METHOD FOR PREDICTING AUTISM SPECTRUM DISORDERS BY CANNABINOID AND CANNABINOID RECEPTOR EXPRESSION

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The inventive method relates to a method for the determination of susceptibility or diagnosis of autism or autism spectrum disorders. Diagnosis or determination of susceptibility determinations are predicated on quantitative analysis of endocannabinoid levels or endocannabinoid receptor expression.
FIG. 1

Time Near Stranger Mouse (sec.)

Acetaminophen Dose (mg/kg)

* *
A.

[Graph showing Anandamide (pmol/g) for No behavior tests and Post sociability tests.]

SAL = saline (a), or saline +/- 10% DMSO

B.

[Graph showing Anandamide (pmol/g) for No behavior tests and Post sociability tests.]

ACM = acetaminophen (200 mg/kg), WIN = WIN 55,212-2 (0.1 mg/kg). N = 5-9 mice.

FIG. 4
Marble Burying

![Bar graph showing marble burying behavior for different treatments.]

Juvenile Development Drug Treatment

FIG. 5
2-AG

Juvenile Development Drug Treatment

FIG. 6
METHOD FOR PREDICTING AUTISM SPECTRUM DISORDERS BY CANNABINOID AND CANNABINOID RECEPTOR EXPRESSION

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/448,891, filed 3 Mar. 2011.

BACKGROUND OF INVENTION

[0002] 1. Field of Invention

[0003] The inventive subject matter relates to a method of diagnosing or predicting the propensity for autism using endogenous cannabinoids and/or cannabinoid receptor expression.

[0004] 2. Background Art

[0005] Autism spectrum disorders (ASD) are a spectrum of psychological conditions characterized by social interaction and communication deficits. Symptoms also include repetitive behavior that appear early in childhood, usually before age 3 years and often are accompanied by abnormalities in cognitive functioning. CDC, MMWR, 58 (SS10:1-20 (2009)) and American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders (4th ed), Washington, D.C. (1994). The prevalence of autism in the United States is approximately 1 in every 110 births (1 in 70 boys). CDC, MMWR, 58 (SS10:1-20 (2009)).

[0006] ASD encompass a range of behaviorally defined conditions. The five forms of ASD, including: Autism; Asperger syndrome; atypical autism (pervasive developmental disorder—not otherwise specified (PDD-NOS)); Rett syndrome; and Childhood Disintegrative Disorder. Asperger syndrome is closest to autism in signs and causes. Rett syndrome and Child Disintegrative Disorder have similar symptoms as autism, but their etiology may be unrelated. Volkmar, et al., J. Child Psychol. Psychiatry, 50: 108-15 (2009).


[0008] Autism can be co-morbid 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%. However, in most cases, the cause of autism is unknown (Fombonne, e., J. Autism Dev. Disord., 33:365-382 (2003)).

SUMMARY OF THE INVENTION

[0009] The current inventions relates to a method of determining indicia of autism or ASD by quantitating endocannabinoid levels.

[0010] An object of the invention is a method of determining the susceptibility of autism or ASD using biological markers comprising measuring endocannabinoid levels.

[0011] Another object of the invention is a method of diagnosing autism or ASD by quantitating endocannabinoid levels in a patient. The endocannabinoids comprise either one or more of Δ^2^-tetrahydrocannabinol (THC); N-arachidonylethanolamine (anandamide); N-palmitoylethanolamine (PEA); cannabidiol (CBD); 2-arachidonoylglycerol (2-AG); and N-oleoylethanolamine (OEA),

[0012] Another object is to measure endocannabinoids subsequent to acute exposure to acetaminophen or sociability testing.

[0013] A further object of the invention is the measurement of endocannabinoid levels, in a diagnostic method or method to determine susceptibility to autism or ASD by quantitating endocannabinoid receptors.

[0014] A still further object of the invention is the determination of susceptibility or diagnosis of autism or autism spectrum disorder by quantitating endocannabinoid synthesis and turnover.

BRIEF DESCRIPTION OF DRAWINGS

[0015] FIG. 1. Acetaminophen was administered by intraperitoneal injection 50 minutes prior to testing in the three-chambered social approach task. Bars show the mean±/−S.E. M. of each treatment group for this and all subsequent graphs. N=6 mice per dose. Acetaminophen doses of 100 mg/kg and 400 mg/kg increased dwelling near confined stranger mice by adult male BTBR.

[0016] FIG. 2. Effect of acetaminophen on social interaction marble burying behavior in BTBR mice. (a) Acetaminophen (ACM, 100 mg/kg) increased dwelling near stranger mice relative to vehicle-control (CTRL, saline±10% DMSO) treated BTBR mice (* p<0.05), while WIN 55, 212-2 (WIN) treatment increased lingering in the arena center (** p<0.05). (b) There was no difference among drug treatment groups in time engaged in social sniff of stranger mice (black bars) or time investigating the empty cup cage (white bars) during social interaction approach testing. (c) Administration of acetaminophen and WIN 55, 212-2 reduced time spent in the chamber of the arena with a novel stranger (* p<0.05), and this in this social novelty test WIN 55, 212-2 reduced dwelling in the arena center (** p<0.05). (d) Marble burying behavior was similar among BTBR mice, irrespective of drug treatment. N=7-14 mice per treatment group.

[0017] FIG. 3. Effect of acetaminophen on social novelty and marble burying behavior in 129S1/SvImJ mice. (a) Drug-treated and control (CTRL) mice spent similar amounts of time by stranger mice in a cage vs. an empty cage (novel object) in the social approach test. However WIN 55, 212-2 (WIN) treatment increased time spent by 129S mice in the arena center (** p<0.05), and reduced their exploratory behavior, as indicated by fewer chamber entries. (b) There was no difference in time spent sniffing stranger mice or empty cages among drug treatment groups during the social approach test. (c) Acetaminophen (ACM) increased dwelling near new strangers relative to controls, while WIN 55, 212-2 increased dwelling in the center chamber during the social novelty test (p<0.05 for each). (d) Acetaminophen treatment increased marble burying behavior relative to control or WIN 55, 212 treatments (p<0.05). N=9 mice per group.

[0019] FIG. 4. Anandamide levels in the frontal cortex of (a) BTBR mice or (b) 129S1/SvImJ mice 70 minutes following injection, or injection plus sociability testing. In (a) and (b), an (*) indicates significantly different from saline-injected controls that were not subjects in sociability testing.

[0020] FIG. 5. Effect of daily injections (×59 days) of early development (18-21 days postnatal) BTBR and C57BL/6 mice of acetaminophen, valproic acid or WIN 55, 212-2 on marble burying behavior.
FIG. 6. 2-AG levels after daily treatment of early postnatal development mice with acetaminophen, valproic acid or WIN 55,212-2

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The following terms are defined:

"Autism Spectrum Disorder" refers to a group of developmental disabilities that includes: Autism; Asperger syndrome; pervasive developmental disorder not otherwise specified (PDD-NOS or atypical autism); Rett syndrome; and childhood disintegrative disorder.

"Autism" refers to an autism spectrum disorder characterized by a neural development disorder leading to impaired social interaction and communication by restricted and repetitive behavior.

"Cannabinoid" refers to a class of chemical compounds including phytocannabinoids, which are oxygen-containing C_{21} hydrocarbons found in the plant species *Cannabis sativa* (i.e., marijuana), including metabolites and synthetic analogues thereof. Cannabinoids include, but are not limited to, cannabidiol, Δ2-tetrahydrocannabinol (THC) and cannabidiol. Cannabinoids as used here also refer to chemical compounds which mimic the actions of phytocannabinoids or have similar structure, such as endocannabinoids. Cannabinoids include compounds that have a high affinity for the cannabinoid receptor, such as THC and those cannabinoids that do not, such as cannabidiol.

Endocannabinoids refer to a class of compounds with similar structure to phytocannabinoids that are found in animals and that activate cannabinoid receptors.

"Diagnostic" refers to identifying the nature of a pathologic condition.

"Susceptibility of autism or autism spectrum disorder" refers to the likelihood of currently exhibiting or exhibiting in the future autism or one of the other autism spectrum disorders.

Endocannabinoids serve as intercellular, neuromodulatory lipid signals. They are involved in a variety of physiological processes including appetite, pain-sensation, mood and memory. Endocannabinoids are derivatives of arachidonic acid, as well as other poly-unsaturated fatty acids.

Endocannabinoids are endogenous ligands for cannabinoid receptors that are also bound by exogenously introduced cannabinoids, such as THC, the psychoactive component of Cannabis sativa (i.e., marijuana). The cannabinoids receptors are classified into two groups, CB1 and CB2.

Endocannabinoids include anandamide (N-arachidonoyl ethanolamine (AEA)), Δ2-tetrahydrocannabinol (THC), N-palmitoyl ethanolamine (PEA), cannabidiol, N-oleoyl ethanolamine (OEA), and 2-arachidonylglycerol (2-AG). AEA is produced mainly by a transacylase-phosphodiesterase-mediated pathway, initiating from the precursor N-arachidonoyl-phosphatidylethanolamine. The biosynthesis of 2-AG proceeds by rapid hydrolysis of inositol phospholipids by specific phospholipase C generating diacylglycerol (DAG), which is converted to 2-AG by a sn-1-DAG lipase.

AEA and 2-AG act primarily via cannabinoid receptors. There are seven trans-membrane spanning receptors that belong to the rhodopsin family of G protein-coupled receptors. The binding of endocannabinoids to CB receptors trigger various signaling pathways, such as the inhibition of adenyly cyclase, the regulation of ionic currents, the activation of focal adhesion kinase, of mitogen-activated protein kinase (MAPK) and of cytosolic phospholipase A2 and the activation of (CB1) or the inhibition (CB2) of nitric oxide synthetase. Both AEA and 2-AG are presumably taken up by cells through a specific carrier, which has not yet been clearly identified. Once inside cells, the cannabinoids are metabolized by multiple pathways.

Plasma endocannabinoid levels have been determined in humans and correlated with a number of maladies, including circulatory dysfunction (Quercioli et al., European Heart Journal 32: 1369-1378 (2011)), and mental health disorders such as anorexia nervosa (Monteleone et al., Neuropsychopharmacology 30: 1216-1221 (2005)).


Cannabinoid receptors can be classified into two known groups, CB1 or CB2. Developmental problems associated with the endocannabinoid system may occur through either of these two receptor classes.

CB1 receptors are located in the central nervous system (CNS), peripheral nervous system, and peripheral organs. In the CNS, CB1 receptors are concentrated in the cerebellum, hippocampus, and the basal ganglia (Drysdale and Platt, Curr. Med. Chem. 10:2719-2732 (2003)), which are areas in the brain implicated as important in autism (Courchesne et al., Neuroreport 16:399-413 (2007), Bauman and Kemper, Int. J. Dev. Neurosci. 23:183-187 (2005)). During fetal development, CB1 receptors activation is important for neuron differentiation and proper axonal migration (Pride et al., Vittam. Horm. 81:139-158 (2009)). In addition, recent studies suggest that CB2 receptors define a unique positive feedback cycle (Harkany et al., Curr. Opin. Neurobiol. 18:338-345 (2008)). Modulation of CB1 cannabinoid receptors may also trigger autism by interrupting normal brain development.

CB2 receptors are primarily located in immune tissues and cells and may serve a regulatory function. CB2 receptors have been implicated with regulation of movement of inflammatory cells to the site of injury (Lunn et al., Br. J. Pharmacol. 153:226-239 (2008)). The activation of CB2 receptors may also slow the progression of Alzheimer’s disease by stimulating beta-amyloid removal by macrophages (Tolon et al., Brain Res. 1283:148-154 (2009)).

In a preferred embodiment, diagnosis of autism spectrum disorder or autism is determined by a method comprising quantitating endocannabinoid levels. In this embodiment, endocannabinoid levels are quantitated from serum samples collected from patients, ensuring against dietary fluctuations in endocannabinoids (Monteleone, et al., Neuropsychopharmacology, 30: 1216-1221 (2005); Habayeb et al., JAMA 299: 1135-1136 (2012); Zozner et al., Anal Bioanal Chem. (online version: doi:10.1007/s00216-011-5725-9) (published 4 Feb. 2012)). Deviations of endocannabinoid lev-
els, compared to normal levels, is indicative of autism spectrum disorder or susceptibility to the disorder. [0039] In another embodiment, endocannabinoid levels can be indirectly assessed by quantitating cannabinoid receptor levels. The embodiment avoids any variability inherent in serum endocannabinoid levels. Quantitation of receptors has the added potential advantage of improved accuracy since low levels of endocannabinoids can induce (i.e., upregulate) endocannabinoid receptors in the brain.

[0040] In a preferred embodiment, a diagnosis or determination of susceptibility to autism or autism spectrum disorders is made in individuals by first administering high doses of acetaminophen (Kozier, et al., Acta Paeadiatr., 95: 1165-71 (2006)), typically 12 mg/kg. Alternatively, individuals are subject to sociability testing. Afterwards, the level of plasma endocannabinoids is quantitated. Endocannabinoids include Δ⁴—tetrahydrocannabinol (THC); N-arachidonylethanolamine (anandamide); N-palmitoylethanolamine (PEA); canabidiol (CBD); 2-arachidonyl glycerol (2-AG); and N-oleylethanolamine (OEA), wherein said diagnosis or determination of susceptibility is made if the quantitated endocannabinoid level of said individual is different from the mean of the range of individuals, of the same age group, without autism or autism spectrum disorders.

[0041] In another embodiment, a diagnosis or determination of susceptibility to autism or autism spectrum disorders is made if the 2-AG level is at least twenty percent (20%), or for AEA, if the AEA level is forty five to seventy five percent (45%-75%), above or below the mean level for non-autistic individuals or individuals without autism spectrum disorders.

Example 1

Anandamide Levels as Indicator of Autistic Behavior

[0042] Autism is associated with impairments in social interaction; communication; and restricted interests and repetitive behavior. The ability to study the physiological and molecular mechanisms associated with this disease is hampered by suitable models. However, several inbred mouse strains demonstrate inherent behaviors paralleling these sociability impairments. These include the strains BTBR and 129S1/SvImJ (Moy, et al, Behav. Brain Res., 176: 4-20 (2007); McFarlane, et al., Genes Brain Behav., 7: 152-163 (2008); Defensor, et al., Behav. Brain Res., 217: 302-308 (2010); Spencer, et al., Autism Res., 4: 40-56 (2011)).


[0045] Studies were conducted to determine the effects of acute administration of acetaminophen on social interaction and endogenous cannabinoid levels in the anterior cingulated region of the frontal cortex. These studies were conducted using the socially-impaired mouse strains BTBR and 129S1/SvImJ. The cingulated cortex was targeted because serotonergic tone in this region is linked to anxiety and emotional states that shape social behavior.

[0046] BTBR T+sc/J, 129S1/SvImJ and C57BL/6 mouse colony founders were originally obtained from the Jackson Laboratory (Bar Harbor, Me., USA). These strains were bred in the animal facilities of the University of Texas Health Science Center at San Antonio through 2 generations. After weaning at 23-25 days of age, male littermates were housed in groups of 4-5 per cage until behavioral testing at 3-4 months of age. Mice had ad libitum access to food (Teklad™ rodent diet, Harlan, Indianapolis, Ind., USA) and water in ventilated clear plastic cages lined with chipped wood bedding. The housing room had a 12 h light/dark cycle (lights on/off at 7:00) and was maintained at 20-22°C.

[0047] Mice were administered acetaminophen (1-400 mg/kg, Sigma, St. Louis, Mo, USA) or 0.9% saline solution by intraperitoneal (i.p.) injection. The cannabinoid agonist WIN55, 212-2 (Ascent Scientific, Princeton, N.J., USA) was dissolved initially in dimethyl-sulfoxide (DMSO Sigma) and was diluted with saline (1:10) to administer 0.1 mg/kg i.p. in 10% DMSO to mice. A sub-group of control mice were treated with 10% DMSO in saline vehicle, these mice did not differ significantly from saline-treated mice in behavioral tests (F₁,₇<1.25; p>0.3 for all parameters), so the two treatment groups were pooled. Injections were given 30 minutes prior to introduction to the testing arena for conditioning, and 50 min prior to behavior testing.

[0048] The three-chamber sociability testing procedure for mice is described in detail in Yang et al. Curr. Protoc. Neurosci., Chapter 8: Unit 8. 26 (2011), our study was conducted in a manner consistent with that protocol. Mice were introduced into the center chamber of an acrylic three-chamber sociability arena, measuring 30x22x61 cm with a light tan bottom, black side walls and two transparent interior walls with slit door openings of 10 cm spaced 17 apart dividing the arena into 3 chambers, for 20 min prior to behavioral testing. As described in Gould et al. J. Neurochem., 116: 291-303 (2011), pre-conditioning was performed under low red light (16 lux) at first for 10 min with the mouse confined in the center chamber, then with the doors opened so the subject could explore the entire arena for 10 min longer. Just prior to testing, subjects were briefly confined in the center chamber while an empty wire cup cage was placed at one end of the arena, and a stranger mouse of the same strain was placed under an identical cup cage at the opposite end. Stranger mice were neither litter- nor cage-mates of the subjects and were housed in a separate ventilated cage rack. Stranger mice were pre-conditioned under cup cages in 3 sessions of 30 min each in the day(s) prior to testing, separated by 1 hour reprises in their home cages. Cup cages were topped with weighted jars (9 cm
The social approach test ended with confinement of the subject into the center chamber and closing of the slot doors. A new stranger mouse was then placed under the empty cup cage, the doors were opened, and behavior was recorded for another 10 min under low red light to assess preference for social novelty. At the end of the test, stranger mice were returned to their home cages for use in subsequent tests, and subject mice were removed from the arena and placed in a marble burying test. The number of both in the center of the arena was counted prior to cleaning the arena with a solution of 70% EtOH and paper towels prior to conditioning and testing the next animal. Digital videos were analyzed for box entries, time in box and social sniffing time by observers blind to mouse strain or drug treatments.

Marble burying was assessed in a dark room (<16 lux) by placing 15 or 20 blue marbles on top of fresh wood chip bedding filled to a depth of 4-5 cm in a 22x22 cm clear acrylic rat cage covered with a filter top. Mice were placed in the cages to bury marbles for 30 min. Marbles that were at least ½ covered by bedding were considered buried, as described previously (Gould et al., J. Neurochem., 116: 291-303 (2011)). Following the marble burying task, mice were sacrificed by cervical dislocation and decapitation; their brains were removed and frozen on powdered dry ice. Cingulate cortex was isolated and stored at −80°C for subsequent measurement of endocannabinoid levels.

To measure levels of the fatty acid amides 2-arachidonyl glycerol (2-AG), anandamide (AEA), and oleoylthanolamide (OEA), frozen cingulate cortex samples were spiked with 50 pmol of [3H]lanamandamide, [3H]oleoylthanolamide and [3H]2-arachidonyl glycerol (internal standards) and processed as described in Hardison et al., Prostaglandins Other Lipid Mediat., 81: 106-112 (2006). Briefly, lipids were extracted by adding methanol/chloroform/water (1:2:1, v/v/v) and the chloroform layer was further purified by solid phase extraction using C18 Bond Elut cartridges (100 mg; Varian, USA). Endocannabinoid-containing fractions were analysed by gas chromatography/chemical ionization mass spectrometry (GC/MS) using an isotope dilution assay as described in Seiller et al. Int. J. Neuropsychopharmacol., 3: 373-386 (2010).

Effects of Acetaminophen in Socially-Deficient Mice

The dose-response relationship for acetaminophen (1,400 mg/kg i.p.) to promote dwelling near a stranger mouse in the three chamber social approach test was initially determined in adult male BTBR mice. The lowest dose of acetaminophen to significantly increase time spent in the chamber with a stranger mouse above that of saline-injected controls was 100 mg/kg (F(4,25)=4.6, p<0.01), as illustrated in Figure 1.

In subsequent three-chamber sociability tests, global repeated-measured ANOVA revealed significant interactions across acute drug treatments, test phase (social approach vs. social novelty) and duration of time spent in each side chamber (F(INTERACTION 2.31)=7.21, p<0.005). In the social approach test, there was a significant interaction between drug treatment and chamber preference in adult male BTBR mice as determined by mixed-model ANOVA (F(INTERACTIONS 2.31)=5.3, p<0.01). Acetaminophen (100 mg/kg) treated BTBR mice spent more time in the chamber with the stranger mouse and less time in the chamber with the empty cage than either vehicle controls (saline10% DMSO) or WIN 55, 212-2 (0.1 mg/kg) treated mice (F(2,31)=4.8, p<0.025) (Figure 2a). However, there were no differences among drug treatment groups in time engaged in social sniff of the stranger mouse, or investigation of the empty cage, during the social approach test (F(2,31)=1.1, p=0.35 for both comparisons, see Figure 2b). Chamber entries did not differ among drug treated BTBR mice, and were on average 41±5 during the 10 min social approach test. In the social novelty test, mixed model ANOVA revealed a significant interaction between drug treatment and chamber preference (F(INTERACTION 2.31)=3.4, p<0.05). Both acetaminophen and WIN 55,212-2 treated mice spent less time in the box with the novel stranger than controls (F(1,31)=40, p<0.001), and WIN 55, 212-2 treated mice spent less time than the other groups in the arena center (F(2,31)=3.4, p<0.05), as shown in Figure 2c. Chamber entries for WIN 55,212-2 treated BTBR mice were lower (19±3) than either vehicle-control (45±5) or acetaminophen-treated (50±7) mice (F(2,31)=5.4, p<0.01) during the social novelty test. Although there was a trend toward WIN 55212 treated mice burying fewer than controls, there was no significant difference in marbles buried by BTBR mice among treatment groups (F(2,17)=2.4, p=0.12, Figure 2d).

Adult male 129S1/SvImJ (129S) mice exhibited global differences in chamber dwelling patterns among drug treatments (F(2,24)=7.4, p<0.003) and across the two sociability test phases (F(1,24)=5.0, p<0.03), without interaction, in repeated-measures ANOVA comparisons. In the social approach test, all groups spent essentially equal time in the chamber with a stranger mouse and in the chamber with an empty cage (Figure 3a). However, 129S mice treated acutely with WIN 55,212-2 (0.1 mg/kg) spent more time than acetaminophen (100 mg/kg) or vehicle (saline10% DMSO) treated mice in the center chamber (F(2,24)=5.0, p<0.02), WIN 55, 212-2 treated mice also made fewer chamber entries on average (9±4), than either acetaminophen treated (21±5) or control mice (27±5) during the social approach test (F(2,24)=3.6, p=0.05). Time spent sniffing the stranger mouse or investigating the empty cage did not differ among drug treatment groups (Figure 3b), although there was a trend toward acetaminophen-treated 129S mice spending more time investigating strangers that did not reach significance (F(2,24)=2.5, p<0.1). In the social novelty phase (Figure 3c), acetaminophen-treated 129S mice spent significantly more time than controls in the arena chamber with a new stranger mouse, while WIN 55,212-2 treatment increased dwelling in the center chamber of the arena (F(2,24)=3.0, p<0.05). The number of chamber entries in the social novelty test was similar across drug treatment groups, and averaged 21±4 for all 129S mice over 10 min. Marble-burying in acetaminophen-treated mice was greater than in vehicle-control or WIN 55,212-2 treated 129S mice (F(2,24)=4.0, p<0.05), as shown in Figure 3d.

BTBR mice that were either treated with acetaminophen and returned to home cages, or were saline-treated subjects in sociability tests had significantly higher anandamide (AEA) levels in frontal cortex (drug effect and interaction F(1,10)=4.11, p=0.05, Fisher’s LSD post hoc p<0.05, N=5-7) than saline-treated controls, but these effects were not additive, as shown in Figure 4a. Oleoylthanolamide (OEA) levels did not differ among treatment groups (effects and interaction F(1,8)=2, p=0.18), and were on average 62±6 pmol/
g. Levels of 2-arachidonylglycerol (2-AG), also did not differ significantly among treatments, in these studies. However, there was a trend (behavior effect $F_{1,20}=3.66$, $p=0.07$) toward the BTBR mice that performed sociability tasks having slightly higher 2-AG levels (4.7±0.3 nmol/g) than those that did not (3.9±0.2 nmol/g).

In contrast, neither sociability testing nor acetylamo-
nophen treatment increased anandamide levels in the frontal cortex of 129Sv/SvImJ (129Sv) mice. Instead, all treatments reduced anandamide levels by ~20% relative to saline-treated, behavior native controls (drug effects $F_{2,25}=4.29$, $p=0.05$; Fisher’s LSD p<0.05), as shown in Fig. 4b. OEA levels were similar among 129Sv treatment groups, and were 82±4.5 pmol/g on average ($F_{2,25}<1.0$, $p=0.33$). However, 2-AG levels were higher ($F_{2,25}=15$, $p<0.001$; Fisher’s LSD p<0.001) in saline-treated sociability test subjects (3.5±0.2 nmol/g) than in all other 129Sv groups (2.3±0.2 nmol/g).

In other studies, in BTBR mice, acetalaminophen had significant affect on 2-AG levels. In these studies, BTBR and C57BL/6J mice, in early (18 to 21 days postnatal) development, were administered either 4 daily saline or 10% DMS/ saline injections; 4 daily injections (i.p.) of 100 mg/kg acetalaminophen; 1 injection (i.p.) of 400 mg/kg valproic acid or 4 daily injections (i.p.) of 0.01 mg/kg of WIN 55,212-2 for 2 months (i.e., 59 days). The mice were then tested for marble burying and evaluated for neurochemical measures. As shown in Fig. 5, acetalaminophen induced significant marble burying behavior. Coincident to this behavior was a diminu-
tion of 2-AG levels, as shown in Fig. 6.

The results show that acute changes in social behav-
ior of adult male mice, with inherently low sociability, are modified by indirect activation of CB1 receptors by elevated levels of the endocannabinoids anandamide in BTBR mice and 2-AG in 129SvImJ mice in the cingulate region of the frontal cortex. As such, the level of anandamide is an indicator of social disfunction associated with autism spectrum disor-
ders.

The pain-relieving properties of acetalaminophen appear to be mediated, in part, through cannabinoid CB1 receptor activation and serotonin (5-HT1) system modulation. Acetalaminophen is unlikely to act as a direct agonist at CB1 receptors, instead the FAAH inhibitor CB1 ligand AM404 is produced through its metabolism, and AM404 increases levels of endogenous cannabinoids such as anandamide and 2-AG in extracellular fluid to activate CB1 receptors indirectly (Högestätt et al., 2005; Bertolini et al., 2006; Schultz, 2010). Based on the results of these studies, quantitation of endocannabinoids, such as anandamide, are of value in diagnosing or determining susceptibility to autism spectrum disorders.

**Example 2**

**Endocannabinoid Receptor Level Analysis**

Cannabinoid receptors modulate serotonin signaling in the cingulated cortex region. Serotoninergic tone is greater in the frontal cortex of fatty acid amide hydrolase (FAAH) knock-out mice. Their social behavior is enhanced, presumably due to higher levels of endogenous cannab-

inoid-agonist anandamide (Cassano, et al., Pchropharmacol-

Endocannabinoids can be indirectly assessed by measuring expression of endocannabinoid receptors. In a preferred embodiment, positron emission tomography (PET) is used to measure CB1 receptor levels in the brain. In other embodiments, PET can be combined with computed tomography (CT) or magnetic resonance imaging (MRI) scans. This embodiment will enable discernment of receptor expression as a function of anatomical structure.

PET is a nuclear imaging technique that produces three-dimensional images of functional processes in the body. Typically, the system is used for the detection of gamma rays emitted indirectly by a position-emitting radionuclide tracer, which is introduced into the body attached to a biologically active molecule.

An embodiment of the inventive method is the analysis of cannabinoid receptors following the introduction of radio-labeled endocannabinoid receptor ligand, such as anandamide or 2-AG, or functional analogs of these molecules, into patients. Although any radio-labeled tracer, suitable for PET can be used, in a preferred embodiment, radio-labeled agonists of CB1 receptors, or their analogs, can be utilized, such as tannabrant (N-((1S,2S)-3-(4-Chlorophenyl)-2-(3-cyanophenyl)-1-methylpropyl)-2-methyl-2-((5-(trifluoromethyl)pyridine-2-yloxy)propionamide). As examples, the following are potential tracer molecules: In other embodi-

The raw data collected through PET (i.e., coincidence events) are then grouped into projection images (i.e., sonograms). After raw data collection, the data is pre-pro-
cessed in order to remove artifacts, such as: random coinci-
dences, estimation and subtraction of scattered photons, detector dead-time correction and detector-sensitivity correction, etc. Reconstruction of the final image is conducted by any number of available algorithms. These include filtered back projection and iterative expectation-maximization algo-
rithms. In a preferred embodiment, correction for differential attenuation of photons will be corrected.

Receptors levels determined through analysis of PET imaging will be directly compared to that for normal, non-autistic levels. Higher cannabinoid receptor levels, compared to receptors levels found in children of similar age, is diagnostic of autism or ASD or susceptibility of disease.

**Example 3**

Diagnosis of Autism or ASD by Endocannabinoid Metabolic Turnover

non-autistic children. Additionally, autistic children also exhibited abnormal cortical asymmetries of serotonin synthesis affecting either the left or right cortex.

[0067] The current method utilizes, as indicia of autism or ASD, direct quantitative analysis of endocannabinoid levels. In one embodiment, serum samples of patients are collected and the endocannabinoid levels quantified by a number of techniques. Examples of quantitation methods include, but are not limited to, liquid chromatographic and mass spectrometry (Palandru, et al, 2011. Chromatogr. B. 887:2052-2060 (2009); Sipe, et al, PLoS ONE 5:e8792 (2010)).

[0068] In an additional embodiment, quantitative assessment of endocannabinoids, is conducted by analysis of turnover of endocannabinoid metabolites. In this embodiment, radio-labeled precursors include any precursor of endocannabinoids. Examples include, but are not limited to, N-arachidonoyl phosphatidylethanolamine (NAPE); arachidonic acid and cephalin (AEA) and/or using the 2-AG precursor diacylglycerol. Analysis of the conversion of these precursors can be quantitatively assessed by any of a number of ways. In one embodiment, assessment is conducted quantitation of radio-labeled endocannabinoids, bound to receptors, by PET. Alternatively, blood samples can be obtained and quantitative assessment of radio-labeled cannabinoids monitored by liquid chromatography and mass spectrometry.

[0069] Alternative to quantitative assessment of metabolites, metabolic byproducts can be analyzed. For example, anandamide, an endocannabinoid, is hydrolyzed by fatty acid amidase hydrolase (FAAH) into free arachidonic acid and ethanolamine. Arachidonic acid can be combined with p-amino phenol, a breakdown product of acetaminophen, to form AM404. In one embodiment, paracetamol can be administered to patients. Serum samples can then be analyzed for quantitative assessment of AM404 levels. Alternatively, radio-labeled paracetamol can administered and AM404 levels analyzed by PET.

[0070] Having described the invention, one of skill in the art will appreciate in the appended claims that many modifications and variations of the present invention are possible, in the light of the above teachings. It is, therefore, to be understood that, within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

What is claimed is:

1. A method of diagnosing or determining susceptibility to developing, autism or autism spectrum disorder comprising administering acetaminophen or applying sociability testing to an individual and quantitating the levels of one or more endocannabinoids in said individual and comparing said level or levels with that of a normal individual, wherein said endocannabinoid is selected from the group consisting of Δ⁹-tetrahydrocannabinol (THC); N-arachidonoyl ethanolamine (anandamide); N-palmitoylethanolamine (PEA); cannabidiol (CBD); 2-arachidonoyl glycerol (2-AG); and N-oleoyl ethanolamine (OEA), wherein said diagnosis or determination of susceptibility is made if the quantitated endocannabinoid level of said individual is different from the mean of the range of individuals, of the same age group, without autism or autism spectrum disorders.

2. The method of claim 1, wherein said method further comprises administering acetaminophen prior to said quantitating endocannabinoid level and wherein a diagnosis or determination of susceptibility is made if the said N-arachidonoyl ethanolamine (anandamide) concentration plasma is changed at least forty five to seventy five percent (45% to 75%) or if the said 2-arachidonoyl glycerol (2-AG) level is changed at least twenty percent (20%) from the mean in non-autistic individuals or individuals without autism spectrum disorders of a similar age group.

3. The method of claim 1, wherein said quantitation of endocannabinoid level is by quantitating cannabinoid receptor levels.

4. The method of claim 1, wherein said endocannabinoid levels are directly quantitated from patient sera.

5. The method of claim 1, wherein said determination of endocannabinoid levels by quantitating the level of AM404 synthesis.

6. The method of claim 1, wherein said endocannabinoid level is quantitated by analysis of endocannabinoid synthesis and turnover comprising the steps:
   a. Administering radio-labeled endocannabinoid precursors into an individual;
   b. Quantitating radio-labeled endocannabinoids

7. The method of claim 3, wherein said cannabinoid receptor levels are quantitated by a method comprising the steps:
   a. Administering radio-labeled endocannabinoid, cannabinoid analog or endocannabinoid precursor into an individual;
   b. Detecting binding of said radio-labeled cannabinoid;
   c. Comparing said binding of the radio-labeled cannabinoid to binding levels of non-ASD or autistic individuals.

8. The method of claim 5, wherein quantitating of AM404 comprises the additional steps comprising:
   a. Administering radio-labeled AM404 precursor to an individual;
   b. Collecting serum samples from said individual;
   c. Quantitating radio-labeled AM404 by liquid chromatography.

9. The method of claim of claim 5, wherein quantitating of AM404 comprises the additional steps comprising:
   a. Administering radio-labeled AM404 precursor to an individual;
   b. Measuring radio-labeled AM404 bound to receptors by positron emission tomography.

10. The method of claim 6, wherein said quantitating of radio-labeled endocannabinoids is by the additional step of quantitating radio-labeled endocannabinoid bound to receptors by positron emission tomography.

11. The method of claim 7, wherein said quantitating of radio-labeled endocannabinoids is by the additional steps:
   a. Collecting sera from individuals;
   b. Quantitating radio-labeled endocannabinoids by liquid chromatography.

12. The method of claim 7, wherein said radio-labeled cannabinoid includes ligands selected from the group consisting of: radio-labeled anandamide or 2-AG or their analogs that are functionally able to bind CB1 receptors.

13. The method of claim 7, wherein said detection of binding is by positron emission tomography.

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