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Acetaminophen Differentially Enhances Social Behavior and Cortical Cannabinoid Levels in Inbred Mice

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Abstract

Supratherapeutic doses of the analgesic acetaminophen (paracetamol) are reported to promote social behavior in Swiss mice. However, we hypothesized that it might not promote sociability in other strains due to cannabinoid CB₁ receptor-mediated inhibition of serotonin (5-HT) transmission in the frontal cortex. We examined the effects of acetaminophen on social and repetitive behaviors in comparison to a cannabinoid agonist, WIN 55,212-2, in two strains of socially-deficient mice, BTBR and 129S1/SvImJ (129S). Acetaminophen (100 mg/kg) enhanced social interactions in BTBR, and social novelty preference and marble burying in 129S at serum levels 70 ng/ml. Following acetaminophen injection or sociability testing, anandamide (AEA) increased in BTBR frontal cortex, while behavior testing increased 2-arachidonyl glycerol (2-AG) levels in 129S frontal cortex. In contrast, WIN 55,212-2 (0.1 mg/kg) did not enhance sociability. Further, we expected CB₁-deficient (+/−) mice to be less social than wild-type, but instead found similar sociability. Given strain differences in endocannabinoid response to acetaminophen, we

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compared cortical CB₁ and 5-HT_{1A} receptor density and function relative to sociable C57BL/6 mice. CB₁ receptor saturation binding (B_{max}= 958±117 fmol/mg protein), and affinity for [³H]CP55,940 (K_D= 3±0.8 nM) was similar in frontal cortex among strains. CP55,940-stimulated [³⁵S]GTPγS binding in cingulate cortex was 136±12, 156±22, and 75±9% above basal in BTBR, 129S and C57BL/6 mice. The acetaminophen metabolite para-aminophenol (1μM) failed to stimulate [³⁵S]GTPγS binding. Hence, it appears that other indirect actions of acetaminophen, including 5-HT receptor agonism, may underlie its sociability promoting properties outweighing any CB₁ mediated suppression by locally-elevated endocannabinoids in these mice.

Keywords

anandamide; autoradiography; CB₁ receptors; marble burying; paracetamol; social interaction

1. Introduction

Deficits in social interaction such as social anxiety, withdrawal, and inattentiveness are symptoms of many psychiatric conditions, including autism, schizophrenia, depression and post-traumatic stress disorder (Pinkham et al., 2008; Derntl et al., 2011; Feldman and Vengrober, 2011). Behaviors paralleling sociability impairments are inherent in several inbred strains of mice, including BTBR and 129S1/SvImJ (Moy et al., 2007; Defensor et al., 2011; Spencer et al., 2011). BTBR mouse social behavior is sensitive to changes in serotonin (5-HT) neurotransmission, since administration of the 5-HT reuptake inhibitor fluoxetine or 5-HT_{1A} agonist buspirone increased their sociability (Gould et al., 2011). However, local application of the endocannabinoid agonist anandamide (AEA) or high doses (50–200 mg/kg) of acetaminophen also promoted social interactions in Swiss mice (Umathe et al., 2009). Hence increases in central 5-HT transmission or endocannabinoid levels can promote social behaviors in mice. Acetaminophen is normally metabolized through sulfation conjugation pathways, but at high doses its metabolic deacetylation products include para-aminophenol and/or N-arachidonoylphenolamine (AM404), an AEA uptake blocker (Beltramo et al., 1997). These metabolites may activate cannabinoid (CB) receptors directly, or indirectly by raising extracellular endogenous CB levels in the brain (Högestätt et al., 2005; Ottani et al., 2006; Bertolini et al., 2006; Mallet et al., 2008). CB agonists such as WIN 55,212-2 can inhibit presynaptic 5-HT release in brain by activating CB₁ receptors found on cell bodies and axons of 5-HT neurons (Nakazi et al., 2000; Lau and Schloss, 2008). Acetaminophen might also be expected to inhibit social behavior via this mechanism, except endocannabinoids have different ligand properties and may be released as mixtures in a region-specific manner, in contrast to systemically-administered agonists.

We aimed to determine if acetaminophen-induced enhancement of Swiss mouse sociability found by Umathe et al. (2009): 1) would occur in socially-deficient strains, and 2) if it is mediated indirectly by AM404, or directly by para-aminophenol action at CB₁ receptors, since its derivatives have some (K_i~200 nM) affinity for them (Sinning et al., 2008). Hence, we examined acetaminophen's effects on social and repetitive behaviors in BTBR and 129S mice, and on endocannabinoid levels in frontal cortex.

Serotonergic tone in the frontal cortex is linked to anxiety and emotional states shaping social behavior (Filipenko et al., 2002; File and Seth, 2003; Boylan et al., 2007; Bartolomucci et al., 2010; Gerretsen et al., 2010). Cannabinoids modulate 5-HT signaling in this region, as exemplified by higher extracellular 5-HT levels in the frontal cortex of CB1 knock-out vs. wild-type mice (Aso et al., 2009). For this reason we measured 5-HT levels after acetaminophen or saline treatment, and compared the density and function of 5-HT_{1A} and CB₁ receptors in the frontal cortex of all strains.

Finally, several human CB₁ receptor gene polymorphisms alter their expression and/or function, and may be associated with social motivation and anxiety disorders (Chakrabarti et al., 2006; Lazary et al., 2009). In mice, CB₁ knock-outs exhibit similar social behavior to wild-types, but not under stressful conditions (Haller et al., 2004). Since CB₁ receptors are intricately involved in brain development, their absence may trigger confounding compensatory alterations in neural function or structure (Trezza et al., 2008; Hoyle et al., 2011). Hence, we compared the sociability of CB₁-deficient (heterozygous) mice to wild-type littermates to determine whether partial loss of CB₁ receptors alone would alter social or repetitive behaviors.

2. Methods

2.1 Mouse Subjects

All procedures involving mice were performed in accordance with guidelines for care and use of laboratory animals (National Institutes of Health), and were approved by the institutional animal care and use committee. BTBR T+tf/J, 129S1/SvImJ and C57BL/6 mouse colony founders were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were bred in the animal facilities of the University of Texas Health Science Center through 2 generations. After weaning (postnatal days 23–25), 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, Harlan, Indianapolis, IN) and water in ventilated clear plastic cages lined with chipped wood bedding. The housing room had a 12h light/dark cycle (lights on/off at 7:00) and was maintained at 20–22°C.

CB₁ knock-out mice on a C57BL/6 background strain were the product of wild-type × heterozygous crosses. To determine their genotypes, a 0.5–0.75 cm section of tail tip was collected from each mouse. Tail tips were added to a solution of 200 µL DirectPCR (Viagen Biotech, Los Angeles, CA) and 20 µL of 20 µg/µL Proteinase K (Sigma Chemical Co., St. Louis, MO), then incubated at 55° C until fully digested (5–14 hours). To extract DNA, samples were centrifuged and the supernatants were mixed with 500 µL isopropanol. Samples were centrifuged again, the supernatant discarded, and 200 µL of genomic buffer was added to the pellet before storage at –20°C. PCR amplification was carried out using 1 µL of DNA sample, 6 µL water, 10 µL AccuStart PCR Supermix (Quanta Biosciences, Gaithersburg, MD), and 0.5 µM of each primer G50: 5'-GCTGTCTCTGGTCTCTTAAA-3', G51: 5'-GGTGTACCTCTGAAAACAGA-3', G54: 5'-CCTACCCGGTAGAATTAGCTT-3' (Invitrogen, Carlsbad, CA). The PCR was carried out in a Techne TC 3000 thermo cycler (Barloworld Scientific, UK): 4 min at 94° C, 35 cycles of 45 sec at 94° C, 45 seconds at 51° C, 60 sec at 72°C, and concluded with 10 min at 72° C. Products were run on 1% agarose gel in 1X TBE buffer at 120V for two hours. The gel was transferred onto a UV Transilluminator (Spectroline, Westbury, NY) for analysis of band placement. A band at 342 bp signified homozygous knockout; 413 bp signified homozygous wild type; both bands signified a heterozygote.

2.2 Drug administration

Mice were administered acetaminophen (1 – 400 mg/kg, Sigma) or 0.9% saline solution by intraperitoneal (i.p.) injection. The cannabinoid agonist WIN55,212-2 (Ascent Scientific, Princeton, NJ) was initially dissolved in dimethyl-sulfoxide (DMSO) and was diluted with saline (1:10) to administer 0.1 mg/kg i.p. in 10% DMSO to mice. A subgroup of controls were treated with 10% DMSO in saline vehicle, they did not differ significantly from saline-treated mice in social and marble burying tests ($F_{1,7} < 1.25$; $p > 0.3$), so these groups were subsequently pooled. Injections were given 30 min prior to introduction into the testing arena, and 50 min prior to testing.

2.3 Behavioral testing and tissue collection

The three-chamber sociability testing procedure for mice was performed as described in Gould et al. (2011). Briefly, pre-conditioning was performed under low red light (16 lux) first for 10 min with the subject confined to the center chamber, then with chamber doors opened so it could explore the whole arena for another 10 min. Just prior to testing, an empty wire cup cage was placed at one end of the arena, and a stranger mouse of the same strain was placed under a cup cage at the opposite end. Stranger mice were pre-conditioned to cup cage confinement in 3 separate 30 min sessions prior to testing, and were neither litter- nor cage-mates of the subjects. Cup cages were topped with weighted jars (9 cm high $\times$ 7 cm diameter) to prevent mice from climbing on top of them. Digital video cameras (Photosmart R742, Hewlett-Packard, Palo Alto, CA) positioned on tripods over the arenas were turned on, the doors were removed and social approach behavior was recorded for 10 min under low red light.

The social approach test ended with subject confinement in the center chamber by closing the doors. A new stranger mouse was placed under the empty cup cage, the doors were re-opened, and behavior was recorded for another 10 min under low red light to assess preference for social novelty. After this test, stranger mice were returned to their home cages for use in subsequent tests, and subjects were removed from the arenas and placed in marble burying tests. Between animal subjects the arena was cleaned with 70% ethanol and dried. Digital videos were analyzed for chamber entries, dwelling and social sniff times by treatment-blind observers.

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 22×42 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 $2/3$ covered by bedding were considered buried, as described previously (Gould et al., 2011).

Following behavior tests, mice were sacrificed by cervical dislocation and decapitation. Trunk blood was collected into tubes containing 25 μ l of 20 mM ethylenediaminetetraacetic acid and centrifuged for 10 min at 5000 rpm, serum was stored at -20°C . Whole brains were frozen on dry ice and stored at -80°C .

2.4 HPLC measurements of acetaminophen in serum and 5-HT in brain

To quantify serum acetaminophen and para-aminophenol, a 100 μ L aliquot of thawed serum was mixed with 200 ng of the internal standard 3-acetamidophenol in 0.05 M aqueous sodium acetate solution (100 μ L). The mixture was extracted with ethyl acetate and the organic extract was dried under nitrogen. The residue from each sample separately was taken again in 0.05 M aqueous sodium acetate solution (200 μ L). An aliquot of 20 μ L from this mixture was injected on a μ -Bondapak, reverse phase C-18, 3.9×300 mm HPLC column (Waters Corporation, Milford, MA) and eluted with 0.05 M aqueous sodium acetate mobile phase. The peaks of acetaminophen and para-aminophenol in biological samples were recognized by co-injection with standard acetaminophen and para-aminophenol separately. Peaks were quantified by a combination of the methods of Wang et al. (1985) and Campanero et al. (1999).

5-HT levels in frontal cortex were measured by HPLC with electrochemical detection as previously reported (O'Connor, 2009), with modifications. Briefly, frozen tissue (20 ± 2 mg) was homogenized and deproteinated in 100 μ l methanol at 4°C , followed by centrifugation through a 0.2 μ m filter column. The resulting supernatants were further diluted in .435mM perchlorate in 13% methanol. Mobile phase consisted of 17.2% acetonitrile, .018mM perchlorate, 0.7 μ M EDTA and 2.54 mg/ml 1-octanesulfonic acid.

Peaks were detected and integrated using a BAS 502 isocratic liquid chromatographic system (Bioanalytical Systems, West Lafayette, IN) with a Ag/AgCl reference electrode and 3mm glassy carbon electrode at +950mV.

2.5 GC-MS measurement of brain endocannabinoids

To measure levels of the fatty acid amides 2 arachidonyl glycerol (2-AG), anandamide (AEA), and oleoylethanolamide (OEA), frontal cortex samples were cut from frozen brain at -20°C , weighed and spiked with 50 pmol of [$^2\text{H}_4$]anandamide, [$^2\text{H}_4$]oleoylethanolamine and [$^2\text{H}_5$]-2-arachidonyl glycerol (internal standards) and processed as in Hardison et al. (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, Palo Alto, CA). Endocannabinoid-containing fractions were analyzed by gas chromatography/chemical ionization mass spectrometry (GC/MS) using isotope dilution as in Seillier et al. (2010).

2.6 Saturation Binding in Frontal Cortex Membrane Preparations

Frontal cortices from 3–4 month old naïve male mice (2 per sample) were homogenized in 40 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4 at 25°C) at 29,000 RPM for 10 sec on a Polytron (Brinkmann-Kinematica, Bohemia, NY). These homogenates were centrifuged at $30,600 \times G$ for 10 min. The supernatants were discarded and pellets re-suspended in 25 ml buffer, and centrifuged at $30,600 \times G$. The final pellets were suspended in 10 ml of buffer. Protein concentration was determined with Bradford reagent (Sigma) by linear regression using bovine serum albumen (BSA) standards on a microplate reader (Synergy HT, Biotek, Winooski, VT).

Saturation binding to 0.003 – 12 nM [^3H] CP 55,940 (Perkin-Elmer, Boston, MA) was carried out in triplicate at 25°C for 2 hours in 50mM Tris buffer supplemented with 5% BSA as in Herkenham et al. (1991). Non-specific binding was defined with 1 μM WIN 55,212-2. Incubation was terminated with 4 ml of 4°C buffer, and labeled homogenates were captured by filtration under vacuum onto glass fiber filters soaked in 0.5% polyethyleneimine with a tissue harvester (Brandel, Gaithersburg, MD). Filters were washed 3 times with 4 ml of 4°C buffer. Bound [^3H] CP55,940 was measured on a liquid scintillation counter (Beckman, Brea, CA) with 61% efficiency. Data were analyzed by non-linear regression using DeltaGraph (Red Rock, Salt Lake City, UT).

2.7 Quantitative Autoradiography

Naïve 3–4 month old male mice were sacrificed by decapitation, their brains were frozen on dry ice, and stored at -80°C . Brains were sectioned 20 μm coronally from 1.3 to 1.6 mm pre-Bregma in a -18°C cryostat (Leica, Bannockburn, IL). Sections were thawed onto gelatin coated slides, desiccated at 4°C for 18–24h, and stored at -80°C .

For CB_1 receptor binding, brain sections on slides were pre-incubated in 50 mM Tris-HCl, 1% bovine serum albumin (BSA) buffer, pH 7.4 at 25°C for 30 min. They were then incubated in that same buffer containing 5 nM [^3H] CP55,940 for 2 h. Nonspecific binding was determined using 0.2 mM WIN 55,212-2 (Ascent Scientific, Princeton, NJ) in the incubation solution for a subgroup of slides. Brain sections were washed in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 % BSA at 4°C for 4 h. This procedure was adapted from Herkenham et al., (1991), except lower concentrations of BSA were used.

To measure 5-HT $_1\text{A}$ receptor density, [^3H] 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT, 2 nM, Perkin-Elmer) binding was performed exactly as described in Gould et al. (2011). Labeled sections were opposed to film (Biomax MR, Kodak, Rochester, NY) with

[³H] standards (American Radiolabeled Chemicals (ARC), St. Louis, MO) for 6 weeks. Autoradiograms were captured as digital images and calibrated using NIH Image-J (NIH, Bethesda, MD) as described previously (Gould et al., 2011).

The methods of Seillier et al. (2010) were used for agonist-stimulated GTP γ S binding. Briefly, sections on slides were equilibrated in buffer containing dithiothreitol (2 mM), pre-incubated in buffer containing 2mM GDP, and incubated in buffer containing 40 pM [³⁵S] GTP γ S in the absence (basal) or presence (agonist-stimulated) of 1 μ M CP 55,940, 8-OH-DPAT or para-aminophenol. Nonspecific binding was defined under basal conditions with 10 μ M GTP γ S. Labeled sections were opposed to film (Biomax MR, Kodak) along with [¹⁴C] standards (ARC) for 48 hours. All autoradiograms were digitally captured and calibrated as described in Gould et al. (2011). Specific agonist-stimulated [³H]GTP γ S binding was expressed as % above basal binding.

2.8 Statistical Analyses

Social interaction behavior was compared among treatment groups, first by global repeated-measures analysis of variance (ANOVA) to compare behavioral tendency shifts between the social interaction and social novelty phases of the test, then by mixed model ANOVA, with significant main effects or interactions (defined as $p < 0.05$) examined post-hoc using Fisher's LSD test. Mean times spent by treated BTBR mice in the chamber with the stranger mouse, as well as acetaminophen, endocannabinoid or 5-HT levels were compared using a two-tailed t-test, and saturation binding parameters were compared using one or two-way ANOVA, with significant effects examined post-hoc using Fisher's LSD test. For autoradiography, mean densities were compared by one-way ANOVA, with significant main effects evaluated by Newman Keul's test. Analyses were performed using Statistica (StatSoft, Tulsa, OK).

3. Results

3.1 Behavior tests: Social approach and marble burying

3.1.1 Acute effects of acetaminophen in BTBR 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 ($F_{4,25} = 4.6$, $p < 0.01$, LSD $p = 0.05$), as illustrated in Figure 1.

In subsequent three-chamber sociability tests, global repeated-measured ANOVA revealed significant interactions among acute drug treatments, test phase (social approach vs. social novelty) and duration of time spent in each side chamber ($F_{\text{INTERACTION } 2,31} = 7.21$, $p < 0.005$). In the social approach test, there was a significant interaction between drug treatment and chamber preference of BTBR mice as determined by mixed-model ANOVA ($F_{\text{INTERACTION } 2,31} = 5.3$, $p = 0.01$). Acetaminophen (100mg/kg) treated mice spent more time in the chamber with the stranger mouse and than either vehicle control (saline $\pm$ 10% DMSO) or WIN 55,212-2 (0.1 mg/kg) treated mice ($F_{2,31} = 4.8$, $p = 0.015$, LSD $p < 0.025$), and less time less time by the empty cage than controls (LSD $p < 0.01$), as shown in Figure 2a. However, there were no differences among 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 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 ($F_{\text{INTERACTION } 2,31} = 3.4$, $p <$

0.05). WIN 55,212-2 treated mice spent less time than others in the arena center ($F_{2,31} = 3.4$, $p < 0.05$, LSD $p < 0.025$), as shown in Figure 2c, tended to spend more time near the old stranger mice ($F_{2,31} = 1.9$, $p = 0.16$, LSD $p = 0.06$), and made fewer chamber entries (19 ± 3) than either vehicle (45 ± 5) or acetaminophen (50 ± 7) treated mice ($F_{2,31} = 5.4$, $p < 0.01$, LSD $p < 0.01$) during social novelty testing. Number of marbles buried did not differ significantly among groups, but a trend toward WIN 55,212-2 treated mice burying less than controls was evident ($F_{2,17} = 2.4$, $p = 0.12$, Figure 2d).

3.1.2 Acute effects of acetaminophen in 129S1/SvImJ mice—Adult male 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 the social approach test, all groups spent essentially equal time in chambers with a stranger and in chambers with an empty cage (Figure 3a). However, 129S mice treated with WIN 55,212-2 (0.1 mg/kg) spent more time than acetaminophen (100 mg/kg) or vehicle (saline $\pm$ 10% DMSO) treated mice in the center ($F_{2,24} = 5.0$, $p < 0.02$, LSD $p < 0.05$). WIN 55,212-2 treated mice also made fewer chamber entries on average (9 ± 4), than control mice (27 ± 5) but not acetaminophen treated mice (21 ± 5) during the social approach test ($F_{2,24} = 3.6$, $p < 0.05$, LSD $p < 0.025$). Time spent sniffing strangers or investigating empty cages 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 ($F_{2,24} > 3.0$, $p < 0.05$, LSD $p < 0.05$ for both). The number of chamber entries in the social novelty test was similar across 129S treatment groups, and averaged 21 ± 4 over 10 min. Marble-burying in acetaminophen-treated mice was greater than in vehicle-control or WIN 55,212-2 treated 129S mice ($F_{2,24} = 4.0$, $p < 0.05$, LSD $p < 0.05$), as shown in Figure 3d.

3.1.3 Cannabinoid receptor 1 (CB₁) deficient mouse behavior—CB₁ receptor heterozygous knock-out mice exhibited similar behavior to wild-type mice in social approach and novelty preference tests, and in the marble burying task, as illustrated in figure 4. Between the social approach and social novelty tests there was a significant interaction between phase of test (approach vs. novelty) and time spent in the end chambers ($F_{\text{INTERACTION } 1,26} = 41$, $P < 0.00001$). This was due to a shift in preference from the side containing the stranger mouse in the initial social approach phase (Figure 4a), to the side containing a new stranger in the subsequent social novelty phase of the test (Figure 4c). There was no significant difference in any other parameter measured in the CB₁ deficient mouse sociability tests ($F_{1,26} < 2.0$, $P = 0.1$). CB₁ wild-type mice made 37 ± 2.7 chamber entries per test, and CB₁ deficient mice made 34 ± 3.3 entries per test during each phase of sociability testing. Marble burying was also similar among CB₁ genotypes, as shown in figure 4d.

3.2 HPLC Analyses

3.2.1 Acetaminophen and para-aminophenol levels in serum—In BTBR mice serum acetaminophen levels were 87 ± 18 , 306 ± 72 , and 1403 ± 142 $\mu\text{g/ml}$, and para-aminophenol levels were 2 ± 0.2 , 2 ± 0.9 and 6 ± 0.2 $\mu\text{g/ml}$, respectively 70 min after injection of 100, 200 and 400 mg/kg acetaminophen. In 129S mice serum acetaminophen levels were 70 ± 5 $\mu\text{g/ml}$ and para-aminophenol levels were 3 ± 0.5 $\mu\text{g/ml}$, 70 min after 100 mg/kg acetaminophen injection.

3.2.2 Serotonin levels in frontal cortex—5-HT levels in the frontal cortex from behaviorally-tested BTBR mice treated with acetaminophen (48 ± 5 ng/mg) or saline (54 ± 4 ng/mg) that were sacrificed 100 min after treatment did not differ significantly ($t = -0.9$, $df = 12$, $p = 0.39$, $N = 7$).

3.3 Endocannabinoid levels in frontal cortex

Measures of endogenous cannabinoids in the frontal cortex from BTBR and 129S mice are summarized in table 1. BTBR mice that were either treated with acetaminophen and returned to home cages, or were saline-treated subjects in sociability tests had higher anandamide (AEA) levels in frontal cortex (drug effect and interaction $F_{1,19} > 4.11$, $p < 0.05$, LSD $p < 0.05$, $N = 5-7$) than saline-treated controls, but these effects were not additive. Levels of 2 arachidonyl glycerol (2-AG) and oleoylethanolamide (OEA) did not differ among treatment groups (effects and interaction $F_{1,8} < 2$, $p > 0.18$). However, there was a trend (behavior effect $F_{1,20} = 3.66$, $p = 0.07$) toward BTBR mice that performed sociability tests 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 behavior testing nor acetaminophen treatment increased AEA in the 129S frontal cortex. Instead, among behavior naïve mice, acetaminophen reduced AEA levels relative to saline controls (drug effect $F_{1,25} = 4.29$, $p < 0.05$; LSD $p < 0.05$). OEA levels were similar among 129S groups ($F_{1,25} < 1.0$, $p = 0.33$). However, 2-AG levels were lower ($F_{1,25} = 15$, $p < 0.001$, LSD $p < 0.001$) in drug treated groups than in vehicle-control behavior test subjects, as shown in table 1.

3.4 CB₁ Receptor saturation binding in frontal cortex

[³H]CP55,940 saturation binding in the frontal cortex revealed similar maximal binding (B_{max} , $F_{2,16} = 1.03$, $p = 0.37$) and dissociation constant values (K_d , $F_{2,16} = 0.72$, $p = 0.49$) for BTBR, C57 and 129S mice. Non-linear regression was used to fit these data to a single-site curve with Hill coefficients = 1. For each mouse strain, CB₁ receptor binding properties are summarized in table 2.

3.5 Quantitative autoradiography: CB₁ receptor density and G-protein coupling capacity

CB₁ receptor density in cingulate cortex, labeled by [³H] CP 55,940 specific binding, did not differ among BTBR, 129S or C57BL/6 mice ($F_{2,21} = 0.78$, $p = 0.47$, Figure 5a). Specific binding of [³H] 8-OH-DPAT to 5-HT_{1A} receptors in this brain region was higher in 129S mice than in other strains ($F_{2,21} = 5.0$, $p < 0.02$, Newman Keul's $p < 0.025$, Figure 5b). When [³⁵S] GTPγS binding was stimulated by 1μM of the CB₁ receptor agonist CP 55,940, C57 mice had lower specific binding than either BTBR or 129S mice ($F_{2,21} = 9.5$, $p < 0.002$, Newman Keul's $p < 0.005$, Figure 5c). In contrast, neither 1μM of para-aminophenol nor 1μM of 8-OH-DPAT were able to produce any [³⁵S] GTPγS binding in excess of 30% over basal binding. Basal binding did not differ among mouse strains ($F_{2,21} = 2.5$, $p = 0.1$) and was on average 225 ± 9 nCi/mg.

4. Discussion

4.1 Sociability promoting effects of acetaminophen

The neural circuitry involved in perception of physical pain and the experience of “social pain” or hurt feelings has been shown to overlap to some extent (Eisenberger et al., 2003; 2006). Magnetic resonance imaging and cerebral blood-flow studies indicate that activity in the anterior cingulate region of the frontal cortex is involved in both pain perception and emotional response to social rejection, and that this activity is suppressed by acetaminophen administration (Baliki et al., 2006; DeWall et al., 2010; Newberg et al., 2011). Consistent

with the idea of shared circuitry, acetaminophen administration is reported to reduce social pain associated with rejection (DeWall et al., 2010), and to promote social interaction (Chibnall et al., 2005) in clinical studies.

Umathe et al. (2009) were the first to describe that acetaminophen and andandamide could dose-dependently enhance social interaction behavior in Swiss mice, and that this effect could be blocked by the CB₁ antagonist AM251. In the present study, we found that acetaminophen also enhanced social behavior in BTBR T+/tf and 129S1/SvImJ mice, two inbred strains with inherently low sociability in three chambered tests. BTBR mice typically spend equal time investigating stranger mice and novel objects in social approach tests, but show a preference for social novelty (Moy et al., 2007). We observed that acetaminophen, but not the CB₁ agonist WIN 55,212-2, increased social interaction by BTBR mice in the social approach test, while WIN 55,212-2 treatment tended to diminish social novelty preference (Figure 2). In contrast, 129S mice normally exhibit both impaired social interaction, and a lack of preference for social novelty (Moy et al., 2007). Acetaminophen and WIN55,212-2 failed to enhance 129S social interaction in the social approach test, however, acetaminophen produced a preference for social novelty in 129S mice (Figure 3). Hence sociability was enhanced by acetaminophen in different ways in each strain.

4.2 Effects of acetaminophen or CB1 expression on marble burying

Marble burying by mice was initially thought to be an index of anxiety and obsessive compulsive behavior (e.g. Ichimaru et al., 1995), but a more recent study has characterized this genetically-variable but inherent behavior more properly as an index of restrictive-repetitive digging behavior, unrelated to novelty, and not correlated with other measures of anxiety (Thomas et al., 2009). In our study, vehicle-treated BTBR mice buried more marbles (13 ± 2 per 30 min) than 129S mice (3 ± 1.2 per 30 min), and acetaminophen only increased marble burying in 129S mice (Figures 2d and 3d). WIN 55,212-treated BTBR mice tended to bury fewer marbles than controls, this trend, which did not reach significance, is consistent with a recent study in which mice treated with a higher dose of WIN 55,212 (1mg/kg) buried significantly fewer marbles than controls (Gomes et al., 2011). Since marble burying is a behavior that is sensitive to various pharmacological effects (e.g. Gould et al., 2011), its use may aid discovery of effective interventions for repetitive behaviors found in various psychiatric disorders.

4.3. Region-specific endocannabinoid induction vs. global full-agonist effects

The acetaminophen-induced increase in BTBR cortical AEA (Table 1) may have contributed to their enhanced social behavior. Paralleling this finding, in fatty acid amide hydrolase (FAAH) knock-out mice AEA tone, 5-HT tone and social behavior are all enhanced (Cassano et al., 2011). In contrast, WIN55,212-2 failed to improve sociability, but instead tended to worsen it in our tests. This second finding is consistent with a study in rats in which inhibition of AEA breakdown increased social play, while WIN55,212-2 reduced it (Trezza and Vanderschuren, 2008). The inconsistent behavioral effects of this full CB₁ agonist and AEA could be due to WIN55,212-2 having a greater affinity for, and agonist effect at CB₁ and/or CB₂ receptors than endogenous cannabinoids (Ashton et al., 2008). Further, WIN55,212-2 may not mimic the regional specificity of endogenous cannabinoids, and may instead recruit areas with deleterious effects on social interaction, or it may have longer-lasting effects (Wiley et al., 2005). Alternatively, the different behavioral effects of these drugs might be attributed to initial variability in cannabinoid or 5-HT tone. For example, withdrawal in phencyclidine-treated rats impairs social behavior, but treatment with the FAAH inhibitor URB597 restores sociability; yet alone it suppressed sociability in naïve rats (Seillier et al., 2010).

It is of interest that the full cannabinoid agonist, WIN 55,212-2, reduced locomotor activity in all of our behavior tests. We selected a 0.1 mg/kg dose through a combination of prior literature indicating a dose 1 mg/kg increased open-arm entries in the elevated plus maze without impairing locomotion (Haller et al., 2004; Patel and Hillard, 2006). However, we found in our own pilot studies that 1 mg/kg WIN 55,212-2 completely inhibited locomotor activity in BTBR and 129S mice, resulting in no chamber entries in sociability tests. Based on this, we reduced the dose to 0.1 mg/kg for these experiments. An earlier study also found that WIN55,212-2 suppressed mouse locomotor activity at low receptor occupancy, with an effective dose of 0.3 mg/kg (Gifford et al., 1999). Yet WIN 55,212-2 had demonstrated sedative effects only at higher doses in other mouse studies (e.g. Cosenza et al., 2000; Rutkowska et al., 2006). Sensitivity to WIN55,212-2 may be strain-specific, such that BTBR and 129S mice are more responsive to it than C57BL/6 mice. This would be consistent with our CP55,940 stimulated GTP γ S binding results in the cingulate cortex (Figure 5c). We did not find any differences in the density of CB₁ receptors in the frontal or cingulate cortex among BTBR, 129S or C57BL/6 mice (Table 2 and Figure 5a), yet we found increased capacity for CB₁ agonist stimulated G-protein coupling in BTBR and 129S mice (Figure 5c). We also found no evidence of para-aminophenol stimulated G-protein coupling activity in the cingulate cortex among the strains we examined.

4.4. Acetaminophen, cannabinoid and 5-HT system interactions

The pain-relieving properties of acetaminophen appear to be mediated, in part, through both cannabinoid CB₁ receptor activation and 5-HT system modulation. Acetaminophen's analgesic effects are blocked by CB₁ receptor antagonists or by complete depletion of CB₁ receptors (Ottani et al., 2006; Dani et al., 2007; Mallet et al., 2008). However, acetaminophen is unlikely to act as a direct CB₁ receptor agonist. Instead the endocannabinoid transporter inhibitor AM404 is produced through its metabolism. AM404 itself has little affinity for CB₁ receptors (Beltramo et al., 1997), but it increases extracellular levels of endogenous cannabinoids such as AEA and 2-AG that activate CB₁ receptors (Högestätt et al., 2005; Bertolini et al., 2006; Schultz, 2010). However, other receptors are implicated in acetaminophen's effects, for example, its analgesic effects are lost in transient receptor potential vanilloid 1 (TRPV1) knock-out mice, and AM404 is a TRPV1 agonist (Mallet et al., 2010). Also, CB₂ receptors in the central nervous system may also modulate such responses (Onaivi et al., 2012).

Endocannabinoids also regulate glutamate signaling and plasticity in the frontal cortex (Lafourcade et al., 2007). Administration of the CB₁ agonists CP55,940, AEA, or WIN55,212-2 suppressed 5-HT release, perhaps by blocking glutamate transmission in the dorsal raphe, which projects neurons into the frontal cortex (Nakazi et al., 2000; Haj-Dahmane and Shen, 2009). Consistent with this, in CB₁ knock-out mice 5-HT negative feedback is lost, and 5-HT neurotransmission is facilitated (Aso et al., 2009).

Yet acetaminophen metabolites such as AM404 may also act as agonists at 5-HT receptors. For example, in a clinical study, antagonists of 5-HT₃ receptors effectively blocked acetaminophen's antinociceptive properties (Pickering et al., 2006). In rats, 5-HT_{1A} and 5-HT_{3/4} antagonists, as well as 5,7-dihydroxytryptamine-induced lesions of bulbospinal 5-HT pathways blocked acetaminophen-induced analgesia (Mallet et al., 2008). When the roles of 5-HT receptors in the analgesic actions of AM404 and acetaminophen were compared, the 5-HT₃ antagonist ondansetron blocked both, while the 5-HT₂ antagonist ketanserin blocked only acetaminophen's effects (Ruggieri et al., 2008). This latter result suggests that acetaminophen metabolites other than AM404 may activate 5-HT₂ receptors. Taken together, these findings suggest that enhancement, not suppression of serotonergic transmission is also involved in acetaminophen's analgesic actions.

Further evidence for this comes from discovery that both acute and sub-chronic acetaminophen administration reduced 5-HT₂ receptor density by 30% in rat frontal cortex (Sandrini et al., 2007), an effect blocked by p-chlorophenylalanine-induced 5-HT depletion in the brain (Pini et al., 1996); and that the 5-HT_{1&2} receptor antagonist methysergide blocked acetaminophen-induced changes in mouse hippocampal long term potentiation (Chen and Bazan, 2003). These findings suggest that acetaminophen is promoting 5-HT neurotransmission, instead of suppressing it, as might be expected to occur through CB₁ receptor activation. These outcomes may stem from high dose-acetaminophen (100 mg/kg) inhibiting the hepatic apoenzyme of tryptophan-2,3-dioxygenase (TDO), thereby increasing conversion of tryptophan to 5-HT in the brain, as was found in rats (Daya and Anoopkumar-Dukie, 2000). Another study concluded that Inhibition of 5-HT breakdown is unlikely to account for increased brain 5-HT levels 45 min after high dose (100–400 mg/kg) acetaminophen administration to rats, since monoamine oxygenase A activity and 5-hydroxyindoleacetic acid levels were unchanged (Courade et al., 2001). 5-HT levels in the frontal cortex of BTBR mice were not in excess of saline treated BTBR controls in frontal cortex 100 min after acetaminophen administration (100 mg/kg) in our study. However, this measurement was made in frozen frontal cortex collected at a later time than in either rat study, and acetaminophen and para-aminophenol may have been substantially cleared by then. It would be of interest to measure 5-HT turnover in the mouse frontal cortex, or extracellular 5-HT levels by microdialysis sooner after acetaminophen is given to see if 5-HT neurotransmission is enhanced. Nevertheless, social behaviors 50–70 min after acetaminophen administration were consistent with enhanced 5-HT neurotransmission, as occurs with 5-HT uptake blockade or buspirone treatment (Gould et al., 2011).

We also demonstrated that the potential for CB₁ agonist stimulated G-protein coupling in frontal cortex is much greater than for 5-HT_{1A} agonists, since we saw no evidence of strong 8-OH-DPAT (5-HT_{1A} agonist) stimulated G-protein activity in the cingulate cortex of any strain (Figure 5c). Yet acetaminophen could be acting at other types of 5-HT receptors, such as 5-HT_{2/3} and/or in other brain regions. For example, in the dorsal hippocampus of BTBR mice, 8-OH-DPAT produces significant increases over basal [³⁵S]GTPγS binding, and buspirone, a 5-HT_{1A} partial-agonist improved their sociability (Gould et al., 2011). Other key brain regions to social behavior that have yet to be identified might also respond similarly to acetaminophen treatment.

4.5. CB₁ deficient mice: Are they on the edge of impaired sociability?

We did not observe differences in sociability or marble burying between CB₁ heterozygous and wild-type mice (Figure 4). This was not surprising, since CB₁ knock-out sociability is similar to wild-type mice under dim lighting (Haller et al., 2004). However with bright lighting or in unfamiliar cages, Haller et al. (2004) found impaired sociability in CB₁ knock-outs. Four common and many additional rare functional CB₁ receptor gene promoter polymorphisms occur in human populations, and some are associated with susceptibility to substance abuse, anxiety disorders and social reward (Zhang et al., 2004; Chakrabarti et al., 2006; Lazary et al., 2009). Subtle differences in CB₁ affinity for endocannabinoids from such polymorphisms may underlie different responses to social cues (Chakrabarti et al., 2006). It is of interest that in CB₁ deficient mice, WIN55,212-2 stimulated [³⁵S]GTPγS binding was only 25% lower than wild-types, despite 50% lower CB₁ receptor density, and region-dependent differences were found in the efficacy/potency of partial-agonists that the authors thought may result from either differential G-protein availability, or activation of different subtypes (Selley et al., 2001). There has been little follow-up on the role CB₁ polymorphisms may play in the impaired social behaviors of autism and schizophrenia, but given the involvement of CB₁ receptors in brain development (Trezza et al., 2008), CB₁ deficient mice may be a useful model in which to study these effects.

5. Conclusions

Acetaminophen administration enhanced social behavior in adult male mice with otherwise inherently low sociability. This may be partially mediated via elevated cortical levels of AEA in BTBR mice and 2-AG in 129S mice. The behavioral effects of acetaminophen are distinct from the full CB₁ agonist WIN 55,212-2 which suppressed locomotor activity, and are consistent with enhanced 5-HT neurotransmission. Our findings suggest that therapeutic interventions that modulate endogenous cannabinoids in the frontal cortex may be useful for treating sociability impairments, while full agonists of CB₁ receptors may be of interest for treating repetitive behaviors.

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Abbreviations

2-AG	2-arachidonyl glycerol
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
ACM	acetaminophen
AEA	anandamide
AM404	N-arachidonyl-phenolamine
ANOVA	analysis of variance
CB	cannabinoid
DMSO	dimethyl-sulfoxide
FAAH	fatty acid amide hydrolase
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
OEA	oleylethanolamide
PCR	polymerase chain reaction
TRPV1	transient receptor potential vanilloid 1 cation channels
Tris	Tris(hydroxymethyl) aminomethane
WIN	WIN55,212-2

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Highlights

- Acetaminophen deacetylation metabolites include para-aminophenol and AM404
- These may increase anandamide in the frontal cortex of socially deficient mice
- Acetaminophen administration enhances mouse social and marble burying behaviors
- In socially-impaired mice cortical CB₁ receptor function, not density, is increased
- Mouse sociability in 3-chamber tests is not altered by CB₁ receptor deficiency

Figure 1. Acetaminophen doses of 100 mg/kg and 400 mg/kg increased dwelling near confined stranger mice by adult male BTBR mice

Acetaminophen was administered by i.p. injection 50 min prior to testing in the three-chambered social approach task. Bars show the mean $\pm$ S.E.M. of each treatment group for this and all subsequent figures. *More time dwelling in the chamber with a stranger mouse than saline controls, $p < 0.05$, $N = 6$ mice per dose.

Figure 2. Acetaminophen increased BTBR mouse social interaction but not marble burying
 (a) Acetaminophen (ACM, 100 mg/kg) increased dwelling near stranger mice relative to vehicle-control (CTRL, saline $\pm$ 10% DMSO) and WIN 55,212-2 (WIN) treated BTBR mice (* $p < 0.05$), and also decreased time spent in the chamber with the empty cage (** $p < 0.05$). (b) There was no difference among groups in time engaged in social sniff of strangers (black bars) or time investigating the empty cage (white bars) during the social interaction test. (c) Administration of WIN 55,212-2 reduced time dwelling in the arena center (***) $p < 0.05$ in tests of preference for social novelty. (d) Marble burying behavior was similar among all BTBR mouse treatment groups. $N = 7-14$ mice per treatment group.

Figure 3. Acetaminophen increased 129S1/SvImJ social novelty preference and marble burying
 (a) Drug-treated and control (CTRL = saline $\pm$ 10% DMSO) mice spent similar time by caged stranger mice vs. empty cages (novel objects) in the social approach test. However WIN 55,212-2 (WIN) treatment (0.1 mg/kg) increased dwelling by subjects in the arena center (** $p < 0.05$), and reduced exploratory behavior, as they made fewer chamber entries. (b) There was no difference between times sniffing stranger mice or empty cages among treated 129S mice in the social interaction test. (c) Acetaminophen (ACM) treatment (100 mg/kg) increased dwelling near new strangers relative to controls (* $p < 0.05$), while WIN only increased center chamber dwelling in the social novelty test (** $p < 0.05$). (d) Acetaminophen treatment increased marble burying relative to control or WIN 55,212 treatments (* $p < 0.05$), $N = 9$ mice per group.

Figure 4. Performance of cannabinoid CB1 receptor-deficient and wild-type mice is similar in sociability and marble-burying tasks

The behaviors of CB₁ heterozygous mice (N = 9) did not differ from that of wild-type mice (N = 19) in (a) social approach or (b) time engaged in social sniff of stranger mice in the three-chambered social interaction test. Both genotypes spent most of the social interaction test investigating the stranger mice. (c) Likewise, CB₁ deficient and wild-type mice exhibited similar preferences for novel strangers over familiar stimulus mice in the social novelty test. (d) Both genotypes buried roughly 33% of the marbles in the bedding over 30 min.

Figure 5. Comparison of CB₁ and 5-HT_{1A} receptor density and agonist-stimulated G-protein coupling in the cingulate cortex indicate higher CP55,940 sensitivity in socially impaired mice, and lack of para-aminophenol CB₁ agonist activity
 (a) CB₁ receptor density, determined with 10 nM [³H] CP55,940 was similar among stains.
 (b) [³H] 8-OH-DPAT binding (2 nM) revealed higher 5-HT_{1A} density in this brain region in 129S mice than in the other strains (*p < 0.05, N = 8).
 (c) CP55,940-stimulated [³⁵S] GTPγS binding was lower in C57 mice than in the socially-impaired BTBR and 129S mice (*p < 0.05, N = 8). Para-aminophenol and 8-OH-DPAT were not significant stimulants of [³⁵S] GTPγS binding in this brain region.

Table 1

Endogenous cannabinoid levels in mouse frontal cortex

a) BTBR		No Behavior (70 min)		After SI/SN (70 min)	
Endocannabinoid:		Saline	ACM	Saline	ACM
AEA (nmol/g)		11 ± 2 ^a	30 ± 7 *	26 ± 3 *	25 ± 3 *
2-AG (pmol/g)		3.8 ± 0.2	4.1 ± 0.3	4.6 ± 0.4	4.8 ± 0.4
OEA (pmol/g)		65 ± 11	43 ± 5	65 ± 2	58 ± 4
b) 129S1/SvImJ		No Behavior (70 min)		After SI/SN and MB (100 min)	
Endocannabinoid:		Saline	ACM	Control	WIN55,212
AEA (nmol/g)		119 ± 5	89 ± 6 *	93 ± 13	98 ± 7
2-AG (pmol/g)		2.2 ± 0.3	2.2 ± 0.2	3.5 ± 0.2	1.4 ± 0.1 *
OEA (pmol/g)		85 ± 5	85 ± 13	83 ± 12	88 ± 9

^aData are mean ± S.E.M. BTBR mice were injected with SAL = saline or ACM = acetaminophen (100 mg/kg) and returned to home cages. After 30 min, half were subjects in 40 min sociability tests and sacrificed afterward, while the others were returned to home cages and sacrificed 40 min later.

* AEA was higher after ACM treatment or sociability tests than untested, SAL controls (p <0.05).

^b129S mice treated with ACM (100 mg/kg), WIN = WIN 55,212-2 (0.1 mg/kg), or CONTROL = saline ± 10% DMSO were returned to home cages for 30 min. Mice treated with ACM or saline and not behavior-tested were sacrificed after 40 min. Other ACM, WIN or CTRL mice were tested in sociability (40 min) and marble burying (30 min) tasks before sacrifice.

* Significantly different from controls (p <0.05), N = 5–9 mice per group.

Table 2Strain comparison of [³H] CP 55,940 binding in frontal cortex

Mouse Strain	N	Bmax (fmol/mg pr.)	Kd (nM)
BTBR	7	1062 ± 238 ^a	2.0 ± 0.5
C57B/L6	6	1351 ± 142	2.5 ± 0.4
129S1/SvImJ	6	965 ± 164	1.7 ± 0.3

^aMean ± S.E.M. The cortical membrane binding comparison indicated similar saturable binding densities and affinities for [³H] CP55, 940 among the strains. N indicates # of independent binding experiments.