MMP-033

New Jersey Department of Health
Medicinal Marijuana Program
PO 360
Trenton, NJ 08625-0360

MEDICINAL MARIJUANA PETITION
(N.J.A.C. 8:64-5.1 et seq.)

INSTRUCTIONS

This petition form is to be used only for requesting approval of an additional medical condition or treatment thereof as a "debilitating medical condition" pursuant to the New Jersey Compassionate Use Medical Marijuana Act, N.J.S.A. 24:6I-3. Only one condition or treatment may be identified per petition form. For additional conditions or treatments, a separate petition form must be submitted.

NOTE: This Petition form tracks the requirements of N.J.A.C. 8:64-5.3. Note that if a petition does not contain all information required by N.J.A.C. 8:64-5.3, the Department will deny the petition and return it to petitioner without further review. For that reason the Department strongly encourages use of the Petition form.

*This completed petition **must** be postmarked **August 1 through August 31, 2016** and sent by **certified mail** to:*

New Jersey Department of Health
Office of Commissioner - Medicinal Marijuana Program
Attention: Michele Stark
369 South Warren Street
Trenton, NJ 08608

Please complete each section of this petition. If there are any supportive documents attached to this petition, you should reference those documents in the text of the petition. If you need additional space for any item, please use a separate piece of paper, number the item accordingly, and attach it to the petition.

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1. **Petitioner Information**

Name: [redacted]
Street Add
City, State,
Telephone
Email Addr

2. **Identify the medical condition or treatment thereof proposed. Please be specific. Do not submit broad categories (such as "mental illness").**

Autism aka Autism Spectrum Disorder (ASD)

3. **Do you wish to address the Medical Marijuana Review Panel regarding your petition?**

☒ Yes, in Person
☐ Yes, by Telephone
☐ No

4. **Do you request that your personally identifiable information or health information remain confidential?**

☐ Yes
☒ No

If you answer "Yes" to Question 4, your name, address, phone number, and email, as well as any medical or health information specific to you, will be redacted from the petition before forwarding to the panel for review.

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OFFICE OF THE CHIEF OF STAFF

5. **Describe the extent to which the condition is generally accepted by the medical community and other experts as a valid, existing medical condition.**

Autism is classified by US and international health organizations as a valid existing medical condition. There are two established accepted systems in the medical community for identifying mental disorders: the ICD and the DSM. Both systems recognize autism as a diagnosed medical condition in their current published guides.
ICD-10 is the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD), a medical classification list by the World Health Organization (WHO). ICD-10 lists various diagnoses within the autism spectrum within the diagnostic code ICD-10-CM F84, Pervasive Developmental Disorders. Autism diagnoses listed in the ICD-10 include Childhood autism (F84.0), Atypical autism (F84.1), Rett Syndrome (F84.2), Asperger syndrome (F84.5), as well as other Pervasive Developmental Disorders related to an Autism Spectrum Disorder (ASD) diagnosis [1].
The Diagnostic and Statistical Manual (DSM) lists mental health disorders recognized by The American Psychiatric Association. The DSM has officially recognized autism as a disorder since 1980 [2]. The current version of the DSM (DSM-V) identifies Autism Spectrum Disorder (ASD) as diagnosis 299.00 and provides standardized criteria to help diagnose ASD. ASD is currently defined by the DSM-5 as a single disorder that includes a number of diagnoses that were originally considered separately (autistic disorder, Asperger's disorder, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified) [3].

6. **If one or more treatments of the condition, rather than the condition itself, are alleged to be the cause of the patient's suffering, describe the extent to which the treatments causing suffering are generally accepted by the medical community and other experts as valid treatments for the condition.**

Currently, there are only two drugs that are FDA-Approved for the treatment of autism: Risperidone (Risperdal®) and Aripiprazole (Abilify™). Both are antipsychotics that are used to treat behavioral symptoms of autism including aggression, self-injurious behavior and severe tantrums [4]. Additionally, as noted in literature from autism awareness organizations and major medical clinics, off-label medical treatments for autism symptoms include other prescription antipsychotics, antidepressants, stimulants, and mood stabilizers [5].

Unfortunately, all prescription medications carry the risk of side effects, and medications prescribed to treat the symptoms of autism can cause dangerous, debilitating, potentially permanent side effects, including heart conditions that can lead to sudden death [6], as described further in Question 7.

See attachment describing other medicines used to treat autism off label. [ATTACH1_Q6.docx]

7. **Describe the extent to which the condition itself and/or the treatments thereof cause severe suffering, such as severe and/or chronic pain, severe nausea and/or vomiting or otherwise severely impair the patient's ability to carry on activities of daily living.**

Both autism itself, as well as its treatments, can cause severe suffering that significantly impacts the patient's quality of life, and impairs the patient's ability to carry on activities of daily living.

Suffering related to the condition itself:

Autism has a number of challenging behaviors that go along with it, as highlighted in the webpage https://iancommunity.org/challenging-behaviors , for example aggression, self-injury, sleep issues, anxiety, focus issues, mood instability, and meltdowns. My child with autism has had many a meltdown, threatened us (mom, dad, sister) verbally, and assaulted us physically. Below I have gathered some testimonies from parents in NJ and other states in regards to autism and medical marijuana.

"As the mother of a teenager with autism and co-founder of MAMMA (Mothers Advocating Medical Marijuana for Autism), I have heard countless heartbreaking stories of suffering by children on the autism spectrum and their families. The first example is physical pain. When my own son was small, he would crawl across the floor while pushing his forehead into the carpet. He also spent hours leaning over furniture to put pressure on his stomach. I know now that these actions were due to brain and gut inflammation...in other words, pain. Children on the spectrum cannot necessarily tell us that they are in pain. I've heard many stories of children who had broken bones from self-injury (wrist, nose, feet) that went undiagnosed for an unknown amount of time. There are children who regularly put their heads through walls, windows, doors and even car windshields. Self-injurious behaviors can be the cause of pain, and/or the result of pain. Actual behaviors vary but include self- biting, hitting, punching, pinching, kicking, head banging, eye poking and skin peeling. Research suggests that these children may feel the self-inflicted pain more acutely than their typical peers. Cannabis is well known to alleviate pain. There are children in (medical marijuana) legal states where their qualifying condition is pain, and when they were treated with cannabis their self-injurious behaviors improved significantly.
Another area of hardship and suffering that cannabis is well known to alleviate is sleep issues. Sleep issues are a hallmark of autism. Many friends of mine have children who sleep less than a few hours a night. One mother who reached out to MAMMA told us her teenage daughter went 30 days without sleeping. What does that do to a person and what does that do to a family or caregivers? Sometimes I find it hard to separate the suffering of the parents from the children. It's a fact that sleep deprivation

has been used as a means of torture. Cannabis would be a welcome intervention to aid sleep deprivation for our kids, and therefore their families.
The last example of suffering that concerns the child with autism as well as the family is aggression. Over 50% of people with ASD will be aggressive in their lifetime. As the mother of a child with aggressive tendencies, it is frightening to be the recipient of aggression no matter how old your child is, because they don't hold back. Hitting, kicking, scratching, head butting, biting, throwing objects, squeezing around the neck– all of these can cause injury and trauma for everybody involved. I wonder about children who have experienced physical restraint by numerous adults when they became out-of-control aggressive. My son was taken down by blocking pads in 3rd grade when he was aggressive at school. Does he remember or have flashbacks? In despair after attacking his mother, a young Texas man retreated to his room, locked the door, and tried to kill himself by putting his head through windows and walls. In my opinion, and in the research literature, these children have suffered through enough trauma to qualify for a diagnosis of PTSD.
Cannabis has been shown to help pain, self-injury, sleep issues, aggression and PTSD. Children with autism are likely to suffer from one or more of these issues, and in my son's case, all of them. Thank you for listening to my story."
-submitted by [redacted]

"My son [redacted] became a pediatric medical marijuana patient in Colorado in 2013. He's severely affected by autism and he has bilateral retroversion causing chronic pain. Pre cannabis, [redacted] would self injure 100-250 times per day. He never slept & often eloped (wandered) at night. We spent about 2 hours per day restraining him. It was awful and we feared what would happen if we couldn't keep him safe. [redacted] first dose of cannabis was a piece of an edible put into a capsule. That night, [redacted] who was non-verbal at 14 years old, spoke spontaneously and appropriately. We were shocked. He went 13 days with no self injuring. [redacted] is doing so well. He isn't beating his head, he isn't hurting himself, and he went back to public school last year for the first time since 2010. I've done everything legally. I AM NOT A CRIMINAL. I love my son and I will always do what is best for him. Autism is a debilitating condition. Cannabis has given him the quality of life that we'd so desperately hoped for."
-submitted by [redacted]

"I am from NJ and would like MM for my son with PDD. He has not been able to tolerate meds and MM could help tremendously."
-submitted by [redacted]

"My 11 year old has ASD as well as anxiety and mood disorder and ADHD. This (medical marijuana) would be a miracle for him! He functions fairly well, but his anxiety gets the better of him and we refuse to give him any drugs, as the side effects are scarier than the behavior. To see ASD as a qualifying condition finally would be an answer to our prayers, and thousands of other parents too."
-submitted by [redacted]

"I'm in [redacted] County, NJ and cannot wait until marijuana is approved for Autism. I have 3 girls on the Spectrum - 14, 12 and 12 years of age. They are very different, but each would greatly benefit from treatment. My girls have issues with anxiety, mild depression, OCD, SIB, stims, the list goes on."
-submitted by [redacted]

"I have a 19 year old boy diagnosed with autism. He has a very weak immune system, and is partially verbal. He gets very agitated and frustrated when he cannot express himself or otherwise make himself understood. This causes the decibel on the anxiety issue to rise for everyone in our household. His sleep pattern is also erratic. I started giving him CBD oil a few months ago. Within days I could see the changes in him. He was not only sleeping through the night, but was also sleeping longer hours. I also noted that he was calmer and happier. Today I noted that his energy level was up as well, as he asked to go out to do laps around our property. This occurred after having been in the pool all morning. I can state for sure that CBD oil is contributing to the positive changes I'm seeing in my son."
-submitted by [redacted]

Suffering related to the treatment of autism:

As discussed above, the current medical treatment for autism includes 2 drugs that are FDA-approved for the treatment of autism, as well as numerous additional drugs that are FDA-approved for conditions or symptoms that are often associated with an autism diagnosis.
According to the FDA's Drug Safety and Medication Guides, which provide FDA-approved information required to be issued with certain prescription drugs, the potential serious side effects of the FDA-Approved autism drug Aripiprazole (Abilify™) include:

- Risk of suicidal thoughts and actions
- Neuroleptic malignant syndrome (NMS) – a rare and serious condition that can lead to death
- Tardive Dyskinesia – uncontrolled body movements
- Hyperglycemia (high blood sugar) and diabetes
- Orthostatic hypertension (decreased blood pressure)
- Low white blood cell count
- Seizures
- Temperature regulation issues
- Difficulty swallowing – risk of aspiration pneumonia

"Most common" side effects of Abilify™ include nausea, vomiting, constipation, headache, blurred vision, upper respiratory illness, dizziness, anxiety, insomnia, akathisia, fatigue, increased or decreased appetite, increased saliva or drooling, insomnia, stuffy nose, weight gain, and uncontrolled movement such as restlessness, tremor, muscle stiffness.
While not all patients will experience any or all of the side effects listed above, FDA-reported side effects this treatment can clearly cause severe suffering.
In order to summarize for the purpose of this petition, the following is an abbreviated list of side effects of additional drugs commonly prescribed for autistic behaviors that can cause suffering, according to the FDA [7]:

Suicidal thoughts or actions, changes in electrical activity of the heart (QT prolongation and Torsade de Pointes), Serotonin Syndrome, agitation, hallucinations, loss of consciousness, coma, coordination problems, muscle twitching (overactive reflexes), racing heartbeat, high blood pressure, low blood pressure, sweating or fever, nausea, vomiting, diarrhea, constipation, muscle rigidity, eye pain, changes in vision, abnormal bleeding, seizures or convulsions, manic episodes, severe trouble sleeping, reckless behavior, changes in appetite or weight, low sodium levels, headache, weakness, confusion, memory problems, panic attacks, increased aggression or violence, acting on dangerous impulses, hyperglycemia (high blood sugar), high fat levels in blood (cholesterol and triglycerides), Neuroleptic Malignant Syndrome, Tardive Dyskinesia, temperature regulation issues, dizziness, fainting, unusual changes in behavior or mood, difficulty swallowing, lack of energy, tremors, restlessness, headache, abdominal pain, pain in arms or legs, hypertension, hypotension, low white blood cell count, abnormal thyroid, sore throat, difficulty moving, drooling, anxiety, heart related problems including sudden death, stroke, and heart attack, new or worse behavior or thought patterns, new or worse bipolar illness, new or worse aggressive behavior or hostility, new psychotic symptoms such as hearing voices (children and teenagers only), circulation problems (peripheral vasculopathy, including Raynaud's phenomenon), slowing of growth (height and weight) in children, priapism, and abuse and dependency.

Attached is a more detailed overview of medication types and specific drugs commonly used to treat symptoms of autism, along with FDA-approved information on side effects. Further references are provided in attachment.

8. **Describe the availability of conventional medical therapies other than those that cause suffering to alleviate suffering caused by the condition and/or the treatment thereof.**

According to Mayo Clinic literature, "No medication can improve the core signs of ASD, but certain medications can help control symptoms."[8] It is fair to say that none of the conventional medical therapies for autism are able to alleviate suffering without the risk of additional suffering. Likewise, as far as treatments to alleviate the suffering caused by drugs intended to treat autism, it can be fairly concluded that pharmaceutical drugs used to treat treatment side-effects such as nausea and vomiting have their own risks and side effects as well. For example, Phenothyazines are antiemetic drugs commonly prescribed drug-induced nausea. However, common adverse effects of Phenothyazines include extrapyramidal reactions, tardive dyskinesia, orthostatic hypertension, and drug-induced Parkinson's syndrome [9].

Attached literature supports our assertion that while the suffering caused by autistic behaviors and symptoms may be improved in some patients through the use of conventional drug therapy, many patients continue to suffer from severe side effects or paradoxical reactions.

Attachment 2 [ATTACH2_Q8.docx] is a chart from the Autism Research Institute (ARI), an organization that has used surveys to collect more than 26,000 parent ratings for drugs used to treat autism since 1967 [10]. The data in the chart demonstrates that although some patients see improved behaviors through drug therapy, many patients experience a paradoxical effect, in which behaviors are actually worsened while taking a drug.

Attachment 3 [ATTACH3_Q8.pdf] is a 2008 study that reviews clinical trials of Risperidone published in the Journal of Neuropsychiatric Disease Treatment. While the study shows that some autistic patients showed "moderate" improvement, it also includes data on serious side effects including tachycardia, and discusses the "unwanted extrapyramidal effects" (UEE) that have been a major drawback in many accepted medical treatments for autism [11].

The purpose of this petition is to help lessen the number of drugs used in autism treatment and the corresponding drugs used to combat the side effects of the first line medications, by instead using cannabis as both a first-line treatment for autism in New Jersey as well as to address the suffering caused by side effects of any pharmaceutical drugs that are used by a patient with autism.

9. **Describe the extent to which evidence that is generally accepted among the medical community and other experts supports a finding that the use of marijuana alleviates suffering caused by the condition and/or the treatment thereof.** *[**Note:** You may attach articles published in peer-reviewed scientific journals reporting the results of research on the effects of marijuana on the medical condition or treatment of the condition and supporting why the medical condition should be added to the list of debilitating medical conditions.]*

   Please find attached articles published in peer-reviewed scientific journals:
   [SCIENCE1]: The Effect of Medicinal Cannabis on Pain and Quality of Life Outcomes in Chronic Pain: A Prospective Open-label Study.
   http://www.ncbi.nlm.nih.gov/pubmed/26889611
   [SCIENCE2]: Use of dronabinol (delta-9-THC) in autism: A prospective single-case-study with an early infantile autistic child
   http://www.cannabis-med.org/data/pdf/en_2010_04_1.pdf
   [SCIENCE3]: Consequences of cannabinoid and monoaminergic system disruption in a mouse model of autism spectrum disorders.
   http://www.ncbi.nlm.nih.gov/pubmed/21886592
   [SCIENCE4]: Autism-associated neuroligin-3 mutations commonly disrupt tonic endocannabinoid signaling.
   http://www.ncbi.nlm.nih.gov/pubmed/23583622
   [SCIENCE5]: Cannabinoid receptor type 2, but not type 1, is up-regulated in peripheral blood mononuclear cells of children affected by autistic disorders.
   http://www.ncbi.nlm.nih.gov/pubmed/23585028
   [SCIENCE6]: Evidence for a Common Endocannabinoid-Related Pathomechanism in Autism Spectrum Disorders
   http://www.ncbi.nlm.nih.gov/pubmed/23664608
   [SCIENCE7]: Variation in the human cannabinoid receptor CNR1 gene modulates gaze duration for happy faces
   http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3155489/
   [SCIENCE8]: Can autism be triggered by acetaminophen activation of the endocannabinoid system?
   http://www.ncbi.nlm.nih.gov/pubmed/20628445
   [SCIENCE9]: The in vitro GcMAF effects on endocannabinoid system transcriptionomics, receptor formation, and cell activity of autism-derived macrophages.
   http://www.ncbi.nlm.nih.gov/pubmed/24739187

10. **Attach letters of support from physicians or other licensed health care professionals knowledgeable about the condition. List below the number of letters attached and identify the authors.**

    Please find attached letters of support from physicians or other licensed health care professionals:
    Dr. Bogner [SUPPORT1A] [SUPPORT1B] and [SUPPORT1C]
    Victoria LaChapelle, LPN [SUPPORT2]
    Ken Wolski, RN [SUPPORT3]

I certify, under penalty of perjury, that I am 18 years of age or older; that the information provided in this petition is true and accurate to the best of my knowledge; and that the attached documents are authentic.

Signature of Petitioner	Date
[redacted]	8/30/16

Notes about the petition formatting

Please note that superscripts are represented in the petition by a number in square brackets, for example [1]. Please refer to the 'SUPERSCRIPTS.docx' attachment, which lists the references that are tied to the superscript number in brackets.

Additionally, attachments for question 6 and 8 are represented by [ATTACH...] in the petition, which points to the corresponding file, for example [ATTACH1_Q6.docx] and file 'ATTACH1_q6.docx' which is labeled ATTACH1.docx in the upper righthand corner of the printed document.

Also, studies have been represented in the petition as [SCIENCE...] and their corresponding file names are science...pdf , for example [SCIENCE3] refers to file 'SCIENCE3 CN-9-209.pdf' which is labeled SCIENCE3.PDF in the upper righthand corner of the printed document.

Finally, letters of support are represented in the petition as [SUPPORT...] and the corresponding files are 'SUPPORT...pdf', for example [SUPPORT2] refers to file 'SUPPORT2 Nurse Letter of Support – LaChapelle.docx' which is labeled as SUPPORT2.docx in the upper righthand corner of the printed document.

[1] World Health Organization, ICD-10 Version 2016. retrieved from: http://apps.who.int/classifications/icd10/browse/2016/en#/F84.0

[2] US Autism and Asperger Association, "What is Autism, Asperger Syndrome, and Pervasive Developmental Disorders," retrieved from http://www.usautism.org/definitions.htm

[3] American Psychiatric Association. Diagnostic and statistical manual of mental disorders. 5th ed. Arlington, VA: American Psychiatric Association; 2013. Retrieved from: http://www.cdc.gov/ncbddd/autism/hcp-dsm.html

[4] Food and Drug Administration. Consumer Updates: Beware of False or Misleading Claims for Treating Autism; November 2015. Retrieved from: http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm394757.htm

[5] Mayo Clinic Staff. Autism spectrum disorder: Treatments and drugs. 2014. Retrieved from: http://www.mayoclinic.org/diseases-conditions/autism-spectrum-disorder/basics/treatment/con-20021148

[6] FDA Medication Guide, 2016. Retrieved from: http://www.fda.gov/Drugs/DrugSafety/ucm085729.htm

[7] FDA Medication Guide, 2016. Retrieved from: http://www.fda.gov/Drugs/DrugSafety/ucm085729.htm

[8] http://www.mayoclinic.org/diseases-conditions/autism-spectrum-disorder/basics/treatment/con-20021148

[9] Michael A. Mancano, PharmD and Jason C. Gallagher, PharmD, BCPS. *Frequently Prescribed Medications: Drugs You Need to Know* (Burlington: Jones & Bartlett Learning, 2014), 203.

[10] Autism Research Institute Parent Ratings of Behavioral Effects of Biomedical Intervention. 2009. Retrieved from http://www.autism.com/pdf/providers/ParentRatings2009.pdf

[11] Canitano, R., Scandurra, V. Risperidone in the treatment of behavioral disorders associated with children and adolescents. Journal of Neuropsychiatric Disease Treatment. 2008 Aug; 4(4): 723–730. Retrieved from: http://www.ncbi.nlm.nih.gov/pubmed/19043516

ATTACHMENT FOR QUESTION 6:

The following is a list of medications commonly prescribed to treat symptoms of autism, classified by group.[1]

Antidepressant drugs and their side effects

Despite the lack of definitive results in clinical testing, a wide variety of antidepressant drugs are used to treat symptoms of autism, including aggression, self-injurious behavior, anxiety, agitation, overactivity, and some stereotypic behaviors.[2] Prescription antidepressants used for autism include:

- SSRIs used in autism: fluvoxamine (Luvox™, Faverin™), fluoxetine (Prozac™, Fontex™, Seromex™, Seronil™, Sarafem™), Sertraline (Zoloft™, Lustral™, Serlain™), Paroxetine (Paxil™, Seroxat™, Aropax™, Deroxat™, Paroxat™), Citalopram (Celexa™, Cipramil™, Emocal™, Sepram™, Seropram™), Escitalopram (Lexapro™, Cipralex™, Esertia™)
- TCAs used in autism: clomipramine (Anafranil™), desipramine (Norpramine™), amitriptyline (Elavil™, Endep™),imipramine (Tofranil™)
- Other antidepressants used in autism include: venlafaxine (Effexor™)

The following is a partial list of FDA reported side effects for the above medications[3]:

- Suicidal thoughts or actions
- Changes in electrical activity of the heart (QT prolongation and Torsade de Pointes)
- Serotonin Syndrome
- Agitation
- Hallucinations
- Loss of consciousness
- Coma
- Coordination problems
- Muscle twitching (overactive reflexes)
- Racing heartbeat
- High blood pressure
- Low blood pressure
- Sweating or fever
- Nausea, vomiting, or diarrhea
- Muscle rigidity
- Eye pain
- Changes in vision
- Abnormal bleeding

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[1] Interactive Autism Network, *Linking the Autism Community and Researchers*, website: "Medications"; https://iancommunity.org/cs/what_do_we_know/medication

[2] Handen, B.L., & Lubetsky, M. (2005). Pharmacotherapy in autism and related disorders. School Psychology Quarterly, 20(2), 155-171.

[3] FDA Medication Guide, Drug Safety, Reference ID: 3596033, 3595809, 3490671, 3642387, 3233597, 3651522, 3209061, 3536021.

- Seizures or convulsions
- Manic episodes
- Severe trouble sleeping
- Reckless behavior
- Changes in appetite or weight
- Low sodium levels
- Headache
- Weakness
- Confusion
- Memory problems
- Panic attacks
- Acting aggressive, being angry, or violent
- Acting on dangerous impulses

Antipsychotic drugs and their side effects

The US Food and Drug Administration (FDA) first approved the antipsychotic medication Risperidone in October 2006 for treatment of autistic behaviors related to irritability, including aggression, self-injury, temper tantrums, and rapid mood changes.[4] Additional antipsychotics currently used to treat autistic behaviors include:

- Additional atypical antipsychotics used in autism: clozapine (Clozaril™), olanzapine (Zyprexa™), quetiapine (Seroquel™), ziprasidone (Geodon™)
- Other antipsychotics commonly used in autism include: haloperidol (Haldol™), chlorpromazine (Thorazine™), apriprazole (Abilify™)

The following is a partial list of FDA reported side effects for the above medications[5]:

- Suicidal thoughts or actions
- Hyperglycemia (high blood sugar)
- High fat levels in blood (cholesterol and triglycerides)
- Weight gain
- Neuroleptic Malignant Syndrome
- Tardive Dyskinesia
- Temperature control issues
- Dizziness
- Fainting
- Difficulty swallowing
- Seizures
- Lack of energy
- Tremors
- Restlessness

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[4] Nagaraj, R., Singhi, P, & Mahli, P. (2006). Risperidone in children with autism: Randomized, placebo-controlled, double-blind study. Journal of Child Neurology, 21(6), 450-5.

[5] FDA Medication Guide, Drug Safety, Reference ID: 3347923, 3397413, 3874224

- Insomnia
- Sleepiness
- Headache
- Abdominal pain
- Pain in arms or legs
- Hypertension
- Hypotension
- Low white blood cell count
- Abnormal thyroid tests
- Nausea
- Vomiting
- Constipation
- Sore throat
- Difficulty moving
- Rapid heartbeat
- Drooling
- Blurred vision
- Anxiety

Stimulants and their side effects

Stimulants commonly prescribed to treat Attention Deficit Hyperactivity Disorder (ADHD) are also used to treat similar symptoms of inattention, overactivity, and impulsivity in patients with autism.[6] The following drugs are commonly prescribed:

- Methylphenidate (Ritalin™) is the most studied and most commonly used psychostimulant.
- Other psychostimulants used in children with autism: amphetamine mixed salts (Adderall™, Adderall XR™), methylphenidate XR (Concerta™, Metadate CD™), dextroamphetamine (Dexedrine™)

The following is a partial list of FDA reported side effects for the above medications[7]:

- Heart-related problems: sudden death in patients who have heart problems or heart defects; stroke and heart attack in adults; increased blood pressure and heart rate
- New or worse behavior and thought problems
- New or worse bipolar illness
- New or worse aggressive behavior or hostility
- New psychotic symptoms such as hearing voices (in children and teenagers)
- Circulation problems (peripheral vasculopathy, including Raynaud's phenomenon)
- Slowing of growth (height and weight) in children
- Seizures
- Eyesight changes

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[6] Interactive Autism Network, Linking the Autism Community and Researchers, website: "Medications"; https://iancommunity.org/cs/what_do_we_know/medication

[7] FDA Medication Guide, Drug Safety, Reference ID: 3734564, 3827706, 3421020, 3734614, 3418238

- Priapism (painful and prolonged erections)
- Headache
- Stomach ache
- Trouble sleeping
- Nausea
- Decreased appetite
- Nervousness
- Abuse and dependency

ARI Publ. 34/March 2009

PARENT RATINGS OF BEHAVIORAL EFFECTS OF BIOMEDICAL INTERVENTIONS

Autism Research Institute • 4182 Adams Avenue • San Diego, CA 92116

The parents of autistic children represent a vast and important reservoir of information on the benefits—and adverse effects—of the large variety of drugs and other interventions that have been tried with their children. Since 1967 the Autism Research Institute has been collecting parent ratings of the usefulness of the many interventions tried on their autistic children.

The following data have been collected from the more than 27,000 parents who have completed our questionnaires designed to collect such information. For the purposes of the present table, the parents responses on a six-point scale have been combined into three categories: "made worse" (ratings 1 and 2), "no effect" (ratings 3 and 4), and "made better" (ratings 5 and 6). The "Better:Worse" column gives the number of children who "Got Better" for each one who "Got Worse."

DRUGS	Parent Ratings: Got Worse[A]	No Effect	Got Better	Better: Worse	No. of Cases[B]
Actos	19%	60%	21%	1.1:1	140
Aderall	43%	26%	31%	0.7:1	894
Amphetamine	47%	28%	25%	0.5:1	1355
Anafranil	32%	39%	29%	1.1:1	440
Antibiotics	33%	50%	18%	0.5:1	2507
Antifungals[C]					
Diflucan	5%	34%	62%	13:1	1214
Nystatin	5%	43%	52%	11:1	1969
Atarax	26%	53%	21%	0.8:1	543
Benadryl	24%	50%	26%	1.1:1	3230
Beta Blocker	18%	51%	31%	1.7:1	306
Buspar	29%	42%	28%	1.0:1	431
Chloral Hydrate	42%	39%	19%	0.5:1	498
Clonidine	22%	32%	46%	2.1:1	1658
Clozapine	38%	43%	19%	0.5:1	170
Cogentin	20%	53%	27%	1.4:1	198
Cylert	45%	35%	19%	0.4:1	634
Depakene[D]					
Behavior	25%	44%	31%	1.2:1	1146
Seizures	12%	33%	55%	4.6:1	761
Desipramine	34%	35%	32%	0.9:1	95
Dilantin[D]					
Behavior	28%	49%	23%	0.8:1	1127
Seizures	16%	37%	47%	3.0:1	454
Fenfluramine	21%	52%	27%	1.3:1	483
Haldol	38%	28%	34%	0.9:1	1222
IVIG	7%	39%	54%	7.6:1	142
Klonapin[D]					
Behavior	31%	40%	29%	0.9:1	270
Seizures	29%	55%	16%	0.6:1	86
Lithium	22%	48%	31%	1.4:1	515
Luvox	31%	37%	32%	1.0:1	251
Mellaril	29%	38%	33%	1.2:1	2108
Mysoline[D]					
Behavior	41%	46%	13%	0.3:1	156
Seizures	21%	55%	24%	1.1:1	85
Naltrexone	18%	49%	33%	1.8:1	350
Low Dose Naltrexone	11%	52%	38%	4.0:1	190
Paxil	34%	32%	35%	1.0:1	471
Phenobarb.[D]					
Behavior	48%	37%	16%	0.3:1	1125
Seizures	18%	44%	38%	2.2:1	543
Prolixin	30%	41%	28%	0.9:1	109
Prozac	33%	32%	35%	1.1:1	1391
Risperidal	21%	26%	54%	2.6:1	1216
Ritalin	45%	26%	29%	0.6:1	4256
Secretin					
Intravenous	7%	50%	43%	6.4:1	597
Transderm.	9%	56%	35%	3.9:1	257
Stelazine	29%	45%	26%	0.9:1	437
Steroids	34%	30%	36%	1.1:1	204
Tegretol[D]					
Behavior	25%	45%	30%	1.2:1	1556
Seizures	14%	33%	53%	3.8:1	872
Thorazine	36%	40%	24%	0.7:1	945
Tofranil	30%	38%	32%	1.1:1	785
Vallum	35%	42%	24%	0.7:1	895
Valtrex	8%	42%	50%	6.7:1	238
Zarontin[D]					
Behavior	34%	48%	18%	0.5:1	164
Seizures	20%	55%	25%	1.2:1	125
Zoloft	35%	33%	31%	0.9:1	579

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Risperidone in the treatment of behavioral disorders associated with autism in children and adolescents

Roberto Canitano
Valeria Scandurra

Division of Child Neuropsychiatry, University Hospital of Siena, Siena, Italy

Abstract: This is a review of the clinical trials investigating the efficacy and safety of risperidone in the treatment of children with autistic spectrum disorders (ASD). The main clinical characteristics are impairment in social skills, communication difficulties, repetitive movements and behaviors, including stereotypies. Pharmacotherapy is mainly directed at the so-called target symptoms, ie, behavioral disorders and the various kinds of repetitions associated with ASD. According to the available data, risperidone seems to be moderately efficacious and safe for treating behavioral disorders. 4 double blind controlled trial. 3 reanalysis studies, and 12 open studies have documented the role of risperidone in children with ASD. Controlled studies have been thoroughly considered in this review.

Keywords: autism, pervasive developmental disorders, risperidone

Introduction

Autism spectrum disorders (ASD) are characterized by impaired development of social interaction and reciprocity, communication difficulties affecting language and nonverbal skills, and repetitive patterns of movements and behaviors which include restricted interests and activities. Although they are not included in the diagnostic criteria, learning disabilities affect as much as two thirds of individuals with ASD (APA 1994).

They are defined as pervasive developmental disorders (PDDs) in DSM IV classification and include the following diagnostic categories: autistic disorder, Asperger disorder, Rett disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified (PDD-NOS) including the different forms of atypical autism not matching the diagnostic criteria for the other PDDs.

Autistic disorder (AD), or autism, is the most common PDD/ASD with a recently estimated prevalence of 13 per 10,000. The etiology of AD is still unknown, although genetic factors are probably involved, and in 5% to 10% of cases there is an identifiable associated known medical condition. The onset of autistic disorder has been set at before the age of 3 years, and other ASDs may have a later onset (APA 1994). The prevalence of Asperger's disorder is approximately 3 per 10,000 and childhood disintegrative disorder is very rare, estimated at 0.2 per 10,000. Rett disorder prevalence is 1 per 15000. The prevalence of all PDDs in recent surveys is about 60 per 10,000 (Fombonne 2005).

Behavioral disturbances are fairly common in these disorders and are very often challenging to treat. Tantrums, aggressive behavior, and overactivity/hyperactivity are frequent from the early phases and may last throughout adulthood, causing serious problems in adaptation. The severity and the development of the various symptoms

Correspondence: Roberto Canitano
Division of Child Neuropsychiatry, University Hospital of Siena, Viale Bracci 14 53100 Siena, Italy
Email R.Canitano@ao-siena.toscana.it

and their clinical features vary on an individual basis. When behavioral problems interfere with general well-being, daily activities, and with the therapeutic program, pharmacological intervention should be considered (Aman 2004). The so-called target symptoms are therefore tackled by pharmacotherapy with a symptom-based approach. Antipsychotics have been used to control aggression, self-injurious behavior and overactivity/hyperactivity in ASD. Typical antipsychotics have been found to be useful in limiting temper tantrums, hyperactivity, and repetitive movements, and they have also been reported to be effective in improving social interaction. Typical antipsychotics have been extensively used clinically in ASD, but they have not been extensively evaluated, with the exception of Campbell's studies with haloperidol (Campbell et al 1997). Unwanted extrapyramidal effects (UEE) have been a major drawback in the use of these agents and the advent of atypical antipsychotics has provided a safer option in the therapeutic approach to ASD. Furthermore, movement disorders are often difficult to evaluate in this patient population since unusual stereotipies and repetitive movements are part of the core disorder, thus it is often difficult to distinguish between movement disorders that are intrinsic to ASD and those that are possibly related to treatment (Campbell et al 1999).

Atypical antipsychotics, such as risperidone, are being studied for the treatment of behavioral symptoms in ASD due to their increased safety and efficacy compared to conventional neuroleptics (Barnard et al 2002). Most data have been collected on the use of risperidone in children aged 5 and older, but there is still a lack of research in preschool children (Chavez et al 2006; West and Waldrop 2006; Jesner et al 2007). This paper reviews the use of risperidone in children and adolescents with ASD, particularly regarding the treatment of associated behavioral disorders.

Method

A MEDLINE/Pubmed search was performed, ending in February 2007, on the use of risperidone in children with ASD. The key words used were: autism, pervasive developmental disorders and risperidone. A total of 4 double-blind, placebo controlled trials were found and 2 additional studies were also found which were based on the reanalysis of the data. The results of these studies have been reviewed and summarized below (Table 1). Furthermore, 12 open label, several observational or retrospective trials and case reports have also been published. They are synthetically presented in Table 2.

Risperidone efficacy and safety in children and adolescents with ASD

- The RUPPAN study (McCracken et al 2002) [ages: 5–17 yrs; risperidone dose: 1.8 mg/day; outcome measurement: ABC-I (and CGI-I); percentage of improvement 69%].

The first RUPPAN (Research Units on Pediatric Psychopharmacology Autism Network) trial was conducted in a group of children with a diagnosis of autistic disorder as defined in the DSM-IV. Additional inclusion criteria were behavioral disturbances: aggression, hyperactivity, self-injury and tantrums in varying combinations. The irritability subscale of the aberrant behavior checklist (ABC) was used to rate these symptoms and a score of at least 18 was required for inclusion in the study (Aman et al 1985). The assessment was carried out twice to confirm that the entry criteria had been met. The two groups were made up of 49 and 52 children, with a total of 82 boys and 19 girls.

The Clinical Global Impression – Severity (CGI-S) scale was used to evaluate the children's symptoms at baseline, and the Clinical Global Impression – Improvement scale (CGI-I) was used to evaluate changes during the trial. Eighteen children were found to have moderate severity on the CGI-S, 55 were rated as having marked severity, 24 had severe impairment, and 1 child had extreme impairment. No statistically significant differences were found in any of the baseline scores between the two treatment groups.

The dosage of risperidone was established on the basis of the weight of patients. The starting dose was 0.25 mg/day if the child's weight was less than 20 kg, and 0.5 mg if the child's weight was between 20 and 45 kg, and in this case the dose was increased to 0.5 mg twice daily on day 4. The titration scheme was the following: for children weighing less than 45 kg, the dose was increased by 0.5 mg according to the clinical response. For children weighing 45 kg or more, dose increase was accelerated without specifying the actual doses. Maximum dosage was 2.5 mg for children weighing less than 45 kg, and 3.5 mg for children weighing 45 kg or more.

Primary outcome measures were the Irritability subscale of the ABC, considering a score of 18 as the population average, and the rating on the CGI-I. A decrease in the irritability score was detected in the risperidone group ($p < 0.001$). Seventy-five percent of children on risperidone were rated as much improved or very much improved on the CGI-I versus 11.5% of children in the placebo group ($p < 0.001$). Participants had to have at least a 25% reduction in the irritability subscale and a rating of much improved or very much

Table 1 Clinical trials of risperidone for children with ASD/PDD

	Design	Pts.(N)	Age (y)	Mean dose (mg/day)	Results
RUPPAN (2002)	8 wk, DB, PC	101	5–17	1.8	Improvement on ABC in irritability, stereotypy, hyperactivity; improved CGI-I
Shea (2004)	Reanalysis 8 wk, DB, PC	79	5–12	1.48	Improvement in irritability, stereotypy hyperactivity, insecurity/ anxiety; oversensitivity in ABC and N-CBRF; also in language and social withdrawal
RUPPAN (2005)	Reanalysis 4 mo, OL, followed for 8 wk, DB, PC discontinuation phase	63	5–17	2.08	Improvement in ABC in irritability; continued improvement on CGI-I; during discontinuation phase, placebo-treated pts. relapsed more often and sooner
McDougle (2005)	Reanalysis of Database from RUPPAN (8 wk, DB, PC)	101	5–17	1.8	No improvement in social and communication skills; improvement in sensory motor behaviors, affective reactions; decreased stereotypy on CY-BOCS
Luby (2006)	6 mo, DB, PC	24	2.5–6	0.5–1.5	Improvement in core symptoms (CARS)
Pandina (2006)	Reanalysis of Database from RUPPAN (8 wk, DB, PC)	55	5–12	1.48	Hyperactivity, aggression and irritability
Nagaray (2006)	6 mo, DB, PC	40	2–9	1*	CARS, C-GAS

Abbreviations: ABC, Aberrant Behavior Checklist; ASD/PDD, autism spectrum disorder/pervasive developmental disorders; CGI-I, Clinical Global Impression-Improvement; CY-BOCS, Children's Yale-Brown Obsessive Compulsive Scale; DB, Double-blind; N-CBRF, Nisonger-Child Behavior Rating Form; OL, open-label; PC, placebo-controlled; R-FRLRS, Ritvo-Freeman Real Life Rating Scale; RUPPAN, Research Units on Pediatrics Psychopharmacology Autism Network; 1 mg, *Fixed Dose.

Table 2 Open-label studies of risperidone in children with autism

	Pts. (n)	Age (ys)	Mean dose (mg/day)	Results
Fisman and Steele (1996)	14	9–17	1.1	13 pts. Improved on CGAS, agitation, anxiety, disruptive behaviors, social awareness
McDougle (1997)	18	5–18	1.8	12 pts. improved on CGI-I, repetitive behavior, aggression, impulsivity
Findling (1997)	6	5–9	1.1	Effective in all 6 pts. in problematic behaviors; improvement on CPRS and CGI-I
Nicolson (1998)	10	4–10	1.3	8 of 10 showed improvement on CPRS and CGI-I
Zuddas (2000)	11	7–17	2.7	Improvement in behavioral symptoms in 10 pts.; results maintained for 12 months
Masi (2001)	24	3.6–6.6	0.5	8 pts. showed improvement on CPRS and CGI-I
Diler (2002)	20	3–7	1.5	13 pts. had a positive response on CGI-I
Malone (2002)	22	3–16	1.8	Significant improvements in CPRS and CGI-I for 10 pts.
Gagliano (2004)	20	3–10	1.3	8 showed improvement on CPRS and CGI-I
Troost (2005)	36	5–17	1.8	24 pts. where considered responders. In the double-blind discontinuation phase, 8 out of 12 on placebo relapsed vs 3 out of 12 on risperidone.
Raush (2005)	13	6–12	1.0	9 pts. had reduction in SAN
Canitano (2006)	11	6–10	0.6	Reduction in frequency of self-injurious behaviors in 9 pts. YAPA-SIB

Abbreviations: C-GAS, Children's Global Assessment Scale; CGI-I, Clinical Global Impression-Improvement; CPRS, Children's Rating Scale; PDD, pervasive developmental disorders; SANS, Scale for Assessment of Negative Symptoms; YAPA-SIB, Yale-Paris Self-Injurious Behavior Scale.

improved in the trial in order to be considered as responders. Accordingly, 34 (69%) of the 49 children were responders in the risperidone group and 6 (12%) of the 52 in placebo group. Hyperactivity and stereotypy subscales of ABC as secondary measures also showed an improvement in the risperidone group.

The most frequently observed side effects were weight gain, increased appetite, fatigue, drowsiness, drooling, dizziness, constipation, tremor, and tachycardia. The average weight gain was 2.7 ± 2.9 kg. Improvement was observed in the majority of children by week 4. Three children in the risperidone group withdrew due to a lack of effectiveness compared to 18 withdrawals in the placebo group.

A 4-month open-label extension of the trial was accepted by 23 (68%) of the children labeled as positive responders and all of them maintained the positive trend during that period.

- The McDougle study (McDougle et al 2005) [reanalysis to assess stereotypy/repetitive behaviors, outcome measurement: CY-BOCS; effect size d = 0.55; 69% percent improvement. Responders were subsequently enrolled in the open label and discontinuation phase].

The RUPPAN database was then used by McDougle and colleagues (2005) to further develop the analysis of risperidone efficacy. The aim of this study was to verify risperidone efficacy on the core symptoms of autism (social impairment, communication deficits, stereotypies). The variation of repetitive movements and behaviors, including stereotypies, is particularly important for the purpose of this review and is presented in detail. A modified version of the Children Yale-Brown obsessive compulsive scales (CY-BOCS) was used to assess these aspects because of the difficulties in language and expression of children with autism. This is a 10 item clinician rated scale normally used for obsessive-compulsive disorder, and the CY-BOCS score in this study was 20 points. At week 8, a significant reduction of repetitive behaviors was observed in the risperidone group (score decrease from 15.51 ± 2.73 to 11.65 ± 4.2, and in the placebo group from 15.18 ± 3.88 to 14.21 ± 4.821, $p = 0.005$).

Patients who had a positive response to the 8 week trial were given the option of entering the 4-month open-label phase of the study. Furthermore, nonresponders to placebo were given the option of participating in an 8-week open-label treatment with risperidone. If they had a positive response, they were given a further option of entering the 4-month open-label phase.

At the end of the 4 months, responders entered the discontinuation phase. They were randomly assigned to continue risperidone treatment or to be tapered off to placebo. A total of 63 patients entered the 4-month open-label phase. The two primary outcome measurements were the irritability subscale on ABC and CGI-I. A total of 51 (81%) subjects completed the open-label phase and all the children continued to show a positive response to the treatment. Twelve children withdrew from this phase, 5 because of loss of efficacy. The most common adverse events were increased appetite and sedation. Of the 51 patients who completed the open-label phase, 38 entered the discontinuation phase. Ten out of 16 patients in the placebo group and 2 out of 16 in the risperidone group had a relapse. The median time periods to relapse were 34 and 57 days in the placebo and risperidone groups, respectively.

- The Shea study (Shea et al 2004) [ages: 5–12 yrs, risperidone dose; 0.06 mg; outcome measurement: ABC-I score; percentage of improvement: 64% of children].

Shea and colleagues (2004) conducted an 8-week double blind, placebo-controlled study of risperidone for the treatment of disruptive behavioral symptoms in children and adolescents with ASD (Schopler et al 1980). The participants had to meet DSM-IV criteria for autism or other PDD and had to have a score of at least 30 on the Childhood Autism Rating Scale (CARS) with or without mental retardation (RUPPAN 2005) The final mean dose was 0.05 mg/kg/day with a maximum mean dose of 0.06 mg/kg/day. Forty-one of the 79 children were randomized to receive risperidone and 39 received placebo, and at the end of the trial, the participants showed 64% and 31% improvement in the ABC irritability subscale, respectively. Notably, a significant decrease in the ABC subscales exploring the core symptoms of ASD, such as inappropriate speech and lethargy/social withdrawal, was also observed. Furthermore, a significant decrease was observed in hyperacitvity/compliance ABC subscales and in the Nisonger Behavior Rating Form (NCBRF) subscales of conduct, insecure/anxious, hyperactive and oversensitivity (Aman et al 1996). With the use of CGI-I, 21 (51%) patients on risperidone were rated as much improved or very much improved compared to only 7 (18%) patients in the placebo group ($p < 0.005$).

Somnolence was the most common side effect in children on risperidone. The mean weight gain in the risperidone group was 2.7 kg, while in the placebo group it was 1.0 kg. Tachycardia, upper respiratory infections, and rhinitis occurred more frequently in the risperidone group. Unwanted

extrapyramidal side effects were reported in 11 patients on risperidone and in 5 on placebo, tremor, and hypokinesia were the most common.

- The Pandina reanalysis study (Pandina et al 2007) [outcome measurement: ABC-I, ABC-I plus CGI-I; effect size: 0.7; composite score responders: 58.3%].

A secondary analysis of the data of Shea's study was conducted by Pandina and colleagues (2007) to further evaluate the efficacy and safety of risperidone treatment (Nicolson et al 1998). The same behavior and clinical assessment measurements used in the RUPP study were carried out and evaluation of the current most disturbing symptoms was also included. Primary efficacy measurement was the irritability subscale of the ABC, and secondary efficacy measurements were the same as those used in Shea's study (ABC subscales scores and N-CBRF subscale scores, The visual analog scale for the most troublesome symptom (VAS-MS) and CGI-I). As a post-hoc analysis, treatment response was determined by identifying composite responders with an improvement of >25% in the ABC-irritability subscale plus a CGI-I score of <2 (eg, much or very much improved) for a composite score. A total of 80 ASD patients were enrolled in Shea's original study, and of these 55 were children with a diagnosis of autistic disorder according to the DSM-IV. A CARS score of >30 was also required for inclusion in the study.

Twenty-five (92.6%) of subjects receiving risperidone and 24 (85.7%) of the placebo group completed 8 weeks of treatment. Subjects dropped out of the risperidone group due to insufficient response (1) and extrapyramidal disorders (1). The changes in the VAS for the most troublesome symptom data were also favorable in the risperidone group. CGI-I and composite response (>25% ABC irritability subscale and CGI-I much or very much improved) endpoint changes further confirmed that children in the risperidone group had undergone a significant improvement. There was also a consistent reduction in hyperactivity and aggression, the most troublesome symptoms mentioned by parents, in the risperidone group.

This secondary analysis showed that risperidone was superior to the placebo in controlling behavioral disturbances in more than half of the participants. Among the adverse events reported, weight gain was mild, perhaps due to the low doses of risperidone used, at a mean dose of 1.37 mg/day vs 1.8 mg/day in the RUPP trial. The small sample size and the brief duration of the study were the major limitations.

Risperidone safety and efficacy in preschool children with ASD: age 2.5–6 years

Studies of the efficacy of risperidone in the treatment of ASD among preschool children have been extremely limited and are mostly case reports or open-label trials. The largest available open-label study was conducted on preschoolers with ASD, aged 3.9–6.6 yrs, by Masi and colleagues (2003). The significant finding was that core social impairment and verbal and nonverbal communication skills improved, which most previous studies had failed to demonstrate. To date, there have been two controlled studies of risperidone in children with ASD.

- Luby's study (Luby et al 2006) [ages 2.5–6 yrs, risperidone dose 0.5–1.5 mg; CARS score; effect size 0.95].

This was the first double blind controlled study on risperidone in the treatment of preschool children with ASD (Jesner et al 2007). The aim of this research was to test the safety and effectiveness of this medication and to discover whether it could improve the core deficits of ASD as well as interfere with behavioral symptoms. Children with PDD-NOS not otherwise specified and those undergoing behavioral treatment were included in the study in order to have a wider population of patients with ASD. Twenty four preschool children aged 2.5–6 yrs who met DSM-IV diagnostic criteria for autism or PDD were recruited for participation. The principal outcome measurements were CARS and the Gillian autism rating scale that were administered at baseline, 2, 4, and 6 months. Socialization and adaptive behavior were assessed at baseline and at the 6-month endpoint with the Vineland adaptive behavior scales. Risperidone was administered in low doses and the final mean daily dose was 0.05 mg/kg. The duration of treatment was 6 months. Safety and efficacy of treatment over the 6-month period was satisfactory. Side effects were relatively mild, as were weight gain, hypersalivation, and higher prolactin levels.

An overall amelioration, measured by means of the difference between CARS scores at baseline and at the end of treatment, was observed in this study in children receiving risperidone compared to those receiving placebo. However, it should be noted that many of the subjects of both groups were undergoing intensive behavioral treatment and different interpretations could be offered regarding the effect of this concomitant treatment. No specific improvements in autism domains were observed as a function of the treatment group, and this is in line with the findings of the other double-blind

trials with risperidone in older children with ASD. This means that the core social and communication impairment of ASD were only mildly, if at all, affected by the treatment. The decrease in stereotypies, repetitive behaviors and hyperactivity obtained with risperidone therapy had a positive effect on the global functioning of children. A lack of objective measures other than symptoms expression and the lack of a more detailed assessment and structured clinical interview for ASD were the major limitations. Furthermore, the small number of subjects did not allow firm conclusions.

- Nagaraj's study (Nagaraj et al 2006) [ages <6 yrs, risperidone dosage 1 mg/day as a fixed dose, 63% improvement on CARS score].

A double-blind placebo controlled study in preschool children with ASD, aged <6 yrs, was conducted by Nagaraj and colleagues (2006) to test the efficacy of risperidone in limiting irritability, aggressive behavior and hyperactivity and in improving the core symptoms of social and communication impairment (McDougle et al 2005). Forty children with a diagnosis of autism according to the DSM IV were enrolled for a 6 month trial with risperidone. The CARS, CGAS, and Global Impression of Parents were used and the mean changes from baseline to the end of 6 months period were the primary outcome measurements. A fixed dose of 1 mg/day was used to obtain more homogenous group data. Twelve of the 19 (63%) children in the risperidone group demonstrated an improvement of at least 20% in CARS however none of the children on placebo showed any favorable variations. On the CGAS, children in the risperidone group showed a score that was increased by at least 20%, and 94% were considered as improved to some extent or considerably improved according to the Global Impression of Parents rating. This study reported a positive outcome regarding the so-called target symptoms and an improvement in social and global functioning. It should be noted that only social responsiveness and nonverbal communication were considered to be improved, but no significant variations were noted in language, speech or in stereotypies. Therefore core social and communication domains were still prominent affected areas of functioning. With regard to the unwanted effects, in this study mild and transient dyskinesia was seen in three children.

Discussion

The pharmacological approach in the use of risperidone for treating behavioral disturbances in children with ASD is on the increase, but the existing data are still limited and much caution is recommended when prescribing it in such a vulnerable population as that of children with ASD.

The four randomized control trials of risperidone have demonstrated the moderate and clinically significant benefits in behavioral disturbances control, including disruptive behaviors, hyperactivity, stereotypies, and self-injury. The decreases in repetitive behaviors and self-injurious behavior (SIB) were particularly noteworthy since they are core symptoms of ASD. On the contrary, social and language impairments were only slightly modified by treatment, although results differed between studies. It is important to mention that patients selected for more severe behaviors at baseline, such as those that are probably enrolled in these studies, may show a greater treatment benefit in target symptoms. Consequently, generalization of these findings is not recommended, and further investigations on broader clinical ASD populations are needed.

The multisite trial by the RUPPAN (2005) showed that repetitive movements and behaviors decreased with treatment, whereas social and language skills remained unchanged (McCracken et al 2002). Parents' reports were in agreement with these findings and supported the empirical data (Arnold et al 2003). However, the studies by Shea and colleagues (2004) and Nagaraj and colleagues (2006) also found the amelioration of some core aspects of communication and social withdrawal, but in the latter the outcome measurements and low numbers were insufficient for drawing strong conclusions. Further research is needed to test this hypothesis and to replicate the above findings in larger populations.

Risperidone doses varied from 0.01 mg/day to 0.05 mg/day, and all the studies started with low doses and then increased them slowly. The initial dose was 0.25 to 0.5 mg/day once or twice daily, with increments of 0.25 mg or 0.5 every three to seven days until a therapeutic response was reached. Therefore, a flexible schedule of dosing is advisable to coincide with characteristics of the child and to minimize unwanted side effects. Dosing guidelines have been provided in labeling for risperidone in many countries for children from 6–16 years of age, but they are not yet available for pre-school children. Therefore, careful monitoring and slow titration of medication are the mainstays of treatment, particularly in younger children.

Weight gain was the most frequent adverse event, ranging from 1 to 10 kg, at a of 2.7 kg in the Shea and colleagues (2004) and McCracken and colleagues (2002) studies and 2.96 kg in preschoolers in the Luby and colleagues study (2006). Weight increase usually stabilizes over time, but it

is more pronounced during the first 2–3 months of therapy. Several potential long term health risks arise with weight gain, such as hypertension, heart disease, diabetes, and dyslipidemia. Diet and physical activity should be suggested during the early phases of treatment with risperidone to limit weight gain. Sedation is another common side effect, but in most studies it is usually referred to as transient and it has not caused withdrawal or any other major effect.

An increase in prolactin levels is very common but monitoring is not required since the higher levels are predictive of clinical symptoms. In the pooled data of the clinical trials the rise in mean values was observed during the first two months of therapy (Findling et al 2003; Anderson et al 2007). It is not yet possible to provide recommended levels of prolactin to be maintained during risperidone treatment due to the lack of data, but clinical monitoring of potentially related symptoms is recommended.

The occurrence of UEE was relatively low in these trials and it must be emphasized that they occur at high doses of risperidone. The maximum mean dose used was 2.08 mg/day, in the low range, and this was probably a conservative approach which limited the occurrence of UEE. Once more, careful clinical monitoring is needed during risperidone treatment because the many common repetitive movements, including complex tics and sterotypies, in children with ASD may mask UEE.

There are still no guidelines for the duration of risperidone treatment in children with ASD. The blinded discontinuation phase of the RUPPAN (2005) study after a 6-month period of double blind risperidone treatment and 4 months of open-label, demonstrated that a relapse in behavioral symptoms was observed in a significant percentage of children on placebo (10 out of 16), 2 months after stopping treatment, and yet this occurred in only 2 out of 16 patients on risperidone.

Several open trials on the use of risperidone in children and adolescents with ASD have been conducted (Table 2). They demonstrated similar overall findings of those obtained by the controlled trials as to the effectiveness of risperidone on three major clinical domains of ASD. Limited effectiveness of risperidone treatment was reported on core language and social impairment in most of them. In the Masi and colleagues (2003) study in preschoolers various degrees of improvement were observed in stereotypies and repetitive behaviors, affect modulation, SIB, and hyperactivity. In these open studies dosage and titration were similar to those used in controlled trials, indicating the general attitude of clinicians to proceed with low titration and to maintain low doses. The principal unwanted effects observed were increased appetite, weight gain and prolactin elevation, are reported in controlled trials (Table 1).

Empirical findings suggest that risperidone should be maintained for a long period, eg, one or more years, using clinical parameters due to the lack of evidence-based guidelines. Treatment studies, including discontinuation studies, suggest is still warranted in ASD after 6 months, but routine periodic re-assessment of the need for continued treatment should be performed. Future investigation should address the important issues of long term follow-up and discontinuation of risperidone, since ASDs are long-lasting lifetime disorders. The main unwanted UEEs to be controlled in long term treatment are tardive dyskinesia and akathisia.

Heterogeneity in study design and evaluation procedures among the different studies limited the critical analysis. Guidelines for pharmacological trials should be applied in future studies in order to obtain more comparable results regarding safety and efficacy of treatment.

Finally the effects on the developing brain are unknown and concern is warranted. It is not known whether or how it could interfere with the maturational processes of the brain. The mechanisms of action that are effective in limiting behavioral disorders in ASD are likewise not yet known and should be investigated. Therefore there are several implications of early risperidone treatment on the neurobiology of development, and further research is needed to elucidate these aspects.

Conclusion

Moderate efficacy and safety of risperidone for treating maladaptive behaviors, including aggression, hyperactivity, self injury and irritability, have been documented in the available studies. Two studies also found some degree of improvement in some of the core features of ASD. Risperidone was promising in preschoolers with ASD also combined with behavioral interventions. Further trials on larger populations and of longer duration are needed for obtaining more information on the tolerability and efficacy of risperidone at a young age. Furthermore, the different biases, such as the tendency to enroll more severe cases in clinical trials, should be thoroughly controlled in future studies as subgroups of children that could be more responsive to treatment have been considered in current research. Efficacy and tolerability of risperidone in the various types of PDDs, including the different degrees of severity of core symptoms, from mild to severe, are still undetermined and should be appropriately addressed. At present much caution is therefore warranted

in this vulnerable population that raises additional concerns and that needs continuous care, especially when receiving pharmacotherapy.

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8/13/16

Christian Bogner, MD, FACOG
Phytoelements,LLC
email: drb@phytoelements.com

Re: Physician expertise letter of support for adding Autism to the qualifying conditions list for the New Jersey Medical Marijuana program

Dear New Jersey Department of Health,

This is a letter in support of adding autism to the list of qualifying conditions in your Medical Marijuana Program

Background

I have worked as physician in the State of Michigan. I am currently a Staff attending at several institutions, including William Beaumont Hospital in Royal Oak and McLaren Oakland Hospital. I have researched autism for the last 10 years and have been looking into the benefits of cannabis for the autism spectrum.

We petitioned to add autism to the qualifying condition list in Michigan in 2015 and the panel which consisted of physicians and other experts voted in favor 4:2. This was mainly due to the overwhelming evidence we presented. Our case was not that it demonstrated to be bulletproof effective for autism (there is no studies yet for obvious reasons), but because of what we know about its safety and adverse profile. Only then we weighed those and see if it is heavy enough to allow it to be legal, so the doctor and patient can work out the best way to do just that without fearing prosecution.

I have spoken at the annual AutismOne conference in Chicago this year about the current understanding of the endocannabinoid system in relation to autism pathophysiology. I was also part of the Holistic Cannabis summit in 2016. We see incredible results countless reports from parents all over the country and in my opinion could actually heal a whole generation if we work together and make the choice for them, our children to explore this treatment modality.

What research exists that shows Cannabis is safe for children affected with Autism?

The first answer would be: What research do you have to counter-demonstrate that it makes the already severe autism condition worse? Especially, when you have such strong anecdotal success stories, including my own personal very close family friends.

As you are well aware, conducting a federally approved study on children with autism seems a thousand times more impossible than even getting an adult study with cannabis approved on a federal level. So then the next question would be:

" What evidence exists that demonstrates that cannabis is safe"?

And then most importantly, we need to ask ourselves, what is the best way to see if we will do harm with it or not? Unless we let these parents try as their personal choice, alongside the guidance of a well trained physician who could recommend medical cannabis, we will not. So hence, let me make a case for it. And this case is based on scientific research conducted at reputable institutions all over the world, including our own Ivy leagues.

Let us begin with the current considerations of medications for the treatment of autism and their side effects. Please note that none of the following medications are FDA approved for the treatment of autism and are used "off-label". This treatment regimen is being used by the leading authority on Autism in the State of Michigan, Dr. Harry Chugani at Children's Hospital of Michigan and appears to be the current "gold standard" along with ABA, speech and OT therapy.

Zyprexa (Olanzapine) *neuroleptic malignant syndrome, severe extrapyramidal symptoms,tardive dyskinesia, dystonia,severe hyperglycemia, diabetes mellitus, hyperlipidemia, stroke, hypotension, syncope, dysphagia, aspiration, hyperthermia, anaphylaxis, angioedema, seizures, priapism, hepatotoxicity, pancreatitis, rhabdomyolysis, anemia, thrombocytopenia, neutropenia,leukopenia,agranulocytosis and more*

Klonopin (Clonazepam)
Respiratory depression, dependency (abuse), seizures, suicidality, hypotension, tachycardia, syncope, blood dyscrasias, hepatomegaly, withdrawl symptoms, impaired coordination, depression, dysarthria, amnesia, confusion, disinhibition, irritability, dystonia, incontinence, LFTs elevated, rash and more

Risperdal (Risperidone)
Severe hypotension, syncope,severe extrapyramidal symptoms, tardive dyskinesia, neuroleptic malignant syndrome, severe hyperglycemia, diabetes mellitus, seizures, stroke, TIA, QT prolongation, hypersensitivity reactions, angioedema, neutropenia, suicidality, hypothermia, hyperthermia, insomnia, dystonia, headache, akathisia, confusion, epistaxis, photosensitivity and more

Abilify (Aripirazole)
Neuroleptic malignant syndrome,extrapyramidal symptoms, tardive dyskinesia, dystonia, stroke, TIA, syncope, hypotension, seizures, severe hyperglycemia, diabetes mellitus, severe dysphagia, aspiration, hyperthermia, hypertension, tachycardia, hemorrhage, intestinal obstruction, cholecystitis, pancreatitis, blood dyscrasias, leukopenia, neutropenia, hypokalemia, hyperkalemia, rhabdomyolysis, suicidality, depression, anxiety, akathisia, incontinence, insomnia, fatigue, fever, dystonia, dyslipidemia, impaired body temperature regulation, xerostomia and more

These adverse reactions happen. Otherwise they would not be on that list. Each one of those reported side effects above could **kill** a person or leave them more debilitated than before. Do we have ANY long term studies on brain development on those medications? Certainly not. Yet, most are ok with prescribing them as a physician to your patients. In 2010, there were roughly 40,000 deaths attributable to medication overdose alone in the United States. An overdose of Cannabis is never lethal. There are 0 reported deaths attributable to cannabis overdose reported in recorded time.

Review of the literature relevant to our topic

The Endocannabioid system and as it relates to Autism, Dr. Christian Bogner, Joe Stone 2014

In this 16 page summary we carefully reviewed 45 papers that add up to about 800 pages of solid scientific data. It is attached to this letter and should be studied carefully in addition to my letter of support. Some of the arguing scientific support for cannabinoids causing NO harm to neuronal development we may highlight these conclusions based mainly on phytocannabinoids (e.g. the cannabinoids from the cannabis plant):

*"**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)"

Official United States Government Evidence

The United States Government filed a patent on the beneficial effects of Cannabinoids in 1999:

Cannabinoids as antioxidants and neuroprotectants – Patent US 6630507 B1 – US department of Health

"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..."

-Source: http://www.google.com/patents/US6630507

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 New Jersey Medical Marijuana Program in Trenton,NJ in 2016:

"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

Source: http://www.iowamedicalmarijuana.org/documents/nc1ch2.aspx#2f

Nixon response to the media 2 days after its release: *"If we move the line to the other side and accept the use of this drug, how can we draw the line against other illegal drugs"?* The Shafer Commission report has never been disproven.

National Institute of Drug Abuse Evidence

The NIH subdivision NIDA (National Institute of Drug Abuse) defends Marijuana's current scheduling. In regards to the long term effects on brain development, it concluded that a study showed that people *"...who started smoking marijuana heavily in their teens and had an ongoing cannabis use disorder lost an average of eight IQ points between ages 13 and 38."* This study was conducted by Meier in 2012. I want to point out its fundamental key concerns:

1. Meier studied an individual between ages 7-13 and measured the IQ. Then he sent out a questionnaire to "*someone who knows the patient well*" when the patient was aged 38 and had that submitted back to Meier. This is a confounding error and not good science. Selection bias and survey sampling bias to name just 2.

2. Only 41 patients of "*heavy chronic use*" were examined, for which the conclusion was drawn that it causes cognitive decline. 41 patients do not reflection the whole population. This study has no correlation to clinical applicability. What makes a study good is power. More study subjects increases your power. This study has absolutely no power.

3. Even if you dissect those 41 patients, the arbitrary SD deviation (0.8) that was set out to be SEVERE cognitive decline was never achieved by any group tested. The heavy users were considered to have only a SD deviation of 0.3, which according to the paper is "only" SMALL decline. But the word SMALL was never mentioned in the ultimate conclusion. Neither was it addressed if an IQ drop of 8 over 30 years has any negative impact on social integration.

source : *http://www.drugabuse.gov/publications/drugfacts/marijuana*

Ironic that on one side there is a 26 page government patent praising its neuroprotective value in the treatment of many neurocognitive degenerative diseases, on the other side the government claims the best defense to keep it from everybody is an 8 page study is easily dismantled and labeled scientific noise for someone able to talk about true evidence. Studies like this are not even debatable. Using this as argument against usage of Cannabis for autism is not only non-sense, but in my opinion unethical. Similar studies have appeared recently and I encourage everyone to examine the study design first before listening to somebody else's conclusion. Please examine how many people were studied and consider all other factors that we were taught as medical professionals to determine what makes a study a good study.

Additional evidence

80% of autistic patients have gastrointestinal problems. There is mounting evidence in the scientific literature about the benefits of even non-psychoactive THC-A (the actual plant does not have the psychoactive delta-9 in it, that happens only with decarboxylation, which is done best with heat) and its healing effects on the gastrointestinal tract.

"Irritable bowel syndrome (IBS) might be counteracted by cannabinoids, at least the motility-related symptoms, where cannabinoids could reduce muscle spasms underlying the symptom of abdominal pain" -The endocannabinoid system in the physiology and pathophysiology of the gastrointestinal tract, Massa and Storr, 2015, J Mol Med (2005) 83: 944–954

Conclusion and final thoughts

When conventional therapies have fallen short and the likely outcome of not intervening is worse than the potential adverse effects of the use of cannabis (as a last-line defense), legal protection should be granted in these extreme cases. Not because Cannabis may or may not be as suitable for a wide range of autistic patients, but because it might prove therapeutic value.

As fellow colleague of each one of you panel members, I really encourage you to study this material and look at the bigger picture. We have clearly been misled for many years in regards to Cannabis. The few studies that demonstrate long term dangers (poorly powered, not clinically applicable) are a little grain on a beach of overwhelming evidence that cannot be ignored anymore. Our own government knows that this works and is trying to patent this plant. Since this can grow in everyone's backyard, one can see why this is hard to patent. Only prohibition has worked so far to keep it from the population. 80% of Americans approve the use of medical marijuana. Almost half of the states of the US have adapted medical marijuana programs for their citizens. It is a controversial situation we have created here for you as panel member, but still, it might be just as controversial if this does not get approval. Because our highest duty and promise to our patients should be only one and that is **DO NO HARM.** With the powerful support of my State's finest experts (Dr. Chugani) and National Experts from Ivy league Universities (Dr. Grinspoon out of Harvard) we need to move forward and help these families. I can assure you, there will be great harm reduction if you chose to vote Yes. Please provide protection for the parents of the affected. We need your vote. We need to set an example to the rest of the world.

We have done our homework. I want you to be confident about voting yes and use any evidence that we presented as your defense for any backlashes this might cause in your current professional career.

Feel free to contact me at any time at drb@phytoelements.com.
Respectfully,

Christian Bogner, MD, FACOG
Clinical Director
Phytoelements,LLC
Detroit, MI

Highlights

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)

The ECS plays a functional role in a number of symptoms and associated diseases of autism. The ECS is a potential target of therapeutic exploitation. We're confident that if you're willing to review the available data that you might be able to lead the way in an attempt to increase the quality of life for those in MI suffering from truly debilitating forms of autism.

The Endocannabinoid System as it Relates to Autism
Joe Stone; Christian Bogner, M.D.

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.

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August 30, 2016

To whom it may concern:

My name is Victoria LaChapelle and I have been a Licensed Practical Nurse for 13 years, working in the areas of Developmental Disability, Mental Health and Public Health. I am writing to recommend that Autism be added as a qualifying condition for the New Jersey Medical Marijuana Program.

As a nurse who has worked with numerous children on the autism spectrum, I have seen firsthand the impact that autism has both on individuals with autism and their families. I have treated patients whose self-harming behaviors associated with autism have led to their hospitalization. For many families, simply dealing with autistic behaviors and symptoms is the main focus of their lives, and they are suffering greatly.

I have also seen firsthand the harms that can result from the use of FDA-approved pharmaceutical medications prescribed for autism. Many of these medications are not approved for use in children, yet are prescribed routinely because families' situations are so desperate that they will try potentially harmful medications because they feel such a compelling need to improve their children's quality of life. Many patients I know have tried numerous prescriptions without seeing any positive results, at great risk to their physical and emotional health.

Since moving to Colorado in 2015, I have had the opportunity to work with patients who are in the state's Medical Marijuana program. I currently work as a private duty nurse for a child with both epilepsy and an autism diagnosis. Her parents and I have been able to watch this child thrive through the use of medical cannabis. She previously had very extreme behaviors and sleep disturbances, as well as cognitive delays. Her autistic behaviors have improved significantly through the use of cannabis, and her sleep patterns are hugely improved as well. Her cognitive abilities continue to improve. It is hard to even put into words the positive changes this has brought to her family.

I understand that in New Jersey, children who qualify for the program due to an epilepsy diagnosis have also had similar experiences with autistic behavior improvements and cognitive gains since beginning medical cannabis treatment. It does not seem fair to me that children who have autism but not epilepsy are currently unable to access medical cannabis in New Jersey.

Please consider adding autism as a qualifying condition to help improve the lives of these children and their families.

Sincerely,

Victoria LaChapelle, LPN
8195 Summerset Dr. Unit H
Colorado Springs, CO 80920
Ph: 608-520-2092

SUPPORT3.PDF

219 Woodside Ave.
Trenton, NJ 08618

August 30, 2016

New Jersey Department of Health
Office of Commissioner - Medicinal Marijuana Program
Attention: Michele Stark
369 South Warren St., Trenton, NJ 08608

Re: Adding Autism as a qualifying condition for marijuana therapy

To Whom It May Concern:

I have practiced as a registered nurse (RN) since 1976, and I am currently licensed to practice in New Jersey (#[redacted]) and Pennsylvania (#[redacted]). I am writing to recommend that Autism be added as a qualifying medical condition in the New Jersey Medicinal Marijuana Program (MMP), pursuant to the Compassionate Use Medical Marijuana Act.

There are no FDA approved medicines for treating the core symptoms of autism – communication difficulties, social challenges, and repetitive behaviors. There are two FDA approved drugs for treating behavioral symptoms associated with autism – risperidone and aripiprazole - but both come with significant risk of dangerous and sometimes permanent side effects. Medical marijuana/cannabis offers doctors a safe alternative to pharmaceuticals to address the complex issue of autism.

A great deal of scientific literature supports the use of cannabis and cannabinoids for the many symptoms and diagnoses within the classification of autism. Notably, the newest clinical literature for epilepsy also shows reduction of autism symptoms.

As the executive director of the Coalition for Medical Marijuana – New Jersey, I have come in contact with numerous families whose children have epilepsy, some of whom are cardholders in New Jersey's MMP. In addition to epilepsy, some of these same patients have also been diagnosed with autism. Their families and caregivers overwhelmingly report that the patients' autistic behaviors and associated cognitive delays have significantly improved since they began using cannabis, and that their suffering due to autism is greatly diminished. I believe that people who have been diagnosed with autism but do not have another condition that qualifies them for the state's MMP should be allowed to join the program in the hopes of realizing the same life-changing benefits.

Sincerely,

RN

Kenneth R. Wolski, RN, MPA
Executive Director, Coalition for Medical Marijuana--New Jersey, Inc. www.cmmnj.org
[redacted]

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Format: Abstract Send to

Clin J Pain. 2016 Feb 17. [Epub ahead of print]

The Effect of Medicinal Cannabis on Pain and Quality of Life Outcomes in Chronic Pain: A Prospective Open-label Study.

Haroutounian S[1], Ratz Y, Ginosar Y, Furmanov K, Saifi F, Meidan R, Davidson E.

Author information

Abstract

OBJECTIVES: The objective this prospective, open-label study was to determine the long-term effect of medicinal cannabis treatment on pain and functional outcomes in subjects with treatment-resistant chronic pain.

METHODS: The primary outcome was change in pain symptom score on the S-TOPS (Treatment Outcomes in Pain Survey - Short Form) questionnaire at 6 months follow-up in intent-to-treat (ITT) population. The secondary outcomes included change in S-TOPS physical, social and emotional disability scales, pain severity and pain interference on brief pain inventory (BPI), sleep problems, and change in opioid consumption.

RESULTS: 274 subjects were approved for treatment; complete baseline data were available for 206 (ITT), and complete follow-up data for 176 subjects. At follow-up, pain symptom score improved from median 83.3 (95% CI 79.2-87.5) to 75.0 (95% CI 70.8-79.2), P<0.001. Pain severity score (7.50 [95% CI 6.75-7.75] to 6.25 [95% CI 5.75-6.75] and pain interference score (8.14 [95% CI 7.28-8.43] to 6.71 [95% CI 6.14-7.14]) improved (both P<0.001), together with most social and emotional disability scores. Opioid consumption at follow-up decreased by 44% (P<0.001). Serious adverse effects led to treatment discontinuation in two subjects.

DISCUSSION: The treatment of chronic pain with medicinal cannabis in this open-label, prospective cohort resulted in improved pain and functional outcomes, and significant reduction in opioid use. The results suggest long-term benefit of cannabis treatment in this group of patients, but the study's non-controlled nature should be considered when extrapolating the results.

PMID: 26889611 DOI: 10.1097/AJP.0000000000000364

[PubMed - as supplied by publisher]

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Cannabinoids 2010;5(4):4-6

Case report

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

René Kurz, Kurt Blaas

Lindengasse 27/10, 1070 Wien, Vienna/Austria

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

Author's address: René Kurz, Rene.Kurz@gmx.at

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

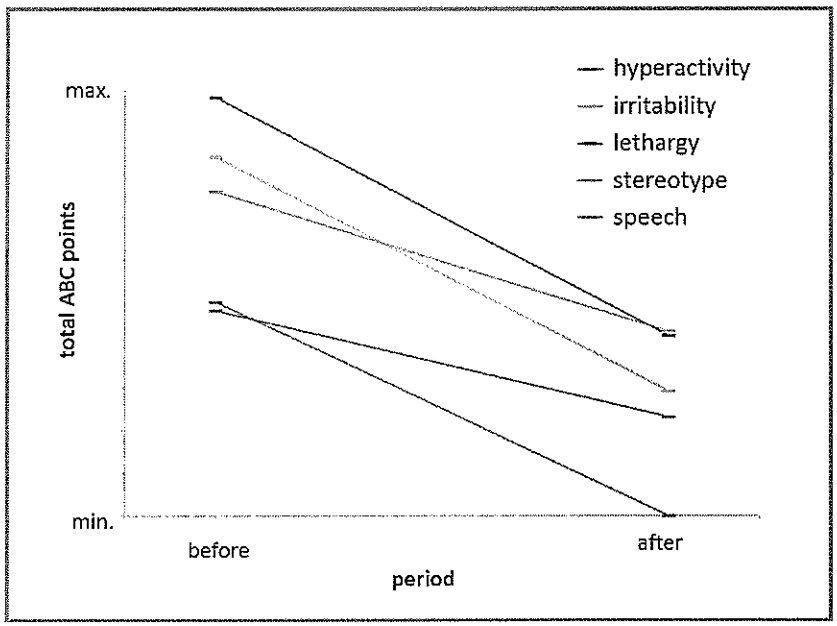

Figure 1. Change of ABC subscales within six months.

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 decreased 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.

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

E.S. Onaivi[1,2,*], R. Benno[1], T. Halpern[1], M. Mehanovic[1], N. Schanz[1], C. Sanders[1], X. Yan[1], H. Ishiguro[2,3], Q-R Liu[4], A.L. Berzal[5], M.P. Viveros[5] and S.F. Ali[6]

[1]*William Paterson University, Wayne, USA;* [2]*Molecular Neurobiology Branch, National Institute on Drug Abuse, NIH, Baltimore, USA;* [3]*Ikeda Hospital, Happoukai Medical Corporation, Ryugasaki, Japan;* [4]*Behavioral Neurobiology Branch, National Institute on Drug Abuse, NIH, Baltimore, USA;* [5]*Departamento de Fisiología (Fisiología Animal II), Facultad de Biología, Universidad Complutense, Madrid, Spain;* [6]*Neurochemistry Laboratory, NCTR/FDA, Jefferson, AR, USA*

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.

*Address correspondence to this author at the Department of Biology, William Paterson University Wayne, NJ 07470, USA; Tel: +1 973-720-3453; Fax: +1 973-720-2338; E-mail: Onaivie@wpunj.edu

1570-159X/11 $58.00+.00

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 was 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 spontaneous wheel running test. The BTBR males had slightly higher 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.

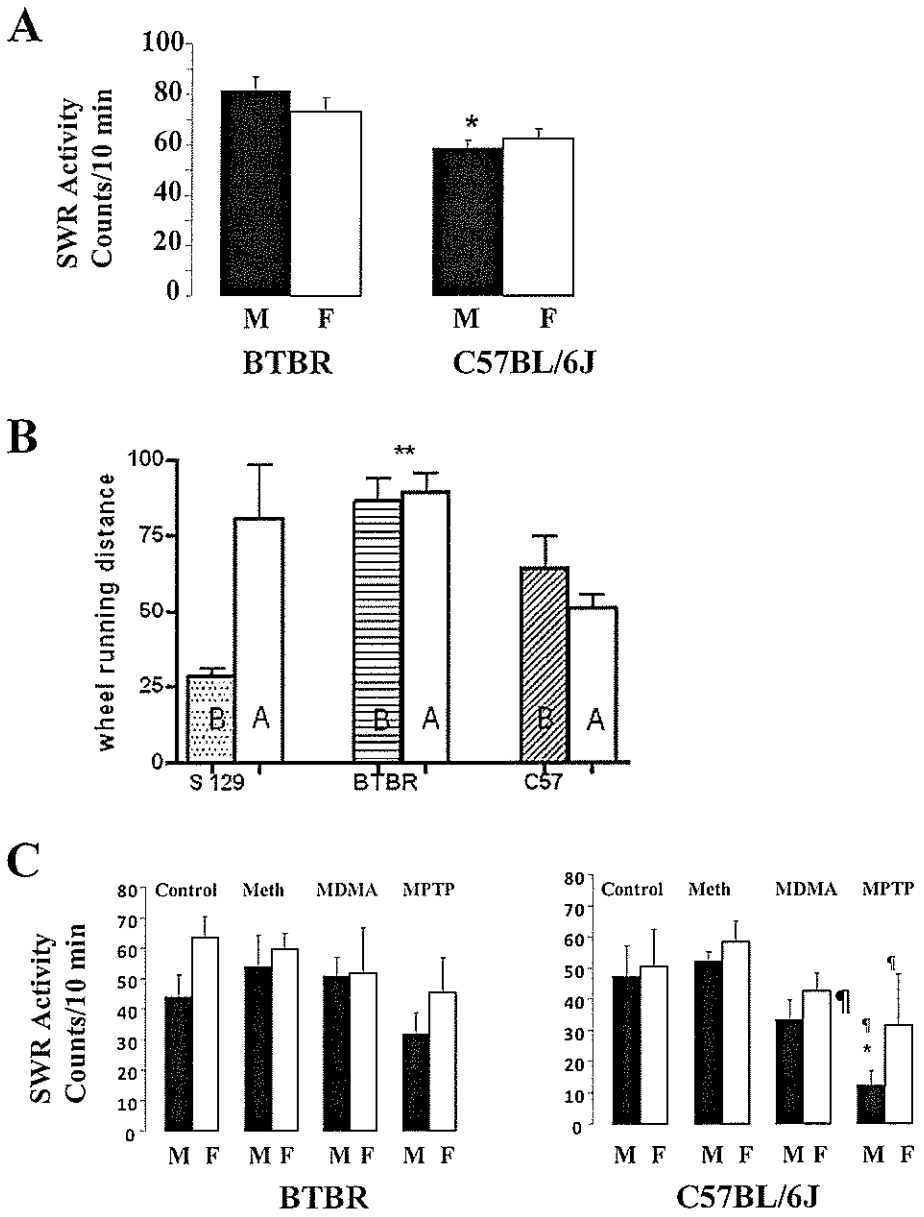

Fig. (1). The effects of psychostimulants (d-amphetamine, Methamphetamine and MDMA), and disruption of monoaminergic system by the neurotoxin (MPTP), in a mouse model of autism spectrum disorders. Panel A shows the basal motor activity of male and female BTBR and C57BL/J mice in the spontaneous wheel 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-

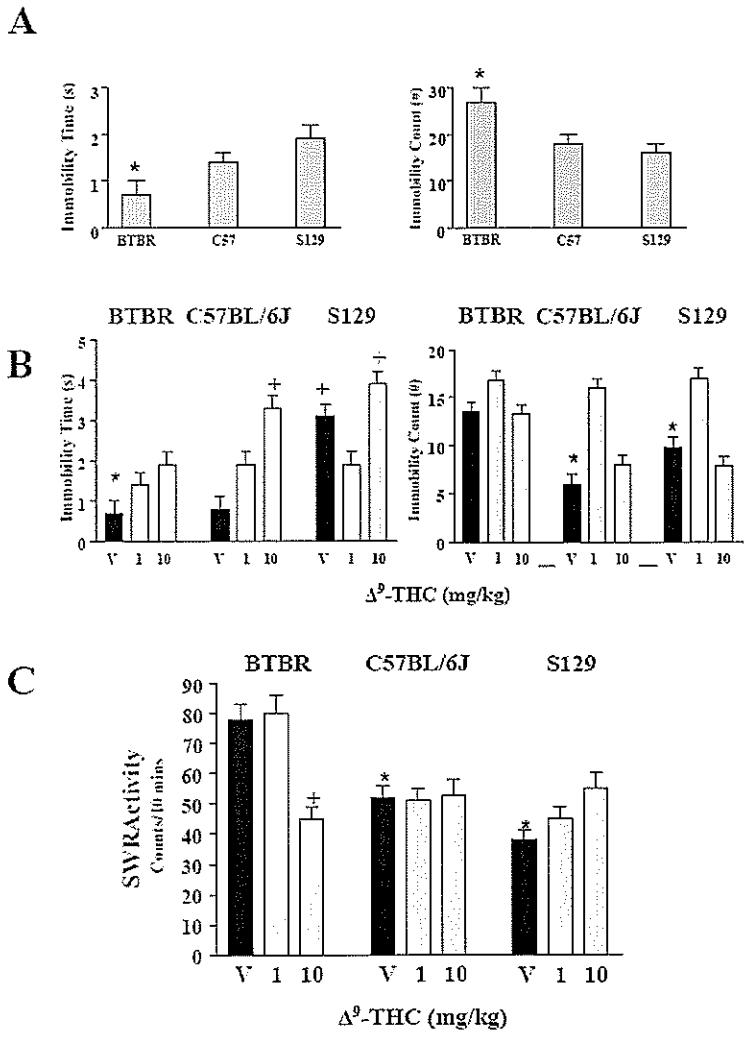

Fig. (**2**). Behavioral effects of BTBR, C57BL/6J and S129 mouse strains in the FST. Panel A shows the basal levels of performance indicated by the time and number of immobility by the three mouse strains in the forced swim test model. Panel B is time and number of immobility after acute treatment of 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.

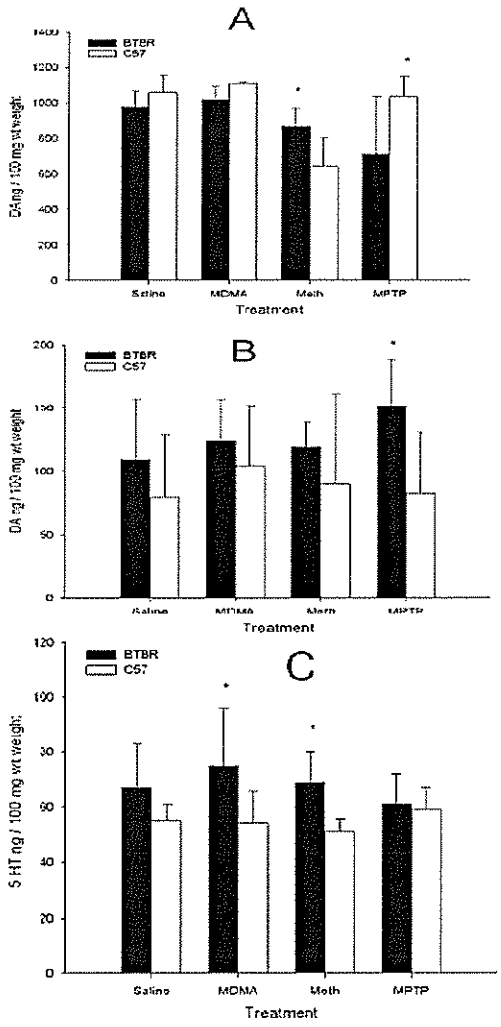

Fig. (3). Neurochemical analysis of dopamine (DA) and serotonin (5HT) levels in striatum and frontal cortex in BTBR and C57BL/6J male and female mice following a single day three times administration of either saline, methamphetamine (10 mg/kg), MDMA (20 mg/kg) or MPTP (20 mg/kg). Regional brain areas 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.

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Autism-Associated Neuroligin-3 Mutations Commonly Disrupt Tonic Endocannabinoid Signaling

Csaba Földy[1,2], **Robert C. Malenka**[2,3], and **Thomas C. Südhof**[1,3,4]

[1]Department of Molecular and Cellular Physiology, Stanford University Medical School, Stanford, CA94305

[2]Nancy Pritzker Laboratory, Stanford University Medical School, Stanford, CA94305

[3]Department of Psychiatry, Stanford University Medical School, Stanford, CA94305

[4]Howard Hughes Medical Institute, Stanford University Medical School, Stanford, CA94305

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

Address for correspondence: C.F. (foldy@stanford.edu) or T.C.S. (tcs1@stanford.edu).

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 ± 1.9 mV, R451C: −19.4 ± 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 $\left(\left|PSC=Q\cdot N\cdot\left|1-\sqrt[N]{1-successes}\right|\right.\right.$ 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 σ_{PR}=0.224 and a σ_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 σ_{PR}=0.09 and a σ_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 σ_{PR}=0.19 and a

σ_Q=2; Q=39 pA and N=6 per model estimates) for NL3 WT, and when P_R=0.26 (together with a σ_{PR}=0.26 and a σ_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.

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 $\left[\mathrm{PSC}=Q \cdot N \cdot \left[1-\sqrt[N]{1-\mathrm{Successes}}\right]\right.$ 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: $\Delta P_R=\pm 0.006$, $\Delta\sigma_{PR}=\pm 0.09$, $\Delta Q=\pm 0.7$ pA and $\Delta\sigma_Q=\pm 0.06$ 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

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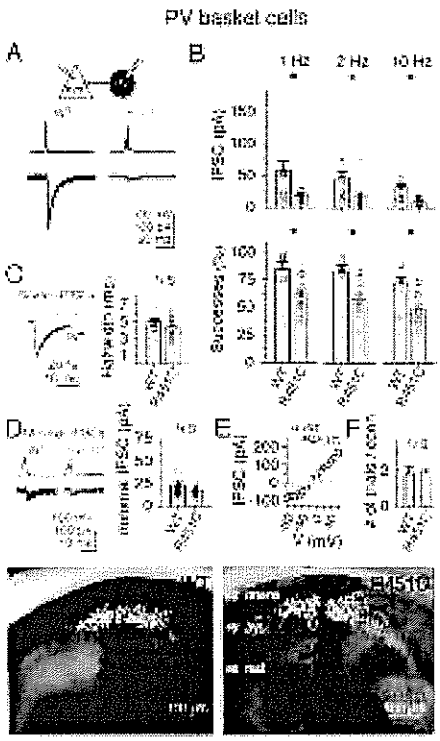

Figure 1. Neuroligin-3 R451C substitution impairs GABAergic synaptic transmission in PV basket cell synapses

(**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.

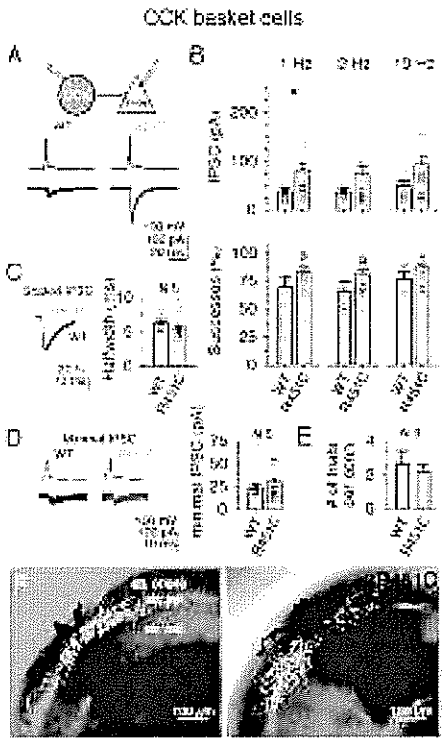

Figure 2. Neuroligin-3 R451C substitution enhances GABAergic synaptic transmission in CCK basket cell synapses

(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.

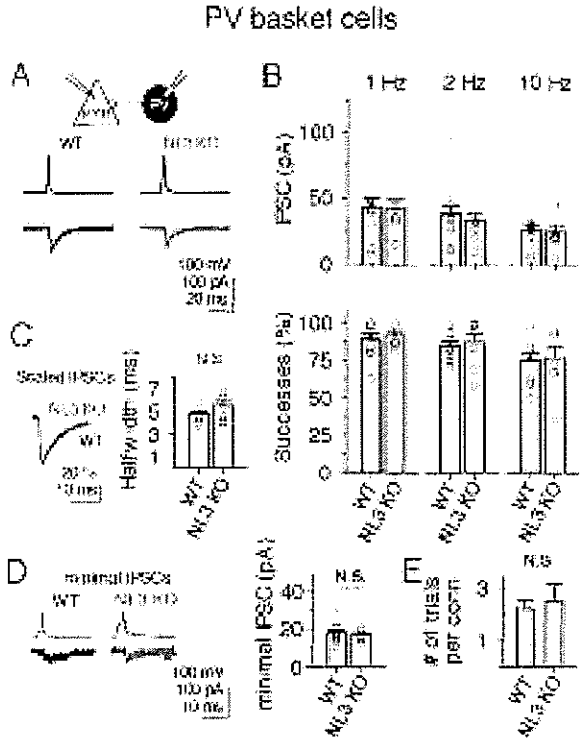

Figure 3. Neuroligin-3 KO does not alter GABAergic transmission in PV basket cell synapses

(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.

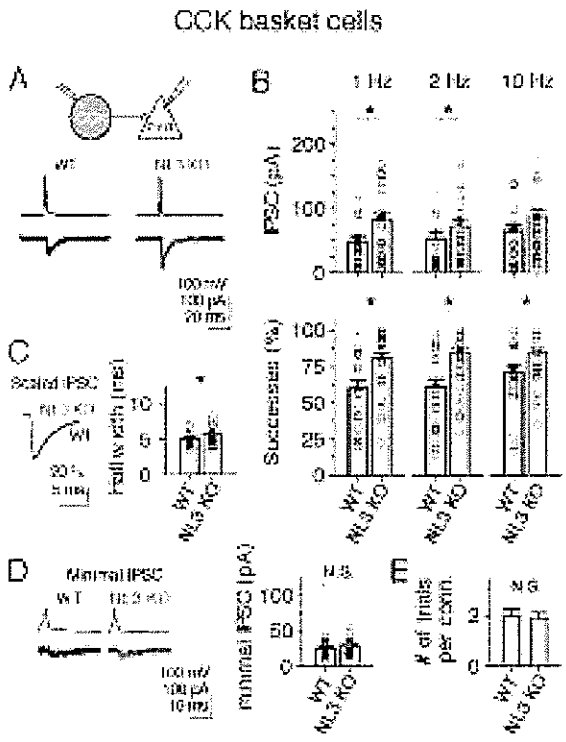

Figure 4. Neuroligin-3 KO enhances GABAergic synaptic transmission in CCK basket cell synapses similar to the R451C KI

(**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.

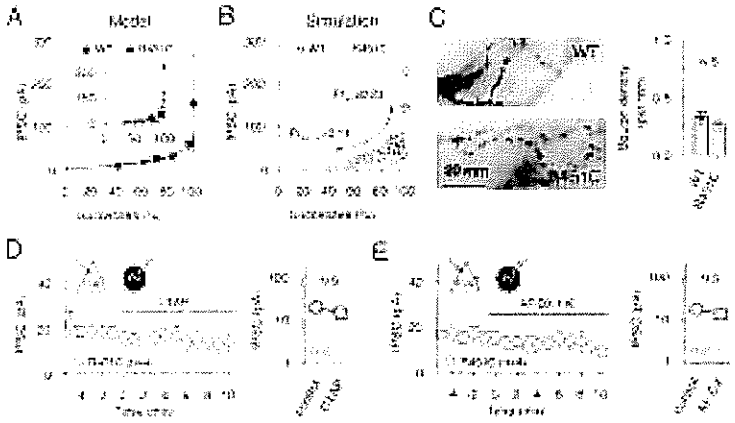

Figure 5. The NL3 R451C KI mutation lowers the probability of GABA release from PV basket cell synapses

(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.

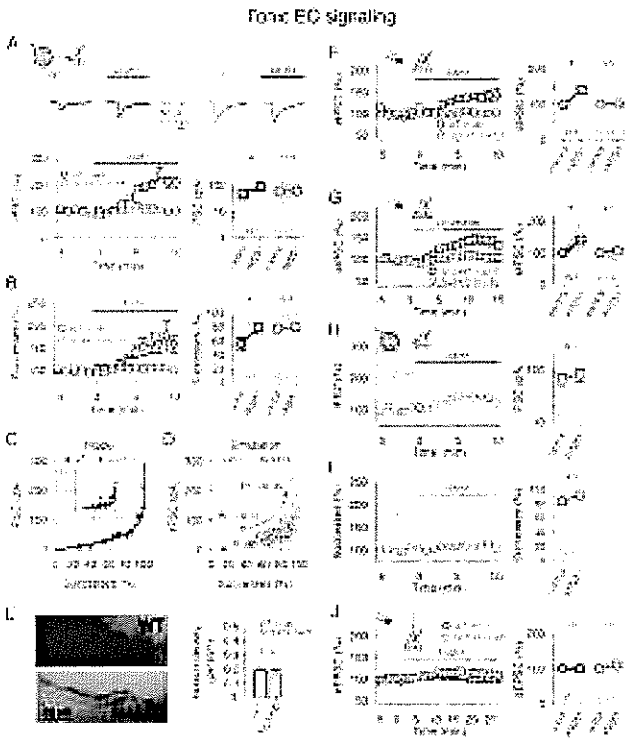

Figure 6. Neuroligin-3 KO and R451C KI mutations impair tonic endocannabinoid signaling

(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.

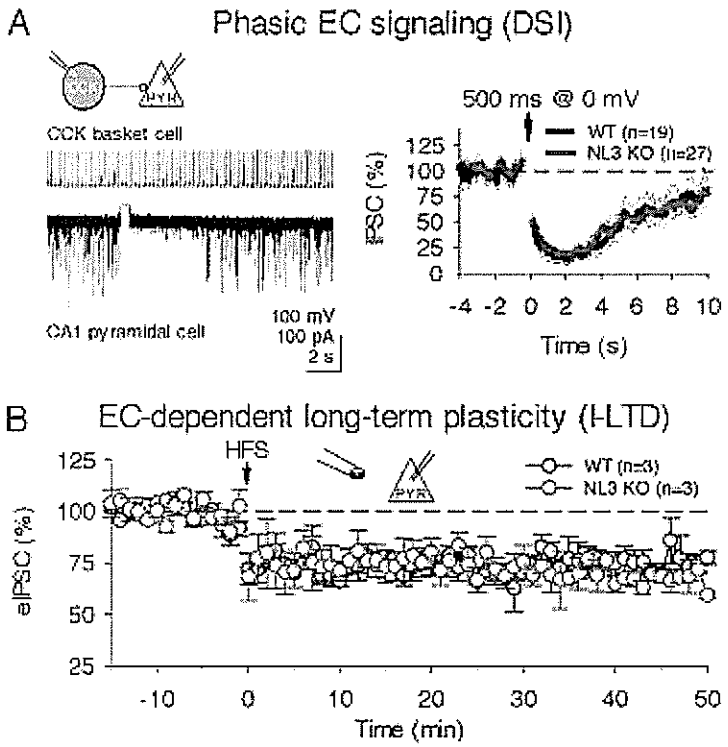

Figure 7. Neuroligin-3 is not required for phasic short-term endocannabinoid signaling (DSI) or long-term endocannabinoid-dependent synaptic plasticity (i-LTD)

(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).

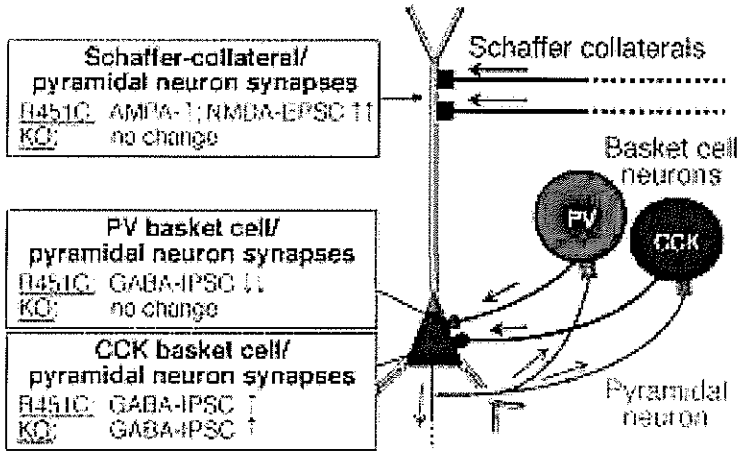

Figure 8. Schematic summary diagram of the effects of the NL3 KO and R451C substitution on three different synapses on pyramidal neurons in the CA1 region of the hippocampus

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.

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ORIGINAL PAPER

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

Dario Siniscalco · Anna Sapone · Catia Giordano · Alessandra Cirillo · Laura de Magistris · Francesco Rossi · Alessio Fasano · James Jeffrey Bradstreet · Sabatino Maione · Nicola Antonucci

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 ± 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 ± 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

D. Siniscalco (✉) · C. Giordano · F. Rossi · S. Maione
Division of Pharmacology, Department of Experimental Medicine, Second University of Naples, via S. Maria di Costantinopoli, 16, 80138 Naples, Italy
e-mail: dariosin@uab.edu

D. Siniscalco
Centre for Autism, La Forza del Silenzio, Caserta, Italy

A. Sapone · L. de Magistris
Department of Internal and Experimental Medicine "Magrassi-Lanzara", Second University of Naples, Naples, Italy

A. Sapone · A. Fasano
Center for Celiac Research and Mucosal Immunology and Biology Research Center, Massachusetts General Hospital East, Charlestown, MA, USA

A. Cirillo
Division of Biotechnology and Molecular Biology "A. Cascino", Department of Experimental Medicine, Second University of Naples, Naples, Italy

A. Fasano
MassGeneral Hospital for Children, Boston, MA, USA

J. J. Bradstreet
International Child Development Resource Center, Cumming, GA, USA

N. Antonucci
Biomedical Centre for Autism Research and Treatment, Bari, Italy

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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, $MgCl_2$, 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; $MgCl_2$ 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 bisbenzimide 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).

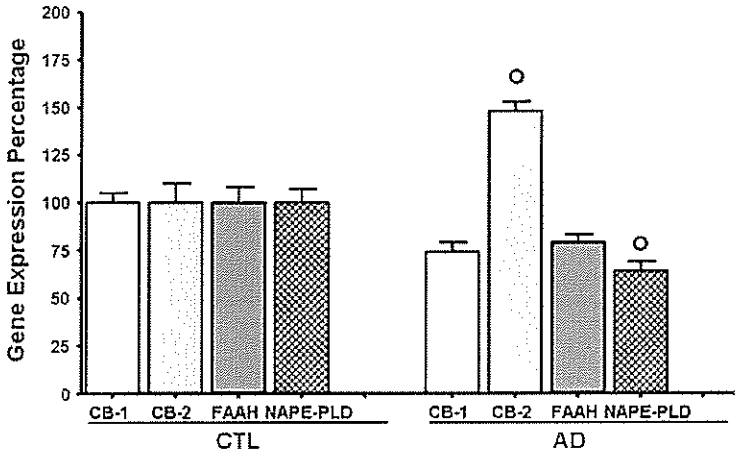

Fig. 1 Over-expression of CB2 receptor gene, but not of CB1 receptor and FAAH enzyme, and down-expression of NAPE-PLD gene in AD-PBMCs. The measured mRNA levels were normalized with respect to *GAPDH* (housekeeping gene) and gene expression values were expressed as a percentage of arbitrary units ± SEM *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,

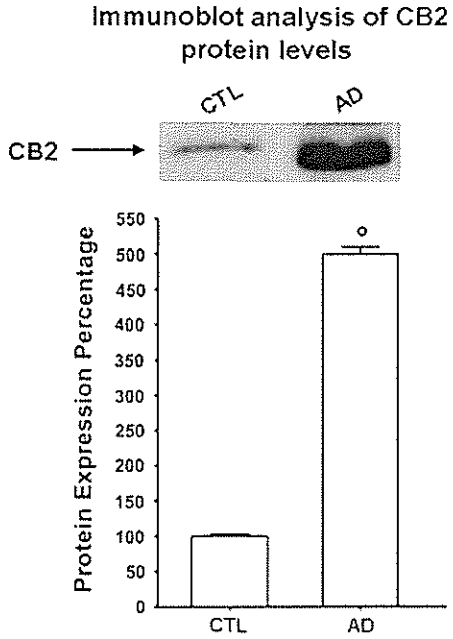

Fig. 2 Representative western blot analysis of CB2 protein levels in the PBMCs obtained from the autistic children and the healthy controls, respectively. *CTL* healthy control subjects, *AD* autistic disorder subjects. The histograms indicate percentage variations in CB2 protein levels in the PBMCs 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 suppression (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 immunomodulatory 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

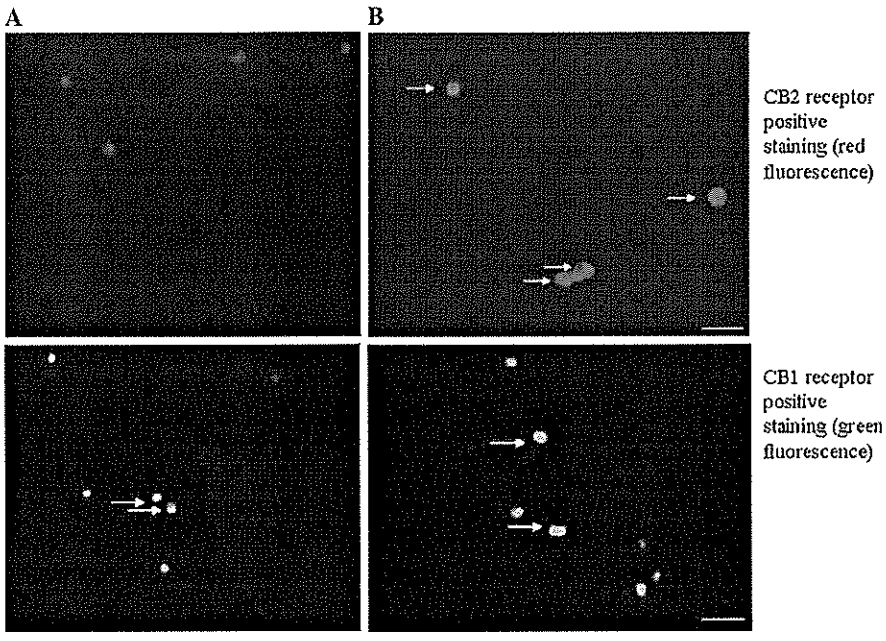

Fig. 3 Representative fluorescent photomicrograph of PBMCs showing immunocytochemistry for CB1 receptor (*bottom*) and CB2 receptor (*top*). *Top Arrows* indicate CB2 positive staining (*red fluorescent*). *Bottom Arrows* indicate CB1 positive staining (*green fluorescent*). To correctly identify cells, 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

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.

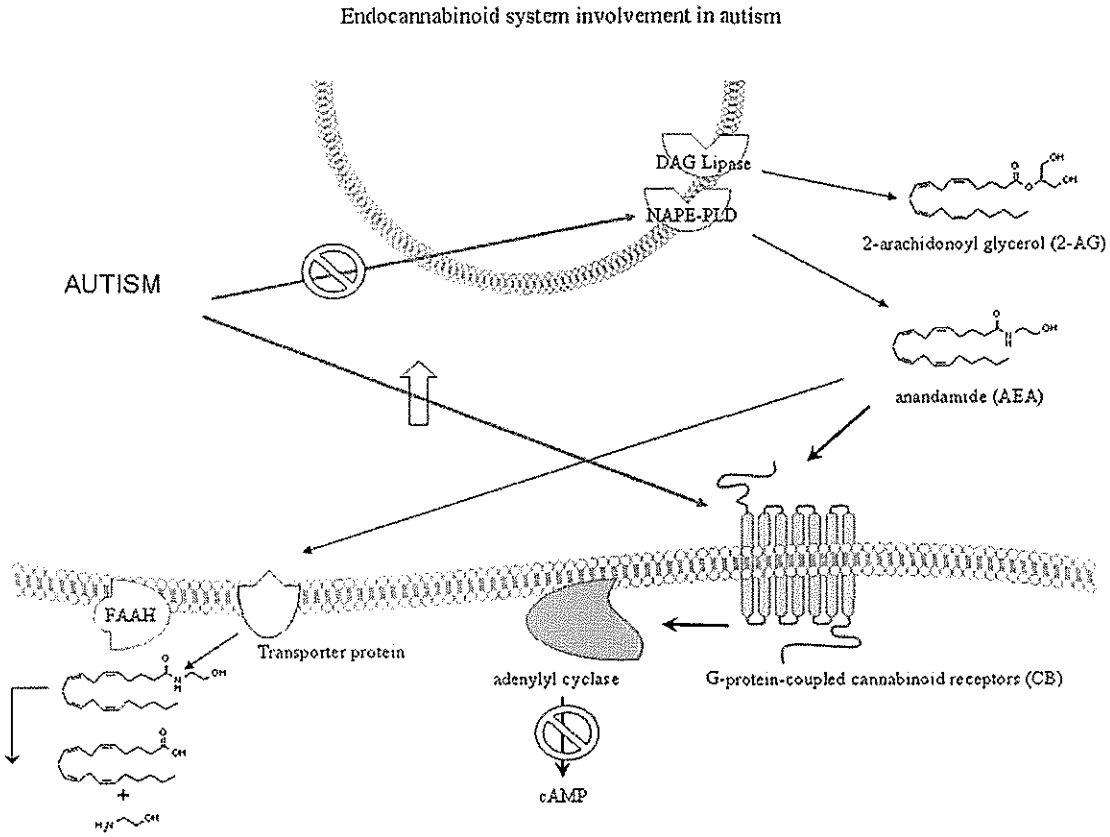

Fig. 4 Endocannabinoids, such as N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG), are synthesized and released upon demand in a receptor-dependent way, through the AEA biosynthetic enzyme *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and the diacylglycerol (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.

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

Dilja D. Krueger[1,*] and Nils Brose[1,*]
[1]Department of Molecular Neurobiology, Max Planck Institute of Experimental Medicine, 37075 Göttingen, Germany
*Correspondence: krueger@em.mpg.de (D.D.K.), brose@em.mpg.de (N.B.)
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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

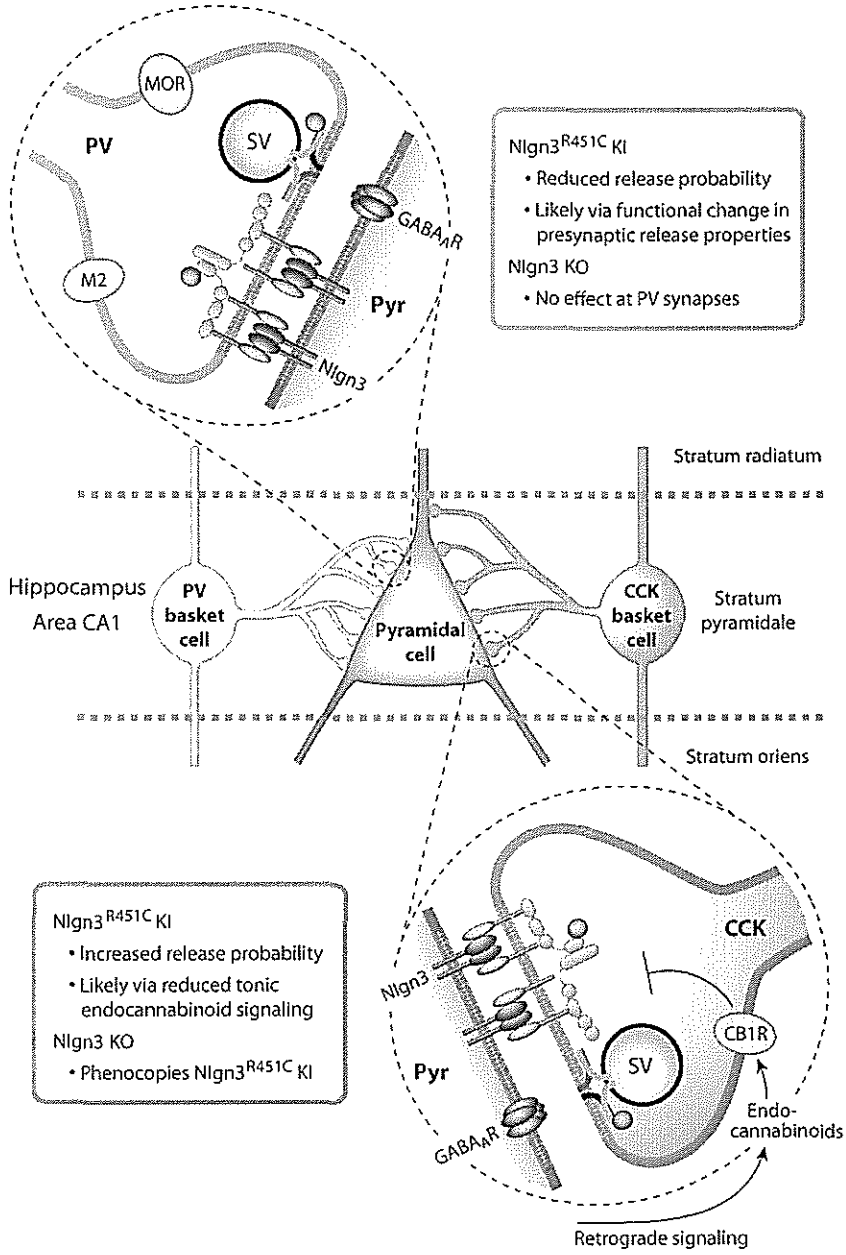

Figure 1. Potential Role of Nlgn3 at Perisomatic Inhibitory Synapses on CA1 Pyramidal Neurons

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 eludicate 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.

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Molecular Autism

RESEARCH Open Access

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

* Correspondence: bhisma@cantab.net
[1]Centre for Integrative Neuroscience and Neurodynamics, School of Psychology and Clinical Language Sciences, University of Reading, Whiteknights, Reading RG6 6AL, UK
Full list of author information is available at the end of the article

[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

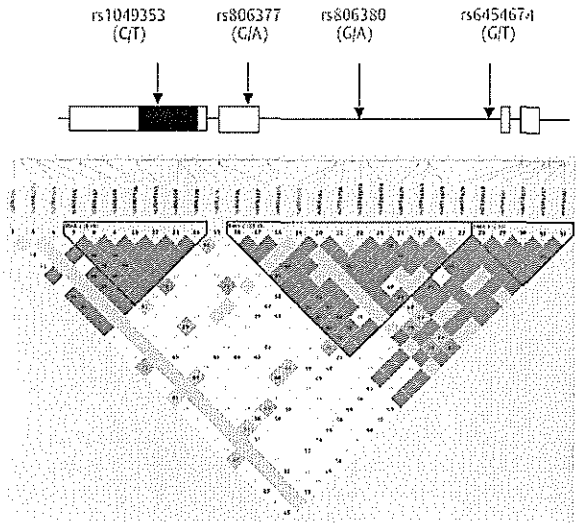

Figure 1 Schematic structure of the cannabinoid receptor *CNR1* gene with all four genotyped single-nucleotide polymorphisms indicated. Top: White boxes indicate untranslated regions, black boxes indicate translated regions and intervening straight line indicates an intronic region. Bottom: The linkage 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 (λ = 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 (χ^2 = 8.88, df = 2, P = 0.011) and rs806380 (χ^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

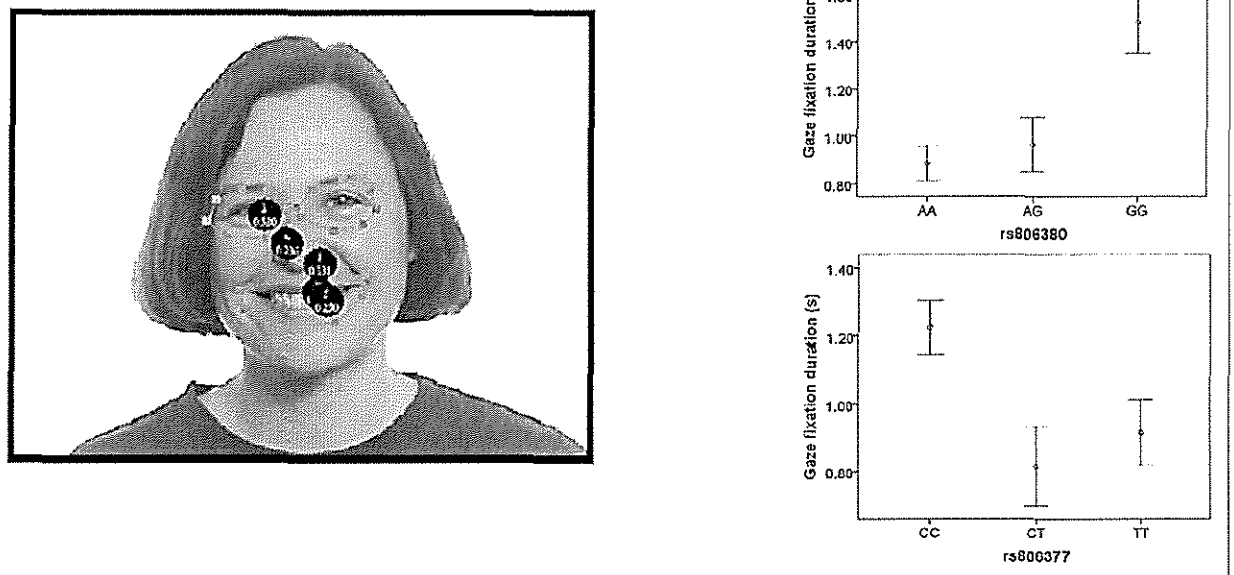

Figure 2 Example gaze trail from a single participant and group mean gaze durations for happy faces classified by genotype. (a) Example gaze trail from a single participant on a still frame from a video stimulus showing a happy expression. The black circles represent fixation points and the amount of 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 consideration the LD between SNPs. This method was implemented 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 significant 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 variations would be associated with differences in gaze fixation duration towards happy faces. This prediction was confirmed: two SNPs in this gene (rs806377 and rs806380) were associated with differences in gaze duration 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 relevant 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 neurotransmission (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].

Abbreviations

CNR1: cannabinoid receptor 1; mRNA: messenger RNA; SNP: single-nucleotide polymorphism.

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Author details
[1]Centre for Integrative Neuroscience and Neurodynamics, School of Psychology and Clinical Language Sciences, University of Reading, Whiteknights, Reading RG6 6AL, UK. [2]Autism Research Centre, Department of Psychiatry, University of Cambridge, Douglas House, 18B Trumpington Road, Cambridge CB2 8AH, UK.

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.

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Can autism be triggered by acetaminophen activation of the endocannabinoid system?

Stephen T. Schultz

Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA
Email: stephen.schultz@med.navy.mil

Acetaminophen use in children has been associated with increased autism risk. Recent evidence suggests that acetaminophen's analgesic actions result from activation of the endocannabinoid system, and activation of this system can have neuromodulatory consequences during development. This investigation was performed to determine if there is evidence to support the hypothesis that acetaminophen use can trigger autism by activation of the endocannabinoid system.

Key words: autistic disorder, autism, acetaminophen, cannabinoid receptors, endocannabinoid system

INTRODUCTION

Autism is a severe developmental disorder defined by social and communication deficits and ritualistic-repetitive behaviors that appear in early childhood (American Psychiatric Association 1994). Autism can be comorbid with tuberous sclerosis (1.2%), fragile X syndrome (0.3%), and congenital rubella syndrome (0.3%), although the attributable proportion of all medical disorders is less than 10%, and in most cases the cause of autism is unknown (Fombonne 2003). Two of the prominent features of autism are immune system dysregulation (Pessah et al. 2008) and abnormal brain neuron organization (Courchesne et al. 2007). In this report we present evidence of a link to autism from acetaminophen use, evidence to show that acetaminophen produces analgesia by activating cannabinoid receptors, and evidence that activation of the cannabinoid receptors may interfere with normal development to trigger autism.

A LINK TO AUTISM FROM ACETAMINOPHEN USE

There are several theories about possible environmental triggers for autism including childhood vaccinations, mercury exposure, and viral infections. Descriptive clinical studies have suggested a link between measles, mumps, rubella (MMR) vaccination and autism/pervasive developmental disorder (Kawashima et al. 2000, Wakefield et al. 2000, Furlano et al. 2001, Singh et al. 2002, Uhlman et al. 2002, Singh and Jensen 2003). Epidemiological studies have not supported the relationship between prevalence of autism and the MMR vaccine (Peltola et al. 1998, Taylor et al. 1999, Dales et al. 2001, Madsen et al. 2002, Chen et al. 2004).

The link between the MMR vaccine and an elevated risk for autism is controversial. However, children are often given acetaminophen if they have symptoms such as fever or irritability, and the MMR vaccination can cause these symptoms (Centers for Disease Control and Prevention 2009). One study showed that administration of acetaminophen after the MMR vaccination is associated with increased risk for autism (Schultz et al. 2008).

A further report compared the features of autism with asthma and suggested a link to acetaminophen use (Becker and Schultz 2009). In this report, events in the history of acetaminophen use were compared with the number of eligible persons with autism from a 1999 report to the legislature by the California DDS (Department of Developmental Services 1999).

The three pathways for the metabolism of acetaminophen are glucuronidation, sulfation, and the

Correspondence should be addressed to S.T. Schultz
Email: stephen.schultz@med.navy.mil

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cytochrome P-450 system. One study of children with autism indicated that these children had a sulfation deficit which causes them to process acetaminophen differently from control children (Alberti et al. 1999). Sulfation is the primary pathway for acetaminophen metabolism until age 10–12 years (Defendi and Tucker 2009). It is possible that children predisposed to developing autism have a sulfation deficit which may lead to increased blood levels of acetaminophen after therapeutic doses of acetaminophen are administered.

EVIDENCE THAT ACETAMINOPHEN PRODUCES ANALGESIA BY ACTIVATING CANNABINOID RECEPTORS

Although acetaminophen has been used as an analgesic for more than a hundred years, its mechanism of action has remained elusive. It has recently been shown by two independent groups (Hogestatt et al. 2005, Bertolini et al. 2006) that acetaminophen produces analgesia by potentiating cannabinoid receptors in the brain. These observations have been confirmed by Mallet and colleagues (2008).

Hogestatt and colleagues have shown that acetaminophen is deacetylated to p-aminophenol which is conjugated with arachidonic acid in the brain and spinal cord by fatty acid amide hydrolase (FAAH). The resulting compound, N-arachidonoylphenolamine inhibits the cellular uptake of anandamide, a naturally occurring endogenous cannabinoid or endocannabinoid. The result is increased levels of endocannabinoids which produce an analgesic effect (Hogestatt et al. 2005).

Bertolini and colleagues (2006) noticed a similarity in the effect of acetaminophen and cannabinoids. Cannabinoids and acetaminophen both have an analgesic action and lower body temperature. They were able to show that blockage of cannabinoid receptor 1 (CB_1) completely prevents the analgesic activity of acetaminophen (Bertolini et al. 2006).

EVIDENCE THAT MODULATION OF THE CANNABINOID SYSTEM MAY INTERFERE WITH NORMAL DEVELOPMENT

The endocannabinoid system plays an important role in the development of the central nervous system and its activation can induce long-lasting functional alterations (Campolongo et al. 2009). Use of cannabis (an exogenous cannabinoid) in the still-maturing brain may produce persistent alterations in brain structure and cognition (Jager and Ramsey 2008). Animal models have revealed the danger of both cannabis abuse and exposure to cannabinoid drugs during brain development (Anavi-Goffer and Mulder 2009). Developmental problems associated with the endocannabinoid system may occur through either of the two known cannabinoid receptors, CB_1 or CB_2.

CB_1 receptors are located in the central nervous system (CNS), peripheral nervous system, and peripheral organs. In the CNS, CB_1 receptors are concentrated in the cerebellum, hippocampus, and the basal ganglia (Drysdale and Platt 2003) which are areas in the brain implicated as dysfunctional in autism (Bauman and Kemper 2005, Courchesne et al. 2007). During fetal life, CB_1 receptors and their associated endocannabinoids are important for neuron differentiation and proper axonal migration (Fride et al. 2009). In addition, recent studies suggest that CB_1 cannabinoid receptors define synapse positioning (Harkany et al. 2008). Modulation of CB_1 cannabinoid receptors could trigger autism by interrupting normal brain development.

CB_2 receptors are primarily located in immune tissues and cells and may serve a regulatory function. CB_2 receptors have been shown to control the movement of inflammatory cells to the site of injury, and CB_2 receptors' reverse agonists may serve as immune system modulators (Lunn et al. 2008). The activation of CB_2 receptors stimulate beta-amyloid removal by macrophages which may slow the progression of Alzheimer's Disease (Tolon et al. 2009). CB_2 receptor agonists attenuate transendothelial migration of monocytes and monocyte-endothelial adhesion (Rajesh et al. 2007).

Monocytes are one of the primary cells of the immune system and differentiate into macrophages and dendritic cells. If the evidence is correct that acetaminophen acts as an activator of cannabinoid receptors, then activating CB_2 receptors could influence the growth of monocytes. Data from our lab indicates that acetaminophen in the media inhibits the cell division of monocytes in a dose dependent manner as assayed with resazurin stain for mitochondrial dehydrogenase activity. Inhibition of growth is noted even at the therapeutic concentration of 20 micrograms per milliliter. If as proposed, children with autism are poor metabolizers of acetaminophen, higher than normal therapeutic levels could be possible with recommended doses which could lead to a greater inhibition of monocytes.

It has been shown in several studies that children with autism have immune system dysregulation (Warren et al. 1996, Jyonouchi et al. 2005, Ashwood et al. 2006, Molloy et al. 2006, Li et al. 2009, Entstrom et al. 2010). This dysregulation includes differential monocyte responses, abnormal T helper cytokine levels, decreased T cell mitogen response, decreased numbers of lymphocytes, and abnormal serum immunoglobulin levels. Many studies have shown that children with autism exhibit autoimmunity, in particular antibodies against brain and central nervous system proteins (Singh et al. 1993, Connolly et al. 1999, Ashwood and Van De Water 2004, Cohly and Panja 2005, Kawashti et al. 2006, Wills et al. 2007, Martin et al. 2008). It is proposed that the immune dysregulation in children with autism is due to the influence of acetaminophen on CB_2 receptors during gestation or in early childhood.

HYPOTHESIS

The hypothesis presented here is that the use of acetaminophen may trigger autism by activating the endocannabinoid system thereby interfering with normal development. Children who are poor metabolizers of acetaminophen may be at higher risk since normal therapeutic doses may lead to higher blood levels in these children.

It has been proposed that the blockage of fever with antipyretics (as acetaminophen) could lead to autism by interfering with normal immunologic development (Torres 2003). Children with autism have reported to have a decrease in autism symptoms when they have a fever (Sullivan 1980, Cotterill 1985, Torres 2003, Curran et al. 2007). It is interesting to note that activation of CB_1 receptors, in addition to providing an analgesic effect, causes a decrease in body temperature (Fraga et al. 2009). This type of effect may be further evidence of endocannabinoid disruption in children with autism.

LIMITATIONS

Other environmental factors may also be involved in triggering autism. For example, low levels of breastfeeding could decrease immune protection in infants by decreasing mother to child transfer of IgA. Decreased immune protection could make a child more vulnerable to viral infection which in theory could lead to autism. Lack of breastfeeding has been shown to be associated with autism (Schultz et al. 2006). This same study found an association between use of infant formula without docosahexaenoic acid or arachidonic acid supplementation and autism. Arachidonic acid metabolism is an integral part of the endocannabinoid system and its disruption could be further evidence of a role for the endocannabinoid system in autism.

CONCLUSION

The purpose of this report was to explore a possible correlation between acetaminophen and autism which acts through activation of the cannabinoid system. If this hypothesis is correct, it opens new avenues of investigation for possible autism treatment including agonists and antagonists of the CB_1 and CB_2 receptors.

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The *in vitro* GcMAF effects on endocannabinoid system transcriptionomics, receptor formation, and cell activity of autism-derived macrophages

Dario Siniscalco[1,2,3*], James Jeffrey Bradstreet[4,5], Alessandra Cirillo[6] and Nicola Antonucci[7]

Abstract

Background: Immune system dysregulation is well-recognized in autism and thought to be part of the etiology of this disorder. The endocannabinoid system is a key regulator of the immune system via the cannabinoid receptor type 2 (CB2R) which is highly expressed on macrophages and microglial cells. We have previously published significant differences in peripheral blood mononuclear cell *CB2R* gene expression in the autism population. The use of the Gc protein-derived Macrophage Activating Factor (GcMAF), an endogenous glycosylated vitamin D binding protein responsible for macrophage cell activation has demonstrated positive effects in the treatment of autistic children. In this current study, we investigated the *in vitro* effects of GcMAF treatment on the endocannabinoid system gene expression, as well as cellular activation in blood monocyte-derived macrophages (BMDMs) from autistic patients compared to age-matched healthy developing controls.

Methods: To achieve these goals, we used biomolecular, biochemical and immunocytochemical methods.

Results: GcMAF treatment was able to normalize the observed differences in dysregulated gene expression of the endocannabinoid system of the autism group. GcMAF also down-regulated the over-activation of BMDMs from autistic children.

Conclusions: This study presents the first observations of GcMAF effects on the transcriptionomics of the endocannabinoid system and expression of CB2R protein. These data point to a potential nexus between endocannabinoids, vitamin D and its transporter proteins, and the immune dysregulations observed with autism.

Keywords: GcMAF, Endocannabinoids, Gene expression, Macrophages, Autism

Introduction

Autism and autism spectrum disorders (ASDs) are complex heterogeneous neurodevelopmental conditions [1], arising from the interaction of genetic and environmental factors [2]. The established symptom categories include dysfunctions in communication skills and social interactions, combined with repetitive, restrictive and stereotypic verbal and non-verbal behaviors. Despite extensive research efforts, the etiopathologies of ASDs remain inadequately understood [3-5]. Early inflammatory processes, including maternal-fetal immune interactions and resultant immunological dysfunctions have been proposed as potential mechanisms [6-9]. The prevailing hypothesis is that some combination of immune factors including maternally-developed antibodies to fetal brain, prime microglia in such a way as to preclude their normal functions of directing neuronal migration and pruning [10,11].

The functional role of Vitamin D in the central nervous system has recently been reviewed and includes neurogenesis, neuroplasticity and a neuroprotection [12]. Vitamin D deficiency has been a demonstrated cause of recurrent pregnancy loss and supplementation with D3 significantly reduces IFN-γ and TNF-α secretion from natural killer (NK) cells [13]. There is a complex interaction between vitamin D and polymorphisms of the vitamin D receptor (VDR) and both the risk of autoimmunity and the responsiveness to vitamin D supplementation

* Correspondence: dariosin@uab.edu
[1]Department of Experimental Medicine, Second University of Naples, via S. Maria di Costantinopoli, 16 - 80138 Naples, Italy
[2]Centre for Autism - La Forza del Silenzio, Caserta 81036, Italy
Full list of author information is available at the end of the article

[14]. In autism, vitamin D deficiency in pregnancy or early childhood appears to contribute significantly to risk [15].

Potentially related to these processes are the recent observations of elevated N-acetylgalactosaminidase (Nagalase) levels in the blood of children with ASDs [16]. Nagalase is an enzyme that catalyzes the deglycosylation of the Gc protein also known as vitamin D3 binding protein (VDBP) rendering it incapable of being converted to the regulatory protein, Gc Macrophage Activating Factor (GcMAF). GcMAF is an immunologically important protein responsible for macrophage activation [17], thus Nagalase diminishes the body's macrophage activating capacity, and elevated Nagalase has been reported in autoimmune disorders including systemic lupus erythematosus (SLE) [18].

We recently demonstrated that a cannabinoid receptor type 2 (CB2R) signalling was significantly upregulated in peripheral blood mononuclear cells (PBMCs) extracted from autistic children, suggesting that endocannabinoid (EC) system dysregulation could be involved in ASD-mediated immune impairments [19].

Using a new methodology of radiolabeling, the CB2R distribution was recently mapped using whole body positron emission tomography (PET) [20]. In healthy subjects without brain related pathology, the CB2R was demonstrated to map to the peripheral lymphoid immune system. Additionally, CB2R is expressed on both macrophages and microglial cells and activation of CB2R has been demonstrated to down-regulate ischemia-induced macrophage-microglial induced inflammation in an animal model [21].

The body produces arachidonate-based lipids, anandamide (N-arachidonoylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) which are binding ligands for the cannabinoid receptors [22]. CB2R appears to have primary immunomodulatory effects and CB2R-specific agonists and phytocannabinioids (for example, cannabidiol (CBD)) lack psychoactive properties [22].

GcMAF treatment seems to ameliorate autistic symptoms in some children [16]; however, the cellular and molecular pathways involved in the apparent therapeutic effect are not understood. We hypothesized that a potential therapeutic mechanism of GcMAF is related to transcriptional regulation of EC genes. We sought to investigate this mechanism *in vitro* using blood monocyte-derived macrophages (BMDMs) from autistic patients and controls.

Materials and methods

Subjects

We investigated 22 children with autism, and compared them to 20 age and sex matched healthy children used as a control group (age ranging 3 to 11 years; mean age: 7.06 ± 1.52 versus 7.38 ± 2.33 in autistic and healthy individuals, respectively). The subjects with autism were recruited into the study from the outpatient Biomedical Center for Autism Research and Treatment, Bari, Italy. Before entering the study, all of the children were administered the Autism Diagnostic Interview-Revised version [23], the Childhood Autism Rating Scales (CARS) [24], and the Autism Diagnostic Observation Schedule-Generic [25] 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) [1]. In addition to meeting the criteria for autistic disorder, subject children were required to score at least 30 points on the CARS scale. Twenty healthy children 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 behavioral disorders, including Pervasive Developmental Disorder - Not Otherwise Specified (PDD-NOS), and 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 autistic subjects nor controls were receiving pharmacological interventions. Other exclusion criteria were celiac disease and/or other major diseases of the intestinal tract, such as inflammatory bowel disease or hepatic disorders.

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

Isolation of peripheral blood mononuclear cells (PBMCs)

Mononuclear cells were extracted as previously described [9,19]. Briefly, less than 10 ml of fresh peripheral blood samples from autistic 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 1077 density gradient (Sigma Chemical, St Louis, MO, USA). Briefly, blood was diluted 1:1 in 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 minutes at room temperature and plasma was removed. Mononuclear cell fraction was harvested and washed twice in PBS. The final pellet was re-suspended in RPMI 1640 complete medium (Lonza, Verviers, Belgium) containing 10% FBS (EuroClone-Celbio, Milan, Italy),

2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Lonza, Verviers, Belgium) and incubated at 37°C with 5% CO_2. Lymphocytes (non-adherent cells) were removed.

Differentiation of macrophages from PBMCs

In order to obtain fully differentiated human blood monocyte-derived macrophages (BMDMs), PBMCs were then cultured for about ten days in the presence of 25 ng/mL recombinant human macrophage colony-stimulating factor (Peprotech, London, UK) [26,27] (Figure 1).

in vitro treatment

GcMAF was added at 0.1 ng/ml final concentration to BMDMs from healthy control and autistic patients. The dose of GcMAF was chosen on the basis of a previous work demonstrating that maximal stimulation of PBMCs was achieved with 0.1 ng/ml [28]. GcMAF was kindly provided by Immuno Biotech (St. Peter Port, Guernsey, UK). Following incubation of the cells for 24 hours in the presence of GcMAF, some cells were lysed for the extraction and analysis of RNA (Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)), and proteins via Western blot analysis; alternatively, other cells were fixed for fluorescence-based immunocytochemistry analysis. Comparisons between pre-post treatment levels of RNA and protein expression were made for both autistic individuals and healthy controls.

RNA extraction and RT-PCR

The RNA was extracted from BMDMs 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).

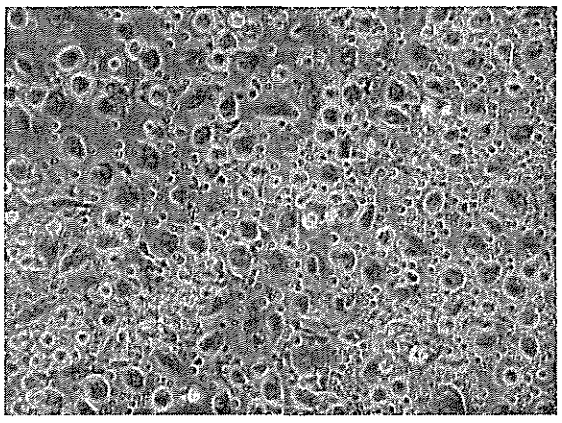

Figure 1 Representative optical photomicrograph of blood monocyte-derived macrophages (BMDMs) *in vitro* culture from autistic children.

The mRNA levels of the EC genes under analysis were measured by RT-PCR amplification, as previously reported [19]. Reverse Transcriptase from Avian Myeloblastosis Virus (AMV-RT; Promega, Madison, WI, USA) was used. For first-strand cDNA synthesis, 200 ng total RNA, random hexamers, dNTPs (Promega, Madison, WI, USA), AMV buffer, AMV-RT and recombinant RNasinribonuclease inhibitor (Promega, Madison, WI, USA) were assembled in diethyl-pyrocarbonate-treated water to a 20 μl final volume and incubated for ten minutes at 65°C and one hour 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, $MgCl_2$, 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 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) [19]. Each RT-PCR was repeated at least three times to achieve the best reproducibility data. 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 [29]. To our knowledge, there is no molecular evidence of variation in *GAPDH* mRNAlevels in autism disorders [19]. 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% agarose gel. A semi-quantitative analysis of mRNA levels was carried out by the Gel Doc EZ UV System (Bio-Rad, Hercules, CA, USA).

Protein extraction and Western blot analysis

For protein extraction, BMDMs were suspended in protein lysis buffer (HEPES 25 mM; EDTA 5 mM; SDS 1%; Triton X-100 1%; PMSF 1 mM; $MgCl_2$ 5 mM; Protease Inhibitor Cocktail (Roche, Mannheim, Germany); Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany)). Protein concentration was determined using the method described by Bradford [30]. For CB2R detection, 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 CB2R (Calbiochem-Merck, Darmstadt, Germany) were used according to the manufacturer's instructions at 1:250 dilutions [19]. The rabbit anti-CB2R

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

Gene	Sense primer (5'-3')	Antisense primer (5'-3')	Annealing temperature (°C)	Product sizes (bp)
CB2R	TTGGCAGCGTGACTATGACC	AGGAAGGCGATGAACAGGAG	55	274
FAAH	GGCCACACCTTCCTACAGAA	GTTTTGCGGTACACCTCGAT	58	218
NAPE-PLD	GAAGCTGGCTTAAGAGTCAC	CCGCATCTATTGGAGGGAGT	60	178
GAPDH	TCACCAGGGCTGCTTTTAAC	GGACTCCACGACGTACTCAG	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).

antibody detects endogenous levels of the human 45 kDa fragment of CB2R 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 CB2R used as a positive control. For mannose receptor detection, each sample was loaded, electrophoresed in a precast gradient 4 to 12% SDS-polyacrylamide gel using Bolt® system (Life Technologies, Monza, Italy) 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 mannose receptor (ab64693 Abcam, Cambridge, UK) were used according to the manufacturer's instructions at 1:1,000 dilutions. 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 Coomassie Blue staining, as previously reported [31]. We used Coomassie Blue staining as equal loading control as this method overcomes the possibility that housekeeping proteins could vary in this pathology or be saturated at the levels of loading [19]. However, we confirmed the signals obtained by Coomassie Blue staining with respect to the signal obtained with anti-β-tubulin monoclonal antibodies (A2066 Sigma Chemical, St Louis, MO, USA; 1:1,000 dilution). The semi-quantitative analysis of protein levels was carried out by the ChemiDoc-It 5000, using VisionWorks Life Science Image Acquisition and Analysis software (UVP, Upland, CA, USA).

Immunocytochemistry

For immunocytochemical analysis, BMDMs were re-suspended at $1x10^6$ cell/mL in RPMI 1640 complete medium (Lonza, Verviers, Belgium) containing 10% FBS (EuroClone-Celbio, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all Lonza, Verviers, Belgium), were plated on slides with a 12-well plate and incubated at 37°C with 5% CO_2. Cells were then fixed with 4% paraformaldehyde fixative. After washing in PBS, non-specific antibody binding was inhibited by incubation for 30 minutes 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 Notch (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or to human Ki67 proliferation marker (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Fluorescent-labeled secondary antibodies (1:1,000; Alexa Fluor 488, 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-labeled slides were viewed with a fluorescence microscope (Leica, Wetzlar, Germany) and with a fluorescence confocal microscope (LSM 710, Zeiss, Oberkochen, Germany). Immunofluorescence images were analyzed with Leica FW4000 software (Leica, Wetzlar, Germany) and with Zen Zeiss software (Zeiss, Oberkochen, Germany). Quantification of Ki67-ir profiles was performed by an observer blind to the treatment. Cell positive profile quantification was performed on each digitized image, and the reported data are the intensity means ± SE on counterstained cells per group. Only bisbenzimide counterstained cells were considered as positive profiles so as to avoid overcounting cells.

Statistical analysis

Biomolecular data are expressed as means ± SEM. ANOVA, followed by Student-Neuman-Keuls *post hoc* test, was used to determine the statistical significance among groups. $P < 0.05$ was considered statistically significant.

Results

GcMAF was able to normalize endocannabinoid system gene dysregulation in blood monocyte-derived macrophages (BMDMs) in autistic children

As we already demonstrated [19], studying EC system gene expression mainly by RT-PCR is far more sensitive for the detection of gene expression than immunocytochemistry [9,32]. We evaluated the GcMAF effects on *NAPE-PLD* (N-acyl phosphatidylethanolamine phospholipase D), a protein-coding gene [GC07M102742]. The gene codes for the enzyme which hydrolyzes N-acyl-phosphatidylethanolamines (NAPEs) to produce N-

acylethanolamines (NAEs) and phosphatidic acid and, specifically, the generation of anandamide (N-arachidonoylethanolamine), the ligand of cannabinoid receptors. When compared to healthy controls, the semiquantitative analysis of BMDM-extracted mRNA levels, measured by RT-PCR amplification, showed an increase in the *NAPE-PLD* gene in BMDMs of autistic patients (mean ± SE of arbitrary units: 1.20 ± 0.34 versus 0.71 ± 0.11, $P < 0.05$, in BMDMs from autistic children and healthy subjects, respectively).

We also evaluated the gene for fatty acid amide hydrolase, (*FAAH*) [GC01P046860]. FAAH is a membrane associated enzyme which hydrolyzes bioactive amides, including the EC, anandamide. We observed that the mRNA levels of the *FAAH* enzyme gene were decreased (mean ± SE of arbitrary units: 0.40 ± 0.08 versus 1.60 ± 0.06, $P < 0.05$, in BMDMs from autistic children and healthy subjects, respectively); the NAPE-PLD/FAAH ratio was significantly increased (mean ± SE of arbitrary units: 3.00 ± 0.84 versus 0.44 ± 0.07 in BMDMs from autistic children compared to healthy subjects, respectively); mRNA levels of *CB2R* gene (mean ± SE of arbitrary units: 0.40 ± 0.01 versus 0.43 ± 0.02, $P > 0.05$, in BMDMs from autistic children and healthy subjects, respectively) were not changed (Figure 2).

In BMDMs of autistic children, GcMAF treatment was able to significantly increase gene expressions both NAPE-PLD (mean ± SE of arbitrary units: 1.20 ± 0.34 and 1.74 ± 0.18, $P < 0.05$, before and after GcMAF treatment in autistic BMDMs, respectively) and FAAH (mean ± SE of arbitrary units: 0.40 ± 0.08 versus 0.95 ± 0.04, $P < 0.05$, before and after GcMAF treatment in autistic BMDMs, respectively), whereas the NAPE-PLD/FAAH ratio was significantly reduced (mean ± SE of arbitrary units: 3.00 ± 0.84 versus 1.83 ± 0.19, before and after GcMAF treatment in autistic BMDMs, respectively). The mRNA levels of *CB2R* gene were not affected by GcMAF treatment (mean ± SE of arbitrary units: 0.40 ± 0.01 versus 0.54 ± 0.01, $P > 0.05$, before and after GcMAF treatment in autistic BMDMs, respectively). No changes were observed in GcMAF treated BMDMs of healthy control children with respect to untreated BMDMs, except for a slight decrease in *NAPE-PLD* gene expression (not affecting NAPE-PLD/FAAH ratio) (Figure 2).

GcMAF affected CB2R protein levels in BMDMs

GcMAF was also able to reduce the protein levels for CB2R in BMDMs from autistic children with respect to treated BMDMs from healthy controls. Since CB2Rs are G protein-coupled receptors, they show post-translational regulation [33]. We therefore determined the protein levels of CB2R by Western blot analysis.

Western blot analysis showed a strong decrease in CB2R protein levels in GcMAF treated BMDMs from autistic children as compared to untreated macrophages (mean ± SE of arbitrary units: 3.24 ± 0.54 versus 1.66 ± 0.39, $P < 0.05$, before and after GcMAF treatment in autistic BMDMs, respectively). Interestingly, GcMAF was also able to decrease CB2R protein levels in GcMAF treated BMDMs from healthy controls as compared to untreated BMDMs (Figure 3) (mean ± SE of arbitrary

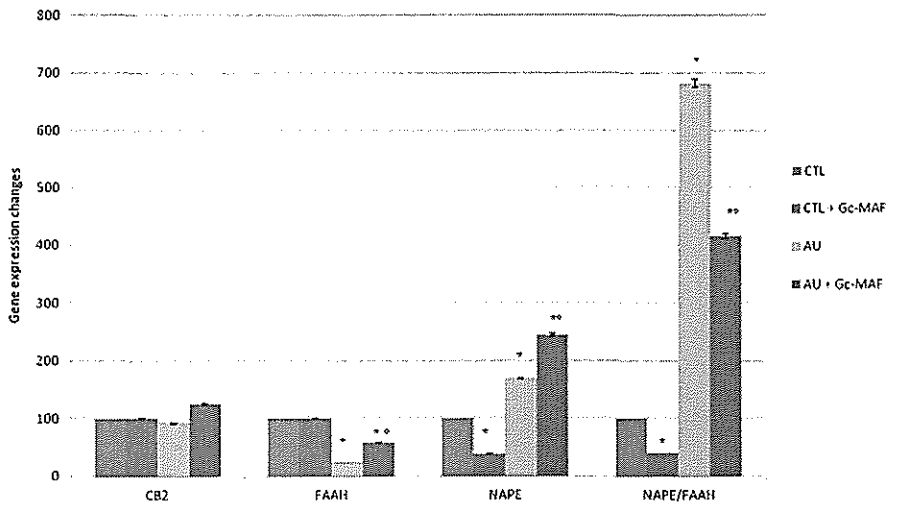

Figure 2 Expression of the enzymes NAPE-PLD and FAAH, and *CB2R* genes in blood monocyte-derived macrophages (BMDMs). The ratio NAPE/FAAH was also reported. The measured mRNA levels were normalized with respect to *GAPDH* (housekeeping gene) and gene expression values were expressed as a percentage of arbitrary units ± SEM. * indicates significant difference versushealthy controls; ° indicates significant difference versus GcMAF untreated autistic BMDMs. *P*-values <0.05 were considered statistically significant. CTL, healthy control subjects;AU, autistic patients. Values were reported in percentage versus healthy control values.

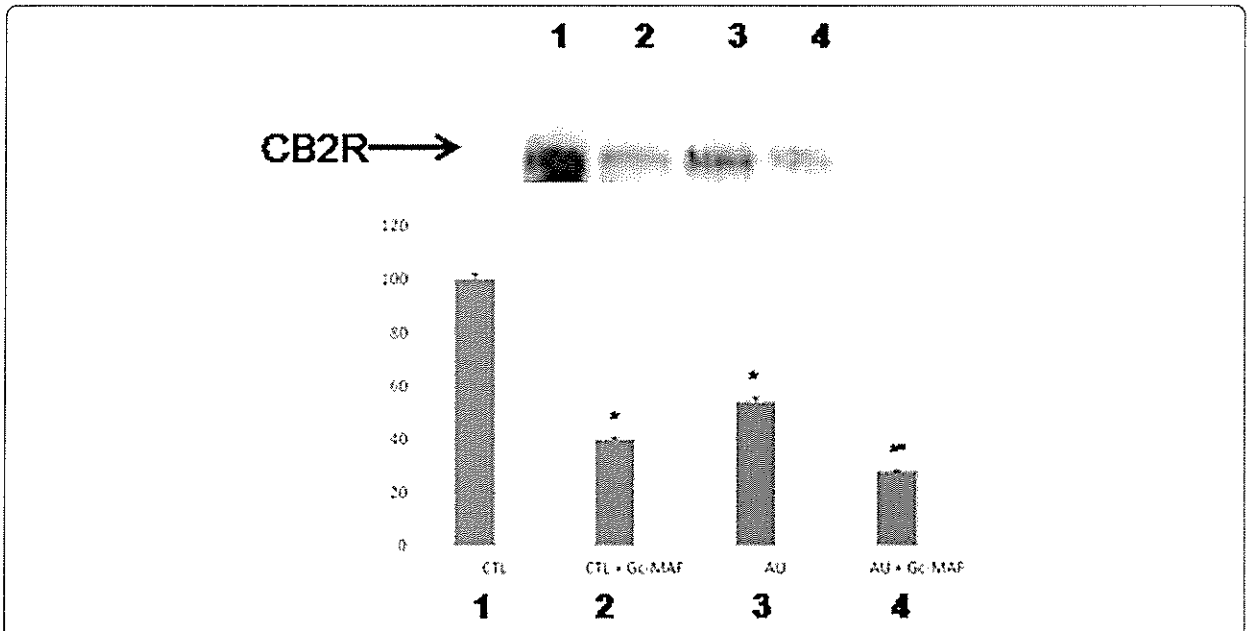

Figure 3 Representative Western blot analysis of CB2R protein levels in the blood monocyte-derived macrophages (BMDMs) obtained from the autistic children and the healthy controls. (1) untreated BMDMs from healthy control subjects; (2) GcMAF-treated BMDMs from healthy control subjects; (3) untreated BMDMs from autistic disorder subjects; (4) Gc-MAF-treated BMDMs from autistic disorder subjects. The histograms indicate percentage variations in CB2R protein levels in the BMDMs. * indicates significant difference versus healthy controls; ° indicates significant difference versusGcMAF untreated autistic BMDMs.

units: 5.97 ± 0.34 versus 2.39 ± 0.43, $P < 0.05$, before and after GcMAF treatment in healthy control BMDMs, respectively).

The difference between the unchanged *CB2R* mRNA levels and the decrease in CB2R protein levels in autistic GcMAF treated cells is not surprising, as we have already shown [19]. Indeed, protein levels and functions are affected by post-translational control. The levels of CB2R in the cell are strictly regulated in a multilevel system of regulation [34], and there is not a direct correlation between mRNA transcripts and protein levels [19].

GcMAF was able to trigger overall macrophage deactivation in autistic samples

In order to check cellular activation, fluorescence-based immunocytochemical analysis on macrophage cell culture was performed. In detail, Notch staining was early carried out. Notch is a protein mainly involved in stem cell maintenance, cell differentiation and cellular homeostasis regulation [35]; however, Notch signaling pathway was reported in activated pro-inflammatory macrophages and it is involved in regulating the expression of il12p40 [36]. Recently, it has been proposed a putative role of Notch signaling in autism [37]. We did not find any changes in Notch immunostaining profiles in GcMAF treated blood monocyte-derived macrophages from autistic children as compared to untreated macrophage cells, as analyzed by fluorescence microscopy (Figure 4).

As macrophages possess proliferation capacity [38], we investigated the effect of GcMAF on this cellular activity through Ki67 proliferation marker immunostaining. Interestingly, GcMAF treatment showed reduction in the immunostaining profiles of the proliferation marker Ki67 in GcMAF treated monocyte-derived macrophages from autistic children, as compared to untreated macrophage cells (Figure 4). We quantified this reduction through confocal fluorescence microscopy, showing a decrease of 23% in GcMAF treated monocyte-derived macrophages from autistic children as compared to untreated macrophage cells (mean ± SE of arbitrary units: 0.32 ± 0.04 versus 0.24 ± 0.06, $P < 0.05$, before and after GcMAF treatment in autistic BMDMs, respectively) (Figure 5). GcMAF was also able to reduce Ki67 immunostaining in BMDMs from healthy controls, as compared to untreated macrophage cells (mean ± SE of arbitrary units: 0.17 ± 0.01 versus 0.012 ± 0.003, $P < 0.05$, before and after GcMAF treatment in healthy control BMDMs, respectively) (picture not shown). It is noteworthy to consider that BMDMs from autistic children were more activated than BMDMs from healthy controls (mean ± SE of arbitrary units: 0.32 ± 0.04 versus 0.17 ± 0.01, $P < 0.05$, in BMDMs from autistic children and healthy subjects, respectively).

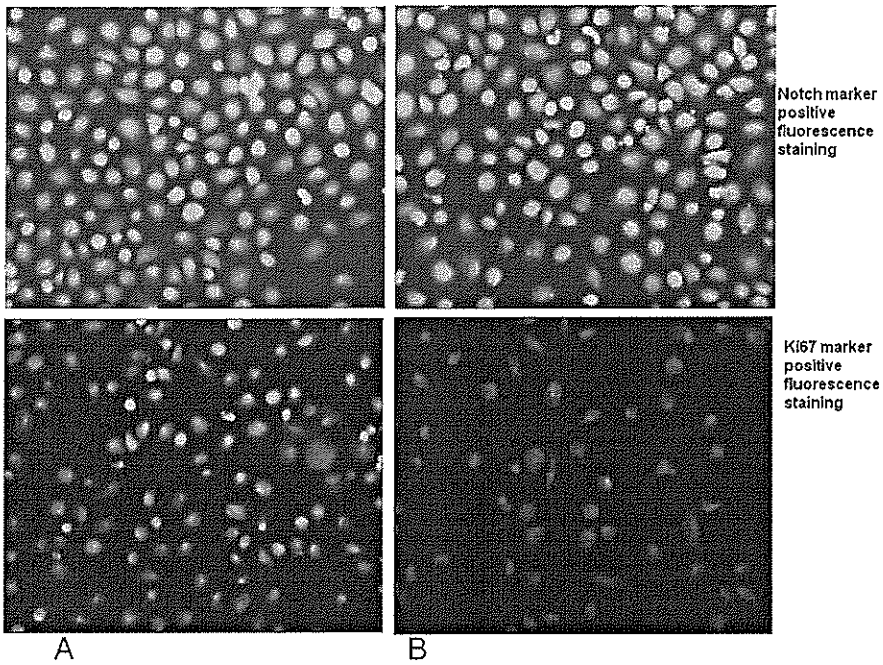

Figure 4 Representative fluorescent photomicrograph of blood monocyte-derived macrophages (BMDMs) from autistic patients showing immunocytochemistry (green fluorescent) for Notch (top) and Ki67 markers (bottom). To correctly identify cells, their nuclei were counterstained with bisbenzimide (blue fluorescence). **(A)** untreated BMDMs; **(B)** GcMAF treated BMDMs.

GcMAF was able to reduce the protein levels of the alternative activated phenotype M2 macrophage marker

To further investigate the GcMAF effect in specific cellular activation in macrophages, we quantified the protein levels of mannose receptor by Western blot analysis. The macrophage mannose receptor (alternative name CD206) mediates the endocytosis of glycoproteins by macrophages and it is considered a specific marker for alternative activated phenotype M2 macrophages [39,40]. Western blot analysis showed a decrease in mannose receptor protein levels in GcMAF treated BMDMs from autistic children as compared to untreated macrophages (mean ± SE of arbitrary units: 28.1 ± 0.41 versus 16.7 ± 0.94, $P < 0.05$, before and after Gc-MAF treatment in autistic BMDMs, respectively) (Figure 6). GcMAF was also able to decrease mannose receptor protein levels in

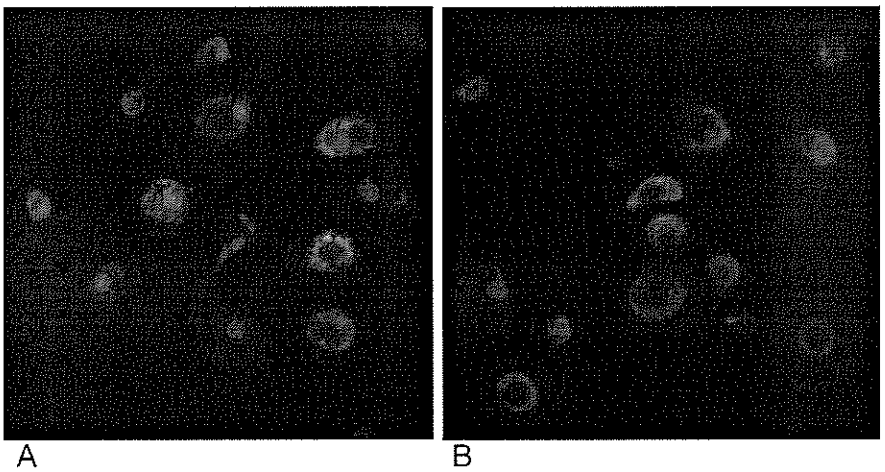

Figure 5 Representative quantitative fluorescent confocal-photomicrograph of blood monocyte-derived macrophages (BMDMs) from autistic patients showing immunocytochemistry (green fluorescent) for Ki67 marker. To correctly identify cells, their nuclei were counterstained with bisbenzimide (blue fluorescence). **(A)** untreated BMDMs; **(B)** Gc-MAF treated BMDMs.

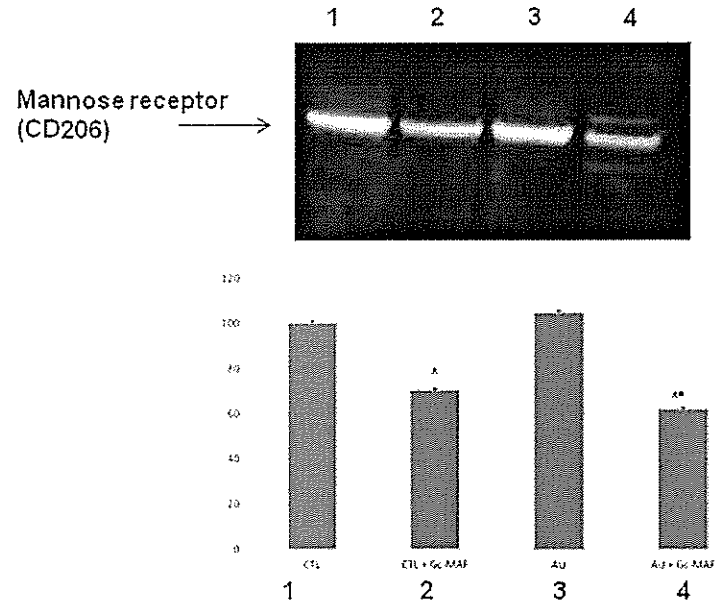

Figure 6 Representative Western blot analysis of mannose receptor protein levels in the blood monocyte-derived macrophages (BMDMs) obtained from the autistic children and the healthy controls. (1) untreated BMDMs from healthy control subjects; (2) GcMAF-treated BMDMs from healthy control subjects; (3) untreated BMDMs from autistic disorder subjects; (4) GcMAF-treated BMDMs from autistic disorder subjects. The histograms indicate percentage variations in mannose receptor protein levels in the BMDMs. * indicates significant difference versus healthy controls; ° indicates significant difference versus GcMAF untreated autistic BMDMs.

GcMAF treated BMDMs from healthy controls as compared to untreated BMDMs (mean ± SE of arbitrary units: 26.8 ± 0.21 versus 18.9 ± 0.47, $P < 0.05$, before and after GcMAF treatment in healthy control BMDMs, respectively) (Figure 6).

Discussion

In this study, we demonstrated for the first time a cannabinoid system mediated biomolecular mechanism and cellular effect of GcMAF in cultured BMDMs from autistic subjects.

Anti-cancer effects of GcMAF have been described since Yamamoto, *et al.*, [41] first demonstrated the vitamin D binding protein macrophage activating effects and the linkage to specific glycosylation of the Gc precursor protein [41]. It has been proposed that GcMAF possesses tumor killing activity through the activation of macrophages [42]. GcMAF-activated macrophages are indeed able to recognize the tumor cell surface abnormalities through a considerable variation of their receptors: in this way, they exert one potent tumoricidal effect [43].

Kanda *et al.*, [44] first described another anti-tumor effect of GcMAF related to its inhibition of angiogenesis, presumably mediated through the CD36 receptor, while Solinas *et al.*, [45] observed the non-psychoactive CB2R-binding cannabinoid, CBD also inhibited neoangiogenesis. Similarly, calcitriol (1,25-dihydroxyvitamin D3), also exerts anti-angiogenesis effects, creating an interesting potential nexus between vitamin D, GcMAF, and the EC system in the etiology and pathology of numerous immune-mediated disorders, including autism [46,47].

In relation to autism, the vitamin D deficiency hypothesis has been extensively investigated, with prenatal and/or early postnatal vitamin D deficiency demonstrating increased risks for development [48,49]. Autism is now considered a multifactorial disease associated with complex genetic and environmental interactions contributing to various risk factors [2]. Moreover, dietary vitamin D seems to be involved in complex epigenetic events. Vitamin D, via its ligand-activated nuclear hormone receptor, is involved in the regulation of pro-inflammatory genes, as well as key cellular events [2]. Nagalase activity is increased in the serum of autistic children [16]. As we previously mentioned, Nagalase is the enzyme responsible for deglycosylation of the vitamin D-binding protein (VDBP), also known as Gc-globulin (group-specific component). Gc-globulin is the precursor of GcMAF, so Nagalase interferes with macrophage regulation by reducing GcMAF production [50]. A predictable consequence of increased Nagalase activity in the serum of children with autism is, therefore, immunosuppression in a way similar to its observed effects in autoimmune patients, for example, SLE [18].

It has been demonstrated that in PBMCs, GcMAF is able to increase the production of the second messenger

cyclic AMP [28]. These data, when combined with our current observations demonstrating GcMAF normalizes EC gene expression, enhance the hypothesis of a potential action of GcMAF on the EC system. Indeed, the EC system is based on receptors coupled to G (i/o) proteins, which are associated with inhibition of cyclic AMP formation [51]. In BMDMs from autistic children, we evaluated whether the involvement of EC signalling could drive a decrease of cyclic AMP. We found an increase in AEA-biosynthetic enzyme NAPE-PLD, together with a decrease in the AEA catabolic enzyme FAAH expressions, indicating an overall increase in the EC AEA levels. AEA is a natural agonist of CB2R and down-regulates cyclic AMP production. Agonist-induced inhibition of adenylyl cyclase in cells expressing human CB2Rs has been demonstrated [52]. Our findings support the influence of GcMAF on the EC system which may result in normalized cycling AMP activity.

Our current findings further support the involvement of the EC system in autism associated immunological disruptions. We previously found that CB2Rs were strongly up-regulated in PBMCs from autistic children [19]. The observations in this present study agree with our hypothesis that the EC system in autism orchestrates the apparent nexus of the peripheral and central neuro-immunologically mediated effects in autism. Interestingly, while in our previous work, the CB2R was over-activated in PBMCs from autistic children, in the present study, we found a decrease in CB2R protein levels in BMDMs from autistic patients. Taking in account the difference between the two-cell systems, as BMDM cells are derived, through differentiation, from PBMCs, this result could indicate a dual role of the CB2R: activation in monocytes to trigger immune imbalance, and deactivation in differentiated macrophages to further persist in the immune dysregulation [19].

The fact that GcMAF was able to reduce Ki67 proliferation marker staining together with a decrease in CD206 positive profiles in BMDMs is not surprising. Indeed, it has been demonstrated that macrophages are altered in autism and this pathology is accompanied by an activation of the macrophages, together with immune alterations and pro-inflammatory cytokines (that is IL-1β) over-production [53,54]. Specifically, Al-Ayadhi and Mostafa [55] found macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC), both were significantly elevated in ASD serum and further demonstrated the level of elevation of both markers directly correlated with the severity of autism [55]. Molloy *et al.*, (2006) also demonstrated a predominately Th2 cytokine shift in the serum of children with autism [56]. In this study, our findings of pre/post Ki67 and CD206 are in agreement with the Th2/M2 macrophage observations and of a tendency toward autoimmunity. Macrophages are not static and can readily shift from immature forms and between M1 and M2 states depending on the local tissue signalling [57].

Recently, a different cohort of Italian children was assessed for anti-brain antibodies [58]. In that research, the presence of specific anti-brain antibody profiles was associated with the severity of cognitive impairment in autism.

The potential for either commensal or pathogenic microbes to trigger immune dysregulation and autoimmunity with resultant neuropsychiatric symptoms was recently reviewed by Hornig [59]. The CB2R profile observed in this study is consistent with these mechanisms and could be tied to either infection or alteration of the gut microbiome as illustrated in a mouse model of autism [60]. In that murine study, alteration in short chain fatty acids as a consequence of valproate exposure, modelled autistic characteristic in the mice.

EC are derived from dietary fatty acids. Several studies illustrate the effect of diet on blood, tissue and brain EC levels [61,62]. So, the measured differences in this study in the CB2R may be the consequence of dietary and/or microbiome changes in the autism population when compared to the controls.

Conclusions

This study demonstrates a biomolecular effect of GcMAF in BMDMs from autistic patients, providing further evidence for a positive use of this molecule in autism treatment. It also seems likely that the CB2R is a potential therapeutic target for ASD interventions. These initial findings will require further study in order to better elucidate the molecular pathways involved in GcMAF effects.

Abbreviations

ASDs: autism spectrum disorder; AEA: N-arachidonoylethanolamide; 2-AG: 2-arachidonoylglycerol; BMDMs: blood monocyte-derived macrophages; CBD: cannabidiol; CB2R: cannabinoid receptor type 2; DSM-IV-TR: Diagnostic and Statistical Manual of Mental Disorders-IV criteria for autism; EC: endocannabinoid; FAAH: fatty acid amide hydrolase; FBS: fetal bovine serum; NK: natural killer; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GcMAF: Gc Macrophage Activating Factor; IL: interleukin; NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D; PET: positron emission tomography; PBMCs: peripheral blood mononuclear cells; PBS: Phosphate buffer saline; PDD-NOS: Pervasive Developmental Disorder - Not Otherwise Specified; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction; RPMI: Roswell Park Memorial Institute; SLE: systemic lupus erythematosus; VDBP: vitamin D3 binding protein.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DS designed the study, carried out the biochemical and immunocytochemical experiments, performed the statistical analysis and wrote the manuscript. JJB participated in the design of the study, wrote the manuscript and edited English language. AC carried out the biomolecular experiments. NA conceived of the study, participated in its design and provided funding support. All authors read and approved the final manuscript.

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Author details
[1]Department of Experimental Medicine, Second University of Naples, via S. Maria di Costantinopoli, 16 - 80138 Naples, Italy. [2]Centre for Autism - La Forza del Silenzio, Caserta 81036, Italy. [3]Cancellautismo- no profit association for autism care, Florence 50132, Italy. [4]Brain Treatment Center of Atlanta, Atlanta, GA 30518, USA. [5]Western University of Health Science, 309 E. Second St., Pomona, CA 91766, USA. [6]Institute of Bioscience and Bioresources, National Research Council of Italy, Naples 80128, Italy. [7]Biomedical Center for Autism Research and Treatment, Bari 70126, Italy.

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