupta, S., Samra, D., & Agrawal, S. (2010). Adaptive and innate immune responses in autism: rationale for therapeutic use of intravenous immunoglobulin. *Journal of Clinical Immunology, 30*, S90–S96.

Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology, 19*(3), 1720–1730.

Hegde, V. L., Nagarkatti, M., & Nagarkatti, P. S. (2010). Cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties. *European Journal of Immunology, 40*(12), 3358–33571.

Högestätt, E. D., Jönsson, B. A., Ermund, A., Andersson, D. A., Björk, H., Alexander, J. P., et al. (2005). Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. *Journal of Biological Chemistry, 280*(36), 31405–31412.

Ishiguro, H., Horiuchi, Y., Ishikawa, M., Koga, M., Imai, K., Suzuki, Y., et al. (2010). Brain cannabinoid CB2 receptor in schizophrenia. *Biological Psychiatry, 67*(10), 974–982.

Jean-Gilles, L., Gran, B., & Constantinescu, C. S. (2010). Interaction between cytokines, cannabinoids and the nervous system. *Immunobiology, 215*(8), 606–610.

Kenny, P. J. (2011). Macrophage cannabinoid receptor goes up in smoke. *Nature Neuroscience, 14,* 1100–1102.

Klein, T. W., & Cabral, G. A. (2006). Cannabinoid-induced immune suppression and modulation of antigen-presenting cells. *Journal of Neuroimmune Pharmacology, 1*(1), 50–64.

Klein, T. W., Newton, C., Larsen, K., Lu, L., Perkins, I., Nong, L., et al. (2003). The cannabinoid system and immune modulation. *Journal of Leukocyte Biology, 74*(4), 486–496.

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X., & Vandenabeele, P. (2002). Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death and Differentiation, 9,* 358–361.

Levy, S. E., Mandell, D. S., & Schultz, R. T. (2009). Autism. *Lancet, 374*(9701), 1627–1638.

Li, X., Chauhan, A., Sheikh, A. M., Patil, S., Chauhan, V., Li, X. M., et al. (2009). Elevated immune response in the brain of autistic patients. *Journal of Neuroimmunology, 207*(1–2), 111–116.

Li, C., Jones, P. M., & Persaud, S. J. (2011). Role of the endocannabinoid system in food intake, energy homeostasis and regulation of the endocrine pancreas. *Pharmacology & Therapeutics, 129*(3), 307–320.

Lord, C., Risi, S., Lambrecht, L., Cook, E. H., Jr, Leventhal, B. L., DiLavore, P. C., et al. (2000). The autism diagnostic observation schedule-generic: A standard measure of social and communication deficits associated with the spectrum of autism. *Journal of Autism and Developmental Disorders, 30*(3), 205–223.

Lord, C., Rutter, M., & Le Couteur, A. (1994). Autism diagnostic interview-revised: A revised version of a diagnostic interview for caregivers of individuals with possible Pervasive Developmental Disorders. *Journal of Autism and Developmental Disorders, 24*(5), 659–685.

Maccarrone, M., De Petrocellis, L., Bari, M., Fezza, F., Salvati, S., Di Marzo, V., et al. (2001). Lipopolysaccharide downregulates fatty acid amide hydrolase expression and increases anandamide levels in human peripheral lymphocytes. *Archives of Biochemistry and Biophysics, 393*(2), 321–328.

Maier, T., Güell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. *FEBS Letters, 583*(24), 3966–3973.

Meyer, S., Temme, C., & Wahle, E. (2004). Messenger RNA turnover in eukaryotes: Pathways and enzymes. *Critical Reviews in Biochemistry and Molecular Biology, 39*(4), 197–216.

Minocci, D., Massei, J., Martino, A., Milianti, M., Piz, L., Di Bello, D., et al. (2011). Genetic association between bipolar disorder and 524A > C (Leu133Ile) polymorphism of CNR2 gene, encoding for CB2 cannabinoid receptor. *Journal of Affective Disorders, 134*(1–3), 427–430.

Molloy, C. A., Morrow, A. L., Meinzen-Derr, J., Schleifer, K., Dienger, K., Manning-Courtney, P., et al. (2006). Elevated cytokine levels in children with autism spectrum disorder. *Journal of Neuroimmunology, 172*(1–2), 198–205.

Mouslech, Z., & Valla, V. (2009). Endocannabinoid system: An overview of its potential in current medical practice. *Neuro Endocrinology Letters, 30*(2), 153–179.

Nagarkatti, P., Pandey, R., Rieder, S. A., Hegde, V. L., & Nagarkatti, M. (2009). Cannabinoids as novel anti-inflammatory drugs. *Future Medicinal Chemistry, 1*(7), 1333–1349.

Nong, L., Newton, C., Cheng, Q., Friedman, H., Roth, M. D., & Klein, T. W. (2002). Altered cannabinoid receptor mRNA expression in peripheral blood mononuclear cells from marijuana smokers. *Journal of Neuroimmunology, 127*(1–2), 169–176.

Onore, C., Enstrom, A., Krakowiak, P., Hertz-Picciotto, I., Hansen, R., Van de Water, J., et al. (2009). Decreased cellular IL-23 but not IL-17 production in children with autism spectrum disorders. *Journal of Neuroimmunology, 216*(1–2), 126–129.

Pacher, P., & Mechoulam, R. (2011). Is lipid signaling through cannabinoid 2 receptors part of a protective system? *Progress in Lipid Research, 50*(2), 193–211.

Pascal, L. E., True, L. D., Campbell, D. S., Deutsch, E. W., Risk, M., Coleman, I. M., et al. (2008). Correlation of mRNA and protein levels: Cell type-specific gene expression of cluster designation antigens in the prostate. *BMC Genomics, 9*, 246.

Peralta, L., Agirregoitia, E., Mendoza, R., Expósito, A., Casis, L., Matorras, R., et al. (2011). Expression and localization of cannabinoid receptors in human immature oocytes and unfertilized metaphase-II oocytes. *Reprod Biomed Online, 23*(3), 372–379.

Pertwee, R. G., Howlett, A. C., Abood, M. E., Alexander, S. P., Di Marzo, V., Elphick, M. R., et al. (2010). Cannabinoid receptors and their ligands: Beyond CB1 and CB2. *Pharmacological Reviews, 62*(4), 588–631.

Rajagopalan, L. E., & Malter, J. S. (1997). Regulation of eukaryotic messenger RNA turnover. *Progress in Nucleic Acid Research and Molecular Biology, 56*, 257–286.

Robinson, S. A., Loiacono, R. E., Christopoulos, A., Sexton, P. M., & Malone, D. T. (2010). The effect of social isolation on rat brain expression of genes associated with endocannabinoid signaling. *Brain Research, 1343*, 153–167.

Romero-Calvo, I., Ocón, B., Martínez-Moya, P., Suárez, M. D., Zarzuelo, A., Martínez-Augustin, O., et al. (2010). Reversible

Consequences of Cannabinoid and Monoaminergic System Disruption in a Mouse Model of Autism Spectrum Disorders

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Abstract: Autism spectrum disorders (ASDs) are heterogenous neurodevelopmental disorders characterized by impairment in social, communication skills and stereotype behaviors. While autism may be uniquely human, there are behavioral characteristics in ASDs that can be mimicked using animal models. We used the BTBR T+tf/J mice that have been shown to exhibit autism-like behavioral phenotypes to 1). Evaluate cannabinoid-induced behavioral changes using forced swim test (FST) and spontaneous wheel running (SWR) activity and 2). Determine the behavioral and neurochemical changes after the administration of MDMA (20 mg/kg), methamphetamine (10 mg/kg) or MPTP (20 mg/kg). We found that the BTBR mice exhibited an enhanced basal spontaneous locomotor behavior in the SWR test and a reduced depressogenic profile. These responses appeared to be enhanced by the prototypic cannabinoid, Δ^9-THC. MDMA and MPTP at the doses used did not modify SWR behavior in the BTBR mice whereas MPTP reduced SWR activity in the control CB57BL/6J mice. In the hippocampus, striatum and frontal cortex, the levels of DA and 5-HT and their metabolites were differentially altered in the BTBR and C57BL/6J mice. Our data provides a basis for further studies in evaluating the role of the cannabinoid and monoaminergic systems in the etiology of ASDs.

Keywords: Cannabinoid, Monoamines, Δ^9-THC, Psychostimulants, MPTP, Behavior, Autism, BTBR T+tf/J mice.

INTRODUCTION

Autism is a behaviorally defined neurodevelopmental disorder characterized by impairments in social interaction and communication and repetitive/stereotyped behaviors [1, 2]. The cause of autism is not completely understood and there is no effective cure. However, genetic and environmental factors and the interaction between genes and environment are known to play a role in Autism Spectrum Disorders (ASDs) [3-7]. A common genetic variant on chromosome 5p14.1 was shown to associate with ASDs using genome-wide association studies [6] and there are currently a number of other autism susceptibility candidate genes (ASCG) that may be involved [7]. New thinking and hypothesis have been generated to include epigenetic mechanisms in ASDs [8, 9]. This is because of the complexity of ASDs and the understanding that alteration of gene function could be due to a polymorphism in DNA sequence or epigenetic programming changes of genes in the interaction with environment without change of DNA sequences [10].

We recognize that the symptoms of ASDs are difficult to model in rodents because of the absence of verbal communication and the variability of symptoms. Nevertheless, a number of relevant behavioral and social changes have been documented in transgenic mouse models of ASDs. Specifically mouse behavioral tests modeling some of the core symptoms of autism have now been established [11]. The goal of this study, was to use the mouse model to determine the role if any of the endocannabinoid system in autism. This was accomplished using the BTBR T+tf/J mice with autism-like behavioral phenotypes. The behavioral, morphological and neurochemical alterations in this model will allow us to test our hypothesis about the causes of autism, and may serve as an index for the evaluation of proposed treatment strategies in combination with other transgenic models. The rationale for this novel hypothesis arises from the discovery that the endocannabinoid system is one of the most abundant physiological control systems in animals and humans. This system is intricately involved with embryo development and growth with limitless interaction with most biological systems including the monoaminergic systems. The endocannabinoid system consists of genes that encode cannabinoid receptors, endogenous ligands that activate these receptors and the enzymes that synthesize, degrade and perhaps reuptake the endocannabinoids [12]. While the endocannabinoid system is ubiquitous and interacts with most biological systems, the role it plays in ASDs is unknown. We recently observed that the basal level of CB2A gene expression in the BTBR T+tf/J mice was upregulated in the cerebellum compared to control mice [13]. Therefore, we have begun studies to determine the behavioral effects of cannabinoid ligands in the BTBR mice in comparison to control groups.

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MATERIALS AND METHODS

Animals

Adult male and female BTBR T+tf/J, C57BL/6J and 129SI/SvImJ (S129) mouse strains were housed in individual cages with access to mouse chow 12 hr in the light and 12 hr in the dark. Experiments were conducted according to standard NIH guidelines and approved by Institutional Animal Care and Use Committee.

Drugs

Δ^9-THC was obtained from our collaborators in NIDA intra-mural program and it was made up in a 1:1:18 solution of alcohol: emulphur: saline. MDMA, methamphetamine, and MPTP were obtained from our FDA collaborators. Animals were injected intra-peritoneal (i.p) using 1.0 and 10 mg/kg doses of Δ^9-THC and the control animals were injected with the vehicle. The doses of MDMA (20 mg/kg), methamphetamine (10 mg/kg), MPTP (20 mg/kg) or d-amphetamine (5 mg/kg) were used. In all experiments all drugs were injected in a volume of 1ml/kg.

EXPERIMENTAL PROCEDURE

Motor Function Test

Spontaneous wheel running monitors were used to access motor activity and function. The standard wheel running activity monitors measures the counts per revolution and was used to access the spontaneous wheel running behavior of naïve mice and following acute treatment with the test compounds and corresponding vehicle used. The wheel running activity of the animals were monitored by the auto-counters, for 10 minutes during the assessment of spontaneous wheel running activity following specific drug pretreatment times. Data was obtained as total number of revolutions over the 10 min evaluation period. The performance of the animals following the acute administration of the test compounds to the mouse strains were compared to their respective vehicle treated controls.

Forced Swim Test

The forced swim test (FST) paradigm was used. It consists of a glass cylinder (16 cm diameter and height 35 cm) filled to a depth 15 cm with water (23-25°C). One glass cylinder was used for each mouse and we tested six mice at a time using six glass cylinders and test observers. In this study a two-day swim test procedure was utilized first to access the basal performance of the different mouse strains. On the first day mice were placed in the glass cylinder with water to the specified depth, and all animals were exposed for 15-min pre-swim test prior to the 5-min forced swim test on day 2. Fresh water was introduced prior to each test. The test sessions were recorded by trained observers for consistent data recording. The observer used stop watches and counters to record immobility times and counts respectively. The data recorded during the 5-min test session were the times the animals were immobile and also the number of immobility counts during the test session. Similar data was obtained for the vehicle treated naïve control animals. During the test session the duration of immobility was defined by the animal's stationary position, and only made the minimal movements necessary to keep the head above water.

Neurochemical Analysis of Dopamine (DA) and Serotonin (5HT) and their Metabolites in Selected Brain Areas

Prior to preparation of animals for selected brain region dissection for neurochemical analysis, animals were scheduled for three saline or three drug injections that were given about 8 hrs apart for one day only. Mice in the different groups were injected with saline (n = 10) or these test compounds (n = 10/per group): methamphetamine (10 mg/g), MDMA (20 mg/kg) or MPTP (20 mg/kg). After the completion of drug or vehicle administration, mice were housed one per cage for two days before the animals were sacrificed two days later, and the striatum, frontal cortex and hippocampus were dissected and frozen at -80°C. All frozen samples were shipped to the FDA for the neurochemical analysis. Briefly, tissues from the different groups were prepared for high performance liquid chromatography (HPLC) combined with electrochemical detection to determine dopamine (DA), 3, 4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5HT) and 5-hydroxyindole acetic acid (5HIAA).

CANNABINOID GENOMIC ANALYSIS IN BTBR MICE

In a previous study Liu *et al.*, 2009, [13], during the analysis of CB2-R gene expression in different brain regions of C57BL/6 mice treated with the mixed cannabinoid agonist WIN55212-2 (2mg/kg) for 7 days, we also analyzed CB2-gene expression in non-injected BTBR mice. This was accomplished by the analysis of CB2A and CB2B gene expression in brain regions, testis and the spleen. Briefly, RNA w isolated using TRIzol reagent and cDNA synthesized using SuperScript III first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). The expression of CB2A and CB2B genes were compared by TaqMan real-time PCR with an ABI PRISM 7900 HT Sequence Detection System, using custom designed Fam-labeled MGB probes and primers for CB2A and CB2B (Applied Biosystems, Foster City, CA). The custom-designed mouse beta-actin Fam-labeled MGB probe was used for normalization [13].

Statistical Analysis

Prism-3 program, version 3.02 (Graphpad Software, Inc., San Diego, CA, USA) was used for statistical analyses, including *t*-tests and analysis of variance (ANOVA). Data from motor function and forced swim tests were subjected to analysis of variance for multiple comparisons followed by Turkey's test where appropriate. For CB1 and CB2 gene expression analysis, unpaired *t*-test was used. The accepted level of significance is $P < 0.05$.

RESULTS

Effects of Δ^9-THC, Psychostimulants and Disruption of Monoaminergic System by MPTP on Motor Activity in the Mouse Model of ASD

The naïve untreated BTBR mice exhibited an enhanced basal locomotor activity as recorded in the spontaneo~ wheel running test. The BTBR males had slightly hig activity than the females and the motor activity of the males of the C57BL/6J were significantly lower ($p<0.05$, N = 10)

than the activity of the BTBR males as shown in Fig. (1A). The effects of d-amphetamine treatment in the three mouse strains varied, with the S129 mouse showing significant locomotor activation compared to both BTBR and C57BL/6J mice as shown in Fig. (1B). A similar response of male and female mice in motor activity was recorded following the acute treatment of BTBR and C57BL/6J, with methamphetamine and MDMA (Fig. (1C)). The motor activity of C57BL/6J male mice was significantly reduced compared to

those of the BTBR mice after treatment with the dopaminergic neurotoxin MPTP as shown in Fig. (1C). However, the motor activity of BTBR mice when compared to those of C57BL/6J and S129 mice were significantly reduced and more sensitive to the higher dose of 10 mg/kg Δ^9-THC used in this study as shown in Fig. (2C). At the doses used in this study Δ^9-THC actually enhanced motor activity in the C57BL/6J and S129 mice which were the control background mice for the BTBR animals.
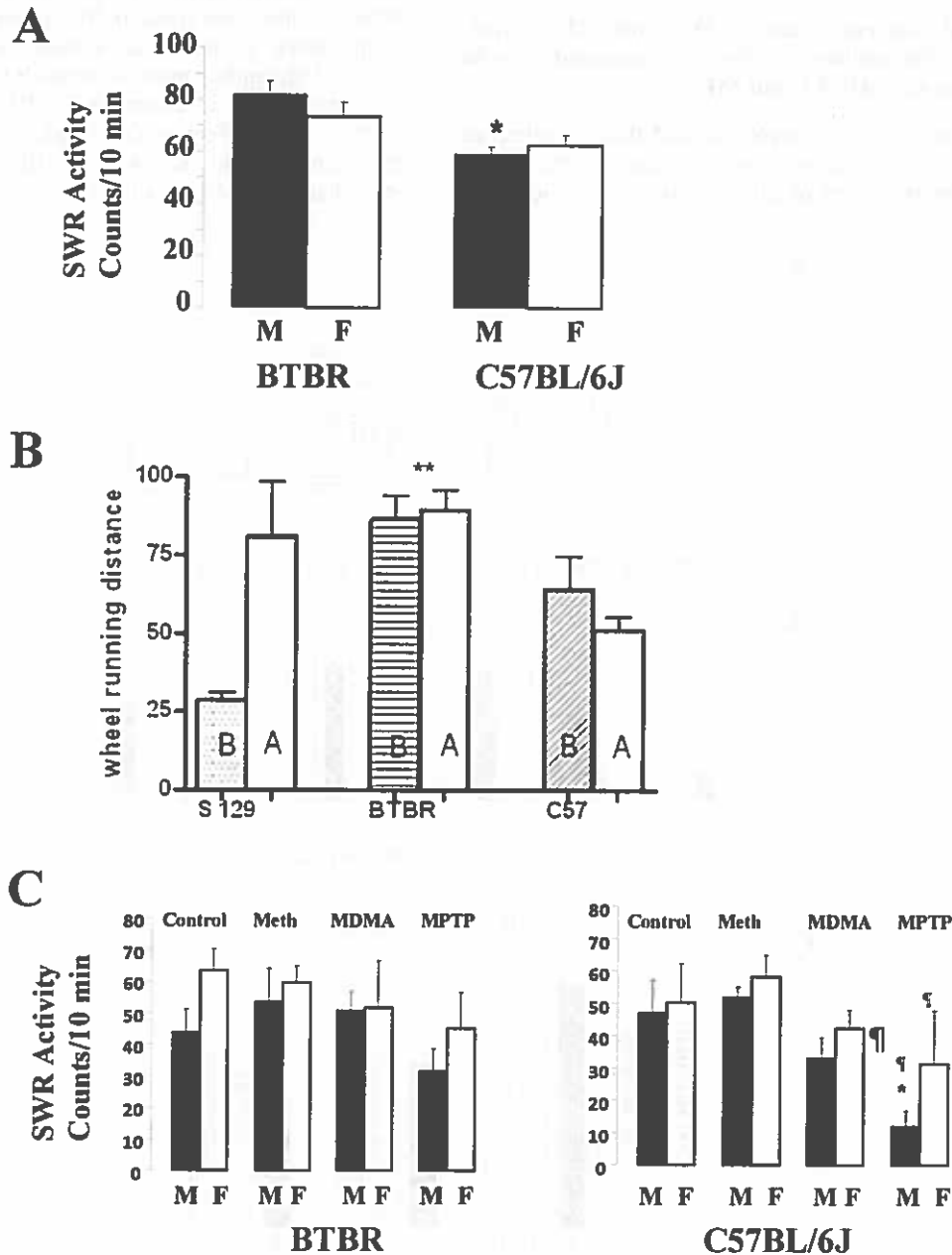
Fig. (1). The effects of psychostimulants (d-amphetamine, Methamphetamine and MDMA), and disruption of monoaminergic system by the neurotoxin (MPTP), in a mouse model of autism spectrum disorders. Panel A shows the basal motor activity of male and female BTBR and C57BL/J mice in the spontaneous wheel running (SWR) monitors; panel B is the effect of acute 10 min treatment with d-amphetamine (5.0 mg/kg) on the performance of male BTBR and the male controls, S129 and C57BL/6J mice. Panel C shows the effects of acute administration of methamphetamine (10 mg/kg), MDMA (20.0 mg/kg) and MPTP (20 mg/kg) in both male and female BTBR and C57BL/6J mice in comparison to their respective controls. The duration of the wheel running behavior was accessed over a 10 min period in all animals tested. * or ¶ represents statistical significance at $p<0.05$ as compared to the same gender.

Behavioral Effects of BTBR, C57BL/6J and S129 Mice in the Forced Swim Test after Treatment with a Cannabinoid, Δ^9-THC:

The naïve BTBR mice demonstrated reduced immobility time and increased immobility count when compared to C57BL/6J and S129 mice in the FST, as shown in Fig. (2A). Surprisingly, in the FST, Δ^9-THC at the doses used did not modify the immobility time and counts of the BTBR mice when compared to the C57BL/6J and S129 mice as shown in Fig. (2B).

Neurochemical Determination of DA and 5HT Levels and their Metabolites after Treatment with Methamphetamine, MDMA and MPTP

The levels of dopamine, serotonin and their metabolites were analyzed in the striatum, frontal cortex and the hippocampus after the treatment of different strains of mice with

methamphetamine, MDMA and MPTP. Data on striatal DA and 5HT levels and frontal cortex 5HT levels are presented in Fig. (3). In this preliminary neurochemical analysis of DA, 5HT and their metabolite levels in the striatum, frontal cortex and hippocampus after the drug treatments, the levels of these monoamines and their metabolites were differentially altered in the BTBR and C57BL/6J mice used, see Fig. (3). The variable levels of monoamines made it difficult to define a specific association of these changes with the underlying features in the mouse model of ASDs. There are however some striking observations that can be gleaned from the effects of the doses used in drug treatments and the analyzed comparative striatal data between BTBR and C57BL/6J mice: Methamphetamine lowered BTBR DA levels relative to controls with no effect on C57BL/6J DA levels whereas MPTP had no effect on DA levels in BTBR mice relative to their controls, but lowered C57BL/6J DA levels. On the other hand MDMA had little on no significant effect on ei-
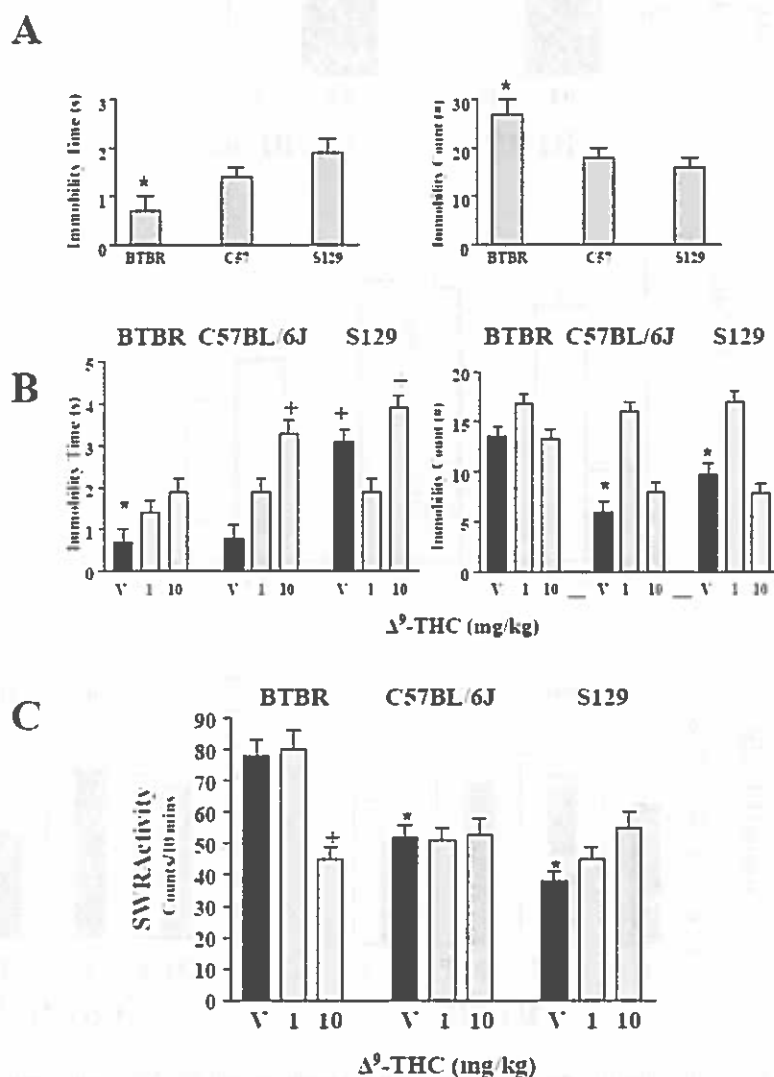
Fig. (2). Behavioral effects of BTBR, C57BL/6J and S129 mouse strains in the FST. Panel A shows the basal levels of performance indicated by the time and number of immobility by the three mouse strains in the forced swim test model. Panel B is time and number of immobility after acute treatment of the mouse strains with Δ^9-THC (1 and 10 mg/kg) in comparison to vehicle treated controls. Panel C shows the influence of acute treatment of the mouse strains with Δ^9-THC (1 and 10 mg/kg) in the spontaneous wheel running activity monitors. * or + represents statistical significance at p<0.05 with strains and drug treatment in the behavioral measures.

ther BTBR or C57BL/6J DA levels in comparison to their respective controls.
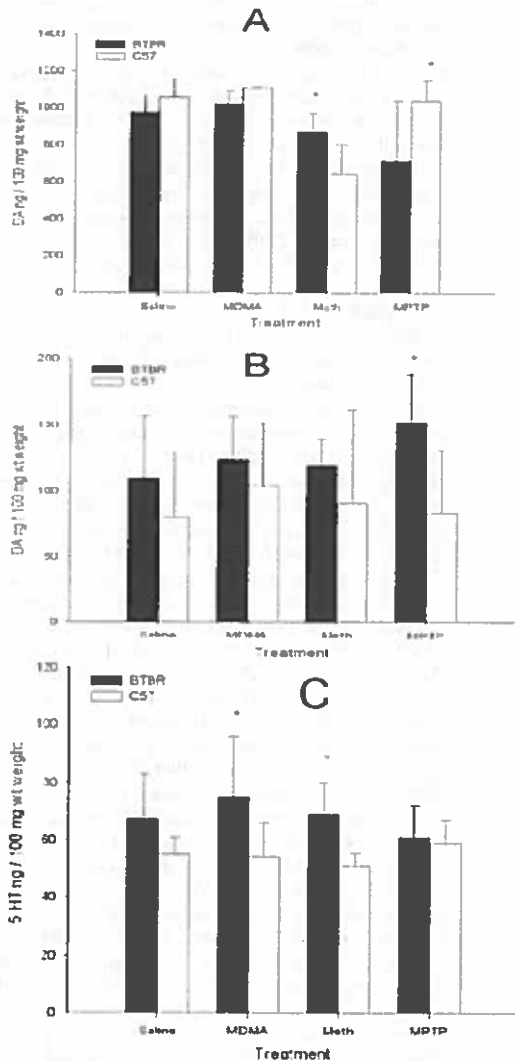
Fig. (3). Neurochemical analysis of dopamine (DA) and serotonin (5HT) levels in striatum and frontal cortex in BTBR and C57BL/6J male and female mice following a single day three times administration of either saline, methamphetamine (10 mg/kg), MDMA (20 mg/kg) or MPTP (20 mg/kg). Regional brain areas were dissected 2 days later. Since there were no significant sex differences the data was collapsed on the variable sex. Panel A is the striatal dopamine level in BTBR relative to C57BL/6J mice. Panel B is the frontal cortex dopamine level in the two strains of mice. Panel C is the striatal serotonin level. *Represents statistical significance at $p < 0.05$ using a least squares means analysis. Significance tests were performed between the two strains of mice for each of the treatments independently.

Cannabinoid CB2A Gene Expression is Upregulated in BTBR Mice

We have previously shown that naive BTBR mice that have been reported to have autism-like behavioral phenotypes have an upregulated higher levels of CB2A gene expression in the cerebellum without treatment with

cannabinoids. This upregulation occurred usually only after sub-acute treatment with a mixed cannabinoid agonist, WIN55212-2 in the C57BL/6J mice [13]. However, no significant changes were observed in other brain regions including frontal cortex and striatum - brain areas evaluated in the current study and the hypothalamus (data not shown). The expression level of CB2B in the mouse brain is lower than CB2A and the mRNA levels could not be reliably measured by TaqMan assay (data not shown).

DISCUSSION

While autism may be uniquely human, we have investigated the consequences of cannabinoid and monoaminergic system disruption in the BTBR T+tf/J mice that have been shown to exhibit autism-like behavioral phenotypes. We report that the BTBR mice exhibited an enhanced basal spontaneous locomotor behavior in the spontaneous wheel running (SWR) test, a measure of locomotor activity, that was reduced by the prototypic cannabinoid, Δ^9-THC. In addition, this enhanced spontaneous wheel running behavior was sexually dimorphic as the motor activity in the naïve male BTBR mice was significantly higher than those of the naïve male C57BL/6J mice without significant alteration in the female mice. Furthermore, the doses of the psychostimulants, d-amphetamine, methamphetamine and MDMA used in this study did not modify the SWR behavior in the BTBR mice whereas MPTP reduced SWR activity in the control CB57BL/6J mice. One characteristic of ASDs is stereotype behavior characterized by high levels of repetitive self-grooming behavior that has recently been shown to be reduced in the BTBR mice by methyl-6-phenylethynl-pyridine (MPEP) – an mGluR5 antagonist [14]. It is tempting to suggest the evaluation of Δ^9-THC or other cannabinoids with reduced psychoactivity in irritability, tantrums and self-injurious behavior associated with autistic individuals. This is because at the low doses used in this study, only the BTBR mice were sensitive to motor depressant effects of Δ^9-THC when compared to those of C57BL/6J and S129 mice. This hypothesis is further supported by our data showing that the BTBR mice were also insensitive to the locomotor activation induced by psychostimulants and the neurotoxic effects of MPTP when compared to those of C57BL/6J and S129 mice.

An unusual behavioral phenotype characterized by exaggerated responses to stress in the BTBR mouse has been demonstrated [15]. The study showed that the BTBR mice had increased levels of the stress hormone corticosterone following tail suspension, and a heightened anxiety response in the plus-maze test, when compared to C57BL/6J mice [15]. In our current study, there were marked strain differences in immobility times and counts in the FST model of depression and BTBR mice displayed a reduced immobility time and an enhanced immobility count compared to the control C57BL/6J and S129 mice. Curiously however, Δ^9-THC at the doses used in this study did not modify the immobility time and counts in BTBR mice when compared to the C57BL/6J and S129 mice whose immobility times and counts were differentially modified dose dependently by Δ^9-THC.

The cause of autism is unknown, but there has been much progress and new knowledge with the environment, epigenetics and genetic factors all playing some role in the etiology of ASDs. For example multiple gene variants and genome-wide copy number variations have been reported in children with ASDs, but not in healthy controls [16]. Data from comparative genomics of autism and schizophrenia support the hypothesis that autism and schizophrenia represent diametric conditions with regard to their genomic underpinnings and phenotypic manifestations [16]. Our data indicating that the BTBR mice have an abnormal regulation of DA functioning with an upregulated CB2A gene expression in naïve BTBR mouse of ASDs [13], and our finding indicating an increased risk of schizophrenia in patients with low CB2 receptor function [17], is in agreement with the hypothesis that autism and schizophrenia represent diametric conditions [16]. Moreover, more research needs to be done to understand the nature of the neurochemical changes recorded in our preliminary study in the hippocampus, striatum and frontal cortex, where the levels of DA and 5-HT and their metabolites were differentially altered in the BTBR and C57BL/6J mice. Thus our data provides a basis for further studies in evaluating the role of the cannabinoid and monoaminergic systems in the etiology of ASDs and whether the BTBR mice can model both schizophrenia and ASDs.

ACKNOWLEDGEMENTS

REFERENCES

[1] King, B.H., Bostic, J.Q. An update on pharmacologic treatments for autism spectrum disorders. *Child Adolesc. Psychiatr. Clin. N. Am.*, **2006**, *15*, 161-175.

[2] Nickels, K.C., Katusic, S.K., Colligan, R.C., Weaver, M.S., Voigt, R.G., Barbaresi, W. J. Stimulant medication treatment of target behaviors in children with autism: A population-based study. *J. Dev. Behav. Pediatr.*, **2008**, *29*, 75-81.

[3] Robinson, P.D., Schutz, C.K., Macciardi, F., White, B.N., Holden, J.A. Genetically determined low maternal serum dopamine b-hydroxylase levels and etiology of autism spectrum disorders. *Am. J. Med. Genet.*, **2001**, *100*, 30-36.

[4] Yrigollen, C.M., Han, S.S., Kochetkova, A., Babitz, B., Chang, J.T., Volkmar, F.R., Leckman, J.F., Grogorenko, E.L. Genes controlling affiliative behavior as candidate genes for autism. *Biol. Psychiatry.*, **2008**, *63*, 911-916.

[5] Page, D.T., Kuti, O.J., Prestia, C., Sur, M. Haploinsufficiency for pten and serotonin transporter cooperatively influences brain size and social behavior. *Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 1989-1994.

[6] Wang, K., Zhang, H., Ma, D., Bucan, M, Glessner, J.T., Abrahams, B.S., Salyakina, D., Imielinski, M., Bradfield, J.P., Sleiman, P.M.A, Kim, C.E., Hou, C., Frackelton, E. Hakonarson, H. Common genetic variants on 5p14.1 associate with autism spectrum disorders. *Nature*, **2009**, *459*, 528-533.

[7] Bill, B.R., Geschwind, D.H. Genetic advances in autism: heterogeneity and convergence on shared pathways. *Curr. Opin. Genet. Dev.*, **2009**, *19*, 271-278.

[8] Mehler, M.F., Purpra, D.P. Autism, fever, epigenetics and the locus coerulus. *Brain Res. Rev.*, **2008**, *59*, 388-392.

[9] Jones, J.R., Skinner, C., Friez, M.J., Schwartz, C.E., Stevenson, R.E. Hypothesis: Dysregulation of methylation of brain expressed genes on the X chromosome and autism spectrum disorders. *Am. J. Med. Genet. Part A.*, **2008**, *146A*, 2213-2220.

[10] Szyf, M. Epigenetics, DNA methylation, and chromatin modifying drugs. *Annu. Rev. Pharmacol. Toxicol.*, **2009**, . *49*, 243-263.

[11] Crawley, J.N. Mouse behavioral assays relevant to the symptoms of autism. *Brain Pathol.*, **2007**, *17*, 448-459.

[12] Onaivi, E.S. Cannabinoid receptors in brain: pharmacogenetics, neuropharmacology, neurotoxicology, and potential therapeutic applications. *Int. Rev. Neurobiol.*, **2009**, *88*, 335-369.

[13] Liu, Q.R., Pan, C.H., Hishimoto, A., Li, C.Y., Xi, Z.X, Llorente-Berzal, A., Viveros, M.P., Ishiguro, H., Arinami, T., Onaivi, E.S., Uhl, G.R. Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Gene Brain Behav.*, **2009**, *8*, 519-530.

[14] Silverman, J.L., Tolu, S.S., Barkan, C.L., Crawley, J.N. Repetitive Self-Grooming Behavior in the BTBR Mouse Model of Autism is Blocked by the mGluR5 Antagonist MPEP. *Neuropsychopharmacology*, **2009**, 1-14. (Online ahead of print).

[15] Benno, R., Smirnova, Y., Vera, S., Liggett, A., Schanz, N. Exaggerated responses to stress in the BTBR T+tf/J mouse: An unusual behavioral phenotype. *Behav. Brain Res.*, **2009**, *197*, 462-465.

[16] Crespi, B., Stead, P., Elliot, M. Comparative genomics of autism and schizophrenia. *Proc. Natl. Acad. Sci. USA*, **2009**, 1-6. Online ahead of print.

[17] Ishiguro, H., Horiuchi, Y., Ishikawa, M., Koga, M., Imai, K., Suzuki, Y., Morikawa, M., Inada, T., Watanabe, Y., Takahashi, M., Someya, T., Ujike, H., Iwata, N., Ozaki, N., Onaivi, E.S, Kunugi, H., Sasaki, T., Itokawa, M., Arai, M., Niizato, K., Iritani, S., Naka, I., Ohashi, J., Kakita, A., Takahashi, H., Nawa, H., Arinami, T. Brain cannabinoid CB2 receptor in schizophrenia. *Biol. Psychiatry*, **2010**, *67*, 974-982.

Jin, P., Zarnescu, D.C., Zhang, F., Pearson, C.E., Lucchesi, J.C., Moses, K., and Warren, S.T. (2003). Neuron 39, 739–747.

Khateb, S., Weisman-Shomer, P., Hershco-Shani, I., Ludwig, A.L., and Fry, M. (2007). Nucleic Acids Res. 35, 5775–5788.

López Castel, A., Cleary, J.D., and Pearson, C.E. (2010). Nat. Rev. Mol. Cell Biol. 11, 165–170.

Lu, C., Lin, L., Tan, H., Wu, H., Sherman, S.L., Gao, F., Jin, P., and Chen, D. (2012). Hum. Mol. Genet. 21, 5039–5047.

Mori, K., Weng, S.M., Arzberger, T., May, S., Rentzsch, K., Kremmer, E., Schmid, B., Kretzschmar, H.A., Cruts, M., Van Broeckhoven, C., et al. (2013). Science 339, 1335–1338.

Napierala, M., Michalowski, D., de Mezer, M., and Krzyzosiak, W.J. (2005). Nucleic Acids Res. 33, 451–463.

Oostra, B.A., and Willemsen, R. (2009). Biochim. Biophys. Acta 1790, 467–477.

Pearson, C.E. (2011). PLoS Genet. 7, e1002018.

Pearson, C.E., Nichol Edamura, K., and Cleary, J.D. (2005). Nat. Rev. Genet. 6, 729–742.

Reddy, K., Zamiri, B., Stanley, S.Y., Macgregor, R.B., Jr., and Pearson, C.E. (2013). J. Biol. Chem. 288, 9860–9866.

Todd, P.K., Oh, S.Y., Krans, A., He, F., Sellier, C., Frazer, M., Renoux, A.J., Chen, K.C., Scaglione, K.M., Basrur, V., et al. (2013). Neuron 78, this issue, 440–455.

Zu, T., Gibbens, B., Doty, N.S., Gomes-Pereira, M., Huguet, A., Stone, M.D., Margolis, J., Peterson, M., Markowski, T.W., Ingram, M.A., et al. (2011). Proc. Natl. Acad. Sci. USA 108, 260–265.

Evidence for a Common Endocannabinoid-Related Pathomechanism in Autism Spectrum Disorders

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In this issue of Neuron, Földy et al. (2013) report that endocannabinoid-mediated signaling at inhibitory synapses is dysregulated in mouse models of autism-associated Neuroligin-3 mutations. These findings carry implications regarding the pathophysiology of autism spectrum disorders and the development of treatment strategies.

The correct wiring of the brain during development is an extremely complex biological process, during which a staggering number of synapses with often very diverse characteristics have to be formed and maintained in a precise and delicate balance. Not surprisingly, therefore, numerous neurodevelopmental and psychiatric diseases appear to be disorders of aberrant synaptogenesis and synapse function, or "synaptopathies." Particularly in the context of autism spectrum disorders (ASDs), an ever-growing number of mutations in genes encoding synaptic proteins have been identified in affected individuals (Murdoch and State, 2013), and major research efforts are currently focusing on strategies to transform this knowledge base into viable treatment strategies.

However, the corresponding challenges are substantial. For example, very little is known about the role of ASD-related synaptic proteins in vivo, e.g., in neuronal circuits that control autism-relevant behavior. Second, many known ASD-related proteins are structural proteins with adhesion or scaffold functions and therefore poor targets for pharmacological intervention with small molecule drugs. Third, many ASD-related mutations lead to a loss of the corresponding protein so that no target for pharmacological intervention remains. Finally, each individual ASD-related mutation is rare, with the vast majority accounting for less than 1% of affected individuals each. In view of these difficulties, the focus in the field of ASD biology has shifted toward the identification of cellular protein-protein interactions or signaling pathways that are common to the various ASD-related proteins and therefore expected to be perturbed by a wide range of ASD-related mutations—with the hope that such pathways may represent more

promising treatment targets than the ASD-linked proteins discovered so far.

One of the synaptic proteins associated with ASDs is Neuroligin-3 (NLGN3), a member of the Neuroligin family of postsynaptic cell adhesion molecules that interact with presynaptic Neurexins to control synapse development and function. Two distinct mutations in NLGN3 have been linked to ASDs, a point mutation resulting in an R451C substitution in the Neurexin-binding domain (Jamain et al., 2003) and a deletion of the NLGN3 gene (Sanders et al., 2011). Studies on the respective mouse models, a Nlgn3^{R451C} knockin (KI) and a Nlgn3 knockout (KO), showed that both mutations cause ASD-related behavioral phenotypes (Radyushkin et al., 2009; Tabuchi et al., 2007) but have strikingly different effects on synapse and network function, with the Nlgn3^{R451C} mutation resulting in a gain-of-function phenotype that is
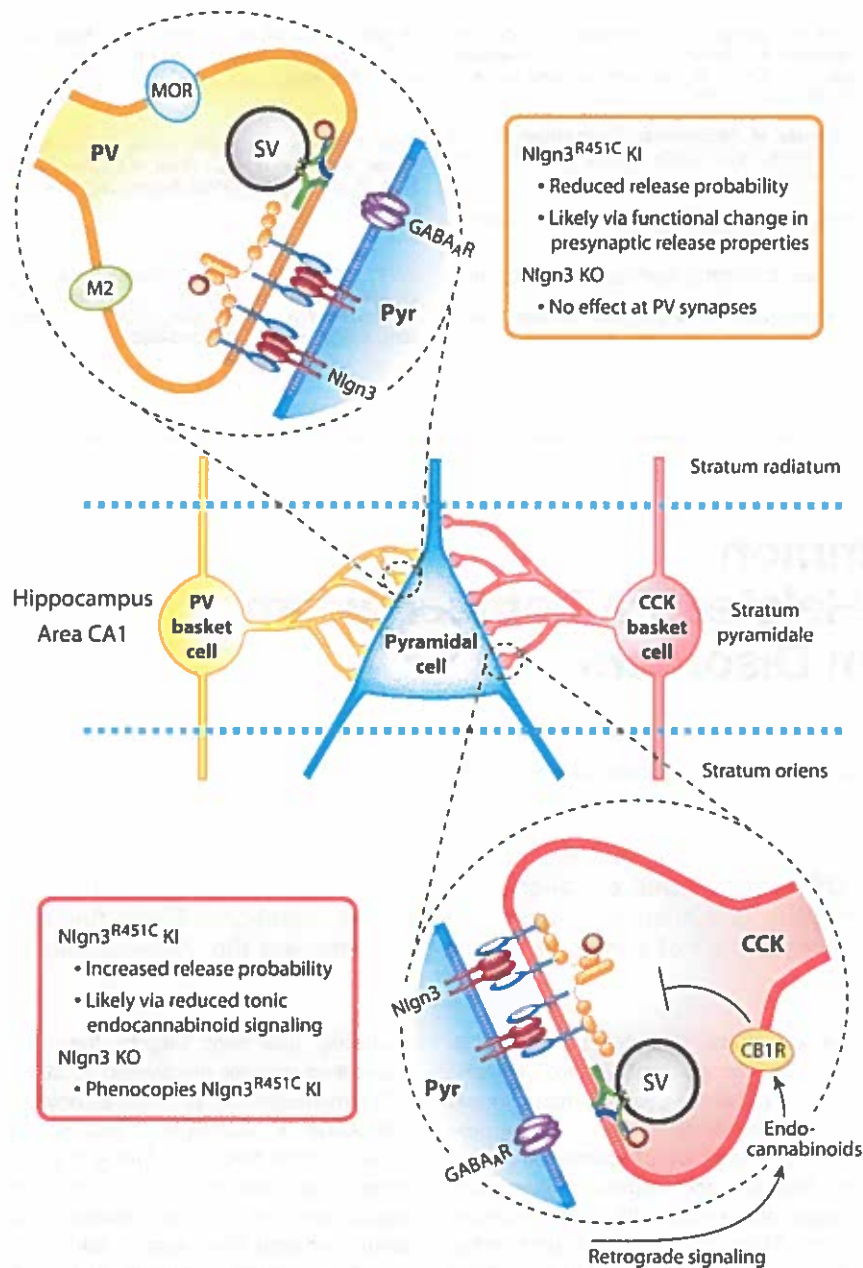
Figure 1. Potential Role of Nlgn3 at Perisomatic Inhibitory Synapses on CA1 Pyramidal Neurons

PV and CCK basket cells form perisomatic inhibitory synapses onto pyramidal neurons. Boxed inserts summarize the data reported by Földy et al. (2013). Expanded views show a model of how Nlgn3 may affect PV basket cell synapses (upper left) and CCK basket cell synapses (lower right) based on these findings. It should be noted that the presence of Nlgn3 at these synapses has not been investigated and is inferred from the data. Abbreviations: Nlgn3, Neuroligin-3; PV, parvalbumin; CCK, cholecystokinin; SV, synaptic vesicle; MOR, μ-opioid receptor; M2, M2 muscarinic acetylcholine receptor; CB1R, cannabinoid receptor type 1.

unrelated to the loss of function caused by the Nlgn3 deletion (Baudouin et al., 2012; Etherton et al., 2011; Tabuchi et al., 2007).

In a new study published in the current issue of *Neuron*, Földy and colleagues searched for common phenotypic changes in Nlgn3^{R451C} KI and Nlgn3 KO

mice that might explain the similar ASD-related behavioral changes in these mouse lines and may therefore be particularly relevant to ASDs (Földy et al., 2013). To this end, the authors investigated GABAergic synaptic transmission in the hippocampus of Nlgn3^{R451C} KI and Nlgn3 KO mice, focusing on the synaptic connections between inhibitory basket cells and pyramidal neurons, which are known to play a fundamental role in the generation of the network oscillations that underlie a number of cognitive functions controlled by the hippocampus (Lisman and Buzsáki, 2008). Two types of basket cells are particularly relevant for this process, parvalbumin-containing (PV) and cholecystokinin-containing (CCK) basket cells, and Földy et al. (2013) employed paired whole-cell recordings to monitor perisomatic synapses formed by each of these inhibitory cell types onto postsynaptic pyramidal neurons (Figure 1).

The authors found that synaptic transmission is substantially impaired at PV basket cell synapses in Nlgn3^{R451C} KI mice, with IPSC amplitudes reduced by ~70%. No such alterations were observed in the Nlgn3 KOs, consistent with the previously published notion that the R451C substitution exerts its influence by a gain-of-function mechanism. Unexpectedly in view of the postsynaptic localization of Nlgn3, this decrease in IPSC amplitude appears to be of presynaptic origin and due to a reduction in presynaptic transmitter release probability. In contrast, no evidence for changes in postsynaptic GABA receptor number or composition, in the total number of synapses, in quantal size or the number of release sites, or in the activation of presynaptic receptors that modulate release probability was observed. The authors conclude that the Nlgn3^{R451C} KI affects the presynaptic transmitter release machinery at PV basket cell synapses through gain-of-function alterations in transsynaptic signaling, although the precise mechanism has yet to be elucidated.

While these experiments provided valuable new insights into the mechanisms by which the R451C substitution might affect Nlgn3 function, they failed to uncover common phenotypic features of the two Nlgn3 mutants that might be related to pathways of particular relevance

for ASD pathophysiology. Hence, Földy et al. (2013) next investigated transmission at CCK basket cell synapses. Unexpectedly, the authors found that the Nlgn3^{R451C} KI phenotype at these CCK basket cell synapses was diametrically opposite to the one found at PV basket cell synapses, with IPSC amplitudes substantially increased rather than decreased. As with the PV basket cell synapses, this phenotypic change is again likely the result of an alteration in presynaptic GABA release probability. However, in the case of the CCK basket cell synapses, the change in IPSC amplitude was phenocopied in the Nlgn3 KO mouse, indicating that it represents a loss-of-function effect that is mechanistically distinct from the one observed at PV basket cell synapses of Nlgn3^{R451C} KI mice.

It was shown previously that GABA release at CCK basket cell synapses can be suppressed by tonic endocannabinoid-mediated activation of presynaptic CB1 receptors, most likely via constitutive release of endocannabinoids from the postsynaptic neuron (Katona and Freund, 2012). The authors therefore tested if the increase in GABA release probability observed at CCK basket cell synapses of Nlgn3^{R451C} KI and Nlgn3 KO mice is caused by a deficiency in tonic endocannabinoid signaling. In support of this notion, bath application of a CB1 receptor antagonist resulted in an increase in IPSC amplitudes at wild-type synapses, but failed to further enhance transmission at Nlgn3^{R451C} KI or NL3 KO or R451C KI synapses, indicating that CB1 receptor signaling was already reduced in the two mutants. Interestingly, Nlgn3 loss-of-function impaired tonic endocannabinoid signaling at all CB1-containing GABAergic synapses throughout the hippocampus, but showed no effect on glutamatergic transmission or on phasic endocannabinoid signaling. These data led the authors to conclude that Nlgn3 is required to specifically localize the release machinery for tonic endocannabinoid release to CB1-containing synapses.

There are several interesting lessons to be learned from this study. First, the observation that the same Nlgn3 mutation can have such different effects on two types of presynapses contacting the same postsynaptic neuron highlights

the fundamental importance of synaptic context in understanding Neuroligin function. The function of Neuroligins and Neurexins is not only diversified by extensive alternative splicing, but also by alternate transsynaptic binding partners such as LRRTMs or N-cadherin (reviewed recently in Krueger et al., 2012). Accordingly, each synapse type may express its own distinct transsynaptic signaling complex, dependent on the identity of both the presynaptic and the postsynaptic neuron. As Földy et al. (2013) discuss, it is conceivable that the Nlgn3^{R451C} substitution may exert distinct effects on the binding affinity to various transsynaptic partners, thereby differentially shifting the composition of the transsynaptic signaling complex at PV basket cell and CCK basket cell synapses. The consequence of this complexity is that it becomes challenging to predict the relevance of a given mutation for ASD-related phenotypes without directly assessing its effects in a synapse-specific and circuitry-specific manner. The use of genetic strategies to selectively target individual types of synapses, as well as methods to elucidate the molecular identity of transsynaptic signaling complexes in a synapse-specific manner, will be essential to fully elucidate the role of Neuroligins in normal synapse development and in disorders of the synapse.

A second key implication arising from the present study is that dysregulation of the endocannabinoid system may play an important role in ASD pathophysiology and may therefore represent a target for pharmacological intervention. A similar strategy was previously employed to identify the metabotropic glutamate receptor (mGluR) signaling pathway as a target for drug development in several mouse models of ASD-related disorders, including fragile X syndrome (Bear et al., 2004), tuberous sclerosis complex (Auerbach et al., 2011), and Nlgn3 deletion (Baudouin et al., 2012), and clinical trials based on these findings are underway. Whether targeting the endocannabinoid system in the context of ASDs will prove to be similarly promising remains to be seen, and additional research will be necessary to build upon this notion. Interestingly, however, aberrant activation of the endocannabinoid system was also recently reported in

a mouse model of fragile X syndrome (Busquets-Garcia et al., 2013; Jung et al., 2012). Together with these findings, the data presented by Földy et al. indicate that further analyses of the link between endocannabinoid signaling and ASDs may provide valuable insights into the pathophysiology and potential treatment strategies for ASDs.

REFERENCES

Auerbach, B.D., Osterweil, E.K., and Bear, M.F. (2011). Nature 480, 63–68.

Baudouin, S.J., Gaudias, J., Gerharz, S., Hatstatt, L., Zhou, K., Punnakkal, P., Tanaka, K.F., Spooren, W., Hen, R., De Zeeuw, C.I., et al. (2012). Science 338, 128–132.

Bear, M.F., Huber, K.M., and Warren, S.T. (2004). Trends Neurosci. 27, 370–377.

Busquets-Garcia, A., Gomis-Gonzalez, M., Guegan, T., Agustin-Pavon, C., Pastor, A., Mato, S., Perez-Samartin, A., Matute, C., de la Torre, R., and Dierssen, M. (2013). Nat. Med. Published online March 31, 2013. http://dx.doi.org/10.1038/nm.3127.

Etherton, M., Földy, C., Sharma, M., Tabuchi, K., Liu, X., Shamloo, M., Malenka, R.C., and Südhof, T.C. (2011). Proc. Natl. Acad. Sci. USA 108, 13764–13769.

Földy, C., Malenka, R.C., and Südhof, T.C. (2013). Neuron 78, this issue, 498–509.

Jamain, S., Quach, H., Betancur, C., Råstam, M., Colineaux, C., Gillberg, I.C., Soderstrom, H., Giros, B., Leboyer, M., Gillberg, C., and Bourgeron, T.; Paris Autism Research International Sibpair Study. (2003). Nat. Genet. 34, 27–29.

Jung, K.-M., Sepers, M., Henstridge, C.M., Lassalle, O., Neuhofer, D., Martin, H., Ginger, M., Frick, A., DiPatrizio, N.V., and Mackie, K. (2012). Nat. Comm. 3, 1080.

Katona, I., and Freund, T.F. (2012). Annu. Rev. Neurosci. 35, 529–558.

Krueger, D.D., Tuffy, L.P., Papadopoulos, T., and Brose, N. (2012). Curr. Opin. Neurobiol. 22, 412–422.

Lisman, J., and Buzsáki, G. (2008). Schizophr. Bull. 34, 974–980.

Murdoch, J.D., and State, M.W. (2013). Curr. Opin. Genet. Dev. Published online March 25, 2013. http://dx.doi.org/10.1016/j.gde.2013.02.003.

Radyushkin, K., Hammerschmidt, K., Boretius, S., Varoqueaux, F., El-Kordi, A., Ronnenberg, A., Winter, D., Frahm, J., Fischer, J., Brose, N., and Ehrenreich, H. (2009). Genes Brain Behav. 8, 416–425.

Sanders, S.J., Ercan-Sencicek, A.G., Hus, V., Luo, R., Murtha, M.T., Moreno-De-Luca, D., Chu, S.H., Moreau, M.P., Gupta, A.R., Thomson, S.A., et al. (2011). Neuron 70, 863–885.

Tabuchi, K., Blundell, J., Etherton, M.R., Hammer, R.E., Liu, X., Powell, C.M., and Südhof, T.C. (2007). Science 318, 71–76.

Case report

Use of dronabinol (delta-9-THC) in autism: A prospective single-case-study with an early infantile autistic child

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Abstract

Objective: To evaluate the effectiveness of dronabinol (delta-9-THC) as supplementary therapy in a child with autistic disorder.

Methods: A child who met the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) criteria for a diagnosis of autistic disorder and who took no other medication during the observation time was included in an open and uncontrolled study. Symptom assessment was performed using the Aberrant Behavior Checklist (ABC) before and after six months of medical treatment.

Result: Compared to baseline, significant improvements were observed for hyperactivity, lethargy, irritability, stereotypy and inappropriate speech at follow-up (p=0.043).

Conclusion: This study showed that the use of dronabinol may be able to reduce the symptoms of autism.

Keywords: early infantile autism, autistic disorder, dronabinol, cannabinoid

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Introduction

Autistic Disorder (also referred to as early infantile autism, childhood autism, Kanner-Syndrome) is a pervasive developmental disorder characterized by marked impairment in social interaction, delayed language, and restricted repertoire of activity and interests (DSM-IV criteria for diagnosis of autistic disorder, 2007) [8][14]. Beside these core symptoms autistic children often show aggression against others and self-injurious behaviour, also have sleep problems and eating disorders. Early infant autism affects 1 of 2000 children, with boys affected three times more often than girls. Autism does not equate with mental retardation, but intelligence is frequently limited (intelligence quotient (IQ) below 70). One quarter of autistic children achieve good results on IQ tests, termed 'high functional autism'. The cause of autism is still not fully explored, but seems to be multifactorial (including genetic, environmental and neurobiochemical disorders) [19]. Cog-

nitive Behavioural Therapy is the gold standard in treating children with early infant autism and is supported by occupational therapy, physical therapy and pharmacological intervention (e.g. antipsychotic drugs) [4][9][12][13][17][18].

Dronabinol, or tetrahydrocannabinol / Δ-9-THC, is a purified cannabinoid. The main accepted field of use is in oncology to reduce nausea and in AIDS to increase appetite, but has also been used in chronic pain patients, inflammatory bowel diseases (Crohn's disease, ulcerative colitis) and multiple sclerosis for muscle relaxation and neuropathic pain [9]. It may also be used for major depression and Tourette's syndrome [1][6][11].

To date there have been no reports of the use of cannabinoids in autism. However, in internet blogs and discussion forums there are many reports of parents who have tried THC for their autistic children, but without medical monitoring and inappropriate administration.

Table 1. Wilcoxon Rank Sum Test for samples / pre- & post-values

Subscales	Before (May 2009)	After (November 2009)
Irritability	38	13
Lethargy	23	11
Stereotype	16	9
Hyperactivity	47	20
Inappropriate speech	6	0
P-value for Wilcoxon rank sum test	0.04311	

There are well known alterations of neurotransmitters in autistic people especially in the cerebral cannabinoid receptor system [5]. We therefore asked whether dronabinol could safely be used in autism and what outcomes can be achieved within an observation period of six months.

Methods

This study involved a six year old boy with early infant autism (F84.0), who was diagnosed in the Pediatric Clinic Graz at the age of three. The diagnosis had been made using DSM-IV criteria (American Psychiatric Association, Diagnostic Manuel of Mental Disorders,

4th Edition) and confirmed by ADOS (Autism Diagnostic Observation Schedule) and ADI (Autism Diagnostic Interview) [2][3]. During the six months of follow-up the child did not start any new therapies or change existing assistance measures.

At beginning and end of this study symptom severity was determined by using the ABC (Aberrant Behavior Checklist) [7]. This is a questionnaire consisting of 52 questions with a rating scale from zero to three (0 ... no problem, 3 ... severe problem) filled out by an examiner together with the parents. Results are stratified in five subscales "hyperactivity" (min.0/max.48), "lethargy"(min.0/max.48), "stereotype"(min.0/max.21), "irritability"(min.0/max.45) and "inappropriate speech"(min.0/max.12). Analysis was done with SPSS (SPSS 2002-10) by using the Wilcoxon Rank Sum Test. Statistical significance was set with $p \leq 0.05$.

The therapy used was dronabinol drops (dronabinol solved in sesame oil). Initial dosage was one drop (0.62mg) in the morning which was gradually increased from day to day.

Results

During the six months follow-up the subject received only dronabinol therapy. The maximum tolerated dose effect was reached at 2-1-3 (two drops in the morning,
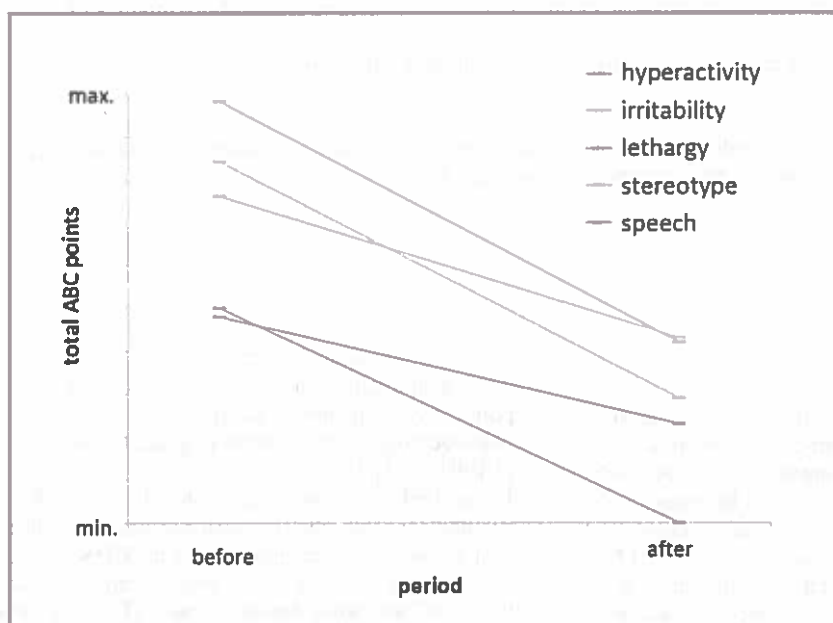
Figure 1. Change of ABC subscales within six months.

one drop midday, three drops in the evening), total daily dose of 3,62 mg dronabinol. No adverse effects were reported during treatment.

The ABC subscales significantly changed over six month (p= 0.04) (see Table 1). Hyperactivity decreased by 27 points, lethargy was reduced by 25 points and irritability by 12 points. Stereotypic behaviour de-

creased by 7 points and inappropriate speech improved by 6 points (see Figure 1).

Discussion

This uncontrolled single case study suggests that dronabinol may reduce symptoms in early infant autism.

This may have been achieved by modifying cannabinoid levels in the central nervous system. Larger controlled studies are needed to explore this effect. Dronabinol will likely not replace cognitive behavioural therapy with early intervention, but we believe that as an additional support it may be effective and better tolerated than many existing antipsychotic drugs.

References

1. Bisogno T, Di Marzo V. Cannabinoid Receptors and Endocannabinoids: Role in Neuroinflammatory and Neurodegenerative Disorders. CNS Neurol Disord Drug Targets. 2010 [in press]
2. Bölte S, Poustka F. Diagnostic Observation Scale for Autistic Disorders: initial results of reliability and validity. Z Kinder Jugendpsychiatr Psychother 2004;32(1):45-50.
3. Bölte S, Poustka F. Psychodiagnostic instruments for the assessment of autism spectrum disorders. Z Kinder Jugendpsychiatr Psychother 2005; 33(1):5-14
4. Campbell JM. Efficacy of behavioral interventions for reducing problem behavior in persons with autism: a quantitative synthesis of single-subject research. Res Dev Disabil 2003;24(2): 120-38.
5. Chakrabarti B, Kent L, Suckling J, Bullmore E, Baron-Cohen S. Variations in the human cannabinoid receptor (CNR1) gene modulate striatal responses to happy faces. Eur J Neurosci 2006; 23(7):1944-8.
6. Crippa JA, Zuardi AW, Hallak JE. Therapeutical use of the cannabinoids in psychiatry. Rev Bras Psiquiatr 2010;32 Suppl 1:S56-66.
7. Demb HB, Brier N, Huron R, Tomor E. The Adolescent Behavior Checklist: normative data and sensitivity and specificity of a screening tool for diagnosable psychiatric disorders in adolescents with mental retardation and other development disabilities. Res Dev Disabil 1994;15(2): 151-65.
8. Dover CJ, Le Couteur A. How to diagnose autism. Arch Dis Child 2007;92(6):540-5.
9. Eikeseth S, Smith T, Jahr E, Eldevik S. Outcome for children with autism who began intensive behavioral treatment between ages 4 and 7: a comparison controlled study. Behav Modif 2007; 31(3):264-78.
10. Grotenhermen F. Cannabinoids in cancer pain. Cannabinoids 2010;5(1):1-3
11. Hasan A, Rothenberger A, Münchau A, Wobrock T, Falkai P, Roessner V. Oral Delta 9-tetrahydrocannabinol improved refractory Gilles de la Tourette syndrome in an adolescent by increasing intracortical inhibition: a case report. J Clin Psychopharmacol 2010;30(2):190-2.
12. Kurz R, Muchitsch E, Feucht M. Cognitive Behavioral Therapy in Children with Autistic Disorder A Prospective Long-time Observational Study. 2010, [in press]
13. Myers SM, Johnson CP; American Academy of Pediatrics Council on Children With Disabilities. Management of children with autism spectrum disorders. Pediatrics 2008;121(4):828-30.
14. Pardo CA, Eberhart CG. The neurobiology of autism. Brain Pathol 2007;17(4):434-47.
15. Patterson SY, Smith V, Jelen M. Behavioural intervention practices for stereotypic and repetitive behaviour in individuals with autism spectrum disorder: a systematic review. Dev Med Child Neurol 2010;52(4):318-27.
16. Posey DJ, McDougle CJ. Pharmacotherapeutic management of autism. Expert Opin Pharmacother 2001;2(4):587-600.
17. Remington B, Hastings RP, Kovshoff H, degli Espinosa F, Jahr E, Brown T, Alsford P, Lemaic M, Ward N. Early intensive behavioral intervention: outcomes for children with autism and their parents after two years. Am J Ment Retard 2007;112(6):418-38.
18. Sallows GO, Graupner TD. Intensive behavioral treatment for children with autism: four-year outcome and predictors. Am J Ment Retard 2005; 110(6):417-38.
19. Trottier G, Srivastava L, Walker CD. Etiology of infantile autism: a review of recent advances in genetic and neurobiological research. J Psychiatry Neurosci 1999;24(2):103-15.Di Marzo V, De Petrocellis L. Plant, synthetic, and endogenous cannabinoids in medicine. Annu Rev Med 2006; 57:553-74.

Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome

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Fragile X syndrome, the most commonly known genetic cause of autism, is due to loss of the fragile X mental retardation protein, which regulates signal transduction at metabotropic glutamate receptor-5 in the brain. Fragile X mental retardation protein deletion in mice enhances metabotropic glutamate receptor-5-dependent long-term depression in the hippocampus and cerebellum. Here we show that a distinct type of metabotropic glutamate receptor-5-dependent long-term depression at excitatory synapses of the ventral striatum and prefrontal cortex, which is mediated by the endocannabinoid 2-arachidonoyl-sn-glycerol, is absent in fragile X mental retardation protein-null mice. In these mutants, the macromolecular complex that links metabotropic glutamate receptor-5 to the 2-arachidonoyl-sn-glycerol-producing enzyme, diacylglycerol lipase-α (endocannabinoid signalosome), is disrupted and metabotropic glutamate receptor-5-dependent 2-arachidonoyl-sn-glycerol formation is compromised. These changes are accompanied by impaired endocannabinoid-dependent long-term depression. Pharmacological enhancement of 2-arachidonoyl-sn-glycerol signalling normalizes this synaptic defect and corrects behavioural abnormalities in fragile X mental retardation protein-deficient mice. The results identify the endocannabinoid signalosome as a molecular substrate for fragile X syndrome, which might be targeted by therapy.

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ragile X syndrome (FRAX), the most frequently known genetic cause of autism, is characterized by a series of physical, cognitive and emotional symptoms that include social deficits and diminished ability to learn[1]. FRAX is due to the mutation of a single gene, called *FMR1* (ref. 2), which encodes for the fragile X mental retardation protein (FMRP)[3]. FMRP regulates the translation and transport of messenger RNAs in dendritic spines of brain neurons, and is involved in modulating signal transduction through type-I metabotropic glutamate (mGlu$_1$ and mGlu$_5$) receptors at glutamatergic synapses throughout the brain. Loss of FMRP causes profound changes in the structure of neuronal dendrites and enhances, in the hippocampus and cerebellum of FMRP-deficient (*fmr1*$^{-/-}$) mice, a form of long-term depression (LTD) that requires mGlu$_5$ receptor-mediated protein synthesis[3–7].

In medium spiny neurons of the ventral striatum and pyramidal neurons of the prefrontal cortex (PFC), activation of mGlu$_5$ receptors initiates a distinct form of LTD, which does not depend on protein synthesis and is affected by the retrograde endocannabinoid (eCB) transmitter, 2-arachidonoyl-*sn*-glycerol (2-AG)[8,9]. Stimulation of mGlu$_5$ receptors releases 2-AG through the sequential recruitment of two enzymes: phospholipase C-β, which produces 1,2-diacyl-*sn*-glycerol, and diacylglycerol lipase-α (DGL-α), which converts 1,2-diacyl-*sn*-glycerol into 2-AG[10–12]. Morphological studies have shown that both phospholipase C-β and DGL-α are localized to the perisynapse, a subdivision of the dendritic spine that forms a 100–200-nm-thick border around the postsynaptic density[13]. mGlu$_5$ receptors are concentrated in this region[14] where they may be physically linked to DGL-α through the adaptor protein Homer[10,15]. This supramolecular complex is likely to have an important role in eCB-mediated transmission at excitatory synapses, by providing a focal point for the generation of a 2-AG pool that is specifically committed to retrograde transmission. The 2-AG produced by this complex can readily cross the synaptic cleft to access CB$_1$ cannabinoid receptors on presynaptic terminals, where the 2-AG-deactivating enzyme monoacylglycerol lipase (MGL) is also found[16,17]. We propose that this complex, which we refer to as 'eCB signalosome', allows for the separation of the 2-AG pool responsible for eCB signalling from other cellular pools of 2-AG, such as those involved in eicosanoid production and phospholipid remodelling.

FMRP deletion is known to be associated with greater eCB-mediated responses at GABAergic synapses of the dorsal striatum and CA1 region of the hippocampus[18,19]. Here, we show that loss of FMRP is accompanied by marked deficits in mGlu$_5$-dependent 2-AG release and eCB-mediated LTD at excitatory synapses of the forebrain, as well as by a structural disorganization in the eCB signalosome. We further show that inhibition of presynaptic MGL activity normalizes eCB-mediated LTD and corrects key behavioural abnormalities in *fmr1*$^{-/-}$ mice, suggesting that pharmacological enhancement of 2-AG signalling might offer a new therapeutic strategy for FRAX.

Results

FMRP binds DGL-α mRNA and controls 2-AG signalling. FMRP regulates protein translation in postsynaptic spines by interacting with mRNAs that often, albeit not always, contain a G-quartet FMRP-binding motif[20]. A bioinformatics search showed that an FMRP-binding sequence is present in DGL-α mRNA (Fig. 1a), but not in mRNAs encoding for structurally or functionally related enzymes such as DGL-β, MGL, *N*-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD) and α/β hydrolase domain-6 (ABHD6) (data not shown). To test whether FMRP binds the DGL-α message, we immunoprecipitated FMRP from mouse forebrain homogenates and used quantitative PCR to measure DGL-α mRNA in the pellets. The pellets contained DGL-α mRNA (Fig. 1b), but no detectable levels of mRNAs encoding for DGL-β or NAPE-PLD (Fig. 1c). As expected, the precipitates

also contained mRNAs encoding for PSD-95 and amyloid precursor protein, which are known to bind FMRP[21,22] (Fig. 1d). Moreover, experiments with rat cortical neurons in primary cultures showed that exposure to the mixed mGlu$_1$/mGlu$_5$ receptor agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) (100 μM) caused the dissociation between FMRP and DGL-α mRNA (Fig. 1e), as previously shown for other FMRP-regulated messages[22].

To examine whether FMRP deletion influences mGlu$_5$-dependent 2-AG signalling, we used mouse brain synaptoneurosomes, a subcellular preparation enriched in resealed postsynaptic spines attached to their corresponding axon terminals[23]. As expected for previous results[10,11], incubation of wild-type synaptoneurosomes with DHPG resulted in a rapid increase in DGL activity, which was dependent on both incubation time and DHPG concentration (Fig. 2a,b), and was accompanied by an elevation in 2-AG levels (Fig. 2c). These effects were not associated with changes in DGL-α content (Fig. 2d) and were not prevented by the translation inhibitor cycloheximide (Supplementary Fig. S1A), indicating that they did not require the synthesis of new DGL-α protein. In synaptoneurosomes prepared from *fmr1*$^{-/-}$ mice, DHPG failed to stimulate DGL activity and 2-AG production (Fig. 2a–c), even though baseline levels of 2-AG were similar to those measured in wild-type controls (Supplementary Fig. S1B). The results suggest that genetic loss of FMRP disrupts mGlu$_5$-dependent 2-AG signalling at excitatory synapses.

FMRP targets DGL-α to the perisynapse. One mechanism through which FMRP deletion might affect receptor-operated 2-AG production is by altering the structure of the supramolecular complex that couples mGlu$_5$ receptors to DGL-α (which we refer to here as 'eCB signalosome'). To test this possibility, we immunostained for mGlu$_5$ or DGL-α brain sections from wild-type and *fmr1*$^{-/-}$ mice. We first focused on the core of the ventral striatum, a brain region in which the eCB system mediates excitatory LTD[8]. Immunoperoxidase labelling for mGlu$_5$ or DGL-α revealed a dense punctuate distribution throughout the neuropil, which appeared to be similar between wild-type and *fmr1*$^{-/-}$ mice (Fig. 3a,b,g,h). Further analyses by immunogold electron microscopy revealed a widespread presence of mGlu$_5$ and DGL-α in postsynaptic profiles—predominantly in dendritic spine heads that received asymmetric, putatively glutamatergic inputs from DGL-α or mGlu$_5$-immunonegative axon terminals (Fig. 3c,d,i,j). To assess whether FMRP deletion alters the subcellular distribution of mGlu$_5$ and DGL-α, in three separate experiments we measured the distance along the plasma membrane between the gold particles attached to DGL-α or mGlu$_5$ and the closest edge of the postsynaptic density. There was no statistically detectable difference in the subcellular distribution of mGlu$_5$ between wild-type and *fmr1*$^{-/-}$ mice (*n* = 294 and 285 synapses for wild-type and *fmr1*$^{-/-}$ mice, respectively; Kolmogorov–Smirnov test, *P* = 0.917) (Fig. 3k). By contrast, the cumulative distribution functions of the subcellular localization of DGL-α were significantly different between the two genotypes (*n* = 259 and 185 synapses for wild-type and *fmr1*$^{-/-}$ mice, respectively; *P* = 0.001) (Fig. 3e). This suggests that FMRP deletion selectively impairs the subcellular targeting of DGL-α in postsynaptic spine heads, without affecting the targeting of mGlu$_5$.

To investigate this phenomenon further, we divided the spine-head plasma membrane into 60-nm bins and calculated the frequency of gold particles within each bin. As previously shown for the hippocampus[13], particles associated with DGL-α were concentrated in the perisynaptic zone, with a gradient that decreased towards the spine neck, and were nearly absent from the intrasynaptic domain (Fig. 3f). This skewed distribution was clearly detectable in the ventral striatum core of wild-type mice, but was totally absent in *fmr1*$^{-/-}$ mutants (Fig. 3f). Swapping all data points in the first
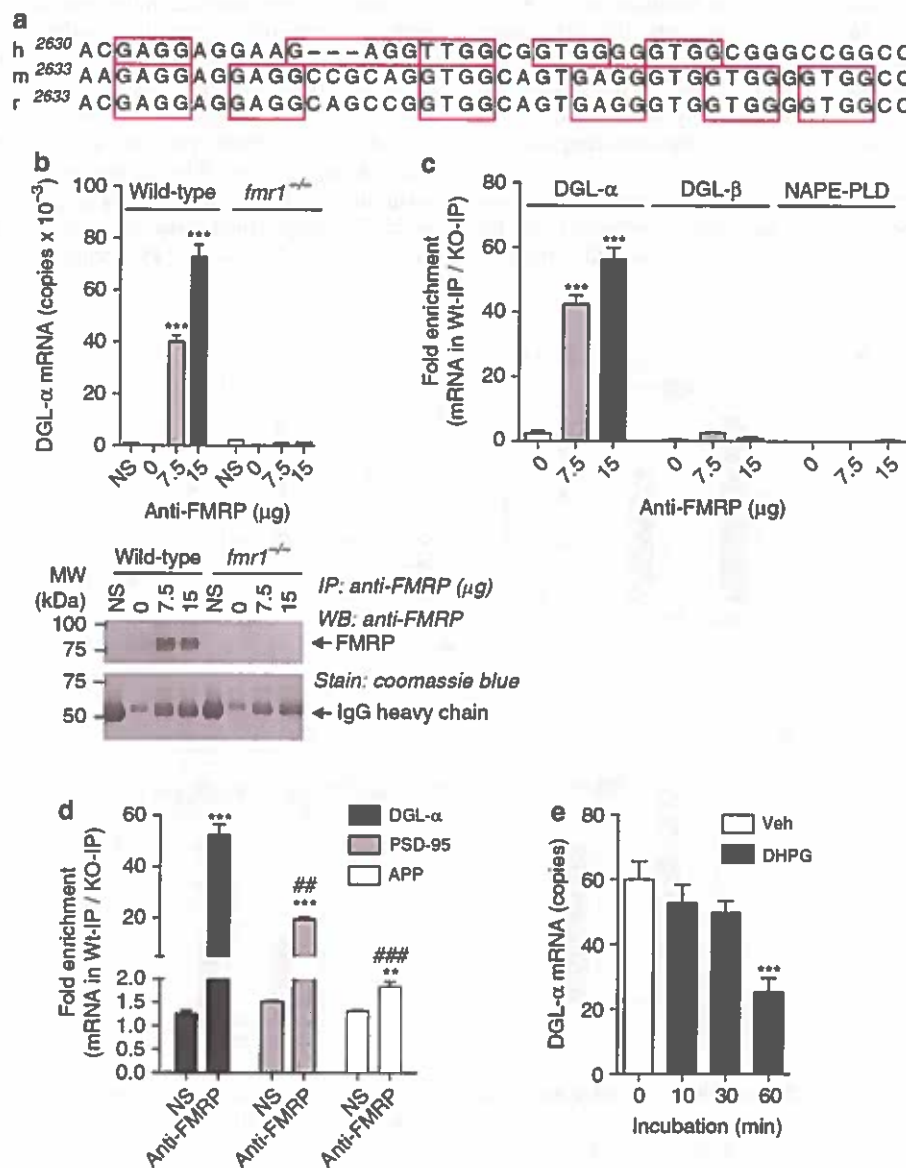
Figure 1 | DGL-α mRNA binds to FMRP. (a) Alignment of the coding region in human (h), mouse (m) and rat (r). DGL-α mRNAs reveal a putative G-quartet sequence within a G-rich region containing several DWGG repeats. The canonical G-quartet motif is DWGG-$N_{(0-5)}$-DWGG-$N_{(0-3)}$-DWGG-$N_{(0-2)}$-DWGG, where D is any nucleotide except C, and W is A or U. The DWGG repeats are boxed in red. (b) Co-immunoprecipitation of FMRP with DGL-α mRNA. Brains from wild-type or $fmr1^{-/-}$ mice were homogenized and centrifuged at 70,000 g for 30 min. The supernatant (1 mg protein) was incubated with the indicated amounts of anti-FMRP antibody or normal serum (NS), and the immunocomplex was precipitated using protein G-sepharose beads. Top, levels of DGL-α mRNA in the immunoprecipitates were quantified by real-time quantitative PCR ($n=3$, ***$P<0.001$). Bottom, a portion of the immunoprecipitate was subjected to SDS–PAGE and western blot analyses to confirm the presence of FMRP. (c,d) Analyses of mRNAs encoding for DGL-α, DGL-β and NAPE-PLD (c) or positive control PSD-95 and amyloid precursor protein (d) in anti-FMRP-immunoprecipitates ($n=3$, **$P<0.01$ and ***$P<0.001$ compared with NS, ##$P<0.01$ and ###$P<0.001$ compared with DGL-α-anti-FMRP). Similar results were obtained using $fmr1^{-/-}$ mice bred on either a FVB.129 background (b,c) or a C57BL/6J background (d). (e) DHPG-induced dissociation of DGL-α mRNA from FMRP in cultured cortical neurons. Rat primary neurons were prepared from embryonic day 18 cortex, as described[55]. Cells were treated with DHPG (100 μM) in culture medium and harvested at the indicated time. Levels of FMRP-bound DGL-α mRNA were determined by anti-FMRP immunoprecipitation and quantitative PCR, as described in Methods ($n=5$, ***$P<0.001$). Results are representative of at least two independent experiments. Significance was determined using two-tailed Student's t-test. Error bars represent s.e.m.

60-nm bin of wild-type mice to the same data set of $fmr1^{-/-}$ animals and vice versa eliminated statistical significance (Kolmogorov–Smirnov test, $P=0.187$ and $P=0.332$ for wild-type to $fmr1^{-/-}$ swap and $fmr1^{-/-}$ to wild-type swap, respectively). As swapping data sets in other bins did not reveal differences, the disappearance of DGL-α labelling from the perisynaptic domain may underlie the difference in the cumulative distribution functions. Furthermore, the same

analytical approach revealed a biased perisynaptic localization of mGlu5 in both wild-type and $fmr1^{-/-}$ mice (Fig. 3l).

To determine whether FMRP deletion influences the subcellular targeting of DGL-α in other forebrain structures, we examined the localization of mGlu5 and DGL-α in the CA1 subfield of the hippocampal formation,[9,13,24,25]. The perisynaptic localization of mGlu5 at hippocampal excitatory synapses was identical between

$fmr1^{-/-}$ and wild-type mice, whereas the localization of DGL-α was markedly impaired in $fmr1^{-/-}$ mutants (Supplementary Fig. S2). An economical interpretation of these findings, which is consistent with our biochemical data, is that FMRP enables the correct targeting of DGL-α and its assembly with mGlu₅ into a functional eCB signalosome at excitatory synapses throughout the forebrain.

FMRP is a core component of the translational machinery in dendritic polyribosomes and has been implicated in the transport and localization of mRNAs to dendrites and synapses[3].

We reasoned therefore that, in the absence of FMRP, DGL-α mRNA might be translated ectopically. Consistent with this prediction, an analysis of 300 synapses per genotype revealed an increased average density of intracellular DGL-α labelling in the ventral striatum of $fmr1^{-/-}$ mice, compared with wild-type animals (0.91 (range: 0.6–1.43) gold particles per μm^2 and 3.02 (range: 2.19–3.63) gold particles per μm^2 in wild-type and $fmr1^{-/-}$ animals, respectively, normalized to background levels) (Fig. 3m). This increase in normalized average density was also reflected in the ratio of intracellularly localized DGL-α (14% (range: 10–17%) and 36% (range:
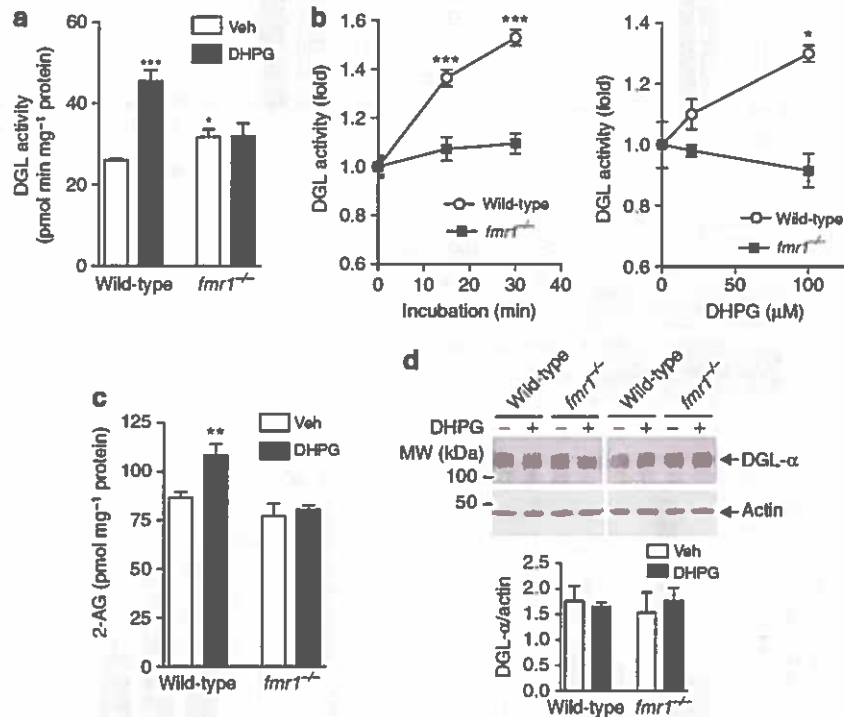
Figure 2 | mGlu₅ receptor-stimulated DGL activity and 2-AG release is disrupted in $fmr1^{-/-}$ mice. (a) Synaptoneurosome (SN) fractions prepared from wild-type or $fmr1^{-/-}$ mice (1 mg ml⁻¹) were incubated at 37 °C for 30 min in the presence of 100 μM DHPG, and DGL activity was measured *in vitro* using 10 μM diheptadecanoylglycerol as a substrate ($n = 4$). (b) DGL activity was measured in SN fractions after incubation with various concentrations of DHPG ($n = 3$). (c) 2-AG levels in SN fractions were measured after 45-min incubation with DHPG (100 μM) or vehicle (Veh, PBS) ($n = 5$–9). (d) After treatment of synaptoneurosomes with DHPG, as described above, levels of DGL-α protein were measured by western blot. Representative images for DGL-α and loading control actin are shown ($n = 6$ each). Experiments were conducted on $fmr1^{-/-}$ mice on C57BL/6J (a,d) or FVB.129 background (b,c). Results are representative of at least two independent experiments. Significance was determined using two-tailed Student's *t*-test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Error bars represent s.e.m.

Figure 3 | Impaired targeting of DGL-α to the perisynaptic domain of excitatory synapses in $fmr1^{-/-}$ mice. DGL-α immunoperoxidase labelling in wild-type (a) and $fmr1^{-/-}$ mice (b) reveals a similar granular staining pattern in the neuropil of the accumbens/ventral striatum (arrowheads). Medium spiny neuron somata are devoid of labelling (asterisks). aco, anterior commissure. Electron micrographs of asymmetric synapses reveal that DGL-α-positive gold particles (arrowheads) are predominantly found perisynaptically, close to the PSD (red arrows) in wild-type animals (c), but not in $fmr1^{-/-}$ synapses (d) s, spine head; b, bouton. (e) Cumulative distribution plot of DGL-α localization in wild-type (solid line, $n = 259$) and $fmr1^{-/-}$ spines (dashed line, $n = 185$). (f) Histogram summarizing the normalized distribution of DGL-α-immunogold on the spine head membrane of wild-type mice (black arrow and black bars, $n = 259$) and $fmr1^{-/-}$ mice (grey arrow and grey bars, $n = 185$). Large arrow = edge of PSD. mGlu₅ immunoperoxidase labelling in wild-type (g) and $fmr1^{-/-}$ animals (h) depicts a similar granular labelling pattern (arrowheads) as DGL-α. Electron micrographs of asymmetric synapses containing mGlu₅-immunogold labelling (arrowhead) revealed that mGlu₅ was predominantly found perisynaptically (red arrows) in both wild-type (i) and $fmr1^{-/-}$ spines (j). (k) Cumulative distribution plot of mGlu₅-immunogold localization in wild-type (solid line, $n = 294$) and $fmr1^{-/-}$ mice (dashed line, $n = 285$). (l) Histogram representing the normalized distribution of mGlu₅-immunogold on spine heads of wild-type (black arrow and black bars, $n = 294$) and $fmr1^{-/-}$ spines (grey arrow and grey bars, $n = 285$). The number of DGL-α-positive spines was higher in $fmr1^{-/-}$ mice; however, the density of spine head membrane labelling was the same in both strains. Instead, a significantly higher density of DGL-α labelling was found within the spine head and neck cytoplasm (arrowheads; m1-m2) and along the spine neck membrane (black arrowheads; n). Symmetrical synapses are considered inhibitory GABAergic inputs and frequently synapse onto spine necks (red arrowhead; n) and dendritic shafts. Importantly, the number of DGL-α positive symmetrical synapses were similar in wild-type (o) and $fmr1^{-/-}$ mice (p). Scale bars: 15 μm in a,b,g and h and 100 nm in c,d,i,j,m-p.

33–46%) of all gold particles found in dendritic spine heads in wild-type and $fmr1^{-/-}$ animals, respectively). In another experiment, we assessed the presence of DGL-α labelling in a second data set of 75 random intact spines per animal. We found that $fmr1^{-/-}$ spines had a higher probability of DGL-α labelling than did wild-type spines (χ^2 test, $P < 0.001$).

In which subcellular structure was DGL-α labelling concentrated? To address this question, we divided each spine into three distinct
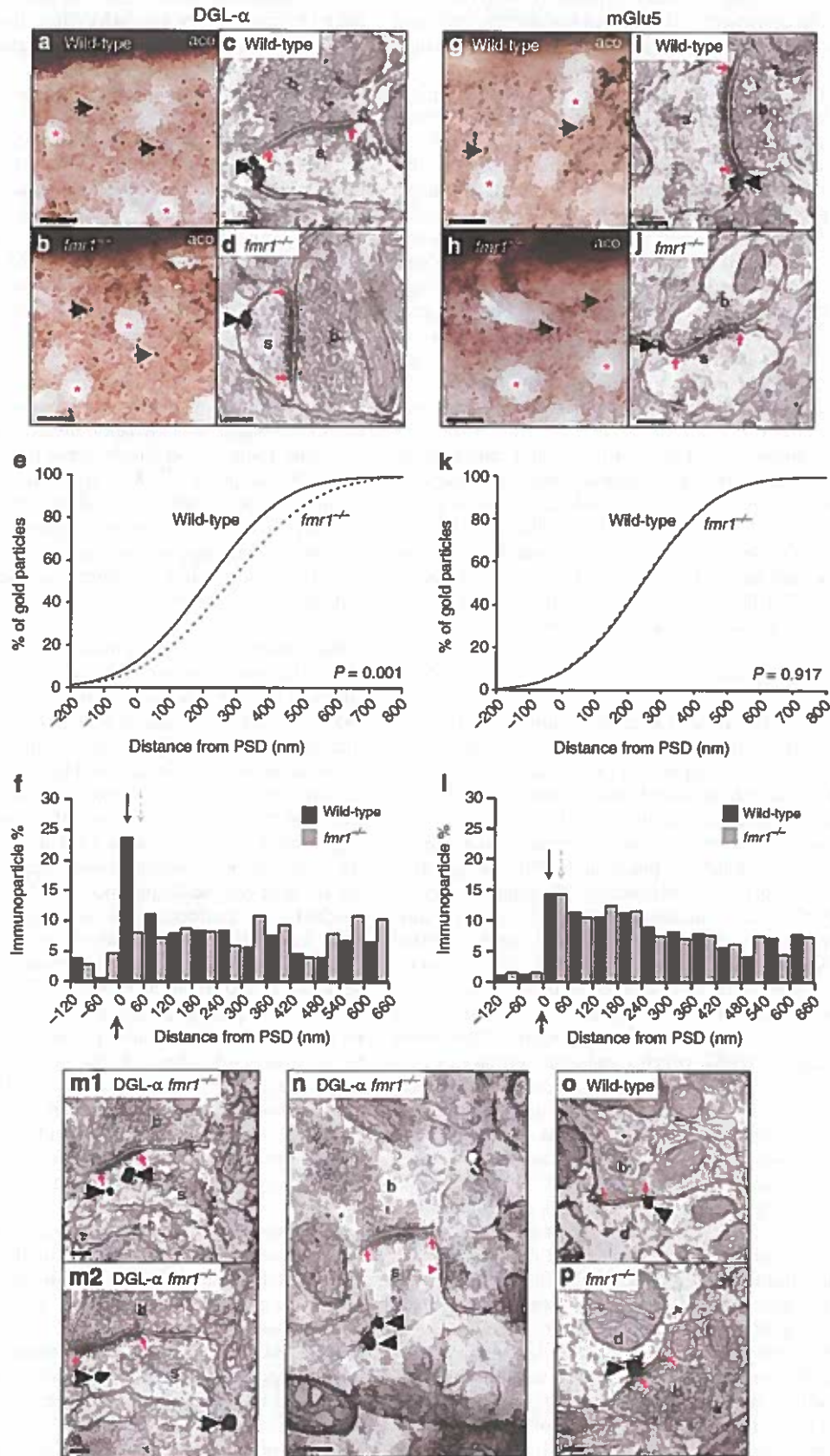
compartments: spine-head membrane, spine-neck membrane, and head and neck cytoplasm. The number of DGL-α gold particles on the spine head membrane was very similar between wild-type and $fmr1^{-/-}$ mice (χ^2 test, $P = 0.696$). By contrast, we found a significantly higher number of DGL-α gold particles in spine neck membrane (χ^2 test, $P < 0.001$) (Fig. 3n) and cytoplasm (χ^2 test, $P = 0.003$) of $fmr1^{-/-}$ mice. In agreement with previous studies[26,27], we found that dendrites in $fmr1^{-/-}$ mice exhibited longer spine necks than wild-type (852 ± 19 and 1030 ± 24 nm in wild-type and $fmr1^{-/-}$ respectively; Kolmogrov–Smirnov test, $P < 0.001$), which might account for the higher number of DGL-α gold particles observed in these animals. However, when we calculated the density of DGL-α labelling for each spine compartment, this was also significantly higher in the $fmr1^{-/-}$ necks than wild-type mice (2.29 (range: 1.63–3.01) gold particles per μm^2 and 4.67 (range: 3.63–5.85) gold particles per μm^2 in wild-type and $fmr1^{-/-}$ animals, respectively). By contrast, the density of DGL-α labelling in the spine-head membrane was similar between the two strains (1.26 (range: 0.43–1.89) gold particles per μm^2 and 1.42 (range: 1.06–1.86) gold particles per μm^2 in wild-type and $fmr1^{-/-}$ animals, respectively), but higher intracellular labelling was observed in $fmr1^{-/-}$ mice (0.4 (range: 0.2–0.8) gold particles per μm^2 and 0.91 (range: 0.78–0.92) gold particles per μm^2 in wild-type and $fmr1^{-/-}$ animals, respectively).

Finally, we examined whether FMRP deletion might affect DGL-α localization at GABAergic synapses, which are also dysfunctional in $fmr1^{-/-}$ mice[18,19]. DGL-α gold particles were found at the postsynaptic plasma membrane of both wild-type (Fig. 3o) and $fmr1^{-/-}$ mice (Fig. 3p), but no statistically detectable difference was noted in their distribution (χ^2 test, $P = 0.6$, $n = 150$). The results suggest that genetic FMRP deletion alters the normal localization of DGL-α at excitatory, but not inhibitory, synapses of the forebrain, causing this enzyme to accumulate ectopically in the neck and cytoplasm of dendritic spines.

FMRP enables eCB-LTD in ventral striatum and PFC. The biochemical and ultrastructural results reported above suggest that FMRP deletion reduces 2-AG-dependent LTD at excitatory synapses of the forebrain. In acutely dissected brain slices from wild-type mice, low-frequency electrical stimulations (10 min, 10 Hz) elicited robust LTD of excitatory postsynaptic currents (EPSCs, Fig. 4a, white symbols) or extracellular field potentials (fEPSP, see Fig. 5a) in medium spiny neurons of the ventral striatum[8]. The induction of LTD was prevented by the DGL-α inhibitor tetrahydrolipstatin (10 μM) (Fig. 4b), as expected from previous work[9,28], and was not affected by pharmacological blockade of protein synthesis (Supplementary Fig. S3). Importantly, the same low-frequency stimulations that caused LTD in wild-type mice failed to do so in $fmr1^{-/-}$ mutants (Figs 4a and 5a, black symbols). Moreover, bath applications of the mixed mGlu$_1$/mGlu$_5$ agonist, DHPG, which consistently elicited LTD in brain slices prepared from wild-type mice, were ineffective in slices obtained from $fmr1^{-/-}$ mutants (Fig. 4c). To evaluate whether the deficits in eCB-LTD observed in $fmr1^{-/-}$ mutants extended beyond the ventral striatum, we took advantage of the fact that this brain region receives major excitatory projections from the medial PFC, a structure where mGlu$_5$ activation also underlies 2-AG-dependent eCB-LTD at synapses of pyramidal neuron layers 2/3 and 5/6 (refs 9,29). Similar to the ventral striatum, eCB-LTD was absent in PFC slices obtained from $fmr1^{-/-}$ mutants (Fig. 4d). Together, these results support the idea that FMRP deletion causes a widespread impairment of mGlu$_5$-dependent LTD at excitatory synapses.

To test the alternative possibility that the LTD deficit observed in $fmr1^{-/-}$ mice might result from impaired CB$_1$ receptor function, we assessed the ability of the cannabinoid agonist CP55940 (0.01–10 μM) to inhibit synaptic transmission in slices of ventral striatum prepared from wild-type or $fmr1^{-/-}$ mice. The drug's potency and

efficacy were identical in the two strains (Supplementary Fig. S4A), indicating that CB$_1$ receptors function normally in $fmr1^{-/-}$ mice. Moreover, tonic eCB signalling did not appear to be affected by FMRP deletion, because the selective CB$_1$ inverse agonist AM251 (4 μM, applied 30 min before testing) did not alter baseline excitatory transmission in the ventral striatum of wild-type or $fmr1^{-/-}$ mice (Supplementary Fig. S4B). Thus, the lack of eCB-dependent plasticity observed in $fmr1^{-/-}$ mice cannot be accounted for by alterations in CB$_1$ function or tonic eCB activity. We also compared the intrinsic properties of ventral striatum medium spiny neurons in wild-type and $fmr1^{-/-}$ mice, and found no difference in medium spiny neurons excitability (Supplementary Fig. S5). Deletion of FMRP may negatively impact the basic properties of ventral striatum excitatory synapses and, to test for this possibility, we recorded AMPA receptor-mediated spontaneous EPSCs (sEPSCs) in medium spiny neurons of both genotypes. The amplitude and frequency of AMPAR sEPSCs were similar in wild-type and $fmr1^{-/-}$ mice (Supplementary Fig. S6). The results rule out the possibility that modifications of excitability or basal synaptic properties of ventral striatum medium spiny neurons may be responsible for impaired eCB-LTD in $fmr1^{-/-}$ mice.

To test whether FMRP might influence intracellular signal transduction pathways mediating other forms of LTD, we also analysed the LTD evoked by presynaptic mGlu$_{2/3}$ receptors, which utilizes the same transduction mechanisms that are engaged by presynaptic CB$_1$ receptors[30,31]. Bath application of the selective mGlu$_{2/3}$ agonist LY379268 (100 nM) produced comparable levels of LTD in wild-type and $fmr1^{-/-}$ mice (Supplementary Fig. S7). Together, our experiments support the conclusion that FMRP enables mGlu$_5$ to initiate retrograde 2-AG signalling and elicit LTD at excitatory synapses of the forebrain.

Enhancement of 2-AG signalling normalizes LTD in $fmr1^{-/-}$ mice. The results reported above suggest that, in $fmr1^{-/-}$ mice, the failure of DGL-α to associate with mGlu$_5$ into a functional eCB signalosome results in a loss of eCB-LTD. We have previously shown that pharmacological blockade of intracellular 2-AG hydrolysis, which is predominantly catalysed by presynaptic MGL[16,32], lowers the threshold for LTD induction at excitatory synapses of the ventral striatum[9,29]. We hypothesized therefore that inhibitors of 2-AG degradation might normalize LTD in $fmr1^{-/-}$ mice by reinforcing 2-AG activity at residual intact synapses or, possibly, extending the reach of ectopically produced 2-AG. To test this possibility, we blocked 2-AG hydrolysis in slices using the potent and irreversible MGL inhibitor, JZL184[33]. Brain slices were incubated with JZL184 (1 μM) for 45–90 min before LTD induction. This treatment was sufficient to restore both synaptic and pharmacologically induced LTD in slices prepared from $fmr1^{-/-}$ mutants (Fig. 5a,b), but had no effect in slices from wild-type controls (Supplementary Fig. S8). In addition, inhibition of the postsynaptic 2-AG-hydrolysing enzyme ABHD6[29] with WWL70 (10 μM, 45–90-min incubation) also restored LTD in slices from ventral striatum of $fmr1^{-/-}$ mice (Fig. 5a,b). The effects of JZL184 and WWL70 indicate that pharmacological blockade of either presynaptic or postsynaptic 2-AG degradation rescues eCB-dependent LTD in $fmr1^{-/-}$ mice.

Enhancing 2-AG corrects behavioural changes in $fmr1^{-/-}$ mice. When bred on a C57BL/6 background, $fmr1^{-/-}$ mice display a behavioural phenotype that is characterized by elevated motor activity in an open field (Fig. 6a,b) and decreased aversion to the open arms of an elevated plus maze[34] (Fig. 6c,d). The MGL inhibitor JZL184 (16 mg kg^{-1}, intraperitoneal) increased brain 2-AG levels (Supplementary Fig. S9A) and corrected these behavioural abnormalities within 6 h of administration (Fig. 6a–d). In $fmr1^{-/-}$ mice, JZL184 reduced the number of squares crossed (Fig. 6a) and the total distance travelled (Fig. 6b) in the open field test.
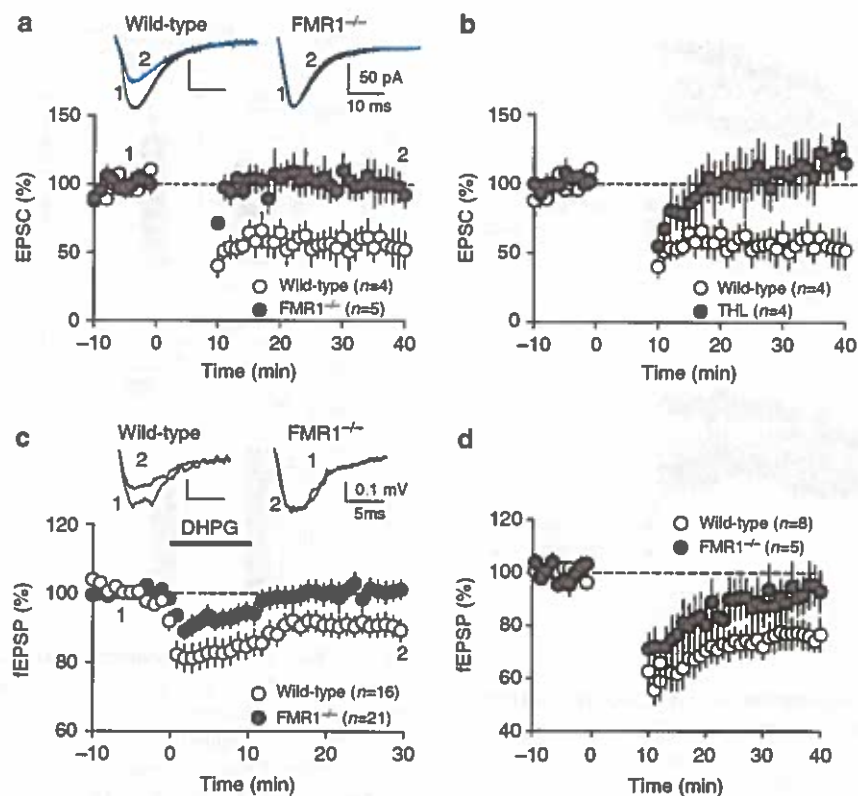
Figure 4 | LTD is abolished in ventral striatum and PFC of *fmr1*^{−/−} mice. (a) Summary graph showing whole-cell evoked EPSC amplitudes. Values are normalized to baseline before the induction of LTD and averaged per minute. Tetanic stimulation (10 min at 10 Hz starting at time 0) of PFC afferent fibres to medium spiny neurons in the ventral striatum induces a robust LTD in wild-type littermates (open symbols, *n* = 4) but not *fmr1*^{−/−} mice (filled symbols, *n* = 5). Here as in all physiology figures, *n* equals the number of animals. (b) Summary graph showing averaged time courses of the experiments in which the 10 min at 10-Hz protocol was given in control ACSF (open symbols, *n* = 4) and after pre-treatment with tetrahydrolipstatin (THL, 10 μM, filled symbols, *n* = 4), an inhibitor of the DGL-α. Graphs show EPSC amplitudes normalized to baseline before the induction of LTD and averaged per minute. (c) Direct pharmacological activation of mGlu$_{1/5}$ with 50 μM (S)-DHPG induces LTD in the ventral striatum of wild-type littermates (open symbols, *n* = 16) but not *fmr1*^{−/−} mice (filled symbols, *n* = 21). Summary graphs show excitatory postsynaptic field potentials (fEPSP) amplitudes. (d) Summary graph showing fEPSP amplitudes recorded in pyramidal neurons of the PFC. Values are normalized to baseline before the induction of LTD and averaged per minute. Tetanic stimulation (10 min at 10 Hz starting at time 0) of layers 2/3 to layers 5/6 pyramidal synapses induces a robust LTD in wild-type littermates (open symbols, *n* = 8) but not *fmr1*^{−/−} mice (filled symbols, *n* = 5). Statistical significance was determined using Mann–Whitney U-test. Error bars represent s.e.m.

The compound also reduced the number of entries and the time spent in the open arm in the elevated plus maze test (Fig. 6c,d and Supplementary Fig. S9C, D). By contrast, JZL184 did not significantly affect open field or elevated plus maze behaviours in wild-type mice (Fig. 6a–d and Supplementary Fig. S9B–D). These experiments suggest that pharmacological enhancement of 2-AG signalling normalizes two key behavioural changes observed in *fmr1*^{−/−} mice.

Discussion

In the present study, we provide three lines of evidence indicating that FMRP exerts a tight regulatory control over 2-AG-dependent retrograde signalling at excitatory synapses of the mouse forebrain. First, we show that an FMRP antibody selectively immunoprecipitates DGL-α mRNA in forebrain synaptoneurosome extracts. This is suggestive of a direct association between the two molecules, possibly through an interaction of FMRP with the G-quartet motif of DGL-α mRNA[35]. Second, we report that the normal perisynaptic colocalization of DGL-α and mGlu$_5$ is severely impaired in *fmr1*^{−/−} mice, indicating that FMRP is necessary for the correct targeting of DGL-α to dendritic spines. Finally, we find that FMRP deletion disrupts both mGlu$_5$-stimulated 2-AG production in synaptoneurosomes and LTD at excitatory synapses of the ventral striatum and PFC. These findings suggest that FMRP may direct the assembling

of DGL-α and mGlu$_5$ into a functional signalosome, and is essential for retrograde 2-AG transmission at glutamatergic synapses (Fig. 7). Underscoring the functional significance of these results, we show that pharmacological blockade of 2-AG degradation reinstates eCB-dependent LTD and corrects key behavioural changes in *fmr1*^{−/−} mice.

The eCB 2-AG is considered to be a primary effector of mGlu$_5$-dependent plasticity in the brain[9,12,28], but the dynamics of 2-AG signalling at central synapses are still unclear. One important question pertains to the mechanism through which mGlu$_5$ activation stimulates production of the 2-AG pool responsible for retrograde eCB transmission. Based on the present results, we hypothesize that the stable association of mGlu$_5$ and DGL-α into a multiprotein complex at the perisynaptic annulus of the dendritic spine provides a structural scaffold that allows for the localized formation of a signalling-competent pool of 2-AG, which may be distinct from the intracellular 2-AG pools involved in phospholipid remodelling and eicosanoid biosynthesis[36,37]. We propose to call this complex 'eCB signalosome', to signify its pivotal role in eCB-mediated retrograde transmission (Fig. 7).

How does the lack of FMRP expression alter the structure of the eCB signalosome? The available data suggest two possibilities. The first is that, in the absence of FMRP, the DGL-α message may
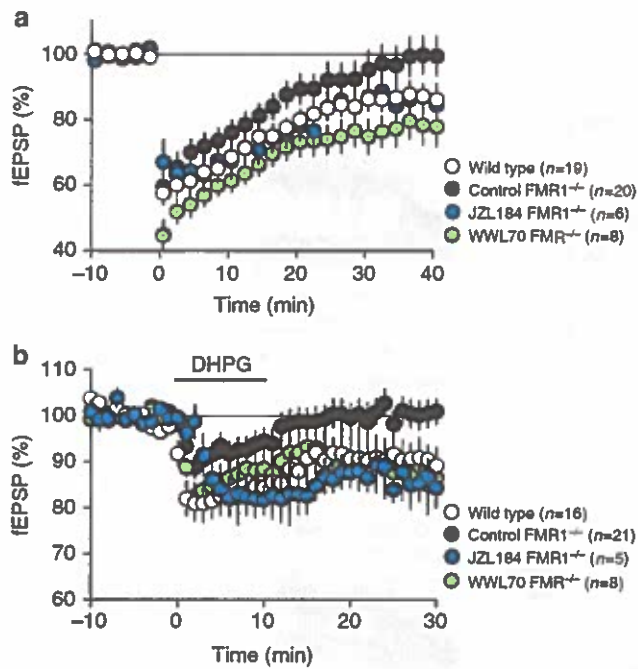
Figure 5 | Inhibitors of 2-AG degradation rescues eCB-dependent LTD in *fmr1*^{-/-} **mice. (a)** Normalized fEPSP amplitude in *fmr1*^{-/-} mice without (n = 20; black symbols) or with JZL184 (1 μM; blue symbols, n = 6) or WWL70 (10 μM; green symbols, n = 8) pretreatment (starting 45–90 min before LTD induction) followed by the LTD induction protocol at time zero. **(b)** Normalized fEPSP amplitude in *fmr1*^{-/-} mice without (n = 20; black symbols) or with JZL184 (1 μM; blue symbols, n = 5) or WWL70 (10 μM; green symbols, n = 8) pretreatment (starting 45–90 min before LTD induction) followed by 10-min bath perfusion with 50 μM (S)-DHPG at time zero. Statistical significance was determined using Mann–Whitney U-test. Error bars represent s.e.m.

Figure 6 | MGL inhibition normalizes behavioural alterations in *fmr1*^{-/-} **mice.** Effects of the MGL inhibitor JZL184 on open field **(a,b)** and elevated plus maze **(c,d)** behaviours. Wild-type and *fmr1*^{-/-} mice received JZL184 (JZL, 16 mg kg^{-1}, intraperitoneal) or vehicle (Veh), and were tested 6 h after injections. Activity in the open field was measured by counting the number of squares crossed **(a)** and total distance travelled **(b)**. In the elevated plus maze test, we measured percentage of open arm entries **(c)** and average time in open per each entry **(d)**. Significance was determined using two-way analysis of variance with post-hoc Student–Newman–Keuls test. NS, not significant; *P < 0.05, and ***P < 0.001 compared with wild-type-Veh; #P < 0.05, and ##P < 0.01 compared with *fmr1*^{-/-}-Veh (n = 11 per each group). Error bars represent s.e.m.

be incorrectly targeted within the dendritic spines, leading to errors in the timing of DGL-α translation or the localization of the translated protein. An error in spatial mRNA delivery caused by FMRP deletion was recently demonstrated for another key postsynaptic protein, calmodulin-activated protein kinase II[38]. A second possibility is that FMRP deletion may produce an alteration in the function of Homer proteins. These scaffolding proteins bind both mGlu5 and DGL-α[10], and single-point mutations in the Homer-2-binding region of DGL-α result in an enzyme that retains functional activity, but is found in intracellular membrane compartments rather than the plasma membrane[10]. Notably, uncoupling of mGlu5 from the postsynaptic Homer scaffold results in the absence of mGlu-activated retrograde 2-AG signalling at excitatory synapses[39]. Previous studies suggest a similar functional uncoupling between mGlu5 and Homer-2 in *fmr1*^{-/-} mice, despite comparable protein levels in synaptoneurosome preparations[40]. In support of this idea, an altered balance in mGlu5 scaffolding between short-form Homer 1a and long-form Homer proteins was found in *fmr1*^{-/-} mice[41]. The hypotheses outlined above will be tested in future experiments.

The mGlu receptor theory of FRAX is based on the seminal observation that *fmr1*^{-/-} mice display a marked enhancement of a form of hippocampal LTD that depends on mGlu receptor-mediated protein synthesis[3,5-7]. Consistent with this theory, the eCB-mediated LTD induced at CA1 GABAergic synapses by pharmacological activation of mGlu5 is similarly enhanced in *fmr1*^{-/-} mice[18]. Likewise, in the dorsal striatum of these mutants mGlu5-mediated eCB activity at GABAergic synapses is increased[19]. The present results, showing that eCB-LTD is abolished in the PFC and

ventral striatum of *fmr1*^{-/-} mice, are only apparently in opposition with those data. Indeed, the notion that different neuron and synapse types adapt differently to the lack of FMRP is supported by a recent study showing that mGlu receptor-dependent LTP is reduced in the lateral amygdala of *fmr1*^{-/-} mice[42]. Although we found no changes in DGL-α density at GABAergic synapses, 2-AG signalling was suggested to be heterosynaptic between glutamatergic and GABAergic synapses[43] (that is, 2-AG released from dendritic spines might activate CB1 receptors on more distant GABAergic terminals). Thus, it is possible that the shifted DGL-α localization gradient from the perisynaptic annulus to the spine neck compartment, where GABAergic synapses are often present (for example see red arrowhead in Fig. 3n), may underlie the opposite alterations in CB1-dependent regulation of synaptic plasticity between these synapses (refs 18,19 and present study). Our findings are in line, therefore, with the core prediction of the mGlu receptor theory—namely, the idea that loss of FMRP alters most consequences of mGlu5 activation[44]. On the other hand, the present results reveal molecular disparities between excitatory and inhibitory synapses of the same brain region, and highlight that distinct forms of LTD may also behave differently in FRAX. While protein synthesis-dependent LTD is enhanced, presynaptic mGlu2/3-dependent LTD is unaffected, and finally the protein synthesis-independent, but eCB-mediated, LTD is absent. This palette of synaptic changes might underlie the complex neurobehavioural abnormalities observed in FRAX patients.

Pharmacological or genetic modulation of mGlu5 signalling normalizes various functional and structural aberrations occurring in
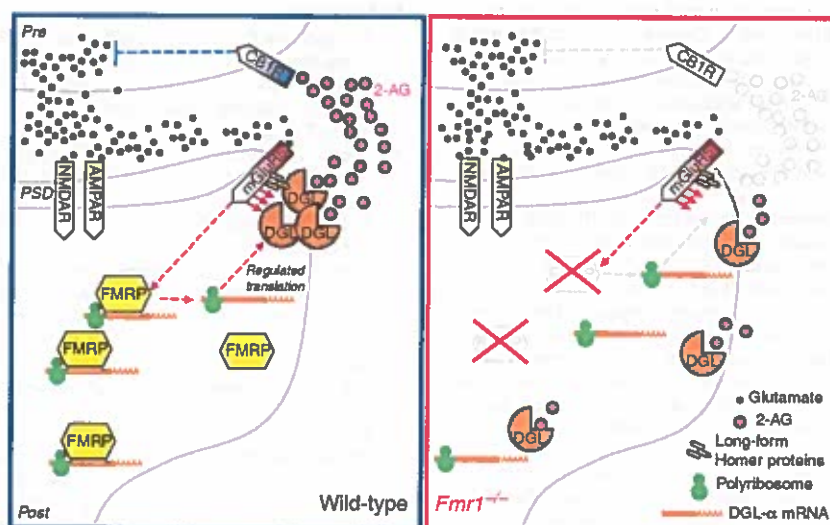
Figure 7 | Hypothetical role of FMRP in eCB-mediated retrograde transmission. At glutamatergic synapses of wild-type mice (*left*), mGlu5 receptors and DGL-α are linked together in a postsynaptic signalling complex (eCB signalosome), which includes the scaffolding protein Homer. Stimulation of mGlu5 receptors (mGluR5) by glutamate (green circles) increases DGL-α activity (red arrows), causing a localized rise in 2-AG levels at the perisynapse. 2-AG (red circles) travels across the synaptic cleft and inhibits glutamate release by stimulating CB1 receptors (CB1R) on nerve endings. We hypothesize that FMRP helps target DGL-α to the eCB signalosome, possibly by positioning the DGL-α or Homer message(s) in specific subcellular nanodomain(s). In *fmr1* $^{-/-}$ mice (*right*), the lack of FMRP expression leads to disruption of this spatial organization, loss of localized 2-AG production and, consequently, impaired retrograde 2-AG-mediated signalling. AMPAR, AMPA receptors; NMDAR, NMDA receptors; PSD, postsynaptic density. Red arrows indicate intracellular signalling pathways triggered by the activation of mGlu5 receptors. Molecular pathways that are impaired in *fmr1* $^{-/-}$ mice are shown in grey.

fmr1 $^{-/-}$ mice—including hippocampal LTD, immature dendritic arbourization and behavioural deficits[44]. However, this approach is unlikely to restore LTD at synapses of the ventral striatum and the PFC, where we find mGlu5-dependent eCB production to be down-regulated. We reasoned therefore that one possible alternative could be to restore eCB-mediated plasticity by reducing the degradation of its main effector, 2-AG. We used JZL184, an irreversible inhibitor of the 2-AG-degrading enzyme MGL[33,45,46]. This compound proved to be effective at restoring eCB-LTD in ventral striatum slices as well as correcting two key behavioural changes observed in *fmr1* $^{-/-}$ mice—elevated motor activity in an open field and decreased aversion to the open arms of an elevated plus maze[34]. Despite these promising findings, the therapeutic potential of JZL184 might be limited by its ability to induce CB1 receptor desensitization[46]. Future experimentation will be required to assess whether other classes of MGL inhibitors (for example, reversible inhibitors)[47] or agents that block additional 2-AG-hydrolysing enzymes, such as ABHD6[29], might normalize behaviour in *fmr1* $^{-/-}$ mice without inducing CB1 desensitization. Supporting the latter possibility, we observed here that acute blockade of ABHD6 re-establishes eCB-LTD in ventral striatum slices from *fmr1* $^{-/-}$ mice.

In conclusion, our results indicate that FMRP expression is necessary for the correct assembling of the eCB signalosome at glutamatergic synapses of the frontal cortex and ventral striatum core, and identify this macromolecular complex as a previously unknown substrate for FRAX, which might be targeted by therapy.

Methods
Physiology. Animals were treated in compliance with the European Communities Council Directive (86/609/EEC) and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were housed, grouped and acclimated to laboratory conditions for 4 days before experiments with 12-h light/dark cycles, and access to food and water *ad libitum*.

Male *fmr1* $^{-/-}$ mice on a C57BL/6J genetic background[48] aged 4 to 12 weeks (*fmr1* $^{-/-}$) were used, with wild-type littermates used as control group. They were anesthetized with isoflurane and decapitated according to institutional regulations.

The brain was sliced (300 μm) in the coronal plane with a vibratome (Integraslice, Campden Instruments, Loughborough, UK) in a sucrose-based solution at 4°C (in mM: 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 21 MgCl$_2$, 0.5 CaCl$_2$ and 1.25 NaH$_2$PO$_4$). Immediately after cutting, slices were stored at room temperature in a low-calcium artificial cerebrospinal fluid (low Ca–ACSF) that contained (in mM): 130 NaCl, 11 glucose, 2.5 KCl, 2.4 MgCl$_2$, 1.2 CaCl$_2$, 23 NaHCO$_3$ and 1.2 NaH$_2$PO$_4$, and was equilibrated with 95% O$_2$/5% CO$_2$ until the time of recording.

Whole-cell patch-clamp of visualized medium spiny neurons, PFC pyramidal neurons and field potential recordings were made in coronal slices containing the ventral striatum or the PFC as previously described[8,9,49,50]. For more details, see Supplementary Methods.

All values are given as mean±s.e.m. For all experiments, n corresponds to the number of animals tested for each condition. Data were analysed using Clampfit 10 (Molecular Devices, Sunnyvale, USA). Unless otherwise stated, the statistical test used was a Mann–Whitney U-test performed with GraphPad Prism (GraphPad Software, La Jolla, CA) and significance determined at $P < 0.05$. The magnitude of LTD was calculated by comparing average responses (30–40 min) after induction to baseline-averaged responses before induction protocol (increased stimulation for 10 min at 10 Hz).

Picrotoxin, THL and anisomycin were from Sigma (St Quentin Fallavier, France). 6,7-dinitroquinoxaline-2,3-dione (DNQX), (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-4-(3-hydroxypropyl)-cyclohexan-1-ol (CP55940), DHPG, AM251, WWL70 and JZL184 were from Tocris (Bristol, UK). Other chemicals were of the highest commercial grade available.

Biochemistry. Male *fmr1* $^{-/-}$ mice (FVB.129P2-Fmr1^{tm1Cgr}/J) and wild-type control mice (FVB.129P2-Pde6b^{+} Tyr^{c-ch}/AntJ) from Jackson Laboratories (Bar Harbor, ME), aged between 6 to 10 weeks, or adult male *fmr1* $^{-/-}$ mice on C57BL/6J background (Fmr1 KO2) were used for the biochemical studies. All procedures met the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

All manipulations of brain homogenates were performed at 4°C[51]. Synaptoneurosomes were prepared from whole brains of wild-type or *fmr1* $^{-/-}$ mice. Briefly, mice were quickly decapitated, brains were removed and homogenized by 20 strokes with a glass-Teflon homogenizer in 10 ml of homogenizing buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 1.53 mM KH$_2$PO$_4$, 212.7 mM glucose, 1 mM DTT, pH7.4, and protease inhibitor) and filtered through a series of polypropylene filters (twice through 100 μm and once through 10 μm (BD Filcon). Filtrates were spun briefly (1,000 g, 15 min) and the pellets were reconstituted in 3 ml of homogenizing buffer at a protein concentration of 1 mg ml^{-1}.

Dissected brain regions were homogenized in methanol (1 ml) containing $[^2H_8]$-2-AG and N-heptadecanoylethanolamide (Cayman Chemicals, Ann Arbor, MI, USA) as internal standards. Protein concentration was determined in the homogenate to normalize samples using the bicinchonic acid protein assay (Pierce, Rockford, IL, USA). Lipids were extracted with chloroform (2 ml) and water (1 ml), and further fractionated by open-bed silica gel column chromatography, as previously described[52]. Lipids were reconstituted in chloroform, loaded onto small glass columns packed with Silica Gel G (60-Å 230–400 Mesh ASTM; Whatman, Clifton, NJ, USA) and washed with 2 ml of chloroform. 2-AG were eluted with 1 ml of chloroform/methanol (9:1, vol/vol). Eluates were dried under N_2 and reconstituted in chloroform/methanol (1:3, 50 μl) for liquid chromatography/mass spectrometry (LC/MS) analyses.

An 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an electrospray ionization interface was used to measure anandamide and 2-AG levels in lipid extracts. Lipids were separated using a XDB Eclipse C18 column (50×4.6 mm i.d., 1.8 μm, Zorbax), eluted with a gradient of methanol in water (from 75 to 85% in 2.5 min, to 90% in 7.5 min, to 100% in 14 min and to 75% in 20 min) at a flow rate of 1.0 ml min^{-1}. Column temperature was kept at 40 °C. MS detection was in the positive ionization mode, capillary voltage was at 3 kV and fragmentor voltage varied from 120 V. N_2 was used as drying gas at a flow rate of 13 l min^{-1} and temperature of 350 °C. Nebulizer pressure was set at 60 PSI. Quantifications were conducted using an isotope-dilution method[52] by monitoring Na$^+$ adducts of the molecular ions ($[M+Na]^+$) in the selected ion-monitoring mode. Quantification limits for 2-AG was 0.4 pmol. For reverse transcriptase-PCR and quantitative PCR and immuno-precipitation, detailed procedures are provided in Supplementary Methods.

Results are expressed as the mean±s.e.m. Statistical significance was evaluated using the Student's t-test.

Animal behaviour. Adult male *fmr1*$^{-/-}$ C57BL/6J mice were used for the animal behaviour studies. For open field and elevated plus maze, detailed procedures provided in Supplementary Methods[53,54].

Anatomy. Experiments were performed according to the guidelines of the institutional ethical code and the Hungarian Act of Animal Care and Experimentation (1998; XXVIII. Section 243/1998). Adult male C57BL/6J mice (three wild-type and three *fmr1*$^{-/-}$ mice (8 weeks old) were deeply anesthetized with a mixture of ketamine–xylazine (25 mg ml^{-1} ketamine, 5 mg ml^{-1} xylazine, 0.1% w/w pipolphen in H_2O; 1 ml per 100 g, intraperitoneal). Animals were then perfused transcardially with 0.9% saline for 2 min, followed by 100 ml of fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 20 min. After perfusion, the brain was removed from the skull, cut into blocks, post-fixed for 2 h and washed in PB. The blocks containing the ventral striatum were sliced into 50-μm-thick coronal sections of the brain with a Leica VTS-1000 vibratome (Vibratome, St Louis, MO, USA).

After slicing and extensive washing in 0.1 M PB, the sections were incubated in 10% sucrose for 15 min and 30% sucrose overnight, followed by freeze thawing over liquid nitrogen four times. Subsequently, all washing steps and dilutions of the antibodies were performed in 0.05 M Tris-buffered saline (TBS), pH 7.4. After extensive washing in TBS, the sections were blocked in 5% normal goat serum for 45 min and then incubated in rabbit anti-DGL-α (1:3,000, 'INT'[13]) or rabbit anti-mGlu5 (1:1,000, AB5675, Millipore, Billerica, MA, USA) for a minimum of 48 h at 4 °C. In the immunoperoxidase staining procedure, after primary antibody incubations, the sections were treated with biotinylated goat anti-rabbit IgG (1:400, Vector Laboratories) for 2 h and after three washes in TBS, with avidin biotinylated–horseradish peroxidase complex (1:500; Elite ABC; Vector Laboratories) for 1.5 h. After extensive washing, the immunoperoxidase reaction was developed using 3,3′-diaminobenzidine as the chromogen. In the immunogold staining procedure, after the primary antibody incubation, the sections were washed extensively in TBS before incubation in 0.8 nm gold-conjugated goat anti-rabbit secondary antibody (1:50; AURION, Wageningen, The Netherlands), overnight at 4 °C. Then sections were silver intensified using the silver enhancement system R-GENT SE-EM according to the kit protocol (AURION). After development of both types of immunolabelling, the sections were treated with osmium tetroxide (0.5% for immunogold labelling, 1% for immunoperoxidase reaction) in 0.1M PB for 20 min (at 4 °C for immunogold labelling) and dehydrated in an ascending series of ethanol and acetonitrile, before being embedded in Durcupan (ACM, Fluka, Buchs, Switzerland). During dehydration, sections were treated with 1% uranyl acetate in 70% ethanol for 15 min (at 4 °C for immunogold labelling). Immunoperoxidase-labelled sections were imaged with a Nikon Eclipse 80i microscope (Nikon Instruments Europe B.V., Amsterdam, The Netherlands) and light micrographs were captured with a DS-Fi1 digital camera (Nikon Instruments Europe B.V., Amsterdam, The Netherlands). For electron microscopy analysis, areas of interest in the ventral striatum core were removed from Durcupan-embedded sections, then re-embedded and re-sectioned. Ultrathin (60 nm) sections were collected on Formvar-coated single-slot grids and stained with lead citrate. Electron micrographs were taken at ×40,000 magnifications with a Hitachi 7100 electron microscope (Tokyo, Japan).

Quantitative analysis of the distribution of DGL-α and mGlu5 in dendritic spines: see Supplementary Methods.

References

1. Penagarikano, O., Mulle, J. G. & Warren, S. T. The pathophysiology of fragile X syndrome. *Annu. Rev. Genomics. Hum. Genet.* 8, 109–129 (2007).
2. Verkerk, A. J. *et al.* Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65, 905–914 (1991).
3. Ronesi, J. A. & Huber, K. M. Metabotropic glutamate receptors and fragile × mental retardation protein: partners in translational regulation at the synapse. *Sci. Signal.* 1, pe6 (2008).
4. Kelleher, R. J. 3rd. & Bear, M. F. The autistic neuron: troubled translation? *Cell* 135, 401–406 (2008).
5. Bear, M. F., Dolen, G., Osterweil, E. & Nagarajan, N. Fragile X: translation in action. *Neuropsychopharmacology* 33, 84–87 (2008).
6. Dolen, G. *et al.* Correction of fragile X syndrome in mice. *Neuron.* 56, 955–962 (2007).
7. Huber, K. M., Gallagher, S. M., Warren, S. T. & Bear, M. F. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc. Natl Acad. Sci. USA* 99, 7746–7750 (2002).
8. Robbe, D., Kopf, M., Remaury, A., Bockaert, J. & Manzoni, O. J. Endogenous cannabinoids mediate long-term synaptic depression in the nucleus accumbens. *Proc. Natl Acad. Sci. USA* 99, 8384–8388 (2002).
9. Lafourcade, M. *et al.* Molecular components and functions of the endocannabinoid system in mouse prefrontal cortex. *PLoS One.* 2, e709 (2007).
10. Jung, K. M. *et al.* A key role for diacylglycerol lipase-alpha in metabotropic glutamate receptor-dependent endocannabinoid mobilization. *Mol. Pharmacol.* 72, 612–621 (2007).
11. Jung, K. M. *et al.* Stimulation of endocannabinoid formation in brain slice cultures through activation of group I metabotropic glutamate receptors. *Mol. Pharmacol.* 68, 1196–1202 (2005).
12. Tanimura, A. *et al.* The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase alpha mediates retrograde suppression of synaptic transmission. *Neuron* 65, 320–327 (2010).
13. Katona, I. *et al.* Molecular composition of the endocannabinoid system at glutamatergic synapses. *J. Neurosci.* 26, 5628–5637 (2006).
14. Lujan, R., Nusser, Z., Roberts, J. D., Shigemoto, R. & Somogyi, P. Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur. J. Neurosci.* 8, 1488–1500 (1996).
15. Won, Y. J., Puhl, H. L. 3rd. & Ikeda, S. R. Molecular reconstruction of mGluR5a-mediated endocannabinoid signaling cascade in single rat sympathetic neurons. *J. Neurosci.* 29, 13603–13612 (2009).
16. Dinh, T. P. *et al.* Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl Acad. Sci. USA* 99, 10819–10824 (2002).
17. Katona, I. & Freund, T. F. Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. *Nat. Med.* 14, 923–930 (2008).
18. Zhang, L. & Alger, B. E. Enhanced endocannabinoid signaling elevates neuronal excitability in fragile X syndrome. *J. Neurosci.* 30, 5724–5729 (2010).
19. Maccarrone, M. *et al.* Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. *Neuropsychopharmacology* 35, 1500–1509 (2010).
20. Brown, V. *et al.* Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. *Cell* 107, 477–487 (2001).
21. Todd, P. K., Mack, K. J. & Malter, J. S. The fragile X mental retardation protein is required for type-I metabotropic glutamate receptor-dependent translation of PSD-95. *Proc. Natl Acad. Sci. USA* 100, 14374–14378 (2003).
22. Westmark, C. J. & Malter, J. S. FMRP mediates mGluR5-dependent translation of amyloid precursor protein. *PLoS Biol.* 5, e52 (2007).
23. Raiteri, L. & Raiteri, M. Synaptosomes still viable after 25 years of superfusion. *Neurochem. Res.* 25, 1265–1274 (2000).
24. Yoshida, T. *et al.* Localization of diacylglycerol lipase-alpha around postsynaptic spine suggests close proximity between production site of an endocannabinoid, 2-arachidonoyl-glycerol, and presynaptic cannabinoid CB1 receptor. *J. Neurosci.* 26, 4740–4751 (2006).
25. Lujan, R., Roberts, J. D., Shigemoto, R., Ohishi, H. & Somogyi, P. Differential plasma membrane distribution of metabotropic glutamate receptors mGluR1 alpha, mGluR2 and mGluR5, relative to neurotransmitter release sites. *J. Chem. Neuroanat.* 13, 219–241 (1997).
26. Bagni, C. & Greenough, W. T. From mRNP trafficking to spine dysmorphogenesis: the roots of fragile X syndrome. *Nat. Rev. Neurosci.* 6, 376–387 (2005).
27. Irwin, S. A., Galvez, R. & Greenough, W. T. Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cereb. Cortex* 10, 1038–1044 (2000).
28. Heifets, B. D. & Castillo, P. E. Endocannabinoid signaling and long-term synaptic plasticity. *Annu. Rev. Physiol.* 71, 283–306 (2009).
29. Marrs, W. R. *et al.* The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. *Nat. Neurosci.* 13, 951–957 (2010).

30. Mato, S., Lafourcade, M., Robbe, D., Bakiri, Y. & Manzoni, O. J. Role of the cyclic-AMP/PKA cascade and of P/Q-type Ca. channels in endocannabinoid-mediated long-term depression in the nucleus accumbens. *Neuropharmacology* 54, 87–94 (2008).

31. Robbe, D., Alonso, G., Chaumont, S., Bockaert, J. & Manzoni, O. J. Role of p/q-Ca2+ channels in metabotropic glutamate receptor 2/3-dependent presynaptic long-term depression at nucleus accumbens synapses. *J. Neurosci.* 22, 4346–4356 (2002).

32. Dinh, T. P., Freund, T. F. & Piomelli, D. A role for monoglyceride lipase in 2-arachidonoylglycerol inactivation. *Chem. Phys. Lipids* 121, 149–158 (2002).

33. Long, J. Z. *et al.* Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat. Chem. Biol.* 5, 37–44 (2009).

34. Yuskaitis, C. J. *et al.* Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of fragile X syndrome. *Biochem. Pharmacol.* 79, 632–646 (2010).

35. Darnell, J. C. *et al.* FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* 146, 247–261 (2011).

36. Piomelli, D. The molecular logic of endocannabinoid signalling. *Nat. Rev. Neurosci.* 4, 873–884 (2003).

37. Prescott, S. M. & Majerus, P. W. The fatty acid composition of phosphatidylinositol from thrombin-stimulated human platelets. *J. Biol. Chem.* 256, 579–582 (1981).

38. Kao, D. I., Aldridge, G. M., Weiler, I. J. & Greenough, W. T. Altered mRNA transport, docking, and protein translation in neurons lacking fragile X mental retardation protein. *Proc. Natl Acad. Sci. USA* 107, 15601–15606 (2010).

39. Roloff, A. M., Anderson, G. R., Martemyanov, K. A. & Thayer, S. A. Homer 1a gates the induction mechanism for endocannabinoid-mediated synaptic plasticity. *J. Neurosci.* 30, 3072–3081 (2010).

40. Ronesi, J. A. & Huber, K. M. Homer interactions are necessary for metabotropic glutamate receptor-induced long-term depression and translational activation. *J. Neurosci.* 28, 543–547 (2008).

41. Ronesi, J. A. *et al.* Disrupted Homer scaffolds mediate abnormal mGluR5 function in a mouse model of fragile X syndrome. *Nat. Neurosci.* 15, 431–440 (2012).

42. Suvrathan, A., Hoeffer, C. A., Wong, H., Klann, E. & Chattarji, S. Characterization and reversal of synaptic defects in the amygdala in a mouse model of fragile X syndrome. *Proc. Natl Acad. Sci. USA* 107, 11591–11596 (2011).

43. Chevaleyre, V. & Castillo, P. E. Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. *Neuron* 38, 461–472 (2003).

44. Krueger, D. D. & Bear, M. F. Toward fulfilling the promise of molecular medicine in fragile X syndrome. *Annu. Rev. Med.* 62, 411–429 (2011).

45. Chanda, P. K. *et al.* Monoacylglycerol lipase activity is a critical modulator of the tone and integrity of the endocannabinoid system. *Mol. Pharmacol.* 78, 996–1003 (2010).

46. Schlosburg, J. E. *et al.* Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat. Neurosci.* 13, 1113–1119 (2010).

47. King, A. R. *et al.* Discovery of potent and reversible monoacylglycerol lipase inhibitors. *Chem. Biol.* 16, 1045–1052 (2009).

48. Pilpel, Y. *et al.* Synaptic ionotropic glutamate receptors and plasticity are developmentally altered in the CA1 field of Fmr1 knockout mice. *J. Physiol.* 587, 787–804 (2009).

49. Lafourcade, M. *et al.* Nutritional omega-3 deficiency abolishes endocannabinoid-mediated neuronal functions. *Nat. Neurosci.* 14, 345–350 (2011).

50. Kasanetz, F. & Manzoni, O. J. Maturation of excitatory synaptic transmission of the rat nucleus accumbens from juvenile to adult. *J. Neurophysiol.* 101, 2516–2527 (2009).

51. Villasana, L. E., Klann, E. & Tejada-Simon, M. V. Rapid isolation of synaptoneurosomes and postsynaptic densities from adult mouse hippocampus. *J. Neurosci. Methods* 158, 30–36 (2006).

52. Astarita, G. & Piomelli, D. Lipidomic analysis of endocannabinoid metabolism in biological samples. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* 877, 2755–2767 (2009).

53. Jarbe, T. U., Andrzejewski, M. E. & DiPatrizio, N. V. Interactions between the CB1 receptor agonist Delta 9-THC and the CB1 receptor antagonist SR-141716 in rats: open-field revisited. *Pharmacol. Biochem. Behav.* 73, 911–919 (2002).

54. Lister, R. G. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl).* 92, 180–185 (1987).

55. Evans, M. S., Collings, M. A. & Brewer, G. J. Electrophysiology of embryonic, adult and aged rat hippocampal neurons in serum-free culture. *J. Neurosci. Methods* 79, 37–46 (1998).

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Author contributions

Additional information

Autism-Associated Neuroligin-3 Mutations Commonly Disrupt Tonic Endocannabinoid Signaling

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Abstract

Neuroligins are postsynaptic cell-adhesion molecules that interact with presynaptic neurexins. Rare mutations in neuroligins and neurexins predispose to autism, including a neuroligin-3 amino-acid substitution (R451C) and a neuroligin-3 deletion. Previous analyses showed that neuroligin-3 R451C-knockin mice exhibit robust synaptic phenotypes, but failed to uncover major changes in neuroligin-3 knockout mice, questioning the notion that a common synaptic mechanism mediates autism pathogenesis in patients with these mutations. Here, we used paired recordings in mice carrying these mutations to measure synaptic transmission at GABAergic synapses formed by hippocampal parvalbumin- and cholecystokinin-expressing basket cells onto pyramidal neurons. We demonstrate that in addition to unique gain-of-function effects produced by the neuroligin-3 R451C-knockin but not the neuroligin-3 knockout mutation, both mutations dramatically impaired tonic but not phasic endocannabinoid signaling. Our data thus suggest that neuroligin-3 is specifically required for tonic endocannabinoid signaling, raising the possibility that alterations in endocannabinoid signaling may contribute to autism pathophysiology.

INTRODUCTION

Neuroligins are postsynaptic cell-adhesion molecules that are expressed in four principal isoforms (neuroligin-1 to -4, abbreviated as NL1 to NL4), and that act as ligands for presynaptic neurexins (Ichtchenko et al., 1995). NL1 is found in excitatory synapses (Song et al., 1999), NL2 in inhibitory synapses (Veroqueaux et al., 2004; Graf et al., 2004), NL3 in both (Budreck and Scheiffele, 2007), and NL4 in glycinergic synapses (Hoon et al., 2011). In humans, more than 30 neuroligin gene mutations have been associated with autism, including a NL3 point mutation (the R451C substitution; Jamain et al., 2003) and a NL3 deletion (Sanders et al., 2011).

Experiments with knockout (KO) mice revealed that neuroligins are essential for synaptic transmission, and suggest that neuroligins organize synapses and determine synapse

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properties (Varoqueaux et al., 2006). Specifically, triple KO mice lacking NL1, NL2, and NL3 die at birth because their synapses – although morphologically normal – exhibit severe impairments in synaptic transmission (Varoqueaux et al., 2006). Moreover, single KO mice lacking either NL1 or NL2 exhibit major deficits in excitatory or inhibitory synaptic transmission, respectively (Chubykin et al., 2007; Gibson et al., 2009; Poulopoulos et al., 2009). NL3 KO mice display changes in spontaneous `mini' synaptic events in the hippocampus (Tabuchi et al., 2007; Etherton et al., 2011a) and in mGluR5 signaling in the cerebellum (Baudouin et al., 2012). Together, these findings are consistent with the notion that neuroligins specify synaptic properties instead of functioning as general `glues' for synapses (Varoqueaux et al., 2006). These conclusions are additionally supported by characterization of another NL3 mutation, the R704C substitution (Etherton et al., 2011b). The R704C substitution corresponds to an autism-associated mutation in NL4 (Zhang et al., 2009) that, when introduced into NL3, selectively altered postsynaptic AMPA-type glutamate receptor levels, confirming that neuroligins contribute to shaping synapse properties.

In contrast to NL3 KO mice, NL3 knockin (KI) mice carrying the R451C substitution that mimics the human autism mutation displayed robust synaptic phenotypes that differed between the somatosensory cortex and hippocampus, and that were absent from NL3 KO mice (Tabuchi et al., 2007; Etherton et al., 2011a; see also Südhof, 2008). Thus, although the R451C substitution destabilizes NL3 (De Jaco et al., 2010) and caused a loss of more than 90% of NL3 protein (Tabuchi et al., 2007), it nevertheless produced a gain-of-function phenotype in at least some synapses. To date, no synaptic phenotype was detected that is shared by the two known autism-associated NL3 mutations, raising the question of how these mutations may actually induce autism.

To gain insight into how different NL3 mutations might contribute to autism pathogenesis, we here followed up on the observation that the NL3 KO increases inhibitory and decreases excitatory spontaneous mini events in the hippocampus (Etherton et al., 2011a). Since the NL3 KO did not alter excitatory synaptic strength in the hippocampus, we hypothesized that the NL3 KO may cause a specific change in a subset of inhibitory synapses. The hippocampus contains multiple at least 21 different types of inhibitory neurons that exhibit specific circuit properties (Klausberger and Somogyi, 2008). Thus, when examining inhibitory synaptic transmission, it is advantageous to investigate specific synapses formed by identified types of inhibitory neurons. To this end, we performed paired recordings that monitor synapses formed by two different defined types of inhibitory basket cells onto the soma and proximal dendrites of pyramidal neurons. One type of basket cell co-expresses presynaptic cannabinoid type-1 (CB1) receptors and the neuropeptide cholecystokinin (CCK; `CCK basket cells'), whereas the other type expresses parvalbumin (PV; `PV basket cells'; Freund, 2003; Freund et al., 2003; Bartos et al., 2007; Klausberger and Somogyi, 2008). The two types of basket cells participate in parallel inhibitory systems that play distinct but complementary roles in network oscillations (Bartos et a., 2007; Klausberger et al., 2005), and have been implicated in neurological and mood disorders (Freund and Katona, 2007; Lisman et al., 2008). In these paired recordings, we sought to identify specific loss-of-function effects that are shared by both the NL3 KO and the R451C KI mutation since both are associated with autism, prompting us to analyze both mutations in parallel.

Our data show that NL3 R451C KI and NL3 KO neurons exhibit distinct phenotypes at synapses formed by PV basket cells, similar to previous observations in other synapses (Tabuchi et al., 2007; Etherton et al., 2011a). Surprisingly, however, we find that at synapses formed by CCK basket cells, the two mutations produced the same phenotype that consisted of a loss of the tonic CB1 receptor-dependent suppression of GABA release that is observed at these synapses (Losonczy et al., 2004; Hentges et al., 2005; Neu et al., 2007; Ali and

Todorova, 2010; Kim and Alger, 2010). This observation identifies NL3 as the first molecule that is selectively essential for tonic endocannabinoid signaling, an enigmatic component of overall endocannabinoid signaling (Alger, 2012). Given the common genetic association of the R451C substitution and NL3 deletion with autism, our data thus suggest that disrupted endocannabinoid signaling may contribute to autism pathophysiology, a tantalizing idea given the great interest in developing therapeutic approaches that modify endocannabinoid signaling in the brain.

RESULTS

R451C KI impairs GABAergic synaptic transmission at PV basket cell synapses

We performed paired whole-cell recordings between presynaptic basket cells and postsynaptic CA1 pyramidal neurons in acute slices from littermate wild-type and R451C KI mice (Tabuchi et al., 2007). In these recordings, we determined the characteristics of synaptic transmission by measuring unitary inhibitory post-synaptic currents (IPSCs) evoked by basket cell action potentials (APs)(see Experimental Procedures for details).

We found that the R451C KI severely impaired synaptic transmission at synapses formed by PV basket cells onto pyramidal neurons (Figs. 1A and 1B). The amplitude of IPSCs was decreased ~70% (failures included), and the success rate with which an AP elicited an IPSC was lowered ~20%. This phenotype was observed independent of whether APs were induced at 1 Hz, 2 Hz, or 10 Hz. In addition, we observed a statistically insignificant decrease in IPSC half-widths (Fig. 1C, WT: 5 ± 0.3 ms, R451C: 4.3 ± 0.2 ms). The impairment of IPSCs in R451C KI neurons was independent of postsynaptic membrane potential (analyzed from −80 to +60 mV; Figs. 1E and S1), and the R451C KI did not affect the reversal potential of PV basket cell-evoked IPSCs (WT: $−18.6 \pm 1.9$ mV, R451C: $−19.4 \pm 2.1$ mV). Moreover, we observed no change in the amplitude of the minimal unitary IPSC that could be evoked by a presynaptic AP, suggesting that single synaptic events elicited similar postsynaptic responses (Fig. 1D; WT: 22.5 ± 3.5 pA, R451C: 16.4 ± 0.8 pA). We also found no change in the number of trials needed to identify synaptically connected pairs of PV basket cell/pyramidal neurons, indicating that the number of pyramidal neurons innervated by individual PV basket cells was not altered (Fig. 1F; WT: 1.8 ± 0.3, R451C: 1.8 ± 0.2 trials per presynaptic basket cell). Finally, we did not detect major morphological changes in the axonal or dendritic arbor of PV basket cells in R451C KI mice (Fig. 1G).

Together, these data show that the R451C KI produces a large impairment in synaptic transmission at synapses formed by PV basket cells onto pyramidal neurons. The lack of a change in the voltage-dependence of IPSCs, the reversal potential, and the minimal unitary IPSC size suggest that the R451C KI did not alter the number of postsynaptic GABA-receptors or disrupt postsynaptic chloride homeostasis, while the lack of change in the IPSC kinetics suggests that the subunit composition of GABA-receptors or the reuptake kinetics of released GABA were not altered significantly. The decrease in the success rate of eliciting an IPSC from PV basket cells suggests that the R451C KI impaired synaptic transmission by a presynaptic mechanism, despite the presumed postsynaptic localization of NL3. Notably, this is the first phenotype of the R451C mutation that entails a decrease in synaptic strength, not an increase as previously observed for global inhibitory synaptic transmission in the somatosensory cortex (Tabuchi et al., 2007) and for both AMPA- and NMDA-receptor mediated excitatory synaptic transmission in the hippocampus (Etherton et al., 2011a).

The R451C KI enhances GABAergic synaptic transmission at CCK basket cell synapses

We next analyzed the properties of transmission at pyramidal synapses formed by CCK basket cells. Surprisingly, here the R451C KI caused a ~100% increase in the IPSC amplitudes and a ~15% increase in the IPSC success rate during 1 Hz stimulation, and a slightly smaller change during 2 and 10 Hz stimulation (Figs. 2A and 2B). The increase in success rate suggests an increase in the presynaptic GABA release probability, which is also a plausible explanation for the increase in IPSC amplitudes. This hypothesis was further supported by the absence of detectable changes in the IPSC half-width, indicating that the GABA-receptor subunit composition or uptake mechanisms were unaltered (Fig. 2C; WT: 6.3 ± 0.4 ms, R451C: 5.4 ± 0.3 ms). Furthermore, the amplitude of minimal unitary IPSCs (Fig. 2D; WT: 23.4 ± 4.3 pA, R451C: 29.6 ± 4 pA) and the rate of finding connected pairs (Fig. 2E; WT: 2.6 ± 0.7, R451C: 2.2 ± 0.3) were similar in wild-type and R451C mutant slices, as was the morphology of their CCK basket cells (Fig. 2F). The phenotype of the R451C mutation in the CCK cell synapses again was more consistent with a presynaptic change (such as increased release probability) than a structural alteration (e.g. increase in synapse density) or postsynaptic effect. Thus, the R451C KI produces opposite changes at two different perisomatic inhibitory synapses, and in both cases the changes appear to involve an ultimately presynaptic mechanism, even though NL3 is a postsynaptic molecule.

A synaptic phenotype of NL3 KO mice

To test whether the R451C KI phenotypes represent gain- or loss-of-function effects, we next performed paired recordings in acute slices from NL3 KO mice, again using littermate wild-type mice as controls. When we analyzed the properties of transmission between PV basket cells and pyramidal neurons, we failed to detect a phenotype. Specifically, the amplitude and success rate of IPSCs were unchanged (Figs. 3A and 3B), as were the half-width of the IPSCs (Fig. 3C; WT: 4.7 ± 0.2 ms, NL3 KO: 5.5 ± 0.4 ms), the size of unitary minimal IPSCs (Fig. 3D; WT: 17.6 ± 1.6 pA, NL3 KO: 16.5 ± 1.1 pA), and the rate of finding connected pairs (Fig. 3E; WT: 2.2 ± 0.3, NL3 KO: 2.5 ± 0.6). These results suggest that the loss of synaptic transmission at this synapse in R451C mutant mice represents an active suppression of synaptic transmission by a gain-of-function activity of R451C-mutant NL3.

We then examined the effect of the NL3 KO on synaptic transmission mediated by inhibitory synapses that were formed by CCK-containing terminals on pyramidal neurons (Fig. 4). Surprisingly, here the NL3 KO phenocopied the R451C KI. Specifically, the NL3 KO caused a significant increase in synaptic strength, as manifested by both an increase in IPSC amplitude and in success rate (Figs. 4A and 4B). In addition, we observed a small increase in IPSC half-width (Fig. 4C; WT: 4.9 ± 0.1 ms, NL3 KO: 5.6 ± 0.2), but no change in the size of unitary minimal IPSCs (Fig. 4D; WT: 25.3 ± 1.7 pA, NL3 KO: 29 ± 2.9 pA), or in the rate of finding synaptically connected pairs of neurons (Fig. 4E; WT: 2 ± 0.2, NL3 KO: 1.8 ± 0.2). The fact that increased synaptic transmission at CCK basket cell synapses is equally observed in NL3 KO and R451C KI neurons shows that it is caused by a loss-of-function mechanism.

NL3 R451C KI lowers the probability of GABA release at PV basket cell synapses

The change in success rates in our paired recordings of synapses with the NL3 R451C KI or the NL3 KO mutations suggests a presynaptic origin for the observed phenotypes, despite the postsynaptic localization of NL3 (Budreck and Scheiffele, 2007). To evaluate whether presynaptic changes alone (such as in the probability of release) could in principle account for the NL3 related phenotypes, we analyzed these phenotypes by modeling and computer simulations.

We first plotted bin-averaged PV basket cell IPSC amplitudes against their corresponding averaged success rates (Fig. 5A; inset shows distribution of individual pairs). We then fitted these data with an equation that relates IPSC amplitudes to the success rate of transmission ($PSC=Q \cdot N \cdot \left[1 - \sqrt[N]{1 - \text{successes}} \right]$ see Experimental Procedures and Figs. S2A–S2C) to estimate characteristic mean quantal size (Q) and number of release sites (N) for WT (n=25) and R451C KI (n=26) populations. The resulting estimates for Q were similar for both genotypes (mean and 95% confidence intervals: 21.9/12.5–31.3 pA and 17.5/16.4–18.6 pA for WT and R451C, respectively), as were the estimates for N (mean and 95% confidence intervals: 7.6/1.6–13.6 and 8.7/0–17.4 for WT and R451C synapses, respectively). These estimates support the notion that the synaptic phenotype in R451C-mutant PV basket cell synapses was not due to a decrease in quantal size (see Fig. 1D), and, limited by the wide confidence interval of estimates, also suggest that the R451C phenotype was not due to a decrease in the number of release sites.

To examine the remaining possibility, namely that a lower neurotransmitter release probability (P_R) underlies the R451C phenotype, we performed computer simulations in which we modeled IPSCs at different P_R values (Figs. 5B and S3). In this computational model, we incorporated a minimal set of synaptic parameters that allowed us to simulate the IPSC amplitudes and success rates, and to compare these parameters to the experimental data. The simulation parameters included, in addition to the number of release sites (N), the mean and the variance of the release probability (P_R and σ_{PR}) and the mean and the variance of the quantal amplitude (Q and σ_Q). For each simulated paired recording, the

computationally determined IPSC (cIPSC) was derived as $cIPC = \sum_{i=1}^{N} p_i \cdot q_i$, and the computationally determined success rate (cSuccesses) was derived as

$cSuccess = \left[1 - \prod_{i=1}^{N} (1 - p_i) \right] \cdot 100$, where p_i and q_i are the probability of release and the quantal amplitude in the i-th release site, respectively (see Experimental Procedures and Figs. S3A–3G).

We started the simulations by using Q and N values estimated from the population quantal analysis (Fig. 5A; see above) to derive values for P_R, σ_{PR}, and σ_Q that result in cIPSCs and cSuccesses which approximate the experimentally determined IPSCs and success rates. For PV basket cell IPSCs in WT neurons, we found that a $P_R=0.23$, together with a $\sigma_{PR}=0.224$ and a $\sigma_Q=2.25$ (Q=21 pA and N=7, per modeling), provided computationally determined cIPSCs and cSuccesses that did not significantly differ from the experimental data (mean difference ± SD for IPSCs: 0±6 pA; for success rates: 0±0.02; t-test, P>0.5 for both). For computer simulation of R451C synapses, we found that much lowered release probabilities, $P_R=0.11$, together with a $\sigma_{PR}=0.09$ and a $\sigma_Q=1.65$ (Q=17 pA and N=8, per modeling) were needed to replicate the experimental data (mean difference ± SD for IPSCs: 0±0.99 pA; for success rates: 0±0.01; t-test, P>0.5 for both). These simulations thus suggest that a ~2-fold decrease in the probability of GABA release could sufficiently explain the NL3 R451C KI phenotype in PV basket cell synapses. These conclusions were further supported by consequent analysis of biocytin-filled axons (Fig. 5C), which also did not indicate a difference in the number of synapses formed by individual PV basket cells (WT: 0.33±0.03 and R451C: 0.26±0.01, synapses per μm, Mann-Whitney RST, P=0.152).

Next, we sought to determine a cause for lower release rates in PV basket cell synapses in the R451C KI mice. We reasoned that such decreases in release rate could be caused by NL3 mutation-driven alterations of the presynaptic release machinery, or alternatively, by over-activation of a presynaptic receptor, such as a neuropeptide receptor, that physiologically suppresses GABA release from these synapses (Freund and Katona, 2007). We addressed

this latter possibility by application of pharmacological agents in paired recording experiments.

Activation of two presynaptic G-protein coupled receptors, namely μ-opioid and M2 muscarinic-receptors, is known to suppress GABA release at PV basket cell synapses (Glickfeld et al., 2008, Szabó et al., 2010). Thus, we tested the effect of the μ-opioid receptor antagonist CTAP (500 nM; Fig. 5D, n=4 pairs) and of the M2 muscarinic-receptor antagonist AF-DX (10 μM; Fig. 5E, n=4 pairs) in paired-recordings of PV basket cell to pyramidal neuron synapses in NL3 R451C KI mice. Neither antagonist increased IPSC amplitudes in paired recordings, indicating that tonic activation of these receptors does not account for the decreased transmission at PV basket cell synapses in R451C KI mice. In additional control experiments, both antagonists reliably reversed the effect of their corresponding agonists, DAMGO (1 μM) and carbachol (5 μM; not shown). Thus, the presence of NL3 the R451C mutation likely induces a functional change in the presynaptic release properties of PV basket cell synapses.

Neuroligin-3 is essential for tonic endocannabinoid signaling at CCK basket cell synapses

Our data suggest that a loss of NL3 function produces an increase in GABA release at synapses formed by CCK basket cells onto pyramidal neurons synapses. CCK basket cell synapses exhibit a distinct feature that offers an immediate hypothesis to account for the observed phenotype. This feature consists of the efficient suppression of GABA release from CCK basket cell terminals by the endocannabinoid-mediated activation of presynaptic CB1 receptors (reviewed in Alger, 2002; Piomelli, 2003; Freund and Katona, 2007).

Endocannabinoids are secreted from postsynaptic pyramidal neurons to activate presynaptic CB1 receptors in two principal modes. Phasic secretion of endocannabinoids is induced by postsynaptic depolarization and/or mGluR5 activation and mediates decreases in synaptic transmission during short- and long-term plasticity. Tonic secretion of endocannabinoids affects synaptic transmission over longer time periods (reviewed in Alger, 2012; Katona and Freund, 2012). A deficiency in tonic endocannabinoid signaling, with or without an effect on phasic endocannabinoid signaling, would be expected to enhance the probability of GABA release, and thus would increase IPSCs similar to what we observed in R451C KI and NL3 KO neurons. Thus, we tested the hypothesis that a loss-of-function of NL3 - either via the KO or via the R451C KI - impairs tonic endocannabinoid signaling.

In wild-type synapses, bath application of 10 μM AM251 (a CB1 receptor antagonist and inverse agonist) caused a ~100% increase in IPSC amplitudes and ~50% increase in success rate (Figs. 6A and 6B; 1 Hz AP firing), reflecting disinhibition of GABA release by blocking tonically active CB1 receptors (Neu et al., 2007). In NL3 KO synapses, strikingly, AM251 did not enhance IPSC amplitudes (Figs. 6A, S4A, and S4B) or success rates of synaptic transmission (Figs. 6B, S4A, and S4B). These findings suggest that IPSC amplitudes in the NL3 KO were larger because these synapses express higher release probabilities due to an apparent lack of tonic CB1 receptor activation.

To evaluate whether differences in the release probability alone, without other possible consequences of NL3 deletion, could explain the observed phenotype, we again used modeling and computer simulations. Fitting of the bin-averaged IPSC – successes data (Figs. 6C and S3A–S3C) resulted in similar Q and N estimates for the NL3 WT and KO data sets (mean and 95% confidence intervals; Q: 39 / 30.8–47.3 and 46.2 / 14.1–78.4 pA, and N: 5.6 / 4.1–7 and 4.4 / −2.9–11.8, for WT and NL3 KO, respectively). Using these parameter estimates in subsequent simulations (Figs. 6D and S3A–S3G), we found that the mean values of simulated IPSC – successes distributions were not significantly different from experimental values (inset in left panel) when $P_R=0.12$ (together with a $\sigma_{PR}=0.19$ and a

$\sigma_Q=2$; Q=39 pA and N=6 per model estimates) for NL3 WT, and when $P_R=0.26$ (together with a $\sigma_{PR}=0.26$ and a $\sigma_Q=2.1$; Q=46.2 pA and N=5 per model estimates) for NL3 KO. In addition, we quantified axonal bouton densities (Fig. 6E), which were not different between the two genotypes (WT: 0.18 ± 0.01 and NL3 KO: 0.18 ± 0.01, per μm, t-test, P=0.779). Together, these analyses suggest that the loss of tonic CB1 receptor activation, and the consequent ~2-fold increase in the probability of GABA release, is sufficient to account for the entire phenotype of the NL3 deletion at these synapses.

We next determined whether the loss of tonic CB1 receptor activation was affecting GABA release only from basket cell synapses, or whether all CB1-containing GABAergic synapses exhibit this phenotype. Thus, we repeated the CB1 receptor blocking experiments by monitoring IPSCs evoked by extracellular stimulation (which will cause GABA release from a broad set of presynaptic fibers that include CB1-receptor-containing axons). Application of AM251 again enhanced IPSCs in CA1 pyramidal cells, but consistently failed to do so in the NL3 KO (Fig. 6F). We also repeated these latter extracellular stimulation experiments with CP 945,598, a CB1 receptor antagonist that is structurally unrelated to AM251. Bath application of CP 945,598 (5 μM) replicated the findings with AM251 (Fig. 6G), independently confirming the absence of tonic EC signaling in NL3 KO mice.

Similar to the NL3 KO, paired recordings from slices prepared from the NL3 R451C KI mice revealed that the effect of AM251 on CCK basket cell IPSCs was greatly reduced (Figs. 6H and 6I). These data suggest that NL3 is essential for the tonic endocannabinoid signaling that inhibits GABA release from CCK basket cell synapses. Futhermore, we tested whether the NL3 KO may alter tonic CB1 receptor-mediated signaling at glutamatergic synapses. We stimulated Schaffer-collateral synapses and recorded from CA1 pyramidal cells (in the presence of 50 μM picrotoxin). However, bath application of AM251 (10 μM) failed to increase EPSC amplitudes in either WT slices or NL3 KO slices (Fig. 6J; see also Hoffmann et al., 2010). Together, these data suggest that NL3-related mutations may impair tonic endocannabinoid signaling at CB1 receptor-containing inhibitory, but not excitatory synapses.

NL3 is not required for phasic endocannabinoid signaling

A loss of tonic endocannabinoid signaling could be due to a specific ablation of tonic endocannabinoid secretion, or to a general block of all endocannabinoid secretion or endocannabinoid sensing, for example due to a removal of CB1 receptors. To differentiate between these possibilities, we examined phasic endocannabinoid signaling in NL3 KO mice. We first analyzed depolarization-induced suppression of inhibition (DSI). During DSI, depolarization of pyramidal neurons induces transient release of endocannabinoids, which retrogradely activate CB1 receptors, leading to powerful blockade of GABA release that can last for several seconds (Pitler and Alger, 1994; Wilson and Nicoll, 2001; Földy et al 2006). These experiments showed that the NL3 KO did not affect the magnitude or time course of DSI, documenting that CB1 receptors were properly localized and phasic endocannabinoid signaling was retained in NL3 KO mice (Fig. 7A). We also tested whether the NL3 KO alters the phasic endocannabinoid signaling that induces a long-term depression of inhibitory synapses (I-LTD; Chevaleyre and Castillo, 2003; reviewed in Castillo et al., 2011). High-frequency extracellular stimulation at the border of strata pyramidale and radiatum reliably induced I-LTD both in wild-type and in NL3 KO mice (Fig. 7B). Thus, the NL3 KO does not block two different forms of synaptic plasticity dependent on phasic endocannabinoid signaling.

DISCUSSION

In the present study, we systematically compared the synaptic effects of two different mutations in NL3 that are associated with autism, and examined in paired recordings inhibitory synapses that are formed by two classes of presynaptic basket cells onto the same class of postsynaptic pyramidal neurons in the hippocampus.

This study had two goals. The first goal was based on the lack of a common phenotype produced by the two NL3 mutations in mice, despite their shared association with autism in humans, prompting us to search for such a common phenotype. As a starting point in this search, we used the altered rate of spontaneous mini activity that we had previously identified in NL3 KO mice (Etherton et al., 2011a). We were led in this search by the notion that the lack of a similar phenotype in R451C-mutant synapses could have been due to confounding gain-of-function effects of the R451C substitution on other subsets of synapses on the same neuron, which may have occluded a common phenotype shared by the R451C KI and NL3 KO neurons. Thus, to search for common phenotypes, we used paired recordings which enabled us to separately monitor defined synapses originating from specific classes of inhibitory basket cells in the hippocampus.

The second goal of this study was stimulated by our earlier results demonstrating that the R451C substitution produced different synaptic phenotypes in distinct brain regions (Tabuchi et al., 2007; Etherton et al., 2011a). These results led us to test whether the NL3 KO and the R451C KI mutations might produce different phenotypes even in distinct synapses formed onto the same postsynaptic neuron. The differences in NL3 phenotypes in different brain regions supported the hypothesis that NL3 does not simply act in establishing synapses as such, but functions to specify synaptic properties depending on the presynaptic partner, a hypothesis that would predict that synapses formed by different presynaptic partners on the same postsynaptic neuron may also exhibit distinct changes in NL3 mutants.

Our study addresses both goals. The results suggest three major conclusions that have implications not only for autism pathophysiology, but also for synapse formation and synaptic endocannabinoid signaling.

First, we unexpectedly found that NL3 is essential for tonic but not phasic endocannabinoid signaling. The mechanisms of tonic endocannabinoid signaling are not well studied - in fact, its very existence as a specific process was unclear (Kim and Alger, 2010; Alger, 2012). Our finding that tonic endocannabinoid signaling is impaired in NL3 KO neurons (and R451C KI neurons) validates this form of endocannabinoid signaling as a specific regulatory process that is not an 'accident' of endocannabinoid leakage or spillover, and identifies NL3 as the only protein known to be specifically required for tonic endocannabinoid secretion. The loss of tonic endocannabinoid signaling is the likely cause for the change in mini-frequency we previously observed in NL3 KO mice (Etherton et al., 2011a). The fact that this phenotype is caused by both the NL3 KO and R451C KI suggests that a loss of tonic endocannabinoid signaling may be a component of autism pathogenesis, and suggests new avenues for potential treatments (Cravatt and Lichtman, 2003; Piomelli, 2003; Katona and Freund, 2008). Although the mechanism by which NL3 acts in tonic endocannabinoid secretion is unknown, it seems likely that NL3 serves to localize the as yet unknown tonic secretory machinery to synapses via trans-synaptic interactions with neurexins. Alternatively, it is conceivable that the NL3 loss-of-function activates an enzyme that selectively degrades ligands of tonic but not the phasic endocannabinoid signal (Alger and Kim, 2011; Alger, 2012).

Second, the R451C mutation causes both gain- and loss-of-function effects (Fig. 8). We previously demonstrated that the R451C KI causes gain-of-function effects when we

compared the phenotype of the NL3 KO and R451C KI mutations in inhibitory synapses in the cortex and excitatory synapses in the hippocampus (Tabuchi et al., 2007; Etherton et al., 2011a). In these synapses, the NL3 KO elicited no major phenotype while the NL3 R451C KI produced specific increases in synaptic transmission. However, R451C loss-of-function effects were not detected in earlier studies, although they are consistent with the fact that the R451C mutation destabilizes NL3 and reduces its levels ~90% (De Jaco et al., 2010; Tabuchi et al., 2007). The present paper now shows that the R451C mutation does indeed also cause loss-of-function phenotypes, thereby reconciling the observation of both this mutation and a NL3 deletion in autism (Jamain et al., 2003; Sanders et al., 2011).

Third, the R451C mutation causes distinct effects on different types of synapses of the same postsynaptic neuron (Fig. 8). The differences between phenotypes induced by the R451C KI suggests that NL3 acts in a context-dependent manner not only in a regional sense (i.e., it has a different phenotype in cortical vs. hippocampal synapses), but also within a brain region. This observation argues against what might be called a `mechanical' view of synaptic cell adhesion whereby a molecule performs the same function in all contexts - instead, the observations on the R451C mutation reveal that NL3 can perform distinct functions, presumably depending on the ligands that are available in a given synapse, a result that is consistent with previous results obtained for neuroligin-2 (Gibson et al., 2009). Moreover, the inhibition of PV-containing synapses by the R451C substitution represents the first time the R451C mutation was found to decrease synaptic strength as in previous studies it always increased synaptic strength (Tabuchi et al., 2007; Etherton et al., 2011a). The powerful size of this effect is again consistent with a major regulatory function of neuroligins in synapses.

The multitude of the effects of the R451C mutation on neurotransmission (Fig. 8) is surprising and supports the notion that neuroligins participate in a balanced array of diverse functions, possibly via interactions with multiple ligands. Specifically, the R451C mutation may act by shifting the activity of NL3 in a fluid interaction network composed of multiple competing trans-synaptic ligands. Our previous studies suggested that at least neuroligin-1 functions as a trans-synaptic cell-adhesion molecule by binding both to neurexins and to as yet unidentified other ligands (Ko et al., 2009). It is possible that the R451C mutation blocks the binding of NL3 to one of the ligands, and/or activates the binding of another ligand, thereby shifting the interaction network.

Although we show here that NL3 is selectively essential for tonic endocannabinoid signaling, this result does not exclude the possibility that NL3 performs other functions. In fact, analogous to other genes such as RIMs (Kaeser et al., 2012), NL3 could perform major functions that are redundantly also performed by other neuroligins. The previous analysis of constitutive neuroligin triple KO mice strongly supports this notion by revealing functional redundancy among neuroligins (Varoqueaux et al., 2006), as does the observation of multiple strong phenotypes produced by the R451C and R704C KI mutations in NL3 (Tabuchi et al., 2007; Etherton et al., 2011a and 2011b). The requirement for NL3 in tonic endocannabinoid signaling affirms the notion that neuroligins specify synapse properties, as NL3 confers onto CCK-containing synapses tonic endocannabinoid signaling without influencing phasic signaling or other synaptic parameters. Tonic endocannabinoid signaling was not previously associated with a specific regulatory mechanism but the link to NL3 revealed here validates the importance of this signaling pathway and suggests a possible endocannabinoid involvement in autism.

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EXPERIMENTAL PROCEDURES

Mouse breeding and genotyping

Mice were genotyped as described previously (Tabuchi et al., 2007, Etherton et al., 2011a). All animal protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee at Stanford University.

Electrophysiology

Hippocampal slices (300 μm) were prepared from 3–4 weeks old NL3 R451C KI and NL3 KO mice. Slices were incubated at 33 °C in sucrose-containing artificial cerebrospinal fluid (ACSF; 85 mM NaCl, 75 mM sucrose, 2.5 mM KCl, 25 mM glucose, 1.25 mM NaH_2PO_4, 4 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 24 mM $NaHCO_3$) for an hour and than incubated in the same solution at room temperature until recording. Electrophysiological recordings were made in ACSF containing 126 mM NaCl, 2.5 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 26 mM $NaHCO_3$. Slices were visualized in an upright microscope (Olympus, BX-61WI) with infrared differential interference contrast optics. Whole cell recordings were obtained from the interneurons with patch pipettes (King Precision Glass, Inc., 3–5 MΩ) filled with internal solution containing 126 mM K-gluconate, 4 mM KCl, 10 mM HEPES, 4 mM Mg-ATP, 0.3 Na-GTP, 10 mM phosphocreatine and 0.2% biocytin (pH 7.2, 270–290 mOsm), and from postsynaptic pyramidal cells containing 40 mM CsCl, 90 mM K-gluconate, 1.8 mM NaCl, 1.7 mM $MgCl_2$, 3.5 mM KCl, 0.05 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.4 mM Na-GTP, 10 mM phosphocreatine (pH 7.2, 270–290 mOsm; in some of the recordings 0.2% biocytin was also added to this solution). All electrophysiological recordings were made at 33 °C, using MultiClamp700B amplifiers (Molecular Devices, Sunnyvale, CA). Signals were filtered at 4 kHz using Bessel filter and digitized at 10 kHz with a Digidata 1440A analog-digital interface (Molecular Devices, Sunnyvale, CA). Series resistance was monitored, and recordings were discarded if the series resistance changed significantly or reached 25 MΩ. The recorded traces were analyzed using Clampfit software (Molecular Devices, Sunnyvale, CA). PV and CCK interneurons were distinguished based on their distinct electrophysiological spiking properties (Földy et al., 2010), and by the presence of DSI in CCK basket cell synapses (see Fig. 7A). IPSCs were individually inspected and included in the analysis based on their onset latency following the presynaptic action potential. For statistical analysis Student's t-test, paired t-test or Mann-Whitney Rank Sum Test (RST) was used, and data are presented as mean ± s.e.m., unless noted otherwise; significance was $P < 0.05$.

Quantal model

Individual basket cells innervate postsynaptic pyramidal cells via multiple release sites (N; Biró et al., 2006; Földy et al., 2010), in which intrinsically variable synaptic parameters (such as quantal size and release probability; Q and P_R respectively) produce a trial-to-trial fluctuation in the IPSC amplitudes. The distribution of these fluctuations can be described by models that are based on binomial statistics and allow estimates of Q and N (Silver, 2003, Biró et al., 2006). In this study, we ought to extend quantal modeling to analyze pooled data from multiple paired-recording experiments of defined synapse populations, and extract mean quantal information that is characteristic to each population. For modeling, we analyzed synapses by quantifying IPSC amplitudes and success rates. Assuming that each synapse population can be described by characteristic mean N and Q values, it is reasonable to assume that the pair-to-pair variability in IPSC amplitudes and success rates is dominated by variability in P_R. In this case, the distribution of IPSC amplitudes and success rates should follow the $\left[PSC = Q \cdot N \cdot \left[1 - \sqrt[N]{1 - Successes} \right] \right]$ model (Eq. 1; see Fig. S2 for more information). For fitting the IPSC model on experimental data, to estimate quantal

parameters, we employed the built-in, unconstrained *NonlinearModelFit* algorithm in Mathematica 8 (Wolfram Research, Inc., Champaign, IL). Note that the basic assumptions of this approach (i.e. the existence of characteristic Q and N values in each synapse population) were supported by the similarity between the observed and predicted IPSC distributions (Eq.1; see Figs. 5A and 6C).

Computational model

In order to gain further qualitative insight into how pre- and postsynaptic changes may contribute to the synaptic phenotypes produced by NL3 mutations, we devised a simple computational model that incorporated five modifiable synaptic parameters: the number of release sites (N) and the mean and variance of the release probability (P_R and σ_{PR}, respectively) and of quantal IPSCs (Q and σ_Q, respectively). Note that non-zero variances were necessary to simulate variability both in the number of successful transmissions (by σ_{PR}) and IPSC amplitudes (by σ_Q). To initialize the simulations, p_i values (that is the release probability of the *i*-th release site) were assigned randomly from a normal probability distribution function with P_R mean and σ_{PR} variance for each release site. In addition, for each release site, q_i values (that is the quantal size in the *i*-th release site) were randomly assigned from a log-normal probability distribution function of mean Q and σ_Q variance parameters (see Supplementary Fig. S3 for more information). Computational IPSCs (cIPSCs) and successes (cSuccesses) were derived as described in the text. For each condition, estimates of Q and N were adopted from the quantal model (Fig. 5A and 6C). Each simulation had the same sample size as the original data, and each simulation was repeated 50 times with random assignments of new p_i and q_i values. For statistical comparisons, we tested the null-hypothesis that the difference between the mean computed and experimental successes and IPSCs were zero; simulation parameters were accepted when P>0.05 using Student's t-test. To estimate the robustness of the resulting simulation parameters, we quantified an average range for each parameter which still justifies the null-hypothesis: $\Delta P_R=\pm0.006$, $\Delta\sigma_{PR}=\pm0.09$, $\Delta Q=\pm0.7$ pA and $\Delta\sigma_Q=\pm0.06$ pA (relative to values presented in the main text). Parameter deviations beyond these ranges independently resulted in statistically significant differences (P<<0.05) between the simulated and experimental distributions. Simulations were implemented and run using Mathematica 8 (Wolfram Research, Inc.).

Neuroanatomy

After recordings, all slices were transferred into a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer. In order to examine the axonal and dendritic arbor of presynaptic basket cell, biocytin-filled cells were visualized after recordings with 3,3-diaminobenzidinetetrahydrochloride (0.015%) using PK-6100 DAB and Vectastain SK-4100 ABC kit (Vector Laboratories, Burlingame, CA). Example basket cells in Figures 1 and 2 were reconstructed using Neurolucida 10 (MBF Bioscience, Williston, VT). For axonal bouton density quantification, axonal segments with corresponding boutons were reconstructed using Neurolicida 10. The length of the axons (which averaged 1180.4±128.4 μm, mean length ± s.e.m., in the reconstructed cells) and bouton numbers were determined using NeuroExplorer (MBF Bioscience, Williston, VT).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

REFERENCES

Ali AB, Todorova M. Asynchronous release of GABA via tonic cannabinoid receptor activation at identified interneuron synapses in rat CA1. Eur. J. Neurosci. 2010; 31:1196–1207. [PubMed: 20345910]

Alger BE. Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. Prog. Neurobiol. 2002; 68:247–286. [PubMed: 12498988]

Alger BE, Kim J. Supply and demand for endocannabinoids. Trends Neurosci. 2011; 34:304–315. [PubMed: 21507493]

Alger BE. Endocannabinoids at the synapse a decade after the dies mirabilis (29 March 2001): what we still do not know. J. Physiol. 2012; 590:2203–2212. [PubMed: 22289914]

Bartos M, Vida I, Jonas P. Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks. Nat. Rev. Neurosci. 2007; 8:45–56. [PubMed: 17180162]

Biró AÁ, Holderith NB, Nusser Z. Release probability-dependent scaling of the postsynaptic responses at single hippocampal GABAergic synapses. J. Neurosci. 2006; 26:12487–12496. [PubMed: 17135411]

Baudouin SJ, Gaudias J, Gerharz S, Hatstatt L, Zhou K, Punnakkal P, Tanaka KF, Spooren W, Hen R, De Zeeuw CI, Vogt K, Scheiffele P. Shared synaptic pathophysiology in syndromic and nonsyndromic rodent models of autism. Science. 2012; 338:128–132. [PubMed: 22983708]

Budreck EC, Scheiffele P. Neuroligin-3 is a neuronal adhesion protein at GABAergic and glutamatergic synapses. Eur. J. Neurosci. 2007; 26:1738–1748. [PubMed: 17897391]

Castillo PE, Chiu CQ, Carroll RC. Long-term plasticity at inhibitory synapses. Curr. Opin. Neurobiol. 2011; 21:328–338. [PubMed: 21334194]

Chadman KK, Gong S, Scattoni ML, Boltuck SE, Gandhy SU, Heintz N, Crawley JN. Minimal aberrant behavioral phenotypes of NL3 R451C knockin mice. Autism Res. 2008; 1:147–158. [PubMed: 19360662]

Chevaleyre V, Castillo PE. Heterosynaptic LTD of hippocampal GABAergic synapses: a novel role of endocannabinoids in regulating excitability. Neuron. 2003; 38:461–472. [PubMed: 12741992]

Chubykin AA, Atasoy D, Etherton MR, Brose N, Kavalali ET, Gibson JR, Südhof TC. Activity-dependent validation of excitatory versus inhibitory synapses by neuroligin-1 versus neuroligin-2. Neuron. 2007; 54:919–931. [PubMed: 17582332]

Cravatt BF, Lichtman AH. Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. Curr. Opin. Chem. Biol. 2003; 7:469–475. [PubMed: 12941421]

De Jaco A, Dubi N, Comoletti D, Taylor P. Folding anomalies of NL3 caused by a mutation in the alpha/beta-hydrolase fold domain. Chem. Biol. Interact. 2010; 187:56–58. [PubMed: 20227402]

Etherton MR, Blaiss CA, Powell CM, Südhof TC. Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. Proc. Natl. Acad. Sci. USA. 2011a; 106:17998–18003. [PubMed: 19822762]

Etherton MR, Tabuchi K, Sharma M, Ko J, Südhof TC. An autism-associated point mutation in the neuroligin cytoplasmic tail selectively impairs AMPA receptor-mediated synaptic transmission in hippocampus. EMBO J. 2011b; 30:2908–2919. [PubMed: 21642956]

Földy C, Neu A, Jones MV, Soltesz I. Presynaptic, activity-dependent modulation of cannabinoid type 1 receptor-mediated inhibition of GABA release. J. Neurosci. 2006; 26:1465–1469. [PubMed: 16452670]

Földy C, Lee SH, Morgan RJ, Soltesz I. Regulation of fast-spiking basket cell synapses by the chloride channel ClC-2. Nat. Neurosci. 2010; 13:1047–1049. [PubMed: 20676104]

Freund TF. Interneuron Diversity series: Rhythm and mood in perisomatic inhibition. Trends Neurosci. 2003; 26:489–495. [PubMed: 12948660]

Freund TF, Katona I, Piomelli D. Role of endogenous cannabinoids in synaptic signaling. Physiol. Rev. 2003; 83:1017–1066. [PubMed: 12843414]

Freund TF, Katona I. Perisomatic inhibition. Neuron. 2007; 56:33–42. [PubMed: 17920013]

Gibson JR, Huber KM, Südhof TC. Neuroligin-2 deletion selectively decreases inhibitory synaptic transmission originating from fast-spiking but not from somatostatin-positive interneurons. J. Neurosci. 2009; 29:13883–13897. [PubMed: 19889999]

Glickfeld LL, Atallah BV, Scanziani M. Complementary modulation of somatic inhibition by opioids and cannabinoids. J. Neurosci. 2008; 28:1824–1832. [PubMed: 18287499]

Graf ER, Zhang X, Jin SX, Linhoff MW, Craig AM. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. Cell. 2004; 119:1013–1026. [PubMed: 15620359]

Hentges ST, Low MJ, Williams JT. Differential regulation of synaptic inputs by constitutively released endocannabinoids and exogenous cannabinoids. J. Neurosci. 2005; 25:9746–9751. [PubMed: 16237178]

Hoffman AF, Laaris N, Kawamura M, Masino SA, Lupica CR. Control of cannabinoid CB1 receptor function on glutamate axon terminals by endogenous adenosine acting at A1 receptors. J. Neurosci. 2010; 30:545–555. [PubMed: 20071517]

Hoon M, Soykan T, Falkenburger B, Hammer M, Patrizi A, Schmidt KF, Sassoè-Pognetto M, Löwel S, Moser T, Taschenberger H, et al. Neuroligin-4 is localized to glycinergicpostsynapses and regulates inhibition in the retina. Proc. Natl. Acad. Sci. USA. 2011; 108:3053–3058. [PubMed: 21282647]

Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Südhof TC. Neuroligin 1: a splice site-specific ligand for beta-neurexins. Cell. 1995; 81:435–443. [PubMed: 7736595]

Ichtchenko K, Nguyen T, Südhof TC. Structures, alternative splicing, and neurexin binding of multiple neuroligins. J. Biol. Chem. 1996; 271:2676–2682. [PubMed: 8576240]

Jamain S, Quach H, Betancur C, Råstam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T, Paris Autism Research International Sibpair Study. Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. Nat. Genet. 2003; 34:27–29. [PubMed: 12669065]

Kaeser PS, Deng L, Fan M, Südhof TC. RIM Genes differentially contribute to organizing presynaptic release sites. Proc. Natl. Acad. Sci. USA. 2012; 109:11830–11835. [PubMed: 22753485]

Katona I, Freund TF. Endocannabinoid signaling as a synaptic circuit breaker in neurological disease. Nat. Med. 2008; 14:923–930. [PubMed: 18776886]

Katona I, Freund TF. Multiple functions of endocannabinoid signaling in the brain. Annu. Rev. Neurosci. 2012; 35:529–558. [PubMed: 22524785]

Kim J, Alger BE. Reduction in endocannabinoid tone is a homeostatic mechanism for specific inhibitory synapses. Nat. Neurosci. 2010; 13:592–600. [PubMed: 20348918]

Klausberger T, Marton LF, O'Neill J, Huck JH, Dalezios Y, Fuentealba P, Suen WY, Papp E, Kaneko T, Watanabe M, et al. Complementary roles of cholecystokinin- and parvalbumin-expressing GABAergic neurons in hippocampal network oscillations. J. Neurosci. 2005; 25:9782–9793. [PubMed: 16237182]

Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science. 2008; 321:53–57. [PubMed: 18599766]

Ko J, Fuccillo MV, Malenka RC, Südhof TC. LRRTM2 functions as a neurexin ligand in promoting excitatory synapse formation. Neuron. 2009; 64:791–798. [PubMed: 20064387]

Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA. Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. Trends Neurosci. 2008; 31:234–242. [PubMed: 18395805]

Losonczy A, Biró AA, Nusser Z. Persistently active cannabinoid receptors mute a subpopulation of hippocampal interneurons. Proc. Natl. Acad. Sci. USA. 2004; 101:1362–1367. [PubMed: 14734812]

Neu A, Földy C, Soltesz I. Postsynaptic origin of CB1-dependent tonic inhibition of GABA release at cholecystokinin-positive basket cell to pyramidal cell synapses in the CA1 region of the rat hippocampus. J. Physiol. 2007; 578:233–47. [PubMed: 17053036]

Piomelli D. The molecular logic of endocannabinoid signalling. Nat. Rev. Neurosci. 2003; 4:873–884. [PubMed: 14595399]

Pitler TA, Alger BE. Depolarization-induced suppression of GABAergic inhibition in rat hippocampal pyramidal cells: G protein involvement in a presynaptic mechanism. Neuron. 1994; 13:1447–1455. [PubMed: 7993636]

Poulopoulos A, Aramuni G, Meyer G, Soykan T, Hoon M, Papadopoulos T, Zhang M, Paarmann I, Fuchs C, Harvey K, et al. Neuroligin 2 drives postsynaptic assembly at perisomatic inhibitory synapses through gephyrin and collybistin. Neuron. 2009; 63:628–642. [PubMed: 19755106]

Sanders SJ, Ercan-Sencicek AG, Hus V, Luo R, Murtha MT, Moreno-De-Luca D, Chu SH, Moreau MP, Gupta AR, Thomson SA, et al. Multiple recurrent de novo CNVs, including duplications of the 7q11.23 Williams syndrome region, are strongly associated with autism. Neuron. 2011; 70:863–885. [PubMed: 21658581]

Silver RA. Estimation of nonuniform quantal parameters with multiple-probability fluctuation analysis: theory, application and limitations. J. Neurosci. Methods. 2003; 130:127–41. [PubMed: 14667542]

Song JY, Ichtchenko K, Südhof TC, Brose N. Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. Proc. Natl. Acad. Sci. USA. 1999; 96:1100–1105. [PubMed: 9927700]

Südhof TC. Neuroligins and neurexins link synaptic function to cognitive disease. Nature. 2008; 455:903–911. [PubMed: 18923512]

Szabó GG, Holderith N, Gulyás AI, Freund TF, Hájos N. Distinct synaptic properties of perisomatic inhibitory cell types and their different modulation by cholinergic receptor activation in the CA3 region of the mouse hippocampus. Eur. J. Neurosci. 2010; 31:2234–2246. [PubMed: 20529124]

Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, Südhof TC. A NL3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science. 2007; 318:71–76. [PubMed: 17823315]

Varoqueaux F, Aramuni G, Rawson RL, Mohrmann R, Missler M, Gottmann K, Zhang W, Südhof TC, Brose N. Neuroligins determine synapse maturation and function. Neuron. 2006; 51:741–754. [PubMed: 16982420]

Wilson RI, Nicoll RA. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature. 2001; 410:588–592. [PubMed: 11279497]

Zhang C, Milunsky JM, Newton S, Ko J, Zhao G, Maher TA, Tager-Flusberg H, Bolliger MF, Carter AS, et al. A neuroligin-4 missense mutation associated with autism impairs neuroligin-4 folding and endoplasmic reticulum export. J. Neurosci. 2009; 29:10843–10854. [PubMed: 19726642]
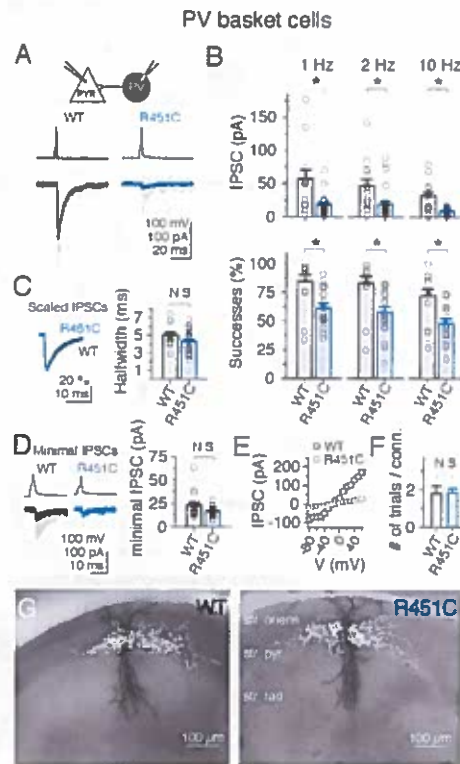
Figure 1. Neuroligin-3 R451C substitution impairs GABAergic synaptic transmission in PV basket cell synapses

(A) Paired recordings of presynaptic APs in PV basket cells (upper traces) that produce unitary IPSCs in CA1 pyramidal cells (lower traces, $V_{holding} = -70$ mV). (B) Comparison of IPSC amplitudes (including failures) and of the percentages of successful transmissions induced by presynaptic APs applied at 1, 5, and 10 Hz in wild-type and R451C-mutant synapses. Open circles represent individual pairs ($n_{WT}=14$, $n_{R451C}=27$, Mann-Whitney RST, P<0.02 for all data sets). (C) The R451C KI mutation did not alter the half width of IPSCs ($n_{WT}=14$, $n_{R451C}=23$, Mann-Whitney RST, P=0.092). (D) Quantification of minimal IPSCs (amplitude of reliably occurring smallest IPSCs in each pair) suggest no change in quantal response in the R451C KI ($n_{WT}=15$, $n_{R451C}=25$, Mann-Whitney RST, P=0.235). (E) Additional paired-recordings show that IPSCs was independent of post-synaptic membrane voltage in R451C KI mice ($n_{WT}=3$, $n_{R451C}=4$). (F) The frequency of finding synaptically coupled pairs was not altered in R451C mice. (G) Neurolucida reconstructions of biocytin-filled basket cells show major reorganization in axonal and dendritic arbor of PV basket cells (str. = stratum, pyr. = pyramidale, rad. = radiatum). See also Figure S1.
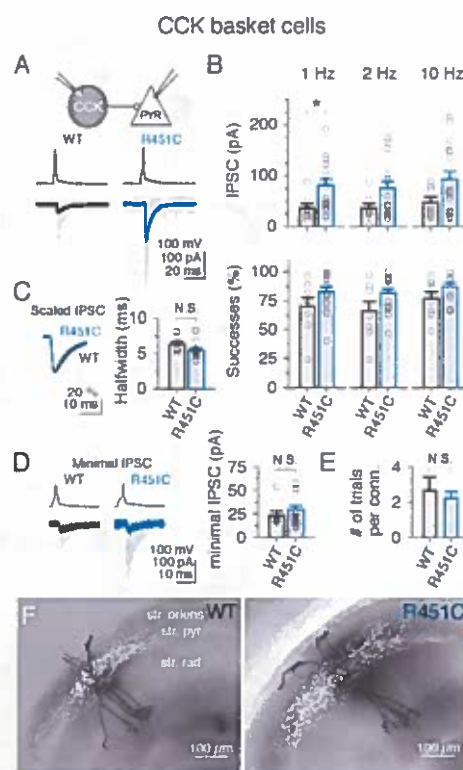
Figure 2. Neuroligin-3 R451C substitution enhances GABAergic synaptic transmission in CCK basket cell synapses

(A) Paired recordings of presynaptic APs in CCK basket cells (upper traces) that produce unitary IPSCs in CA1 pyramidal cells (lower traces, $V_{holding} = -70$ mV). (B) Comparison of IPSC amplitudes (including failures) and of the percentages of successful transmissions induced by presynaptic APs applied at 1, 5, and 10 Hz in wild-type and R451C-mutant synapses. Open circles represent individual pairs (n_{WT}=8, n_{R451C}=17, Mann-Whitney RST, P=0.013 at 1 Hz IPSCs and P>0.08 in all other data sets). (C & D) No change in IPSC halfwidth (n_{WT}=8, n_{R451C}=15, Mann-Whitney RST, P=0.098) and no increase in the minimal IPSC amplitudes in R451C KI (n_{WT}=8, n_{R451C}=15, Mann-Whitney RST, P=0.5) suggest that the enhanced IPSC amplitudes in R451C KIs is not due to changes in quantal GABA receptor responses. (E) The frequency of finding synaptically coupled pairs was not altered in R451C mice. (F) Neurolucida reconstructions of biocytin-filled basket cells show no major reorganization in axonal and dendritic arbor of CCK basket cells.
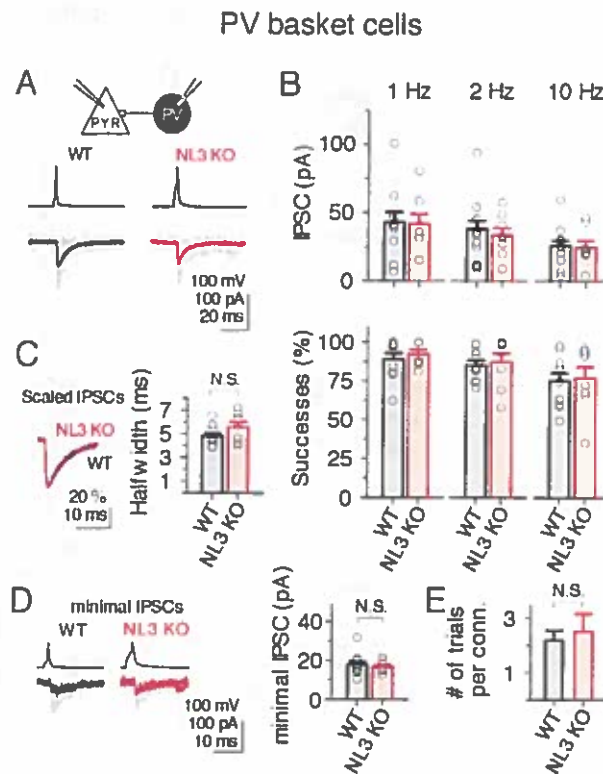
Figure 3. Neuroligin-3 KO does not alter GABAergic transmission in PV basket cell synapses
(A & B) Paired-recording data show that IPSC amplitudes and transmission rates were unaltered in NL3 KO mice compared to WT littermates (n_{WT}=12, n_{KO}=8, Mann-Whitney RST, P>0.32 in all data set). (C & D) Quantification of IPSC halfwidth and minimal IPSC amplitudes suggest no changes in postsynaptic GABA receptor subunit composition. (E) The frequency of finding connected pairs was similar in NL3 WT and KO mice.
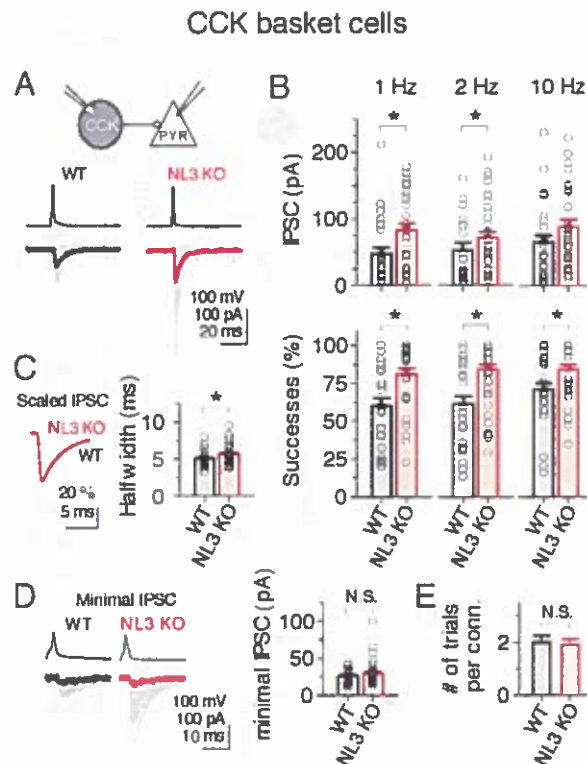
Figure 4. Neuroligin-3 KO enhances GABAergic synaptic transmission in CCK basket cell synapses similar to the R451C KI

(A & B) Paired recording data show that IPSC amplitudes and transmission rates were enhanced in CCK basket cell to CA1 pyramidal neuron synapses at multiple AP firing frequencies ($n_{WT}=28$, $n_{KO}=36$, Mann-Whitney RST, P=0.12 at 10 Hz IPSCs, and P<0.03 for all other data set). (C) Increase in IPSC halfwidth in KO suggest possible subunit reorganization of GABA receptor subunits in NL3 KO ($n_{WT}=28$, $n_{KO}=35$, Mann-Whitney RST, P=0.021). (D & E) No change in minimal IPSC amplitudes ($n_{WT}=28$, $n_{KO}=35$, Mann-Whitney RST, P=0.885), and in the frequency of finding connected pairs between CCK basket cells and pyramidal cells.
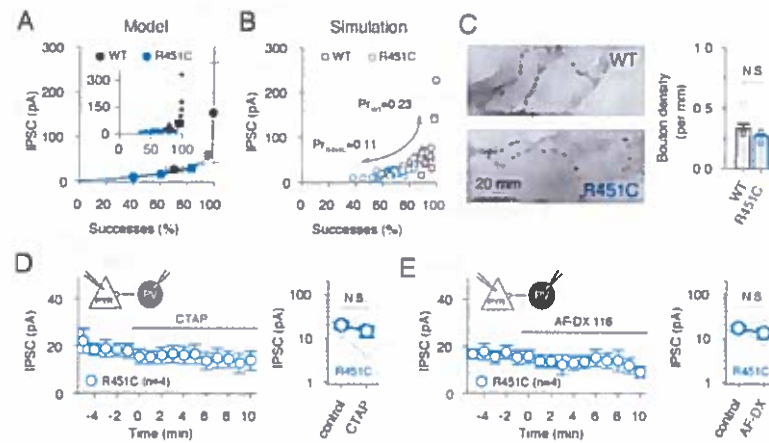
Figure 5. The NL3 R451C KI mutation lowers the probability of GABA release from PV basket cell synapses

(A) Averaged PV basket cell IPSCs (same data as in Fig. 1) are plotted against their corresponding averaged success rates (WT data were pooled from wild-type littermates of R451C KI and NL3 KO mice). Data were fitted to the equation
$$\left[PSC = Q \cdot N \cdot \left[1 - \sqrt[N]{1 - Successes} \right] \right]$$ to estimate the mean quantal size (Q) and number of release sites (N) for each synapse population. Solid lines indicate best fit (black: WT, blue: R451C KI). Inset shows the distribution of individual data points. (B) Computer simulations of PV basket cell IPSCs. Simulation results for WT (open black circles) and R451C KI (open blue circles) were not significantly different (in mean IPSCs and successes) from their corresponding experimental IPSCs datasets when P_R was set to 0.23 and 0.11, respectively, in the model (see main text for further parameters). (C) Light microscopy analysis of the bouton density of PV basket cell axons. Left: example of axonal segments for axons in WT and R451C KI mice. Right: summary data from WT (n=7) and R451C KI (n=8) mice. P=0.152, Mann-Whitney RST. (D) Bath application of μ-opioid receptor antagonist CTAP (500 nM) in paired recording experiments between PV basket and pyramidal cells in R451C KI mice (n=4 pairs). Averaged time course (left) and time averaged means (right) of the 4 recordings did not show statistically significant effect of μ-opioid receptor antagonist on IPSCs. (E) Bath application of M2 muscarinic-receptor antagonist AF-DX 116 (10 μM) in paired recording experiments between PV basket and pyramidal cells in R451C KI mice (n=4 pairs). Averaged time course (left) and time averaged means (right) of the 4 recordings did not show statistically significant effect of μ-opioid receptor antagonist on IPSCs. Averaged data presented as mean ± s.e.m. See also Figure S2 and S3.
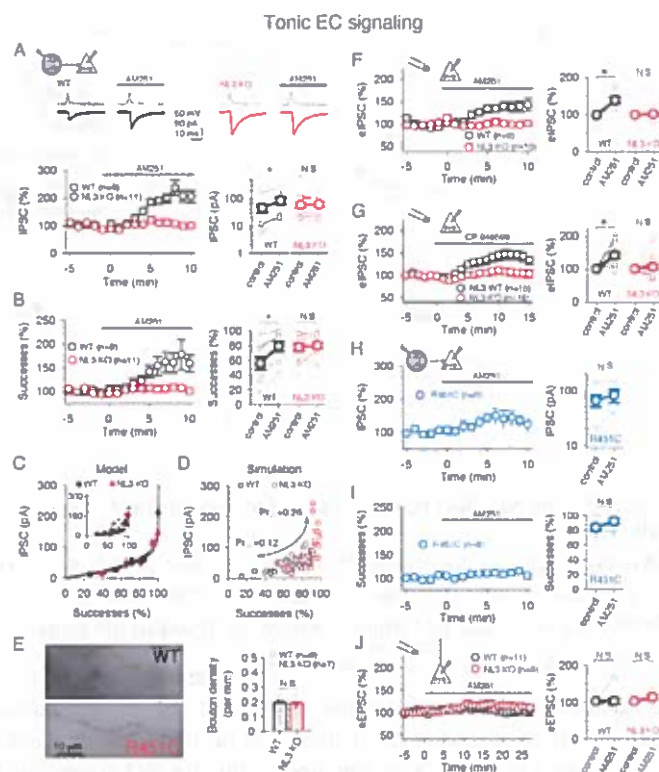
Figure 6. Neuroligin-3 KO and R451C KI mutations impair tonic endocannabinoid signaling
(A) Representative paired recordings (upper traces) and normalized time-courses (lower left panel) demonstrate that bath application of 10 μM AM251 enhances IPSCs in WT, but not in NL3 KO mice. Lower right panel: IPSC changes (failures included) in each paired-recording experiment (`control': average data for minutes 1–5, `AM251': for minutes 6–10; n_{WT}=9, P=0.004; n_{NL3KO}=11, P=0.268, paired T-test). (B) Left panel: time-courses of AM251 wash-in suggest that the lack of effect of AM251 on IPSCs was due to the failure of AM251 in increasing the number of successful transmissions. Right panel: AM251 reliably increased the number of successes in WT, but not in NL3 KO mice (n_{WT}=9, P<0.001; n_{NL3KO}=11, P=0.79, paired T-test). (C) Averaged CCK basket cell IPSCs (same data as in Fig. 4) are plotted against their corresponding averaged success rates (WT data were pooled from wild-type littermates of R451C KI and NL3 KO mice). Data were fitted to the equation $\left[PSC = Q \cdot N \cdot \left[1 - \sqrt[N]{1 - Successes} \right] \right]$ to estimate the mean quantal size (Q) and number of release sites (N) for each synapse population. Solid lines indicate best fit (black: WT, red: NL3 KO). Inset shows the distribution of individual data points. (D) Computer simulations of CCK basket cell IPSCs. Simulation results for WT (open black circles) and NL3 KO (open red circles) were not significantly different (in mean IPSCs and successes) from their corresponding experimental IPSCs datasets when P_R was set to 0.26 and 0.12, respectively, in the model (see main text for further parameters). (E) Light microscopy analysis of the bouton density of CCK basket cell axons. Left: example of axonal segments for axons in WT and NL3 KO mice. Right: summary data from WT (n=6) and NL3 KO (n=7) mice. P=0.779, t-test. (F) Time-course of the effect of the AM251 wash-in on extracellulary evoked IPSCs (eIPSC; left panel), and averaged data in each experiment (right panel) show increase in eIPSC amplitude in WT, but not in NL3 KO mice ($V_{pyramidal}$= −70 mV, 1 Hz stimulation, in the presence of 5 μM NBQX and 10 μM D-AP5; n_{WT}=6, P=0.008; n_{NL3KO}=10, P=0.63, paired t-test). (G) Time-course of the effect of the CP945598 wash-in on extracellulary evoked IPSCs (eIPSC; left panel), and averaged data in each experiment

(right panel) show increase in eIPSC amplitude in WT, but not in NL3 KO mice ($V_{pyramidal}$= −70 mV, 1 Hz stimulation, in the presence of 5 μM NBQX and 10 μM D-AP5; n_{WT}=15, P=0.0005; n_{NL3KO}=18, P=0.41, paired t-test). (**H & I**) Paired recordings of IPSC amplitudes and success rates in response to 10 μM AM251 in R451C KI mice. Left panels: time-course of the experiments. Right panels: absolute changes in each pair (n_{WT}=6, P=0.07; n_{R451C}=10, P=0.072, paired T-test). (**J**) Time-course of the effect of 10 μM AM251 wash-in on extracellularly evoked EPSCs (eEPSC; left panel), and averaged data in each experiment (right panel) in WT and NL3 KO mice ($V_{pyramidal}$= −70 mV, 1 Hz stimulation, in the presence of 50 μM picrotoxin; n_{WT}=11, P>0.05; n_{NL3KO}=8, P>0.05, paired t-test). See also Figure S2, S3 and S4.
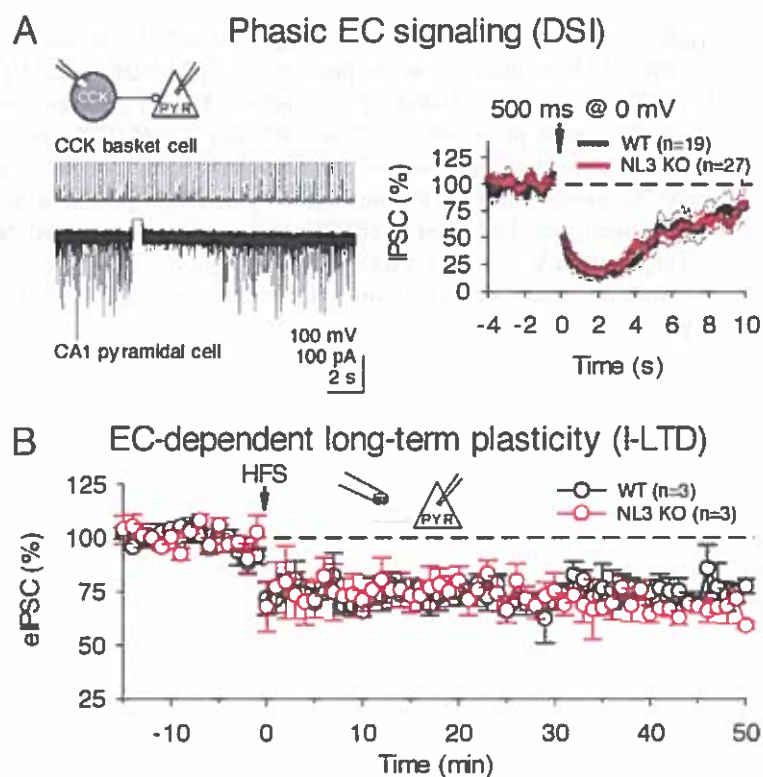
Figure 7. Neuroligin-3 is not required for phasic short-term endocannabinoid signaling (DSI) or long-term endocannabinoid-dependent synaptic plasticity (i-LTD)

(A) Paired recordings show that DSI induced by phasic endocannabinoid signaling was unaffected in NL3 KO (left panel: example of DSI, note the transient suppression of IPSCs after brief depolarization in the pyramidal cell; right panel: averaged time-course of DSI in WT and NL3 KO). (B) Deletion of NL3 does not affect the magnitude or time-course of the endocannabinoid-dependent I-LTD ($V_{pyramidal}$ = +10 mV, inter-stimulus interval 20 s, $[Cl_{pipette}]$ = 4 mM, in presence of 5 µM NBQX and 10 µM D-AP5).
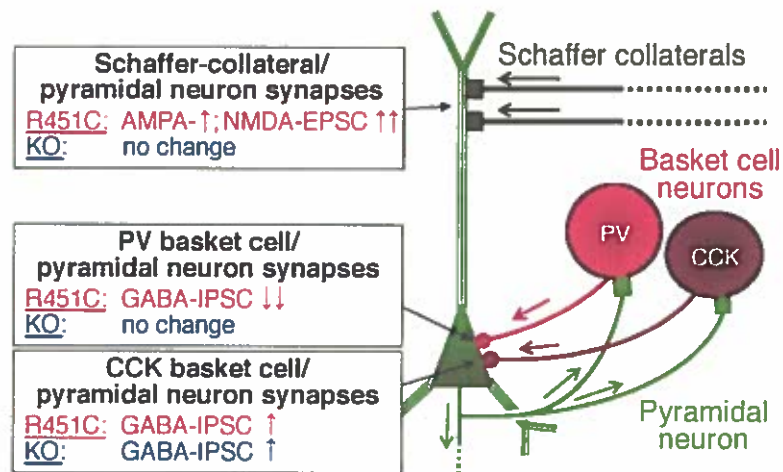
Figure 8. Schematic summary diagram of the effects of the NL3 KO and R451C substitution on three different synapses on pyramidal neurons in the CA1 region of the hippocampus
The diagram depicts a pyramidal neuron (green) receiving inputs from Schaffer collateral fibers and two different types of basket cell neurons (PV, parvalbumin; CCK, cholecystokinin). The changes observed in NL3 R451C knockin and KO mice are described on the left.

Review

The Basolateral Amygdala γ-Aminobutyric Acidergic System in Health and Disease

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The brain comprises an excitatory/inhibitory neuronal network that maintains a finely tuned balance of activity critical for normal functioning. Excitatory activity in the basolateral amygdala (BLA), a brain region that plays a central role in emotion and motivational processing, is tightly regulated by a relatively small population of γ-aminobutyric acid (GABA) inhibitory neurons. Disruption in GABAergic inhibition in the BLA can occur when there is a loss of local GABAergic interneurons, an alteration in $GABA_A$ receptor activation, or a dysregulation of mechanisms that modulate BLA GABAergic inhibition. Disruptions in GABAergic control of the BLA emerge during development, in aging populations, or after trauma, ultimately resulting in hyperexcitability. BLA hyperexcitability manifests behaviorally as an increase in anxiety, emotional dysregulation, or development of seizure activity. This Review discusses the anatomy, development, and physiology of the GABAergic system in the BLA and circuits that modulate GABAergic inhibition, including the dopaminergic, serotonergic, noradrenergic, and cholinergic systems. We highlight how alterations in various neurotransmitter receptors, including the acid-sensing ion channel 1a, cannabinoid receptor 1, and glutamate receptor subtypes, expressed on BLA interneurons, modulate GABAergic transmission and how defects of these systems affect inhibitory tonus within the BLA. Finally, we discuss alterations in the BLA GABAergic system in neurodevelopmental (autism/fragile X syndrome) and neurodegenerative (Alzheimer's disease) diseases and after the development of epilepsy, anxiety, and traumatic brain injury. A more complete understanding of the intrinsic excitatory/inhibitory circuit balance of the amygdala and how imbalances in inhibitory control contribute to excessive BLA excitability will guide the development of novel therapeutic approaches in neuropsychiatric diseases. © 2015 Wiley Periodicals, Inc.

Key words: basolateral amygdala; GABA; autism; Alzheimer's disease; anxiety; epilepsy

The brain comprises a highly complex network of excitatory and inhibitory circuits that maintains exquisite balance in network activity. Hyperexcitability arises when there is an imbalance between excitation and inhibition

SIGNIFICANCE:

Deficits in the brain inhibitory systems can occur at any stage of life. The resulting hyperexcitability leads to the development of neurological and/or neuropsychiatric diseases. We assess how the loss of inhibitory synaptic transmission and mechanisms that modulate inhibition in the basolateral amygdala lead to increased anxiety. In addition, we examine how different diseases including autism/fragile X syndrome, Alzheimer's disease, traumatic brain injury, and epilepsy result in amygdalar hyperexcitability. By evaluating how deficiencies in inhibition within the amygdala contribute to these diseases, future research may be directed toward developing new therapies for reducing excitability that may alleviate the behavioral symptomology of neurologic diseases.

(E/I), often as a result of deficiencies or disruption in γ-aminobutyric acid (GABA) inhibitory system control. Hyperexcitability of the amygdala, in particular, can be strongly associated with anxiety, hypervigilance, and an inability to regulate emotions. Acquired deficiencies in the GABAergic inhibitory system have been observed after traumatic brain injury (TBI; Reger et al., 2012; Almeida-Suhett et al., 2014; Depue et al., 2014; Guerriero et al., 2015) and status epilepticus (SE; Gean et al., 1989; Fritsch et al., 2009; Prager et al., 2014b). In addition, amygdala hyperexcitability resulting in anxiety has been observed in neuropsychiatric disorders, such as posttraumatic stress disorder (PTSD; Nuss, 2015; Truitt et al., 2009), as well as in neurodevelopmental disorders, including autism/fragile X syndrome (Olmos-Serrano et al., 2010; El-Ansary and Al-Ayadhi, 2014; Martin et al., 2014), and in neurodegenerative disorders, such as Alzheimer's disease (AD; Klein et al., 2014; Palop and Mucke, 2010).

Hyperexcitability of the basolateral nucleus of the amygdala (BLA) is associated with increased anxiety and often occurs in parallel with various neurodevelopmental, neurodegenerative, and neuropsychiatric disorders. The GABAergic inhibitory system is one target of therapeutic treatments to reduce anxiety and maintain homeostasis. For example, benzodiazepines, which allosterically enhance postsynaptic actions of GABA at the inhibitory type A GABA receptor (GABA_A receptor), are one first-line treatment for anxiety (Farb and Ratner, 2014) and seizure disorders. However, in many cases, benzodiazepines are ineffective and/or exacerbate symptoms, as has been observed in seizure models when, for example, administration of diazepam initially suppresses seizures but leads to rebound seizures that are similar to or longer in duration than those of animals that do not receive the anticonvulsant (Apland et al., 2014). Thus, the efficacy of current treatments targeting the GABAergic system has been called into question, and new therapeutic targets merit preclinical investigation.

This Review discusses the BLA GABAergic system in health and disease, focusing on five diseases, autism/fragile X, AD, epilepsy, TBI, and anxiety and trauma- or stressor-related disorders (such as PTSD) because these disorders are prime examples of acquired amygdala dysfunctions that occur during development, during aging, or after injury. First, we review the anatomy and development of the GABAergic system in the BLA and the different ways in which GABAergic inhibitory synaptic transmission is modulated. Second, we review how local GABAergic inhibitory neurotransmission in the BLA is altered in disease. Through the study of how deficiencies in the GABAergic inhibitory system in the amygdala contribute to disease outcomes, future research may be directed at developing new therapies to reduce excitability or to increase inhibition.

THE GABAERGIC SYSTEM IN THE BLA

GABAergic Interneurons

The amygdala is located in the medial temporal lobe and is made up of 13 subnuclei (for a comprehensive review of the anatomical connections of the rat and human amygdala see Pitkanen, 2000; Sah et al., 2003; Whalen and Phelps, 2009). The BLA makes up a large component of this network, receiving input from cortical and subcortical structures. The BLA, which generally comprises the lateral and basal portions, contains two main types of neurons, glutamatergic (pyramidal) principal neurons and GABAergic interneurons (McDonald, 1992; Pare and Smith, 1998). Principal neurons constitute the majority of the neurons in the BLA (80–85%), whereas GABAergic interneurons form ~15–20% of the neuronal population (Sah et al., 2003; Spampanato et al., 2011). GABAergic interneurons can be subdivided into those that express calbindin (CB) or calretinin (CR) and can be further subdivided into groups by neuropeptide expression (i.e., vasoactive intestinal peptide [VIP] and/or cholecystokinin [CCK]) or by the expression of the calcium-binding protein parvalbumin (PV; Kemppainen and Pitkanen, 2000; McDonald and Mascagni, 2001a, 2002; Mascagni and McDonald, 2003; Davila et al., 2008; Table I). PV-immunopositive neurons make up about 40% of GABAergic interneurons and are the main source of the perisomatic innervation of principal cells, suggesting that their primary role is in feedback inhibition. CR interneurons make up about 25–30% of BLA GABAergic interneurons and innervate primarily other interneurons (McDonald and Mascagni, 2001a; Muller et al., 2003, 2006; Capogna, 2014).

GABA_A Receptor Structure and Function

GABAergic inhibitory synaptic transmission plays a central role in the regulation of amygdala excitability. Pathological disruption of GABA_A receptors causes a disruption of the E/I balance and has been increasingly implicated in neurological and neurodegenerative diseases (Deidda et al., 2014). Fast inhibitory synaptic transmission within the central nervous system is mediated by the GABA_A receptor, a heteropentameric chloride-permeable, GABA-gated member of the cys-loop superfamily of ligand-gated ion channels. GABA_A receptors are formed from limited combinations of subunits that have diverse structural and functional properties (α1–6, β1–3, γ1–3, δ, ε, θ, and π; Olsen and Sieghart, 2009).

Proper maturation of the GABAergic system in the BLA is essential in neurodevelopment. Dysfunction in the development of the GABAergic inhibitory system within the BLA may be associated with neurodevelopmental diseases, such as autism or fragile X. In rat, the development of the mature GABAergic system in the BLA takes place between postnatal day (P) 14 and P30 with the emergence of PV interneurons (Berdel and Morys, 2000; Davila et al., 2008), an increase in the density of GABAergic fibers, and a decrease in the density of GABAergic cell bodies (Brummelte et al., 2007). Concurrently, GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) reach maturity between P21 and P28. Simultaneously, the reversal potential of GABA_A receptors expressed in principal neurons shifts from −55 mV at P7 to −70 mV by P21. This increase in hyperpolarization

TABLE I. Summary of Systems Modulating BLA GABAergic Inhibition*

| Immunohistochemistry | | | | |
General	Specific	Firing patterns	Innervation of GABAergic interneurons	Receptor subtypes and roles in modulating GABAergic transmission
CB	PV	Fast spiking, stuttering, nonadapting, adapting	~50% Cortical, < 1% thalamic to CB interneurons, VTA, SN, DRN, SI, VP of basal forebrain (cholinergic and GABAergic)	D1, ↑ firing, induces rhythmic oscillations; D2, ↓ presynaptic GABA release; 5-HT$_{2A}$, ↑ excitability; GABA$_B$, ↓ excitability
	CCK (VIP⁻)	Nonadapting, burst adapting		5-HT$_{3A}$, ↑ excitability but rapidly desensitizing; α1 AR and α2 AR, ↑ AP firing and IPSCs; CB1, ↓ excitability; GABA$_B$, ↓ excitability
	SOM/NPY/NK$_{1r}$		DRN	5-HT$_{1A}$ (NPY, NK$_{1r}$), ↓ presynaptic GABA release; 5-HT$_{2C}$ (NPY), ↑ excitability; α1 AR and α2 AR, ↑ AP firing and IPSCs; GABA$_B$, ↓ excitability
CR	CCK (VIP⁺)	Adapting	VTA and SN (<PV interneurons)	5-HT$_{3A}$, ↑ excitability but rapidly desensitizing; α1 AR and α2 AR, ↑ AP firing and IPSCs; GABA$_B$, ↓ excitability
Not localized to specific GABAergic interneuronal subpopulations			LC, NTS	M1 mAChR, ↑ excitability; M2 mAChR, ↓ excitability; α$_7$ and α$_4$β$_2$ nAChR, ↑ excitability; ASIC1A, ↑ excitability; AMPA lacking GluR2 and NMDA, ↑ excitability; GluK1, ↑ presynaptic GABA release (dose dependent)

*Note that no study has differentiated receptor localization to VIP⁺ or VIP⁻. Therefore, we have placed the receptor modulating VIP⁺ or VIP⁻ in each category. For citations see text.

may be due, in part, to a switch from a greater expression of sodium-potassium-chloride cotransporter 1, which accumulates intracellular chloride and renders GABA$_A$ receptors excitatory, or to an increase in the potassium-chloride cotransporter 2, which extrudes chloride from the cell, rendering GABA$_A$ receptors inhibitory (Ben-Ari et al., 2012; Ehrlich et al., 2013). In addition, a decrease in rise-time and decay-time constant occurs because of a change in the GABA$_A$ receptor subunit composition (from primarily the α2 subunit to the α1 subunit; Ehrlich et al., 2013). This shift results in a GABAergic shunt that limits the extent of BLA activation (see below; Rainnie et al., 1991b).

The composition of GABA$_A$ receptors has been found to be quite diverse because their subunit assembly makes their roles significantly different, depending on the timing of activation and subcellular localization (Pouille and Scanziani, 2001; Marowsky et al., 2004). The BLA of mature animals contains α1 and α2 subunits of the GABA$_A$ receptor; α1 subunit-containing GABA$_A$ receptors are expressed primarily at the somal level of PV GABAergic interneurons but also exhibit coimmunoreactivity with the β2/3 subunits (McDonald and Mascagni, 2004). Alternatively, GABA$_A$ receptors on principal neu-

rons contain primarily the α2 subunit, which is predominantly responsible for the benzodiazepine allosteric potentiation of inhibitory currents (Marowsky et al., 2004). In addition, principal neurons in the BLA contain γ2 subunits, which likely contribute to the formation of α2βxγ2 pentameric GABA$_A$ receptors, which contribute to fast inhibitory synaptic transmission (Esmaeili et al., 2009). Extrasynaptically, the GABA$_A$ receptor in the BLA is made up primarily of the α3 subunit, which strongly mediates tonic GABAergic currents (Marowsky et al., 2012). However, the α5 subunit, which is diazepam sensitive and shapes the decay phase of the inhibitory postsynaptic currents (Marowsky et al., 2004), and the δ subunit, both of which are hallmark subunits that contribute to tonic inhibition (Farrant and Nusser, 2005), are also expressed in the BLA, though not as strongly as the α3 subunit (Marowsky et al., 2012).

Temporal Dynamics and Intra-Amygdala Regulation of Excitatory Activity

GABAergic interneurons can be differentiated by their firing properties. PV interneurons fire primarily short duration, nonadapting action potentials (Rainnie et al., 2006; Woodruff and Sah, 2007b), whereas CB-

expressing GABAergic interneurons fire broad action potentials, display firing adaptation, and synapse primarily with somata (Jasnow et al., 2009; see Table I). Other interneurons expressing somatostatin (SOM), VIP, CR, and CCK also target dendrites or somata (Mascagni and McDonald, 2003; Muller et al., 2007a). Although GABAergic interneurons constitute only a fraction of the total neuronal population, they tightly regulate network excitability and lead to a low resting firing rate of principal neurons (Pare and Gaudreau, 1996; Lang and Pare, 1997; Woodruff and Sah, 2007a).

The regulation of excitatory activity by local GABAergic interneurons is influenced by the firing properties (Rainnie et al., 1991b; Lang and Pare, 1997). Most BLA GABAergic interneurons fire short-duration action potentials with small spike frequency adaptation in response to prolonged depolarization, although specific subpopulations of GABAergic interneurons have different firing patterns (see Table I). Principal neurons, by comparison, show spike frequency adaptation and prolonged afterhyperpolarization in response to prolonged depolarizing currents (Rainnie et al., 1991a,b; Pare et al., 1995; Sah et al., 2003). The axonal morphology of BLA GABAergic interneurons also allows for tight inhibitory control over principal neurons. GABAergic interneuron axons branch, on average, two to six times, forming relatively dense terminal and collateral fields with principal neurons (Millhouse and DeOlmos, 1983; Smith et al., 1998). BLA GABAergic projections participate in either feedback inhibition or transient disinhibition of principal neurons. Indeed, PV interneurons receiving strong excitatory local inputs from BLA projection neurons appear to be involved in feedback inhibition (Smith et al., 2000; Unal et al., 2014), whereas intercalated interneurons, which have recently been found to project to the BLA (Manko et al., 2011), appear to target PV- and CB-immunoreactive GABAergic interneurons and are likely to disinhibit principal cells transiently (Bienvenu et al., 2015).

The regulation of the firing rate by GABAergic interneurons controls the flow of information from the BLA, and evidence indicates that local inhibitory circuits in the amygdala mediate its functioning. Activation of the GABAergic system appears to play a central role in the synchronization of spiking activity. This synchronization can coordinate and enhance the effects of input signals, which precisely allows the activation of glutamatergic activity to drive behavioral responses (Courtin et al., 2014; Herry and Johansen, 2014). For example, the initiation and expression of fear requires synchronization of amygdala activity, among other regions (Stujenske et al., 2014). Ongoing research has revealed that the θ and the faster γ oscillations may be fundamental to circuits underlying sensory processing and cognitive functions and that changes in emotional states may be mediated by alterations in BLA γ coupling to θ frequency inputs (Fries, 2009; Stujenske et al., 2014). The activity of PV interneurons has been implicated in θ synchrony within the medial prefrontal cortex (PFC; Courtin et al., 2014) because suppression of PV interneuronal activity in the

PFC is necessary to disinhibit prefrontal projection neurons to the BLA, thereby synchronizing their firing by resetting local θ oscillations. Although this work has not yet been confirmed in the amygdala it has been hypothesized that inhibiting PV interneurons in the BLA might also synchronize activity and enhance fear responses (Stujenske et al., 2014).

MODULATION OF GABA$_A$ RECEPTOR-MEDIATED INHIBITORY SYNAPTIC TRANSMISSION IN THE BLA

GABAergic inhibition in the BLA is modulated by afferents from both cortical and subcortical brain regions (Fig. 1A). In most cases, afferents from these regions project to both principal neurons and GABAergic interneurons. In some cases, it has been determined that projections are directed to particular subpopulations of neurons. This section reviews the afferent projections that modulate and facilitate GABAergic inhibitory synaptic transmission in the BLA. More specifically, we discuss how activation of different receptor types modulates the release of GABA from the presynaptic terminal or alters the excitation of GABAergic interneurons (Fig. 1B).

Cortical and Thalamic Regulation of BLA GABAergic Interneurons

The BLA receives extensive cortical and thalamic projections, which synapse onto both principal neurons and GABAergic interneurons. Stimulating afferents from either the cortical or the thalamic pathways have been found to monosynaptically activate BLA and lateral amygdala (LA) GABAergic interneurons, primarily in a feedforward manner (Rainnie et al., 1991b; Washburn and Moises, 1992; Lang and Pare, 1998; Szinyei et al., 2000). Recent studies have identified to which type of interneuron cortical and thalamic inputs project. Unal and colleagues (2014) found that BLA interneurons expressing CB receive about half of the cortical inputs to local-circuit cells of the BLA and constitute a major source of feedforward inhibition, whereas thalamic inputs form less than 1% of synapses on interneurons (Carlsen and Heimer, 1988; LeDoux et al., 1991). There appears to be a possible discrepancy in the regulation of GABAergic interneurons in the LA vs. the BLA. Cortical inputs to the BLA regulate primarily CB-expressing interneurons, whereas GABAergic interneurons in the LA respond equally to both cortical and thalamic pathways (Szinyei et al., 2000; Unal et al., 2014).

Dopaminergic Afferents

The BLA receives dense dopaminergic innervation from the ventral tegmental area (VTA) and the substantia nigra (SN; Fallon and Ciofi, 1992; Asan, 1997). VTA and SN projections synapse on BLA principal (projection) neurons and PV- and CR-immunopositive GABAergic interneurons (Brinley-Reed and McDonald, 1999; Pinard et al., 2008). However, compared with CR-immunopositive interneurons, PV interneurons appear to be the preferential
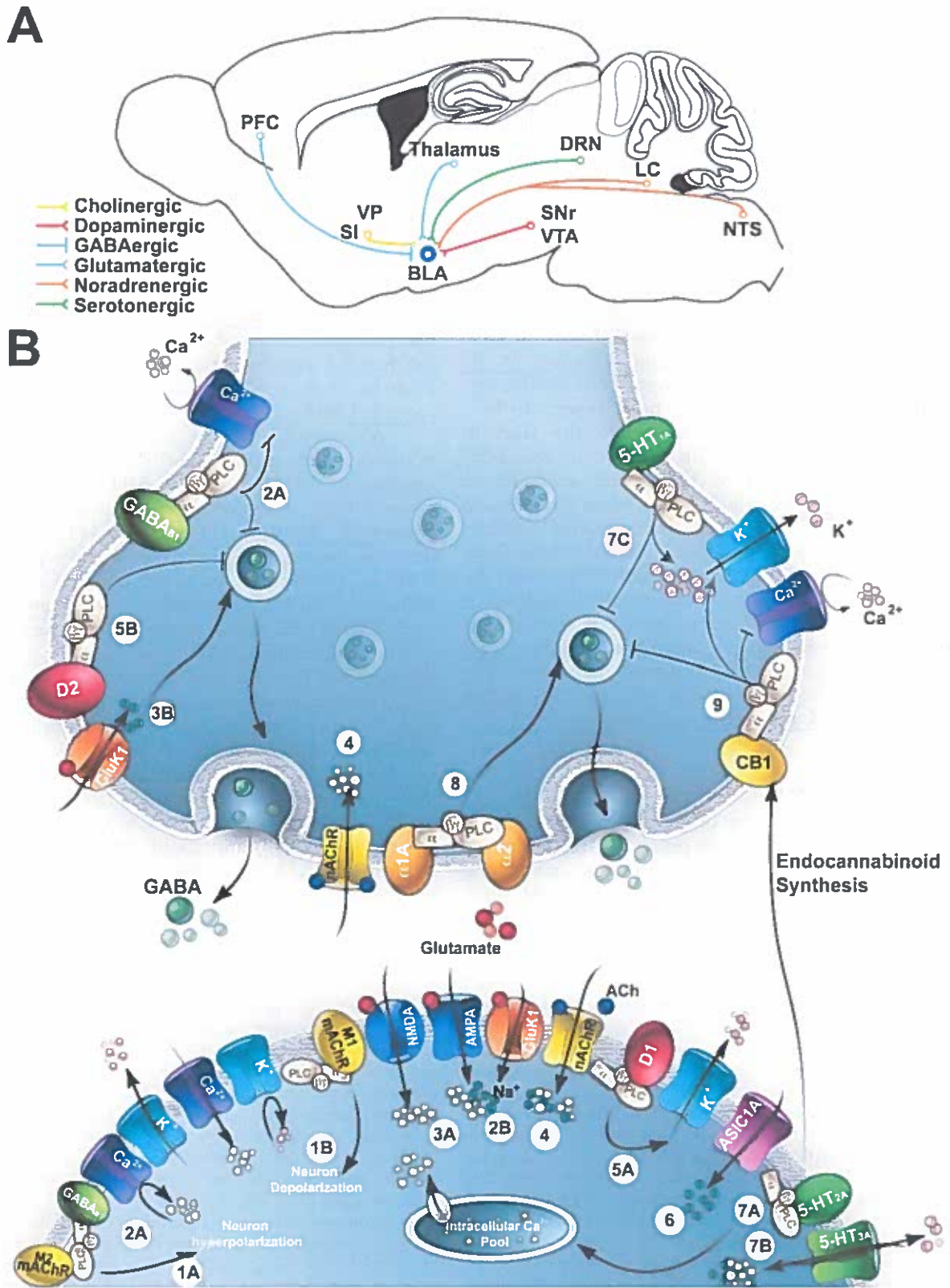
Fig. 1.

target of dopaminergic synapses in the BLA (Pinard et al., 2008). By projecting to principal neurons and GABAergic interneurons, dopamine (DA) influences the activity of both excitatory and inhibitory cell types within the BLA (Rosenkranz and Grace, 1999; Kroner et al., 2005). Via activation of D1 receptors, DA increases excitability and evoked firing of principal neurons by reducing slowly inactivating K^+ currents, whereas activation of D2 receptors increases input resistance. Moreover, D1 receptor activation increases evoked firing in fast-spiking BLA interneurons and the frequency of spontaneous IPSCs (sIPSCs; Kroner et al., 2005). Activation of DA receptors has also been found to induce rhythmic inhibitory oscillations (Loretan et al., 2004; Ohshiro et al., 2011), although increases in excitatory transmission are required to precede GABAergic interneuronal burst firing (Ohshiro et al., 2011). Although DA fibers synapse onto both GABAergic interneurons and principal neurons, there appears to be a net increase in excitatory activity within the BLA in response to DA application. This increase in excitatory activity may be the result of 1) reduced activation of GABAergic interneurons, which occurs when activation of D2 receptors on GABAergic interneurons causes a reduction in the probability of presynaptic quantal release (Seamans et al., 2001); 2) amygdala disinhibition and the subsequent increase in excitatory activity, which may occur when DA suppresses GABA release from PV interneurons onto principal neurons but not interneurons (Chu et al., 2012); or 3) DA increasing the excitatory drive onto disinhibitory interneurons, which would subsequently increase excitatory activity (Kemppainen and Pitkanen, 2000; Bissiere et al., 2003).

Serotonergic Afferents

Serotonergic projections originating from the dorsal raphé nucleus (DRN) innervate primarily BLA principal neurons, PV interneurons, and interneurons containing neuropeptide Y (NPY), a subgroup of CB and SOM interneurons (Ma et al., 1991; Muller et al., 2007b). Postsynaptically, serotonin (5-HT) neurotransmission leads to the activation of 5-HT receptors, which are grouped into seven families (5-HT$_{1-7}$). 5-HT$_{1A}$ receptors, which are $G_{i/o}$ protein coupled, have been localized to principal neurons (Stein et al., 2000) and the presynaptic terminal of GABAergic interneurons within the BLA (Kishimoto et al., 2000). 5-HT$_{1A}$ receptor activation inhibits the discharge rate of principal neurons by inducing hyperpolarization and reduces GABA release from presynaptic terminals by activating potassium channels (Kishimoto et al., 2000; Stein et al., 2000). Alternatively, 5-HT$_2$ receptors, and more specifically the 5-HT$_{2A}$ and the 5-HT$_{2C}$ receptors, are $G_{q/11}$-coupled membrane receptors that increase intracellular Ca^{2+} levels and increase interneuronal excitability (Jiang et al., 2009; Bonn et al., 2012). The 5-HT$_{3A}$ receptor is a ligand-gated sodium, potassium, and calcium channel that also increases interneuronal excitability but leads to a rapidly desensitizing depolarization (Rainnie, 1999; Mascagni and McDonald, 2007; Gharedaghi et al., 2014).

Although all 5-HT receptors have been documented in the amygdala, most (~65–70%) GABA-immunoreactive neurons in the BLA exhibit 5-HT$_{2A}$ immunoreactivity; fewer 5-HT$_{2A}$ receptors are present on principal neurons (Bombardi, 2011). 5-HT$_{2A}$ and 5-HT$_{3A}$ receptors have been localized to specific interneuronal types in the BLA. 5-HT$_{2A}$ receptors are found primarily on PV interneurons within the BLA (McDonald and Mascagni, 2007; Bombardi, 2011) and tightly control glutamatergic output by perisomatic inhibition (Muller et al., 2005; Holmes, 2008), whereas 5-HT$_{3A}$ receptors are expressed primarily on the CCK interneuronal subpopulation (Mascagni and McDonald, 2007), which constitutes only a small proportion of GABAergic interneurons in the BLA (Mascagni and McDonald, 2003). In contrast, 5-HT$_{1A}$, which is expressed in low to moderate concentrations in the BLA (Asan et al., 2013), coexpresses with ~50% of NPY interneurons (Bonn et al., 2013) and about one-third of neurokinin-1 receptor (NK$_{1r}$) interneurons (Hafizi et al., 2012), whereas ~30–40% of NPY interneurons express the excitatory 5-HT$_{2C}$ receptor subtype (Bonn et al., 2012, 2013). 5-HT$_{1A}$ and 5-HT$_3$ receptors have been localized on

Fig. 1. Modulation of GABAergic inhibitory synaptic transmission in the BLA. A: Schematic representation of GABAergic projections from the PFC and glutamatergic projections from the thalamus. In addition, GABAergic interneurons in the BLA receive cholinergic projections from the SI and the VP, dopaminergic projections from the VTA and the SN, noradrenergic projections from the LC and the NTS, and serotonergic projections from the DRN. B: Schematic representation of receptors modulating GABAergic inhibitory synaptic transmission in the BLA. Postsynaptic M2 mAChRs (1A) and GABA$_B$ receptors (2A) hyperpolarize GABAergic interneurons by reducing voltage-gated Ca^{2+} channels and increasing K^+ channel conductance. M1 mAChRs (1B) increase excitability (though primarily expressed on principal neurons) by suppressing several K^+ currents and increasing voltage-gated Ca^{2+} conductance. Activation of presynaptic GABA$_B$ receptors (2B) inhibits neurotransmitter release on both excitatory and inhibitory synapses by inhibiting voltage-gated Ca^{2+} channels and, possibly, by interacting with vesicular release machinery. Activation of postsynaptic NMDARs or AMPARs (3A) on interneurons increases excitability. Activation of GluK$_1$-containing kainate receptors (3B) depolarizes interneurons by increasing the presynaptic release of GABA or increasing excitability via activation of postsynaptic GluK$_1$ receptors. Activation of α_7 nAChRs and/or $\alpha_4\beta_2$ nAChRs (4) presynaptically modulates GABA release or regulates neuronal activity by the position on interneurons. Dopaminergic projections (5A) activate postsynaptic D1 receptors, which increases excitability by reducing slowly inactivating K^+ currents, whereas D2 receptors (5B) reduce presynaptic release of GABA. Activation of ASIC1A receptors (6) increases interneuronal excitability. Postsynaptically, activation of 5-HT$_2$ (7A) and 5-HT$_{3A}$ (7B) receptors increases interneuronal excitability via an increase in intracellular Ca^{2+} concentrations or increasing the interneuronal excitability, respectively. Activation of presynaptic 5-HT$_{1A}$ receptors (7C) reduces quantal release and increases hyperpolarization. Activation of $\alpha1$ and $\alpha2$ receptors (8) depolarize interneurons, subsequently increasing action potential firing and enhancing inhibitory synaptic transmission. Activation of CB1 receptors (9) on CCK interneurons reduces presynaptic release by inhibiting voltage-gated Ca^{2+} channels and activating voltage-gated K^+ channels.

GABAergic nerve terminals in the BLA (Koyama et al., 1999, 2000), where activation of these receptors inhibits or facilitates miniature IPSC (mIPSC) frequency without effects on mIPSC amplitude, respectively (Koyama et al., 2002).

Noradrenergic Afferents

The BLA receives extensive noradrenergic (NA; norepinephrine) innervation from the locus coeruleus (LC) and nucleus of the solitary tract (NTS; Pitkanen, 2000; Williams et al., 2000), which synapse onto GABAergic interneurons (Li et al., 2002). NA released from LC terminals activates three distinct classes of adrenoreceptors (AR; $\alpha 1$, $\alpha 2$, and ß AR) that have multiple subtypes and appear to be more potent modulators of GABAergic inhibitory synaptic transmission than DA (Miyajima et al., 2010). Although no study has yet anatomically identified the type of interneuron to which the receptor subunits are localized in the BLA, electrophysiological evidence indicates that $\alpha 1$ and $\alpha 2$ AR activation depolarizes SOM- and CCK-positive interneurons, resulting in action potential firing and enhanced inhibitory synaptic transmission (Braga et al., 2004b; Buffalari and Grace, 2007; Kaneko et al., 2008). In addition to direct enhancement of inhibitory activity by LC afferents, NA enhancement of inhibitory activity in the BLA occurs indirectly. Indeed, activation of ß1 and ß3 ARs in lateral paracapsular (LPCS) interneurons, which are a distinct class of GABAergic interneurons bordering the BLA and external capsule and are thought to provide cortical feedforward inhibition to the BLA (Marowsky et al., 2005), enhances LPCS GABAergic inhibition of the BLA (Silberman et al., 2010, 2012).

Cholinergic Afferents

The BLA is extensively innervated by fibers from the substantia innominata (SI; nucleus basalis magnocellularis) and ventral pallidum (VP) of the basal forebrain (Emson et al., 1979; Woolf et al., 1984; Carlsen et al., 1985; Zaborszky et al., 1986). The extensive innervation leads to some of the highest levels of choline acetyltransferase, the synthesizing enzyme for acetylcholine (ACh), and acetylcholinesterase (AChE), the hydrolyzing enzyme for ACh, in the BLA compared with other brain regions (Ben-Ari et al., 1977; Prager et al., 2013). The basal forebrain projects both cholinergic and noncholinergic neurons to the BLA. Recent evidence indicates that ~10-15% of basal forebrain neurons projecting to the BLA are PV-immunopositive GABAergic interneurons (Mascagni and McDonald, 2009), which target primarily PV interneurons in the BLA but also target principal neurons (McDonald et al., 2011). In comparison, ~75-80% of the basal forebrain projection neurons are cholinergic (Carlsen et al., 1985; Zaborszky et al., 1986; Mascagni and McDonald, 2009), project primarily to dendritic shafts and spines of BLA principal neurons, and innervate PV GABAergic interneurons to a small extent (~7% of postsynaptic targets; Muller et al., 2011).

Stimulation of afferents from basal forebrain cholinergic neurons leads to the release of ACh, which extensively regulates neuronal excitability by acting on muscarinic (mAChR) and nicotinic (nAChR) acetylcholine receptors, both of which are abundant in the BLA (Mash and Potter, 1986; Swanson et al., 1987; Hill et al., 1993; Zhu et al., 2005; Pidoplichko et al., 2013). mAChRs are G-protein-coupled receptors that have five subtypes, designated M1–5. M1, M3, and M5 receptors couple preferentially to $G_{q/11}$ proteins, which subsequently initiate signaling cascades that mobilize intracellular Ca^{2+}, whereas M2 and M4 receptors couple to $G_{i/o}$ proteins, which subsequently hyperpolarize neurons or inhibit neurotransmitter release (for review see Alger et al., 2014). Although the BLA expresses M1–M4 mAChRs (Mash and Potter, 1986; Cortes et al., 1987; McDonald and Mascagni, 2010), M1 mAChR appears to be the predominant mAChR subtype in the amygdala. M1 mAChRs are localized primarily to principal neurons and appear to increase excitability of BLA principal cells resulting from the suppression of several potassium currents, whereas M1 immunoreactivity has been observed at low levels on GABAergic interneurons (McDonald and Mascagni, 2010; Muller et al., 2013). In contrast, M2 mAChRs are expressed on interneurons within the BLA and lead predominantly to hyperpolarization of GABAergic interneurons (McDonald and Mascagni, 2011).

In the brain, nAChRs are ligand-gated ion channels permeable to cations, including Ca^{2+}, that produce membrane depolarization and postsynaptic excitation or stimulation of neurotransmitter release (Dani and Bertrand, 2007). nAChRs comprise nine different subunits (α_{2-7} and β_{2-4}) that combine as either homomeric or heteromeric pentameric receptors (Dani and Bertrand, 2007; Yakel, 2013). The homomeric α_7 and the heteromeric $\alpha_4\beta_2$ are the two major subtypes of nAChRs found in the mammalian brain (Gotti et al., 2009); they have previously been found to be expressed in the BLA (Hill et al., 1993; Seguela et al., 1993) and appear to regulate neuronal excitability by presynaptically modulating neurotransmitter release or directly regulating neuronal activity by their position on somatodendritic sites of interneurons or principal neurons (Klein and Yakel, 2006; Pidoplichko et al., 2013). In addition, $\alpha_3\beta_4$ nAChRs are also present on GABAergic interneurons and enhance GABAergic inhibitory synaptic transmission (Zhu et al., 2005).

The functional activity and subsequent modulation of either inhibition or excitation by mAChRs and nAChRs in the BLA appear to diverge from anatomical evidence. Indeed, although α_7 nAChRs are present on GABAergic interneurons and principal neurons and enhance both excitatory and inhibitory synaptic transmission, their activation powerfully modulates GABAergic inhibition, resulting in a net reduction in BLA excitability (Pidoplichko et al., 2013). Moreover, optogenetic activation of basal forebrain inputs to the BLA during periods of neuronal quiescence does not trigger excitatory responses; rather, muscarinic activation increases the inhibitory response, which may be a result of the contrasting

spatiotemporal profile of cholinergic receptor activation (Unal et al., 2015). Light-induced activation of basal forebrain inputs transiently silences cells, which is followed by a longer-duration inhibitory postsynaptic potential (IPSP; Unal et al., 2015). This suggests that the early IPSP is due to activation of nicotinic receptors and, from the results of Pidoplichko and colleagues (2013), presumptively α_7 nAChR activation, although the subunit configuration was not tested by Unal and colleagues (2015). In contrast, the late IPSP was mediated by M1 and not by M2 mAChR activation. It is important to emphasize that this inhibitory effect occurred only in quiescent principal neurons. During periods of strong activation, mAChR inhibition appeared to be overwhelmed, and M1-mediated excitation predominated (Unal et al., 2015).

Glutamate Receptors

GABAergic interneuronal excitability in the BLA is regulated, in part, by principal neurons within the BLA. Glutamatergic inputs make dual-component synapses with both fast α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and slower N-methyl-D-aspartate receptors (NMDARs), which are present on the postsynaptic membrane of interneurons and principal neurons (McDonald, 1992; Mahanty and Sah, 1998; Smith et al., 1998; Weisskopf and LeDoux, 1999; Sah et al., 2003). Glutamatergic inputs to interneurons express AMPARs that have rapid kinetics and strong inward rectification, indicating calcium permeability and a lack of the GluA2 subunit (Mahanty and Sah, 1998; Polepalli et al., 2010). Interneurons also express a heterogeneous population of NMDARs. These cells can be separated into groups that lack NR2B NMDAR subunits (Mahanty and Sah, 1998; Polepalli et al., 2010), express NMDARs that contain mostly NR2B subunits, and have fast decay kinetics (Williams, 1993; Polepalli et al., 2010) or express NMDARs that have slow kinetics and contain mostly GluN1/GluN2B heterodimers (Szinyei et al., 2003; Polepalli et al., 2010; Spampanato et al., 2011).

The heterogeneity of the subunits of AMPARs and NMDARs within specific populations of interneurons is essential for the regulation of feedforward inhibition to principal cells. Polepalli and colleagues (2010) demonstrated, for example, that long-term potentiation to interneurons is restricted to interneurons that contain GluR2-lacking AMPAR at the postsynaptic membrane. Only these neurons provided feedforward inhibition to principal cells, and, although this has not been specifically tested, it is likely that the CB-immunopositive interneurons are the subpopulation of interneurons that provides the feedforward inhibition to principal cells (Unal et al., 2014).

In addition to AMPARs and NMDARs, kainate receptors represent a distinct class of ionotropic glutamate receptors that are preferentially activated by kainic acid. Kainate receptors consist of five different subunits, $GluK_{1-3}$ and KA_{1-2} (Chittajallu et al., 1999). $GluK_{1-3}$ subunits form functional homomeric and heteromeric channels, whereas the KA subunits generate only func-

tional receptors with distinct physiological properties when combined with the $GluK_{1-3}$ subunits (Herb et al., 1992; Schiffer et al., 1997). Although kainate receptors are not widely distributed in the brain, the BLA has a markedly high expression of $GluK_1R$ subunit (Braga et al., 2003). Kainate receptors, and in particular the $GluK_1$ subunit-containing kainate receptor, have been found to enhance excitatory synaptic transmission in the BLA (Li and Rogawski, 1998) by modulating pre- and postsynaptic release of glutamate (Jiang et al., 2001; Braga et al., 2004a). Moreover, presynaptic $GluK_1$-containing kainate receptors have also been found to modulate GABA release in the BLA in a bidirectional manner. At low concentrations, activation of high-affinity $GluK_1$-containing kainate receptors depolarizes both principal neurons and GABAergic interneurons, which leads to a substantial increase in GABA release. However, high concentrations of agonists activate low-affinity presynaptic $GluK_1$-containing kainate receptors, which again depolarize both GABAergic interneurons and principal neurons but suppress evoked GABA release, leading to an enhancement in BLA network excitability (Braga et al., 2003; Aroniadou-Anderjaska et al., 2007, 2012).

GABA$_B$ Receptors

The late or slow component of inhibitory synaptic transmission is mediated by activation of GABA$_B$ receptors, which comprise the GABA$_{B1}$ and GABA$_{B2}$ subunits (Craig and McBain, 2014). Both GABA$_{B1}$ and GABA$_{B2}$ mRNA are expressed in the BLA (Bischoff et al., 1999; Durkin et al., 1999; Clark et al., 2000). Postsynaptically, the $G_{i/o}$-protein-coupled GABA$_B$ receptors (primarily the GABA$_{B1B}$ isoform) mediate hyperpolarization of postsynaptic membranes by inhibiting voltage-gated Ca^{2+} activation and activating inwardly rectifying potassium channels (Rainnie et al., 1991b; Sugita et al., 1993; Couve et al., 2000). Presynaptic GABA$_B$ receptors, primarily the GABA$_{B1A}$ isoform, on the other hand, have been found to inhibit neurotransmitter release on both excitatory and inhibitory synapses by inhibiting voltage-gated Ca^{2+} channels and, possibly, by interacting with vesicular release machinery (Yamada et al., 1999; Szinyei et al., 2000; Gassmann and Bettler, 2012).

Anatomically, GABA$_{B1}$ receptors are found in many amygdala nuclei. However, in the BLA, GABA$_B$ receptor immunoreactivity is found primarily on GABAergic interneurons; very few principal neuronal somata in the BLA exhibit immunoreactivity for GABA$_{B1}$ receptors (McDonald et al., 2004). However, the light staining found in the neuropil likely is due to the staining of dendritic shafts and spines belonging to pyramidal cells (McDonald et al., 2004). Among the subpopulations of GABAergic interneurons, GABA$_{B1}$ receptor immunoreactivity is found primarily on large CCK$^+$ neurons but is also found to a lesser extent on small CCK$^+$ interneurons. In addition, GABA$_{B1}$ receptor immunoreactivity is found on the remaining subpopulations of GABAergic interneurons in the BLA (e.g., SOM, PV, and VIP neurons;

TABLE II. Summary of Alterations in the BLA GABAergic System in Disease*

	Anxiety/trauma	Autism/fragile X	Alzheimer's disease	Epilepsy	TBI
Behavioral symptoms	Anxiety, hypervigilance, stress, fear	Impaired social interaction, language/communication deficits, repetitive/restricted interest, aggression, anxiety, epilepsy	Memory impairment, impaired fear conditioning, anxiety, epilepsy	Seizures, status epilepticus	Cognitive and emotional deficits, posttraumatic epilepsy (PTE), anxiety, increased fear conditioning, PTSD
GABA interneurons	↓ SOM/NK$_{1r}$	↓ Synaptic number but no interneuronal loss	↓ Total number of interneurons	↓ Total number of interneurons; ↓ density of SOM interneurons	↓ Total number of interneurons
GAD/GABA	↑ Tonic/phasic IPSC; ↓ GABA release	↓ GABA metabolism; ↓ GAD65/67; ↓ tonic/phasic IPSC	No change in IPSC of 1- or 7-month-old apoE4 mice (in LA); ↑ IPSC in 18–20-month-old apoE4 mice (in LA) but <↑ in EPSC	↓ sIPSC frequency and amplitude; ↓ mIPSC amplitude	↓ sIPSC frequency and amplitude; ↓ mIPSC frequency and amplitude
GABA$_A$ receptors	↑ α2 in highly anxious rats	Delayed maturation of α1 and α2; ↓ α5, δ		↑ α1	↓ α1, β2, γ2

*For citations see text.

McDonald et al., 2004). More recently, GABA$_{B1}$ immunoreactivity has been examined at the synapse in the LA. Evidence indicates that GABA$_{B1}$ receptors are localized to the extrasynaptic terminal of both interneurons and principal neurons. Although most receptors are found in the sensory afferent terminals of principal neurons (Pan et al., 2009), where they act to reduce glutamate release, GABA$_B$ receptors are also present on inhibitory inputs to principal neurons, where they act as autoreceptors (Szinyei et al., 2000).

Alterations in the modulation of inhibitory and excitatory synaptic transmission during development might be due, in part, to the activation of GABA$_B$ receptors, which are functionally expressed early in development (Bosch and Ehrlich, 2015). Indeed, the GABA$_B$ receptor, which mediates paired-pulse depression (PPD) of sensory evoked IPSCs (Szinyei et al., 2000), is differentially regulated during development; intra-BLA inhibitory synapses show pronounced PPD in the first 2 weeks of development, but this is reduced by the third week (Ehrlich et al., 2013). In other words, the contribution of PPD by GABA$_B$ receptors may be due primarily to activation of GABA$_B$ receptors in infancy, whereas PPD in older animals is only partially controlled by GABA$_B$ receptors. Moreover, Bosch and Ehrlich (2015) found that presynaptic GABA$_B$ receptors, which are present on sensory inputs to LA principal neurons, are activated as early as P8. Finally, tonic presynaptic control of IPSCs and excitatory postsynaptic currents (EPSCs) in the LA appears to be mediated by GABA$_B$ receptors and is likely permitted by ambient GABA that also emerge in adolescence (Bosch and Ehrlich, 2015).

Acid-Sensing Ion Channels

Acid-sensing ion channels (ASICs), in particular the ASIC1a splice variant, are highly expressed in the BLA (Waldmann et al., 1997; Biagini et al., 2001). ASICs are heterotrimeric or homotrimeric proton-gated channels activated by extracellular acidosis, intracellular pH, and other factors (Wemmie et al., 2013). Until recently, the precise role of ASIC1a activation in the BLA remained unknown. ASIC1a was thought to promote hyperexcitability because it was found to reduce fear and to have antidepressant and anxiolytic effects (Coryell et al., 2007, 2009; Dwyer et al., 2009; Ziemann et al., 2009). Indeed, electrophysiological evidence indicates that ASIC1a channels are present on principal neurons within the BLA and are activated by ammonium or by lowering extracellular pH, which increases glutamatergic activity (Pidoplichko et al., 2014). However, ASIC1a is also found on GABAergic interneurons within the BLA, and its activation increases GABAergic activity. The increase in GABA$_A$ receptor-mediated inhibitory synaptic transmission seems to predominate, suppressing overall excitability (Pidoplichko et al., 2014), which may be a result of the intrinsic organization of the BLA. Much of the excitatory input within the BLA is directed onto interneurons,

which subsequently project back onto principal cells (Lang and Pare, 1997; Smith et al., 1998).

Cannabinoid Receptors

Cannabinoids exert their effects by the activation of two known cannabinoid receptor subtypes, the cannabinoid type 1 (CB1) receptor and the cannabinoid type 2 receptor (CB2). The CB1 receptor, which is expressed primarily presynaptically and is activated by retrograde transmission of endogenous cannabinoids, is coupled to $G_{i/o}$ proteins. Activation of CB1 receptors decreases excitability of the presynaptic terminal by closing calcium (n and P/Q type) channels, increasing G-protein-coupled inwardly rectifying potassium channels, and decreasing cyclic adenosine monophosphate-dependent sodium conductance (Pertwee, 1997; Schlicker and Kathmann, 2001). The CB2 receptor is also coupled to $G_{i/o}$ proteins, but its expression is restricted primarily to immunological tissues peripherally, and is implicated in immunological functions (Schatz et al., 1997).

CB1 receptors are widely distributed in the brain but are present in relatively high concentrations in the BLA and, in particular, are present on the presynaptic terminal of CCK interneurons (Katona et al., 2001; McDonald and Mascagni, 2001b), which densely innervate principal neurons (McDonald and Pearson, 1989). Activation of the presynaptic CB1 receptors on CCK GABAergic interneurons reduces the amplitude of sIPSCs but does not affect mIPSCs (Katona et al., 2001) because CB1 receptors reduce GABA release via blockade of presynaptic N-type Ca^{2+} channels (Wilson et al., 2001). Activation of CB1 receptors has also been found to reduce excitatory synaptic transmission in the LA and decrease basal synaptic transmission, indicating that, in the LA, CB1 modulation of neuronal activity is determined by CB1 receptors expressed on principal neurons (Azad et al., 2003). In addition to regulating GABA release, CB1 receptor activation appears to be essential for the expression of postsynaptic $GABA_A$ receptors. Expression of $\alpha1$ and $\alpha2$ subunits of the $GABA_A$ receptor is reduced in the amygdala of $CB1^{-/-}$ mice. This reduction in subunit expression may be the result of a developmental neuroadaptation that compensates for the overstimulation of postsynaptic receptors resulting from the lack of inhibitory presynaptic activity exerted by CB1 receptors (Diana and Bregestovski, 2005; Uriguen et al., 2011).

GABAERGIC CIRCUIT DYSFUNCTION WITHIN THE BLA

A functional BLA GABAergic system is essential throughout one's life. Deficiencies in GABAergic inhibitory synaptic transmission are associated with neurodevelopmental diseases, such as autism or fragile X syndrome, and also appear in neurodegenerative diseases, such as Alzheimer's disease. In addition, deficiencies in the GABAergic system can appear as a result of brain trauma, such as after TBI, or may be acquired after SE. In this section, we first provide an overview with respect to how a reduction in

GABAergic inhibitory synaptic transmission within the BLA is associated with the development of anxiety. We then provide an example of a neurodevelopmental and neurodegenerative disorder that results in deficiencies in the GABAergic system within the BLA (see Table II). In addition, we provide two examples of acquired GABAergic deficiencies. It must be noted that genetic variations may be an underlying factor in deficiencies of GABAergic inhibitory synaptic transmission. Unless genetic variations are directly involved in changing GABAergic function within the amygdala, we do not address these variations.

Anxiety and PTSD

Anxiety and stress-related disorders, such as PTSD, develop when individuals are exposed to situations eliciting extreme stress or fear (Heim and Nemeroff, 2001; van der Kolk, 2003). These disorders are commonly associated with amygdala hyperactivity (Terburg et al., 2012; Nuss, 2015) and are often treated by administering benzodiazepines, which mediate their actions via $GABA_A$ receptors (Smith, 2001). However, in many cases, the treatment of anxiety disorders with benzodiazepine-like compounds may be ineffective, potentially because of deficits in GABA release, GABAergic interneuronal loss in the BLA, or alterations in $GABA_A$ receptor functionality (Farb and Ratner, 2014).

The loss of GABAergic interneurons or reductions in glutamate decarboxylase (GAD), an enzyme that catalyzes the decarboxylation of glutamate into GABA, may lead to deficits in the presynaptic release of GABA and contribute to increased anxiety and associated BLA hyperexcitability. Indeed, excess reductions in GAD, such as occur when knocking out one of the GAD isoforms (GAD65), lead to reduced phasic and tonic inhibition and subsequently result in BLA hyperexcitability, increased anxiety, and pathological fear memory reminiscent of PTSD (Walls et al., 2010; Lange et al., 2014; Muller et al., 2015).

Selectively targeting GABAergic interneurons in the BLA has recently been investigated for the development of anxiety-like behavior as well as fear learning. Lesions to GABAergic interneurons that contain NK_{1r}, which colocalize with interneurons containing NPY, SOM, and CB, have been found to increase anxiety-like behaviors in rats (Truitt et al., 2007, 2009). It is notable that NK_{1r}-containing interneurons account for only ~3% of the total population of GABAergic interneurons in the BLA. The loss of NK_{1r}-containing interneurons does not result in a significant reduction in the total number of interneurons (Truitt et al., 2009). However, selective ablation of these interneurons and in particular the SOM GABAergic interneurons, which include approximately half of the NK_{1r}-immunoreactive interneurons, likely disinhibits the synchronizing activity of projection neurons and may impair feedforward inhibition (Truitt et al., 2009). By comparison, increasing the number of GABAergic interneurons in the BLA will reduce anxiety, and animals that had increased inhibitory neurons were

less sensitive to unlearned fear, although they could still acquire conditioned fear responses (Cunningham et al., 2009). Although anxiety appears to be regulated in part by NK$_{1r}$-containing interneurons, PV and SOM GABAergic interneurons bidirectionally regulate the acquisition of a fear memory through two distinct mechanisms. During an auditory cue, PV interneurons are excited through direct sensory input from the auditory thalamus and cortex and indirectly disinhibit principal neurons via inhibition of SOM neurons. However, during an aversive footshock, both PV and SOM interneurons are inhibited, most likely via the activation of other interneuron subtypes that contact both PV and SOM interneurons, suggesting that the interneurons exhibit distinct temporal dynamics that correlate with specific behavioral differences (Wolff et al., 2014).

Impaired GABA release, disinhibition of GABAergic interneurons, or deficiences in the activation of postsynaptic GABAA receptors may result in anxiety-like behavior and increased fear responses. Pharmacological alterations of GABA$_A$ receptor activity by microinjection of GABA$_A$ receptor agonists or antagonists induce anxiolytic or anxiogenic-like effects, respectively (Da Cunha et al., 1992; Sanders and Shekhar, 1995; Zangrossi et al., 1999; Barbalho et al., 2009). Moreover, highly anxious rats exhibit an increase in the expression of the α2 subunit of the GABA$_A$ receptor in the BLA (Lehner et al., 2010; Skorzewska et al., 2014). However, it remains unknown whether alterations in the expression of other GABA$_A$ subunits also contribute to increased anxiety. In all, these results indicate that deficiencies in GABAergic inhibitory synaptic transmission within the BLA contribute to BLA hyperexcitability and the subsequent development of anxiety- and trauma-related disorders.

Autism Spectrum Disorders and Fragile X Syndrome

Autism spectrum disorders (ASDs), which include fragile X syndrome, are a group of neurodevelopmental syndromes that are often associated with aggression, anxiety, and epilepsy (Parikh et al., 2008; Tuchman and Cuccaro, 2011). Emerging evidence indicates a glutamatergic/GABAergic imbalance in multiple brain regions, including the amygdala, with greater levels of glutamatergic and reduced GABAergic activity, which results in the manifestation of symptoms associated with ASD (Coghlan et al., 2012; El-Ansary and Al-Ayadhi, 2014). The amygdala has recently been implicated in ASD, including fragile X (Suvrathan and Chattarji, 2011), because increased activation of the left amygdala has been reported in functional magnetic resonance imaging studies of fragile X patients (Watson et al., 2008).

Environmental models of autism or fragile X knockout (KO) mice revealed deficiencies in the GABAergic system within the BLA. The reduced GABAergic inhibition appears to be a result of deficits in synaptic transmission and GABA metabolism and not the result of a loss of GABAergic interneurons (Kim et al., 2014). Indeed, in a fragile X mental retardation 1 (FMR1) gene KO model of fragile X syndrome, the total number of neurons, including GABA-immunopositive interneurons in the BLA, was unaffected. Similarly, human studies of autistic children have shown little morphological alterations in the BLA compared with developmentally typical children (for review see Blatt, 2012). However, there appears to be a significant decrease in the total number of BLA inhibitory synaptic connections, indicating aberrant circuit development (Olmos-Serrano et al., 2010). Moreover, in the BLA of FMR1 KO mice, reductions in GAD1 mRNA and protein expression for GAD65/67 were observed and were associated with reduced presynaptic GABA release (Olmos-Serrano et al., 2010).

Although the overall number of amygdala GABAergic interneurons remains stable, mechanisms that modulate the activation of GABAergic interneurons may be impaired in ASD and fragile X models. For example, FMR1 KO mice have reduced activation of SOM-expressing low-threshold spiking interneurons in layers II and III of the somatosensory cortex, causing reduced spike synchronization of BLA principal neurons (Paluszkiewicz et al., 2011b); reductions in spike synchronization from the cortex could subsequently impair neuronal activity in the BLA required in the expression of fear (Courtin et al., 2014) and also lead to hyperresponsivity within the amygdala (Rauch et al., 2006). In addition, in a rat model of ASD, reductions in dendritic morphology, including spine density, or distal connectivity between the PFC and the BLA may lead to impaired cortical BLA regulation (Bringas et al., 2013).

Deficits in GABAergic inhibitory synaptic transmission in ASD and fragile X appear to be also a result of genetic variations in genes coding for particular subunits of the GABA$_A$ receptor, as has been documented throughout multiple brain regions in ASD (Fatemi et al., 2010; Coghlan et al., 2012) and in fragile X (Deidda et al., 2014). In the amygdala of ASD and fragile X models, delays in the maturation of postsynaptic GABA$_A$ receptors (Vislay et al., 2013) may lead to reductions in phasic and tonic GABAergic inhibitory synaptic transmission (D'Hulst et al., 2006; Olmos-Serrano et al., 2010). In the fragile X model, the timing of the developmental expression of the α1 and the α2 GABA$_A$ receptor subunits was delayed, which in turn may have impaired the switch in GABA polarity (He et al., 2014) and proper neuronal connections in wiring of local neuron networks in the BLA (Cossart, 2011; Paluszkiewicz et al., 2011a).

In addition to the deficits in phasic (synaptic) inhibition, tonic inhibition, which is mediated by extrasynaptic GABA$_A$ receptors containing either the α5 or the δ subunit, is also compromised in the BLA of FMR1 KO mice (Martin et al., 2014) and in related ASDs (Zhang et al., 2008). Tonic inhibition, maintained by low levels of ambient GABA in the extrasynaptic space (Farrant and Nusser, 2005), provides a persistent inhibitory conductance that regulates the E/I balance (Semyanov et al., 2004). The reduction in the expression of the α5 subunit of the GABA$_A$ receptor narrows the integration window

necessary for feedforward inhibition. Moreover, because of the reduced GABA release, more synchronized afferent inputs must be generated to result in an action potential and to modulate the integration of postsynaptic excitatory and inhibitory potentials (Pouille and Scanziani, 2001; Gabernet et al., 2005).

Alzheimer's Disease

AD is associated with severe neuronal loss, with a predilection for brain regions within the medial temporal lobe, including the amygdala (Arnold et al., 1991; Braak and Braak, 1991). Recent studies have shown that, in symptomatic AD patients, the basomedial and lateral nuclei of the amygdala, display between 14% and 60% volumetric loss compared with controls as well as nonuniform shape changes (Cavedo et al., 2011, 2014; Poulin et al., 2011; Miller et al., 2015). In addition, postmortem studies have revealed that, although there is damage throughout the amygdala, the degree of atrophy and neurofibrillary tangles of amygdala nuclei is greater in the corticomedial group than in the BLA, suggesting that perhaps there is a selective loss of neurons that contributes to the loss in amygdala volume (Tsuchiya and Kosaka, 1990).

Although the overall loss of neurons, contributing to volumetric changes, has been observed in the amygdala, it remains unknown whether GABAergic interneuronal subpopulations or specific subunits of the GABA$_A$ receptor are targeted in the BLA and contribute to the observed deficits in fear learning and increased anxiety. Indeed, a loss of GABAergic interneurons or alterations in the expression of GABA$_A$ receptor subunits in the amygdala is possible, given that it has been observed that in the canine PFC PV- or CR-immunoreactive interneurons are resistant to neuronal death, whereas CB-positive interneurons are depleted (Pugliese et al., 2004); in the mouse dentate gyrus, significantly fewer SOM-immunopositive neurons are observed, whereas in the cornus ammonis 1 hippocampal region there is a significant loss of PV and SOM interneurons (Levenga et al., 2013). In addition, as observed in the hippocampus (see Mizukami et al., 1998; Armstrong et al., 2003; Iwakiri et al., 2009), GABA$_A$ receptor subunit expression might also be reduced in the BLA, which could contribute to amygdala hyperactivity.

Although it may be hypothesized that there are alterations to the expression of GABA$_A$ receptors and the interneuronal population in the BLA, only one study has examined alterations to the E/I balance in an AD model and focused this examination on the LA. In a study using the apolipoprotein E4 (apoE4)-targeted replacement mouse to model AD (Wang et al., 2005), 1- or 7-month-old mice expressing apoE4 displayed reduced excitatory synaptic transmission in the LA but no changes in inhibitory synaptic transmission (Klein et al., 2010). However, aged (18–20 months) apoE4 animals displayed significant increases in both inhibitory and excitatory synaptic transmission and an increased seizure phenotype

(Hunter et al., 2012; Klein et al., 2014), suggesting that increased excitatory synaptic transmission predominates. The results indicate that it is unlikely that, in the LA, there are alterations in the subunit composition of the GABA$_A$ receptor; rather, increased excitatory transmission may be the result of alterations in the presynaptic release of GABA. Although it remains unknown why animals display increased excitatory activity in addition to the increased inhibitory activity, one hypothesis is that the increased excitatory activity seen in apoE4 mice may be the result of a loss of inhibition from extrinsic afferent cortical inputs (Swanson and Petrovich, 1998; Klein et al., 2014) or deficiencies in neuromodulatory mechanisms such as the loss of GABAergic interneurons but not cholinergic neurons in the basal forebrain (Loreth et al., 2012).

Epilepsy and Seizures

As a principal circuit projecting to many brain regions, hyperexcitability within the amygdala may be one source of seizure generation (Prager et al., 2013). For instance, spontaneous bursting activity has been found to appear first in the BLA of kindled animals (White and Price, 1993; Smith and Dudek, 1997), and seizure generation after a nerve agent exposure occurs only if AChE activity is significantly impaired in the amygdala compared with other seizurogenic brain regions (McDonough et al., 1987; Prager et al., 2013). The amygdala receives monosynaptic inputs from many frontal and temporal cortical areas that are known to generate and propagate seizure activity (Pitkanen et al., 1998). The convergence of input onto specific nuclei can then recruit a large number of neurons from interdivisional network connections, which may contribute to ictal-like activity within different amygdala nuclei. Efferents from amygdala nuclei may subsequently provide routes by which the amygdala can recruit other brain regions and lead to seizure propagation (Hirsch et al., 1997; Pitkanen et al., 1998; Pitkanen, 2000).

The loss of neurons in the amygdala and subsequent reductions in amygdalar volume further indicate the amygdala's role in the generation and propagation of seizures. The amygdala has previously been found to be severely damaged in patients with temporal lobe epilepsy and in both adults and children who experience SE (Pitkanen et al., 1998). Although often occurring in combination with hippocampal damage, neuronal loss has been observed in the amygdala without any apparent damage to the hippocampus (Hudson et al., 1993; Miller et al., 1994; Pitkanen et al., 1998). The loss of neurons in the amygdala has also been observed in animal models, the BLA being among the most damaged nuclei (Tuunanen et al., 1996; Apland et al., 2010; Figueiredo et al., 2011; Prager et al., 2013, 2014a). The loss of GABAergic interneurons in the amygdala in different seizure models has also been examined. Seizures or SE causes between 37% and 64% of GABAergic interneurons in the BLA to die, irrespective of the seizure model, although the loss of GABAergic interneurons was delayed by 7 days in animals

that developed SE after a nerve agent exposure (Callahan et al., 1991; Figueiredo et al., 2011; Prager et al., 2014a). Tunnanen and colleagues (1997) found a 35% decrease in the density of SOM-immunoreactive neurons in a kindling model.

Kindling- or nerve agent-induced SE causes long-lasting changes in synaptic transmission in the BLA, including impaired feedforward GABAergic inhibition and disinhibition of excitatory circuits (Rainnie et al., 1991a, 1992) and network reorganization resulting in BLA hyperexcitability (Smith and Dudek, 1997; Prager et al., 2014a). The loss of feedforward inhibition has been observed indirectly as a significant increase in paired-pulse facilitation beginning 24 hr after SE and continuing up to 30 days after nerve agent exposure (Zinebi et al., 2001; Prager et al., 2014a) and directly as a prolonged reduction in $GABA_A$ receptor-mediated inhibitory synaptic transmission, which likely was due to the loss of GABAergic interneurons in the BLA (Prager et al., 2014b). The loss of inhibitory synaptic transmission has been found to cause a concomitant increase in excitatory synaptic transmission (Prager et al., 2014b), which is associated with an increase in both NMDAR- and non-NMDAR-mediated glutamatergic transmission (Gean et al., 1989; Rainnie et al., 1992; Shoji et al., 1998).

Although alterations in GABAergic synaptic transmission have been observed in the amygdala after nerve agent-induced SE, reductions in $GABA_A$ receptor-mediated IPSCs appear to be model specific. After nerve agent-induced SE, there was a significant reduction in the frequency but not the amplitude of $GABA_A$ receptor-mediated mIPSCs (Prager et al., 2014b), indicating that the deficits in inhibitory synaptic transmission resulted from the loss of GABAergic interneurons. However, 7–10 days after kainate acid-induced SE, there was an increase in $\alpha 1$ subunit and GAD expression but a reduction in tonic inhibition (Fritsch et al., 2009). Although few studies have addressed how the stoichiometry of $GABA_A$ receptor subunits changes in the BLA after prolonged SE, it is well known that the expression of $GABA_A$ subunits is altered in other brain regions, such as the hippocampus (Mohler, 2006; Ferando and Mody, 2012). Thus, perhaps in some cases of epilepsy, alterations in the stoichiometry of $GABA_A$ receptor subunits may contribute to impaired inhibition in the BLA, whereas in other cases the loss of GABAergic inhibition may be the result of the death of interneurons.

Traumatic Brain Injury

Similarly to many other disorders, TBI can affect many brain regions, including the amygdala, and the disruption in neuronal excitability in surrounding regions may ultimately alter the homeostasis of the amygdala. The disruption in the E/I balance stems from an initial rise in glutamate release, which is responsible for excitotoxicity, and also from a delayed disruption of excitatory glutamate circuits, which may underlie the cognitive and motor deficits observed after TBI (Guerriero et al., 2015). Altera-

tions in both glutamatergic and GABAergic synaptic transmission and the expression of their corresponding receptors have been observed after TBI in many brain regions, including the BLA (Almeida-Suhett et al., 2014; Guerriero et al., 2015), although this work is in its infant stages. An increase in the NR1 subunit of the NMDAR has been observed in the amygdala 2 weeks after injury (Reger et al., 2012), and reductions in the $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits of the $GABA_A$ receptor were observed 7 days after a mild TBI (Almeida-Suhett et al., 2014). Moreover, even when there is no overt neuronal death in the BLA, a delayed loss of GABAergic interneurons is observed after a mild TBI, which may contribute to increased anxiety-like behavior (Almeida-Suhett et al., 2014) and enhanced fear conditioning (Reger et al., 2012).

CONCLUSIONS AND FUTURE DIRECTIONS

Although alterations in GABAergic inhibitory synaptic transmission in different diseases have been reviewed separately, it cannot go unstated that, in many cases, comorbidity occurs within many of these diseases. For example, estimates of comorbidity between PTSD and some types of TBI, including combat-related TBI, are as high as 73% (Hoge et al., 2008; Taylor et al., 2012). Moreover, epilepsy is often found to occur with diseases, including autism/fragile X (Berry-Kravis et al., 2010; Khetrapal, 2010), schizophrenia (Kandratavicius et al., 2012), AD (Palop et al., 2007; Chan et al., 2015), and anxiety disorders (Trimble and Van Elst, 2003; Vazquez and Devinsky, 2003). Indeed, many of these disorders have comorbidities, and often these comorbidities involve deficiencies within the BLA GABAergic system.

We are not arguing in this Review that amygdala hyperactivity results in the development of symptoms associated with the diseases discussed above. Rather, this Review seeks to provide evidence that reduced GABAergic inhibition and alterations in the mechanisms that modulate GABAergic inhibition contribute, in part, to amygdalar hyperexcitability; BLA hyperexcitability is common among these disorders and may lead to comorbid behavioral deficits. The extensive innervation of the amygdala by multiple brain regions has revealed that specific pathways modulate GABAergic inhibitory synaptic transmission and that these pathways may be disrupted in different diseases.

GABAergic activity in the BLA is modulated by dopaminergic, serotonergic, noradrenergic, and cholinergic activation as well as by the activation of various glutamate receptor subtypes and the CB1 and ASIC1a receptors. For the diseases discussed, deficiencies in the release of monoamines or ACh or alterations in glutamatergic receptor activity can lead to reduced modulation of GABAergic inhibition and, more locally, greater excitation via deficiencies in either feedforward or feedback inhibitory mechanisms. Moreover, because many of these systems are interconnected, deficiencies in one system may result in a cascading effect, which could contribute to disinhibition of excitatory neurons in the BLA and,

subsequently, increased anxiety-like behavior or increased seizure activity. However, the data in many cases are not conclusive. Much remains unknown with respect to how alterations in neurotransmitter release, receptor activation, and stoichiometry contribute to the behavioral deficits and increased anxiety often associated with these disorders.

E/I balance in the amygdala is dependent on functional neuromodulatory mechanisms and local interneuronal regulation. Neuromodulation is ineffective when there is a substantial loss of GABAergic interneurons, as has been observed in the amygdala in various neurological and neuropsychiatric disorders. In some cases, it is known that a specific class of interneurons is differentially affected, but in most cases it remains unknown what type of interneuron is most susceptible to cell death. Moreover, the loss of GABAergic interneurons may be delayed compared with the death of principal neurons. The immediate death may be due to the excitotoxic effects that occur with glutamatergic excitotoxicity (Zhou et al., 2013); however, one hypothesis is that the delayed loss of GABAergic interneurons is due to an upregulation of D-serine, an endogenous coagonist for NMDARs (Liu et al., 2009). Alternatively, even if there is no interneuronal degeneration, deficits in GAD may reduce the synthesis of GABA, which could subsequently reduce the concentration of GABA released in the synapse and impair inhibitory synaptic transmission. Alternatively, in many of the diseases discussed, alterations in expression of GABA$_A$ receptor subunits have been observed in the BLA. Although it is unknown whether these changes are transient or permanent, it can be assumed that alterations in the subunit stoichiometry may lead to reduced tonic and phasic inhibitory synaptic transmission.

This Review has two major themes. First, we have summarized the neuromodulatory systems that modulate GABA$_A$ receptor-mediated inhibitory synaptic transmission. Second, we have discussed how reduced GABAergic inhibition in the BLA throughout the life span can contribute to the behavioral manifestation of symptoms associated with autism and fragile X, AD, epilepsy, TBI, and anxiety- or stress-related disorders. In each case, results indicate that BLA hyperexcitability is associated with deficits in mechanisms that modulate GABAergic inhibitory synaptic transmission, loss of GABAergic interneurons, or alterations in GABA$_A$ receptor subunit expression. However, in many of the diseases discussed above, much remains unknown with respect to why the amygdala is hyperexcitable. By understanding how the GABAergic system is impaired, future research can target the functional aspects of the GABA$_A$ receptor for potential therapeutic options. Future research might also develop new therapies that induce the growth of interneurons in specific brain regions or target and reduce excitation of the glutamatergic system. The latter option has been implemented after a nerve agent-induced seizure, for example, in which administering a GluK$_1$ antagonist prevented neurodegeneration and associated increases in anxiety or seizure activity (Figueiredo et al., 2011; Prager et al.,

2015). Overall, identifying the alterations to the inhibitory system and the mechanisms that modulate inhibitory synaptic transmission is a fundamental prerequisite for the design of effective and well-tolerated therapeutic treatments for these and other neurological and neuropsychiatric disorders.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests

ROLE OF AUTHORS

All authors take responsibility for the integrity and accuracy of this article. Drafting of the manuscript and critical revisions of the article: EMP, HCB, GHW, MFMB

REFERENCES

Alger BE, Nagode DA, Tang AH. 2014. Muscarinic cholinergic receptors modulate inhibitory synaptic rhythms in hippocampus and neocortex. Front Synaptic Neurosci 6:18.

Almeida-Suhett CP, Prager EM, Pidoplichko V, Figueiredo TH, Marini AM, Li Z, Eiden LE, Braga MF. 2014. Reduced GABAergic inhibition in the basolateral amygdala and the development of anxiety-like behaviors after mild traumatic brain injury. PLoS One 9:e102627.

Apland JP, Figueiredo TH, Qashu F, Aroniadou-Anderjaska V, Souza AP, Braga MF. 2010. Higher susceptibility of the ventral versus the dorsal hippocampus and the posteroventral versus anterodorsal amygdala to soman-induced neuropathology. Neurotoxicology 31:485–492.

Apland JP, Aroniadou-Anderjaska V, Figueiredo TH, Rossetti F, Miller SL, Braga MF. 2014. The limitations of diazepam as a treatment for nerve agent-induced seizures and neuropathology in rats: comparison with UBP302. J Pharmacol Exp Ther 351:359–372.

Armstrong DM, Sheffield R, Mishizen-Eberz AJ, Carter TL, Rissman RA, Mizukami K, Ikonomovic MD. 2003. Plasticity of glutamate and GABA$_A$ receptors in the hippocampus of patients with Alzheimer's disease. Cell Mol Neurobiol 23:491–505.

Arnold SE, Hyman BT, Flory J, Damasio AR, Van Hoesen GW. 1991. The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. Cereb Cortex 1:103–116.

Aroniadou-Anderjaska V, Qashu F, Braga MF. 2007. Mechanisms regulating GABAergic inhibitory transmission in the basolateral amygdala: implications for epilepsy and anxiety disorders. Amino Acids 32:305–315.

Aroniadou-Anderjaska V, Pidoplichko VI, Figueiredo TH, Almeida-Suhett CP, Prager EM, Braga MF. 2012. Presynaptic facilitation of glutamate release in the basolateral amygdala: a mechanism for the anxiogenic and seizurogenic function of GluK$_1$ receptors. Neuroscience 221: 157–169.

Asan E. 1997. Ultrastructural features of tyrosine-hydroxylase-immunoreactive afferents and their targets in the rat amygdala. Cell Tissue Res 288:449–469.

Asan E, Steinke M, Lesch KP. 2013. Serotonergic innervation of the amygdala: targets, receptors, and implications for stress and anxiety. Histochem Cell Biol 139:785–813.

Azad SC, Eder M, Marsicano G, Lutz B, Zieglgansberger W, Rammes G. 2003. Activation of the cannabinoid receptor type 1 decreases

glutamatergic and GABAergic synaptic transmission in the lateral amygdala of the mouse. Learn Mem 10:116–128.

Barbalho CA, Nunes-de-Souza RL, Canto-de-Souza A. 2009. Similar anxiolytic-like effects following intra-amygdala infusions of benzodiazepine receptor agonist and antagonist: evidence for the release of an endogenous benzodiazepine inverse agonist in mice exposed to elevated plus-maze test. Brain Res 1267:65–76.

Ben-Ari Y, Zigmond RE, Shute CC, Lewis PR. 1977. Regional distribution of choline acetyltransferase and acetylcholinesterase within the amygdaloid complex and stria terminalis system. Brain Res 120:435–444.

Ben-Ari Y, Khalilov I, Kahle KT, Cherubini E. 2012. The GABA excitatory/inhibitory shift in brain maturation and neurological disorders. Neuroscientist 18:467–486.

Berdel B, Morys J. 2000. Expression of calbindin-D28k and parvalbumin during development of rat's basolateral amygdaloid complex. Int J Dev Neurosci 18:501–513.

Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey DB. 2010. Seizures in fragile X syndrome: characteristics and comorbid diagnoses. Am J Intellect Dev Disabil 115:461–472.

Biagini G, Babinski K, Avoli M, Marcinkiewicz M, Seguela P. 2001. Regional and subunit-specific downregulation of acid-sensing ion channels in the pilocarpine model of epilepsy. Neurobiol Dis 8:45–58.

Bienvenu TC, Busti D, Micklem BR, Mansouri M, Magill PJ, Ferraguti F, Capogna M. 2015. Large intercalated neurons of amygdala relay noxious sensory information. J Neurosci 35:2044–2057.

Bischoff S, Leonhard S, Reymann N, Schuler V, Shigemoto R, Kaupmann K, Bettler B. 1999. Spatial distribution of $GABA_BR1$ receptor mRNA and binding sites in the rat brain. J Comp Neurol 412:1–16.

Bissiere S, Humeau Y, Luthi A. 2003. Dopamine gates LTP induction in lateral amygdala by suppressing feedforward inhibition. Nat Neurosci 6:587–592.

Blatt GJ. 2012. The neuropathology of autism. Scientifica 2012:703675.

Bombardi C. 2011. Distribution of 5-HT2A receptor immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. Brain Res 1370:112–128.

Bonn M, Schmitt A, Asan E. 2012. Double and triple in situ hybridization for coexpression studies: combined fluorescent and chromogenic detection of neuropeptide Y (NPY) and serotonin receptor subtype mRNAs expressed at different abundance levels. Histochem Cell Biol 137:11–24.

Bonn M, Schmitt A, Lesch KP, Van Bockstaele EJ, Asan E. 2013. Serotonergic innervation and serotonin receptor expression of NPY-producing neurons in the rat lateral and basolateral amygdaloid nuclei. Brain Struct Funct 218:421–435.

Bosch D, Ehrlich I. 2015. Postnatal maturation of GABAergic modulation of sensory inputs onto lateral amygdala principal neurons. J Physiol 593:4387–4409.

Braak H, Braak E. 1991. Neuropathological staging of Alzheimer-related changes. Acta Neuropathol 82:239–259.

Braga MF, Aroniadou-Anderjaska V, Xie J, Li H. 2003. Bidirectional modulation of GABA release by presynaptic glutamate receptor 5 kainate receptors in the basolateral amygdala. J Neurosci 23:442–452.

Braga MF, Aroniadou-Anderjaska V, Li H. 2004a. The physiological role of kainate receptors in the amygdala. Mol Neurobiol 30:127–141.

Braga MF, Aroniadou-Anderjaska V, Manion ST, Hough CJ, Li H. 2004b. Stress impairs $alpha_{1A}$ adrenoceptor-mediated noradrenergic facilitation of GABAergic transmission in the basolateral amygdala. Neuropsychopharmacology 29:45–58.

Bringas ME, Carvajal-Flores FN, Lopez-Ramirez TA, Atzori M, Flores G. 2013. Rearrangement of the dendritic morphology in limbic regions and altered exploratory behavior in a rat model of autism spectrum disorder. Neuroscience 241:170–187.

Brinley-Reed M, McDonald AJ. 1999. Evidence that dopaminergic axons provide a dense innervation of specific neuronal subpopulations in the rat basolateral amygdala. Brain Res 850:127–135.

Brummelte S, Witte V, Teuchert-Noodt G. 2007. Postnatal development of GABA and calbindin cells and fibers in the prefrontal cortex and basolateral amygdala of gerbils (Meriones unguiculatus). Int J Dev Neurosci 25:191–200.

Buffalari DM, Grace AA. 2007. Noradrenergic modulation of basolateral amygdala neuronal activity: opposing influences of alpha-2 and beta receptor activation. J Neurosci 27:12358–12366.

Callahan PM, Paris JM, Cunningham KA, Shinnick-Gallagher P. 1991. Decrease of GABA-immunoreactive neurons in the amygdala after electrical kindling in the rat. Brain Res 555:335–339.

Capogna M. 2014. GABAergic cell type diversity in the basolateral amygdala. Curr Opin Neurobiol 26:110–116.

Carlsen J, Heimer L. 1988. The basolateral amygdaloid complex as a cortical-like structure. Brain Res 441:377–380.

Carlsen J, Zaborszky L, Heimer L. 1985. Cholinergic projections from the basal forebrain to the basolateral amygdaloid complex: a combined retrograde fluorescent and immunohistochemical study. J Comp Neurol 234:155–167.

Cavedo E, Boccardi M, Ganzola R, Canu E, Beltramello A, Caltagirone C, Thompson PM, Frisoni GB. 2011. Local amygdala structural differences with 3T MRI in patients with Alzheimer disease. Neurology 76:727–733.

Cavedo E, Pievani M, Boccardi M, Galluzzi S, Bocchetta M, Bonetti M, Thompson PM, Frisoni GB. 2014. Medial temporal atrophy in early- and late-onset Alzheimer's disease. Neurobiol Aging 35:2004–2012.

Chan J, Jones NC, Bush AI, O'Brien TJ, Kwan P. 2015. A mouse model of Alzheimer's disease displays increased susceptibility to kindling and seizure-associated death. Epilepsia 56:e73–e77.

Chittajallu R, Braithwaite SP, Clarke VR, Henley JM. 1999. Kainate receptors: subunits, synaptic localization, and function. Trends Pharmacol Sci 20:26–35.

Chu HY, Ito W, Li J, Morozov A. 2012. Target-specific suppression of GABA release from parvalbumin interneurons in the basolateral amygdala by dopamine. J Neurosci 32:14815–14820.

Clark JA, Mezey E, Lam AS, Bonner TI. 2000. Distribution of the $GABA_B$ receptor subunit gb2 in rat CNS. Brain Res 860:41–52.

Coghlan S, Horder J, Inkster B, Mendez MA, Murphy DG, Nutt DJ. 2012. GABA system dysfunction in autism and related disorders: from synapse to symptoms. Neurosci Biobehav Rev 36:2044–2055.

Cortes R, Probst A, Palacios JM. 1987. Quantitative light microscopic autoradiographic localization of cholinergic muscarinic receptors in the human brain: forebrain. Neuroscience 20:65–107.

Coryell MW, Ziemann AE, Westmoreland PJ, Haenfler JM, Kurjakovic Z, Zha XM, Price M, Schnizler MK, Wemmie JA. 2007. Targeting ASIC1a reduces innate fear and alters neuronal activity in the fear circuit. Biol Psychiatry 62:1140–1148.

Coryell MW, Wunsch AM, Haenfler JM, Allen JE, Schnizler M, Ziemann AE, Cook MN, Dunning JP, Price MP, Rainier JD, Liu Z, Light AR, Langbehn DR, Wemmie JA. 2009. Acid-sensing ion channel-1a in the amygdala, a novel therapeutic target in depression-related behavior. J Neurosci 29:5381–5388.

Cossart R. 2011. The maturation of cortical interneuron diversity: how multiple developmental journeys shape the emergence of proper network function. Curr Opin Neurobiol 21:160–168.

Courtin J, Chaudun F, Rozeske RR, Karalis N, Gonzalez-Campo C, Wurtz H, Abdi A, Baufreton J, Bienvenu TC, Herry C. 2014. Prefrontal parvalbumin interneurons shape neuronal activity to drive fear expression. Nature 505:92–96.

Couve A, Moss SJ, Pangalos MN. 2000. $GABA_B$ receptors: a new paradigm in G protein signaling. Mol Cell Neurosci 16:296–312.

Craig MT, McBain CJ. 2014. The emerging role of $GABA_B$ receptors as regulators of network dynamics: fast actions from a "slow" receptor? Curr Opin Neurobiol 26:15–21.

Cunningham MG, Connor CM, Carlezon WA Jr, Meloni E. 2009. Amygdalar GABAergic-rich neural grafts attenuate anxiety-like behavior in rats. Behav Brain Res 205:146–153.

Da Cunha C, Wolfman C, Levi de Stein M, Ruschel AC, Izquierdo I, Medina JH. 1992. Anxiogenic effects of the intra-amygdala injection of flumazenil, a benzodiazepine receptor antagonist. Funct Neurol 7:401–405.

Dani JA, Bertrand D. 2007. Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. Annu Rev Pharmacol Toxicol 47:699–729.

Davila JC, Olmos L, Legaz I, Medina L, Guirado S, Real MA. 2008. Dynamic patterns of colocalization of calbindin, parvalbumin, and GABA in subpopulations of mouse basolateral amygdalar cells during development. J Chem Neuroanat 35:67–76.

Deidda G, Bozarth IF, Cancedda L. 2014. Modulation of GABAergic transmission in development and neurodevelopmental disorders: investigating physiology and pathology to gain therapeutic perspectives. Front Cell Neurosci 8:119.

Depue BE, Olson-Madden JH, Smolker HR, Rajamani M, Brenner LA, Banich MT. 2014. Reduced amygdala volume is associated with deficits in inhibitory control: a voxel- and surface-based morphometric analysis of comorbid PTSD/mild TBI. Biomed Res Int 2014:691505.

D'Hulst C, De Geest N, Reeve SP, Van Dam D, De Deyn PP, Hassan BA, Kooy RF. 2006. Decreased expression of the GABAA receptor in fragile X syndrome. Brain Res 1121:238–245.

Diana MA, Bregestovski P. 2005. Calcium and endocannabinoids in the modulation of inhibitory synaptic transmission. Cell Calcium 37:497–505.

Durkin MM, Gunwaldsen CA, Borowsky B, Jones KA, Branchek TA. 1999. An in situ hybridization study of the distribution of the GABA$_{B2}$ protein mRNA in the rat CNS. Brain Res Mol Brain Res 71:185–200.

Dwyer JM, Rizzo SJ, Neal SJ, Lin Q, Jow F, Arias RL, Rosenzweig-Lipson S, Dunlop J, Beyer CE. 2009. Acid-sensing ion channel (ASIC) inhibitors exhibit anxiolytic-like activity in preclinical pharmacological models. Psychopharmacology 203:41–52.

Ehrlich DE, Ryan SJ, Hazra R, Guo JD, Rainnie DG. 2013. Postnatal maturation of GABAergic transmission in the rat basolateral amygdala. J Neurophysiol 110:926–941.

El-Ansary A, Al-Ayadhi L. 2014. GABAergic/glutamatergic imbalance relative to excessive neuroinflammation in autism spectrum disorders. J Neuroinflammation 11:189.

Emson PC, Paxinos G, Le Gal La Salle G, Ben-Ari Y, Silver A. 1979. Choline acetyltransferase and acetylcholinesterase containing projections from the basal forebrain to the amygdaloid complex of the rat. Brain Res 165:271–282.

Esmaeili A, Lynch JW, Sah P. 2009. GABAA receptors containing gamma1 subunits contribute to inhibitory transmission in the central amygdala. J Neurophysiol 101:341–349.

Fallon J, Ciofi P. 1992. Distribution of monoamines within the amygdala. In: Aggleton J, editor. The amygdala. New York: Academic Press.

Farb DH, Ratner MH. 2014. Targeting the modulation of neural circuitry for the treatment of anxiety disorders. Pharmacol Rev 66:1002–1032.

Farrant M, Nusser Z. 2005. Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors. Nat Rev Neurosci 6:215–229.

Fatemi SH, Reutiman TJ, Folsom TD, Rooney RJ, Patel DH, Thuras PD. 2010. mRNA and protein levels for GABAA alpha4, alpha5, beta1, and GABABR1 receptors are altered in brains from subjects with autism. J Autism Dev Disord 40:743–750.

Ferando I, Mody I. 2012. GABAA receptor modulation by neurosteroids in models of temporal lobe epilepsies. Epilepsia 53(Suppl 9):89–101.

Figueiredo TH, Qashu F, Apland JP, Aroniadou-Anderjaska V, Souza AP, Braga MF. 2011. The GluK1 (GluR5) kainate/alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist LY293558 reduces soman-induced seizures and neuropathology. J Pharmacol Exp Ther 336:303–312.

Fries P. 2009. Neuronal gamma-band synchronization as a fundamental process in cortical computation. Annu Rev Neurosci 32:209–224.

Fritsch B, Qashu F, Figueiredo TH, Aroniadou-Anderjaska V, Rogawski MA, Braga MF. 2009. Pathological alterations in GABAergic interneurons and reduced tonic inhibition in the basolateral amygdala during epileptogenesis. Neuroscience 163:415–429.

Gabernet L, Jadhav SP, Feldman DE, Carandini M, Scanziani M. 2005. Somatosensory integration controlled by dynamic thalamocortical feedforward inhibition. Neuron 48:315–327.

Gassmann M, Bettler B. 2012. Regulation of neuronal GABAB receptor functions by subunit composition. Nat Rev Neurosci 13:380–394.

Gean PW, Shinnick-Gallagher P, Anderson AC. 1989. Spontaneous epileptiform activity and alteration of GABA- and of NMDA-mediated neurotransmission in amygdala neurons kindled in vivo. Brain Res 494:177–181.

Gharedaghi MH, Seyedabadi M, Ghia JE, Dehpour AR, Rahimian R. 2014. The role of different serotonin receptor subtypes in seizure susceptibility. Exp Brain Res 232:347–367.

Gotti C, Clementi F, Fornari A, Gaimarri A, Guiducci S, Manfredi I, Moretti M, Pedrazzi P, Pucci L, Zoli M. 2009. Structural and functional diversity of native brain neuronal nicotinic receptors. Biochem Pharmacol 78:703–711.

Guerriero RM, Giza CC, Rotenberg A. 2015. Glutamate and GABA imbalance following traumatic brain injury. Curr Neurol Neurosci Rep 15:545.

Hafizi S, Serres F, Pei Q, Totterdell S, Sharp T. 2012. Evidence for the differential colocalization of neurokinin-1 receptors with 5-HT receptor subtypes in rat forebrain. J Psychopharmacol 26:505–515.

He Q, Nomura T, Xu J, Contractor A. 2014. The developmental switch in GABA polarity is delayed in fragile X mice. J Neurosci 34:446–450.

Heim C, Nemeroff CB. 2001. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49:1023–1039.

Herb A, Burnashev N, Werner P, Sakmann B, Wisden W, Seeburg PH. 1992. The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. Neuron 8:775–785.

Herry C, Johansen JP. 2014. Encoding of fear learning and memory in distributed neuronal circuits. Nat Neurosci 17:1644–1654.

Hill JA Jr, Zoli M, Bourgeois JP, Changeux JP. 1993. Immunocytochemical localization of a neuronal nicotinic receptor: the beta 2-subunit. J Neurosci 13:1551–1568.

Hirsch E, Danober L, Simler S, Pereira de Vasconcelos A, Maton B, Nehlig A, Marescaux C, Vergnes M. 1997. The amygdala is critical for seizure propagation from brainstem to forebrain. Neuroscience 77:975–984.

Hoge CW, Castro CA, Messer SC, McGurk D, Cotting DI, Koffman RL. 2008. Combat duty in Iraq and Afghanistan, mental health problems, and barriers to care. US Army Med Dep J:7–17.

Holmes A. 2008. Genetic variation in corticoamygdala serotonin function and risk for stress-related disease. Neurosci Biobehav Rev 32:1293–1314.

Hudson LP, Munoz DG, Miller L, McLachlan RS, Girvin JP, Blume WT. 1993. Amygdaloid sclerosis in temporal lobe epilepsy. Ann Neurol 33:622–631.

Hunter JM, Cirrito JR, Restivo JL, Kinley RD, Sullivan PM, Holtzman DM, Koger D, Delong C, Lin S, Zhao L, Liu F, Bales K, Paul SM. 2012. Emergence of a seizure phenotype in aged apolipoprotein epsilon 4 targeted replacement mice. Brain Res 1467:120–132.

Iwakiri M, Mizukami K, Ikonomovic MD, Ishikawa M, Abrahamson EE, DeKosky ST, Asada T. 2009. An immunohistochemical study of GABA A receptor gamma subunits in Alzheimer's disease hippocampus: relationship to neurofibrillary tangle progression. Neuropathology 29:263–269.

Jasnow AM, Ressler KJ, Hammack SE, Chhatwal JP, Rainnie DG. 2009. Distinct subtypes of cholecystokinin (CCK)-containing interneurons of

the basolateral amygdala identified using a CCK promoter-specific lentivirus. J Neurophysiol 101:1494–1506.

Jiang L, Xu J, Nedergaard M, Kang J. 2001. A kainate receptor increases the efficacy of GABAergic synapses. Neuron 30:503–513.

Jiang X, Xing G, Yang C, Verma A, Zhang L, Li H. 2009. Stress impairs 5-HT2A receptor-mediated serotonergic facilitation of GABA release in juvenile rat basolateral amygdala. Neuropsychopharmacology 34:410–423.

Kandratavicius L, Lopes-Aguiar C, Bueno-Junior LS, Romcy-Pereira RN, Hallak JE, Leite JP. 2012. Psychiatric comorbidities in temporal lobe epilepsy: possible relationships between psychotic disorders and involvement of limbic circuits. Rev Bras Psiquiatr 34:454–466.

Kaneko K, Tamamaki N, Owada H, Kakizaki T, Kume N, Totsuka M, Yamamoto T, Yawo H, Yagi T, Obata K, Yanagawa Y. 2008. Noradrenergic excitation of a subpopulation of GABAergic cells in the basolateral amygdala via both activation of nonselective cationic conductance and suppression of resting K^+ conductance: a study using glutamate decarboxylase 67-green fluorescent protein knockin mice. Neuroscience 157:781–797.

Katona I, Rancz EA, Acsady L, Ledent C, Mackie K, Hajos N, Freund TF. 2001. Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. J Neurosci 21: 9506–9518.

Kemppainen S, Pitkanen A. 2000. Distribution of parvalbumin, calretinin, and calbindin-D(28k) immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid. J Comp Neurol 426:441–467.

Khetrapal N. 2010. Overlap of autism and seizures: understanding cognitive comorbidity. Mens Sana Monogr 8:122–128.

Kim SY, Burris J, Bassal F, Koldewyn K, Chattarji S, Tassone F, Hessl D, Rivera SM. 2014. Fear-specific amygdala function in children and adolescents on the fragile x spectrum: a dosage response of the *FMR1* gene. Cereb Cortex 24:600–613.

Kishimoto K, Koyama S, Akaike N. 2000. Presynaptic modulation of synaptic gamma-aminobutyric acid transmission by tandospirone in rat basolateral amygdala. Eur J Pharmacol 407:257–265.

Klein RC, Yakel JL. 2006. Functional somatodendritic alpha7-containing nicotinic acetylcholine receptors in the rat basolateral amygdala complex. J Physiol 576:865–872.

Klein RC, Mace BE, Moore SD, Sullivan PM. 2010. Progressive loss of synaptic integrity in human apolipoprotein E4 targeted replacement mice and attenuation by apolipoprotein E2. Neuroscience 171:1265–1272.

Klein RC, Acheson SK, Mace BE, Sullivan PM, Moore SD. 2014. Altered neurotransmission in the lateral amygdala in aged human apoE4 targeted replacement mice. Neurobiol Aging 35:2046–2052.

Koyama S, Kubo C, Rhee JS, Akaike N. 1999. Presynaptic serotonergic inhibition of GABAergic synaptic transmission in mechanically dissociated rat basolateral amygdala neurons. J Physiol 518:525–538.

Koyama S, Matsumoto N, Kubo C, Akaike N. 2000. Presynaptic 5-HT3 receptor-mediated modulation of synaptic GABA release in the mechanically dissociated rat amygdala neurons. J Physiol 529:373–383.

Koyama S, Matsumoto N, Murakami N, Kubo C, Nabekura J, Akaike N. 2002. Role of presynaptic 5-HT1A and 5-HT3 receptors in modulation of synaptic GABA transmission in dissociated rat basolateral amygdala neurons. Life Sci 72:375–387.

Kroner S, Rosenkranz JA, Grace AA, Barrionuevo G. 2005. Dopamine modulates excitability of basolateral amygdala neurons in vitro. J Neurophysiol 93:1598–1610.

Lang EJ, Pare D. 1997. Similar inhibitory processes dominate the responses of cat lateral amygdaloid projection neurons to their various afferents. J Neurophysiol 77:341–352.

Lang EJ, Pare D. 1998. Synaptic responsiveness of interneurons of the cat lateral amygdaloid nucleus. Neuroscience 83:877–889.

Lange MD, Jungling K, Paulukat L, Vieler M, Gaburro S, Sosulina L, Blaesse P, Sreepathi HK, Ferraguti F, Pape HC. 2014. Glutamic acid decarboxylase 65: a link between GABAergic synaptic plasticity in the lateral amygdala and conditioned fear generalization. Neuropsychopharmacology 39:2211–2220.

LeDoux JE, Farb CR, Milner TA. 1991. Ultrastructure and synaptic associations of auditory thalamoamygdala projections in the rat. Exp Brain Res 85:577–586.

Lehner M, Wislowska-Stanek A, Skorzewska A, Maciejak P, Szyndler J, Turzynska D, Sobolewska A, Plaznik A. 2010. Differences in the density of GABA-A receptor alpha-2 subunits and gephyrin in brain structures of rats selected for low and high anxiety in basal and fear-stimulated conditions in a model of contextual fear conditioning. Neurobiol Learn Mem 94:499–508.

Levenga J, Krishnamurthy P, Rajamohamedsait H, Wong H, Franke TF, Cain P, Sigurdsson EM, Hoeffer CA. 2013. Tau pathology induces loss of GABAergic interneurons leading to altered synaptic plasticity and behavioral impairments. Acta Neuropathol Commun 1:34.

Li H, Rogawski MA. 1998. GluR5 kainate receptor mediated synaptic transmission in rat basolateral amygdala in vitro. Neuropharmacology 37:1279–1286.

Li R, Nishijo H, Ono T, Ohtani Y, Ohtani O. 2002. Synapses on GABAergic neurons in the basolateral nucleus of the rat amygdala: double-labeling immunoelectron microscopy. Synapse 43:42–50.

Liu YH, Wang L, Wei LC, Huang YG, Chen LW. 2009. Upregulation of D-serine might induce GABAergic neuronal degeneration in the cerebral cortex and hippocampus in the mouse pilocarpine model of epilepsy. Neurochem Res 34:1209–1218.

Loretan K, Bissiere S, Luthi A. 2004. Dopaminergic modulation of spontaneous inhibitory network activity in the lateral amygdala. Neuropharmacology 47:631–639.

Loreth D, Ozmen L, Revel FG, Knoflach F, Wetzel P, Frotscher M, Metzger F, Kretz O. 2012. Selective degeneration of septal and hippocampal GABAergic neurons in a mouse model of amyloidosis and tauopathy. Neurobiol Dis 47:1–12.

Ma QP, Yin GF, Ai MK, Han JS. 1991. Serotonergic projections from the nucleus raphe dorsalis to the amygdala in the rat. Neurosci Lett 134:21–24.

Mahanty NK, Sah P. 1998. Calcium-permeable AMPA receptors mediate long-term potentiation in interneurons in the amygdala. Nature 394: 683–687.

Manko M, Geracitano R, Capogna M. 2011. Functional connectivity of the main intercalated nucleus of the mouse amygdala. J Physiol 589: 1911–1925.

Marowsky A, Fritschy JM, Vogt KE. 2004. Functional mapping of GABA A receptor subtypes in the amygdala. Eur J Neurosci 20:1281–1289.

Marowsky A, Yanagawa Y, Obata K, Vogt KE. 2005. A specialized subclass of interneurons mediates dopaminergic facilitation of amygdala function. Neuron 48:1025–1037.

Marowsky A, Rudolph U, Fritschy JM, Arand M. 2012. Tonic inhibition in principal cells of the amygdala: a central role for alpha3 subunit-containing $GABA_A$ receptors. J Neurosci 32:8611–8619.

Martin BS, Corbin JG, Huntsman MM. 2014. Deficient tonic GABAergic conductance and synaptic balance in the fragile X syndrome amygdala. J Neurophysiol 112:890–902.

Mascagni F, McDonald AJ. 2003. Immunohistochemical characterization of cholecystokinin-containing neurons in the rat basolateral amygdala. Brain Res 976:171–184.

Mascagni F, McDonald AJ. 2007. A novel subpopulation of 5-HT type 3A receptor subunit immunoreactive interneurons in the rat basolateral amygdala. Neuroscience 144:1015–1024.

Mascagni F, McDonald AJ. 2009. Parvalbumin-immunoreactive neurons and GABAergic neurons of the basal forebrain project to the rat basolateral amygdala. Neuroscience 160:805–812.

Mash DC, Potter LT. 1986. Autoradiographic localization of M1 and M2 muscarine receptors in the rat brain. Neuroscience 19:551–564.

McDonald A. 1992. Cell types and intrinsic connections of the amygdala. In: Aggleton J, editor. The amygdala: neurobiological aspects of emotion, memory, and mental dysfunction. New York: Wiley-Liss.

McDonald AJ, Mascagni F. 2001a. Colocalization of calcium-binding proteins and GABA in neurons of the rat basolateral amygdala. Neuroscience 105:681–693.

McDonald AJ, Mascagni F. 2001b. Localization of the CB1 type cannabinoid receptor in the rat basolateral amygdala: high concentrations in a subpopulation of cholecystokinin-containing interneurons. Neuroscience 107:641–652.

McDonald AJ, Mascagni F. 2002. Immunohistochemical characterization of somatostatin-containing interneurons in the rat basolateral amygdala. Brain Res 943:237–244.

McDonald AJ, Mascagni F. 2004. Parvalbumin-containing interneurons in the basolateral amygdala express high levels of the alpha1 subunit of the GABA$_A$ receptor. J Comp Neurol 473:137–146.

McDonald AJ, Mascagni F. 2007. Neuronal localization of 5-HT type 2A receptor immunoreactivity in the rat basolateral amygdala. Neuroscience 146:306–320.

McDonald AJ, Mascagni F. 2010. Neuronal localization of m1 muscarinic receptor immunoreactivity in the rat basolateral amygdala. Brain Struct Funct 215:37–48.

McDonald AJ, Mascagni F. 2011. Neuronal localization of M2 muscarinic receptor immunoreactivity in the rat amygdala. Neuroscience 196:49–65.

McDonald AJ, Pearson JC. 1989. Coexistence of GABA and peptide immunoreactivity in nonpyramidal neurons of the basolateral amygdala. Neurosci Lett 100:53–58.

McDonald AJ, Mascagni F, Muller JF. 2004. Immunocytochemical localization of GABA$_B$R1 receptor subunits in the basolateral amygdala. Brain Res 1018:147–158.

McDonald AJ, Muller JF, Mascagni F. 2011. Postsynaptic targets of GABAergic basal forebrain projections to the basolateral amygdala. Neuroscience 183:144–159.

McDonough JH Jr, McLeod CG Jr, Nipwoda MT. 1987. Direct microinjection of soman or VX into the amygdala produces repetitive limbic convulsions and neuropathology. Brain Res 435:123–137.

Miller LA, McLachlan RS, Bouwer MS, Hudson LP, Munoz DG. 1994. Amygdalar sclerosis: preoperative indicators and outcome after temporal lobectomy. J Neurol Neurosurg Psychiatry 57:1099–1105.

Miller MI, Younes L, Ratnanather JT, Brown T, Trinh H, Lee DS, Tward D, Mahon PB, Mori S, Albert M, Team BR. 2015. Amygdalar atrophy in symptomatic Alzheimer's disease based on diffeomorphometry: the BIOCARD cohort. Neurobiol Aging 36(Suppl 1):S3–S10.

Millhouse OE, DeOlmos J. 1983. Neuronal configurations in lateral and basolateral amygdala. Neuroscience 10:1269–1300.

Miyajima M, Ozaki M, Wada K, Sekiguchi M. 2010. Noradrenaline-induced spontaneous inhibitory postsynaptic currents in mouse basolateral nucleus of amygdala pyramidal neurons: comparison with dopamine-induced currents. Neurosci Lett 480:167–172.

Mizukami K, Ikonomovic MD, Grayson DR, Sheffield R, Armstrong DM. 1998. Immunohistochemical study of GABA$_A$ receptor alpha1 subunit in the hippocampal formation of aged brains with Alzheimer-related neuropathologic changes. Brain Res 799:148–155.

Mohler H. 2006. GABAA receptors in central nervous system disease: anxiety, epilepsy, and insomnia. J Recept Signal Transduct Res 26:731–740.

Muller JF, Mascagni F, McDonald AJ. 2003. Synaptic connections of distinct interneuronal subpopulations in the rat basolateral amygdalar nucleus. J Comp Neurol 456:217–236.

Muller JF, Mascagni F, McDonald AJ. 2005. Coupled networks of parvalbumin-immunoreactive interneurons in the rat basolateral amygdala. J Neurosci 25:7366–7376.

Muller JF, Mascagni F, McDonald AJ. 2006. Pyramidal cells of the rat basolateral amygdala: synaptology and innervation by parvalbumin-immunoreactive interneurons. J Comp Neurol 494:635–650.

Muller JF, Mascagni F, McDonald AJ. 2007a. Postsynaptic targets of somatostatin-containing interneurons in the rat basolateral amygdala. J Comp Neurol 500:513–529.

Muller JF, Mascagni F, McDonald AJ. 2007b. Serotonin-immunoreactive axon terminals innervate pyramidal cells and interneurons in the rat basolateral amygdala. J Comp Neurol 505:314–335.

Muller JF, Mascagni F, McDonald AJ. 2011. Cholinergic innervation of pyramidal cells and parvalbumin-immunoreactive interneurons in the rat basolateral amygdala. J Comp Neurol 519:790–805.

Muller JF, Mascagni F, Zaric V, McDonald AJ. 2013. Muscarinic cholinergic receptor M1 in the rat basolateral amygdala: ultrastructural localization and synaptic relationships to cholinergic axons. J Comp Neurol 521:1743–1759.

Muller I, Caliskan G, Stork O. 2015. The GAD65 knock out mouse: a model for GABAergic processes in fear- and stress-induced psychopathology. Genes Brain Behav 14:37–45.

Nuss P. 2015. Anxiety disorders and GABA neurotransmission: a disturbance of modulation. Neuropsychiatr Dis Treat 11:165–175.

Ohshiro H, Kubota S, Murakoshi T. 2011. Dopaminergic modulation of oscillatory network inhibition in the rat basolateral amygdala depends on initial activity state. Neuropharmacology 61:857–866.

Olmos-Serrano JL, Paluszkiewicz SM, Martin BS, Kaufmann WE, Corbin JG, Huntsman MM. 2010. Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. J Neurosci 30:9929–9938.

Olsen RW, Sieghart W. 2009. GABA A receptors: subtypes provide diversity of function and pharmacology. Neuropharmacology 56:141–148.

Palop JJ, Mucke L. 2010. Synaptic depression and aberrant excitatory network activity in Alzheimer's disease: two faces of the same coin? Neuromol Med 12:48–55.

Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, Yoo J, Ho KO, Yu GQ, Kreitzer A, Finkbeiner S, Noebels JL, Mucke L. 2007. Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. Neuron 55:697–711.

Paluszkiewicz SM, Martin BS, Huntsman MM. 2011a. Fragile X syndrome: the GABAergic system and circuit dysfunction. Dev Neurosci 33:349–364.

Paluszkiewicz SM, Olmos-Serrano JL, Corbin JG, Huntsman MM. 2011b. Impaired inhibitory control of cortical synchronization in fragile X syndrome. J Neurophysiol 106:2264–2272.

Pan BX, Dong Y, Ito W, Yanagawa Y, Shigemoto R, Morozov A. 2009. Selective gating of glutamatergic inputs to excitatory neurons of amygdala by presynaptic GABAb receptor. Neuron 61:917–929.

Pare D, Gaudreau H. 1996. Projection cells and interneurons of the lateral and basolateral amygdala: distinct firing patterns and differential relation to theta and delta rhythms in conscious cats. J Neurosci 16:3334–3350.

Pare D, Smith Y. 1998. Intrinsic circuitry of the amygdaloid complex: common principles of organization in rats and cats. Trends Neurosci 21:240–241.

Pare D, Pape HC, Dong J. 1995. Bursting and oscillating neurons of the cat basolateral amygdaloid complex in vivo: electrophysiological properties and morphological features. J Neurophysiol 74:1179–1191.

Parikh MS, Kolevzon A, Hollander E. 2008. Psychopharmacology of aggression in children and adolescents with autism: a critical review of efficacy and tolerability. J Child Adolesc Psychopharmacol 18:157–178.

Pertwee RG. 1997. Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacol Ther 74:129–180.

Pidoplichko VI, Prager EM, Aroniadou-Anderjaska V, Braga MF. 2013. α7-Containing nicotinic acetylcholine receptors on interneurons of the basolateral amygdala and their role in the regulation of the network excitability. J Neurophysiol 110:2358–2369.

Pidoplichko VI, Aroniadou-Anderjaska V, Prager EM, Figueiredo TH, Almeida-Suhett CP, Miller SL, Braga MF. 2014. ASIC1a activation enhances inhibition in the basolateral amygdala and reduces anxiety. J Neurosci 34:3130–3141.

Pinard CR, Muller JF, Mascagni F, McDonald AJ. 2008. Dopaminergic innervation of interneurons in the rat basolateral amygdala. Neuroscience 157:850–863.

Pitkanen A. 2000. Connectivity of the rat amygdaloid complex. In: Aggleton J, editor. The amygdala: a functional analysis. Oxford: Oxford University Press.

Pitkanen A, Tuunanen J, Kalviainen R, Partanen K, Salmenpera T. 1998. Amygdala damage in experimental and human temporal lobe epilepsy. Epilepsy Res 32:233–253.

Polepalli JS, Sullivan RK, Yanagawa Y, Sah P. 2010. A specific class of interneuron mediates inhibitory plasticity in the lateral amygdala. J Neurosci 30:14619–14629.

Pouille F, Scanziani M. 2001. Enforcement of temporal fidelity in pyramidal cells by somatic feedforward inhibition. Science 293:1159–1163.

Poulin SP, Dautoff R, Morris JC, Barrett LF, Dickerson BC, Alzheimer's Disease Neuroimaging Initiative. 2011. Amygdala atrophy is prominent in early Alzheimer's disease and relates to symptom severity. Psychiatry Res 194:7–13.

Prager EM, Aroniadou-Anderjaska V, Almeida-Suhett CP, Figueiredo TH, Apland JP, Braga MF. 2013. Acetylcholinesterase inhibition in the basolateral amygdala plays a key role in the induction of status epilepticus after soman exposure. Neurotoxicology 38:84–90.

Prager EM, Aroniadou-Anderjaska V, Almeida-Suhett CP, Figueiredo TH, Apland JP, Rossetti F, Olsen CH, Braga MF. 2014a. The recovery of acetylcholinesterase activity and the progression of neuropathological and pathophysiological alterations in the rat basolateral amygdala after soman-induced status epilepticus: relation to anxiety-like behavior. Neuropharmacology 81:64–74.

Prager EM, Pidoplichko VI, Aroniadou-Anderjaska V, Apland JP, Braga MF. 2014b. Pathophysiological mechanisms underlying increased anxiety after soman exposure: reduced GABAergic inhibition in the basolateral amygdala. Neurotoxicology 44:335–343.

Prager EM, Figueiredo TH, Long RP 2nd, Aroniadou-Anderjaska V, Apland JP, Braga MF. 2015. LY293558 prevents soman-induced pathophysiological alterations in the basolateral amygdala and the development of anxiety. Neuropharmacology 89:11–18.

Pugliese M, Carrasco JL, Geloso MC, Mascort J, Michetti F, Mahy N. 2004. Gamma-aminobutyric acidergic interneuron vulnerability to aging in canine prefrontal cortex. J Neurosci Res 77:913–920.

Rainnie DG. 1999. Serotonergic modulation of neurotransmission in the rat basolateral amygdala. J Neurophysiol 82:69–85.

Rainnie DG, Asprodini EK, Shinnick-Gallagher P. 1991a. Excitatory transmission in the basolateral amygdala. J Neurophysiol 66:986–998.

Rainnie DG, Asprodini EK, Shinnick-Gallagher P. 1991b. Inhibitory transmission in the basolateral amygdala. J Neurophysiol 66:999–1009.

Rainnie DG, Asprodini EK, Shinnick-Gallagher P. 1992. Kindling-induced long-lasting changes in synaptic transmission in the basolateral amygdala. J Neurophysiol 67:443–454.

Rainnie DG, Mania I, Mascagni F, McDonald AJ. 2006. Physiological and morphological characterization of parvalbumin-containing interneurons of the rat basolateral amygdala. J Comp Neurol 498:142–161.

Rauch SL, Shin LM, Phelps EA. 2006. Neurocircuitry models of posttraumatic stress disorder and extinction: human neuroimaging research—past, present, and future. Biol Psychiatry 60:376–382.

Reger ML, Poulos AM, Buen F, Giza CC, Hovda DA, Fanselow MS. 2012. Concussive brain injury enhances fear learning and excitatory processes in the amygdala. Biol Psychiatry 71:335–343.

Rosenkranz JA, Grace AA. 1999. Modulation of basolateral amygdala neuronal firing and afferent drive by dopamine receptor activation in vivo. J Neurosci 19:11027–11039.

Sah P, Faber ES, Lopez De Armentia M, Power J. 2003. The amygdaloid complex: anatomy and physiology. Physiol Rev 83:803–834.

Sanders SK, Shekhar A. 1995. Regulation of anxiety by GABA$_A$ receptors in the rat amygdala. Pharmacol Biochem Behav 52:701–706.

Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE. 1997. Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol Appl Pharmacol 142:278–287.

Schiffer HH, Swanson GT, Heinemann SF. 1997. Rat GluR7 and a carboxy-terminal splice variant, GluR7b, are functional kainate receptor subunits with a low sensitivity to glutamate. Neuron 19:1141–1146.

Schlicker E, Kathmann M. 2001. Modulation of transmitter release via presynaptic cannabinoid receptors. Trends Pharmacol Sci 22:565–572.

Seamans JK, Gorelova N, Durstewitz D, Yang CR. 2001. Bidirectional dopamine modulation of GABAergic inhibition in prefrontal cortical pyramidal neurons. J Neurosci 21:3628–3638.

Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW. 1993. Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13:596–604.

Semyanov A, Walker MC, Kullmann DM, Silver RA. 2004. Tonically active GABA A receptors: modulating gain and maintaining the tone. Trends Neurosci 27:262–269.

Shoji Y, Tanaka E, Yamamoto S, Maeda H, Higashi H. 1998. Mechanisms underlying the enhancement of excitatory synaptic transmission in basolateral amygdala neurons of the kindling rat. J Neurophysiol 80:638–646.

Silberman Y, Ariwodola OJ, Chappell AM, Yorgason JT, Weiner JL. 2010. Lateral paracapsular GABAergic synapses in the basolateral amygdala contribute to the anxiolytic effects of beta 3 adrenoceptor activation. Neuropsychopharmacology 35:1886–1896.

Silberman Y, Ariwodola OJ, Weiner JL. 2012. β1-Adrenoceptor activation is required for ethanol enhancement of lateral paracapsular GABAergic synapses in the rat basolateral amygdala. J Pharmacol Exp Ther 343:451–459.

Skorzewska A, Lehner M, Wislowska-Stanek A, Krzascik P, Ziemba A, Plaznik A. 2014. The effect of chronic administration of corticosterone on anxiety- and depression-like behavior and the expression of GABA-A receptor alpha-2 subunits in brain structures of low- and high-anxiety rats. Horm Behav 65:6–13.

Smith BN, Dudek FE. 1997. Enhanced population responses in the basolateral amygdala of kainate-treated, epileptic rats in vitro. Neuroscience Lett 222:1–4.

Smith TA. 2001. Type A gamma-aminobutyric acid (GABA$_A$) receptor subunits and benzodiazepine binding: significance to clinical syndromes and their treatment. Br J Biomed Sci 58:111–121.

Smith Y, Pare JF, Pare D. 1998. Cat intraamygdaloid inhibitory network: ultrastructural organization of parvalbumin-immunoreactive elements. J Comp Neurol 391:164–179.

Smith Y, Pare JF, Pare D. 2000. Differential innervation of parvalbumin-immunoreactive interneurons of the basolateral amygdaloid complex by cortical and intrinsic inputs. J Comp Neurol 416:496–508.

Spampanato J, Polepalli J, Sah P. 2011. Interneurons in the basolateral amygdala. Neuropharmacology 60:765–773.

Stein C, Davidowa H, Albrecht D. 2000. 5-HT(1A) receptor-mediated inhibition and 5-HT$_2$ as well as 5-HT$_3$ receptor-mediated excitation in different subdivisions of the rat amygdala. Synapse 38:328–337.

Stujenske JM, Likhtik E, Topiwala MA, Gordon JA. 2014. Fear and safety engage competing patterns of theta-gamma coupling in the basolateral amygdala. Neuron 83:919–933.

Sugita S, Tanaka E, North RA. 1993. Membrane properties and synaptic potentials of three types of neurone in rat lateral amygdala. J Physiol 460:705–718.

Acetaminophen Use for Fever in Children Associated with Autism Spectrum Disorder

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Abstract

Autism Spectrum Disorder (ASD) is characterized by persistent deficits in social communication and restrictive behavior, interests, and activities. Our previous case-control study showed that use of acetaminophen at age 12–18 months is associated with increased likelihood for ASD (OR 8.37, 95% CI 2.08–33.7). In this study, we again show that acetaminophen use is associated with ASD (p = 0.013). Because these children are older than in our first study, the association is reversed; fewer children with ASD vs. non-ASD children use acetaminophen as a "first choice" compared to "never use" (OR 0.165, 95% CI 0.045, 0.599). We found significantly more children with ASD vs. non- ASD children change to the use of ibuprofen when acetaminophen is not effective at reducing fever (p = 0.033) and theorize this change in use is due to endocannabinoid system dysfunction. We also found that children with ASD vs. non-ASD children are significantly more likely to show an increase in sociability when they have a fever (p = 0.037) and theorize that this increase is due to anandamide activation of the endocannabinoid system in ASD children with low endocannabinoid tone from early acetaminophen use. In light of this we recommend that acetaminophen use be reviewed for safety in children.

Keywords

Autism; Autism spectrum disorder; Acetaminophen; Anandamide; Endocannabinoid; Cannabinoid; Fever; Medication

Introduction

Autism Spectrum Disorder (ASD), as defined by the Diagnostic and Treatment Manual for Mental Disorders, Fifth Edition (DSM-5), is characterized by persistent deficits in social communication and interaction and restricted-repetitive patterns of behavior, interests, or activities. These symptoms manifest in early childhood, and produce clinically significant developmental impairment [1]. Many children with ASD share traits of Attention Deficit/

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hyperactivity disorder (ADHD) and epilepsy is comorbid with ASD in an estimated 20–25% of cases [1–4]. Some features of ASD, commonly called autism or autistic disorder, are seen in genetic and chromosomal abnormalities such as fragile X syndrome, Down syndrome, and genomic insertions and deletions; however, most cases of ASD have an unknown etiology. Two of the prominent clinical features of ASD are immune system dysregulation and abnormal brain connectivity [5–7]. The US Centers for Disease Control and Prevention (CDC) estimates that ASD occurs in one of every 68 children in the US, while the National Health Interview Survey puts the estimate of ASD higher with one child affected out of every 45 children aged 3–17 years in the US [8,9].

Recently it has been shown that more than half of ASD cases are attributable to environmental factors [10]. A subset of children with ASD undergo a period of apparently normal development followed by a regression in development [11]. Since children with regression in development did not initially manifest ASD features, they may have been more likely influenced by drugs or other environmental exposures. In our own data, regression was featured in 38% of cases, and we were able to show an increased likelihood for ASD from acetaminophen use, which was higher in children with regression [12]. Prenatal and perinatal use of acetaminophen, also known as paracetamol, was linked to ASD in an ecological study [13]. We have theorized that acetaminophen disruption of the endocannabinoid system may underlie some of the increased likelihood for ASD, particularly in children with genetically compromised primary conjugation pathways that normally metabolize this drug [14].

Acetaminophen is one of three analgesics derived from aniline dye; the others, acetanilide and phenacetin, have been discontinued due to side effects [15]. Although acetaminophen has been used as an analgesic for more than a hundred years, its mechanism of action was unclear. It has now been shown that acetaminophen produces analgesia by acting in the brain as an indirect agonist at cannabinoid receptors through conversion of the acetaminophen metabolite p-aminophenol to N-arachidonoylaminophenol (AM404) which inhibits the reuptake of anandamide [15–17]. Blocking cannabinoid receptors completely eliminates the analgesic effect of acetaminophen [15,18].

We have shown in a case-control study that use of acetaminophen early in life is associated with increased likelihood for ASD [12]. We showed in this study that children who used acetaminophen at age 12 to 18 months vs. those who did not were eight times more likely to have ASD when all children were considered and nearly 21 times more likely to have ASD when limiting cases to children with regression in development. Ibuprofen use at age 12 to 18 months was not significantly associated with ASD for either of these groups.

In a later report we showed that the events in the history of acetaminophen use were related to the number of children with ASD [19]. In this report, acetaminophen history was linked with the number of eligible persons with autism by birth year from a 1999 report to the legislature by the California Department of Developmental Services. We showed that the number of children with ASD greatly increased after the CDC issued a warning against using aspirin for children's fever in 1980. We further showed separate decreases in the number of children with ASD born after highly publicized incidents in 1982 and 1986 where

acetaminophen capsules were laced with cyanide in the US. These incidents caused precipitous declines in acetaminophen sales.

To continue investigating whether ASD is associated with acetaminophen use, we searched for available data which described acetaminophen use in older children with and without ASD. In 2015, we discovered data to re-test our ASD-acetaminophen link in children. The current study was undertaken to determine if acetaminophen use for fever in older children was associated with ASD.

Materials and Methods

Our data source was the National Database for Autism Research (NDAR) of the National Institute of Mental Health (NIMH) which is one of the institutes in the National Institutes of Health (NIH) in the US. Approval for use of this data was obtained from NIMH and the University of Texas Health Science Center San Antonio. The data we chose to use was from the study entitled "Association between pupillary light reflex and sensory behaviors in children with autism spectrum disorders." This de-identified data contained information on ASD diagnoses and over-the-counter medications used to treat fever. The selection criteria for these children may be found in the 2013 paper by Daluwatte et al. [20].

The diagnosis of ASD in our data was listed as confirmed by testing the children with the Autism Diagnostic Observation Schedule (ADOS) or the Autism Diagnostic Interview-Revised (ADI-R). In order to focus on the effect of environmental exposures, only children with ASD and without known genetic disease were included as cases. Genetic diseases linked to ASD which were excluded include fragile X syndrome, Down Syndrome, tuberous sclerosis, Rett Syndrome, and known ASD-linked chromosome insertions and deletions. After these exclusions, we obtained information concerning 155 children with ASD and 154 non-ASD children. We further limited our data analysis to the individuals for whom information regarding acetaminophen use for fevers was available which yielded 118 case children with ASD and 79 control children.

SPSS version 23 for Windows was used for all statistical analyses unless otherwise noted. Chi square tests were used to determine whether fever medication use was associated with ASD. Age at time of interview was tested for association with ASD by ANOVA. Logistic regression modeling was used to produce age-adjusted models for levels of fever medication use in children with ASD compared to children without ASD.

Results

Table 1 shows the characteristics of children in our study. We limited our dataset to children with information regarding acetaminophen use for fevers. However, some of the other questions analyzed were answered by fewer respondents. Therefore for clarity, the number of cases and controls available for analysis of each question is listed in the table. There was no significant difference in the ages of the children, mean 131 months for cases and 135 months for controls. Acetaminophen use for fevers was significantly different for cases compared to controls ($p = 0.013$). Ibuprofen use for fevers was not significantly different between cases and controls ($p = 0.570$). Aspirin use for fevers was extremely low in both

cases and controls; a total of four children were reported to ever use aspirin, and no comparative analysis was possible for aspirin use. Compared to control children, the frequency of fevers showed a trend for increasing rate in case children ($p = 0.057$), and case children were significantly more likely to show better social interaction when experiencing a fever ($p = 0.037$).

Table 2 shows the results of age-adjusted logistic regression models for three levels of acetaminophen and ibuprofen use compared to never or rarely use in case children with ASD vs. control children without ASD. Using acetaminophen as a first choice was 83% less likely in children with ASD (OR 0.165, 95% CI 0.045, 0.599) while use of acetaminophen if other medication doesn't bring down fever was 82% less likely in children with ASD (OR 0.183, 95% CI 0.050, 0.675). Using only acetaminophen for fever showed a non-significant negative trend with a p value of 0.065. There was no significant difference in the three levels of ibuprofen use between cases and controls.

Table 3 shows the use of ibuprofen if acetaminophen doesn't bring down fever vs. rarely or never use ibuprofen for children with ASD vs. non-ASD children while limiting the analysis to children who use acetaminophen as first choice. A significantly higher number of children with ASD vs. non-ASD children used ibuprofen to bring down their fever vs. rarely or never using ibuprofen ($p = 0.033$). This result indicates that significantly more children with ASD vs non-ASD children switch to the use of ibuprofen if acetaminophen does not bring down their fever.

Discussion

As seen in Table 1, the use of acetaminophen in children with ASD compared to control children was still significantly different in the current study as it was in our 2008 study. However, as seen in Table 2, the association direction seen in our current study for acetaminophen use is opposite to our previous results which asked about acetaminophen use in young children. In our current study, older children with ASD compared to control children were significantly less likely to use acetaminophen for fever; whereas, in our 2008 study, younger children with ASD compared to control children were significantly more likely to use acetaminophen at 12–18 months of age and after the MMR vaccination. If we consider that early use of acetaminophen may be responsible for endocannabinoid system dysfunction, this could result in acetaminophen losing effectiveness. In this case, the results we found in older children are to be expected.

We have shown that the acetaminophen metabolites AM404 and p-aminophenol are toxic for mouse embryonic cortical neurons [21]. AM404 increases brain endocannabinoid levels by decreasing the re-uptake of anandamide [15]. We have further shown that acetaminophen differentially changes social behavior in adult male black and tan brachury tufted (BTBR) mice, a commonly used mouse model of behavioral traits of autism [22]. Neonatal exposure to acetaminophen affects cognitive function and reduces its analgesic and anxiolytic response in adult male mice and in our lab produced long-lasting immune system changes [23–25].

As we saw in our 2008 study, the use of ibuprofen in the current study is not significantly different for children with ASD vs. non-ASD children, although the use of ibuprofen has now increased from 56% to 87%. The children in our current study are older on average than our first study, 11 years vs. 7.5 years, and parents would have time to switch to a different anti-pyretic drug for their children if acetaminophen is no longer effective. We have shown in Table 3 that children with ASD vs. non-ASD children are significantly ($p = 0.033$) more likely to switch to the use of ibuprofen if acetaminophen doesn't bring down their fever. The reasons for this reversal in analgesic use seen in our current study for children with ASD vs. non-ASD children could be a decrease in acetaminophen effectiveness due to endocannabinoid system dysfunction.

The endocannabinoid system plays a key role in the development of the central nervous system and its activation can induce long-lasting functional alterations [26]. Use of the exogenous cannabinoid tetrahydrocannabinol in the still-maturing brain may produce persistent alterations in brain structure and cognition [27]. Animal models have revealed the danger of both cannabis abuse and exposure to cannabinoid drugs during brain development [28,29].

Dysfunction of the endocannabinoid system can occur through either of the two classic cannabinoid receptors, CB1 or CB2. CB1 receptors are primarily located in the central nervous system (CNS) and are concentrated in the cerebellum, hippocampus, and the basal ganglia which are areas in the brain implicated as dysfunctional in ASD [30,31]. It has been demonstrated that during fetal life, CB1 receptors and their associated endocannabinoids provide axon guidance cues and are responsible for synaptogenesis [32–34]. Children with ASD have been shown to have abnormal brain connectivity which could be due to lack of CB1 axon guidance [7].

CB2 receptors are primarily located on immune system cells and serve a regulatory function. CB2 receptors have been shown to control the movement of inflammatory cells to the site of injury, and CB2 receptors' reverse agonists may serve as immune system modulators [35]. CB2 receptor agonists reduce transendothelial migration of monocytes by interfering with endothelial adhesion [36]. It has been shown in many studies that children with ASD have immune system dysregulation [37–43]. This dysregulation includes differential monocyte responses, abnormal cytokine levels, decreased T cell mitogen response, decreased numbers of lymphocytes, and abnormal serum immunoglobulin levels. Other studies have shown that children with ASD exhibit abnormal antibodies against brain and central nervous system proteins [44–50] and increased plasma pro-inflammatory cytokine levels [51]. These problems could be due to dysregulation of the immune system through CB2 receptors.

Siniscalco et al. in a 2013 landmark paper were able to confirm endocannabinoid system dysfunction in ASD vs healthy subjects by showing in peripheral blood mononuclear cells that the mRNA and protein for CB2 was significantly increased and mRNA for the gene that synthesizes anandamide, N-acylphosphatidyl-ethanolamine-hydrolyzing phospholipase D (NAPE-PLD), was significantly decreased [52]. This upregulation of CB2 receptors and downregulation of NAPE-PLD in these immune system cells indicates endocannabinoid system dysfunction in children with ASD. This dysfunction could result from insufficient

endocannabinoid system tone. Also in 2013, Földy et al. found that neuroligin-3 mutations associated with autism commonly disrupt tonic endocannabinoid signaling, providing further evidence of endocannabinoid system involvement in ASD [53].

The endocannabinoid system participates in fever generation by increasing anandamide activation of CB1 receptors [54,55]. We have shown in Table 1 that fever is associated with a significant increase in social interaction in children with ASD compared to non-ASD children. The increase in social interaction seen in this study could be due to fever producing a normalization of endocannabinoid tone in a system that has been made dysfunctional by acetaminophen use in children with ASD.

Kerr et al. have demonstrated dysfunction in the endocannabinoid system in the rat valproic acid model of autism. Alterations include reduced expression in the frontal cortex of PPARα and GPR55 and in the hippocampus of PPARγ and GPR55, which are additional receptor targets of the endocannabinoids. They found increased tissue levels of anandamide and palmitoylethanolamide (PEA) in the hippocampus after social interaction [56]. PEA is found abundantly in the central nervous system, especially in neurons and glial cells, and is a dietary supplement available without a prescription in Europe [57]. Administration of PEA has been shown to increase tissue levels of anandamide and increase activation of the endocannabinoid system [58]. PEA attenuates seizures in rats through CB1 and CB2 cannabinoid receptors [59]. Antonucci and colleagues in 2015 reported two case studies of boys with ASD in which PEA supplementation reduced inflammatory markers and produced rapid clinically significant improvements. With the success of these cases, they have recommended appropriate double-blind controlled clinical trials to further explore the potential of PEA as a treatment for ASD [60].

The warning in 1980 by the CDC against aspirin use in children has been very effective. As physicians began recommending acetaminophen as an alternative antipyretic drug, sales of children's aspirin precipitously declined beginning in 1980 and were replaced with sales of acetaminophen [61]. As seen in our current study only four children were ever given aspirin for fever. However, since the initial CDC warning against the use of aspirin for fever in children, reports have been published casting doubt on the initial studies which associate children's use of aspirin with Reye Syndrome [62–64]. As reported in an excellent review by Schrör in 2007, the attribution of Reye Syndrome to aspirin use in children was not sufficiently supported by research [65,66]. We have shown that the current rise in cases of autism began in 1980 in the US, which is the same year that the CDC warned against the use of aspirin in US children [19]. There is no good evidence that acetaminophen is superior to aspirin for use in children, and we have shown evidence that acetaminophen use is associated with ASD. We recommend that the use of acetaminophen in children be reviewed for safety. Also, the strong warning against the use of aspirin for fever in children should be reviewed.

Conclusion

In summary, we have presented evidence for the association of acetaminophen use with ASD. Our theory of how this may occur can be explained in the following illustration.

Suppose a susceptible young boy has a fever due to a viral infection or after the MMR vaccination. His parents give him acetaminophen which increases endocannabinoid stimulation in his brain making him feel better and bringing down his fever. But the increased activation of the endocannabinoid system also decreases immune system function which prolongs the illness and leads to even more acetaminophen use. Eventually, the boy recovers but his endocannabinoid system has been dysregulated to a lower level to compensate for the prolonged over-activation. Now the neurons in his brain are not getting the proper guidance for their growth through CB1 receptors and further suffer from increased inflammation due to lack of CB2 regulation in immune system cells. The boy develops ASD. When the boy gets a fever, his parents again give him acetaminophen but it no longer works well since his endocannabinoid tone is at a low level, and his parents switch to ibuprofen. Also, when he gets a fever, the increased anandamide levels briefly increase endocannabinoid tone and improve his sociability. After the fever, the endocannabinoid tone again drops back to low levels and his sociability decreases again. His condition, however, may be reversible with new cannabinoid medications to increase endocannabinoid system activation and allow his brain to slowly recover. Research needs to be conducted to see if PEA, cannabidiol, or other cannabinoids will be effective treatments for ASD.

Acknowledgments

References

1. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders. 5. American Psychiatric Publishing; 2013. Autism Spectrum Disorder, 299.00 (F84.0); p. 50-59.

2. Ronald A, Simonoff E, Kuntsi J, Asherson P, Plomin R. Evidence for overlapping genetic influences on autistic and ADHD behaviours in a community twin sample. J Child Psychol Psychiatry. 2008; 49:535–542. [PubMed: 18221348]

3. Taurines R, Schwenck C, Westerwald E, Sachse M, Siniatchkin M, et al. ADHD and autism: differential diagnosis or overlapping traits? A selective review. Atten Defic Hyperact Disord. 2012; 4:115–139. [PubMed: 22851255]

4. Canitano R. Epilepsy in autism spectrum disorders. Eur Child Adolesc Psychiatry. 2007; 16:61–66. [PubMed: 16932856]

5. Onore C, Careaga M, Ashwood P. The role of immune dysfunction in the pathophysiology of autism. Brain Behav Immun. 2012; 26:383–392. [PubMed: 21906670]

6. Xu N, Li X, Zhong Y. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. Mediators Inflamm. 2015; 2015:531518. [PubMed: 25729218]

7. McFadden K, Minshew NJ. Evidence for dysregulation of axonal growth and guidance in the etiology of ASD. Front Hum Neurosci. 2013; 7:671. [PubMed: 24155705]

8. US Centers for Disease Control and Prevention. Prevalence of Autism Spectrum Disorder Among Children Aged 8 Years — Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2010, Surveillance Summaries. Morbidity and Mortality Weekly Report. 2010; 63(SS02):1–21.

9. Zablotsky B, Black LI, Maenner MJ, Schieve LA, Blumberg SJ. Estimated Prevalence of Autism and Other Developmental Disabilities Following Questionnaire Changes in the 2014 National Health Interview Survey. National Health Statistics Reports Number. 2015; 87:1–21.

10. Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, et al. Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry. 2011; 68:1095–1102. [PubMed: 21727249]

11. Lord C, Shulman C, DiLavore P. Regression and word loss in autistic spectrum disorders. J Child Psychol Psychiatry. 2004; 45:936–955. [PubMed: 15225337]

12. Schultz ST, Klonoff-Cohen HS, Wingard DL, Akshoomoff NA, Macera CA, et al. Acetaminophen (paracetamol) use, measles-mumps-rubella vaccination, and autistic disorder: the results of a parent survey. Autism. 2008; 12:293–307. [PubMed: 18445737]

13. Bauer AZ, Kriebel D. Prenatal and perinatal analgesic exposure and autism: an ecological link. Environ Health. 2013; 12:41. [PubMed: 23656698]

14. Schultz ST. Can autism be triggered by acetaminophen activation of the endocannabinoid system? Acta Neurobiol Exp (Wars). 2010; 70:227–231. [PubMed: 20628445]

15. Bertolini A, Ferrari A, Ottani A, Guerzoni S, Tacchi R, et al. Paracetamol: new vistas of an old drug. CNS Drug Rev. 2006; 12:250–275. [PubMed: 17227290]

16. Högestätt ED, Jönsson BA, Ermund A, Andersson DA, Björk H, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J Biol Chem. 2005; 280:31405–31412. [PubMed: 15987694]

17. Mallet C, Daulhac L, Bonnefont J, Ledent C, Etienne M, et al. Endocannabinoid and serotonergic systems are needed for acetaminophen-induced analgesia. Pain. 2008; 139:190–200. [PubMed: 18485596]

18. Ottani A, Leone S, Sandrini M, Ferrari A, Bertolini A. The analgesic activity of paracetamol is prevented by the blockade of cannabinoid CB1 receptors. Eur J Pharmacol. 2006; 531:280–281. [PubMed: 16438952]

19. Becker KG, Schultz ST. Similarities in features of autism and asthma and a possible link to acetaminophen use. Med Hypotheses. 2010; 74:7–11. [PubMed: 19748189]

20. Daluwatte C, Miles JH, Christ SE, Beversdorf DQ, Takahashi TN, et al. Atypical pupillary light reflex and heart rate variability in children with autism spectrum disorder. J Autism Dev Disord. 2013; 43:1910–1925. [PubMed: 23248075]

21. Schultz S, DeSilva M, Gu TT, Qiang M, Whang K. Effects of the analgesic acetaminophen (paracetamol) and its para-aminophenol metabolite on viability of mouse-cultured cortical neurons. Basic Clin Pharmacol Toxicol. 2012; 110:141–144. [PubMed: 21771276]

22. Gould GG, Seillier A, Weiss G, Giuffrida A, Burke TF, et al. Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. Prog Neuropsychopharmacol Biol Psychiatry. 2012; 38:260–269. [PubMed: 22542870]

23. Gould, GG.; Smolik, CM.; Gu, TT.; Vitela, M.; Valdez, M., et al. Early juvenile exposure to high-dose acetaminophen impacts adult murine social behavior in a strain dependent manner. The Toxicologist: Supplement to Toxicological Sciences 51st Annual Meeting of the Society of Toxicology; San Francisco, CA. 2012; 2012.

24. Viberg H, Eriksson P, Gordh T, Fredriksson A. Paracetamol (acetaminophen) administration during neonatal brain development affects cognitive function and alters its analgesic and anxiolytic response in adult male mice. Toxicological Sciences. 2014; 138:139–147. [PubMed: 24361869]

25. Edwards, MT.; Gould, GG.; Alvarez, R.; Schultz, ST. Early Juvenile exposure of mice to high-dose acetaminophen confers elevated serum eotaxin levels and sociability impairments in adulthood. The Toxicologist: Supplement to Toxicological Sciences, 55th Annual Meeting of the Society of Toxicology; New Orleans, LA. 2016; 2016.

26. Campolongo P, Trezza V, Palmery M, Trabace L, Cuomo V. Developmental exposure to cannabinoids causes subtle and enduring neurofunctional alterations. Int Rev Neurobiol. 2009; 85:117–133. [PubMed: 19607965]

27. Jager G, Ramsey NF. Long-term consequences of adolescent cannabis exposure on the development of cognition, brain structure and function: an overview of animal and human research. Curr Drug Abuse Rev. 2008; 1:114–123. [PubMed: 19630711]

28. Anavi-Goffer S, Mulder J. The polarised life of the endocannabinoid system in CNS development. Chembiochem. 2009; 10:1591–1598. [PubMed: 19533710]

29. Drysdale AJ, Platt B. Cannabinoids: mechanisms and therapeutic applications in the CNS. Curr Med Chem. 2003; 10:2719–2732. [PubMed: 14529462]

30. Bauman ML, Kemper TL. Neuroanatomic observations of the brain in autism: a review and future directions. Int J Dev Neurosci. 2005; 23:183–187. [PubMed: 15749244]

31. Ecker C, Bookheimer SY, Murphy DG. Neuroimaging in autism spectrum disorder: brain structure and function across the lifespan. Lancet Neurol. 2015; 14:1121–1134. [PubMed: 25891007]

32. Berghuis P, Rajnicek AM, Morozov YM, Ross RA, Mulder J, et al. Hardwiring the brain: endocannabinoids shape neuronal connectivity. Science. 2007; 316:1212–1216. [PubMed: 17525344]

33. Harkany T, Mackie K, Doherty P. Wiring and firing neuronal networks: endocannabinoids take center stage. Curr Opin Neurobiol. 2008; 18:338–345. [PubMed: 18801434]

34. Fride E, Gobshtis FE, Dahan H, Weller A, Giuffrida A, et al. The endocannabinoid system during development: emphasis on perinatal events and delayed effects. Vitam Horm. 2009; 81:139–158. [PubMed: 19647111]

35. Lunn CA, Reich EP, Fine JS, Lavey B, Kozlowski JA, et al. Biology and therapeutic potential of cannabinoid CB2 receptor inverse agonists. Br J Pharmacol. 2008; 153:226–239. [PubMed: 17906679]

36. Rajesh M, Mukhopadhyay P, Bátkai S, Haskó G, Liaudet L, et al. CB2-receptor stimulation attenuates TNF-alpha-induced human endothelial cell activation, transendothelial migration of monocytes, and monocyte endothelial adhesion. Am J Physiol Heart Circ Physiol. 2007; 293:H2210–H2218. [PubMed: 17660390]

37. Enstrom AM, Onore CE, Van de Water JA, Ashwood P. Differential monocyte responses to TLR ligands in children with autism spectrum disorders. Brain Behav Immun. 2010; 24:64–71. [PubMed: 19666104]

38. Ashwood P, Wills S, Van de Water J. The immune response in autism: a new frontier for autism research. J Leukoc Biol. 2006; 80:1–15. [PubMed: 16698940]

39. Warren RP, Singh VK, Averett RE, Odell JD, Maciulis A, et al. Immunogenetic studies in autism and related disorders. Mol Chem Neuropathol. 1996; 28:77–81. [PubMed: 8871944]

40. Jyonouchi H, Geng L, Ruby A, Zimmerman-Bier B. Dysregulated innate immune responses in young children with autism spectrum disorders: their relationship to gastrointestinal symptoms and dietary intervention. Neuropsychobiology. 2005; 51:77–85. [PubMed: 15741748]

41. Molloy CA, Morrow AL, Meinzen-Derr J, Schleifer K, Dienger K, et al. Elevated cytokine levels in children with autism spectrum disorder. J Neuroimmunol. 2006; 172:198–205. [PubMed: 16360218]

42. Li X, Chauhan A, Sheikh AM, Patil S, Chauhan V, et al. Elevated immune response in the brain of autistic patients. J Neuroimmunol. 2009; 207:111–116. [PubMed: 19157572]

43. Krakowiak P, Goines PE, Tancredi DJ, Ashwood P, Hansen RL, et al. Neonatal Cytokine Profiles Associated with Autism Spectrum Disorder. Biol Psychiatry. 2015

44. Ashwood P, Van de Water J. Is autism an autoimmune disease? Autoimmun Rev. 2004; 3:557–562. [PubMed: 15546805]

45. Martin LA, Ashwood P, Braunschweig D, Cabanlit M, Van de Water J, et al. Stereotypies and hyperactivity in rhesus monkeys exposed to IgG from mothers of children with autism. Brain Behav Immun. 2008; 22:806–816. [PubMed: 18262386]

46. Cohly HH, Panja A. Immunological findings in autism. Int Rev Neurobiol. 2005; 71:317–341. [PubMed: 16512356]

47. Connolly AM, Chez MG, Pestronk A, Arnold ST, Mehta S, et al. Serum autoantibodies to brain in Landau-Kleffner variant, autism, and other neurologic disorders. J Pediatr. 1999; 134:607–613. [PubMed: 10228297]

48. Singh VK, Warren RP, Odell JD, Warren WL, Cole P. Antibodies to myelin basic protein in children with autistic behavior. Brain Behav Immun. 1993; 7:97–103. [PubMed: 7682457]

49. Wills S, Cabanlit M, Bennett J, Ashwood P, Amaral D, et al. Autoantibodies in autism spectrum disorders (ASD). Ann N Y Acad Sci. 2007; 1107:79–91. [PubMed: 17804535]

50. Kawashti MI, Amin OR, Rowehy NG. Possible immunological disorders in autism: concomitant autoimmunity and immune tolerance. Egypt J Immunol. 2006; 13:99–104. [PubMed: 17974154]

51. Ashwood P, Krakowiak P, Hertz-Picciotto I, Hansen R, Pessah I, et al. Elevated plasma cytokines in autism spectrum disorders provide evidence of immune dysfunction and are associated with impaired behavioral outcome. Brain Behav Immun. 2011; 25:40–5. [PubMed: 20705131]

52. Siniscalco D, Sapone A, Giordano C, Cirillo A, de Magistris L, et al. Cannabinoid receptor type 1, but not type 2, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders. J Autism Dev Disord. 2013; 43:2686–2695. [PubMed: 23585028]

53. Földy C, Malenka RC, Südhof TC. Autism-associated neuroligin-3 mutations commonly disrupt tonic endocannabinoid signaling. Neuron. 2013; 78:498–509. [PubMed: 23583622]

54. Fraga D, Zanoni CI, Rae GA, Parada CA, Souza GE. Endogenous cannabinoids induce fever through the activation of CB1 receptors. Br J Pharmacol. 2009; 157:1494–1501. [PubMed: 19681872]

55. Fraga D, Zanoni CI, Zampronio AR, Parada CA, Rae GA, et al. Endocannabinoids, through opioids and prostaglandins, contribute to fever induced by key pyrogenic mediators. Brain Behav Immun. 2015 S0889-1591: 00470–00475.

56. Kerr DM, Downey L, Conboy M, Finn DP, Roche M. Alterations in the endocannabinoid system in the rat valproic acid model of autism. Behav Brain Res. 2013; 249:124–132. [PubMed: 23643692]

57. Mattace Raso G, Russo R, Calignano A, Meli R. Palmitoylethanolamide in CNS health and disease. Pharmacol Res. 2014; 86:32–41. [PubMed: 24844438]

58. Jonsson KO, Vandevoorde S, Lambert DM, Tiger G, Fowler CJ. Effects of homologues and analogues of palmitoylethanolamide upon the inactivation of the endocannabinoid anandamide. Br J Pharmacol. 2001; 133:1263–1275. [PubMed: 11498512]

59. Aghaei I, Rostampour M, Shabani M, Naderi N4, Motamedi F, et al. Palmitoylethanolamide attenuates PTZ-induced seizures through CB1 and CB2 receptors. Epilepsy Res. 2015; 117:23–28. [PubMed: 26370914]

60. Antonucci N, Cirillo A, Siniscalco D. Beneficial Effects of Palmitoylethanolamide on Expressive Language, Cognition, and Behaviors in Autism: A Report of Two Cases. Case Rep Psychiatry. 2015; 2015:325061. [PubMed: 26491593]

61. Arrowsmith JB, Kennedy DL, Kuritsky JN, Faich GA. National patterns of aspirin use and Reye syndrome reporting, United States, 1980 to 1985. Pediatrics. 1987; 79:858–863. [PubMed: 3588140]

62. Arrowsmith JB, Kennedy DL, Kuritsky JN, Faich GA. National patterns of aspirin use and Reye syndrome reporting, United States, 1980 to 1985. Pediatrics. 1987; 79:858–863. [PubMed: 3588140]

63. Gauthier M, Guay J, Lacroix J, Lortie A. Reye's syndrome. A reappraisal of diagnosis in 49 presumptive cases. Am J Dis Child. 1989; 143:1181–1185. [PubMed: 2801659]

64. Casteels-Van Daele M, Van Geet C, Wouters C, Eggermont E. Reye syndrome revisited: a descriptive term covering a group of heterogeneous disorders. Eur J Pediatr. 2000; 159:641–648. [PubMed: 11014461]

65. Orlowski JP, Hanhan UA, Fiallos MR. Is aspirin a cause of Reye's syndrome? A case against. Drug Saf. 2002; 25:225–231. [PubMed: 11994026]

66. Schrör K. Aspirin and Reye syndrome: a review of the evidence. Paediatr Drugs. 2007; 9:195–204. [PubMed: 17523700]

Table 1

Characteristics of children in the 2015 ASD -acetaminophen study by case-control status.

Variable	Cases	Controls	p value[*]
n = Cases, Controls	Mean (SD)	Mean (SD)	
Age (months) (n = 118, 79)	131.3 (43.6)	134.9 (39.6)	0.563[1]
	% (n)	% (n)	
Acetaminophen use for fever (n = 118, 79)			0.013[2]
Only use this	15.3 (18)	12.7 (10)	
First choice	35.6 (42)	46.8 (37)	
Use if other medication doesn't bring down fever	31.4 (37)	36.7 (29)	
Rarely or never use	17.8 (21)	3.8 (3)	
Ibuprofen use for fever (n = 110, 73)			0.57
Only use this	9.1 (10)	4.1 (3)	
First choice	44.5 (49)	50.7 (37)	
Use if other medication doesn't bring down fever	33.6 (37)	32.8 (24)	
Rarely or never use	12.7 (14)	12.3 (9)	
Aspirin use for fever (n = 90, 51)			1.000[2]
Only use this	1.1 (1)	0.0 (0)	
First choice	0.0 (0)	0.0 (0)	
Use if other medication doesn't bring down fever	2.2 (2)	2.0 (1)	
Rarely or never use	96.7 (87)	98.0 (50)	
Frequency of Fevers (n = 117, 78)			0.057
Rarely (< 4 times / year)	85.5 (100)	93.6 (73)	
Sometimes (5–8 times / year)	10.3 (12)	6.4 (5)	
Often (>12 times / year)	4.3 (5)	0.0 (0)	
Social Interaction Better with Fever (n = 118, 79)			0.037[2]
No	81.4 (96)	92.4 (73)	
Yes	18.6 (22)	7.6 (6)	

[*] p values by Pearson chi square unless otherwise noted. A p value of less than 0.05 was considered significant and is marked in bold.

[1] F test p value.

[2] Fisher's exact test p value, two sided.

Table 2

Age adjusted associations of acetaminophen or ibuprofen use for fever in children with ASD compared to control children by logistic regression.

Variable	B	Odds Ratio	95% Confidence Interval	p value[*]
Acetaminophen use for fever compared to rarely or never use:				
Only use this	− 1.352	0.259	0.062, 1.088	0.065
First choice	− 1.804	0.165	0.045, 0.599	0.006
Use if other medication doesn't bring down fever	− 1.697	0.183	0.050, 0.675	0.011
Ibuprofen use for fever compared to rarely or never use:				
Only use this	0.782	2.186	0.467, 10.235	0.321
First choice	−0.16	0.852	0.333, 2.183	0.739
Use if other medication doesn't bring down fever	0.001	1.001	0.374, 2.679	0.998

[*] p value by logistic regression likelihood ratio with a value less than 0.05 considered significant and listed in bold.

Table 3

Use of ibuprofen if acetaminophen doesn't bring down fever vs. rarely or never use ibuprofen for children with or without autism spectrum disorder while limiting the analysis to children who use acetaminophen as first choice.

	For children who use acetaminophen as first choice:		
	Use of ibuprofen if acetaminophen doesn't bring down fever	Rarely or Never use ibuprofen	p value[*] 0.033
Children with ASD	33	0	
Children without ASD	22	4	

[*]
p value by mid-P exact test, OpenEpi Open Source Epidemiologic Statistics for Public Health, Version 3.03a

i behalf of a grower
ous charges, I was
the court in good
that his 30 pounds
lla amounted to a
i supply of CBD for

acid). **"There is 90 times more CBD in a pound of cannabis buds than there is Vitamin C in a pound of oranges," according to Dr. Courtney, "and CBD is twice as potent an anti-oxidant." Crystal CBD courtesy of THC-Pharm, Frankfurt, Germany. Vitamin C courtesy of Trader Joe's, Santa Rosa CA.**

reated with cannabis, continued

Condition			Count	Code	Diagnosis
Chronic Fatigue			4	379.91	Ocular pain
Amphetamine Dep.			4	721.2	Thoracic DJD
Persistent Insomnia			4	720.0	Ankylosing Spondylitis
Multiple joint pains			4	346.2	Cluster Headaches
Bruxism			3	353.0	Thoracic Outlet Syndrome
Lower Extremity DJD			3	595.1	Interstitial Cystitis, chronic
Knee, DJD			3	307.23	Tourette's Syndrome
Arthropathy, Degenerative+			3	251	Hypoglycemia(s)
Shoulder Injury Unspec			2	717.7	Knee, patellar chondromal
Osgood-Schlatter			2	717.7	Patellar chondromalacia
Epilepsy, Grand Mal			2	787.01	Vomiting
Shoulder, DiD			2	782.0	Myofacial Pain Syndrome**
Multiple Sclerosis			2	344.00	Quadriplegia(s), unspec
Post Polio Syndrome			2	337.2x	Reflex Sympath Dystrophy
Lupus			2	310.9	Brain Trauma
Thyroiditis			2	250.2	Diabetes, Adult uncontrolled
Graves Disease			2	296.2	Menopausal depression
Diabetic Neuropathy			2	296.2	Major Depression, Sgl Epis
Diabetic Gastroparesis			2	305.2	Cannabis Abuse
Spinal Stenosis			2	042	AIDS-Related Illness
Pain, Abdominal			2	733.0	Osteoporosis
Opiate Dependence+			2	362.5	Macular Degeneration**
Raynaud's Disease			2	696.0	Psoriatic Arthritis
Shingles (Herpes Zoster)			2	558.9	Colitis, unspecified +
Plantar fasciitis			2	725	Polymyalgia rheumatica
F m/Wrist/Hand pain			2	300.22	Agoraphobia
Colon diverticulitis			2	054.10	Genital Herpes
Back Sprain			2	577.1	Pancreatitis
Herpetic infection of penis			2	218.9	Uterine Fibroids
Diarrhea			2	359	Muscular Dystrophies
Colitis, Ulcerative			1	070.52	Hepatitis, Viral B chronic
Scleroderma			1	722.83	Post Lamin. SyndLumbar
Bipolar Disorder, manic			1	733.00	Osteoporosis
			1	737.41	Cervical kyphosis
			1	296.5	Bipolar Disorder, depressed
			1	732.1	Legg Calve Perthes Dis

CE UPDATE: WILLIAM TOY, MD

(Grass Valley), began conducting cannabis consultations part-time
d full-time (40 weeks/year) since 2004.

als issued to date: approximately 2,000

sly self-medicating >99%.

ould not have come to my office and paid money to have the approval
not had a good experience.

mmon conditions:

ble pain > 50%. The most common causes are failed orthopedic sur-
lving the spine and peripheral joints) and orthopedic problems (se-
:is, degenerative spinal disease, spinal injury).

ntestinal problems 15-20% (including irritable bowel syndrome,
lcerative colitis, failed GI surgery).

gical problems c. 10% (including spinal cord injury and neuropa-

torrhea and menopause-related problems c. 10%

ncies c. 10% (for nausea, pain and depression)

10%

d oth ainful conditions about the mouth c. 5%

tory arthropathies and collagen disease, including HLA-positive
5%

reported: Approximately 80-90% of patients in the pain group report
iate intake by at least 50%. Several patients completely removed
s from Oxycontin. Cannabis is very good for treating anxiety and
ssociated with various conditions

---

lent behavior directed at self and others is a particularly difficult symptom. Current treatment modalities include family education, behavior modification, special education services, a wide variety of psychotropic medications and institutionalization in some cases.

I have recently evaluated two children whose families have decided to use cannabis in lieu of standard medications for treatment of autism. They report dramatic improvements.

E.S.

E.S. is a nine-year-old adopted male. He is the product of an uncomplicated full-term pregnancy delivered by C-section. The neonatal period was unremarkable. Severe behavioral problems were noted beginning at 20 months, ultimately leading to a diagnosis of Autism.

Despite aggressive treatment with behavioral interventions and multiple medications including the atypical antipsychotics, severe behavior problems, especially violent behavior, persisted.

His teachers have noted major improvements in learning and socialization.

Encouraged by internet reports and desperate for an alternative, the parents began using small amounts of cannabis concentrate administered in yogurt. The results were immediate and dramatic. Violent outbursts became rare, self-stimulation stopped completely. The child became calmer and more focused. His teachers have noted major improvements in learning and socialization. these positive changes have persisted for more than a year, while the dose of cannabis has remained stable. The parents report no adverse effects of cannabis use, but

PRACTICE UPDATE
PHILIP A. DENNEY, MD
AND ROBERT SULLIVAN, MD

Drs. Denney and Sullivan share a practice in Carmichael. Denney spent most of his career in Family Practice, Sullivan in Emergency Medicine. (He is a Fellow of the American College of Emergency Physicians.) By opening offices in Lake Forest (Orange County) in 2004 and Redding (Butte County) in 2005, they helped bring the medical marijuana movement to parts of the state that had been underserved by pro-cannabis doctors. In 2008 they decided to consolidate closer to home.

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lence and aggressiveness. At a months, a diagnosis of Pervasive opmental Delay was made. At a months a diagnosis of Autism Sp Disorder was made. Despite agg intervention and multiple psych medications, severe violent be persisted. The family described m injuries to B.T. and his caregiv the need for around-the-clock assi Violent behavior ultimately pre any school attendance at all.

Complete elimination o lent behavior and a marke crease in affection and c eration.

Noting internet reports of s with cannabis for Autism and con about the use of psychotropic m tion for their son, the parents dec try cannabis. The results were dr with complete elimination of v behavior and a marked increase fection and cooperation. B.T. ha able to return to school and is des as a "different child." He has bec to eliminate his prescribed medic completely. Mom administers servings of cornbread made wit nabis-infused oil and adamantly any adverse effects.

Conclusion

In summary, these two cases s a potentially significant therapeu for the cannabinoids in the treatn Autism Spectrum Disorder. Its saf lack of toxicity may make canna attractive alternative to psychc medications for many children. I tion, study of the endocannabino tem as it relates to Autism may le better understanding of both.

ROBERT SULLIVAN trying to co the Sacramento City Council th nabis has valid medical uses. He de pa-tients coping with migraines, c pain, and nausea. "These sympto erode your life *en toto*. When yo something that really works, is
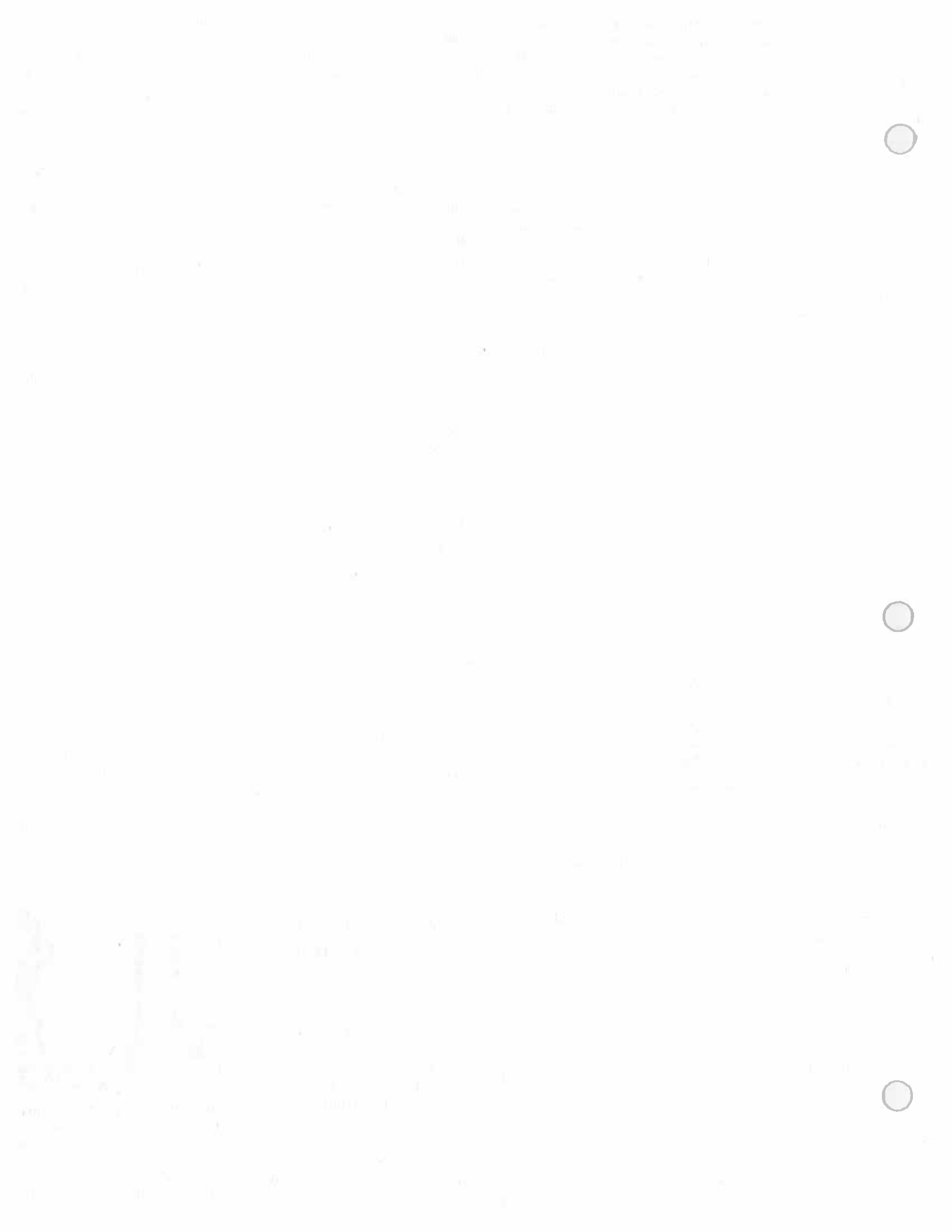
Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

Research report

Alterations in the endocannabinoid system in the rat valproic acid model of autism

CrossMark

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HIGHLIGHTS

- Prenatal VPA exposure elicits autistic-like behaviour during adolescence.
- Social exposure increases hippocampal anandamide levels in VPA exposed rats.
- DAGLα and MAGL expression is reduced in the cerebellum and hippocampus of VPA exposed rats.
- PPARα and GPR55 mRNA expression in the cortex is reduced in VPA exposed rats.
- VPA exposed rats exhibit reduced PPARγ and GPR55 mRNA expression in the hippocampus.

ARTICLE INFO

ABSTRACT

The endocannabinoid system plays a crucial role in regulating emotionality and social behaviour, however it is unknown whether this system plays a role in symptoms associated with autism spectrum disorders. The current study evaluated if alterations in the endocannabinoid system accompany behavioural changes in the valproic acid (VPA) rat model of autism. Adolescent rats prenatally exposed to VPA exhibited impaired social investigatory behaviour, hypoalgesia and reduced lococmotor activity on exposure to a novel aversive arena. Levels of the endocananbinoids, anandamide (AEA) and 2-arachidonylglycerol (2-AG) in the hippocampus, frontal cortex or cerebellum were not altered in VPA- versus saline-exposed animals. However, the expression of mRNA for diacylglycerol lipase α, the enzyme primarily responsible for the synthesis of 2-AG, was reduced in the cerebellum of VPA-exposed rats. Furthermore, while the expression of mRNA for the 2-AG-catabolising enzyme monoacylglycerol lipase was reduced, the activity of this enzyme was increased, in the hippocampus of VPA-exposed animals. CB_1 or CB_2 receptor expression was not altered in any of the regions examined, however VPA-exposed rats exhibited reduced PPARα and GPR55 expression in the frontal cortex and PPARγ and GPR55 expression in the hippocampus, additional receptor targets of the endocannabinoids. Furthermore, tissue levels of the fatty acid amide hydrolase substrates, AEA, oleoylethanolamide and palmitoylethanolamide, were higher in the hippocampus of VPA-exposed rats immediately following social exposure. These data indicate that prenatal VPA exposure is associated with alterations in the brain's endocannabinoid system and support the hypothesis that endocannabinoid dysfunction may underlie behavioural abnormalities observed in autism spectrum disorders.

1. Introduction

Autism is a neurodevelopmental disorder characterised by impaired social interaction, deficits in communication and restrictive, repetitive stereotyped patterns of behaviours. The aetiology of this disorder remains unknown, although several genetic and environmental factors have been identified which play a role in this spectrum of disorders. Prenatal exposure to teratogenic agents such as valproic acid (VPA) has been implicated in the pathogenesis of autism [1–3] and knowledge of this association has led to the development of a widely used and validated preclinical model of autism.

Exposure of prenatal rats to VPA impairs neural tube closure and results in behavioural aberrations such as reduced social behaviour, lower sensitivity to pain and increased anxiety and fear in adolescent and adult rats [4–7], behaviours analogous to those observed clinically. Anatomical alterations such as diminished number of cerebellar purkinje and cranial neurons [8,9], enhanced synaptic plasticity of the prefrontal cortex [10] and amygdala [7,11], alterations in monoamine and amino acid neurotransmission [6,12,13] and immunological alterations [14] have also been reported in the model.

Increasing evidence suggests a role for the endocannabinoid system in social and emotional processing [15,16], however there is a paucity of studies directly examining the role of this system in autism. Comprised of the G-protein coupled CB_1 and CB_2 receptors, the endogenous cannabinoid ligands (endocannabinoids) including anandamide (AEA) and 2-archidonylglygerol (2-AG) and the enzymes responsible for the synthesis and catabolism of the endocannabinoids, the neuroanatomical distribution of this system means that it is well positioned to modulate affective and social responding. A recent review has suggested metabolism of acetaminophen (paracetamol) to N-arachidonoylphenolamine (AM404) [17], an AEA reuptake inhibitor, results in enhanced AEA tone which may alter neuronal development and immunological function during critical neurodevelopmental phases possibly predisposing certain children to developing autism [18]. However, to date no detailed studies have been carried out investigating the link between acetaminophen, the endocannabinoid system and the development of autism. Polymorphisms in the gene encoding the CB_1 receptor, CNR1, have been shown to modulate striatal responses [19] and gaze duration [20] to social reward cues, indicating that subtle changes in endocannabinoid affinity at the CB_1 receptors due to these polymorphisms may underlie deficits in social reward processing such as observed in autism. Preclinical studies have indicated that social play behaviour enhances AEA levels in several brain regions including the amygdala, nucleus accumbens [21] and striatum [22] and that enhancing endogenous AEA tone following pharmacological inhibition of fatty acid amide hydrolyse (FAAH), the enzyme primarily responsible for the catabolism of this endocannabinoid [23], or inhibition of AEA reuptake, and subsequent CB_1 receptor activation results in enhanced social play behaviour [24,25]. In comparison, direct activation of CB_1 receptors with the potent agonist WIN55,212-2 reduces social behaviour [24]. The differential effects of global CB_1 receptor activation and enhancing AEA tone on social play behaviour have been proposed to be due to the selective activation of CB_1 receptors in brain regions involved in social and emotional responding following FAAH inhibition [21,24]. However, it should be noted that in addition to increasing AEA levels, FAAH inhibition also increases N-acylethanolamines such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), although the role of these N-acylethanolamines on social and emotional behavioural responding remains to be been investigated. Recent studies have demonstrated enhanced cortical levels of AEA, but not 2-AG, following social exposure in BTBR mice, [26], a mouse strain known to exhibit an autistic-like behavioural phenotype [27]. Agonist-induced $GTP\gamma S$ binding of CB_1 receptors is enhanced in the BTBR mouse [26] and pharmacological activation of CB1/2 receptors has been shown to attenuate the hyperlocomotor activity displayed by these mice [26,28]. Central activity of diacylglycerol lipase (DAGL)α and monoacylglycerol lipase (MAGL), the enzymes responsible for the synthesis and catabolism of 2-AG respectively [29,30], have been reported to be enhanced in the fmr−/− mouse [31,32], a model of fragile X syndrome, the most common genetic form of autism. In addition, pharmacological inhibition of MAGL and subsequent augmentation of endogenous 2-AG levels, results in the normalisation of locomotor and anxiety-related behavioural changes in

fmr−/− mice [32]. As highlighted, several lines of evidence suggest a potential role for the endocannabinoid system in autism, however a detailed profile of the system in a validated preclinical model is lacking.

The aim of the present study was to examine if the autistic-like behavioural changes exhibited by adolescent rats prenatally exposed to VPA are associated with endocannabinoid dysfunction in discrete brain regions known to modulate emotional and social behaviour. In addition to examining changes in endocannabinoid and N-acylethanolamine levels, and the expression of genes regulating the synthesis and catabolism of AEA and 2-AG, the expression of CB_1 and CB_2 receptors and other targets of the endocannabinoid system including peroxisome proliferator-activated receptor (PPAR)α, PPARγ and GPR55 [33,34] were examined.

2. Materials and methods

2.1. Animals

Male and female Sprague-Dawley rats (200–300 g; Charles River Laboratories, UK) were mated following determination of the oestrus phase of the reproductive cycle. The presence of spermatozoa in vaginal smears indicated the first day of gestation (G0.5). Following copulation, female rats were housed singly and maintained at constant temperature ($21 \pm 2 °C$) and humidity (30–35%) under standard lighting conditions (12:12 h light–dark, lights on from 07:00 to 19:00 h). Food and water were available ad libitum. Experimental protocols were carried out under approval from the Animal Care and Research Ethics Committee at NUI Galway and under licence from the Irish Department of Health and Children, in compliance with the European Communities Council directive 86/609.

On gestational day 12.5 (G12.5), female rats received a single subcutaneous injection of sodium valproate (VPA) (Sigma, Dublin, Ireland) (600 mg/kg) or saline vehicle. The dose and time of administration was chosen based on studies demonstrating that this regime elicits autistic-like behavioural changes in offspring [5]. Females were allowed to raise their own litters and pups which were weaned on postnatal day (PND) 21. Following weaning, rats of either sex were housed separately in groups of 3–6 per cage.

2.2. Experimental design

A schematic representation of the experimental design is presented in Fig. 1.

2.2.1. Experiment 1: behavioural profile of the VPA model and associated changes in the endocannabinoid system

Behavioural testing was carried out during adolescence between PND 33 and 40. The sequence of testing remained constant, and involved the sociability test (saline-treated n = 16; VPA treated n = 14) followed by the hot plate test, followed by the open field and elevated plus maze test (saline-treated n = 10; VPA treated n = 8) and was modelled on the study design described by Schneider and colleagues [5]. All behavioural testing was carried out by an experimenter blinded to treatment. Seventy-two hours following the final behavioural test (PND 43) animals were killed by decapitation, the brain removed and discrete brain regions including the frontal cortex, hippocampus and cerebellum dissected out and snap frozen on dry ice. The frontal cortex was considered cortical tissue rostral to the central sulcus and included regions such as the prefrontal cortex, premotor cortex and motor cortex. All regions of the cerebellum (cerebro-, spino- and vertibular) were included in the cerebellar tissue samples that were processed. The aforementioned regions have been implicated in autistic-like symptoms and alterations in these regions have previously been demonstrated in the VPA model of autism [8,13,35]. Brain regions were stored at −80 °C until assayed for endocannabinoid and N-acylethanolamine levels, and mRNA expression of endocannabinoid related genes.

2.2.2. Experiment 2: endocannabinoid and N-acylethanolamine levels in discrete brain regions in VPA-exposed animals following exposure to the sociability test

Immediately following the sociability test, a subset of animals (saline-treated n = 6; VPA treated n = 6) were killed by decapitation, the frontal cortex, hippocampus and cerebellum excised, snap frozen on dry ice and stored at −80 °C until assayed for endocannabinoid and N-acylethanolamine levels.

2.3. Behavioural testing

2.3.1. Sociability test

The sociability test was conducted in a novel 3-chamber apparatus which allows for the measurement of social approach and social preference [36,37]. In brief, animals were placed into a novel arena (80 cm × 31.5 cm) composed of three communicating chambers separated by Perspex walls with central openings allowing access to all chambers for 5 min. Distance moved (cm) and time spent (s) in the
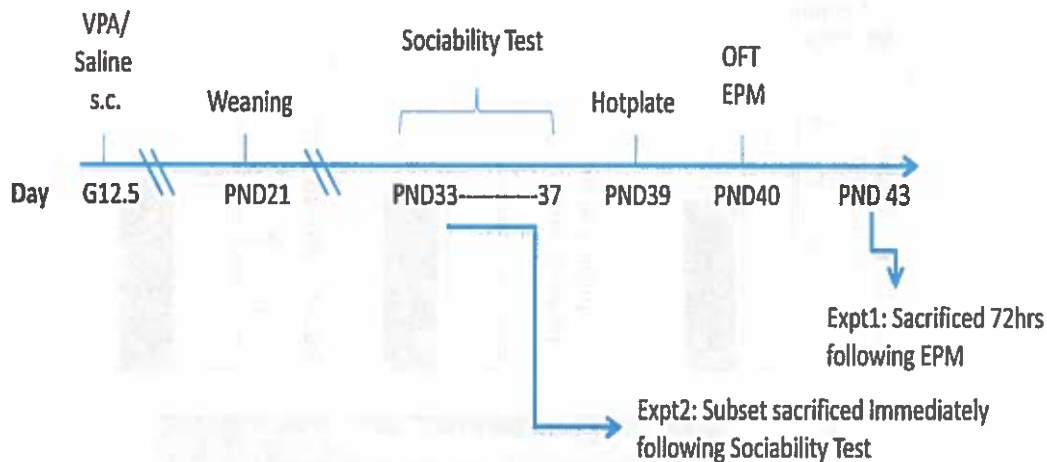
Fig. 1. Schematic representation depicting the experimental design. Behavioural testing occurred during adolescence (PND 33–40). G12.5: gestational day 12.5, EPM elevated plus maze, OFT open field test, PND: postnatal day, s.c.: subcutaneous injection.

various compartments was assessed during this time to evaluate general locomotor activity and ensure that animals did not have a preference for a particular side of the arena. Following this acclimatisation period, animals were briefly confined to the central chamber while an unfamiliar rat confined in a small wire cage was placed in one of the outer chambers. An identical empty wire cage was placed in the other chamber. The unfamiliar rat was randomly assigned to either the right or left chamber of the arena. The test animal was then allowed to explore the arena/chambers for a further 10 min. Distance moved in the arena, time spent engaging in investigatory behaviour with the novel rat and frequency of investigatory behaviour with the novel rat was evaluated with the aid of EthoVision XT software (Noldus Netherlands) in order to examine social approach and preference. All testing occurred during the dark phase (21:00–03:00 h) under red light illumination.

2.3.2. Hot plate test

The hot plate test was used to assess nociceptive responding to a noxious thermal stimulus. On the test day (10:00–12:00 h), the animal was taken from its home cage and placed directly onto a hot plate (IITC Life Science Inc, California, USA) heated to $55 \pm 1\,°C$. Thermal nociception was measured as the time elapsed (i.e. latency to respond (s)) between placement of the animal on the surface of the hot plate and when the animal first licked either of its hind paws, with a cut-off time of 40 s to avoid tissue damage.

2.3.3. Open field test

On the experimental day, each animal was removed from the home cage during the light phase (between 10:00 h and 15:00 h) and placed singly into a brightly lit (lux 300–400) novel open field environment (diameter 75 cm) where behaviour was assessed using a computerised video tracking system (EthoVision XT, Noldus Netherlands) for a 5 min period. Behaviours assessed included locomotor activity (distance moved: cm) and duration of time spent (seconds; s) in the centre zone (45 cm diameter), an indication of anxiety-related behaviour.

2.3.4. Elevated plus maze

Immediately following exposure to the open field, animals were placed directly onto the elevated plus maze. This 4-arm maze consisted of two open (lux 90) and two closed (30 cm high wall, lux 30) arms (50 cm length × 12 cm wide) forming a plus shape, elevated approximately 50 cm from the floor. Each rat was placed in the centre of the maze facing an open arm and allowed to freely explore for 5 min. Time (s) in the open and closed arms was assessed over the trial with the aid of EthoVision XT video tracking system (Noldus Netherlands).

2.4. Quantitation of endocannabinoids and N-acylethanolamine levels using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Quantitation of endocannabinoids and N-acylethanolamines was essentially as described previously [38–41]. In brief, samples were homogenised in 400 μL 100% acetonitrile containing deuterated internal standards (0.014 nmol AEA-d8, 0.48 nmol 2-AG-d8, 0.016 nmol PEA-d4, 0.015 nmol OEA-d2). Lyophilised samples were re-suspended in 40 μL 65% acetonitrile and separated by reversed-phase gradient elution initially with a mobile phase of 65% acetonitrile and 0.1% formic acid which was ramped linearly up to 100% acetonitrile and 0.1% formic acid over 10 min and held at this for a further to 20 min. Under these conditions, AEA, 2-AG, PEA and OEA eluted at the following retention times: 11.4 min, 12.9 min, 14.4 min and 15.0 min respectively. Analyte detection was carried out in electrospray-positive ionisation and multiple reaction monitoring (MRM) mode on an Agilent 1100 HPLC

system coupled to a triple quadrupole 6460 mass spectrometer (Agilent Technologies Ltd, Cork, Ireland). Quantitation of each analyte was performed by ratiometric analysis and expressed as nmol or pmols per gram of tissue. The limit of quantification was 1.3 pmol/g, 12.1 pmol/g, 1.5 pmol/g, and 1.4 pmol/g for AEA, 2-AG, PEA and OEA respectively.

2.5. Enzyme and receptor mRNA expression using quantitative real-time PCR

As previously described [38,40], RNA was extracted from cortical, hippocampal or cerebellar tissue using NucleoSpin RNA II total RNA isolation kit (Macherey-Nagel, Germany) and reverse transcribed into cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, UK). Taqman gene expression assays (Applied Biosystems, UK) containing forward and reverse primers and a FAM-labelled MGB Taqman probe were used to quantify the gene of interest and real-time PCR was performed using an ABI Prism 7500 instrument (Applied Biosystems, UK). Assay IDs for the genes examined were as follows: NAPE-PLD (Rn01786262_m1), DAGLα (Rn01454304_m1), DAGLβ (Rn01453775_m1), FAAH (Rn00577086_m1), MAGL (Rn00593297_m1), CB₁ (Rn00562880_m1), CB₂ (Rn03993699_s1), PPARα (Rn00566193_m1), PPARγ (Rn00440945_m1), and GPR55 (Rn03037213_s1). PCR was performed using Taqman Universal PCR Master Mix and samples were run in duplicate. The cycling conditions were 90 °C for 10 min and 40 cycles of 90 °C for 15 min followed by 60 °C for 1 min. β-Actin was used as an endogenous control to normalise gene expression data. Relative gene expression was calculated using the $^{\Delta\Delta}$CT method.

2.6. FAAH and MAGL enzyme activity assay

Enzyme activity assays were conducted essentially as previously described [40,42]. In brief, hippocampal tissue was weighed (~20 mg), homogenised in 1 ml of TE buffer (50 mM Tris, 1 mM EDTA, pH7.4) and centrifuged at $14,000 \times g$ for 15 min. The pellet was resuspended in 1 ml of TE buffer, centrifuged and resuspended in a final volume of TE buffer to give a 1:1000 dilution (FAAH determination) or 1:5000 dilution (MAGL determination) of the initial wet hippocampal tissue weight. 90 μl of sample aliquots or blanks were pre-incubated with 5 μl of Hanks/Hepes buffer (116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂·2H₂O, 25 mM HEPES, 0.8 mM MgSO₄, 1 mM NaH₂PO₄·2H₂O) pH 7.4, containing 1 mg/ml defatted albumin for 30 min at 37 °C. After pre-incubation, FAAH substrate (5 μl: 40 μM AEA containing 2 μCi [³H]-AEA; American Radiolabelled Chemicals) or MAGL substrate (5 μl: 2 mM 2-OG containing 3.75 μCi 2-oleoyl-[³H]-glycerol; American Radiolabelled Chemicals) was added to the samples to give a final [³H]-AEA concentration of 2 μM or [³H]-2-OG concentration of 100 μM. The reactions were allowed to proceed for 15 min at 37 °C, following which 300 μl of stop solution (8% w/v charcoal in 0.5 M HCl) was added. Samples were allowed to stand for 20 min, centrifuged at $14,000 \times g$ for 5 min and 200 μl of the supernatant was used for liquid scintillation counting. Homogenates were assayed in triplicate. Data were expressed as pmol/min/g for FAAH activity or nmol/min/g for MAGL activity.

2.7. Statistical analysis

SPSS statistical package was used to analyse all data. Normality and homogeneity of variance was assessed using Shapiro–Wilk and Levene test, respectively. All data were analysed using unpaired t-test to compare effect of prenatal saline- vs. VPA-exposure. Data were considered significant when $P < 0.05$. Results expressed as group means + standard error of the mean (SEM).
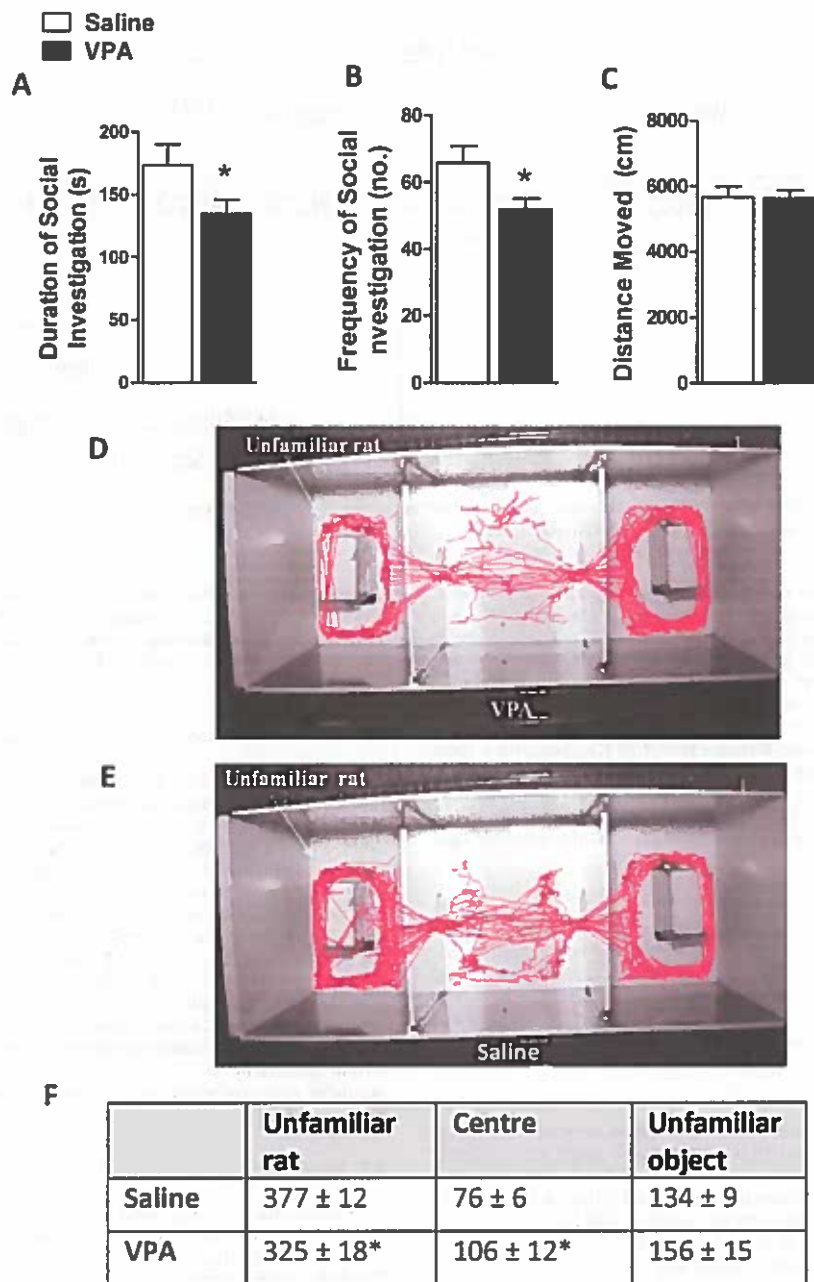
Fig. 2. Rats prenatally exposed to VPA exhibit reduced time and frequency of investigative behaviour towards an unfamiliar con-specific rat. (A) Duration and (B) frequency of social investigatory behaviour of control and VPA-exposed rats in the sociability test (*$P < 0.05$ vs saline-treated counterpart). (C) Distance moved did not differ between the groups over the course of the test. Representative images demonstrating track tracing movements of (D) VPA- and (E) saline-exposed rats in the sociability test. (F) Time spent (s) of saline and VPA exposed animals in each the 3 chambers of the test area over the 10 min trial period. Data expressed as mean + SEM. $n = 14$–16 per group.

3. Results

3.1. Behavioural phenotyping of adolescent rats exposed prenatally to VPA

Analysis of behaviour during the acclimatisation period of adolescent rats to the novel 3-chamber sociability area prior to the introduction of an unfamiliar con-specific rat revealed that prenatal exposure to VPA did not alter locomotor activity (saline: 2482 ± 144 cm vs. VPA: 2435 ± 77 cm) or time spent in either side of the arena (time in left side: saline 103 ± 8 s vs VPA 112 ± 12 s; time in right side: saline 99 ± 10 s vs VPA 85 ± 12 s). Following the introduction of the unfamiliar rat and novel object (empty wire container) into 3-chamber test arena, analysis revealed that time in the chamber containing the unfamiliar rat and the time and frequency of investigatory behaviours towards the stimulus animal was significantly less in VPA-exposed rats when compared to controls ($P < 0.05$; Fig. 2A, B, D, E, F). This decrease was accompanied by an increase in the time spend in the central chamber but (Fig. 2F) was not related to alterations in locomotor activity as distance moved in the arena over the test period did not differ between the groups (Fig. 2C).

In the hotplate test, VPA-exposed animals exhibited a significant increase in latency to respond ($P < 0.01$) when compared to control animals (Fig. 3A), indicating the development of heat hypoalgesia in the model.

Fig. 3. Rats prenatally exposed to VPA exhibit thermal hypoalgesia and reduced locomotor activity in open field test. (A) VPA-exposed rats exhibit an increased latency to lick the hindpaws in the hotplate test (**$P < 0.01$ vs saline). (B) Distance moved and (C) duration of time in the centre arena of a novel brightly lit open field is reduced in VPA-exposed animals when compared to saline-treated controls (*$P < 0.05$ vs saline). There was no significant difference between VPA- and saline- exposed rats in terms of % time on the open arms of the elevated plus maze. Data expressed as mean + SEM. $n = 8$–10 per group.

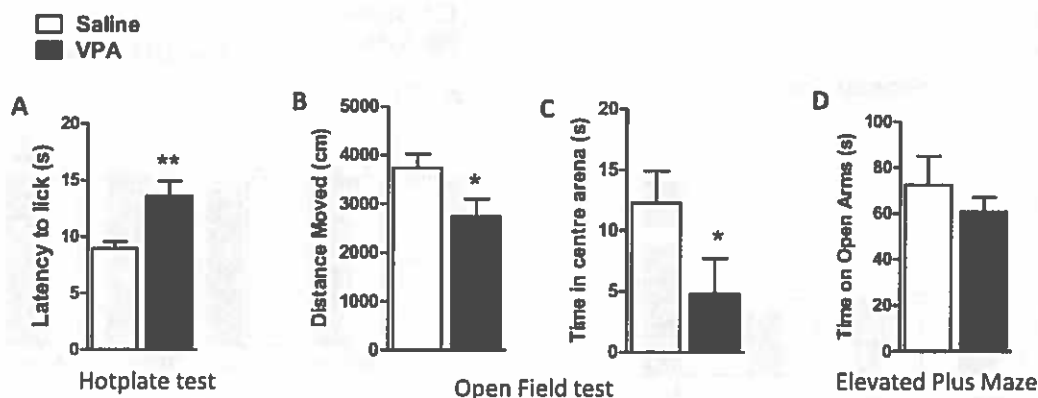On exposure to a novel brightly lit aversive open field arena, VPA-exposed rats exhibited reduced locomotor activity as demonstrated by a decrease in distance moved when compared to saline-treated counterparts ($P < 0.05$ Fig. 3B). Furthermore, the duration of time spent in the central arena of the open field arena was also reduced ($P < 0.05$ Fig. 3C), indicative of an anxiety-related phenotype. In order to further investigate possible anxiety-related behaviour, VPA-exposed rats were placed on the elevated plus maze. Time spent on the open (Fig. 3D) and closed (saline: 113 ± 11 s vs. VPA: 101 ± 6 s) arms of the test arena did not differ between VPA- and saline-exposed rats.

3.2. Endocannabinoid and N-acylethanolamine levels in discrete brain regions do not differ between animals prenatally exposed to VPA or saline

Although 2-AG levels in the frontal cortex were slighted reduced in VPA-exposed animals (Fig. 4A), this effect failed to reach statistical significance ($P = 0.06$). Levels of the endocannabinoids, AEA and 2-AG, or the N-acylethanolamines PEA and OEA, in the frontal cortex (Fig. 4A), hippocampus (Fig. 4B) or cerebellum (Fig. 4C) did not differ between VPA and saline-exposed rats.

3.3. Prenatal VPA exposure reduces expression and enhances activity of MAGL in the hippocampus

Evaluating the expression of genes which encode for the synthetic and catabolic enzymes of the endocannabinoid system in discrete brain regions revealed that VPA-exposed rats exhibit reduced MAGL ($P < 0.05$ Fig. 5B) and DAGLα ($P < 0.01$ Fig. 5C) mRNA in the hippocampus and cerebellum respectively, when compared to saline-treated counterparts. There was no significant difference in expression of synthetic or catabolic enzymes in the frontal cortex between VPA- and saline-exposed rats (Fig. 5A). As VPA-exposed animals exhibit reduced MAGL mRNA expression in the hippocampus (Fig. 5B), we investigated if altered MAGL activity may account for the lack of change in 2-AG levels observed in VPA exposed rats (Fig. 4B). In accordance with this, MAGL (saline: 588 ± 36 nmol/min/g vs VPA 786 ± 64 nmol/min/g, $P < 0.05$), but not FAAH (saline: 951 ± 42 pmol/min/g vs VPA 951 ± 67 pmol/min/g), activity was enhanced in the hippocampus of VPA-exposed rats ($P < 0.01$ vs saline-treated counterparts).

3.4. PPAR and GPR55 expression is reduced in the frontal cortex and hippocampus of VPA-exposed rats

Neither CB_1 nor CB_2 receptor gene expression in the frontal cortex, hippocampus or cerebellum differed between VPA- or saline-exposed rats (Fig. 6). As endocannabinoids are known to have affinity and activity at additional non-cannabinoid receptor targets, the effect of prenatal VPA exposure on PPARα/γ and GPR55 was assessed. The expression of PPARα ($P < 0.05$) and PPARγ ($P < 0.01$) was reduced in the frontal cortex and hippocampus respectively, of VPA-exposed rats when compared to saline-treated counterparts (Fig. 6A and B). In addition, GPR55 expression was reduced in the frontal cortex ($P < 0.01$) and hippocampus ($P < 0.05$), but not cerebellum, of VPA-exposed rats (Fig. 6A and B).

3.5. Social exposure enhances FAAH substrates in the hippocampus of VPA exposed rats

Following the sociability test a subset of rats were sacrificed in order to determine if the social deficits observed in VPA-exposed animals are accompanied by alterations in endocannabinoid levels in discrete brain regions. While neither endocannabinoid nor N-acylethanolamine levels were altered in the frontal cortex (Fig. 7A) or cerebellum (Fig. 7C) of VPA-exposed animals following the sociability test, the FAAH substrates, AEA ($P < 0.05$), OEA ($P < 0.05$) and PEA ($P < 0.05$), were increased in the hippocampus (Fig. 7B) when compared to saline-treated counterparts.

4. Discussion

The results of the present studies demonstrate that rats prenatally exposed to VPA exhibit autistic-like behavioural changes including reduced sociability, increased anxiety-related behaviour in an open field and reduced sensitivity to noxious stimuli, behavioural changes accompanied by alterations in various components of the endocannabinoid system. Specifically, VPA-exposed animals exhibited reduced expression of the 2-AG synthesising enzyme DAGLα in the cerebellum, reduced expression and enhanced activity of the 2-AG catabolising enzyme MAGL in the hippocampus, reduced expression of mRNA for PPARα and GPR55, endocannabinoid receptor targets, in the frontal cortex, and reduced expression of PPARγ and GPR55 mRNA in the hippocampus. In addition, the FAAH substrates, AEA, OEA and PEA were enhanced in the hippocampus of VPA-exposed animals following the sociability test. Thus, dysfunction in the endocannabinoid
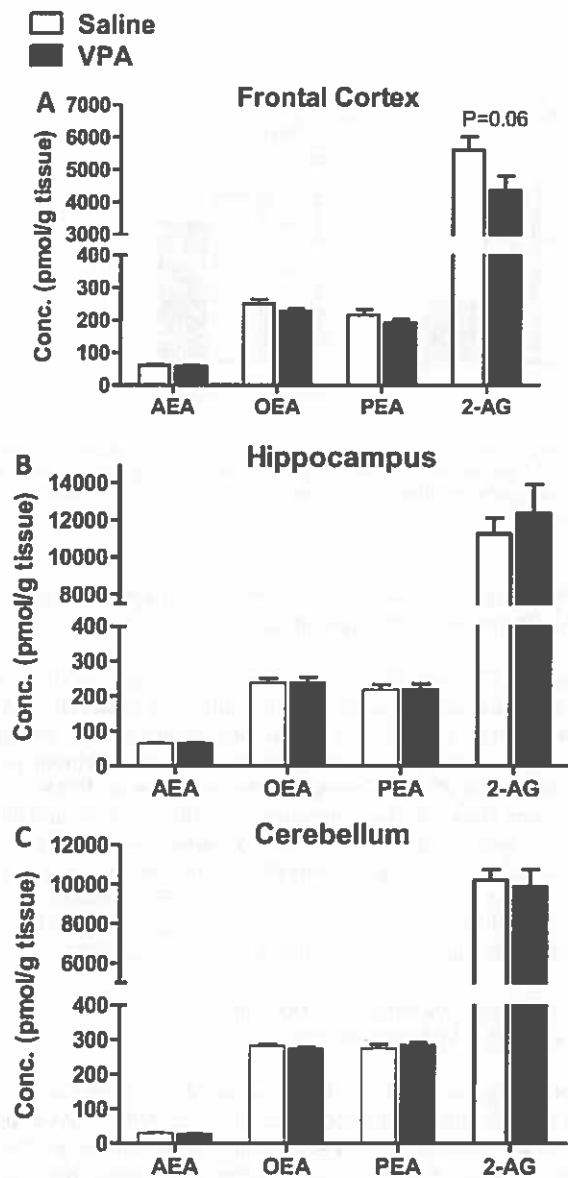
Fig. 4. Endocannabinoid and *N*-acylethanolamine levels in the (A) frontal cortex, (B) hippocampus or (C) cerebellum did not differ between VPA or saline-exposed rats. AEA: anandamide, 2-AG: 2-arachidonyl glycerol, PEA: *N*-palmitoylethanolamide, OEA: *N*-oleoylethanolamide. Data expressed as mean + SEM. *n* = 8 per group.

Fig. 5. Expression of genes encoding for the enzymes involved in the synthesis and catabolism of endocannabinoids in discrete brain regions. VPA-exposed rats exhibit a decrease in the expression of (B) MAGL mRNA in the hippocampus and (C) DAGLα mRNA in the cerebellum, when compared to saline-treated controls (*$P < 0.05$). (A) No change was observed in gene expression in the frontal cortex between the groups. NAPE-PLD: *N*-acyl phosphatidylethanolamine phospholipase D; DAGL: diacylglycerol lipase, FAAH: fatty acid amide hydrolase, MAGL: monoacylglycerol lipase. Data expressed as mean % change from saline-treated control + SEM. *n* = 8 per group.

system may underlie some of the autistic-like behavioural changes observed in the VPA rat model.

Impaired social behaviour, a core symptom of autism spectrum disorders, has been repeatedly demonstrated both in adult and adolescent rats that have been exposed prenatally to VPA [5,7,13,43]. In accordance with these findings, the present study demonstrated that VPA-exposed animals exhibited reduced time and frequency interacting with an unfamiliar con-specific rat in the 3-chamber sociability test during adolescence. VPA-exposed animals did not exhibit altered locomotor activity during the acclimatisation or testing phase of the sociability test, confirming that alterations in social behaviour are not related to motor impairments. However, it has been proposed that enhanced anxiety and fear processing may exacerbate an aversion to environmental interactions typical of social conditions [7], thus leading to impaired social behaviours as seen in the VPA model. Anxiety-related behaviour in this study were assessed in the open field and elevated plus maze and revealed

that locomotor activity and time in the centre of the test arena was reduced in VPA-exposed animals on exposure to a novel brightly lit open field environment. In comparison, previous studies have demonstrated that VPA-exposed animals exhibit increased locomotor activity in an open field test [12,14,44], however, experimental conditions such as size of the test arena, lighting conditions and periods of testing differed significantly from those used in the current study. It appears that aversive stressful conditions, as employed in the open field test used in the current study, elicit anxiety or fear-related behaviour in VPA-exposed animals. Similarly, several studies have demonstrated that VPA-exposed animals exhibit reduced open arm entries and time on the open arms in the elevated plus maze, indicative of enhanced anxiety-related behaviour [6,7,14,35]. Although we failed to observe such changes in the present study, it is possible that performing the elevated plus maze test immediately following exposure to the open field, where anxiety-related behaviour was evident, may have reduced the aversive, anxiety-provoking nature of this test. Autistic

Fig. 6. VPA-exposed rats exhibit a decrease in the expression of genes encoding for receptor targets of the endocannabinoid system in the (A) frontal cortex and (B) hippocampus (**P<0.01 *P<0.05 vs saline-exposed rats). (C) No change was observed in gene expression in the cerebellum between the groups. CB₁: cannabinoid receptor 1, PPAR: peroxisome proliferator–activator receptor, GPR55: G-protein receptor 55. Data expressed as mean % change from saline-treated control + SEM. n = 8 per group.

Fig. 7. Endocannabinoid and N-acylethanolamine levels in the (A) frontal cortex, (B) hippocampus and (C) cerebellum immediately following exposure of saline- or VPA-exposed rats to the sociability test. AEA: anandamide, 2-AG: 2-arachidonyl glycerol, PEA: N-palmitoylethanolamide, OEA: N-oleoylethanolamide. Data expressed as mean + SEM. *P<0.05 vs saline-exposed rats. n = 6 per group.

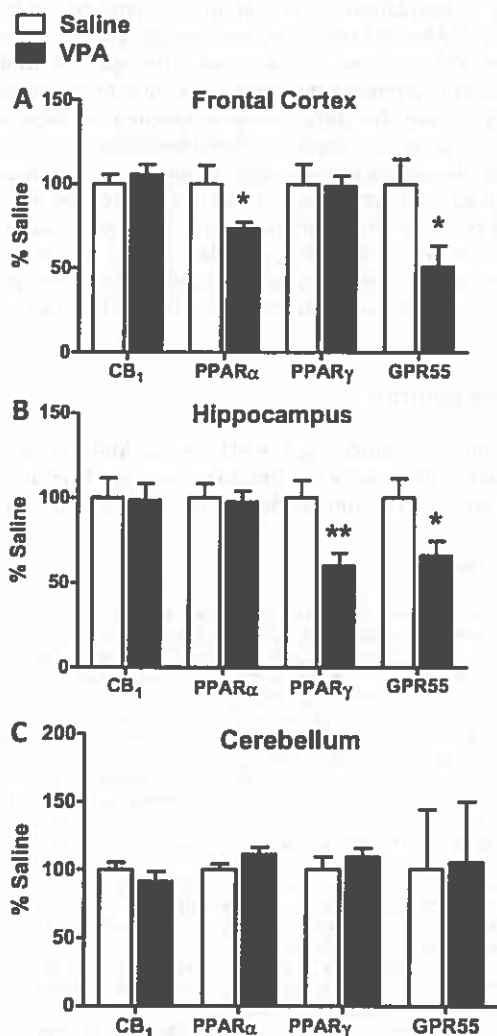patients exhibit reduced sensitivity to painful stimuli [45,46], a phenotype also observed in various pre-clinical models including prenatal exposure to VPA [4,5,7,14,35,47]. In accordance with these data, the present study demonstrated that adolescent rats prenatally exposed to VPA exhibited thermal hypoalgesia in the hotplate test. Together, the present study confirms that exposure to VPA during a critical stage in neo-natal development (G12.5) induces a behavioural phenotype during adolescence similar to that observed in autism, further highlighting the validity of this model.

In addition to behavioural alterations, morphological [7,12,35], neurotransmitter/neuropeptide [6,12,13,35] and immune changes [14,35,47] have been reported in VPA-exposed rats. The endocannabinoid system has been demonstrated to play a role in a wide variety of physiological processes including social and emotional behaviour, nociception and anxiety/fear [16,22,24]. Enhanced DAGL activity in the prefrontal cortex and striatum, enhanced MAGL activity in the striatum and unaltered 2-AG levels have been reported in the fmr−/− model of fragile X syndrome [31,32]. However, to the best of our knowledge, the present study

is the first to examine if post-mortem alterations in the endocannabinoid system are evident in a non-genetic model of autism. Our results demonstrate reduced expression of the 2-AG synthesising enzyme DAGLα in the cerebellum, reduced expression and enhanced activity of the 2-AG catabolising enzyme MAGL in the hippocampus, and unaltered central 2-AG concentrations, in VPA-exposed rats. Thus, under resting conditions, homeostasis in the endocannabinoid system may allow for the maintenance of steady state 2-AG levels in the brain. However, under certain conditions, changes in the ability to synthesise or metabolise 2-AG may lead to altered levels of 2-AG, modulation of neurotransmission and altered behavioural responding. Similar to that previously reported following social interaction [22], 2-AG levels were unaltered in any of the brain regions examined following the sociability test, and therefore alterations in the mobilisation or catabolism of this endocannabinoid may not underlie the social deficits observed in the VPA model. It is however possible that, 2-AG levels were altered during the test period and had returned to levels similar to controls by the end of the trial, or were altered in brain regions other than those investigated. Furthermore, it remains possible that alterations in 2-AG tone may play a role in one or more of the other

behavioural changes observed in the model such as hypoalgesia, stereotypic and anxiety-related behaviour. Augmentation of 2-AG levels by pharmacological inhibition of MAGL results in the normalisation of enhanced locomotor and anxiety-related behavioural changes in *fmr−/−* mice [32], although effects on social behaviour were not investigated. The authors indicate that the behavioural effects mediated by enhanced 2-AG are most likely via CB_1 receptor activation. In accordance with this, administration of the cannabinoid CB_1/CB_2 receptor agonists Δ^9-THC and WIN55,212-2 reduced the hyperlocomotor activity of BTBR mice [26,28], a mouse strain also known to exhibit autistic-like behaviours. Further studies are required in order to decipher if enhancing central 2-AG tone and consequently CB_1 receptor activation may ameliorate some of the behavioural changes in VPA-exposed animals.

Although 2-AG levels were unaltered following exposure to the sociability test, the FAAH substrates AEA, OEA and PEA were increased in the hippocampus of VPA-exposed animals. BTBR mice have been reported to exhibit increased cortical AEA, but not 2-AG or OEA, levels following exposure to the sociability test [26], however it is unknown if alterations also exist in other brain regions. Social play behaviour enhances AEA levels in the amygdala, nucleus accumbens [21] and striatum [22], but not in the prefrontal cortex or hippocampus. Pharmacological and genetic inhibition of FAAH [24,48], inhibition of AEA transport [49] and central administration of AEA [25] enhances social behaviour, indicating that enhanced endocannabinoid activity facilitates social play behaviour. Additional studies have revealed that enhanced AEA tone in the basolateral amygdala and nucleus accumbens [21] but not piriform cortex [50] mediates social interactive behaviour. In comparison, broad central activation of CB_1 receptors impairs social play behaviour [24]. The authors suggest that enhancing AEA levels and activating CB_1 receptors in brain circuits regulating social behaviour facilitates social play, however broad excitation of central CB_1 receptors interferes with the normal excitation of complex social acts [21,24], possibly by interfering with cognitive functions required for normal social interactions [51]. It should also be noted that the experimental conditions (social interactions vs sociability) and test subjects (naive rats vs VPA-exposed rats) used in the latter studies are significantly different to those used in the present study and alterations in endocannabinoid levels in brain regions such as the nucleus accumbens, amygdala or striatum cannot be ruled out. However, the role of the hippocampus in cognition is well recognised with a wealth of data demonstrating that CB_1 receptor activation reduces, while blockade enhances, cognitive performance [51]. As such, it is possible that AEA-induced activation of CB_1 receptors in the hippocampus of VPA-exposed animals during the sociability test results in impaired cognitive ability and subsequent deficits in social investigatory behaviour. Increased OEA and PEA levels as observed following the sociability test, may compete with AEA at the FAAH catalytic site leading to reduced catabolism of AEA, increased levels and subsequent enhanced activity at the CB_1 receptor. Alternatively, as neither OEA nor PEA exhibit affinity for CB_1 receptors, it is possible that competition with the FAAH substrates for binding at PPARs, shunts AEA activity back onto the CB_1 receptor. Some of the behavioural changes may also be mediated by AEA activation of alternative receptor targets to CB_1 or direct activation of PPARs by OEA or PEA. The present study demonstrated a reduced expression of PPARγ and GRP55 in the hippocampus of VPA-exposed animals. Although the role of PPARs or GPR55 on social behaviour is unknown, recent data indicate that activation of hippocampal PPARγ enhances cognitive performance [52]. Thus, downregulation of PPARγ in the hippocampus of VPA-exposed rats may result in reduced cognitive performance and impaired behavioural responding to stressful situations. PPARα activation by OEA or selective agonists facilitates

memory consolidation via noradrenergic activation of the amygdala [53]. PPARα and GPR55 expression are reduced in the frontal cortex of VPA-exposed animals, and although endocannabinoid levels were not altered in this region, altered activity at these receptors may account for some of the behavioural changes observed such as hypoalgesia or anxiety-related behaviour.

In conclusion, the present data demonstrates alterations in the endocannabinoid system in adolescent rats exposed prenatally to VPA, effects which may underlie some of the behavioural changes observed in the model. Thus, modulation of the endocannabinoid system may provide a novel pharmacological target for the treatment of behavioural traits associated with autism spectrum disorders.

Acknowledgements

References

[1] Williams PG, Hersh JH. A male with fetal valproate syndrome and autism. Developmental Medicine and Child Neurology 1997;39:632–4.

[2] Williams G, King J, Cunningham M, Stephan M, Kerr B, Hersh JH. Fetal valproate syndrome and autism: additional evidence of an association. Developmental Medicine and Child Neurology 2001;43:202–6.

[3] Christianson AL, Chesler N, Kromberg JG. Fetal valproate syndrome: clinical and neuro-developmental features in two sibling pairs. Developmental Medicine and Child Neurology 1994;36:361–9.

[4] Schneider T, Labuz D, Przewlocki R. Nociceptive changes in rats after prenatal exposure to valproic acid. Polish Journal of Pharmacology 2001;53:531–4.

[5] Schneider T, Przewlocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology 2005;30:80–9.

[6] Schneider T, Ziolkowska B, Gieryk A, Tyminska A, Przewlocki R. Prenatal exposure to valproic acid disturbs the enkephalinergic system functioning, basal hedonic tone, and emotional responses in an animal model of autism. Psychopharmacology 2007;193:547–55.

[7] Markram K, Rinaldi T, La Mendola D, Sandi C, Markram H. Abnormal fear conditioning and amygdala processing in an animal model of autism. Neuropsychopharmacology 2008;33:901–12.

[8] Ingram JL, Peckham SM, Tisdale B, Rodier PM. Prenatal exposure of rats to valproic acid reproduces the cerebellar anomalies associated with autism. Neurotoxicology and Teratology 2000;22:319–24.

[9] Rodier PM, Ingram JL, Tisdale B, Croog VJ. Linking etiologies in humans and animal models: studies of autism. Reproductive Toxicology 1997;11:417–22.

[10] Sui L, Chen M. Prenatal exposure to valproic acid enhances synaptic plasticity in the medial prefrontal cortex and fear memories. Brain Research Bulletin 2012;87:556–63.

[11] Rinaldi T, Perrodin C, Markram H. Hyper-connectivity and hyper-plasticity in the medial prefrontal cortex in the valproic acid animal model of autism. Frontiers in Neural Circuits 2008;2:1–7.

[12] Kim KC, Kim P, Go HS, Choi CS, Park JH, Kim HJ, et al. Male-specific alteration in excitatory postsynaptic development and social interaction in prenatal valproic acid exposure model of autism spectrum disorder. Journal of Neurochemistry 2013;124:832–43.

[13] Dufour-Rainfray D, Vourc'h P, Le Guisquet AM, Garreau L, Ternant D, Bodard S, et al. Behavior and serotonergic disorders in rats exposed prenatally to valproate: a model for autism. Neuroscience Letters 2010;470:55–9.

[14] Schneider T, Roman A, Basta-Kaim A, Kubera M, Budziszewska B, Schneider K, et al. Gender-specific behavioral and immunological alterations in an animal model of autism induced by prenatal exposure to valproic acid. Psychoneuroendocrinology 2008;33:728–40.

[15] Lutz B. Endocannabinoid signals in the control of emotion. Current Opinion in Pharmacology 2009;9:46–52.

[16] Viveros MP, Marco EM, Llorente R, Lopez-Gallardo M. Endocannabinoid system and synaptic plasticity: implications for emotional responses. Neural Plasticity 2007;2007:1–12.

[17] Hogestatt ED, Jonsson BA, Ermund A, Andersson DA, Bjork H, Alexander JP, et al. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. The Journal of Biological Chemistry 2005;280:31405–12.

[18] Schultz ST. Can autism be triggered by acetaminophen activation of the endocannabinoid system? Acta Neurobiologiae Experimentalis (Warsaw) 2010;70:227–31.

[19] Chakrabarti B, Kent L, Suckling J, Bullmore E, Baron-Cohen S. Variations in the human cannabinoid receptor (CNR1) gene modulate striatal responses to happy faces. European Journal of Neuroscience 2006;23:1944–8.

[20] Chakrabarti B, Baron-Cohen S. Variation in the human cannabinoid receptor CNR1 gene modulates gaze duration for happy faces. Molecular Autism 2011;2:1–7.

[21] Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LW, Pasterkamp RJ, et al. Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. Journal of Neuroscience 2012;32:14899–908.

[22] Marco EM, Rapino C, Caprioli A, Borsini F, Maccarrone M, Laviola G. Social encounter with a novel partner in adolescent rats: activation of the central endocannabinoid system. Behavioral Brain Research 2011;220:140–5.

[23] Di Marzo V, Bisogno T, Sugiura T, Melck D, De Petrocellis L. The novel endogenous cannabinoid 2-arachidonoylglycerol is inactivated by neuronal- and basophil-like cells: connections with anandamide. Biochemical Journal 1998;331(Pt 1):15–9.

[24] Trezza V, Vanderschuren LJ. Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology 2008;197:217–27.

[25] Umathe SN, Manna SS, Utturwar KS, Jain NS. Endocannabinoids mediate anxiolytic-like effect of acetaminophen via CB1 receptors. Progress in Neuropsychopharmacology and Biological Psychiatry 2009;33:1191–9.

[26] Gould GG, Seillier A, Weiss G, Giuffrida A, Burke TF, Hensler JG, et al. Acetaminophen differentially enhances social behavior and cortical cannabinoid levels in inbred mice. Progress in Neuropsychopharmacology and Biological Psychiatry 2012;38:260–9.

[27] Meyza, K.Z., Defensor, E.B., Jensen, A.L., Corley, M.J., Pearson, B.L., Pobbe, R.L., et al. The BTBR T(+)tf/J mouse model for autism spectrum disorders–in search of biomarkers. Behavioral Brain Research, http://dx.doi.org/10.1016/j.bbr.2012.07.021, in press.

[28] Onaivi ES, Benno R, Halpern T, Mehanovic M, Schanz N, Sanders C, et al. Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders. Current Neuropharmacology 2011;9:209–14.

[29] Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL, et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. Proceedings of the National Academy of Sciences of United States of America 2002;99:10819–24.

[30] Gao Y, Vasilyev DV, Goncalves MB, Howell FV, Hobbs C, Reisenberg M, et al. Loss of retrograde endocannabinoid signaling and reduced adult neurogenesis in diacylglycerol lipase knock-out mice. Journal of Neuroscience 2010;30:2017–24.

[31] Maccarrone M, Rossi S, Bari M, De Chiara V, Rapino C, Musella A, et al. Abnormal mGlu5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. Neuropsychopharmacology 2010;35:1500–9.

[32] Jung KM, Sepers M, Henstridge CM, Lassalle O, Neuhofer D, Martin H, et al. Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. Nature Communications 2012;3:1–11.

[33] Sun Y, Alexander SP, Kendall DA, Bennett AJ. Cannabinoids and PPARalpha signalling. Biochemical Society Transactions 2006;34:1095–7.

[34] Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, et al. The orphan receptor GPR55 is a novel cannabinoid receptor. British Journal of Pharmacology 2007;152:1092–101.

[35] Sandhya T, Sowjanya J, Veeresh B. Bacopa monniera (L.) Wettst ameliorates behavioral alterations and oxidative markers in sodium valproate induced autism in rats. Neurochemical Research 2012;37:1121–31.

[36] Crawley JN. Designing mouse behavioral tasks relevant to autistic-like behaviors. Mental Retardation and Developmental Disabilities Research Reviews 2004;10:248–58.

[37] Nadler JJ, Moy SS, Dold G, Trang D, Simmons N, Perez A, et al. Automated apparatus for quantitation of social approach behaviors in mice. Genes, Brain and Behavior 2004;3:303–14.

[38] Kerr DM, Burke NN, Ford GK, Connor TJ, Harhen B, Egan LJ, et al. Pharmacological inhibition of endocannabinoid degradation modulates the expression of inflammatory mediators in the hypothalamus following an immunological stressor. Neuroscience 2012;1:53–63.

[39] Olango WM, Roche M, Ford GK, Harhen B, Finn DP. The endocannabinoid system in the rat dorsolateral periaqueductal grey mediates fear-conditioned analgesia and controls fear expression in the presence of nociceptive tone. British Journal of Pharmacology 2012;165:2549–60.

[40] Kerr, D.M., Harhan, B., Okine, B.N., Egan, L.J., Finn, D.P., Roche, M. The monoacylglycerol lipase inhibitor JZL184 attenuates LPS-induced increases in cytokine expression in the rat frontal cortex and plasma: differential mechanisms of action. British Journal of Pharmacology, http://dx.doi.org/10.1111/j.1476-5381.2012.02237, in press.

[41] Ford GK, Kieran S, Dolan K, Harhen B, Finn DP. A role for the ventral hippocampal endocannabinoid system in fear-conditioned analgesia and fear responding in the presence of nociceptive tone in rats. Pain 2011;152:2495–504.

[42] Cable JC, Tan GD, Alexander SP, O'Sullivan SE. The activity of the endocannabinoid metabolising enzyme fatty acid amide hydrolase in subcutaneous adipocytes correlates with BMI in metabolically healthy humans. Lipids in Health and Disease 2011;10:129.

[43] Kim KC, Kim P, Go HS, Choi CS, Yang SI, Cheong JH, et al. The critical period of valproate exposure to induce autistic symptoms in Sprague-Dawley rats. Toxicology Letters 2011;201:137–42.

[44] Kim P, Park JH, Kwon KJ, Kim KC, Kim HJ, Lee JM, et al. Effects of Korean red ginseng extracts on neural tube defects and impairment of social interaction induced by prenatal exposure to valproic acid. Food and Chemical Toxicology 2013;51:288–96.

[45] Militerni R, Puglisi-Allegra S, Pascucci T, Bravaccio C, Falco C, Fico C. Pain reactivity in children with autistic disorder. Journal of the Intellectual Disability Research 2000;44:394–5.

[46] Tordjman S, Anderson GM, Botbol M, Brailly-Tabard S, Perez-Diaz F, Graignic R, et al. Pain reactivity and plasma beta-endorphin in children and adolescents with autistic disorder. PLoS ONE 2009;4:e5289.

[47] Zhang Y, Sun Y, Wang F, Wang Z, Peng Y, Li R. Downregulating the canonical Wnt/beta-catenin signaling pathway attenuates the susceptibility to autism-like phenotypes by decreasing oxidative stress. Neurochemical Research 2012;37:1409–19.

[48] Cassano T, Gaetani S, Macheda T, Laconca L, Romano A, Morgese MG, et al. Evaluation of the emotional phenotype and serotonergic neurotransmission of fatty acid amide hydrolase-deficient mice. Psychopharmacology 2011;214:465–76.

[49] Trezza V, Vanderschuren LJ. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. Journal of Pharmacology and Experimental Therapeutics 2009;328:343–50.

[50] Zenko M, Zhu Y, Dremencov E, Ren W, Xu L, Zhang X. Requirement for the endocannabinoid system in social interaction impairment induced by coactivation of dopamine D1 and D2 receptors in the piriform cortex. Journal of Neuroscience Research 2011;89:1245–58.

[51] Egerton A, Allison C, Brett RR, Pratt JA. Cannabinoids and prefrontal cortical function: insights from preclinical studies. Neuroscience and Biobehavioral Reviews 2006;30:680–95.

[52] Denner LA, Rodriguez-Rivera J, Haidacher SJ, Jahrling JB, Carmical JR, Hernandez CM, et al. Cognitive enhancement with rosiglitazone links the hippocampal PPARgamma and ERK MAPK signaling pathways. The Journal of Neuroscience 2012;32:16725–35.

[53] Campolongo P, Roozendaal B, Trezza V, Cuomo V, Astarita G, Fu J, et al. Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. Proceedings of the National Academy of Sciences of United States of America 2009;106:8027–31.

Molecular Autism

Variation in the human cannabinoid receptor *CNR1* gene modulates gaze duration for happy faces

Bhismadev Chakrabarti[1,2*] and Simon Baron-Cohen[2]

Abstract

Background: From an early age, humans look longer at preferred stimuli and also typically look longer at facial expressions of emotion, particularly happy faces. Atypical gaze patterns towards social stimuli are common in autism spectrum conditions (ASC). However, it is unknown whether gaze fixation patterns have any genetic basis. In this study, we tested whether variations in the cannabinoid receptor 1 (*CNR1*) gene are associated with gaze duration towards happy faces. This gene was selected because *CNR1* is a key component of the endocannabinoid system, which is involved in processing reward, and in our previous functional magnetic resonance imaging (fMRI) study, we found that variations in *CNR1* modulate the striatal response to happy (but not disgust) faces. The striatum is involved in guiding gaze to rewarding aspects of a visual scene. We aimed to validate and extend this result in another sample using a different technique (gaze tracking).

Methods: A total of 30 volunteers (13 males and 17 females) from the general population observed dynamic emotional expressions on a screen while their eye movements were recorded. They were genotyped for the identical four single-nucleotide polymorphisms (SNPs) in the *CNR1* gene tested in our earlier fMRI study.

Results: Two SNPs (rs806377 and rs806380) were associated with differential gaze duration for happy (but not disgust) faces. Importantly, the allelic groups associated with a greater striatal response to happy faces in the fMRI study were associated with longer gaze duration at happy faces.

Conclusions: These results suggest that *CNR1* variations modulate the striatal function that underlies the perception of signals of social reward, such as happy faces. This suggests that *CNR1* is a key element in the molecular architecture of perception of certain basic emotions. This may have implications for understanding neurodevelopmental conditions marked by atypical eye contact and facial emotion processing, such as ASC.

Background

Vision is the primary sensory modality in primates, reflected by the visual cortex being the largest of all the sensory cortices. Our eyes perform quick orienting movements ('saccades') towards interesting features of stimuli in the external world [1]. In general, we tend to look longer at more rewarding stimuli [2]. This rationale lies behind the 'preferential looking' technique in infancy research, where gaze duration and direction are assumed to reflect visual preference [2-6]. Gaze not only informs

us about normative variation in the visual processing of stimuli but also is relevant to the understanding of complex neurodevelopmental conditions such as autism spectrum conditions (ASC), which are characterised by atypical gaze fixation patterns [7,8]. This has led to the suggestion that gaze fixation patterns could constitute potential endophenotypes for such conditions. Gaze patterns show high test-retest reliability as well as a moderate to high heritability when tested in twins [9-11], suggesting a significant genetic contribution. This raises the possibility that variation in candidate genes underlie normative variation in gaze patterns.

The measure of particular interest to us is the duration of gaze fixation, given the evidence that people with ASC show reduced gaze fixation towards social stimuli

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[8,12-15]. Research in primates suggests that the striatal region plays a major role in directing gaze [16]. The striatum is thought to encode a 'value map' of the visual stimuli. Both ventral striatal neurons as well as a subpopulation of caudate neurons encode reward magnitude of the stimuli [17,18]. This 'value map', in addition to further frontal cortical inputs, is then passed to the lateral intraparietal area (LIP), where a fine-tuned map of 'relative expected subjective value' is created. The LIP projects to the frontal eye fields, which send excitatory projections to the caudate nucleus. A subset of neurons from the caudate nucleus inhibit the substantia nigra and consequently disinhibit the superior colliculus, which in turn controls the gaze control nuclei in the brainstem, leading to a gaze shift [19].

One of the key molecular systems involved in the functioning of the striatal circuit is the endocannabinoid system. It is a neuropeptidergic circuit involved in reward processing and works in tandem with the mesolimbic dopaminergic system [20]. Expressed selectively in the brain, the cannabinoid receptor 1 (CNR1) is the best-studied molecule of this system. Immunolocalisation studies in rats and humans have indicated high *CNR1* expression levels in the striatum, a region known for its central role in reward processing [20-24]. CNR1 is believed to modulate striatal dopamine release through a trans-synaptic mechanism involving both GABAergic and glutamatergic synapses and is expressed strongly in the caudate, putamen, globus pallidus internal and substantia nigra, as well as in the shell of the nucleus accumbens [25]. Phasic release of striatal dopamine is the primary mechanism encoding for reward [26].

Recent studies have suggested abnormalities in ASC in striatal volume [27,28], connectivity [29] and activity in response to social stimuli [30]. In addition, a gene expression study of postmortem brain tissue of people with ASC found reduced expression of *CNR1* [31]. In view of the atypical gaze behaviour of people with ASC, together with the observed striatal atypicalities, it is reasonable to examine the phenotype of gaze patterns as a function of variation in genes expressed in the striatum.

As gaze fixation is linked to striatal activity [16,17,19], we might expect that molecular variation in the genes involved in striatal function would be associated with differences in gaze towards socially rewarding stimuli. Using functional magnetic resonance imaging (fMRI), we previously found genetic variation in *CNR1* modulated activity in the striatal region while watching happy (but not disgust) faces [32]. This result has been independently replicated in larger samples [33]. In the current study, we aimed to conduct an identical experiment using gaze-tracking in a new sample of volunteers. Specifically, we tested whether *CNR1* genetic variation influences gaze duration towards happy faces. To ensure that

this was closely matched to the original fMRI experiment, we also analysed gaze fixation duration for disgust expressions as a function of *CNR1* genetic variation. Disgust faces are potential signals of 'nonreward', in contrast to rewarding happy faces, and hence provide a high-level control condition (that is, matched for face-specific qualities, such as configural features, as well as more general visual qualities of the stimuli, such as colour, shape and luminosity) for our experiment. We predicted that variation (single-nucleotide polymorphisms (SNPs)) in the *CNR1* gene would be significantly associated with individual variability in gaze duration towards happy but not disgust faces.

Methods

Participants

A total of 30 student volunteers (13 males and 17 females; 29 right-handed and 1 left-handed; mean age ± SD, 24.1 ± 3.41 years old) were recruited by advertisement from the local universities. Participants were included only if all four grandparents were of Caucasian European ancestry to avoid genetic heterogeneity between different populations. Participants were also excluded if they reported any history of psychiatric diagnoses or regular drug abuse. They were equated for educational background in that all had completed high school and were studying towards a college degree. All had normal (or corrected to normal) vision. The study was approved by the Psychology Research Ethics Committee of the University of Cambridge.

Buccal swabs were collected from all participants, and DNA was extracted. The four SNPs of choice were identical to those selected in our earlier fMRI study (rs1049353, rs806377, rs806380 and rs6454674), chosen to ensure a minor allele frequency > 0.2 in a Caucasian population and to cover as much of the gene as possible (see Figure 1) [32]. The DNA was genotyped by Geneservice, Inc. (Cambridge, UK) using standard TaqMan™ assays (Applied Biosystems, Inc., California, USA). Genotyping for these SNPs failed for two of these participants, resulting in a sample size of 28 participants for the final analysis.

Procedure

The stimuli were taken from the *Mindreading*™ set developed by Baron-Cohen *et al.* [34], since dynamic facial expressions of emotion are assumed to be more ecologically valid than static photographs. The *Mindreading* set consists of video, audio and textual examples for 412 different emotions arranged into 24 emotion groups and organised according to six different developmental levels (based on emotions recognised in childhood through adulthood). These stimuli have been validated in typical populations and in people with ASC

Figure 1 Schematic structure of the cannabinoid receptor *CNR1* gene with all four genotyped single-nucleotide polymorphisms indicated. Top: White boxes indicate untranslated regions, black boxes indicate translated regions and intervening straight line indicates an intronic region. Bottom: The linkage disequilibrium structure of the gene in the Caucasian (CEU) population is shown (using the publicly available HapMap version 3, release R2, database available at http://hapmap.ncbi.nlm.nih.gov/.

[35-38]. These stimuli were chosen over other existing available stimuli because *Mindreading* stimuli comprise dynamic emotional expressions whilst alternatives (such as the Ekman and Friesen set [39], the Karolinska Directed Emotional Faces set [40] and the NimStim set [41]) comprise static expressions. The *Mindreading* stimuli have excellent interrater reliability and external validity [36,38] (stimuli are available at http://www.jkp.com/mindreading/).

Participants were seated comfortably at a fixed distance of 60 cm from the screen and were instructed to keep movement to a minimum. Participants watched 80 videoclips (three seconds each and sixteen clips for each of the five emotions) presented in a pseudorandom order using GazeTracker™ software (DynaVox Inc., Virginia, USA) with an interstimulus interval of six seconds. Participants were shown a fixation cross during the interstimulus interval. All stimuli were centred on a 19-inch monitor and occupied 70% of the screen area. To ensure that participants were attending to the stimulus, they were asked to say aloud what emotion they thought was being displayed (choosing one of five emotion words: 'happy', 'sad', 'angry', 'disgust' or 'fear'). Their responses were recorded by the experimenter.

The Eye Response Interface Computer Aid camera (ERICA; http://www.eyeresponse.com/) was used to measure fixation time at each point at 60 Hz. ERICA
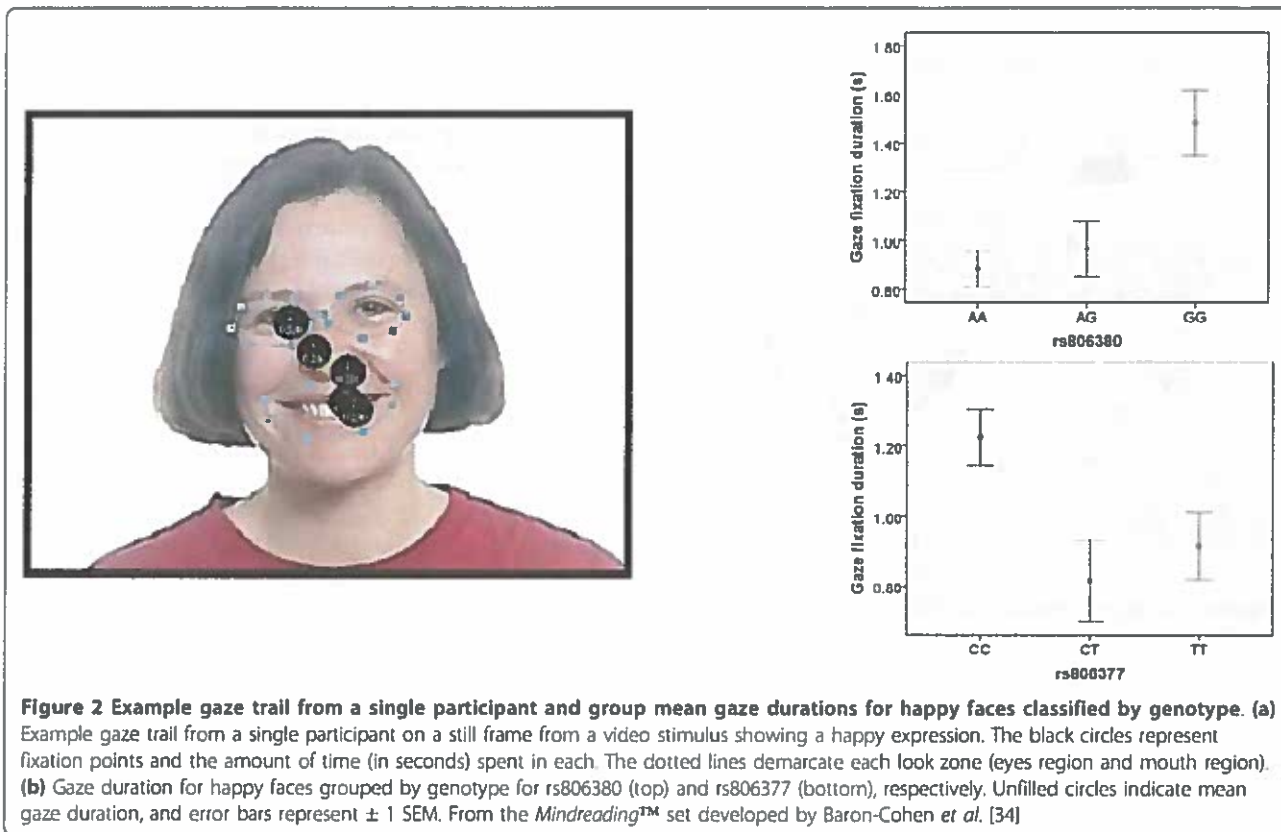
uses reflected low-frequency infrared rays ($\lambda = 880$ nm) to map macrosaccades and fixation times at each point. The data were preprocessed using GazeTracker™ software. To ensure that the measured gaze duration was specific to the socially informative regions of the emotion expressions [35], 'look zones' were manually drawn around the eyes (the eyebrows and lower eyelids) and mouth region (the region from the bottom of the nose to the bottom of the lower lip) of all stimuli (see Figure 2). All look zones were 'dynamic'; that is, they tracked the eyes and the mouth regions while allowing for head movement of the actors.

The sum of duration of all fixations was recorded for each look zone. A fixation was defined as a continuous gaze for 100 ms within a 40-pixel diameter (corresponding to a 1.3° visual angle), which was in line with parameters used in similar studies [15]. Gaze duration for each expression was calculated by summing the mean fixation time for eyes and mouth regions. Different regions of the face (that is, eyes and mouth) are relevant for processing different basic emotions [12,42-44]. Thus it is not ideal to compare the fixation time to the eyes region for happy and disgust faces, since disgust faces are associated with greater gaze duration to the mouth region. Hence, total fixation time across eyes and mouth regions was used as the dependent variable.

Results

Both happy and disgust expressions were recognised with > 80% accuracy. Genetic association was measured using the UNPHASED programme (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased), which computes the retrospective likelihood, that is, the probability of observing different genotypes given an observed distribution of a quantitative trait [45]. The two dependent variables (gaze duration for all happy and all disgust faces) and the genotypes for all four SNPs were included in a single analysis. This analysis revealed a significant association of the gaze duration for happy faces with rs806377 ($\chi^2 = 8.88$, $df = 2$, $P = 0.011$) and rs806380 ($\chi^2 = 8.46$, $df = 2$, $P = 0.014$). No significant associations (at $P \leq 0.05$) were noted for gaze duration for disgust faces (nominal $p_{rs806377} = 0.104$ and nominal $p_{rs806380} = 0.086$). To test whether the observed lack of significant association with disgust faces was due to one video clip that was misclassified by a majority of the participants, the data were reanalysed after removing all fixation data associated with this one video clip. This revealed an identical pattern of results, with a nominal $p_{rs806377} = 0.111$ and a nominal $p_{rs806380} = 0.105$.

However, when multiple SNPs are in linkage disequilibrium (LD), hypothesis tests in single-locus analyses are not independent. To take this into account, Li and Ji

Figure 2 Example gaze trail from a single participant and group mean gaze durations for happy faces classified by genotype. (a)
Example gaze trail from a single participant on a still frame from a video stimulus showing a happy expression. The black circles represent
fixation points and the amount of time (in seconds) spent in each. The dotted lines demarcate each look zone (eyes region and mouth region).
(b) Gaze duration for happy faces grouped by genotype for rs806380 (top) and rs806377 (bottom), respectively. Unfilled circles indicate mean
gaze duration, and error bars represent ± 1 SEM. From the *Mindreading*™ set developed by Baron-Cohen *et al.* [34]

[46] proposed a method for estimating the true number
of independent tests (M_{eff}), which takes into considera-
tion the LD between SNPs. This method was implemen-
ted using the SNPSpD software programme [47], which
revealed that M_{eff} was 3 in the current sample. The
Bonferroni correction for three independent tests gave a
corrected $P = 0.033$ for rs806377 and a corrected $P =$ of
0.042 for rs806380 for association with gaze duration for
happy faces.

To further analyse genotypic differences for each SNP
that were significantly associated with gaze duration for
happy faces, *post hoc* *t*-tests were conducted. In
rs806377, the CC genotype was associated with longer
gaze duration than the CT genotype ($t = 2.92$, $P < 0.025$
with the Bonferroni correction). In rs806380, the GG
genotype was associated with longer gaze duration than
the AA genotype ($t = 2.78$, $P < 0.05$ with the Bonferroni
correction) (see Figure 2).

The main effects of all possible haplotypes were tested
with various possible window sizes (two, three and four
marker combinations) using UNPHASED software.
None of these haplotypic association tests were signifi-
cant at $P < 0.05$. While the small sample size did not
allow for a robust test of sex differences in this genetic
association, we report the nominal P values for these
tests for the sake of completeness. rs806377 was

significantly associated with the gaze duration for happy
faces in both females ($P = 0.021$) and males ($P = 0.004$).
Additionally, in males, rs806380 ($P = 0.019$) and
rs1049353 ($P = 0.004$) were found to be associated with
gaze duration for happy faces.

Discussion

In this experiment, we predicted that *CNR1* genetic var-
iations would be associated with differences in gaze fixa-
tion duration towards happy faces. This prediction was
confirmed: two SNPs in this gene (rs806377 and
rs806380) were associated with differences in gaze dura-
tion for happy (but not disgust) faces. This finding fits
well with the established role of the *CNR1* gene in
reward processes [20] and is consistent with the results
of fMRI studies [32,33] in showing that this gene is a
component of the molecular architecture of social
reward processing. Social reward processing has been
suggested to be impaired in people with ASC [48-50],
particularly as reflected in atypical gaze patterns towards
social stimuli. Hence the current results could be rele-
vant to understanding the genetic underpinning of the
social behavioural symptoms in people with ASC.

A comparison of these results with those from our
earlier fMRI study reveals that for the SNP rs806377,
the allelic group (CC) associated with the highest striatal

response is also associated with the longest gaze duration for happy faces. For rs806380, the allelic group associated with the highest striatal response (GG) is also associated with the longest gaze duration for happy faces. rs806377 is located in an untranslated region (UTR) of the gene (Figure 1), and rs806380 was found to be in significant LD with a 5'-UTR SNP (rs78074274) using CandiSNPer [51]. The observed effects can thus be mediated by either or both of these UTR SNPs by potentially altering gene transcription and/or mRNA stability. Since the fMRI and gaze duration data come from largely independent samples (only three of thirty participants were common to both studies), it is likely that the observed genetic differences reflect real effects.

We interpret the genetically linked biasing of visual perception in terms of individual differences in the reward circuitry. The two processes of increased visual preference (indicated by longer gaze duration) and increased striatal response for happy faces are linked in a positive feedback loop [5]. We tend to look longer at preferred stimuli, which in turn increases our preference/'reward value' for these stimuli. Consequently, we interpret the observed effect in biasing visual perception of social stimuli in terms of differences in the individual reward circuitry. Whether such intrinsic differences in reward circuitry change the formation and nature of 'saliency/value maps' formed during gaze fixation is a question for future research [19,52].

A second broader question for future research is whether the observed *CNR1* genotypic differences in fixation duration for happy faces is specific to social rewards or whether this holds true for all classes of rewards. Variation in *CNR1* has been linked to polysubstance abuse and associated with increased activity in reward-processing areas of the brain in response to drug cues for both marijuana and alcohol addicts [53,54]. Hence it is possible that the observed genotypic differences in the general population may extend to other classes of rewards. Crucially, however, a reduced experience of rewards in response to social stimuli such as happy faces (as has been suggested by Dawson *et al.* [48] to apply to ASC) has more far-reaching consequences, since if an infant is looking less at happy faces and is finding them less rewarding, this will make social interactions less reinforcing, which in turn can exacerbate the social difficulties observed in people with ASC.

It is possible that a number of genes, each of small to medium effect size, determine the striatal response to social stimuli such as happy faces. Other potential candidate genes might include those involved in the oxytocin-vasopressin system (*OXTR*, *AVPR1A* and *AVPR1B*) as well as those coding for key proteins involved in neutrotransmission (for example, *MAOA* and *GABRB3*)

[55]. We speculate that these genes have an additive effect and might potentially underlie complex traits related to social functioning [56]. In a larger population-based genetic association study of empathy, we found a nominally significant association of *CNR1* genetic variation with the Empathy Quotient [55,57]. Additionally, reduced expression of *CNR1* was found in postmortem brain tissue of individuals with ASC [31]. Together, these findings further support the implication that variation in the *CNR1* gene modulates the response to social stimuli such as happy faces.

However, the current findings should be interpreted with caution, since, in the absence of any expression data, any functional role for the SNPs can only be speculative; that is, the observed SNP effects may be caused by being in LD with other functional polymorphisms and/or through mechanisms that affect mRNA stability or splicing as mentioned earlier. However, the observation of genetic differences in two separate (albeit small) samples using an identical paradigm with two different techniques points towards a putative role played by *CNR1* in the response to happy faces.

Conclusions

In this study, we tested whether common variants in the *CNR1* gene modulate gaze duration towards happy faces. We found that two SNPs in this gene were significantly associated with gaze duration for happy (but not disgust) faces. This result is consistent with that of our previous fMRI study [32]. Specifically, the allelic groups that were found to be associated with the strongest striatal response in our fMRI study were associated with the longest gaze duration for happy faces in the current sample. This finding suggests a role for *CNR1* in social reward processing and could have significance for clinical conditions such ASC, which are marked by a deficit in social reward processing as well as atypical responses to facial expressions of emotion [35,36,49].

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Authors' contributions
BC designed and ran the experiment, analysed the data and wrote the paper. SBC provided intellectual input at all stages and cowrote the paper. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

References
1. Moschovakis A, Highstein SM: The anatomy and physiology of primate neurons that control rapid eye movements. *Annu Rev Neurosci* 1994, 17:465-488.
2. Calvo M, Avero P: Time course of attentional bias to emotional scenes in anxiety: gaze direction and duration. *Cogn Emot* 2005, 19:433-451.
3. Butterworth G, Jarrett N: What minds have in common is space: spatial mechanisms serving joint visual attention in infancy. *Br J Dev Psychol* 1991, 9:55-72.
4. Calvo M, Lang P: Gaze patterns when looking at emotional pictures: motivationally biased attention. *Motiv Emot* 2004, 28:221-243.
5. Shimojo S, Simion C, Shimojo E, Scheier C: Gaze bias both reflects and influences preference. *Nat Neurosci* 2003, 6:1317-1322.
6. Connellan J, Baron-Cohen S, Wheelwright S, Batki A, Ahluwalia J: Sex differences in human neonatal social perception. *Infant Behav Dev* 2001, 23:113-118.
7. Fletcher-Watson S, Leekam SR, Benson V, Frank MC, Findlay JM: Eye-movements reveal attention to social information in autism spectrum disorder. *Neuropsychologia* 2009, 47:248-257.
8. Klin A, Jones W, Schulz R, Volkmar F, Cohen DJ: Visual fixation patterns during viewing of naturalistic social situations as predictors of social competence in individuals with autism. *Arch Gen Psychiatry* 2002, 9:809-816.
9. Katsanis J, Taylor J, Iacono WG, Hammer MA: Heritability of different measures of smooth pursuit eye tracking dysfunction: a study of normal twins. *Psychophysiology* 2000, 37:724-730.
10. Iacono WG: Eye tracking in normal twins. *Behav Genet* 1982, 12:517-526.
11. Greenwood TA, Braff DL, Light GA, Cadenhead KS, Calkins ME, Dobie DJ, Freedman R, Green MF, Gur RE, Gur RC, Mintz J, Nuechterlein KH, Olincy A, Radant AD, Seidman LJ, Siever LJ, Silverman JM, Stone WS, Swerdlow NR, Tsuang DW, Tsuang MT, Turetsky BI, Schork NJ: Initial heritability analyses of endophenotypic measures for schizophrenia: the Consortium on the Genetics of Schizophrenia. *Arch Gen Psychiatry* 2007, 64:1242-1250.
12. Baron-Cohen S, Wheelwright S, Jolliffe T: Is there a "language of the eyes"? Evidence from normal adults and adults with autism or Asperger syndrome. *Vis Cogn* 1997, 4:311-331.
13. Joliffe T, Mortimore C, Robertson M: Another advanced test of theory of mind: evidence from very high functioning adults with autism or Asperger syndrome. *J Child Psychol Psychiatry* 1997, 38:813-822.
14. Swettenham J, Baron-Cohen S, Charman T, Cox A, Baird G, Drew A, Rees L, Wheelwright S: The frequency and distribution of spontaneous attention shifts between social and non-social stimuli in autistic, typically developing, and non-autistic developmentally delayed infants. *J Child Psychol Psychiatry* 1998, 9:747-753.
15. Senju A, Southgate V, White S, Frith U: Mindblind eyes: an absence of spontaneous theory of mind in Asperger syndrome. *Science* 2009, 325:883-885.
16. Hikosaka O: Basal ganglia mechanisms of reward-oriented eye movement. *Ann N Y Acad Sci* 2007, 1104:229-249.
17. Lau B, Glimcher PW: Action and outcome encoding in the primate caudate nucleus. *J Neurosci* 2007, 27:14502-14514.
18. Cai X, Kim S, Lee D: Heterogeneous coding of temporally discounted values in the dorsal and ventral striatum during intertemporal choice. *Neuron* 2011, 69:170-182.
19. Trommershäuser J, Glimcher PW, Gegenfurtner KR: Visual processing, learning and feedback in the primate eye movement system. *Trends Neurosci* 2009, 32:583-590.
20. Gardner EL, Vorel SR: Cannabinoid transmission and reward-related events. *Neurobiol Dis* 1998, 5:502-533.
21. Freund TF, Katona I, Piomelli D: Role of endogenous cannabinoids in synaptic signaling. *Physiol Rev* 2003, 83:1017-1066.
22. Fusco FR, Martorana A, Giampà C, De March Z, Farini D, D'Angelo V, Sancesario G, Bernardi G: Immunolocalization of CB1 receptor in rat striatal neurons: a confocal microscopy study. *Synapse* 2004, 53:159-167.
23. Hurley MJ, Mash DC, Jenner P: Expression of cannabinoid CB1 receptor mRNA in basal ganglia of normal and parkinsonian human brain. *J Neural Transm* 2003, 110:1279-1288.
24. Haber SN, Knutson B: The reward circuit: linking primate anatomy and human imaging. *Neuropsychopharmacology* 2010, 35:4-26.
25. van der Stelt M, Di Marzo V: The endocannabinoid system in the basal ganglia and in the mesolimbic reward system: implications for neurological and psychiatric disorders. *Eur J Pharmacol* 2003, 480:133-150.
26. Schultz W: Getting formal with dopamine and reward. *Neuron* 2002, 36:241-263.
27. Langen M, Schnack HG, Nederveen H, Bos D, Lahuis BE, de Jonge MV, van Engeland H, Durston S: Changes in the developmental trajectories of striatum in autism. *Biol Psychiatry* 2009, 66:327-333.
28. Estes A, Shaw DW, Sparks BF, Friedman S, Giedd JN, Dawson G, Bryan M, Dager SR: Basal ganglia morphometry and repetitive behavior in young children with autism spectrum disorder. *Autism Res* .
29. Di Martino A, Kelly C, Grzadzinski R, Zuo XN, Mennes M, Mairena MA, Lord C, Castellanos FX, Milham MP: Aberrant striatal functional connectivity in children with autism. *Biol Psychiatry* 2011, 69:847-856.
30. Weng SJ, Carrasco M, Swartz JR, Wiggins JL, Kurapati N, Liberzon I, Risi S, Lord C, Monk CS: Neural activation to emotional faces in adolescents with autism spectrum disorders. *J Child Psychol Psychiatry* 2011, 52:296-305.
31. Purcell A, Jeon O, Zimmerman A, Blue M, Pevsner J: Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* 2001, 57:1618-1628.
32. Chakrabarti B, Kent L, Suckling J, Bullmore E, Baron-Cohen S: Variations in the human cannabinoid receptor (*CNR1*) gene modulate striatal responses to happy faces. *Eur J Neurosci* 2006, 23:1944-1948.
33. Domschke K, Dannlowski U, Ohrmann P, Lawford B, Bauer J, Kugel H, Heindel W, Young R, Morris P, Arolt V, Deckert J, Suslow T, Baune BT: Cannabinoid receptor 1 (*CNR1*) gene: impact on antidepressant treatment response and emotion processing in major depression. *Eur Neuropsychopharmacol* 2008, 18:751-759.
34. Baron-Cohen S, Golan O, Wheelwright S, Hill JJ: *Mindreading: The Interactive Guide to Emotions* London: Jessica Kingsley Ltd; 2004.
35. El Kaliouby R, Robinson P, Keates S: Temporal context and the recognition of emotion from facial expression. *Proceedings of HCI International Conference* 2003, Springer-Verlag.
36. Golan O, Baron-Cohen S: Systemizing empathy: teaching adults with Asperger's syndrome/high functioning autism to recognize emotions using interactive multimedia. *Dev Psychopathol* 2006, 18:591-617.
37. Chakrabarti B, Bullmore E, Baron-Cohen S: Empathizing with basic emotions: common and discrete neural substrates. *Soc Neurosci* 2006, 1:364-384.
38. Golan O, Baron-Cohen S, Hill J: The Cambridge Mindreading (CAM) Face-Voice Battery: testing complex emotion recognition in adults with and without Asperger syndrome. *J Autism Dev Disord* 2006, 36:169-183.
39. Ekman P, Friesen W: *Pictures of Facial Affect* Palo Alto: Consulting Psychologists Press; 1976.
40. Lundqvist D, Flykt A, Öhman A: The Karolinska Directed Emotional Faces-KDEF, CD ROM from Department of Clinical Neuroscience, Psychology section, Karolinska Institutet. Stockholm: Karolinska Institutet; 1998.
41. Tottenham N, Tanaka JW, Leon AC, McCarry T, Nurse M, Hare TA, Marcus DJ, Westerlund A, Casey BJ, Nelson C: The NimStim set of facial expressions: judgments from untrained research participants. *Psychiatry Res* 2009, 168:242-249.

42. Chakrabarti B: Individual differences in human emotion perception: neuroimaging, genetic and behavioural investigations. *PhD thesis* Department of Psychiatry, School of Clinical Medicine, University of Cambridge; 2007.
43. Garner M, Büchel C: Amygdala activation predicts gaze toward fearful eyes. *J Neurosci* 2009, 29:9123-9126.
44. Kliemann D, Dziobek I, Hatri A, Steimke R, Heekeren HR: Atypical reflexive gaze patterns on emotional faces in autism spectrum disorders. *J Neurosci* 2010, 30:12281-12287.
45. Dudbridge F: Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Hum Hered* 2008, 66:87-98.
46. Li J, Ji L: Adjusting multiple testing in multilocus analyses using the eigenvalues of a correlation matrix. *Heredity* 2005, 95:221-227.
47. Nyholt DR: A simple correction for multiple testing for SNPs in linkage disequilibrium with each other. *Am J Hum Genet* 2004, 74:765-769.
48. Dawson G, Carver L, Meltzoff AN, Panagiotides H, McPartland J, Webb SJ: Neural correlates of face and object recognition in young children with autism spectrum disorder, developmental delay, and typical development. *Child Dev* 2002, 73:700-717.
49. Scott-Van Zeeland AA, Dapretto M, Ghahremani DG, Poldrack RA, Bookheimer SY: Reward processing in autism. *Autism Res* 2010, 3:53-67.
50. Kohls G, Peltzer J, Herpertz-Dahlmann B, Konrad K: Differential effects of social and non social reward on response inhibition in children and adolescents. *Dev Sci* 2009, 12:614-625.
51. Schmitt AO, Aßmus J, Bortfeldt RH, Brockmann GA: CandiSNPer: a web tool for the identification of candidate SNPs for causal variants. *Bioinformatics* 2010, 26:969-970.
52. Henderson J: Human gaze control during real-world scene perception. *Trends Cogn Sci* 2003, 7:498-504.
53. Filbey FM, Schacht JP, Myers US, Chavez RS, Hutchison KE: Individual and additive effects of the *CNR1* and *FAAH* genes on brain response to marijuana cues. *Neuropsychopharmacology* 2009, 35:967-975.
54. Hutchison KE, Haughey H, Niculescu M, Schacht J, Kaiser A, Stitzel J, Horton WJ, Filbey F: The incentive salience of alcohol: translating the effects of genetic variant in *CNR1*. *Arch Gen Psychiatry* 2008, 65:841-850.
55. Chakrabarti B, Dudbridge F, Kent L, Wheelwright S, Hill-Cawthorne G, Allison C, Banerjee-Basu S, Baron-Cohen S: Genes related to sex steroids, neural growth, and social-emotional behavior are associated with autistic traits, empathy, and Asperger syndrome. *Autism Res* 2009, 2:157-177.
56. Ebstein RP, Israel S, Chew SH, Zhong S, Knafo A: Genetics of human social behavior. *Neuron* 2010, 65:831-844.
57. Baron-Cohen S, Wheelwright S: The Empathy Quotient: an investigation of adults with Asperger syndrome or high functioning autism, and normal sex differences. *J Autism Dev Disord* 2004, 34:163-175.

REVIEW

Endocannabinoid signaling in social functioning: an RDoC perspective

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Core deficits in social functioning are associated with various neuropsychiatric and neurodevelopmental disorders, yet biomarker identification and the development of effective pharmacological interventions has been limited. Recent data suggest the intriguing possibility that endogenous cannabinoids, a class of lipid neuromodulators generally implicated in the regulation of neurotransmitter release, may contribute to species-typical social functioning. Systematic study of the endogenous cannabinoid signaling could, therefore, yield novel approaches to understand the neurobiological underpinnings of atypical social functioning. This article provides a critical review of the major components of the endogenous cannabinoid system (for example, primary receptors and effectors—Δ9-tetrahydrocannabinol, cannabidiol, anandamide and 2-arachidonoylglycerol) and the contributions of cannabinoid signaling to social functioning. Data are evaluated in the context of Research Domain Criteria constructs (for example, anxiety, chronic stress, reward learning, motivation, declarative and working memory, affiliation and attachment, and social communication) to enable interrogation of endogenous cannabinoid signaling in social functioning across diagnostic categories. The empirical evidence reviewed strongly supports the role for dysregulated cannabinoid signaling in the pathophysiology of social functioning deficits observed in brain disorders, such as autism spectrum disorder, schizophrenia, major depressive disorder, posttraumatic stress disorder and bipolar disorder. Moreover, these findings indicate that the endogenous cannabinoid system holds exceptional promise as a biological marker of, and potential treatment target for, neuropsychiatric and neurodevelopmental disorders characterized by impairments in social functioning.

INTRODUCTION

Social functioning impairments are frequently cited as core features of neuropsychiatric and neurodevelopmental disorders, yet progress in biomarker identification and development of targeted pharmacotherapies has been extremely limited. Delays in progress are attributed to social deficit heterogeneity and use of the Diagnostic and Statistical Manual of Mental Disorders (DSM) for investigational research.[1–3] The DSM is a categorical classification system intended to provide clinicians with a common language for diagnosis, but its use in empirical research confers a 'top-down' design. Clinicians categorize patients phenomenologically using the DSM, then researchers attempt to identify pathophysiological mechanisms in patient-participants. However, congruence in phenomenology between patients does not necessarily signify similarity in endophenotypes or genotypes, confounding explicit links to pathology.[3,4] To address the disparity in our current understanding of brain dysfunction with clinical phenomenology, a paradigm shift towards a dimensional 'bottom-up' design in research has been adopted. The Research Domain Criteria (RDoC) is a translational research approach that utilizes a matrix framework to facilitate studies that cut across diagnostic boundaries by probing functional constructs associated with neural circuits. The RDoC provides a comprehensive infrastructure to interrogate social functioning impairments and strongly emphasizes the appropriate experimental classification of patients to support direct translation of research findings for clinical use.[2]

Social competence is an emergent property, inter- and intra-dependent on functional integrity of three of the RDoC domains: negative valence systems, positive valence systems and cognitive systems. Initial stages of social interaction require overcoming negative valence systems (for example, fear, anxiety) to initiate the interaction and are reinforced by positive valence systems (for example, reward learning, reward valuation). Cognitive Systems (that is, attention, perception, working memory) guide the exchange after social interaction has commenced. Social process systems (that is, affiliation and attachment, social communication, perception of self and others) exert supramodal control to coordinate germane practices. Dysfunction in one construct intrinsically affects social information processing and impacts the ability to function typically. In addition, the RDoC framework encourages experimental inquiry at multiple levels of analysis (that is, genes, molecules, cells, circuits, physiology, behavior) to foster research within and across constructs, which fundamentally highlights neuromodulatory systems that operate in multiple constructs, such as the cannabinoid system. The functional heterogeneity of the endogenous cannabinoid system recapitulates the diversity of social functioning abilities in neuropsychiatric and neurodevelopmental disorder patients and parallels dimensional analysis of the RDoC.

The first reports on cannabinoid involvement in social functioning are from the nineteenth century psychiatrist Dr. Jacques Moreau de Tours for the treatment of distributed 'neurological

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dysregulation' and 'social alienation'.[5–7] Dr. Moreau noted similarities from experiences in healthy humans after ingesting North African hashish (which contains 'very high concentration of THC [Δ9-tetrahydrocannabinol]'[5]) with the diversity of behaviors in neurological dysfunction.[6] Moreover, first-person reports from hashish users detail traits that neatly map onto RDoC constructs: for example, 'fluctuations of emotions' (negative valence), extreme 'happiness, excitement' (positive valence), 'errors of time and space…and illusions and hallucinations'[6] (arousal/regulatory), 'irresistible impulses…and dissociation of ideas' (cognitive domain).[8,9] However, current research is focused on the use of inhibitors of endogenous cannabinoid degradation (to enhance signaling) in the study of reward-related processing in social interactions.[10–14] The present article reviews the basic biology of the endogenous cannabinoid system and the roles of its relevant components across each RDoC construct as it pertains to social functioning. Data reviewed within each RDoC construct are applicable to multiple neuropsychiatric and neurodevelopmental disorders, the most notable of which are highlighted as exemplars at the conclusion of each section. With development of diagnostic assessments and treatment options for social functioning deficits limited due to inherent phenotypic heterogeneity, methodical research on the endogenous cannabinoid system has high potential to provide inroads in identifying underlying mechanisms shared across disorders.

ENDOCANNABINOID SIGNALING: EFFECTORS IN NEUROPSYCHIATRIC AND NEURODEVELOPMENTAL DISORDERS

Receptors

Cannabinergic effects is chiefly mediated by stimulation of cannabinoid receptors, type 1 and 2 (CB1R and CB2R),[15] which are distributed throughout the central nervous system[8,16] (see Table 1). Both types are seven-transmembrane G-protein-coupled receptors. These receptors stimulate $G_{i/o}$ proteins in the regulation of ion channels, inhibit adenylyl cyclase, which through downstream signaling increase cyclic AMP in the modulation of neurotransmission, activate protein kinase A to govern cellular function and regulate mitogen-activated protein kinases in control of transcriptional factors.[8,17,18] Mainly expressed in presynaptic axonal segments of inhibitory and excitatory neurons of the brain, CB1Rs are implicated in regulation of synaptic strength.[16,17] CB1Rs control the probability of neurotransmitter release at glutamate and γ-aminobutyric acid (GABA) synapses, mediating GABA suppression or glutamate release and reuptake,[18,19] thereby contributing to the homeostatic maintenance of the brain's excitatory-inhibitory balance.[17,20,21] CB1R activation also suppresses release of serotonin, dopamine, acetylcholine and noradrenaline,[18,21] thereby mediating the characteristic cognitive and antidepressant effects.[16,18] CB2Rs, in contrast, were initially considered a peripheral nervous system receptor as they were originally from immune cells and gene expression levels were highest in inflammatory responses.[15,22] The presence of brain CB2Rs was first detected after harm, insult or neuroinflammation, which breaks down the blood–brain barrier and allows for non-neuronal immunocytes to infiltrate the central nervous system, thereby increasing CB2R expression.[23] Accordingly, expression levels of brain CB2R represent brain health,[22,23] as very low expression indicates a fit brain.[23,24] At homeostasis, low CB2R expression[25] is observed in cell bodies and dendrites of neural progenitor cells, neurons, oligodendrocytes, astrocytes[23,26] and stimulated microglia—'resident macrophages of the central nervous system'.[22] Activation of CB2Rs upregulates cell-surface factors in regeneration and degeneration, functions in migration and proliferation, as well as neural cell maturation and survival,[16,23,27] but the underlying mechanisms remain unclear.

Ligands

Elucidation of the delta-9 double bond in plant-derived cannabinoid (phytocannabinoid), Δ9-tetrahydrocannabinol (THC), in the early 1960s instigated the search for endogenously synthesized cannabinoids (endocannabinoids) and led to the detection of anandamide (AEA) and 2-arachidonoylglycerol (2-AG).[65,66] Each cannabinoid class and type is distinct in mechanisms of action, behavioral profiles and action in neural circuits. Phytocannabinoids are produced in secretory cells of glandular trichomes at high concentrations in unfertilized female Cannabis flowers. THC is a low potency partial agonist at CB1R and CB2R.[8,65] The non-psychoactive analog of THC, cannabidiol (CBD), is of burgeoning clinical interest for its very low efficacy and partial agonism at CB1R and CB2R. At low molar concentrations, CBD acts as an antagonist or inverse agonist (particularly at CB2R) to limit THC effects and contributes to the upregulation of endogenous cannabinoid signaling.[67] Clinically, CBD is a neuroprotectant used as an anti-inflammatory, anticonvulsant, antiepileptic and antipsychotic.[8] CBD's effects are attributed to the full agonism it exhibits for non-cannabinoid G-protein-coupled receptors (for example, peroxisome proliferator-activated and nuclear receptors) and interactions with serotonergic, adenosinergic and vanilloid systems.[8,15] Notably, the most efficacious therapeutics are observed with formulations combining THC and CBD (two to four times greater than single cannabinoid preparations[68]) as CBD enhances the therapeutic action of THC by potentiating its psychotropic effects, augmenting THC tolerability, and widening the therapeutic window.[67] Enhanced efficacy is attributed to the entourage effect,[65,68] the synergistic action of the more than 480 biologically active and inactive compounds in Cannabis. It is likely that the as-yet identified major and minor cannabinoids in plant extracts may further enhance the therapeutic benefit observed with THC and CBD through improved stimulation of the endogenous cannabinoid system.[65]

Endocannabinoids (eCBs) are small (< 400 Da) lipophilic activity-dependent retrograde messengers in the brain. Produced post-synaptically 'on demand' through de novo synthesis from membrane phospholipids in response to increased intracellular Ca^{2+} via depolarization,[69] eCBs act presynaptically to inhibit GABA and glutamate release.[17] The physiological endpoints of AEA and 2-AG are comparable to THC,[69] but are functionally and temporally distinct from one another. AEA has high binding affinity (CB1R: $K_i = 239$ nmol l^{-1}, CB2R: 440 nmol l^{-1}),[70] but low efficacy for cannabinoid receptors. It is most active during steady-state conditions and regulates basal synaptic neurotransmission. Data from rodent autism spectrum disorder (ASD) models demonstrate that genetic mutations in the neuroligin gene disrupt tonic eCB signaling[71] and intimate the importance of eCB signaling mode (tonic or phasic) in neurodevelopment and neuropsychiatric disorders. 2-AG is the more prevalent eCB (~4 nmol g^{-1} in brain tissue vs AEA at < 100 pmol g^{-1})[70] and regulates synaptic plasticity.[17,69] It is representative of 'phasic' signaling evoked in sustained depolarization. The greater overall efficacy and binding affinity of 2-AG (CB1R: $K_i = 3424$ nmol l^{-1}, CB2R: 1194 nmol l^{-1}),[70] particularly at CB2R,[72] is likely related to the enhanced native tone and role in adaptive response. Inactivation of AEA and 2-AG is primarily through enzymatic degradation. AEA is transported across the transmembrane proteins[47,73] and hydrolyzed via fatty acid amide hydrolase (FAAH),[47] whereas 2-AG can be hydrolyzed by FAAH or monoacylglycerol lipase.[74]

ENDOCANNABINOID SIGNALING IN SOCIAL FUNCTIONING: AN RDOC PERSPECTIVE

The endocannabinoid system provides a consummate model to examine social functioning deficits across multiple clinical populations. The distribution of eCB components is congruent

Table 1. Endocannabinoid system component in RDoC domains

RDoC construct	Mediating circuit	CB1R in circuitry	CB2R in circuitry	2-AG	AEA
Anxiety	Interpret: BNST, HPC, amygdala. Evaluate and response initiation: PFC, NAc, VTA, hypothalamus[28,29]	BNST[a], [a]HPC, amygdala, [a]PFC, [a]NAc medium spiny neurons, hypothalamus[a,5,8,18]	Microglia,[30] VTA and NAc dopaminergic neurons[16,31]	↑in brain → anxiolytic[30]	↓in brain → anxiogenic[30]
Chronic stress	HPA, amygdala–medial PFC–striatum[32–34]	[a]PFC, [a]HPC, [a]NAc, amygdala, HPA[a,19,35]	Rats: amygdala, HPC, PFC, hypothalamus[25]	Amygdalar impairment in synthesis;[30] ↑in PFC[36]	↓in amygdala (via↑FAAH)[30]
Motivation	Orbitofrontal cortex, amygdala, VTA, NAc[37–39]	Amygdala, [a]HPC, VTA and [a]NAc dopaminergic neurons[38,40,41]	VTA dopaminergic neurons[31,40]	↑in NAc → ↓ dopamine[38,41,42]	↑in NAc → ↑dopamine
Reward learning	VTA, NAc, PFC, hypothalamus, amygdala[40,42]	[a]NAc medium spiny neurons, [a]HPC, amygdala, VTA and [a]NAc dopaminergic neurons, hypothalamus[a], [a]PFC[40,42,43]	VTA dopaminergic neurons[31]	↑in NAc → ↓ dopamine[38,41,42]	↑in NAc → ↑dopamine
Declarative memory	PFC, amygdala, HPC (dentate gyrus, CA1, CA3), VTA, NAc[44]	[a]PFC, amygdala, GABAergic interneurons, [a]HPC (dentate gyrus, CA1 and CA3)[18,19]	HPC interneuron cholecystokinin (+) basket cells[45]	↓HPC CB1R binding → ↑GABA[45,46]	HPC[1B]↑(via ↓FAAH) →↑acquisition[47,48]
Working memory	PFC–parietal–cingulate–thalamus–NAc, HPC[49,50]	[a]NAc medium spiny neuron, GABAergic hippocampal interneurons[46]	HPC[23]	↑HPC CB2R binding → ↓encoding/consolidation[51]	↑ (via ↓FAAH) →↑acquisition[47,48]
Affiliation and attachment	Amygdala, VTA, NAc, PVN, SON, HPA, BNST[52–54]	PVN,[34] SON,[55] HPA,[a,13] BNST[a], amygdala, NAc[12,56]	HPC[23,56]	↑in HPC → ↓ care[54,57] ↑in amygdala → ↓play[36]	Play; ↑amygdala and NAc[12,36]
Social communication	Amygdala, NAc, orbitofrontal cortex, cortico–basal ganglia–thalamo network[58]	HPC, amygdala, NAc[59,60]	Not yet known	Not yet known	↑ (via ↓FAAH) →↑USVs[61–63]

Abbreviations: AEA, anandamide; 2-AG, 2-arachidonoylglycerol; BNST, bed nucleus of the stria terminalis; FAAH, fatty acid amide hydrolase; GABA, glutamate and γ-aminobutyric acid; HPA, hypothalamus–pituitary–adrenal gland axis; HPC, hippocampus; NAc, nucleus accumbens; PFC, prefrontal cortex; PVN, paraventricular nucleus; RDoC, Research Domain Criteria; SON, supraoptic nucleus; USV, ultrasonic vocalization; VTA, ventral tegmental area. CB1R is the most abundant G-protein-coupled receptor ([a]highest levels/densities, [b]lowest) in the mammalian brain, contributing to cannabinergic effects on [c]movement, affective responding, cognition, temperature, appetite and neuroendocrine function.[6,18] Human mRNA expression for a CB2R isoform has been found in amygdala, striatum, HPC, cortex and cerebellum.[25] [a]with rodents showing additional expression in the cerebral cortex, brain stem, thalamic nuclei and periaqueductal grey.[64] AEA binding elicits sub-maximal receptor signaling, whereas 2-AG binds with much greater efficacy,[42] necessary to produce robust phasic signaling responses.

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with affected neuroanatomy in neuropsychiatric and neuro-developmental disorders with social functioning impairments (see Table 1). Changes in eCB components and function contribute to impairments in RDoC constructs critical to social behavior, resulting in a spectrum of atypical social endopheno-types. The next sections review the role of the eCB system in RDoC constructs subserving social functioning and which are characteristically observed as affected in neuropsychiatric and neurodevelopmental disorders.

eCB SIGNALING AND NEGATIVE VALENCE SYSTEMS
Anxiety
Anxiety, fear, and stress as terms are often semantically interchangeable, though each is independently characterized by the nature of the eliciting event and magnitude of response.[28,29] Anxiety is elicited by putatively dangerous, uncertain, imminent situations or events that lead to an acute behavioral response in preparation for threats to individual integrity that is dispropor-tional in intensity or chronicity.[29] Anxiety is physiologically manifested as an exaggerated startle response, increased muscle tension, decreased motion, avoidance behaviors and autonomic hyperactivity. With regard to social functioning, pathological anxiety can impede competent functioning in the absence of triggering stimuli. Major components of the neurocircuitry mediating anxiety, such as the prefrontal cortex (PFC), hippocam-pus, amygdala and hypothalamus are rich in CB1R and CB2R expression (see Table 1). In animal models, genetic deletion of CB1R increases anxiety-like behaviors, but only under highly aversive conditions, whereas deletion of CB2R modulate vulner-ability to anxiogensis.[30,75] Overexpression of CB2Rs in mouse models increases resistance to anxiogenic stimuli, mediated by increased 2-AG and GABA signaling.[30] Exogenous cannabinoids exert an inverted U-shaped effect on anxiety, that is both anxiolytic and anxiogenic, dependent on concentration and context.[76] Acute administration of THC and CBD in preclinical models and patient populations commonly produces an anxiolytic response, likely due to inhibition of the glutamatergic firing in neural networks involved in anxiety.[28,29] Similarly, eCB signaling, such as blockade of AEA hydrolysis[77,78] or inhibition of monoacylglycerol lipase[8] also elicits an anxiolytic response. Anxiogensis can be observed via eCB signaling suppression of glutamate outflow in the hippocampus and periaqueductal gray, as well as in its inhibition of corticolimbic release of noradrenaline, dopamine and serotonin.[30,76] These data are pertinent to a myriad of neurodevelopmental and neuropsychiatric disorders (for example, posttraumatic stress disorder, depression, schizophrenia, obsessive compulsive disorder, ASD, bipolar disorder, phobias and so on), but further study is needed to clarify how best to leverage neurocircuitry modulation with precision to ensure anxiolytic function of cannabinergic pharmacologic interventions and the potential restoration of homeostatic eCB signaling.

Chronic stress
Stress is an adaptive response to a specific internal or external stressor in preparation for injury or threat. Definitions of stress emphasize the physiological and emotional consequences to relate acute parallel activation of autonomic and endocrine systems. Chronic stress is a response to persistent stressors, like social threat or defeat, which reduces social motivation and social interactions by intensifying fear towards (an emotional reaction) and avoidance (anxiogenic behavior) of unknown conspecifics.[32,79] Across development, protracted exposure to stressors imposes a progressive pattern of dysfunction in social functioning, beginning as asociality and culminating in antisociality.[79] Persistent activation (>4 weeks, ≤48 weeks) of stress neurocircuitry breaks down homeostatic balance[32,33] and

creates a 'hypocannabinergic state' through downregulation of CB1R expression in the hippocampus, hypothalamus, striatum and dorsal root ganglion.[33,34] No significant change in CB2R expression was observed in three separate mouse lines, following exposure to chronic stressors, but without analogous data in humans, few conclusions can be made about CB2R function under chronic stress.[25] Alterations in CB1R expression are concurrent with changes in eCB signaling in the hippocampus, striatum, dorsal raphe nucleus, hypothalamus, nucleus accumbens (NAc), PFC (at GABAergic terminals) and the amygdala. In the absence of stressors, AEA tone suppresses activity of the hypothalamic–pituitary–adrenal (HPA) axis (the chief modulator of the stress response[80,81]) and disruption of this suppression initiates HPA-axis activation.[35,81] Chronic stressor exposure impairs AEA signaling and elevates 2-AG content within the amygdala (and increases glucocorticoid hormone secretion), hippocampus, hypothalamus and PFC,[33] likely due to altered FAAH and monoacylglycerol lipase-mediated hydrolysis, as the synthesis of these eCBs is unimpaired.[34,82] Glucocorticoid feedback inhibition of HPA-axis activity is suggested to occur on two temporal scales, rapidly via the PFC and 2-AG tone enhancement, or less rapidly via negative feedback inhibition at the paraventricular nucleus of the hypothalamus.[34,82] Glucocorticoids then normalize the amygdalar AEA levels to support basal HPA function.[34] The enhancement of 2-AG signaling is perceived as an attempt to habituate to the chronic stress exposure and may be crucial in understanding how to properly engage eCB signaling in the modulation of the HPA axis to guarantee a positive adaptive response.[34] However, the presence of a mechanism to examine maladaptive responses to chronic stress exposure highlights novel areas for social deficits in which exposure to a chronic stressor is unavoidable (for example, social anxiety and mood disorders).

eCB SIGNALING AND POSITIVE VALENCE SYSTEMS
Reward learning
Reward attainment is one of the only RDoC constructs to explicitly detail eCBs as candidate modulators of reward learning, valuation and processing.[43] Recent data linking reward and social neuro-peptides with eCBs highlight a novel, potential intervention specific to social functioning deficits.[13] Adaptive reinforcement of social interactions requires long-term synaptic plasticity at excitatory synapses of the NAc and is dependent on oxytocin (OT), a neuropeptide that regulates prosocial behavior and its dysregulation has been implicated in social impairments.[83,84] In the examination of neural circuits of socialization, Wei et al.[13] demonstrated an obligatory role of AEA in a socially isolated rodent model. Salience and reward were modulated by AEA mobilization in the NAc and hippocampus via activation of OT receptors by endogenously released OT following social contact. Additional experiments to control for the anxiogenic induction of AEA release during stressful social situations found the observed effects were independent of the stress response and specific to social reward.[13] Data suggest that OT acts as a social reinforce-ment signal to induce long-term depression in medium spiny neurons and requires specific activation of CB1Rs by AEA to regulate social incentive salience. These preclinical data provide insight into the mechanisms associated with OT in social functioning in clinical populations. If improvement in social functioning requires OT-evoked AEA signaling as data suggest, then enhancement of OT to improve social deficits must be sufficient to engage AEA mobilization for proper functioning of the neural circuit. Researchers have advocated that these findings are of particular relevance to ASD, although these results can also be broadly applied to neuropsychiatric disorders characterized by dysregulated OT and impaired social functioning (for example, schizophrenia and mood, anxiety, or personality disorders[85,86]).

Social motivation

Social motivation leads to enhancement of social development by promoting social interactions. Abnormal motivation precludes proficient social interaction and has negative consequences on the acquisition of higher-order social abilities.[37] The inability to separate motivation from social interest has contributed to the assumption that deficits in social functioning are a cause of disrupted social interest, as opposed to a consequence of them.[37] Motivation is necessary to attend to social information, seek and engage in social interaction, as well as foster and maintain social bonds, whereas interest does not necessitate action. Individual differences in social motivation are associated with alterations in the activity of neurocircuitry that overlap with reward learning; high social motivation is correlated with enhanced activation of the amygdala and orbital frontal cortex, whereas weaker activation is related to lower social motivation. Opioid and dopaminergic neurons in the ventral tegmental area have complementary roles in motivated behaviors and require eCB signaling to fine-tune dopamine release in incentive-related reward learning.[40,52] Concomitantly, enhanced motivation is observed following CB1R stimulation and with increased AEA signaling in the amygdala, NAc and dorsal striatum and 2-AG in the NAc.[38,41,42] Diminished motivation is associated with CB1R blockade.[38,39] Wei *et al.*[87] suggest increased AEA content in the forebrain in animal models of ASD can ameliorate social motivation deficits. Extending previous results in rats in which modulation of CB1R or genetic removal of FAAH negatively impacted social interactions,[79,88] researchers examined the influence of AEA on social behaviors via FAAH modulation.[13] A social approach task and elevated plus maze were used to assess the effects of increased AEA (via FAAH inhibition) on social motivation in BTBR mice (an idiopathic ASD model with known deficits in social approach, reciprocal social interaction, and juvenile play) and Fragile X Syndrome mice (a syndromic ASD model with persistent social approach deficits). Increased AEA signaling in the forebrains of ASD mouse models correlated with increased time in the social chamber and preferential interaction with novel animals compared with untreated and control mice.[87] Effects were not associated with decreased anxiogenic responses, as no change in behavior on the elevated plus maze was observed. Improved prosocial behaviors were 'generalizable'[87] and suggest common neural circuitry of social motivation between idiopathic and syndromic ASD.

eCB SIGNALING AND COGNITIVE SYSTEMS

Declarative memory

Declarative memory (that is, encoding, consolidation, storage and retrieval of factual information[44]) supports social interactions by providing biographical and episodic recall.[49] It functions as a store of experience-outcomes and integrates previous conclusions with new input, as well as emotion, motivation and perception.[44] Stimulation of cannabinoid receptors in hippocampal circuits diminishes glutamate release to below-threshold levels, inhibiting long-term potentiation necessary for encoding.[89,90] In animal models, stimulation of receptors before or after learning induces impaired performance on water maze, contextual fear conditioning and object recognition memory assessments.[89,91] In humans, deficits were not observed in the retrieval of previously stored information, and learning impairments were transient as after a 3-month abstinence from phytocannabinoids, deficits were no longer observed.[8,92] Importantly, cannabinergic modulation of cognitive effects can vary on the basis of the use of exogenous or endogenous cannabinoids. AEA and 2-AG are both robust modulators of early-stage acquisition, consolidation and extinction,[49,66,90] but it is the enhancement of 2-AG that is correlated with disrupted encoding in spatial memory.[51,93,94] An abundance of evidence demonstrates transient, dose-dependent

THC-induced memory impairments (with a tolerance effect in heavy users) and the contrasting absence of memory deficits following CBD administration. Data also suggest that CBD is protective against THC-induced impairments in episodic and spatial memory.[65,66,92] Explicit control of eCB signaling in declarative memory for social functioning deficits must account for individual differences in abilities. Brain disorders characterized by difficulties in uncontrolled recall (for example, ASD, posttraumatic stress disorder, phobia disorders) may benefit from control over extinction, whereas disrupting short-term memory consolidation may be advantageous for other disorders (for example, OCD, anxiety disorders).

Working memory

More immediate information processing involves working memory, which actively maintains and updates relevant information, but is capacity-limited.[50] In social interactions, working memory tracks social information, like the characteristics of, or relationships among, people necessary to competently socially interact. THC exposure in humans negatively impacts working memory via CB1R activation and inhibition of AEA reuptake.[48,51] Correspondingly, rodent models with upregulated CB1R expression in the PFC, as well as CB1R knockout mice, demonstrate changes in cognitive flexibility. Low doses of CB1R antagonists improved task switching (a measure of cognitive flexibility) and inhibitory control via inhibition of PFC glutamatergic activity, whereas CB1R agonists increased impulsive behaviors. A neuroimaging study[90] suggests that THC impacts activity in cerebral inhibition response circuits causing increased hyperactivity in the PFC and anterior cingulate cortices. Acute administration of THC reduces response inhibition[49,89] (that is, increases behavioral impulsivity) and causes hyperactivity at dopaminergic synapses in the PFC.[90] Data from animal models suggest that cannabinoid signaling interacts with dopaminergic, GABAergic and glutamatergic pathways to mediate behavioral changes. Data in FAAH knockout mice demonstrate that AEA-biased tone improves acquisition in working memory tasks, but the effects are transient and do not persist into later trials.[65,66] This evidence suggests that strategies to improve components of working memory (for disorders such as prosopagnosia, schizophrenia and/or depression) should focus on control of AEA tone and local modulation of 2-AG signaling.

eCB SIGNALING AND SOCIAL PROCESSING SYSTEMS

Affiliation and attachment

Maternal care is a newborn's first social experience and variation in maternal care has been shown to dramatically influence social development.[56,57] Mouse pups that receive intensive maternal care demonstrate enhanced maternal behavior in adulthood demonstrating the enduring effects of maternal care on adult social functioning.[95,96] Disruption of the mother–infant bond has lasting consequences on neuroendocrine and cognitive functioning, increasing the risk for subsequent psychopathology.[56] OT is a primary regulator of social behavior,[53,83,84] particularly in maternal care,[53,54] and is associated with eCB signaling.[57,95,97] Schechter *et al.*[57,95] performed several studies examining eCB and OT signaling in maternal care and demonstrated that genetic ablation of CB1R negatively affected maternal care. This impairment correlated with decreased hippocampal OT receptor expression and increased hippocampal levels of 2-AG.[54,57,95] Results are consistent with observations from socially isolated animals. These studies demonstrated that region-specific CB1R expression in the supraoptic nucleus of socially isolated animals can reduce social play and social interaction compared with those that were pair-housed and handled daily.[36,55] Changes in receptor density affect GABAergic and glutamatergic input to OT-synthesizing neurons for mobilization of OT release,[55,57] which is necessary for social

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bonding. Valproate-exposed rats, another animal model of social deficits used in ASD research, demonstrate similarly reduced sociability, but changes in CB1R expression were also correlated with reduced hippocampal 2-AG expression.[98] Moreover, after social play, increased AEA and 2-AG levels in the NAc and the amygdala were observed.[12,88] These data demonstrate that perturbation of eCB signaling is an elegant potential causal effector in the regulation of maternal care and affiliative behaviors. These results are pertinent to better understanding how cannabinoid modulation contributes to the neurocircuitry of psychiatric disorders related to early-life adversity, such as depression, bipolar disorder and schizophrenia.[56,96]

Social communication

In humans, high concentrations of cannabinoid receptors are found in the left hemisphere cortical regions that are associated with verbal language function,[16,18] which suggests a role for eCB signaling in social communication. However, current findings only supports a role for eCB signaling in nonverbal motor-related aspects of social communication (that is, eye-gaze or body language). Genetic variants (that is, single-nucleotide polymorphisms) in CB1Rs are correlated with greater gaze duration to happy faces and are considered a putative endophenotype for communication deficits, particularly in ASD research.[99] Animal models of social communication impairments demonstrate a direct connection to cannabinergic function, which interacts with the forkhead box (FOXP) protein family.[58] Interestingly, FOXP are instrumental in ultrasonic vocalizations (USVs) production and vocal learning.[58,100] In rodents, USVs serve a communicatory function to elicit social interaction[61,62] or to share socially relevant information with known conspecifics.[63] Moreover, USVs are developmental- and context-dependent[62] relating identity, emotionality,[61–63] receptivity to affiliative and sexual behaviors, and changes in the environment.[56] Persistent activation of CB1R, via agonists or inhibitors of enzymatic degradation, decrease emission of USVs in rodent pups. Cannabinoids modulate USVs induced by maternal separation in pups,[61] whereas in adult rodents, cannabinoids increase the emission of USVs when exposed to anxiogenic stimuli.[61,63] Moreover, CB1R knockout mice have low USV emission throughout development,[56] which parallels the communication impairments associated with disorders such as ASD.[62] For clinical populations with impairments in language acquisition, especially nonverbal patients with social deficits, cannabinergic modulation of USVs offers a novel and viable area of biomedical intervention in support of communication acquisition and development.[59,60,100]

CONCLUSIONS

The diversity of social functioning deficits present in neuropsychiatric and neurodevelopmental disorders confounds the efficacy and specificity of treatment for affected individuals. However, the recent shift in psychiatric research towards examining the underlying dysregulated neural circuits of brain disorders has allowed consideration herein of novel aspects of the functionally diverse eCB signaling system. This article reviewed the eCB signaling system and the role of its components (that is, cannabinoid receptors, functional ligands) in varied, but convergent, RDoC domains of social functioning. From a molecular level of analysis, the cannabinoid system is implicated in negative and positive valence systems to restore homeostatic balance, increase the salience of reward learning and motivate social interactions. Behavioral and physiological effects of the eCB system are observed in cognitive systems. Social process systems show evidence of cannabinergic modulation, and as an emergent domain, rely on appropriate eCB function in the aforementioned RDoC domains. Although further work is needed to clarify the

discrete roles of cannabinoid signaling in each RDoC domain, the extant evidence strongly supports the contribution of dysregulated cannabinoid signaling to the pathophysiology of social functioning impairments. With regard to the development of pharmacological interventions, although there is a diverse set of methods to enhance endogenous signaling or receptor stimulation *in vivo*, the lack of evidence on direct endocannabinoid (AEA or 2-AG)[66] highlights the need for further research. Moreover, in the pursuit of precision health,[2] although each component of the eCB system can be targeted for therapeutic development, appropriate targeting with respect to the intersection of the eCB system and a disorder's neurocircuitry must be carefully considered to achieve optimal outcomes. It is clear, though, that the eCB system holds exceptional promise as a biological marker of, and treatment target[6] for, neuropsychiatric and neurodevelopmental disorders characterized by pronounced abnormalities in social functioning.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 American Psychiatric Association. DSM 5. American Psychiatric Association: Washington, DC 2013.
2 Insel TR. The NIMH Research Domain Criteria (RDoC) Project: precision medicine for psychiatry. *Am J Psychiatry* 2014; 171: 395–397.
3 Insel T, Cuthbert B, Garvey M, Heinssen R, Pine DS, Quinn K et al. Research domain criteria (RDoC): toward a new classification framework for research on mental disorders. *Am J Psychiatry* 2010; 167: 748–751.
4 Casey BJ, Craddock N, Cuthbert BN, Hyman SE, Lee FS, Ressler KJ. DSM-5 and RDoC: progress in psychiatry research? *Nat Rev Neurosci* 2013; 14: 810–814.
5 Mechoulam R. Cannabis—a valuable drug that deserves better treatment. *Mayo Clin Proc* 2012; 87: 107–109.
6 Moreau de Tours JJ. Du hachisch et de l'aliénation mentale: études psychologiques 1845. Fortin, Masson: Paris, France. Available from http://archive.org/details/duhachischetdela00more (accessed 21 June 2016).
7 Moreau de Tours JJ. *Hashish and Mental Illness*. Raven Press: New York, NY, USA, 1973.
8 Mechoulam R, Parker LA. The endocannabinoid system and the brain. *Annu Rev Psychol* 2013; 64: 21–47.
9 Kalant OJ. Moreau, hashish, and hallucinations. *Int J Addict* 1971; 6: 553–560.
10 Trezza V, Vanderschuren LJ. Cannabinoid and opioid modulation of social play behavior in adolescent rats: differential behavioral mechanisms. *Eur Neuropsychopharmacol* 2008; 18: 519–530.
11 Trezza V, Vanderschuren LJ. Divergent effects of anandamide transporter inhibitors with different target selectivity on social play behavior in adolescent rats. *J Pharmacol Exp Ther* 2009; 328: 343–350.
12 Trezza V, Damsteegt R, Manduca A, Petrosino S, Van Kerkhof LW, Pasterkamp RJ et al. Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. *J Neurosci* 2012; 32: 14899–14908.
13 Wei D, Lee D, Cox CD, Karsten CA, Peñagarikano O, Geschwind DH et al. Endocannabinoid signaling mediates oxytocin-driven social reward. *Proc Natl Acad Sci USA* 2015; 112: 14084–14089.
14 Qin M, Zeidler Z, Moulton K, Krych L, Xia Z, Smith CB. Endocannabinoid-mediated improvement on a test of aversive memory in a mouse model of fragile X syndrome. *Behav Brain Res* 2015; 291: 164–171.
15 Howlett AC, Bidaut-Russell M, Devane WA, Melvin LS, Johnson MR, Herkenham M. The cannabinoid receptor: biochemical, anatomical and behavioral characterization. *Trends Neurosci* 1990; 13: 420–423.
16 Mackie K. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol* 2005; 168: 299–325.
17 Castillo PE, Younts TJ, Chávez AE, Hashimotodani Y. Endocannabinoid signaling and synaptic function. *Neuron* 2012; 76: 70–81.
18 Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002; 54: 161–202.
19 Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J Neurosci* 1991; 11: 563–583.
20 Eichler SA, Meier JC. E-I balance and human diseases—from molecules to networking. *Front Mol Neurosci* 2008; 1: 2.

21 den Boon FS, Werkman TR, Schaafsma-Zhao Q, Houthuijs K, Vitalis T, Kruse CG et al. Activation of type-1 cannabinoid receptor shifts the balance between excitation and inhibition towards excitation in layer II/III pyramidal neurons of the rat prelimbic cortex. Pflüg Arch Eur J Physiol 2015; 467: 1551–1564.

22 Cabral GA, Griffin-Thomas L. Emerging role of the CB2 cannabinoid receptor in immune regulation and therapeutic prospects. Expert Rev Mol Med 2009; 11: e3.

23 Atwood BK, Mackie K. CB2: a cannabinoid receptor with an identity crisis. Br J Pharmacol 2010; 160: 467–479.

24 Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993; 365: 61–65.

25 Roche M, Finn DP. Brain CB2 receptors: implications for neuropsychiatric disorders. Pharmaceuticals 2010; 3: 2517–2553.

26 Dhopeshwarkar A, Mackie K. CB2 cannabinoid receptors as a therapeutic target—what does the future hold? Mol Pharmacol 2014; 86: 430–437.

27 Yang K, Cao F, Sheikh AM, Malik M, Wen G, Wei H et al. Up-regulation of Ras/Raf/ERK1/2 signaling impairs cultured neuronal cell migration, neurogenesis, synapse formation, and dendritic spine development. Brain Struct Funct 2013; 218: 669–682.

28 Calhoon GG, Tye KM. Resolving the neural circuits of anxiety. Nat Neurosci 2015; 18: 1394–1404.

29 Tovote P, Fadok JP, Lüthi A. Neuronal circuits for fear and anxiety. Nat Rev Neurosci 2015; 16: 317–331.

30 Lutz B, Marsicano G, Maldonado R, Hillard CJ. The endocannabinoid system in guarding against fear, anxiety and stress. Nat Rev Neurosci 2015; 16: 705–718.

31 Zhang H-Y, Gao M, Liu Q-R, Bi G-H, Li X, Yang H-J et al. Cannabinoid CB2 receptors modulate midbrain dopamine neuronal activity and dopamine-related behavior in mice. Proc Natl Acad Sci USA 2014; 111: E5007–E5015.

32 Radley J, Morilak D, Viau V, Campeau S. Chronic stress and brain plasticity: mechanisms underlying adaptive and maladaptive changes and implications for stress-related CNS disorders. Neurosci Biobehav Rev 2015; 58: 79–91.

33 Morena M, Patel S, Bains JS, Hill MN. Neurobiological interactions between stress and the endocannabinoid system. Neuropsychopharmacology 2016; 41: 80–102.

34 Hill MN, Tasker JG. Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. Neuroscience 2012; 204: 5–16.

35 Hill MN, McLaughlin RJ, Morrish AC, Viau V, Floresco SB, Hillard CJ et al. Suppression of amygdalar endocannabinoid signaling by stress contributes to activation of the hypothalamic-pituitary-adrenal axis. Neuropsychopharmacology 2009; 34: 2733–2745.

36 Sciolino NR, Bortolato M, Eisenstein SA, Fu J, Oveisi F, Hohmann AG et al. Social isolation and chronic handling alter endocannabinoid signaling and behavioral reactivity to context in adult rats. Neuroscience 2010; 168: 371–386.

37 Chevallier C, Kohls G, Troiani V, Brodkin ES, Schultz RT. The social motivation theory of autism. Trends Cogn Sci 2012; 16: 231–239.

38 Campolongo P, Fattore L. Cannabinoid Modulation of Emotion, Memory, and Motivation. Springer: New York, 2015.

39 Achterberg EJM, van Kerkhof LWM, Servadio M, van Swieten MMH, Houwing DJ, Aalderink M et al. Contrasting roles of dopamine and noradrenaline in the motivational properties of social play behavior in rats. Neuropsychopharmacology 2015; 41: 858–868.

40 Sagheddu C, Muntoni AL, Pistis M, Melis M. Endocannabinoid signaling in motivation, reward, and addiction: influences on mesocorticolimbic dopamine function. Int Rev Neurobiol 2015; 125: 257–302.

41 López HH. Cannabinoid-hormone interactions in the regulation of motivational processes. Horm Behav 2010; 58: 100–110.

42 Parsons LH, Hurd YL. Endocannabinoid signalling in reward and addiction. Nat Rev Neurosci 2015; 16: 579–594.

43 Panagis G, Mackey B, Vlachou S. Cannabinoid regulation of brain reward processing with an emphasis on the role of CB1 receptors: a step back into the future. Front Psychiatry 2014; 5: 92.

44 Eichenbaum H. A cortical-hippocampal system for declarative memory. Nat Rev Neurosci 2000; 1: 41–50.

45 Du H, Kwon I-K, Kim J. Neuregulin-1 impairs the long-term depression of hippocampal inhibitory synapses by facilitating the degradation of endocannabinoid 2-AG. J Neurosci 2013; 33: 15022–15031.

46 Albayram Ö, Passlick S, Bilkei-Gorzo A, Zimmer A, Steinhäuser C. Physiological impact of CB1 receptor expression by hippocampal GABAergic interneurons. Pflugers Arch 2016; 468: 727–737.

47 Cravatt BF, Lichtman AH. Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. Curr Opin Chem Biol 2003; 7: 469–475.

48 Panlilio LV, Thorndike EB, Nikas SP, Alapafuja SO, Bandiera T, Cravatt BF et al. Effects of fatty acid amide hydrolase (FAAH) inhibitors on working memory in rats. Psychopharmacology (Berl) 2015; 233: 1879–1888.

49 Hampson RE, Deadwyler SA. Role of cannabinoid receptors in memory storage. Neurobiol Dis 1998; 5: 474–482.

50 Baddeley A. Working memory: looking back and looking forward. Nat Rev Neurosci 2003; 4: 829–839.

51 Griebel G, Pichat P, Beeské S, Leroy T, Redon N, Jacquet A et al. Selective blockade of the hydrolysis of the endocannabinoid 2-arachidonoylglycerol impairs learning and memory performance while producing antinociceptive activity in rodents. Sci Rep 2015; 5: 7642.

52 Trezza V, Baarendse PJ, Vanderschuren LJ. The pleasures of play: pharmacological insights into social reward mechanisms. Trends Pharmacol Sci 2010; 31: 463–469.

53 Donaldson ZR, Young LJ. Oxytocin, vasopressin, and the neurogenetics of sociality. Science 2008; 322: 900–904.

54 Scott N, Prigge M, Yizhar O, Kimchi T. A sexually dimorphic hypothalamic circuit controls maternal care and oxytocin secretion. Nature 2015; 525: 519–522.

55 Di S, Boudaba C, Popescu IR, Weng F-J, Harris C, Marcheselli VL et al. Activity-dependent release and actions of endocannabinoids in the rat hypothalamic supraoptic nucleus. J Physiol 2005; 569: 751–760.

56 Manduca A, Campolongo P, Trezza V. Cannabinoid modulation of mother-infant interaction: is it just about milk? Rev Neurosci 2012; 23: 707–722.

57 Schechter M, Weller A, Pittel Z, Gross M, Zimmer A, Pinhasov A. Endocannabinoid receptor deficiency affects maternal care and alters the dam's hippocampal oxytocin receptor and brain-derived neurotrophic factor expression. J Neuroendocrinol 2013; 25: 898–909.

58 Lai CS, Fisher SE, Hurst JA, Vargha-Khadem F, Monaco AP. A forkhead-domain gene is mutated in a severe speech and language disorder. Nature 2001; 413: 519–523.

59 Soderstrom K, Johnson F. Zebra finch CB1 cannabinoid receptor: pharmacology and in vivo and in vitro effects of activation. J Pharmacol Exp Ther 2001; 297: 189–197.

60 Soderstrom K, Johnson F. CB1 cannabinoid receptor expression in brain regions associated with zebra finch song control. Brain Res 2000; 857: 151–157.

61 McGregor IS, Dastur FN, McLellan RA, Brown RE. Cannabinoid modulation of rat pup ultrasonic vocalizations. Eur J Pharmacol 1996; 313: 43–49.

62 Wöhr M. Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: detailed spectrographic analyses and developmental profiles. Neurosci Biobehav Rev 2014; 43: 199–212.

63 Manduca A, Vanderschuren LJ, Trezza V. Cannabinoid modulation of rodent ultrasonic vocalizations in a social context: communicative and rewarding properties. In: Campolongo P, Fattore L (eds). Cannabinoid Modulation of Emotion, Memory, and Motivation. Springer: New York, NY, USA, 2015, pp 225–243.

64 Devane WA, Dysarz FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 1988; 34: 605–613.

65 Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol 2011; 163: 1344–1364.

66 Mechoulam R, Hanuš LO, Pertwee R, Howlett AC. Early phytocannabinoid chemistry to endocannabinoids and beyond. Nat Rev Neurosci 2014; 15: 757–764.

67 Karniol IG, Carlini EA. Pharmacological interaction between cannabidiol and Δ9-tetrahydrocannabinol. Psychopharmacologia 1973; 33: 53–70.

68 Ben-Shabat S, Fride E, Sheskin T, Tamiri T, Rhee MH, Vogel Z et al. An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. Eur J Pharmacol 1998; 353: 23–31.

69 Battista N, Di Tommaso M, Bari M, Maccarone M. The endocannabinoid system: an overview. Front Behav Neurosci 2012; 6: 9.

70 Fisar Z. Phytocannabinoids and endocannabinoids. Curr Drug Abuse Rev 2009; 2: 51–75.

71 Földy C, Malenka RC, Südhof TC. Autism-associated neuroligin-3 mutations commonly disrupt tonic endocannabinoid signaling. Neuron 2013; 78: 498–509.

72 Ueda N, Tsuboi K, Uyama T, Ohnishi T. Biosynthesis and degradation of the endocannabinoid 2-arachidonoylglycerol. Biofactors 2011; 37: 1–7.

73 Elmes MW, Kaczocha M, Berger WT, Leung K, Ralph BP, Wang L et al. Fatty acid-binding proteins (FABPs) are intracellular carriers for Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). J Biol Chem 2015; 290: 8711–8721.

74 Dinh TP, Carpenter D, Leslie FM, Freund TF, Katona I, Sensi SL et al. Brain monoglyceride lipase participating in endocannabinoid inactivation. Proc Natl Acad Sci USA 2002; 99: 10819–10824.

75 García-Gutiérrez MS, García-Bueno B, Zoppi S, Leza JC, Manzanares J. Chronic blockade of cannabinoid CB2 receptors induces anxiolytic-like actions associated with alterations in GABAA receptors. Br J Pharmacol 2012; 165: 951–964.

76 Tambaro S, Bortolato M. Cannabinoid-related agents in the treatment of anxiety disorders: current knowledge and future perspectives. Recent Pat CNS Drug Discov 2012; 7: 25–40.

77 Gobbi G, Bambico FR, Mangieri R, Bortolato M, Campolongo P, Solinas M et al. Antidepressant-like activity and modulation of brain monoaminergic transmission by blockade of anandamide hydrolysis. Proc Natl Acad Sci USA 2005; 102: 18620–18625.

8

78 Kathuria S, Gaetani S, Fegley D, Valiño F, Duranti A, Tontini A et al. Modulation of anxiety through blockade of anandamide hydrolysis. Nat Med 2003; 9: 76–81.

79 Sandi C, Haller J. Stress and the social brain: behavioural effects and neurobiological mechanisms. Nat Rev Neurosci 2015; 16: 290–304.

80 Steiner MA, Wotjak CT. Role of the endocannabinoid system in regulation of the hypothalamic-pituitary-adrenocortical axis. Prog Brain Res 2008; 170: 397–432.

81 Hill MN, Patel S, Campolongo P, Tasker JG, Wotjak CT, Bains JS. Functional interactions between stress and the endocannabinoid system: from synaptic signaling to behavioral output. J Neurosci 2010; 30: 14980–14986.

82 Tasker JG, Herman JP. Mechanisms of rapid glucocorticoid feedback inhibition of the hypothalamic–pituitary–adrenal axis. Stress 2011; 14: 398–406.

83 Parker KJ, Garner JP, Libove RA, Hyde SA, Hornbeak KB, Carson DS et al. Plasma oxytocin concentrations and OXTR polymorphisms predict social impairments in children with and without autism spectrum disorder. Proc Natl Acad Sci USA 2014; 111: 12258–12263.

84 Baribeau DA, Anagnostou E. Oxytocin and vasopressin: linking pituitary neuropeptides and their receptors to social neurocircuits. Front Neurosci 2015; 9: 335.

85 Yuen KW, Garner JP, Carson DS, Keller J, Lembke A, Hyde SA et al. Plasma oxytocin concentrations are lower in depressed vs. healthy control women and are independent of cortisol. J Psychiatr Res 2014; 51: 30–36.

86 Carson DS, Berquist SW, Trujillo TH, Garner JP, Hannah SL, Hyde SA et al. Cerebrospinal fluid and plasma oxytocin concentrations are positively correlated and negatively predict anxiety in children. Mol Psychiatry 2014; 20: 1085–1090.

87 Wei D, Dinh D, Lee D, Li D, Anguren A, Moreno-Sanz G et al. Enhancement of anandamide-mediated endocannabinoid signaling corrects autism-related social impairment. Cannabis Cannabinoid Res 2016; 1: 81–89.

88 Trezza V, Vanderschuren LJ. Bidirectional cannabinoid modulation of social behavior in adolescent rats. Psychopharmacology (Berl) 2008; 197: 217–227.

89 Goodman J, Packard MG. The influence of cannabinoids on learning and memory processes of the dorsal striatum. Neurobiol Learn Mem 2015; 125: 1–14.

90 Fagundo AB, de la Torre R, Jiménez-Murcia S, Agüera Z, Pastor A, Casanueva FF et al. Modulation of the endocannabinoids N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) on executive functions in humans. PLoS One 2013; 8: e66387.

91 Sullivan JM. Cellular and molecular mechanisms underlying learning and memory impairments produced by cannabinoids. Learn Mem 2000; 7: 132–139.

92 Bossong MG, Jansma JM, van Hell HH, Jager G, Kahn RS, Ramsey NF. Default mode network in the effects of Δ9-Tetrahydrocannabinol (THC) on human executive function. PLoS One 2013; 8: e70074.

93 Wise LE, Long KA, Abdullah RA, Long JZ, Cravatt BF, Lichtman AH. Dual fatty acid amide hydrolase and monoacylglycerol lipase blockade produces THC-like Morris water maze deficits in mice. ACS Chem Neurosci 2012; 3: 369–378.

94 Goonawardena AV, Sesay J, Sexton CA, Riedel G, Hampson RE. Pharmacological elevation of anandamide impairs short-term memory by altering the neurophysiology in the hippocampus. Neuropharmacology 2011; 61: 1016–1025.

95 Schechter M, Pinhasov A, Weller A, Fride E. Blocking the postpartum mouse dam's CB1 receptors impairs maternal behavior as well as offspring development and their adult social–emotional behavior. Behav Brain Res 2012; 226: 481–492.

96 Starr-Phillips EJ, Beery AK. Natural variation in maternal care shapes adult social behavior in rats. Dev Psychobiol 2014; 56: 1017–1026.

97 Rettori V, Zubilete M, Fernandez-Solari J, Burdet B, De Laurentiis A. Novel interactions between the endocannabinoid system and oxytocin. Exp Clin Endocrinol Diabetes 2010; 118: 118.

98 Kerr DM, Downey L, Conboy M, Finn DP, Roche M. Alterations in the endocannabinoid system in the rat valproic acid model of autism. Behav Brain Res 2013; 249: 124–132.

99 Chakrabarti B, Persico A, Battista N, Maccarrone M. Endocannabinoid signaling in autism. Neurotherapeutics 2015; 12: 837–847.

100 Soderstrom K, Luo B. Late-postnatal cannabinoid exposure persistently increases FoxP2 expression within zebra finch striatum. Dev Neurobiol 2010; 70: 195–203.

Promoting social behavior with oxytocin in high-functioning autism spectrum disorders

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Social adaptation requires specific cognitive and emotional competences. Individuals with high-functioning autism or with Asperger syndrome cannot understand or engage in social situations despite preserved intellectual abilities. Recently, it has been suggested that oxytocin, a hormone known to promote mother-infant bonds, may be implicated in the social deficit of autism. We investigated the behavioral effects of oxytocin in 13 subjects with autism. In a simulated ball game where participants interacted with fictitious partners, we found that after oxytocin inhalation, patients exhibited stronger interactions with the most socially cooperative partner and reported enhanced feelings of trust and preference. Also, during free viewing of pictures of faces, oxytocin selectively increased patients' gazing time on the socially informative region of the face, namely the eyes. Thus, under oxytocin, patients respond more strongly to others and exhibit more appropriate social behavior and affect, suggesting a therapeutic potential of oxytocin through its action on a core dimension of autism.

cognition | neurodevelopmental disorder | recovery | Syntocinon

Our brain is endowed with the ability to detect and respond to simple social signals such as eye contact, as well as to infer from more complex behaviors intrinsically social qualities of other people such as fairness or cooperation. Individuals suffering from high-functioning autism spectrum disorders (HF-ASD), a neurodevelopmental disorder, are impaired in understanding social cues and in responding to them. These patients generally have normal language or general intellectual abilities, yet in everyday life they avoid eye contact (1–3) and do not spontaneously interact with people (4). On formal tests of social cognitive skill, they show specific impairments in understanding the intentions of others (1, 5) and lack of fast intuitive judgments about social contexts (4).

The pathogenesis of autism is unclear, although mutations in genes implicated in synaptogenesis have been identified (6, 7) and different neurochemical, neurophysiological, and neuropathological abnormalities have been demonstrated in these patients (8). An interesting current hypothesis has implicated oxytocin in the etiology of autism, and in particular in the social disorders that are the hallmark of HF-ASD (8–10).

Oxytocin is a hormone synthesized in the hypothalamus. Best known for its facilitatory role in parturition and lactation, oxytocin is also involved in the regulation of emotions and has receptors distributed in various brain regions including the limbic system and amygdala (11, 12). In mammals, it has been associated specifically with the development of prosocial behavior such as mother-infant attachment, grooming, approach behavior, sexual activity, and stress regulation (13, 14). Oxytocin anomalies have been reported in children with autism. They have significantly lower plasma oxytocin levels compared to control subjects (15) and fail to show the normal developmental increase in oxytocin blood levels. Moreover, plasma samples are associated with higher oxytocin precursor levels, suggesting that autism may be related to anomalies in the way this hormone is synthesized (16).

Experimental manipulation of brain oxytocin levels in healthy human subjects confirms its involvement in the expression of human affiliative social behavior (17). In a simulated economic investment game, subjects who received an intranasal spray of oxytocin were more inclined, as compared to a placebo control group, to trust another player by sending him money with no guarantee of reciprocation, suggesting that oxytocin acts on brain circuits that promote social proximity and affiliation with peers (17). Recently, it has been shown that oxytocin facilitates recognition of memorized faces and strengthens the encoding of social stimuli (18, 19). Moreover, oxytocin has been reported to increase the time spent looking at socially important cues, such as the eyes, when viewing pictures of human faces (20). In the light of the above findings, a key question regarding both the role of oxytocin in the nervous system and the pathophysiology of social disorders in autism is whether administration of oxytocin can influence social interaction behavior in individuals with autism. We investigated the effects of intranasal oxytocin on the social behavior of 13 patients suffering from HF-ASD and compared these effects to a placebo condition and to the behavior of matched healthy subjects. Two different behavioral measures were used: (*i*) decision making and affect in a social interaction game, and (*ii*) eye movement recordings during a face perception task. We also measured plasma oxytocin levels in patients before and after nasal spray intake, to establish whether patients displayed physiological abnormalities in oxytocin and to verify the effectiveness of the nasal administration procedure in enhancing plasma oxytocin levels.

Results

Social Ball Tossing Game. We used a social interaction task inspired by the Cyberball game (21) in which the participant engages in a multiround ball-toss game over a computer network with three fictitious partners (Fig. 1*A Inset*). In our variant game, we manipulated the amount of reciprocation exhibited by the three fictitious players. The critical task manipulation was the probability that each of the three fictitious players would throw the ball to the participant, which allowed us to create different cooperative behavior profiles (good, bad, and neutral) (*Materials and Methods* and *SI Materials and Methods*).

Oxytocin Effect on Social Decision. The behavioral decision variable of interest in this task is the participant's ball-toss choices. Under placebo treatment, patients showed little evidence that they discriminated the three players' cooperative profiles. Whereas healthy subjects sent significantly more balls to the good than to the bad (Wilcoxon test, $z = 3$, $P < 0.003$) or neutral player ($z =$
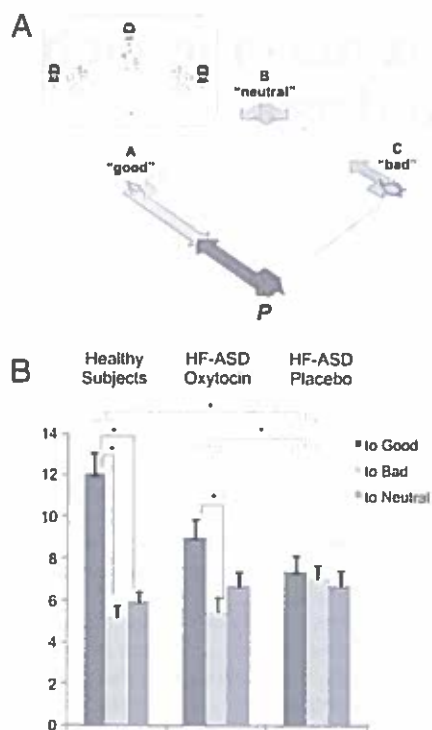
Fig. 1. Cyberball game and ball-toss distributions toward each of the three partners. (A) Schematic representation of the modified Cyberball game. On successive trials, the role of the participant P alternates between turns as observer of ball exchanges between two of the other players, as ball recipient, and as ball sender. The behavior of players A, B, and C is computer-generated so as to define three different profiles from P's standpoint: an includer or good profile, a neutral profile, and an excluder or bad profile. The length of the gray arrows is proportional to the number of balls sent by a given player to each of the other players. The profiles represent the average behavior over the entire game but, rather than being fixed from the beginning, they were tuned progressively using an algorithm described in SI Text. Black arrows represent the behavior of a representative healthy subject. (B) Ball-toss distributions for healthy subjects and for patients with HF-ASD treated with oxytocin or placebo. Under oxytocin, there was a nearly significant trend in the number of balls sent toward the good as compared to the neutral player (significant trend, $z = 1.82$, $P = 0.06$; two-tailed) (middle) (mean and SEM; * indicates significant difference at $P < 0.05$ or better on posthoc pairwise comparisons).

2.76, $P < 0.005$) (Fig. 1B Left), patients under placebo responded in the same manner to all players ($z = 0.36$, $P = 0.72$; $z = 0.2$, $P = 0.84$) (Fig. 1B Right). In striking contrast, oxytocin intake led patients to engage more often with the good player and to send significantly more balls to this player as compared to the bad one ($z = 2.04$, $P < 0.041$; two-tailed) (Fig. 1B Middle). When comparing directly the effects of placebo and oxytocin, we also found a significantly larger difference in the number of balls sent to the good versus the bad player in the oxytocin condition ($z = 1.99$, $P < 0.047$; two-tailed). Finally, the difference in performance (number of balls sent to the good versus the bad player) between the control subjects and patients, which was significant under placebo (Mann–Whitney U test: $z = 3.1$, $P < 0.0021$), disappeared when the comparison was made with the oxytocin treatment condition ($z = 1.62$, $P = 0.11$).

A finer-grained image of the patients' decision making was obtained by examining the distribution of ball tosses over time. Data were binned with respect to intervals defined by player A's turns, as it was through the observation of A's behavior that the participant could learn to cooperate with him more than with the other two players. The first six tosses by A were unbiased; hence

the probability of the participant receiving the ball $p[A \rightarrow P] = 0.33$. On the next four tosses, A increased the proportion of balls sent to the participant up to $p[A \rightarrow P] = 0.50$. From the 11th toss on, A sent all of its balls to P, that is, $p[A \rightarrow P] = 1.0$. Over the same period, player C started to exclude P, revealing himself as the bad player. The effect of these biases on the behavior of the participants is illustrated in Fig. 2. Because preliminary analyses in both healthy subjects and patients failed to reveal any differences in behavior toward the neutral versus the bad player, we focus on good and bad players only. Both healthy subjects and patients under oxytocin begin to cooperate preferentially with the good player at about the same time, with the cumulative number of balls sent to the good and bad players diverging significantly in the 15–17 interval (Fig. 2 A and B; first of two consecutive significant bins for the difference between good and bad, healthy subjects: $z = 2.7$, $P < 0.007$; oxytocin: $z = 2.04$, $P < 0.041$; two-tailed, Wilcoxon test). By contrast, under placebo, the patients' cumulative ball-toss curves never diverged significantly (Fig. 2C).

Oxytocin Effect on Emotions. The emotional response of the patients to the fictitious players' personality was assessed after completion of the task using a seven-point rating scale. These emotional self-ratings were consistent with their decision behavior under both treatment conditions. Whereas feelings (trust and preference) expressed toward the three fictitious players did not differ in the placebo condition (Friedman's ANOVA, respectively: $\chi^2 = 2.39$, $P = 0.3$; $\chi^2 = 1.19$, $P = 0.55$), patients reported that they trusted more and showed stronger preference for the good than the bad player after playing under oxytocin (Friedman's ANOVA, respectively: $\chi^2 = 17.89$, $P < 0.0002$; $\chi^2 = 13.63$, $P < 0.001$; posthoc pairwise comparisons $P < 0.05$; Fig. 3). No significant differences were found between feelings toward the neutral and the other two players.

One question which could be raised about the effect of oxytocin on ball-toss choices is whether it mainly acted on social engagement or on the perception of monetary rewards. To address this issue, we tested a new group of seven HF-ASD patients on the same ball-toss game but modified the contextual framing of the task to eliminate any reference to monetary incentives. The task conditions and oxytocin administration procedures were exactly the same as in the original version except that subjects were instructed that the goal of the task was to play a friendly ball-toss game with other players, but no monetary reward was promised and the participant did not receive any feedback about the number of balls he/she received. They were only told that whenever they tossed the ball to someone, that player could either send it back or toss it to another player. Following completion of the task, the participants again estimated their feelings of "trust" and "preference" with respect to the fictitious players. Despite the smaller size of the patient sample, we again found a significant, positive effect of oxytocin on the participant's capacities to discriminate between the two extreme player profiles (Fig. S1). Comparing directly the effects of placebo and oxytocin, we found a significantly larger difference in number of balls sent to the good versus the bad player in the oxytocin condition ($z = 1.99$, $P < 0.047$; two-tailed) (Fig. S1). Consistent with this behavior, patients also reported that they felt more trust toward the good than the bad player under oxytocin ($z = 2.11$, $P < 0.035$; two-tailed), whereas under placebo, there was no such difference ($z = 0.59$, $P > 0.58$). In the oxytocin condition, a similar trend was found in the feelings of preference but it did not reach significance ($z = 1.57$, $P = 0.11$).

Visual Scanning of Faces. To strengthen our observations of the effects of oxytocin on the processing of socially relevant information, we investigated how patients looked at a fundamental social stimulus, such as human faces. Participants examined
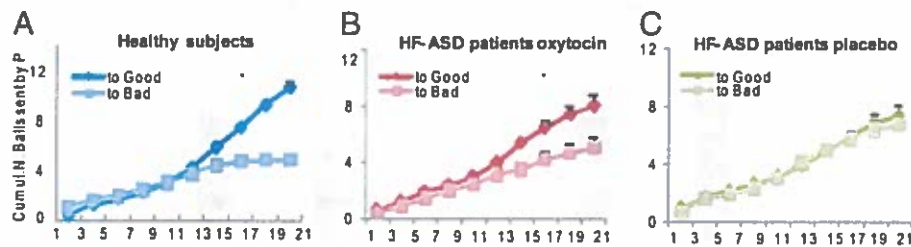
Fig. 2. Time course of ball tosses during the Cyberball game. Cumulative number of balls sent by the participant P to players A (good) and C (bad) in regularly spaced bins, for healthy subjects and for patients with HF-ASD under oxytocin and under placebo. Each data point falls in a bin defined by an interval between player A's tosses n and $n+2$. The participant had, on average, three ball-toss opportunities in each interval. The transition in player A's behavior from unbiased (equal probability of throwing the ball to each player) to positively biased toward P (100% probability of throwing the ball to P) was progressive (mean and SEM; * indicates significant difference at $P < 0.05$ or better for the first of two significant consecutive bins).

pictures of faces presented one at a time on a computer monitor while their eye movements were being recorded. The participants' task was to report either the gender (male/female) or the gaze direction (direct/averted) of the depicted face (Fig. S2). Offline, we computed the total fixation time inside each of six regions of interest (eyes, nose, mouth, forehead, cheeks, and outside of facial contour) and the number of saccades (rapid displacements of the line of gaze) elicited by the face stimuli.

Normal subjects directed their gaze preferentially within the contours of the faces [gender identification (GI): $80\% \pm 2.09$; gaze direction (GD): $84\% \pm 1.74$; Tables S1 and S2]. By contrast, patients under placebo spent significantly less time looking directly at the faces as compared to healthy subjects ($53\% \pm 7.2$ in GI: Mann–Whitney U test, $z = 2.67, P < 0.0077$; $41\% \pm 8.04$ in GD: $z = 4.08, P < 0.00005$). More detailed analysis of scanning pattern by regions of interest shows that they specifically avoided the eye region ($z = 2.88, P < 0.004$; $z = 3.48, P < 0.0005$ for GI and GD, respectively). Interestingly, during gaze direction judgments, patients also produced more saccades than healthy subjects (GD: $z = 2.45, P < 0.015$). This increase in saccade rate was only present during epochs when patients looked at faces directly and not when they explored the rest of the display (GD:

$z = 0.71, P > 0.47$). Such underexploration of the face and eyes, in association with high saccade frequency, implies that patients explored these images hastily by means of multiple brief fixations, probably with high levels of anxiety and discomfort. Saccade frequency was not increased during gender decisions (GI: $z = 1.36, P > 0.17$), possibly because, in contrast to gaze direction judgment, it does not depend critically upon attending to the eye region of the faces.

Oxytocin modified how patients responded to pictures of faces, as compared to the placebo condition. Total gaze time over the face increased significantly under both task conditions (GI: $z = 2.27, P < 0.023$; GD: $z = 2.19, P < 0.029$; two-tailed Wilcoxon test; Fig. 4 *A* and *B Left*). Broken down by region of interest, the effects of oxytocin are found to be largely accounted for by an increased fixation time over the eye region (GI: $z = 2.12, P < 0.04$; nearly significant trend for GD: $z = 1.88, P = 0.059$; Fig. 4 *C* and *D*). No effects of oxytocin were observed over the other regions of interest (mouth + nose, GI: $z = 1.18, P > 0.23$; GD: $z = 1.41, P > 0.15$; forehead + cheeks, GI: $z = 39, P > 0.69$; GD: $z = 0.86, P > 0.38$) (Fig. 4 *C* and *D Right*). Finally, oxytocin significantly reduced the abnormally high saccade frequency observed under placebo during gaze direction judgments ($z = 2.12, P < 0.03$; two-tailed; Table S2).

Although oxytocin significantly enhanced patients' visual scanning of faces, as compared to the placebo condition, their gaze time on the face and eye region remained significantly lower than that of healthy subjects for all comparisons with the exception of whole-face scanning in the gender identification condition (Mann–Whitney U test; face: GD: $z = 3.21, P < 0.002$; GI: $z = 1.96, P > 0.05$; eye: GI: $z = 2.77, P < 0.006$; GD: $z = 2.99, P < 0.003$). The fact that oxytocin did not fully restore a normal visual exploration pattern in patients is discussed below in relation to the magnitude of the changes in blood oxytocin.

In summary, under oxytocin, patients with HF-ASD spent more time looking at the face pictures and, specifically, at the eye region. The accompanying decrease in saccade frequency, that is, the increase in the average duration of individual fixations, suggests that oxytocin may reduce the fear or anxiety induced by face stimuli in these patients.

We tested for a possible effect of treatment order by comparing patients' performance between the two visits in the placebo and the oxytocin condition. No differences were found between the two visits on any of the dependent variables measured in the ball-tossing and face-scanning tasks (Mann–Whitney U test; $z = 0, P = 1$). We also found that oxytocin's effect on patients' performance during both tasks was not related to a simple mood effect (see *SI Results*).

Fig. 3. Subjective postexperimental rating under oxytocin and placebo treatment. Rating (1–7) of subjective feeling states toward the three players in the placebo and oxytocin-treated patients with HF-ASD (mean and SEM; * indicates significant difference at $P < 0.05$ or better on posthoc pairwise comparisons).

Plasma Oxytocin Levels. Baseline plasma oxytocin concentration in patients (1.08 pg/mL ± 1.04) was significantly below the values observed in a normative group of healthy subjects (7.28 pg/mL $\pm$
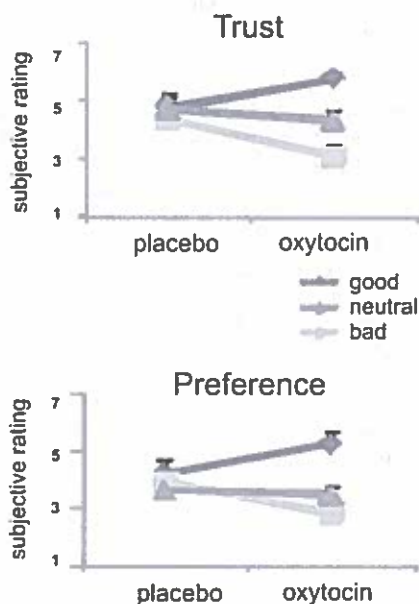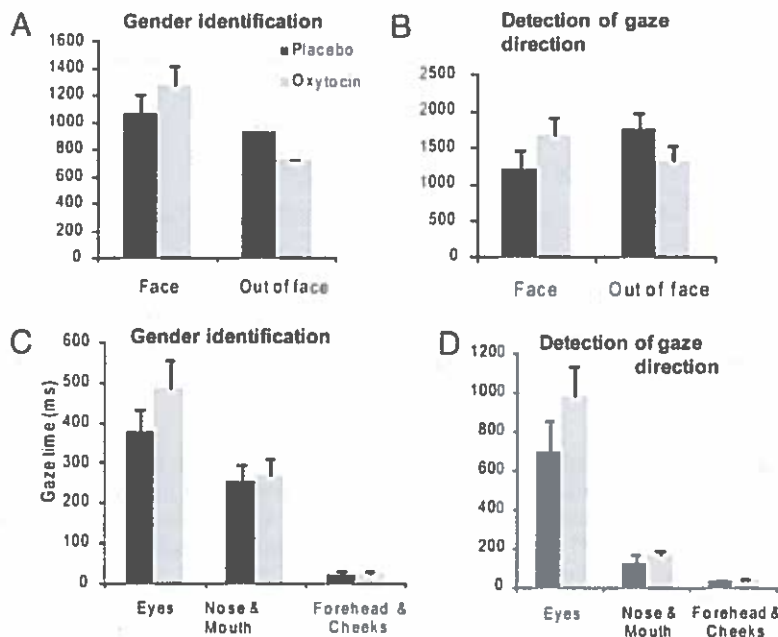
Fig. 4. Mean gaze time spent on different regions of interest for patients with HF-ASD under placebo and oxytocin treatment. (*A* and *B*) Gaze time spent on the face and outside the face region under oxytocin and placebo when patients had to identify the face's gender (male/female) and face's gaze direction (direct/averted), respectively. (*C* and *D*) Gaze time spent on main regions of interest: the eyes, nose and mouth, and other regions such as forehead and cheeks during gender identification and gaze direction detection, respectively. * indicates significant difference at *P* < 0.05 or better on Wilcoxon test.

4.49) (Mann–Whitney *U* test; *z* = 4.69, *P* < 0.0001). A second measurement made 10 min after nasal administration of a dose of 24 IU of Syntocinon spray showed a significant increase in plasma oxytocin concentration (2.66 pg/mL ± 2.2) (Wilcoxon test; *z* = 1.88, *P* < 0.02; Fig. S3), indicating successful assimilation of the substance (*SI Materials and Methods*).

Individual Variability in Response to Oxytocin. Although the social symptoms of HF-ASD can be diagnosed on the basis of well-established, reliable criteria, it is unclear whether these symptoms are related to a common etiological process. A degree of heterogeneity in responsiveness to oxytocin treatment can thus be expected in such a patient group. Inspection of individual performances revealed that some patients responded strongly to oxytocin, others more weakly, and some not at all (Table S3). Furthermore, oxytocin effects on the social game and on the face perception tasks were only weakly correlated (GI: *r* = 0.23; GD: *r* = 0.54, *P* > 0.05), indicating that the two tasks are sensitive to different aspects of social information processing. Indeed, although explicit social engagement is required in the ball game, visual inspection of facial stimuli may involve more implicit, automatic mechanisms. Also, looking directly at large face stimuli may be more threatening to some patients than interacting with other people via a computer network, whereas for others, the dynamical aspect of the social interaction may be more difficult to apprehend than the simple perceptual decision required by the face task. Possible relationships between oxytocin effects and general clinical data on patients were investigated. We found no significant correlation between patients' performance with the Autism Diagnostic Interview-Revised (ADI-R) (ball game: *r* = −0.31; GI: *r* = 0.03; GD: *r* = 0.35; all *P* > 0.05), IQ (ball game: *r* = 0.43; GI: *r* = −0.43; GD: *r* = −0.24; all *P* > 0.05), or age (ball game: *r* = 0.25; GI: *r* = −0.2; GD: *r* = −0.09; all *P* > 0.05). Different authors have suggested that patients with autism may display different social interaction styles (15, 22). According to these authors, one can distinguish between "aloof" individuals who avoid physical proximity with others and actively reject social contact, "passive" individuals

who do not reject approaches but neither engage in social relations, and "active-but-odd" individuals who display approach behavior but in a somewhat inappropriate or one-sided manner. We examined whether such qualitative differences in social interaction profiles might account for the variability in the response to oxytocin treatment. Patients in our study were assigned to one of those three categories based on clinical records and parent interviews (6 patients were classified as aloof, 7 as active-but-odd, none as passive). Interestingly, 6/8 patients who showed positive changes on the ball game under oxytocin had been labeled as active-but-odd, whereas 4/5 who showed no positive change were of the aloof type.

Discussion

In this study, we investigated whether oxytocin could modify how high-functioning autistic patients process social signals and social feedback. Oxytocin was shown to enhance visual scanning of faces and, in particular, of the eye region, as compared to a placebo condition. Eye contact between individuals can be considered a basic form of social aptitude. Previous studies in normal individuals indicate that oxytocin enhances processing of facial stimuli (20) and the ability to infer others' mental states from the eye region (23). Here we further demonstrate that oxytocin promotes a first level of prosocial approach by overturning what constitutes a core deficit of patients with HF-ASD, namely the lack of eye contact. How does oxytocin facilitate patients' prosocial behavior? The present data provide some suggestions of the neural mechanisms mediating these effects. It has been proposed that oxytocin enhances affiliation partly by reducing fear of social unfaithfulness and by suppressing avoidance behavior (14) and that it reduces the activity of the amygdala, resulting in a decrease of fear responses (14, 24, 25). It is possible that patients with autism possess latent social skills and that oxytocin may thus favor social engagement behavior by suppressing fear and mistrust.

The results from the ball-tossing task suggest the possibility of other mechanisms underlying the effects of oxytocin on social interaction. In this simple game simulating social exchanges,

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patients tested under placebo conditions did not take into account the behavior of other players and showed no differential emotional responses to the different players. By contrast, under oxytocin, these patients engaged more often in exchanges with the player who reciprocated strongly, less often with the player who reciprocated weakly, and they exhibited emotional responses congruent with this behavior. Thus, oxytocin enhanced patients' ability to process socially relevant cues and acquire their meaning in an interactive context. A study conducted in normal subjects showed that oxytocin increases trust of others in the absence of any certainty of reciprocation (17), hinting at the possibility that oxytocin may promote indiscriminate prosocial behavior and "blind" trust. The task used here to study the effects of oxytocin on autistic social difficulties was different in that it involved multiple iterations in which the participant was presented with successive feedback from partners that were endowed with different reciprocating tendencies. Therefore, the behavior exhibited by the participants could evolve over time through a learning process which can be interpreted within a social reinforcement learning framework, with social inclusion acting as reinforcer. The fact that oxytocin allowed recognition of the partner who was willing to reciprocate the most cannot be explained only in terms of prosocial attitudes such as reduced fear or increased approach and trust. The patients' ability to discriminate between the good and bad partners shows that oxytocin facilitated learning, which may in turn result from an increased drive for social affiliation or from an enhancement of reinforcers satisfying this drive. This hypothesis is consistent with data from animal and human studies. Animal studies show that oxytocin promotes social bonding behavior. In rodents, oxytocin has been reported to enhance social recognition, as indicated by a decrease of exploration behavior toward a conspecific during a second encounter (13). Moreover, in oxytocin knockout mice, social memory is impaired but recovered after a single shot of the hormone before initial social encountering takes place. Finally, in humans, different studies have shown that oxytocin improves recognition memory of social relevant cues (19) (i.e., faces) and memory of positive social information (i.e., happy faces) (18).

One question that can be raised is whether oxytocin mainly acted by enhancing sensitivity to social rewards (i.e., to being sent the ball, a social engagement gesture) or by enhancing sensitivity to the accompanying nonsocial reward (the monetary value of the received ball). Motivation for money could not have been the main factor determining their choices. Both healthy subjects and patients were biased toward adopting a prosocial attitude because they sent fewer balls to the good player and more balls to the bad player than would be predicted by an optimal reward-seeking strategy such as the matching law (26). More direct evidence that, in this task, oxytocin is acting on social motivation comes from a second ball-toss game that was performed by an independent group of HF-ASD patients, in which ball exchange was not associated with monetary reward. Also in this case, oxytocin enhanced the propensity to interact with the reciprocating partner, as compared to placebo. This is in keeping with results obtained in a similar social task showing that normal subjects preferred to avoid being excluded from the game even when, as a consequence, they ended up *losing* money (27).

Although previous studies have shown that oxytocin can reduce repetitive behavior in subjects with autism (28) and enhance the comprehension of affective speech (29), here we demonstrated that oxytocin can promote social approach and social comprehension in patients with autism. Individual variability was observed in the effects of oxytocin on the social tasks used in this study. Nevertheless, the results from the ball-toss game were statistically robust and found in two independent groups of HF-ASD patients. More work will be needed to understand the relationship between changes in social behavior induced by oxytocin administration in individuals with autism

and local changes in brain oxytocin metabolism. This could be accomplished using functional imaging techniques. Finally, our results highlight the therapeutic potential of oxytocin through its action on core deficits of patients with HF-ASD such as affiliation and cooperative behavior. Although the effect we measured here is certainly transient, it serves to show that these patients are quite able to engage in social relationships. Future research is necessary to investigate whether a long-term intake of oxytocin may improve real-life social functioning of these patients.

Materials and Methods

Participants. A group of 13 adults (11 men and 2 women, mean age = 26, range = 17–39) with a clinical diagnosis of Asperger syndrome (AS) ($n = 10$) or high-functioning autism (HFA) ($n = 3$) according to Diagnostic and Statistical Manual-Revision 4 (DSM-IV R) (American Psychiatric Association, 2000) and ASDI (Asperger Syndrome Diagnostic Interview) (30) were recruited from the expert centers (Foundation FondaMental), Chenevier-Mondor Hospital in Créteil, France. Interviews with parents or caregivers using the ADI-R (Autism Diagnostic Interview-Revised) (31) (Table S4) confirmed the diagnoses. As part of the checking process, the French translation of A-TAC (autism, tics, AD-HD, and other comorbidities) (32) was completed by the parents. Patients received verbal and performance IQ tests (WAIS-III) and all showed average to above average estimates of intelligence (Table S4). Patients were medication-free for at least 2 weeks before and throughout the study (*SI Materials and Methods*). A second group of patients was recruited to test oxytocin effects on a social ball-toss game involving no monetary incentives. It included seven new HF-ASD patients (7 men, mean age = 28, range = 18–38; verbal IQ: 96 ± 15.85, performance IQ: 87 ± 20.57, total IQ: 92 ± 17.47; ADI-R: social interaction 12.6 ± 7.21, communication 6.7 ± 3.73, repetitive behaviors 3.1 ± 2.03) with a clinical diagnosis of AS ($n = 4$) or HFA ($n = 1$) or pervasive developmental disorder-not otherwise specified (PDD-NOS) ($n = 2$). The study also included a control group of 13 healthy subjects matched for chronological age and sex to the patients (11 men and 2 women, mean age = 26, range = 18–40). The study was approved by the Local Ethical Committee (Centre Léon Berard, Lyon IV). The French Agency (Agence Française de Sécurité Sanitaire des Produits de Santé) competent for clinical trials on a medicinal product for human use also gave its approval.

Behavioral Experiments. *Social ball-tossing game.* During this variant version of the Cyberball game, three players depicted by cartoon characters and their corresponding photographs were presented on a touch-sensitive computer display. The participant (player P) was featured by an additional cartoon representing a pair of animated hands assuming a first-person perspective. Each trial consisted of a single ball exchange depicted by a short animation showing one player handling the ball, and a few seconds later another player catching the ball. If a trial ended with the participant as recipient, he/she became the next trial's sender and had to address the ball to player A, B, or C by touching the corresponding photograph. At game start, probabilities were homogeneous for all players, that is, the participant had a probability $P = 1/3$ of receiving the ball from any of the three players. After a predetermined number of rounds, player profiles diverged such that player A (the "good" profile) sent, on average, 70% of its played balls to the participant (P), player B (the "neutral" profile) sent 30% of its played balls to P, and player C (the "bad" profile) sent 10% of its played balls to P. These proportions are represented by the length of the gray arrows in Fig. 1A. The game included a monetary incentive to enhance the participant's cognitive engagement in the task. Any player receiving the ball earned 2€ (see *SI Materials and Methods* for details about the algorithm used to dynamically set the three player profiles). To optimize cognitive engagement in the task, the participant was told that each ball received was worth 2 euros, and that when returning the ball two outcomes were possible: either the recipient would toss the ball back to the participant, generating further income, or toss it to another player, earning that player 2 euros. The participant's cumulative gains were displayed on the screen and he/she was led to expect a percentage of the gains at the end of the game. The participant was instructed that the game ended after a total of 80 tosses. The main dependent variable in this experiment was the distribution of the participant's toss choices between players A, B, and C (illustrated for a representative healthy subject by the black arrows in Fig. 1A). Also, following completion of the task, the participant estimated, using a subjective seven-point rating scale, their sentiments of "trust" and "preference" with respect to the fictitious players.

Face perception tasks. Pictures of faces were presented on the 17-inch video display of a Tobii 1750 eye tracker and patients' gaze movements were

analyzed offline using ClearView software. Five regions of interest (ROI) on the face were defined: the two eyes, nose, mouth region, forehead, and the two cheeks. A sixth ROI consisted of the portion of the image outside the contour of the face. For each picture, gaze fixation time (in milliseconds) was computed for each of the six ROIs. The number of saccadic eye movements (rapid changes in gaze direction) made for two ROIs, inside and outside the facial contours, was computed (a finer-grained parcellation of the face yields too few eye movement samples) and converted into saccade frequency = (number of saccades)/(total fixation time in ROI) (*SI Materials and Methods*).

Procedure. The study used a randomized, placebo-controlled, double-blind within-subject experimental design. Patients received oxytocin and placebo during two visits to the lab separated by 7 days. They were tested on the social ball-tossing game and on face perception tasks and completed a number of rating scales following the ball game on each visit (Table S5). Healthy subjects were tested on a single visit. General affect was measured after oxytocin and after placebo intake for each participant using the PANAS scale to assess the possible mood-altering effects of oxytocin (*SI Materials and Methods*).

Statistics. Statistical analyses were conducted on the behavioral data in the social ball-tossing game, eye movement measurements, and plasma levels of oxytocin. Nonparametric tests were used because the distribution of the data (number of balls, gaze time, oxytocin levels) was non-Gaussian (*SI Materials*

and Methods). Comparisons between groups and drug treatment conditions were made using nonparametric ANOVA (Friedman) as well as Wilcoxon signed-rank and Mann–Whitney rank-sum tests. The sequence effect of treatment was tested using Mann–Whitney rank tests. All tests were evaluated against two-tailed probabilities.

Physiological Measures. *Oxytocin administration.* Each subject served as his or her own control and received oxytocin and placebo with a 1-week interval, in a balanced within-subject design. Participants received a single intranasal dose of 24 IU oxytocin (Syntocinon Spray; Novartis; three puffs per nostril, each with 4 IU oxytocin) or placebo 50 min before the start of the experiments (see *SI Materials and Methods* for the details on the measurement of oxytocin).

1. Baron-Cohen S, Wheelwright S, Hill J, Raste Y, Plumb I (2001) The "Reading the Mind in the Eyes" Test revised version: A study with normal adults, and adults with Asperger syndrome or high-functioning autism. *J Child Psychol Psychiatry* 42:241–251.
2. Dalton KM, et al. (2005) Gaze fixation and the neural circuitry of face processing in autism. *Nat Neurosci* 8:519–526.
3. Klin A, Jones W, Schultz R, Volkmar F, Cohen D (2002) Visual fixation patterns during viewing of naturalistic social situations as predictors of social competence in individuals with autism. *Arch Gen Psychiatry* 59:809–816.
4. Volkmar FR, Klin A (2000) *Asperger's syndrome*, eds Klin A, Volkmar F, Sparrow SS (Guilford, New York), pp 25–71.
5. Frith U (2004) Emanuel Miller lecture: Confusions and controversies about Asperger syndrome. *J Child Psychol Psychiatry* 45:672–686.
6. Durand CM, et al. (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet* 39:25–27.
7. Jamain S, et al.; Paris Autism Research International Sibpair Study (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27–29.
8. Waterhouse L, Fein D, Modahl C (1996) Neurofunctional mechanisms in autism. *Psychol Rev* 103:457–489.
9. Bartz JA, Hollander E (2006) The neuroscience of affiliation: Forging links between basic and clinical research on neuropeptides and social behavior. *Horm Behav* 50: 518–528.
10. Insel TR (1997) A neurobiological basis of social attachment. *Am J Psychiatry* 154: 726–735.
11. Huber D, Veinante P, Stoop R (2005) Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala. *Science* 308:245–248.
12. Landgraf R, Neumann ID (2004) Vasopressin and oxytocin release within the brain: A dynamic concept of multiple and variable modes of neuropeptide communication. *Front Neuroendocrinol* 25:150–176.
13. Ferguson JN, Young LJ, Insel TR (2002) The neuroendocrine basis of social recognition. *Front Neuroendocrinol* 23:200–224.
14. Lim MM, Young LJ (2006) Neuropeptidergic regulation of affiliative behavior and social bonding in animals. *Horm Behav* 50:506–517.
15. Modahl C, et al. (1998) Plasma oxytocin levels in autistic children. *Biol Psychiatry* 43: 270–277.
16. Green L, et al. (2001) Oxytocin and autistic disorder: Alterations in peptide forms. *Biol Psychiatry* 50:609–613.

17. Kosfeld M, Heinrichs M, Zak PJ, Fischbacher U, Fehr E (2005) Oxytocin increases trust in humans. *Nature* 435:673–676.
18. Guastella AJ, Mitchell PB, Mathews F (2008) Oxytocin enhances the encoding of positive social memories in humans. *Biol Psychiatry* 64:256–258.
19. Savaskan E, Ehrhardt R, Schulz A, Walter M, Schächinger H (2008) Post-learning intranasal oxytocin modulates human memory for facial identity. *Psychoneuroendocrinology* 33: 368–374.
20. Guastella AJ, Mitchell PB, Dadds MR (2008) Oxytocin increases gaze to the eye region of human faces. *Biol Psychiatry* 63:3–5.
21. Williams KD, Cheung CK, Choi W (2000) Cyberostracism: Effects of being ignored over the Internet. *J Pers Soc Psychol* 79:748–762.
22. Wing L, Gould J (1979) Severe impairments of social interaction and associated abnormalities in children: Epidemiology and classification. *J Autism Dev Disord* 9:11–23.
23. Domes G, Heinrichs M, Michel A, Berger C, Herpertz SC (2007) Oxytocin improves "mind-reading" in humans. *Biol Psychiatry* 61:731–733.
24. Kirsch P, et al. (2005) Oxytocin modulates neural circuitry for social cognition and fear in humans. *J Neurosci* 25:11489–11493.
25. Petrovic P, Kalisch R, Singer T, Dolan RJ (2008) Oxytocin attenuates affective evaluations of conditioned faces and amygdala activity. *J Neurosci* 28:6607–6615.
26. Herrnstein, R.J. (1961) Relative and absolute strength of response as a function of frequency of reinforcement. *J Exp Anal Behav* 4:267–272.
27. van Beest I, Williams KD (2006) When inclusion costs and ostracism pays, ostracism still hurts. *J Pers Soc Psychol* 91:918–928.
28. Hollander E, et al. (2003) Oxytocin infusion reduces repetitive behaviors in adults with autistic and Asperger's disorders. *Neuropsychopharmacology* 28:193–198.
29. Hollander E, et al. (2007) Oxytocin increases retention of social cognition in autism. *Biol Psychiatry* 61:498–503.
30. Gillberg C, Gillberg C, Råstam M, Wentz E (2001) The Asperger Syndrome (and high-functioning autism) Diagnostic Interview (ASDI): A preliminary study of a new structured clinical interview. *Autism* 5:57–66.
31. Lord C, Rutter M, Le Couteur A (1994) Autism Diagnostic Interview-Revised: A revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord* 24:659–685.
32. Hansson SL, et al. (2005) Psychiatric telephone interview with parents for screening of childhood autism—tics, attention-deficit hyperactivity disorder and other comorbidities (A-TAC): Preliminary reliability and validity. *Br J Psychol* 187:262–267.

ORIGINAL ARTICLE

On the application of cannabis in paediatrics and epileptology

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Abstract

An initial report on the therapeutic application of delta 9-THC (THC) (Dronabinol, Marinol) in 8 children resp. adolescents suffering from the following conditions, is given: neurodegenerative disease, mitochondriopathy, posthypoxic state, epilepsy, posttraumatic reaction. THC effected reduced spasticity, improved dystonia, increased initiative (with low dose), increased interest in the surroundings, and anticonvulsive action. The doses ranged from 0.04 to 0.12 mg/kg body weight a day. The medication was given as an oily solution orally in 7 patients, via percutaneous gastroenterostomy tube in one patient.

At higher doses disinhibition and increased restlessness were observed. In several cases treatment was discontinued and in none of them discontinuing resulted in any problems.

The possibility that THC-induced effects on ion channels and transmitters may explain its therapeutic activity seen in epileptic patients is discussed.

Casuistics

1. In the case of the boy P. G., treatment was begun with delta 9-THC (THC) at the age of 8 years and 9 months and continued until shortly before his death at the age of 9 years and 4 months. Approximately 0.07 mg THC/kg body weight a day was administered in two doses via percutaneous gastroenterostomy tube. The aim was to lessen the severity of spasticity brought on by neuronal ceroid lipofuscinosis, Jansky-Bielschowsky variant, which was causing the boy to suffer and to make it difficult to care for him. The treatment brought about a noticeable reduction in spasticity. Prior treatment with a combination of baclofen and tetrazepam had been unsatisfactory owing to the degree of spasticity. There was no noticeable worsening of the myoclonia symptomatic of the disease. Moreover, the patient's mother observed that the boy seemed more awake. This increased alertness may be ascribed to the discontinuation (without adverse reaction) of meperidine. Following the initiation of treatment, it was observed that the boy turned his head with greater precision towards his mother and laughed when she spoke to him. He seemed happier, although mood swings were also observed. "He would still have a smile on his face when suddenly he would seem to weep". These changes however did not fail to leave their mark on the interaction between mother and son: Sometimes the boy's mother was sadder than previously owing to her awareness that the loss of her increasingly alert son nevertheless was in-

evitable. It is impossible to evaluate the effect of THC treatment on the boy's epileptic seizures owing to the progression of the disease and modifications made to his antiepileptic treatment [1].

2. In the case of L. S., a 12 year-old girl with spasticity arising from mitochondriopathy, to whom approximately 0.09 mg THC/kg body weight a day orally was administered in two doses, the parents reported the following: their child became "more relaxed, more interested, more alert, more interested in her surroundings". L. spent "half an hour investigating her ear, as if it was the first time she had ever noticed it". Nodding spasms and tonic seizures improved considerably. Despite this, a temporary increase in seizure severity was observed.

3. The mother of the 12 year-old girl K. D., who suffers from severe spasticity and seizures as a result of severe hypoxia (foetomaternal transfusion) and who 0.07 mg THC/kg body weight a day orally in two doses was given, reported that she became "relaxed, less stiff, completely happy, open to everything". Whereas before initiation of the treatment she did not show any reaction when exposed to bad smells (her parents are farmers), after that her mimic behaviour demonstrated, that she would smell. In the case of this child, there was also a noticeable reduction in the number of epileptic seizures, heretofore unsatisfactorily treated with valproic acid. Versive seizures with nystagmus became less frequent, but when they occurred any tonic-clonic seizures were "extreme".

4. A 14 year-old girl, A. K., with neuronal ceroid lipofuscinosis, Spielmeyer-Vogt variant, was given 0.04 mg THC/kg body weight a day orally in two doses. In the case of this patient, the aim is to lessen her gait disturbance, manifested by problems of initiation of movements and a stiffening over longer distances ("no ground-covering steps"). L-dopa and amantadine had proved only partially successful: they only improved the initiation. During THC therapy, her gait improved considerably. The stiffness in the left leg lessened and the patient was able to cross the street again. The problem of starting off was not affected by THC. There was another improvement to observe: The girl suddenly developed initiative (setting the breakfast table of her own accord and changing her clothes when she wet herself). Her concentration when playing also improved slightly. Despite the progression of the disease, the number of focal seizures that progressed to grand mal seizures was slightly lower.

5. The case of the 13 year-old boy C. D. is characterised by spasticity, athetosis, myoclonia, and epileptic seizures of uncertain aetiology. He was given 0.14 mg THC/kg body weight a day orally in two doses. His parents reported that: the boy "has become more awake, he speaks more, makes more eye contact, takes part in things more, is more alert. It's great, he's more conscious of everything. For instance, in the past when touching him, he would continue to bite. He is happier,

he laughs more, is more relaxed". A definite influence on the epileptic seizures (both focal and primary generalised) was not observed. There was a reduction in the severity but not in the frequency of myoclonia.

6. A 11 year-old girl, S. P., suffered a spinal contusion (Th11-Th12) with total paraplegia following a traffic accident. She also had a frontal skull fracture and suspected haemorrhaging near the clivus. Owing to the severity of injuries to the abdomen, a subtotal ileum resection was carried out. Despite psychotherapy, the patient developed an eating disorder – without, however, losing weight. This seemed to indicate post-traumatic reaction, although the influence of organic factors remained difficult to assess. She was given 0.09–0.12 mg THC/kg body weight a day, orally administered in two doses. During treatment vomiting decreased. She said, that she was hungry, ate more and started to drink again. Her weight remained constant. The girl became "more accessible, for the first time open to therapy", was no longer on a " ‚No- trip", and "emerged from her previously destructive attitude". She could look others in the eye and was happier. On increasing the dosage, the patient demonstrated a high degree of associative thinking and verbal disinhibition concerning sexual contents. After three months treatment was stopped and there were no symptoms of withdrawal. Even after discontinuing the medication, the patient's body weight remained stable and her mood improved.

7. The youngest patient, a boy J. H., aged 3 years and 10 months became paraplegic as the result of a traffic accident. During his stay in hospital he became considerably withdrawn and ate little. He was given a brief course of treatment using 1 mg THC a day orally – to good effect. The improvement did not seem attributable solely to adjusting to the new environment.

8. A 14 year-old boy, M. Ö., suffers from severe idiopathic early infantile grand mal epilepsy with tonic-clonic seizures and falling. Owing to the modification of antiepileptic treatment, the influence of THC (0.12 mg/kg body weight a day orally given) on the epileptic seizures is impossible to assess. Appetite, playfulness, and mood improved. An epilepsy clinic claimed the boy's restlessness was attributable to the THC medication. However, restlessness was already present before this treatment was established. Therefore, ending the THC medication effected only a slight reduction in the degree of restlessness. Discontinuation of the medication caused no apparent difficulties.

Discussion

The following insights may be derived from the case reports:

1. THC also is a valuable means of treating children and adolescents.

2. Effects, side – effects and averaged daily doses of THC can be summarized as follows: reduced spasticity (0.09mg/kg body weight a day), improved dystonia within the context of a neurodegenerative disease affecting the basal ganglia (0.04mg/kg body weight a day), increased initiative (0.04 mg/kg body weight a day), improved posttraumatic reaction (0.09 mg/kg body weight a day), increased interest in surroundings (0.1mg/kg body weight a day), anticonvulsive action (0.07 mg/kg body weight a day), aided discontinuation of meperidine (approximately 0.07 mg/kg body weight a day), disinhibition concerning thinking and speaking (0.12mg/kg body weight a day), slight increase in preexisting restlessness (0.12mg/kg body weight a day).

3. In several of the cases treatment was discontinued and in none of them this caused any signs of withdrawal..

Looking for most suitable dosages of THC in paediatrics requires further investigation. Optimal doses seem to be varying greatly according to indication. Thus, when compared to doses recommended for treatment of cytostatica-induced emesis (more than 4mg/kg body weight a day [2]), THC doses applied in the described patients were much lower. Side effects of THC in children (mood changes) differ from those in adults (drowsiness, dizziness and in rare cases anxiety) [3]. CB1-receptors increase gradually during postnatal development, so psychotropic side effects in young children may be minor [4]. The effects of cannabinoids on the CNS are often biphasic. When administered to rodents, lower doses increased activity, while higher doses induced sedation and cataleptic behaviour [5]. This observation may explain the increased initiative of A.K., when lower doses (0.04mg/kg body weight a day) are given. It may be interesting to note, that augmented initiative may be explained by findings from positron-emission-tomography revealing improvement in blood circulation to the anterior insula and orbitofrontal and temporopolar cortices [6 and 7]). On the other hand, since autistic behaviour was diminished by higher doses (0.1mg/kg body weight a day), it may be speculated, that THC counteracted inhibitory mechanisms underlying autism.

The following observations are interesting to note: In the patients S.P. and J.H. THC improved posttraumatic reaction consistent with findings, that endocannabinoids extinct aversive memories (demonstrating actions on the basolateral amygdala) [8]. There is an ethical discussion, if it is justified, to extinct memories by drugs [9]. Apart from the ethical question, remembering traumatic experiences may result in suffering and obsession and may inhibit autonomy. In the patient K.D. reaction upon bad smells increased. A similar observation only, as far as I know, has been described by the astronomer Carl Sagan, who experienced with cannabis: "The enjoyment of food is amplified; tastes and aromas emerge that for some reason we ordinarily seem to be too busy to notice. I am able to give my full attention to the sensation." [10]. It may be speculated, that this amplification is not only due to changed attention, but is due to changes in olfactory system, too. The positron-emission-tomography study cited above [6] gives hints that regions of the brain processing olfactory stimuli participate in improved blood circulation, such as amygdala (again), which is involved in aversive olfactory sensations. An other indication is, that in the bulbus olfactorius of the rat the expression of CB1-receptor protein has been demonstrated [11]. In contrast to this hypothesis there has been found no difference in olfactory identification tasks between groups of cannabis users, former cannabis users and drug free controls [12]. Systematic evaluation seems to be much promising. There could be applied the sniffin' sticks – method [13]. Interestingly, preliminary evidence suggests, that cannabinoids are able to improve night vision, another form of sensory function [14].

Concerning the antiepileptic effects of cannabinoids to this point of time there is no systematic knowledge. But there are anecdotal reports even from medieval times: Al Badri (1443–1489) reports, that Ali ben Makki had helped the epileptic Zahir ad din Mohammed ben Ismail ben al Wakil with music and folia of the cannabis plant: the patient had forgot (!) his illness [15].

In addition to these clinical observations future insights as well into ion channel and transmitter mechanisms of epilepsy as into cannabinoid actions will benefit clinical advances in this area.

THC can have a proconvulsive and an anticonvulsive effect. Which one is generated depends on the dose and the type of seizure. It is effective in treating some forms of partial and generalised convulsive epilepsies, but it has no effect on other types of partial epilepsies and petit mal absences [16]. In terms of its antiepileptic effect, cannabidiol, the other major cannabinoid in the cannabis plant, is by far the more interesting substance. It has anticonvulsive properties without demonstrating any proconvulsive effect. In animal petit mal absence models, however, it has been found to block the effectiveness of antiepileptic drugs [17].

Six of the eight patients treated with THC in the present report suffered from epilepsy. In two of the six (L.S. and K.D.), the frequency of seizures decreased considerably upon THC administration. In one patient (A.K.), the frequency of seizures did not increase and severity of seizures remained constant (except for the last seizure), despite the progression of the principal

disease. In one patient (C.D.), a definite influence of THC on seizure activity could not be assessed. Evaluation of two patients proved impossible: in the case of the first because of marked progression of the principal disease (P.G.), in the second (M.Ö.) because of an extensive change in the antiepileptic medication. The effect of THC treatment on the epilepsies of L. S. and K. D. was most impressive. Suffering fundamental pyruvate dehydrogenase deficiency, L. S. demonstrated clinical nodding spasms and tonic seizures. EEG records revealed a left parietotemporal spike-wave and sharp-slow wave focus with generalisation. K. D. suffered from residual symptomatology following severe postnatal hypoxia. Seizures were to classify as versive ones accompanied by nystagmus. EEG records revealed a right temporal sharp-wave and sharp-slow wave focus. In the case of both patients, there was a temporary increase in apparent seizure severity. A.K. demonstrated focal initiated grand mal seizures, her EEG records revealed generalised and multifocal spike waves.

A number of animal experiments may explain the anticonvulsive effect of cannabinoids:

In rat hippocampal neurons, WIN 55.212-2 (WIN are non classical synthetic analogues of cannabinoids) inhibits N and P/Q-type calcium channels [18] regulated by G proteins. In cats L-type calcium channels of cerebral arterial muscle cells are inhibited by CB 1 – receptor [19]. Calcium channels play a role in the initiation and spread of epileptic activity. According to new findings L-type calcium channels play an important role in epileptogenesis [20]. In the case of generalised convulsive seizures non-T – type channels come into play [21]. On the other hand, T –type channels are not inhibited by low cannabinoid concentrations [22]. These channels play an active role in petit mal absences [21].

Inward leading sodium channels, which initiate depolarisation, are inhibited by THC in mouse neuroblastoma cells [23]. Increased sodium conductance is a contributing factor of epileptogenesis.

In addition, CB1 receptors mediate an increase of the outward current of potassium via A channels in hippocampal neuron cultures [24], thereby stabilising the membrane potential of excited cells.

Glutamate release in rat hippocampal cells is reduced [25] by CP54.939, CP55.940 and WIN55.212-2.

GABA, which produces an outward current of chloride that changes direction at –60 mV, thus preventing the depolarisation threshold from being reached [26], is an important transmitter effecting inhibition of epileptogenesis. In rat globus pallidus, GABA reabsorption is blocked by nabilon [27]. Globus pallidus neurons are able to generate epileptic activity [28].

Cannabinoids reduce the potassium efflux via M channels in rat hippocampal slices. This fact could play a role in convulsant action of cannabinoids.

There are no hints, that cannabinoids influence AMPA-receptors [30], which play an important role in epileptogenesis [20].

It has been demonstrated by electric shock model in mice, that the endogenous cannabinoid system inhibits the epileptic excitability of the CNS [31]. In this model NMDA-receptors are involved [21]. Anandamide reduces glutamate release in rat hippocampal cells. Arachidonic acid and its metabolites, the eicosanoids, though produced upon degradation of anandamide and 2-arachidonylglycerol [32], exert different action from that of cannabinoids (e.g. decreasing of potassium outward currents via A-channels) [29]. So the arachidonic acid must be rapidly removed to prevent convulsant effects (and it seems to be reincorporated into membrane phospholipids) [32].

The potential benefit of HU 211, a synthetic non psychactive, non CB1-receptor binding cannabinoid in catastrophic epilepsies and in status epilepticus should be examined. In brain injury a neurotoxicity reducing effect has been observed [33]. It should be considered, however, that apoptosis of cells belonging to an epileptic focus is advantageous for the surviving of the surrounding cells [34].

Further effects of THC which can exert influence on seizure activity must be taken into account:

the influences of THC on vigilance and sleep structure (reducing REM-sleep) [35], the endocrine system (increasing melatonin secretion [35], reducing production of gestagenic hormones [36]), and the immune system.

Under cannabinoid influence, the EEG revealed a "desynchronisation" [39]. On examining 8 healthy volunteers other researchers could not establish any change under CBD influence [38].

Conclusion

THC should be considered in the treatment of neurodegenerative disease or posthypoxic state or posttraumatic reaction not only in adults but also in children or adolescents. Hopefully, future studies of the anticonvulsive effects of cannabinoids will support the current assessment and lead to new antiepileptic drugs.

Acknowledgement

REFERENCES

1 Lorenz R. A casuistic rationale for the treatment of spastic and myocloni in a childhood neurodegenerative disease: neuronal ceroidlipofuscinosis of the type Jansky-Bielschowsky. Neuroendocrinology Letters 2002; 23:387–390.

2 Prod Info Marinol (R). 1999.

3 Abrahamov A, Abrahamov A, Mechoulam R. An efficient new cannabinoid antiemetic in pediatric oncology. L i f e s c i e n c e s 1995; 56: 2097–2102.

4 Fride E. The Endocannabinoid-CB Receptor System: Importance for development and in pediatric disease. Neuroendocrinology Letters 2004; 25. In press.

5 Dewey WL. Cannabinoid Pharmacology. Pharmacological reviews 1986; 38 (2):151–178.

6 O`Leary DS, Block RJ, Kreppel JA, Flaum M, Schultz SK, Andreasen NC, Ponto LB, Watkins GL, Hurtig RR, Hichwa RD. Effects of smoking marijuana on brain perfusion and cognition. Neuropsychopharmacology 2002; 26(6):802–816.

7 O`Leary DS. Personal communication (30.04.03).

8 Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, Hermann H, Tang J, Hofmann D, Ziegelgänsberger W, di Marzo V, Lutz B. The endogenous cannabinoid system controls extinction of aversive memories. Nature 2002; VOL?:530–534.

9 Kass L. Citated in: Frankfurter Allgemeine 20.12.03.

10 Grinspoon L. Marihuana reconsidered. Quick American Archives 1999; VOL?:112.

11 Pettit DA, Harrison MP, Olson JM, Spencer RF, Cabral GA. Immunohistochemical localization of the neural cannabinoid receptor in rat. Journal Neuroscience Research 1998; 51(3):391– 402.

12 Skosnik PD, Spatz – Glenn L, Park S. Cannabis use is associated with schizotypy and attentional disinhibition. Schizophr Res 2001; 48(1):83 – 92.

13 Kobal G, Klimek L, Wolfensberger M, Gudziol H, Temmel A, Owen CM, Seeber H, Pauli E, Hummel T. Multicenter investigation of 1036 subjects using a standardized method for the assessment of olfactory function combining tests of odor identification, odor discrimination, and olfactory thresholds. Eur Arch Otorhinolaryngol 2000; 257:205 – 211.

14 Russo E. Cannabis improves night vision: a pilot study of visual threshold and dark adaptometry in kif smokers in the Rif region of northern Morocco. Lecture at IACM-meeting in Cologne 2003–12–29.

15 Al Badri. Ms. ar. 3544, fol 7b. Paris.

16 Gordon E, Devinsky O. Alcohol and marijuana: effects on epilepsy and use by patients with epilepsy. Epilepsia 2001; 42: 1266–1272.

17 Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. J. Neurophysiol 1997; 78 (1):43–50.

18 Gebremedhin D, Lange AR, Campbell WB, Hillard CJ, Harder DR. Cannabinoid CB 1-receptor of cat cerebral arterial muscle functions to inhibit L-type Calcium channel current. Am J Physiol 276; H 2085 – H 2093.

19 Speckmann EJ. Personal communication (23.07.03).

20 Siemes H, Bourgeois BFD. Anfälle und Epilepsien bei Kindern und Jugendlichen. Thieme, 2001.

21 Mackie K, Devane W, Hille B. Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N 18 neuroblastoma cells. Mol. Pharmacol 1993; 44:498–503.

22 Turkanis S.A, Pastlow LM, Karler R. Delta-9-tetrahydrocannabinol depresses inward sodium current in mouse neuroblastoma cells. Neuropharmacology, 1991, 30:73–77.

23 Pertwee RG. Angriffspunkte und Wirkungsmechanismen. in: Grotenhermen F. Cannabis und Cannabinoide. Hans Huber 2001.

24 Shen M, Piser TM, Seybold VS, Thayer SA. Cannabinoid receptor agonists inhibit glutamatergic synaptic transmission in rat hippocampal cultures. Journal of Neuroscience 1996; 16 (14): 4322–4334.

25 Kandel ER, Schwartz JH, Jessell TM. Neurowissenschaften. Spektrum 1996.

26. Sieradzan KA, Fox SH, Hill M, Dick JPR, Crossmann AR, Brotchie JM. Cannabinoids reduce levodopa-induced dyskinesia in Parkinson`s disease: a pilot study. Neurology 2001; 57:2108–2111.

26 Sawamura A, Hashizume K, Tanaka T. Electrophysiological, behavioral and metabolical features of globus pallidus seizures induced by a microinjection of kainic acid in rats. Brain Res 2002; 935 (1–2):1–8.

27 Schweitzer P. Cannabinoids decrease the K + M-current in hippocampal CA1-neurons. Journal of Neuroscience 2000; 20:51–58.

29 Pertwee R. Personal communication (07.08.03).

30 Wallace M, Martin B, De Lorenzo R. Evidence for a physiological role of endocannabinoids in the modulation of seizure threshold and severity. Eur Journal of Pharmacology 2002; 452 (3): 295–301.

31 Mechoulam R, Fride E, di Marzo V. Endocannabinoids. Eur Journal of Pharmacology 1998; 359:1–18.

32 Shohami E, Gallily R, Mechoulam R, Bass R, Ben-Hur T. Cytokine production in the brain following closed head injury: dexanabinol (HU-211) is a novel TNF-alpha inhibitor and an effective neuroprotectant. J Neuroimmunol 1997; 72 (2):169–177.

33 Rothwell NJ, Strijbos PJLM. Cytokines in neurodegeneration and repair. Int. J. Devl. Neuroscience 1995; VOL?:179–185.

34 Grotenhermen F. Die Wirkungen von Cannabis und der Cannabinoide. In: Grotenhermen F. Cannabis und Cannabinoide. Huber 2001.

35 Murphy L. Hormonelles System und Reproduktio. In: Grotenhermen F. In: Cannabis und Cannabinoide. Huber 2001.

36 Zschocke S. Klinische Elektroenzephalographie. Springer; 2002.

37 Cunha JM, Carlini EA, Pereira AE, Ramos OL, Pimentel C, Gagliardi R, Sonvito WL, Lander N, Mechulam R. Chronic administration of cannabidiol to healthy volunteers and epileptic patients. Pharmacology 1980; 21:175–185.