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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project goal is to test the potential of organic cation transporter (OCT) blockade to improve social behavior in inbred mice exhibiting low sociability, specifically BTBR_T+tf/J, and 129S1/SvImJ strains. OCT blockade is also being examined in sociable C57BL/6 and DBA/1J mice. The OCT blocker decynium-22 (D-22) is compared in efficacy to the serotonin reuptake inhibitor fluoxetine, and the antipsychotic risperidone. The latter two are commonly used to treat autism symptoms, but do little to improve social behavior. Experiments assessing D-22 pharmacokinetics in mouse brain and serum were performed for specific aim 1; analysis of samples is still underway. However, D-22 injected systemically can enter the brain, and has a clearance half-life of approximately 30-40 min. Blockade of [^{3}H] serotonin uptake in synaptosomes revealed that D-22 is effective at nanomolar concentrations, and works independently of fluoxetine for aim 2. Chronoamperometry to compare serotonin uptake rates was performed *in vivo* at micromolar concentrations, and clearance was most rapid in BTBR mice. Finally, behavioral studies of the effects of D-22, risperidone and fluoxetine on sociability, social dominance, and marble burying tests are underway for aim 3. Preliminary findings indicate that D-22 can enhance social behavior, and that sociability testing increases serum corticosterone levels.

15. SUBJECT TERMS
autism, auxiliary neurotransmitter transporters, corticosteroids, decynium-22, impulsivity, inbred mice, organic cation transporters, repetitive behavior, serotonin, social behavior

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Introduction:

Impaired social behavior is a hallmark symptom of autism spectrum disorders that is also prominent in other psychiatric disorders such as schizophrenia and depression. Deficits in social interaction are particularly devastating to affected individuals and their families because verbal and non-verbal social interactions and communication are essential to the fabric of human existence. Clinical and basic research evidence indicates that dysfunctional regulation of serotonin (5-HT) neurotransmission, such that insufficient or otherwise dysfunctional 5-HT neurotransmission is common in autism [1,2,3,4,5,6]. This mechanism may underlie or contribute to this behavioral pathology that has proven very difficult to treat with available pharmacological interventions. Common drug treatments for autism, including the antipsychotic risperidone (a 5-HT$_2$/D$_2$ receptor antagonist), or the selective 5-HT reuptake inhibitor (SSRI) fluoxetine are only partly effective at best, and are even less beneficial in the 40-60% of patients with deficiencies in serotonin transporter (SERT) expression or function [3,7,8,9,10,11,12]. Hence, the discovery of alternative therapeutic targets to improve social behavior in autism and in other psychiatric disorders would be clearly beneficial for many patients and their families.

Aside from 5-HT reuptake by SERT into serotonin neurons ('uptake 1'), 5-HT is also cleared from extracellular fluid in the brain via auxiliary transporters collectively known as 'uptake 2' (Figure 1). Among uptake 2 transporters is organic cation transporter 3 (OCT3), which is blocked by corticosteroids and is expressed on neurons and glia in the brain [13,14]. We have discovered that OCT3 in mice removes 5-HT from extracellular fluid with lower affinity but higher capacity than SERT [15]. Due to this, we have been investigating the effects of OCT3 as part of 'uptake 2' blockade on depression-like behaviors, and it appears to enhance the antidepressant-like effects of selective serotonin reuptake inhibitors or SSRIs [16]. Given that SSRIs are the only drug class that has had some success in improving impaired sociability [8], and that elevated corticosterone was found in autistic children who voluntarily engaged in social interactions [17], we hypothesized that 'uptake 2' and/or OCT3 blockade might be an effective strategy to enhance social behavior.

In searching for new drug targets it is important to know what potential side effects their extended blockade might have, and based on the limited studies conducted so far, it appears that long-term OCT3 blockade is likely to be safe. For example, OCT3 -/- mice function normally in a wide range of physiological and behavioral measures [18]. Importantly, pharmacokinetic studies in OCT3 -/- mice using the organic cation [^{3}H] MPP+ did not reveal significant differences in concentrations in small intestine, liver, kidney, brain and placenta in these mice compared to wild-type mice [19]. These data suggest that cations can be efficiently transported and metabolized in the absence of OCT3. In our ongoing studies with the potent 'uptake 2'/OCT3 blocker decynium-22 (D-22) we see that doses above 1 mg/kg have sedating effects, but doses below that do not adversely impact locomotor behavior in mice. While targeting OCTs is an alternative approach to regulating brain 5-HT levels when SERT is deficient or less functional, such blockers may also be useful for a broader patient population, including adolescents with ASD, schizophrenia, depression and related disorders.

Blockade of these newly discovered 'uptake 2' monoamine transporters in brain remains a promising novel approach and potential refinement over SERT or other monoamine transporter uptake blocking drugs because of the differences in affinity (nearly 1000 fold) between 'uptake 1' and 'uptake 2'. As such this mechanism for enhancing 5-HT transmission might have better efficacy not only in patients who are not benefiting from SSRI treatments, but also in a wider range of patients suffering from sociability impairments characteristic of autism. For this reason, the effects of 'uptake 2' blockade in inbred strains of mice with inherent deficits in sociability, specifically BTBR and 129S1/SvImJ, and in more sociable strains C57BL/6 and DBA1 [20] is being investigated. Co-administration of D-22 with fluoxetine or risperidone might also improve social behavior, and this possibility is also being explored *in vitro* and *in vivo* in the ongoing study.

The primary goal of this project is to understand the role of 'uptake 2' transporters, and more specifically OCT3s, play in shaping social behavior through their regulation of 5-HT availability via uptake. The first specific aim is to determine the pharmacokinetics for D-22 in mouse brain and blood. The second is to quantify the extent of 5-HT uptake by OCT3 and SERT, and determine how D-22, fluoxetine or risperidone inhibits 5-HT uptake in hippocampal synaptosomes, and by *in vivo* chronoamperometry. The key objective is to assess the relative efficacy of D-22, alone or in combination with risperidone or fluoxetine, to improve social behavior in sociability tests and to reduce dominance in tube tests for mice. Accomplishment of these aims will provide critical information regarding potential efficacy of OCT3 blockade by D-22 to increase 5-HT neurotransmission and to treat social interaction deficits in psychiatric disorders where these symptoms are problematic.

Body:

<u>Specific Aim 1, pharmacokinetics of D-22 in mouse brain and blood:</u>

Initially to determine whether systemically injected decynium-22 (D-22) could cross the blood brain barrier, the PI performed intraperitoneal (i.p.) injections of 10 mg/kg D-22 dissolved in a 10% DMSO, 90% saline solution into three male C57BL/6 mice. Thirty minutes later the mice were sacrificed and measurement by HPLC (Javors Lab) revealed that roughly 50 pg/mg of D-22 was found in the brain [16]. This published HPLC procedure will be the same one used to perform analysis of D-22 levels in the brain and blood for the present study (see Appendix A). Another small pilot study was conducted to determine D-22 clearance rate. In adult male BTBR mice D-22 (injected at 1 mg/kg i.p.) had a half-life 35 min, based on sacrifice points at 5, 20 or 50 min after injection (Figure 2). This preliminary data guided subsequent modifications to the design of our pharmacokinetic studies, specifically the 4 hr 12 hr and 24 hour time-points were dropped in favor of more times under 1 hour, since D-22 was not likely to be detectable in serum by HPLC beyond 2 hours.

To commence the pharmacokinetic analyses, 18 of each BTBR, C57BL/6 and 129S1/SvImJ (129S) mice we injected with 10 mg/kg D-22 (dissolved in a 10% DMSO, 90% saline solution) and sacrificed after i.p. injection at 15, 30, 45, 60 or 120 min, with 3 mice per time-point. Many of the mice at the 60 and 120 min time-points exhibited toxic effects, they were lethargic or unconscious, particularly those of the C57BL/6 strain, and were cold to the touch, although respiration was evident from movement of the ribcage and air movement at the mouth. Also one each of the 129S (120 min) and C57BL/6 (60 min) mice died shortly before their appointed blood collection times, which was to be expected as this dose was two orders of magnitude above the therapeutic dose. However it was of interest that almost all of the lethargic mice could still right themselves if placed on their backs. The resultant clearance curve from blood is shown in Figure 3. Due to high variability half-time estimates could not be resolved with linear regression for this concentration of D-22. However the concentration and clearance of D-22 from brain tissue at this concentration should provide valuable information about occupancy at its key binding sites, including the OCT3. Analysis of brain tissue is still underway.

In the next round of experiments 18 BTBR and 18 129S mice were injected i.p. with D-22 at 1 mg/kg (in 10% DMSO, 90% saline vehicle) and were sacrificed at 0, 15, 30, 45, 60 or 120 min. In this group some of the locomotor activity was reduced in the 129S mice at 60 min, but otherwise no adverse effects were noted. The serum and brain samples were collected and are ready for HPLC analysis to be performed. There were insufficient numbers of C57BL/6 and DBA1 mice to utilize in this round of pharmacokinetic analysis of D-22.

In the next year we plan to complete the analysis of the 1mg/kg dose in C57BL/6 and DBA mice, increase the sample size at this dose to 6 for all strains, and begin to examine clearance and brain content at 0.1 mg/kg. Due to limits of detection, it is unlikely that HPLC analysis can be performed at doses below 0.1 mg/kg.

Further, we have undertaken the comparative analysis of serum corticosterone (CORT) levels between strains at baseline, and following social interaction tests. Recently we published a manuscript showing that baseline CORT levels were higher in BTBR mice than in C57 or 129S1/SvImJ mice, and that social interaction testing itself raises serum CORT levels in both adults and adolescent mice that are 35 days old (see Appendix B). This is important, since previous studies in our laboratory and in others indicate that CORT can inhibit 5-HT uptake *in vivo*, most likely via uptake 2 transporters [14,15]. Under the auspices of this project, we compared serum CORT levels between adult male BTBR and C57BL/6 mice after behavior testing using an ELISA kit, and we discovered that with means of (109 ± 11 and 78 ± 6 ng/ml, respectively, they were significantly different (two-tailed t = 2.42, df =8, p < 0.05). We have collected serum samples from most mice that were used for uptake assays and behavioral tests and have frozen them for further measures of CORT levels, and have obtained EIA kits and are ready to analyze more of these samples through November and December 2013.

<u>Specific Aim 2, effects of D-22 on serotonin uptake in vitro and in vivo:</u>

To measure the ability of D-22 to block [^{3}H] 5-HT uptake *in vitro*, initially [^{3}H] 5-HT saturation uptake experiments were run *in vitro* in brain synaptosomes harvested from BTBR, C57BL/6 and 129S mice, and maximum velocity of uptake (Vmax) in these strains was 285 ± 16, 297 ± 53, and 303 ± 42 fmol/mg/min and the Km was 31 ± 3, 39 ± 9, 46 ± 13 nM, respectively (Figure 4). In these assays, 20 µM of the SSRI fluoxetine was used to define non-specific binding, which was roughly 30% of total binding. A similar level of non-specific binding was achieved by performing the uptake in the presence of 1 µM of unlabeled serotonin. Sample sizes

for these assays range from 4 129S to 10 BTBR mice. This analysis is ongoing, and we have yet to examine uptake in DBA1 mice.

Subsequently we have begun to perform competition experiments designed to compare blockade of high-affinity [^{3}H] 5-HT uptake by D-22 to fluoxetine in all four mouse strains. Since co-administration of D-22 with risperidone is of interest as a therapeutic intervention for autism, we also studied that interaction. The competition isotherms are shown in Figure 5, and so far only 2-3 experiments have been run for each strain. However, we can at this time report that fluoxetine blocks [^{3}H] 5-HT uptake in the nM range, while D-22 blocks it in the μM range, and also that in general 5-HT uptake is more efficiently blocked by fluoxetine in BTBR and DBA mice than it is in C57BL/6 mice. This may be because of a single gene mutation that impairs SERT function in C57BL/6 mice, which is not present in the other strains [21]. The reason for relatively inefficient blockade of 5-HT uptake by fluoxetine in 129S mice in is not clear at this time, but the sample sizes are still rather low. More experiments (4-5) must be done to determine Ki values with better accuracy.

In vivo, we have examined the clearance of exogenously applied 5-HT locally into the CA3 region of the hippocampus of anesthetized mice by pressure-ejection using high-speed chronoamperometry. Decay of the resultant oxidation current was detected by a Nafion-coated carbon fiber electrode in close proximity (150-200 mm) to the pipette. The linear slope of the oxidation decay curve from 20% to 60% of maximal signal amplitude (Tc) was the parameter compared among strains as an index of 5-HT clearance rate (Figure 6). Tc values were comparable among strains at low 5-HT concentrations, but it appears that 5-HT clearance may be more rapid in BTBR and 129S than in C57BL mice at high, but physiologically relevant 5-HT concentrations. In the next year we hope to compare the ability of locally-applied D-22 alone and in combination with an SSRI to inhibit 5-HT clearance in the brains of BTBR and C57BL/6 mice, and perhaps also in 129S and DBA1 mice.

We have also made some progress toward developing a protocol to measure [^{3}H] histamine uptake in mouse brain synaptosomes. As we have been measuring [^{3}H] 5-HT uptake blockade in the hippocampus, we have used the cerebellum of some of those mice to examine the saturation binding properties of histamine, and its blockade by D-22. In sum, we did get time-dependent [^{3}H] histamine uptake (Vmax = 17 ± 35 fmol/mg pr./min, Km = 7.327, which is consistent with the only similar prior publication in rat brains [22]. However, we have also observed that ≈ 80% of that is non-specific uptake when defined with 10 μM D-22 (N=3), which may indicate that D-22 has little ability to block the slow rate of histamine uptake in our synaptosomal preparations. However, recently using cultured human and rat astrocytes it was discovered that histamine uptake rates are an order of magnitude higher than in synaptosomal preparations, and this uptake is blocked to a modest (20-30%) extent by D-22, but not by corticosterone, and that OCT3 plays a relatively minor role to glial histamine uptake in comparison to the plasma membrane monoamine transporter [23, 24]. Hence we are investigating the use of a Percoll gradient to obtain separate preparations of mouse brain gliosomes and synaptosomes to determine their relative roles in histamine uptake. We also intend to assess the extent to which histamine uptake is blocked by D-22 and corticosterone in those preparations.

Specific Aim 3, effects of D-22 on murine social behavior:

Two tests have been used to assess social behavior in the four strains of mice, the three-chamber sociability test and the tube test for social dominance. The sociability tests are designed to assess preference for social interaction in the first phase with a choice of stranger mouse vs. novel object, and preference for social novelty with a choice between a previously investigated stranger mouse and a new novel stranger in the second phase. Endpoints include time in each chamber, number of chamber entries, and time spent sniffing stranger mice. These social interaction tests are typically conducted under low red light (16 lux) in the absence of observers, to reduce anxiety levels in the mice, and to promote their social behavior.

For the sociability tests, at the outset of this project we rapidly generated more C57BL/6 and BTBR mice than 129S or DBA1. C57BL/6 mice typically exhibit pro-social behaviors such as more time spent sniffing or dwelling nearby stranger mice than BTBR mice do in these tests [20,25]. However, in contrast to studies utilizing transgenic mice, it is not clear what the appropriate controls are for inbred strains and social behavior. However, the precedent has been to choose one social and one non-social strain [26]. Further research into the background of the socially-deficient BTBR mouse revealed some interesting information. Specifically, hybrid crosses of BTBR and C57BL/6 (BTBRxBL6) produce F1 males with peripheral (non-hepatic) insulin resistance [27,28]. The social behavior of these F1 hybrids was not previously characterized.

We examined BTBRxBL6 F1 social behavior to uncover possible heritability properties of social behavior impairments. We hypothesized that it could correspond with the previously described pattern of insulin-resistance, in that male offspring from BTBR dams bred with C57BL/6 sires had the most insulin resistance [27]. Alternatively, BTBRxBL6 F1 males might exhibit intermediate impairments in social behavior, assuming that such behavior is a complex trait controlled by many genes. We found evidence suggesting that either a dominant an/or maternally-based inheritance pattern for sociability impairments occurred when these strains were crossed (Figure 7). Specifically, the behavior of BTBRxBL6 F1 males did not differ from that of BTBR males, and both were less sociable than C57BL/6 when time in chambers was the main parameter. However when social sniffing time was considered, there was very little difference between the strains or their hybrid.

Further, the atypical antipsychotic risperidone is often used in the treatment of autism to control aggression, yet typically it does not improve social interaction behavior [8]. Corresponding with this, previously we found that risperidone (0.1 mg/kg) failed to improve social behavior, but it reduced mobility and marble burying in BTBR mice [29]. Hence we examined a lower dose in these three mouse lines, and found that risperidone (0.01 mg/kg) worsened social behavior in C57BL/6 mice, but had no effect in BTBRxBL6 F1 or BTBR mice when chamber entries were considered (Figure 7). In general it appeared that the dose of 0.01 mg/kg of risperidone inhibited locomotor activity in C57BL/6 mice generally, as evidenced by their reduced chamber entries during the test and marble burying behavior after the test was completed. Hence C57BL/6 may be more sensitive to the effects of risperidone than either BTBR or hybrid mice. Based on this and the monoamine uptake data that we have generated, we are in the process of preparing a manuscript to submit for peer-review featuring this social behavior data of the hybrid as a key component.

We also used the C57BL/6, BTBR and their F1 hybrids, along with 129S1/SvImJ mice to examine social dominance using the tube test for social dominance [30]. Mice were placed in a tube (the diameter of which prevented the mice from turning around) facing each-other with a mesh divider placed between them. The divider was removed, and the mice were observed until one advanced and the other retreated by backing out of the tube. A win was tallied as 1, a loss as 0 and if there was no mouse backing out after 3 min, it counted as a draw = 0.05. The time to complete each match was also recorded, and the mice were paired against 6-7 mice from each strain. Mice were never used consecutively in matches, instead they were placed in holding cages with wood chip bedding until their next match, but were tested against 6 other mice per day of testing. All mice were tested against litter or cage-mates of their own strain first, and then were tested against other strains. Statistical comparisons consisted of Kruskal Wallace non-parametric ANOVA, followed by Mann-Whitney U tests for match outcome (% wins), or analysis of variance with Fisher's LSD post-hoc for time in match. We found that overall 129S mice were dominant over BTBR and BL6, but not over the F1 mice ($p<0.05$) (Figure 8). This data will also go into the manuscript characterizing the social behavior of the hybrid mice. It will also set the stage for drug treatment comparisons in this test by providing a point of reference (the baseline data). We continue to examine social dominance using this protocol in drug-treated mice, with ongoing experimentation and analysis underway.

Over the summer three-chamber sociability tests were also conducted in C57BL/6 and DBA1 mice. In these studies, we examined the effects of acute treatment with 10 mg/kg fluoxetine. Also, because DBA1 mice have been reported to carry a gene polymorphism impairing the ability of tryptophan hydroxylase 2 to convert tryptophan into 5-HT in the brain, we administered a diet devoid of tryptophan to the mice for 24 hours to compare the impact of serotonin depletion in these animals. In terms of baseline behavior, the C57BL/6 and DBA1 mice were similar, as shown in Figure 9. However fluoxetine treatment (10 mg/kg) worsened the social behavior of DBA1 mice, while tryptophan depletion worsened the behavior of C57BL/6 mice, presumably by impairing their ability to synthesize 5-HT de novo. Based on this it seems C57BL/6 mice may be more prone to low 5-HT tone, while DBA1 mice might already have high levels of 5-HT available. Studies are now underway

to assess the effects of D-22 administration on social behavior in C57BL/6, DBA1 and 129S1/SvImJ mice. However, we have continued to examine the dose-dependent effects of D-22 in BTBR mice.

So far we have found that 0.01 - 0.1 mg/kg of D-22 has the greatest capacity to improve social sniffing behavior in the BTBR inbred mouse strain (Figure 10). We have also been conducting studies to examine the effects of combined D-22 and fluoxetine, or D-22 and risperidone administration on the social behavior of all four strains of mice. All social behavior data is analyzed first by a global repeated measures ANOVA, followed by two-way ANOVA, Fisher's LSD post-hoc tests and paired t-tests to compare time in chambers and sniff time as described previously [20,25,26,29]. These behavioral tests, data collection and analysis will be conducted in all 4 strains and will continue through the next year of study. We have also been collecting brain tissue for measurement of OCT3 and PMAT levels that will be analyzed next year using western blotting.

Quarterly progress meetings among the PI and collaborators have been held and will continue to take place through the duration of the study. We anticipate the preparation and submission for peer review of two to three manuscripts based on this work.

Key Research Accomplishments:

1. Completion of Task 1: Institutional regulatory approvals granted for proposed research, mouse colonies established and producing sufficient numbers for experiments.

2. Task 2, aim 1 activities are well underway, the pharmacokinetic studies of D-22 in serum and brain were completed for 10 and 1 mg/kg, and plans are in place to analyze 0.1 mg/kg. HPLC analyses continue to be performed.

3. Task 2, aim 3 social behavior studies examining the effects of D-22 alone, in comparison to fluoxetine and risperidone, or combined with fluoxetine and risperidone are underway in 4 strains of mice. The 3-chamber sociability tests and tube tests for social dominance seem to be providing much needed insight for translational studies.

4. Task 2, serum has been saved for measurements of serum corticosterone.

5. Task 3 [3H] serotonin uptake protocol is finalized, and competition assays are being performed in all mouse strains.

6. Task 3 [3H] histamine uptake protocol is being developed that may distinguish glial and synaptosomal contributions.

7. Task 3 initial in vivo chronoamperometric comparisons of 5-HT clearance in the CA3 of hippocampus have been made among 3 inbred strains of mice.

8. Task 4. Combined D-22 and risperidone and D-22 and fluoxetine behavior and in vitro uptake experiments are being performed.

9. Progress report meetings have been held every 3 months.

10. The PI will be presenting a poster on some of these findings at the 2013 Society for Neuroscience meeting.

Reportable Outcomes:

Abstracts and Poster Presentations:

Program#: 547.01 Poster#: OO16 Author: Georgianna Gould Title: Targeting serotonin uptake to ameliorate social behavioral deficiencies in pre-clinical models. Presentation Time: Tuesday, Nov 12, 8:00 AM – 9:00 AM, Society for Neuroscience annual meeting, San Diego, CA, Nov 9-13, 2013.

Abstract #920. Smolik C., Javors M., Hensler, J.G., Koek, W. Daws, L.C., Gould G.G. (2013). Effects of "Uptake 2" Blockade and/or Risperidone on Murine 5-HT Uptake, Social and Repetitive Behavior. Poster presentation, 52nd Annual Society of Toxicology Meeting, San Antonio, TX March 10-14. In the Toxicologist (Supplement to Toxicological Sciences) Vol 132, issue 1.

Employment Based on the Award

Corey M. Smolik, a former University of Texas at San Antonio Biology undergraduate student, who was interning in the lab under the University of Texas Health Science Center START UPs program (R25 GM097632) was hired as a full-time research assistant by Dr. Gould to analyze videos of social behavior and to assist with performance of uptake assays.

Conclusion: Among inbred mouse strains, it appears as though there are differences in the capacity of 'uptake 2' transporters to remove 4-HT from extracellular fluid in the brain. This type of uptake is effectively blocked by D-22 in the low µM range, and it seems to differ in its blocking capacity from fluoxetine, which targets serotonin transporters. D-22 may have a short half-life in mice, but it tends to improve social behavior when administered systemically. The outcome of combined D-22 and fluoxetine blockade are of great interest, since we will determine if D-22 may be useful as a SSRI booster. It also appears that BTBR and C57BL/6 mice may be less dominant than, for example, 129S mice. We look forward to developing histamine as a tool to assess the relative contribution of 'uptake 2' transporters to 5-HT uptake.

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Figure 1. Major extracellular serotonin clearance mechanisms in the brain. Serotonin (5-HT) is taken up by several transporters with different uptake capacities, including the organic cation transporter 3 (OCT3).
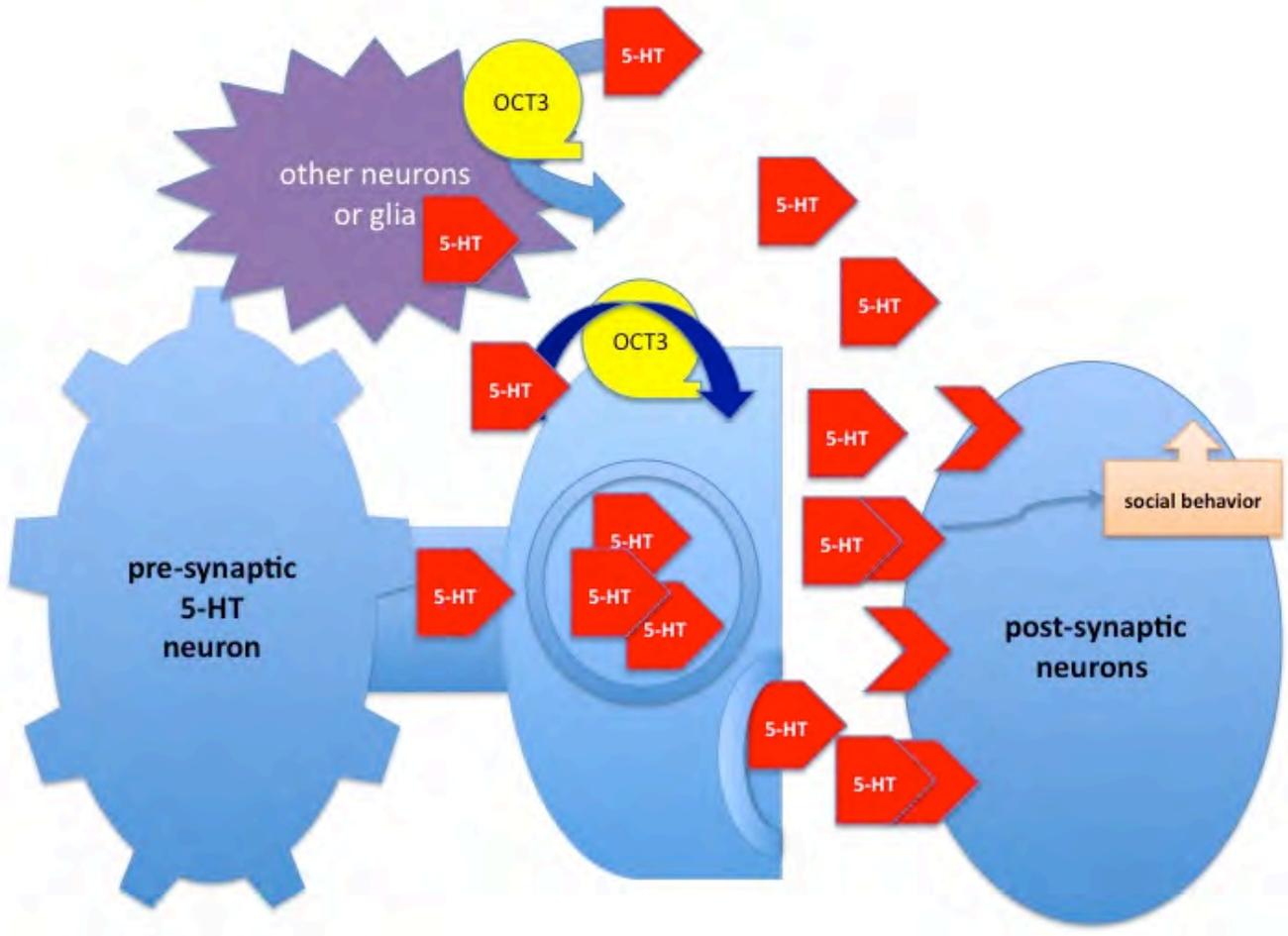
Figure 2. Clearance of 1 mg/kg decynium 22 (D-22) from adult male BTBR mice. The half-time for clearance was approximately 35 min, N =3 mice per point.
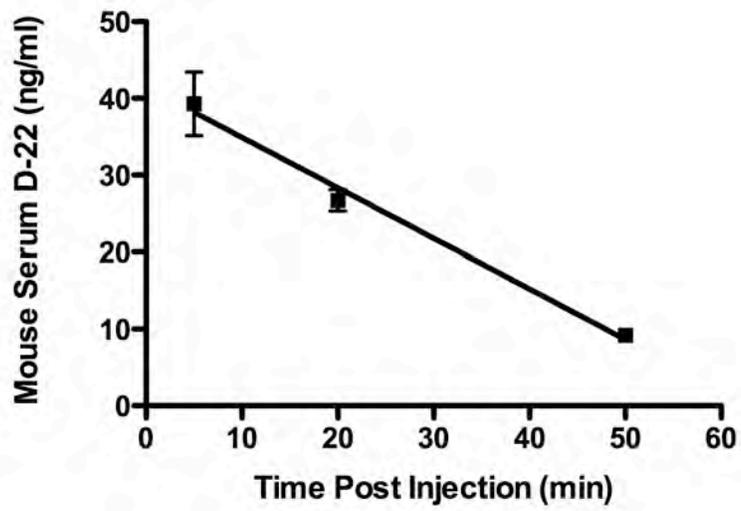
Figure 3. Clearance of D-22 (10 mg/kg) from serum in three mouse strains.
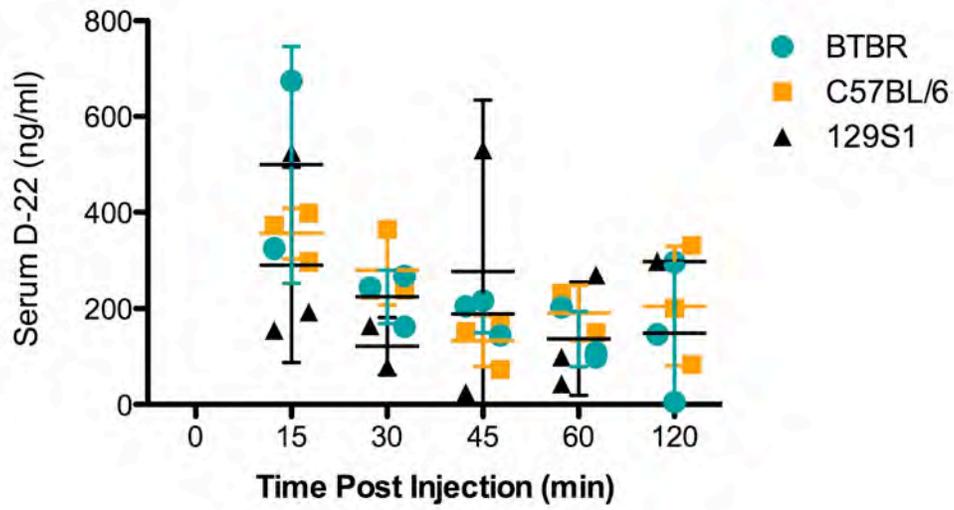
Figure 4. [³H] 5-HT Uptake saturation and blockade of 5-HT uptake in these strains by D-22.
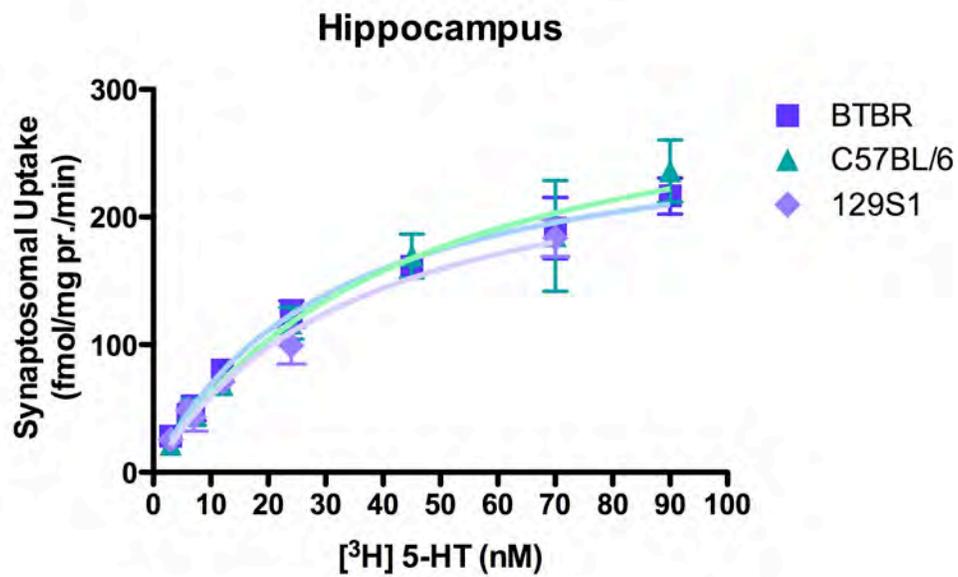
Figure 5. Comparison of [³H] 5-HT uptake blockade by D-22 and fluoxetine in hippocampal synaptosomes from different strains of inbred mice. Risperidone did not exhibit any appreciable ability to block [³H] 5-HT uptake on its own in a prior, unpublished study. Capacity to block 5-HT uptake was at least two orders of magnitude greater for fluoxetine than D-22. Fluoxetine also appeared to work more efficiently in BTBR and DBA mice than it did in C57BL/6 or 129S mice. Based on the variability in these experiments, at least 4-5 more replicates must be run to provide a reasonably accurate measure of these parameters.
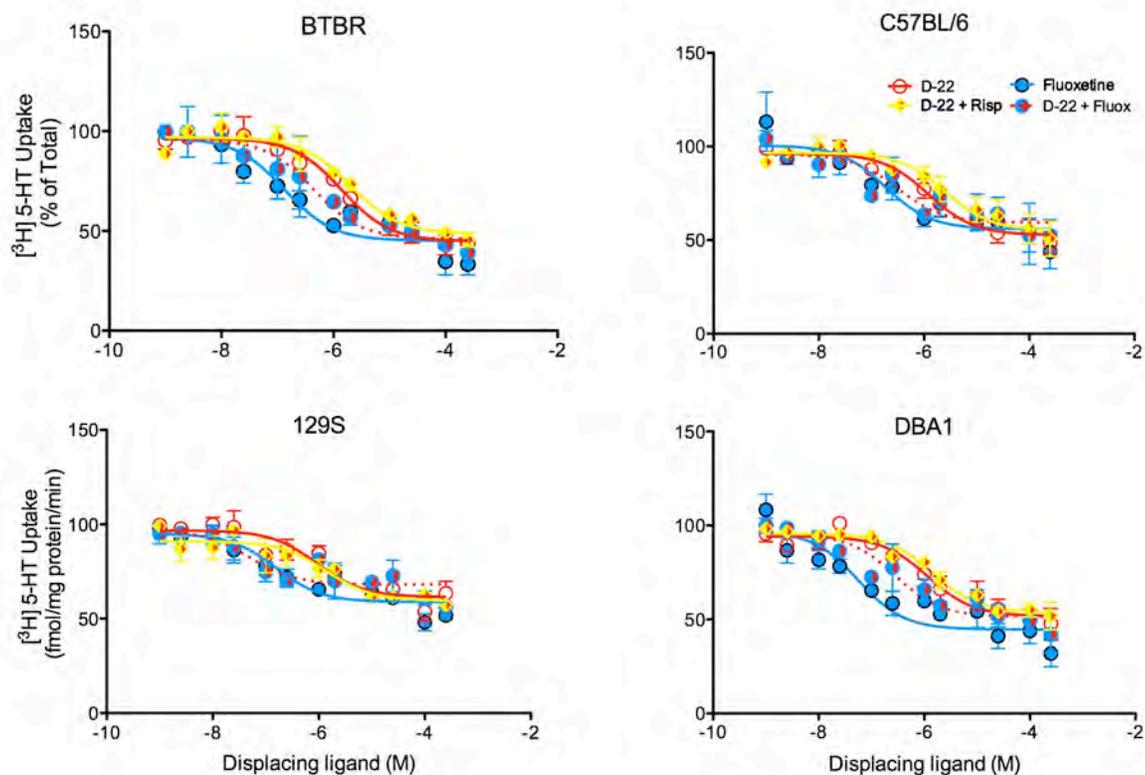
Figure 6. Chronoamperometric measurement of 5-HT clearance rate in CA3 of hippocampus. Exogenous 5-HT was applied via pressure-ejection through a micropipette and its clearance rate, indicated by Tc, was measured. When lesser amounts of 5-HT were applied, in the range of 100 nM -4 µM, clearance rates were similar. However, when 6 µM was applied, 5-HT clearance in C57 was slower than in BTBR or 129S mice.
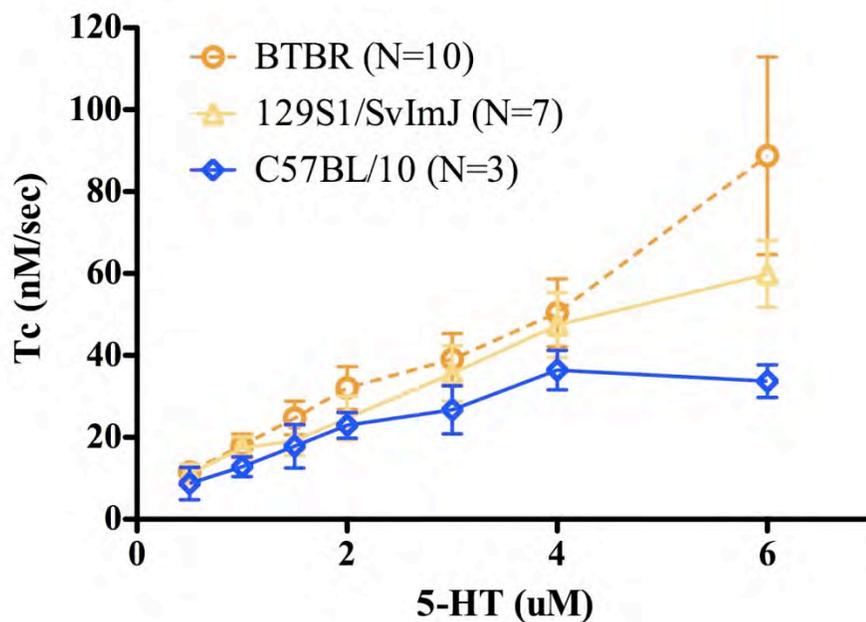
Figure 7. Behavior of mice in three-chamber social interaction tests. Drug naïve and risperidone (+ RISP, 0.01 mg/kg) treated male BTBR, C57BL/6 mice and their F1 hybrids were examined. (a) Only male C57BL/6 mice had a preference for social interaction as indicated by significantly more time spent in the chamber with the stranger mouse than novel object (*p <0.05), but this preference was not displayed by C57BL/6 mice given an i.p. injection of risperidone; they instead spent more time in the arena center (**p < 0.05). (b) All strains exhibited a baseline preference for social interaction (*p <0.05), which was abolished in all but hybrids by risperidone treatment. (c) Naïve hybrids and risperidone-treated BTBR and C57BL/6 mice had chamber times that indicated preference for social novelty (*p <0.05). (d) C57BL/6 and hybrid mice, and all mice treated with risperidone had significant preference for social novelty (*p <0.05) when sniffing time was monitored, although sniffing time was diminished in all risperidone-treated mice (b and d). (e) The total number of chamber entries and (f) the number of marbles buried after sociability tests was significantly reduced by risperidone treatment in C57BL/6 mice (*p <0.05, N = 7-19 per treatment group).
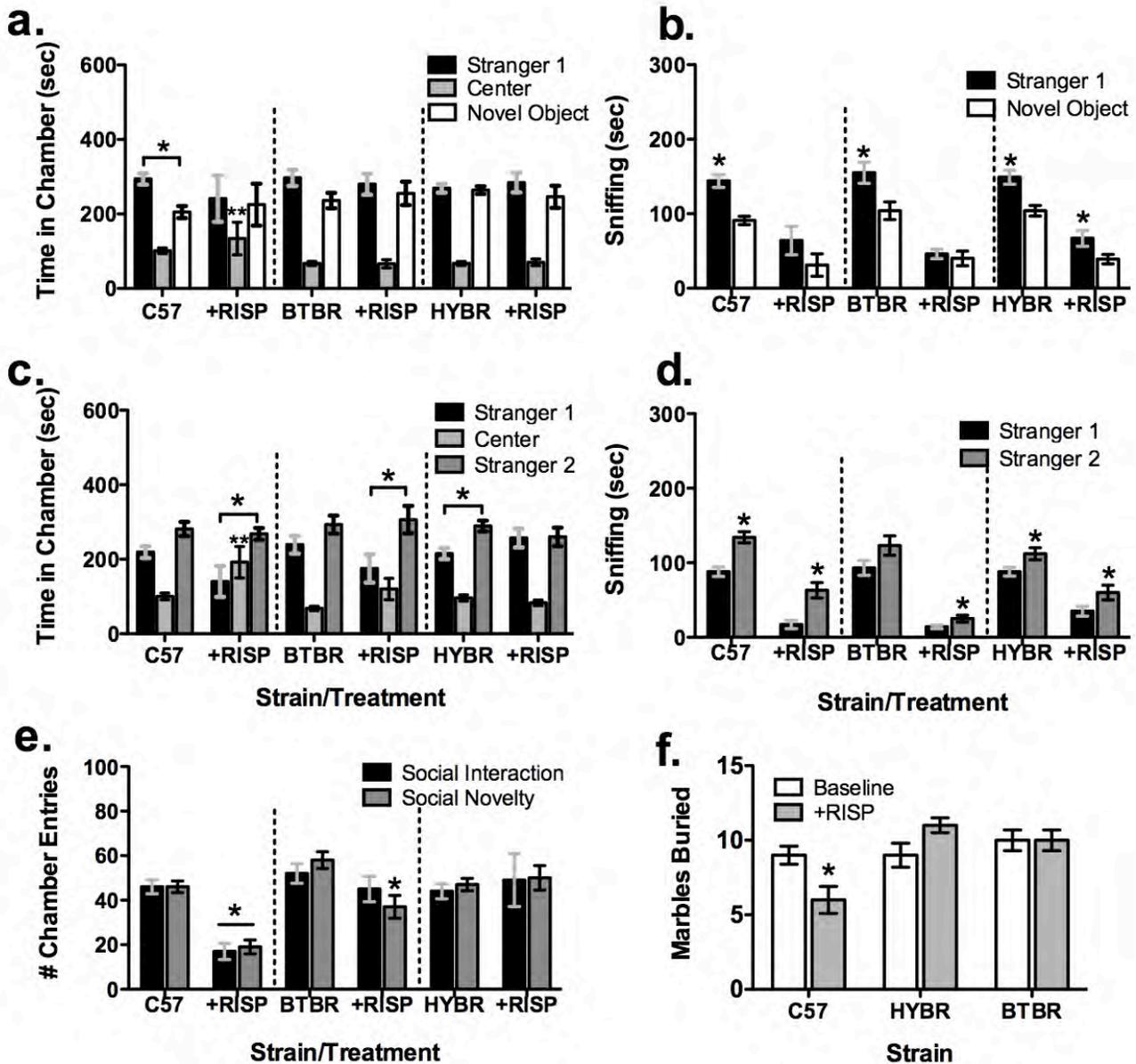
Figure 8. Comparison of mouse strain behavior in the tube-test for social dominance. In the top graph, BTBR an C57BL/6 mice had significantly ($p<0.05$) fewer wins over 129S mice than either hybrids or the 129S mice against themselves. In the lower graph, the longest matches were between 129S mice when paired against themselves.
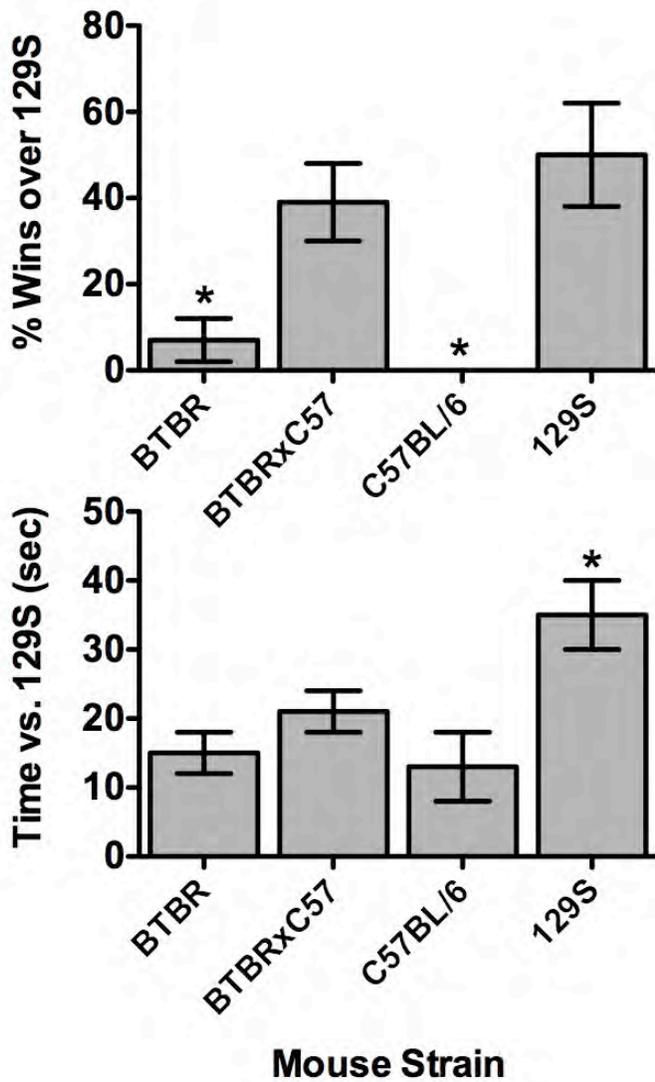
Figure 9. Comparison of social behavior between C57BL/6 and DBA1 mice in three-chambered tests. (a) Time spent in the chambers of the social interaction test was similar in C57BL/6 and DBA1 mice injected with saline. However fluoxetine significantly impaired sociability in DBA1 mice, while tryptophan depletion (-TRP) impaired it in C57BL6 mice. (b) Both C57BL/6 and DBA1 mice exhibited a preference for social novelty, which was abolished by fluoxetine treatment in DBA1 mice and by –TRP in C57BL/6 mice. Social sniffing during the social interaction (c) and social novelty (b) tests were similar among strains, and were reduced by fluoxetine, in this round of experiments neither strain exhibited a preference for social interaction or novelty. C57BL/6 mice made more chamber or box entries in the interaction (e) and novelty (f) phase of the sociability tests. Self grooming (g) was increased by fluoxetine treatment in DBA1 mice, but was otherwise similar among strains. For all graphs *p < 0.05 by ANOVA and Fisher's LSD post hoc, N = 4-7.
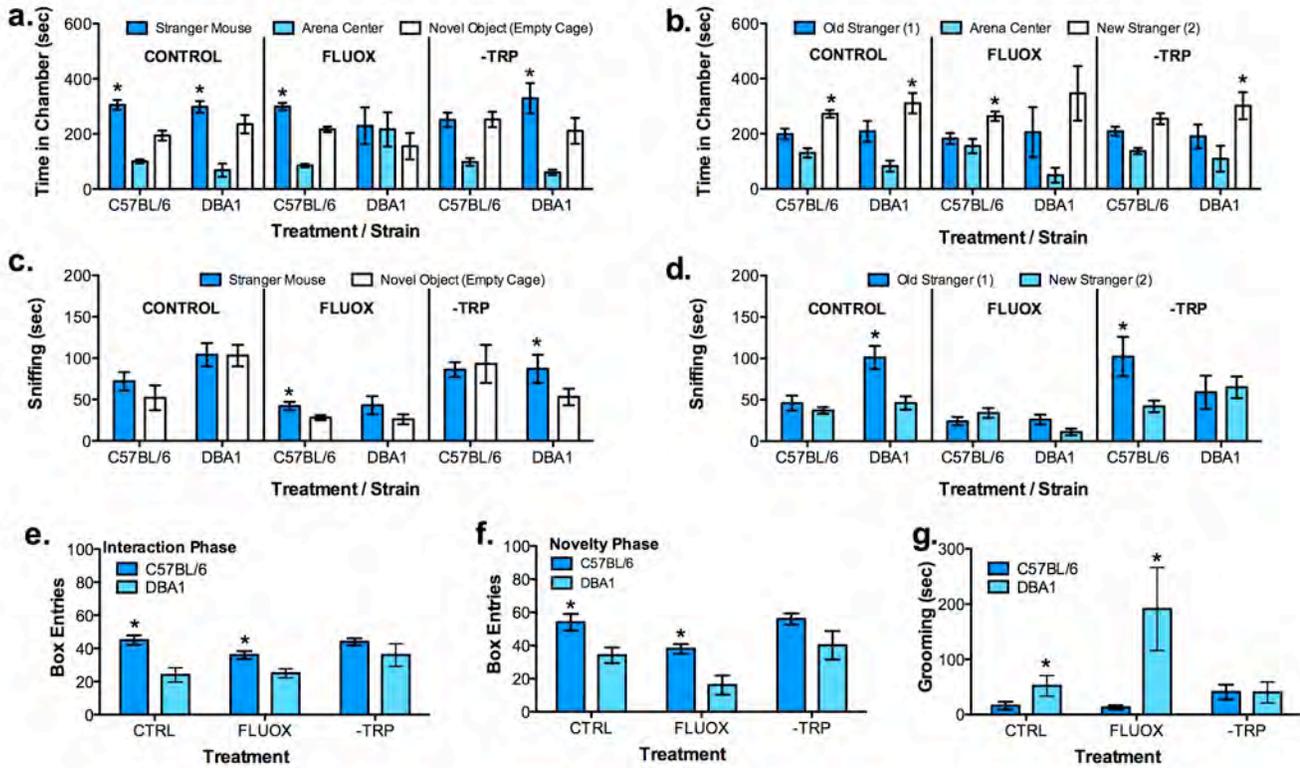
Figure 10. Dose-dependent improvement of BTBR mouse social behavior in the first phase of the social interaction test by D-22. (a) When examining chamber entries there was little difference between dwelling time in the chambers, aside from the increase in time spent in the center arena by mice dosed with 1 mg/kg of D-22. However, social sniff time of stranger mice (b) was significantly increased by 0.01 – 0.1 mg/kg D-22.
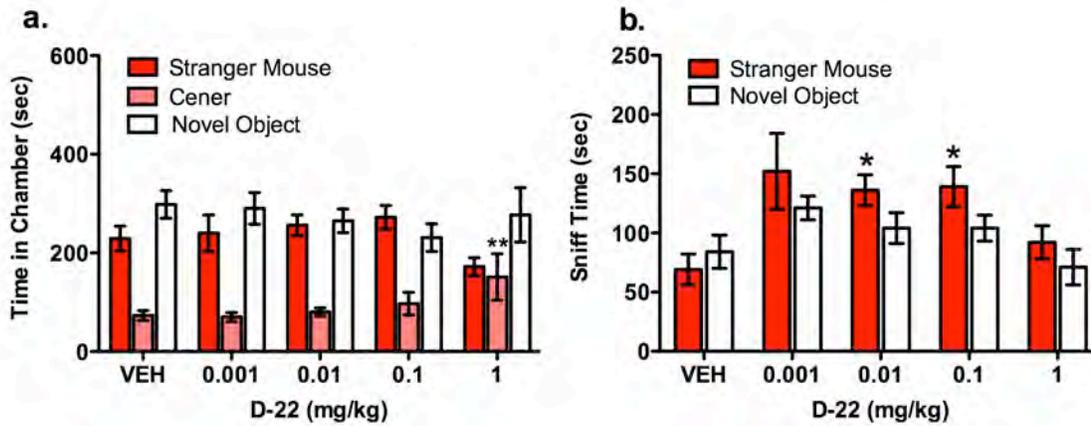
Appendix A

Systems/Circuits

Decynium-22 Enhances SSRI-Induced Antidepressant-Like Effects in Mice: Uncovering Novel Targets to Treat Depression

Rebecca E. Horton,[1] Deana M. Apple,[1,2] W. Anthony Owens,[1] Nicole L. Baganz,[1] Sonia Cano,[2] Nathan C. Mitchell,[1] Melissa Vitela,[1] Georgianna G. Gould,[1] Wouter Koek,[2,3]* and Lynette C. Daws[1,3]*

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Mood disorders cause much suffering and lost productivity worldwide, compounded by the fact that many patients are not effectively treated by currently available medications. The most commonly prescribed antidepressant drugs are the selective serotonin (5-HT) reuptake inhibitors (SSRIs), which act by blocking the high-affinity 5-HT transporter (SERT). The increase in extracellular 5-HT produced by SSRIs is thought to be critical to initiate downstream events needed for therapeutic effects. A potential explanation for their limited therapeutic efficacy is the recently characterized presence of low-affinity, high-capacity transporters for 5-HT in brain [i.e., organic cation transporters (OCTs) and plasma membrane monoamine transporter], which may limit the ability of SSRIs to increase extracellular 5-HT. Decynium-22 (D-22) is a blocker of these transporters, and using this compound we uncovered a significant role for OCTs in 5-HT uptake in mice genetically modified to have reduced or no SERT expression (Baganz et al., 2008). This raised the possibility that pharmacological inactivation of D-22-sensitive transporters might enhance the neurochemical and behavioral effects of SSRIs. Here we show that in wild-type mice D-22 enhances the effects of the SSRI fluvoxamine to inhibit 5-HT clearance and to produce antidepressant-like activity. This antidepressant-like activity of D-22 was attenuated in OCT3 KO mice, whereas the effect of D-22 to inhibit 5-HT clearance in the CA3 region of hippocampus persisted. Our findings point to OCT3, as well as other D-22-sensitive transporters, as novel targets for new antidepressant drugs with improved therapeutic potential.

Introduction

Dysfunction of the serotonin (5-HT) system is thought to underlie many affective disorders, primary among them, depression (Charney et al., 1981). The most commonly prescribed antidepressant medications are selective serotonin reuptake inhibitors (SSRIs). SSRIs act to block activity of the high-affinity serotonin transporter (SERT) and prevent the uptake of 5-HT from extracellular fluid into nerve terminals (Blakely et al., 1998). The ensuing increase in extracellular 5-HT is considered to be an important element in triggering the downstream events needed to produce therapeutic effects. However, a major problem in the

treatment of depression is that many patients experience modest or no therapeutic benefit (Kirsch et al., 2008), indicating the need for alternative approaches to treat individuals who respond poorly to SSRIs.

Organic cation transporters (i.e., OCT1, OCT2, and OCT3) and the plasma membrane monoamine transporter (PMAT) are low-affinity transporters for 5-HT, but unlike SERT, have a high capacity to transport 5-HT (Gründemann et al., 1998; Engel et al., 2004; Amphoux et al., 2006; Koepsell et al., 2007; Baganz et al., 2008; Duan and Wang, 2010). Decynium-22 (D-22) (originally described by Schömig et al., 1993), is a blocker of OCTs and PMAT, which inhibits 5-HT uptake into cell lines expressing OCT1, OCT2, OCT3, or PMAT as well as 5-HT uptake into brain synaptosomes (Engel et al., 2004; Engel and Wang, 2005; Gasser et al., 2006; Schömig et al., 2006; Duan and Wang, 2010; Hagan et al., 2011). These reports raise the possibility that low-affinity, high-capacity D-22-sensitive transporters for 5-HT may account, at least in part, for the poor clinical efficacy of SSRIs by preventing extracellular 5-HT levels reaching those required to trigger the cascade of downstream events needed for therapeutic benefit (Daws et al., 2013).

Support for this idea comes from studies in mice genetically modified to lack SERT or to express half as many SERTs as wild-type mice. In these SERT mutant mice, administration of D-22 inhibits 5-HT clearance from extracellular fluid in hippocampus and produces antidepressant-like effects (Baganz et al., 2008). In

contrast, D-22 does not produce these effects in wild-type mice, perhaps not surprisingly, since these mice have a full complement of functioning SERT controlling 5-HT uptake. Thus it appears that D-22-sensitive uptake mechanisms become more important in regulating 5-HT uptake and behavior when SERT function is genetically inactivated or impaired (Baganz et al., 2008). This raises the possibility that D-22-sensitive transporters for 5-HT may also play a more prominent role in 5-HT clearance when the high-affinity SERT is pharmacologically inactivated. If so, by limiting the increase of 5-HT in extracellular fluid that follows treatment with an SSRI, D-22-sensitive transporters could provide a mechanistic basis for poor therapeutic outcome in many patients. Thus, blockade of D-22-sensitive transporters might be a novel way to increase the therapeutic efficacy of currently available antidepressant drugs. Here we report that D-22 augments the ability of the SSRI, fluvoxamine, to inhibit 5-HT uptake and to produce antidepressant-like effects, providing support for D-22-sensitive transporters as novel targets for new antidepressant drugs.

Materials and Methods

Animals. Adult (>60-d-old; 25–30 g) male C57BL/6 mice, or OCT3 knock-out (KO) mice (bred on a C57BL/6 background, originally developed by Zwart et al. (2001) and generously provided to us by Drs. Kim Tieu, Bruno Giros, and Sophie Gautron) were used for all experiments. All animal procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All measures were taken to limit the number of animals used and to minimize animal discomfort.

High-speed chronoamperometry. In vivo chronoamperometry was performed according to methods described in detail previously (Daws and Toney, 2007; Baganz et al., 2008). We assemble our own carbon fiber electrodes and a detailed description can be found in Williams et al. (2007). Recording electrode/micropipette assemblies were constructed using a single carbon fiber (30 μm diameter; Specialty Materials), which was sealed inside fused silica tubing (Schott, North America). We based our procedure for electrode construction on modifications of published methods (Gerhardt, 1995; Perez and Andrews, 2005). Carbon fiber electrodes were coated with Nafion (5% solution; Aldrich Chemical), to prevent interference from anionic substances in extracellular fluid as previously described (Daws and Toney, 2007). Electrodes were tested for sensitivity to the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA; 250 μM) and calibrated with accumulating concentrations of 5-HT (0–3 μM). Only electrodes displaying a selectivity ratio for 5-HT over 5-HIAA greater than 500:1 and a linear response ($r^2 \geq 0.9$) to 5-HT were used.

The electrochemical recording assembly consisted of a Nafion-coated, single carbon fiber electrode attached to a four-barreled micropipette such that their tips were separated by ~200 μm. Barrels were filled with combinations of either 5-HT (200 μM), D-22 (10 μM), fluvoxamine (400 μM), a combination of D-22 and fluvoxamine, or phosphate-buffered saline (PBS). We elected to use fluvoxamine primarily to allow direct comparison with over a decade of chronoamperometry data reported in the literature, including those from our own lab, as well as others who have used fluvoxamine routinely (Daws et al., 1998; Benmansour et al., 1999, 2002, 2012; Montañez et al., 2003; Daws et al., 2005; Baganz et al., 2008). All compounds were prepared in 0.1 M PBS with 100 μM ascorbic acid added as an antioxidant and the pH adjusted to 7.4. The electrode-micropipette recording assembly was lowered into the CA3 region of the dorsal hippocampus (anteroposterior, −1.94 from bregma; mediolateral, +2.0 from midline; dorsoventral, −2.0 from dura) (Franklin and Paxinos, 1997) of anesthetized mice. For all experiments, mice were anesthetized by intraperitoneal injection (2 ml/kg body weight) of a mixture of chloralose (35 mg/kg) and urethane (350 mg/kg). A tube was inserted into the trachea to facilitate breathing and

mice were then placed into a stereotaxic frame. Body temperature was maintained at 36−37°C by a water circulated heating pad.

High-speed chronoamperometric recordings were made using the FAST-12 and FAST-16 systems (Quanteon). Oxidation potentials consisted of 100 ms pulses of +0.55 V. Each pulse was separated by a 900 ms interval during which the resting potential was maintained at 0.0 V. Voltage at the active electrode was applied with respect to an Ag/AgCl reference electrode positioned in the extracellular fluid of the ipsilateral superficial cortex. The oxidation and reduction currents were digitally integrated during the last 80 ms of each 100 ms voltage pulse.

At the conclusion of the experiment, an electrolytic lesion was made to mark the placement of the electrode tip. The brain was removed, rapidly frozen on dry ice, and stored at −80°C until use. At this time brains were thawed to −15°C and sliced into 20-μm-thick sections for histological verification of electrode localization. Only data from mice in which the electrode was confirmed to be in the CA3 region of the hippocampus were included in the analyses.

Effects of local and systemic drug administration on 5-HT clearance. Exogenous 5-HT was applied into the CA3 region of the hippocampus by pressure ejection (5–25 psi for 0.25–3.0 s). Advantages of this approach are that clearance can be measured without an associated "release" component and that measurements can be made *in vivo* with excellent temporal (milliseconds) resolution. Serotonin was pressure ejected into hippocampus to achieve concentrations at the recording electrode of ~0.5 μM. Once reproducible 5-HT electrochemical signals were obtained, drug or vehicle was applied either locally into the CA3 region of hippocampus, or intraperitoneally, before the next application of 5-HT. For locally administered drugs, 5-HT was pressure ejected 2 min after drug or vehicle to allow sufficient time for drugs to diffuse to the recording site. Serotonin was applied again at 10 min intervals after drug or vehicle until the signal returned to predrug status. The 10 min time interval ensured that each signal produced by local application of 5-HT had completely cleared before the next ejection of 5-HT, drug or vehicle. For systemic administration, exogenous 5-HT was applied 30 min following intraperitoneal administration of drugs or vehicle so as to correspond to the timing used in the tail suspension test (TST). Two signal parameters were analyzed: the peak signal amplitude and the T_{80} time course parameter. T_{80} is defined as the time for the signal to decline by 80% of the peak signal amplitude.

TST. The TST was conducted based on the original description by Steru et al. (1985). In dose–response studies, mice received an intraperitoneal injection of D-22 (0.01–0.32 mg/kg), fluvoxamine (0.32–32 mg/kg), or saline. In drug interaction studies, mice received an intraperitoneal injection of either D-22 (0.1 or 0.32 mg/kg) or saline 60 min before testing, and received a second intraperitoneal injection of either fluvoxamine (10 mg/kg) or saline 30 min before testing; immediately after each injection, they were placed in an observation cage. Thirty minutes after the second injection, the distal end of the tail was fastened to a flat aluminum (2 × 0.3 × 10 cm) bar using adhesive tape at a 90 degree angle to the longitudinal axis of the mouse tail and the aluminum bar, with a distance of 3–4 cm between the base of the tail and the edge of the bar. A hole opposite the taped end of the bar was used to secure the bar to a hook in the ceiling of a visually isolated white test box (40 × 40 × 40 cm). Each mouse was suspended by its tail for 6 min, allowing the ventral surface and front and hindlimbs to be video recorded using a digital camera facing the test box. Total time immobile was measured (in seconds) during the entire 6 min test period. Immobility was defined as the absence of initiated movements, and included passive swaying of the body. A mouse was excluded from the experiments if it climbed and held to its tail or the aluminum bar for a period of 3 s or longer.

Serotonin syndrome. Mice were given saline or a combination of D-22 and fluvoxamine, using the same doses and injection-test intervals as used in the drug interaction study in the TST. In addition, separate groups of mice received 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 10 mg/kg), 5-hydroxy-L-tryptophan (5-HTP, 100 mg/kg), or their vehicles (saline and 5% Tween 80, respectively). Each of the five groups received two intraperitoneal injections, 30 min apart, as follows: (1) D-22 followed by fluvoxamine, (2) saline followed by 8-OH-DPAT, (3) saline followed by 5-HTP, (4) saline followed by saline and (5) saline followed by 5% Tween 80. Immediately after the first injection, animals

were placed in a transparent observation cage with a mirror positioned beneath it to allow simultaneous viewing of both the side and ventral surface of the mouse. Mice were observed for possible occurrence of elements of the 5-HT syndrome during five, 6 min periods, starting at 15, 30, 60, 120, and 240 min after the second injection. Each animal was recorded on video until immediately after the observation period that started at 120 min. The animal was then returned to the home cage where it was observed at 240 min. During each 6 min period mice were observed for the occurrence of the following signs: hindlimb abduction, low body posture, Straub tail, backward movement, tremor, head weaving, and forepaw treading, according to methods adapted from Fox et al. (2008). The presence (1) or absence (0) of each sign was assessed during each min of the 6 min observation period, allowing an animal to receive, for each of the signs, a score of 0–6 at each observation period and an overall score of 30 for all five observation periods.

HPLC analysis of D-22 in brain. HPLC was used to assay for D-22 in brain. D-22 (10 mg/kg) was injected intraperitoneally and mice were killed 30 min later. Brain tissue for samples, calibrators, and controls were weighed into polypropylene tubes, then 10 times the volume of a 70% acetonitrile solution was added. Samples were homogenized by sonication and then 500 μl of each was placed in clean polypropylene tubes. Twenty microliters of diazepam (10 μg/ml) for internal standard was added to each sample. Next, samples were shaken for 10 min, centrifuged at 3200 g for 20 min at 18°C in a Beckman Allegra X-15R centrifuge. The supernatant was decanted into clean tubes, and dried under a stream of nitrogen. Samples were resuspended with 100 μl of mobile phase buffer, and microfilterfuged at 3200 g for 10 min. Fifty microliters of sample was injected into the HPLC system.

The HPLC system included a Waters model 510 pump, a Waters model 717 sample injector, a Waters model 2487 UV detector for HPLC equipped with a deuterium lamp (320/520 nm), and an Alltima 150 mm × 4.6 mm C18 column (5 U). Samples were analyzed at a fixed wavelength of 520 nm, a time constant of 1 s, and a range of 0.1 AUFS on the UV detector. The mobile phase contained 65% methanol, and 35% of a solution of 12 mM KH_2PO_4, pH 6.7. The flow rate of the mobile phase was 1.5 ml/min. D-22 eluted at 8.7 min and diazepam at 10.1 min. The peak area ratio of D-22 and the internal standard diazepam were determined using the Waters Empower chromatography software. D-22 concentration was determined by comparing peak area ratio (D22/diazepam) against the linear regression of ratios of calibrator samples from a 6 point calibrator curve. The concentrations of D-22 in brain were expressed as pg D-22/mg brain wet weight.

High purity-grade acetonitrile and methanol were purchased from Burdick and Jackson. Water used in the assay was purified with a Milli-Q Water System (Millipore). Potassium phosphate was obtained from Fisher Scientific. Hydrochloric acid was obtained from JT Baker Chemical. The internal standard, diazepam, was obtained from Sigma.

Statistics. Statistical analyses were performed using Prism 5.04 (GraphPad). Data were analyzed using one-way ANOVA, followed by Newman–Keuls or Dunnett's multiple-comparison tests. All data were expressed as mean ± SEM, and $p < 0.05$ was considered statistically significant.

Drugs. Serotonin, 5-HIAA, fluvoxamine, urethane, α-chloralose, D-22, 8-OH-DPAT HBr, and 5-HTP were purchased from Sigma-Aldrich. All compounds were injected intraperitoneally in a volume of 5–20 ml/kg. Doses are expressed as mg salt weight per kg body weight.

Results

Intrahippocampally applied D-22 enhances the ability of the SSRI fluvoxamine to inhibit 5-HT clearance from extracellular fluid

In wild-type mice, we previously found that D-22 could inhibit 5-HT clearance from CA3 region of hippocampus, but only when extracellular 5-HT levels were in a range that recruited low-affinity, high-capacity transporters for 5-HT (Baganz et al., 2008, 2010). At lower, SERT-recruiting 5-HT concentrations, D-22 had no effect on 5-HT clearance, a result not especially surprising given the high affinity of SERT for 5-HT. However, in the presence of an SSRI, we reasoned that D-22-sensitive transporters

may exert a more prominent role in 5-HT uptake and therefore, limit the ability of SSRIs to prolong the time 5-HT remains in extracellular fluid. This led us to hypothesize that D-22 should enhance the inhibiting effect of an SSRI on 5-HT clearance. To test this hypothesis we used high-speed chronoamperometry, according to well established protocols (Daws and Toney, 2007; Baganz et al., 2008, 2010), to measure clearance of locally applied 5-HT from the CA3 region of the hippocampus of anesthetized mice, before and after intrahippocampal administration of fluvoxamine, D-22, both drugs given together, or vehicle. The amount of fluvoxamine delivered (54 pmol) was based on previous dose–response studies showing maximal inhibition of 5-HT clearance at this dose (Baganz et al., 2010). The amount of D-22 delivered (1.4 pmol) is the maximum that can be delivered locally without interfering with the properties of the carbon fiber recording electrode. D-22 (1.4 pmol) has no effect on 5-HT clearance in the CA3 region of hippocampus in wild-type mice but inhibits 5-HT clearance in mice lacking SERT, suggesting that the 5-HT clearance inhibiting effect of D-22 is mediated at sites other than SERT, putatively OCT3 (Baganz et al., 2008). Serotonin was pressure ejected to achieve reproducible signals with amplitudes of ~0.5 μM, a concentration that we have previously found to recruit predominantly SERT-mediated 5-HT clearance (Daws et al., 2005; Baganz et al., 2008, 2010). Once reproducible signals were obtained, drug or vehicle was locally ejected into hippocampus and then 2 min later 5-HT was applied again. Representative traces are shown in Figure 1A. As expected, and consistent with other reports using similar methodology (Daws et al., 1998, 2000, 2005; Benmansour et al., 1999, 2002), fluvoxamine prolonged 5-HT clearance time (Fig. 1A,B), without producing any significant effect on signal amplitude (Fig. 1A,C). Likewise, replicating our previous study (Baganz et al., 2008), D-22 was without effect on 5-HT clearance time or signal amplitude (Fig. 1A–C). However, D-22 robustly increased the ability of fluvoxamine to inhibit 5-HT clearance. This effect was apparent not only in terms of prolonging 5-HT clearance time (Fig. 1A,B), but also in terms of increasing the amplitude of the 5-HT signal (Fig. 1A,C). Baseline (i.e., predrug) T_{80} values did not differ significantly ($p > 0.20$) among groups and were 101 ± 9, 116 ± 8, 107 ± 15, and 114 ± 6 s for vehicle, D-22, fluvoxamine, and D-22+fluvoxamine groups, respectively. One-way ANOVA revealed an effect of treatment on the percentage change from baseline 5-HT clearance time, driven by significant effects of fluvoxamine, and the combination of fluvoxamine and D-22 to slow 5-HT clearance ($F_{(3,35)} = 12.07$; $p < 0.0001$; Fig. 1A,B). Likewise, baseline (i.e., predrug) signal amplitude values did not differ significantly ($p > 0.50$) among groups and were 0.66 ± 0.04, 0.56 ± 0.04, 0.65 ± 0.04, and 0.58 ± 0.06 μM for vehicle, D-22, fluvoxamine, and D-22+fluvoxamine groups, respectively. One-way ANOVA revealed an effect of treatment on the percentage change from baseline in 5-HT signal amplitude, driven by the marked ability of fluvoxamine, in combination with D-22, to increase signal amplitude ($F_{(3,35)} = 3.85$; $p < 0.05$; Fig. 1A,C).

It is worth noting that we conducted these *in vivo* electrochemical studies in the hippocampus for three key reasons. First, it is a limbic structure considered to be important in the therapeutic response to antidepressant drugs (Campbell and Macqueen, 2004). Second, it is a region where mechanisms contributing to 5-HT clearance *in vivo* have been best characterized to date (Daws et al., 1998, 2000, 2005; Baganz et al., 2008). Third, and especially relevant to the present study are reports that D-22-sensitive OCTs and PMAT are located in the hippocampus. Indeed, the availability of selective antibodies has made it possible
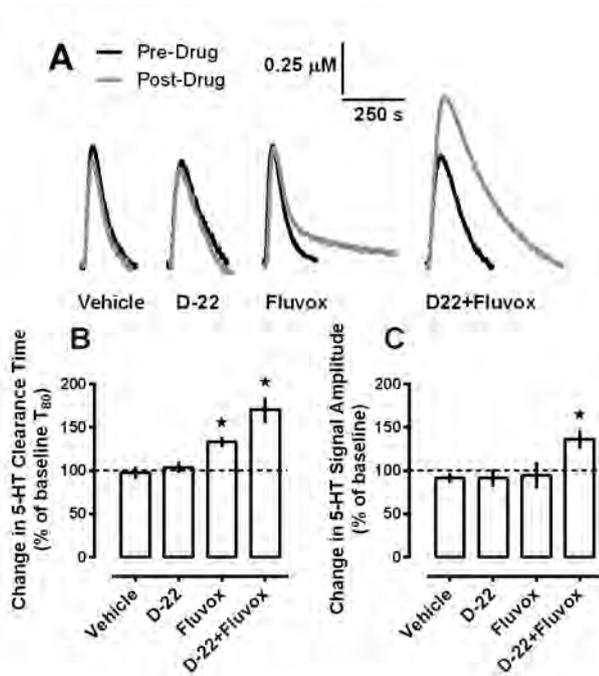
Figure 1. Local blockade of D-22-sensitive transporters enhances fluvoxamine-induced inhibition of 5-HT clearance in mouse hippocampus *in vivo*. *A*, Representative oxidation currents (converted to micromolar values) produced by pressure ejection of 5-HT into the CA3 region of the hippocampus before (black line) and 2 min after (gray line) local application of saline, D-22 (1.4 pmol), fluvoxamine (54 pmol), or fluvoxamine and D-22 given together. Raw tracings are superimposed for ease of comparison. Note the marked increase in both clearance time and signal amplitude after local application of D-22 and fluvoxamine when given together. *B*, The effect of hippocampally applied fluvoxamine to slow 5-HT clearance is enhanced by concurrent administration of D-22; one-way ANOVA with Newman–Keuls *post hoc* comparison, *$p < 0.05$ versus vehicle. *C*, Hippocampally applied fluvoxamine, in combination with D-22, increases 5-HT signal amplitude; one-way ANOVA with Newman–Keuls *post hoc* comparison, *$p < 0.05$ versus vehicle. Data are expressed as mean ± SEM percentage of baseline values with $n = 9$–10 for each drug treatment.

Figure 2. Fluvoxamine, but not D-22, dose dependently reduces immobility time in the TST. Time spent immobile was measured in seconds, and was expressed as a percentage of the corresponding vehicle control group. Time spent immobile for vehicle control groups did not differ significantly ($p > 0.50$) and were 116 ± 19 and 118 ± 19 s for fluvoxamine and D-22 control groups, respectively. *A*, Fluvoxamine, administered 30 min before testing, dose dependently decreased time spent immobile, producing a near maximal effect at the highest dose tested. *B*, D-22, given 60 min before testing, had no significant effect on time spent immobile at any of the doses tested. Data are expressed as mean ± SEM, $n = 7$–9 for each dose tested; one-way ANOVA with Dunnett's multiple-comparison *post hoc* test, *$p < 0.05$.

to map the distribution of OCT subtypes and PMAT in brain (Amphoux et al., 2006; Dahlin et al., 2007; Vialou et al., 2007; Baganz et al., 2008; Lamhonwah et al., 2008; Cui et al., 2009; Gasser et al., 2009; Bacq et al., 2012).

The finding that locally administered D-22 can markedly enhance fluvoxamine-induced inhibition of 5-HT clearance in hippocampus suggests that the combination of these drugs might produce similar effects on 5-HT clearance when administered systemically and, importantly, enhance the antidepressant-like effect of fluvoxamine. The next set of experiments was designed to test this hypothesis.

D-22 enhances fluvoxamine-induced antidepressant-like effects in the TST

The ability of intrahippocampally applied D-22 to enhance fluvoxamine-induced inhibition of 5-HT clearance supports the idea that this drug combination might also produce greater antidepressant-like effects than either drug given alone. To this end, we studied the effects of systemic administration of D-22 and fluvoxamine in the TST, which measures antidepressant-like activity in mice (Steru et al., 1985; Cryan et al., 2005). We then examined how time spent immobile in the TST related to drug-induced inhibition of 5-HT clearance in hippocampus.

Consistent with the literature (Fujishiro et al., 2002; Cryan et al., 2005), fluvoxamine, given 30 min before testing, dose de-

pendently reduced time spent immobile in the TST (Fig. 2A; $F_{(3,28)} = 5.75$, $p < 0.01$). Previously, we found that 0.001 mg/kg D-22 decreased immobility in SERT-deficient mice, but not in wild-type mice, and decreased immobility more extensively at 60 min than at 30 min after intraperitoneal administration (Baganz et al., 2008). Here, we examined the effects of D-22 60 min after intraperitoneal administration in wild-type mice at doses ranging from 0.01 to 0.32 mg/kg. At these doses, D-22 did not significantly affect immobility (Fig. 2B; $p > 0.60$). This result was not especially surprising, given the presence of fully functioning, high-affinity SERT in these mice, which serve to maintain extracellular 5-HT at concentrations below those where low-affinity, high-capacity transporters for 5-HT can exert more influence. Doses >0.32 mg/kg, which in preliminary experiments appeared to decrease locomotion, were not tested.

To test our hypothesis that D-22 would enhance the antidepressant-like effect of fluvoxamine, we selected a dose of each drug that did not produce significant effects on time spent immobile in the TST (i.e., 0.1 mg/kg D-22, and 10 mg/kg fluvoxamine given 60 and 30 min before testing, respectively). Consistent with the results shown in Figure 2, treatment with either D-22 (0.1 mg/kg i.p.) or fluvoxamine (10 mg/kg, i.p.) alone produced no statistically significant decrease in time spent immobile relative to saline-treated control mice (Fig. 3A). However, cotreatment with D-22 and fluvoxamine markedly decreased time spent immobile in the TST (one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$).

To examine whether the marked antidepressant-like effect of the combination of D-22 and fluvoxamine was related to an increased ability of this drug combination to inhibit 5-HT uptake, we used high-speed chronoamperometry to study 5-HT clearance in the CA3 region of hippocampus under the same doses and injection-test intervals as used in the TST. Consistent with behavioral data in Figure 3A, D-22 had no effect on clearance of intrahippocampally applied 5-HT from extracellular fluid (Fig. 3B). Interestingly, systemically administered fluvoxamine (10 mg/kg), which did not produce statistically significant effects in the TST, robustly inhibited clearance of intrahippocampally applied 5-HT. D-22 enhanced fluvoxamine-induced inhibition

of 5-HT clearance (one-way ANOVA, $F_{(3,27)} = 15.47$, $p < 0.05$) and of time spent immobile in the TST (one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$). Thus our own data (Fig. 3C), and those of others (David et al., 2003), suggest a positive correlation between a drug's ability to inhibit 5-HT uptake and to produce antidepressant-like effects.

D-22 crosses the blood–brain barrier
The finding that both intrahippocampal and peripheral (i.p.) administration of D-22 produced similar inhibition of 5-HT clearance in hippocampus suggests that systemically administered D-22 probably exerts its effects on 5-HT clearance and behavior via central actions. To ensure that D-22 crossed the blood–brain barrier, we quantified D-22 in whole brains of three mice injected intraperitoneally with 10 mg/kg D-22 and three control mice injected with an equal volume of vehicle. Thirty minutes after the injection, the brain concentration of D-22 was 56 ± 27 pg/mg, confirming that D-22 reaches the brain following systemic administration.

D-22 enhances fluvoxamine-induced inhibition of 5-HT clearance without inducing elements of the serotonin syndrome
Drugs or drug combinations that produce excessive serotonergic activity can induce the serotonin syndrome (Sternbach, 1991; Kalueff et al., 2007). In humans, symptoms include hyperthermia, tachycardia, and rhabdomyolysis, which can result in death (Lane and Baldwin, 1997; Ener et al., 2003). Thus, an important consideration relating to our finding that D-22 enhances the ability of fluvoxamine to prevent 5-HT reuptake is that this drug combination conceivably could induce elements of the serotonin syndrome. In mice the serotonin syndrome consists of hindlimb abduction, low body posture, Straub tail, tremor, backward movement, head weaving, and forepaw treading (Fox et al., 2007). In the present study we treated mice with D-22 and fluvoxamine using the same doses and injection-test intervals as in the TST to examine whether this combination of D-22 and fluvoxamine, which produced maximal antidepressant-like activity, also induced elements of the serotonin syndrome. Different groups of mice were treated with either vehicle, 8-OH-DPAT (10 mg/kg i.p.), an agonist at 5-HT1A receptors that produces the serotonin syndrome in mice (Middlemiss and Fozard, 1983; Blackburn et al., 1984; Hensler and Truett, 1998; Fox et al., 2007), or 5-HTP (100 mg/kg i.p.), the precursor to 5-HT that also produces the serotonin syndrome in mice (Fox et al., 2007, 2008). Mice were then observed for 6 min periods, starting at 15, 30, 60, 120, and 240 min after the second injection. During each 6 min observation period, mice were scored at 1 min intervals for the presence or absence of serotonin syndrome elements, according to methods adapted from Fox et al., (2007). Results for the observation period starting 30 min after the second injection (i.e., the time corresponding to scoring in the TST) are shown in Table 1. As expected, the positive controls, 8-OH-DPAT and 5-HTP, produced hindlimb abduction and low body posture. In addition, 8-OH-DPAT produced Straub tail, tremor, and backward
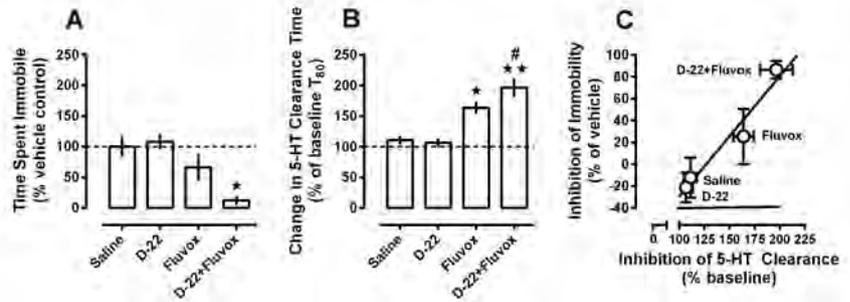
Figure 3. D-22 enhances the antidepressant-like and 5-HT clearance-inhibiting effects of fluvoxamine following systemic administration in wild-type C57BL/6 mice. **A**, Doses of D-22 (0.1 mg/kg) and fluvoxamine (10 mg/kg), which by themselves did not significantly change time spent immobile in the TST compared with saline-injected control mice, markedly decreased immobility time when given together. Data are expressed as mean $\pm$ SEM percentage of time (s) immobile under saline control conditions (112 $\pm$ 18 s); $n = 7$–10 per treatment condition; one-way ANOVA, $F_{(3,31)} = 6.92$, $p < 0.05$; Newman–Keuls post hoc test, *$p < 0.01$. **B**, In a separate group of mice, using the same doses and injection-test intervals used for the TST, systemic administration of D-22 enhanced fluvoxamine-induced inhibition of 5-HT clearance in hippocampus. Data are expressed as a percentage of baseline T_{80} values (i.e., prevehicle or drug injection T_{80} clearance times for 5-HT) $\pm$ SEM; $n = 6$–9 per treatment condition; one-way ANOVA, $F_{(3,27)} = 15.47$, $p < 0.05$; Newman–Keuls post hoc test, *$p < 0.01$, **$p < 0.001$ versus saline; #$p < 0.05$ versus fluvoxamine. **C**, Regression analysis shows a strong, positive, correlation ($r^2 = 0.95$, $p = 0.025$) between the ability of a given treatment to inhibit 5-HT clearance in hippocampus and to produce antidepressant-like effects in the TST.

movement. Essentially none of these signs were observed in vehicle-treated animals. Importantly, the combination of D-22 and fluvoxamine also did not significantly produce any element of the serotonin syndrome, at any time during the 6 min test, 30 min following the second injection. Likewise, no elements of the serotonin syndrome were observed throughout the entire 4 h observation period following the combination of D-22 and fluvoxamine, suggesting that at these doses, the combination treatment does not produce side effects typically associated with excess activation of the serotonin system.

Enhancement by D-22 of fluvoxamine-induced antidepressant-like effects in the tail suspension test is attenuated in OCT3 knock-out mice
Our data show that D-22 can enhance the antidepressant-like activity of fluvoxamine. D-22 has affinity for OCT1, 2, and 3 subtypes as well as PMAT (Amphoux et al., 2006; Schömig et al., 2006; Koepsell et al., 2007; Duan and Wang, 2010). Our next studies therefore sought to gain insight into which of these transporters might be necessary for this action of D-22. OCT1 has limited ability to transport 5-HT and is not densely expressed in hippocampus (Amphoux et al., 2006; Schömig et al., 2006; Koepsell et al., 2007). OCT2 expression in brain is confined primarily to subventricular regions, though it is expressed in hippocampus (Wu et al., 1998, 1999; Amphoux et al., 2006; Bacq et al., 2012). OCT3 and PMAT are richly expressed in limbic regions, including hippocampus (Gründemann et al., 1998; Dahlin et al., 2007; Baganz et al., 2008; Gasser et al., 2009). Based on these reports and because our previous studies support a role for OCT3 in mediating the effects of D-22 on immobility time in the TST (Baganz et al., 2008, 2010), we turned to OCT3 KO mice to investigate the requirement of this transporter for D-22 to enhance the effects of fluvoxamine in the TST. Behavioral and neurochemical characterization of OCT3 KO mice has been reported previously (Vialou et al., 2008, Cui et al., 2009; Wultsch et al., 2009).

Using the same dosing and timing regimen as before, we found that like wild-type C57BL/6 mice, D-22 (0.1 mg/kg) and fluvoxamine (10 mg/kg), when given alone to OCT3 KO mice,

Table 1. D-22 together with fluvoxamine does not produce elements of the serotonin syndrome, unlike the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT and the 5-HT precursor, 5-HTP

Element of the 5-HT syndrome	Saline (Tween 80)	D-22 (0.1 mg/kg) + fluvoxamine (10 mg/kg)	8-OH-DPAT (10 mg/kg)	5-HTP (100 mg/kg)	F[4,18]	p value
Hindlimb abduction	0.2 ± 0.2 (2.0 ± 1.5)	0.2 ± 0.2	5.0 ± 1.0*	4.2 ± 1.1*	7.4	0.001
Low body posture	0 ± 0 (0 ± 0)	0 ± 0	4.4 ± 1.2*	4.2 ± 0.9*	10.7	0.001
Straub tail	0 ± 0 (0 ± 0)	0 ± 0	2.0 ± 0.9*	1.6 ± 0.6	4.4	0.012
Tremor	0 ± 0 (0 ± 0)	0 ± 0	1.6 ± 0.7*	0.4 ± 0.3	4.0	0.017
Backward movement	0 ± 0 (0 ± 0)	0.2 ± 0.2	1.0 ± 0.5*	0 ± 0	3.4	0.031
Head weaving	0 ± 0 (0 ± 0)	0 ± 0	0.6 ± 0.6	0 ± 0	0.9	0.495
Forepaw treading	0 ± 0 (0 ± 0)	0 ± 0	0.2 ± 0.2	0 ± 0	0.9	0.495

Data are expressed as mean score ± SEM; n = 5 per treatment group, except 5% Tween 80 vehicle, n = 3; one-way ANOVA, followed by multiple comparisons with Newman–Keuls test. *Significantly different from saline, 5% Tween 80 vehicle and from D-22 + fluvoxamine.
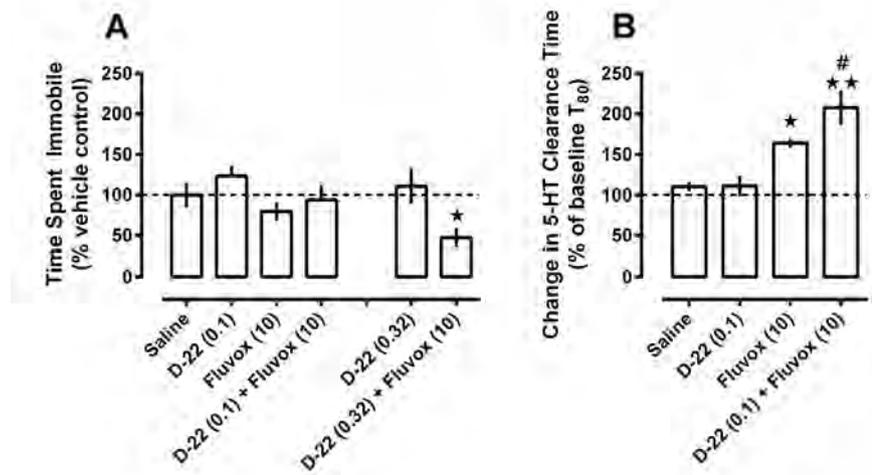
Figure 4. D-22 enhancement of the antidepressant-like effect of fluvoxamine in the TST is attenuated in OCT3 KO mice, whereas D-22 enhancement of the 5-HT clearance inhibiting effect fluvoxamine remains intact. **A**, Doses of D-22 (0.1 and 0.32 mg/kg) and fluvoxamine (10 mg/kg) by themselves did not significantly change time spent immobile in the TST compared with saline-injected control mice. In contrast to wild-type mice (Fig. 3A), a dose of 0.1 mg/kg D-22 did not decrease immobility time when given together with fluvoxamine; however, a significant reduction in immobility time resulted when a higher dose of D-22 (0.32 mg/kg) was given with fluvoxamine. Data are expressed as mean ± SEM percentage of time (s) immobile under saline control conditions (127 ± 19 s); n = 8–11 per treatment condition; one-way ANOVA, $F_{(3,35)}$ = 3.17, p < 0.015; Newman–Keuls post hoc test, *p < 0.05, from saline and D-22 counterparts. **B**, In a separate group of mice, using the injection-test intervals as for the TST, systemic administration of D-22 (0.1 mg/kg) enhanced fluvoxamine (10 mg/kg)-induced inhibition of 5-HT clearance in hippocampus. Data are expressed as a percentage of baseline T_{80} values (i.e., prevehicle or drug injection T_{80} clearance times for 5-HT) ± SEM; n = 6–7 per treatment condition; one-way ANOVA, $F_{(3,25)}$ = 12.34, p < 0.01; Newman–Keuls post hoc test, *p < 0.05, **p < 0.001 versus saline; #p < 0.05 versus fluvoxamine.

did not produce significant effects on time spent immobile compared with saline-treated mice. However, in contrast to wild-type mice, we found that D-22 failed to enhance the ability of fluvoxamine to reduce time spent immobile in the TST in OCT3 KO mice (Fig. 4A). These results suggest a key role for OCT3 in mediating this effect of D-22.

To confirm that this result was not due to reduced sensitivity of OCT3 KO mice to fluvoxamine, we performed dose–response studies and found that, like in wild-type mice, 10 mg/kg fluvoxamine was the largest dose that did not have significant effects by itself in the TST in OCT3 KO mice. Immobility time following vehicle injection in OCT3 KO mice was 127 ± 19 s (n = 10) (compared with 112 ± 18 s in wild-type mice, Fig. 3, legend). Similar to wild-type mice, fluvoxamine at doses of 3.2 and 10.0 mg/kg did not significantly influence time spent immobile in OCT3 KO mice (123 ± 14 s, n = 8, and 101 ± 15 s, n = 11, respectively), but significantly reduced immobility time at a dose of 32.0 mg/kg (47 ± 14 s, n = 8, one-way ANOVA, $F_{(3,33)}$ = 4.81,

p < 0.05). These data indicate that the inability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST in OCT3 KO mice was not due to a reduction in sensitivity of these mice to fluvoxamine.

We next investigated if a higher dose of D-22 (0.32 mg/kg) would enhance the antidepressant-like effect of fluvoxamine (10 mg/kg). This dose of D-22 was the highest that did not decrease locomotion in wild-type mice. We found that this dose of D-22 did enhance the ability of fluvoxamine to reduce time spent immobile in OCT3 KO mice (Fig. 4A; one-way ANOVA, $F_{(3,35)}$ = 3.49, p < 0.05); however, the magnitude of this enhancement was not as pronounced as that produced by the lower dose of D-22 in wild-type mice (Fig. 3A). In OCT3 KO mice given D-22 (0.32 mg/kg) together with fluvoxamine, time spent immobile decreased to 60 ± 15 s (47 ± 12% vehicle control, n = 10), whereas in wild-type mice D-22 (0.1 mg/kg) given in combination with fluvoxamine decreased immobility time essentially maximally (to 14 ± 8 s, or 12 ± 7% vehicle control, n = 7). An unpaired t test showed this difference to be significant ($t_{(15)}$ = 2.29, p < 0.05). These data suggest that OCT3 plays a predominant role in mediating the ability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST, but that D-22 can also produce this effect by actions at sites other than OCT3, albeit with lesser potency.

To examine whether the loss of antidepressant-like effect of the combination of D-22 (0.1 mg/kg) and fluvoxamine in the TST was associated with a reduced ability of the drug combination to inhibit 5-HT uptake in OCT3 KO mice, we used high-speed chronoamperometry to measure 5-HT clearance in the CA3 region of hippocampus under the same dose and injection-test interval as used previously. In contrast to the behavioral data, this drug combination inhibited 5-HT clearance in OCT3 KO mice similarly to that in wild-type mice (compare Figs. 3B, 4B). As we found in wild-type mice, D-22 (0.1 mg/kg) had no effect on 5-HT clearance, whereas fluvoxamine (10 mg/kg), which did not produce statistically significant effects in the TST, significantly inhibited clearance of intrahippocampally applied 5-HT in OCT3 KO mice. D-22 (0.1 mg/kg) enhanced fluvoxamine-induced inhibi-

tion of 5-HT clearance in OCT3 KO mice (one-way ANOVA, $F_{(3,25)} = 12.34$, $p < 0.05$).

Baseline (i.e., predrug) T_{80} values in OCT3 KO mice did not differ significantly ($p > 0.13$) among treatment groups and averaged 86 ± 6 s ($n = 26$), a value comparable to that obtained in wild-type mice (90 ± 4 s, $n = 69$). Likewise, baseline (i.e., predrug) signal amplitude values did not differ significantly ($p > 0.40$) among OCT3 KO mice treatment groups and averaged 0.63 ± 0.03 μM, comparable to that in wild-type mice (0.64 ± 0.02 μM).

Discussion

The major finding of the present studies is that the 5-HT clearance inhibiting and antidepressant-like effects of the SSRI fluvoxamine can be significantly augmented by coadministration of D-22. These data suggest that D-22-sensitive transporters may limit the ability of SSRIs to increase extracellular 5-HT and thus, limit their ability to produce antidepressant effects. D-22-sensitive transporters, such as OCTs and PMAT, may be useful targets for the development of new antidepressant medications to improve the therapeutic utility of SSRIs.

Implications for understanding the relationship between the ability of SSRIs to inhibit serotonin clearance and to produce antidepressant-like effects

An especially intriguing finding was the significant effect of the combination of fluvoxamine and D-22 to increase both the time course for 5-HT clearance and signal amplitude produced by 5-HT locally applied into the CA3 region of hippocampus. Previously, using similar approaches, we and others have not routinely observed an increase in amplitude of the 5-HT signal following administration of fluvoxamine. This has been a puzzling observation given that one would expect blockade of a transporter to increase both the clearance time and signal amplitude of exogenously applied neurotransmitter. Indeed, this is true for dopamine (DA) signals following blockade of the DA transporter (DAT) with drugs such as cocaine and nomifensine in DAT-rich regions, such as striatum and nucleus accumbens (Zahniser et al., 1999). This paradoxical lack of effect of SSRIs to increase 5-HT signal amplitude may well be attributed to the presence of D-22-sensitive transporters (OCTs and PMAT) and their expression level relative to SERT in hippocampus. As discussed earlier, these low-affinity, high-capacity transporters may become more effective at clearing 5-HT when extracellular 5-HT concentrations rise. Thus, following fluvoxamine, D-22-sensitive transporters may serve to prevent the 5-HT signal amplitude from increasing. Consistent with this notion is the observation that the effect of fluvoxamine on 5-HT clearance is most pronounced toward the tail of the signal, i.e., when the 5-HT concentration has fallen below the "reach" of low-affinity, high-capacity D-22-sensitive transporters (Fig. 1A). Thus, the combined effect of an SSRI and blocker of D-22-sensitive transporters is to not only further increase the duration that 5-HT remains in the extracellular fluid, but also to further increase the concentration (Daws et al., 2013).

Increased extracellular levels of 5-HT are considered to be an important trigger for ultimate therapeutic benefit in humans. Supporting this idea, our findings in wild-type mice show that antidepressant-like activity in the TST is positively correlated with the ability of a drug, or drug combination, to inhibit clearance of 5-HT in CA3 region of hippocampus (Fig. 3C). However, this was not the case in OCT3 KO mice, where the ability of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST was greatly attenuated, and yet inhibition of 5-HT clearance in the CA3 region of hippocampus was equivalent to that in wild-type mice. Thus, OCT3 appears to play a prominent role in mediating effects of D-22 in the TST, but does not appear to be involved in mediating the effect of D-22 to enhance fluvoxamine-induced inhibition of 5-HT clearance in the CA3 region of hippocampus. Together, these results indicate that antidepressant-like effects of a given treatment in the TST are not necessarily related to its ability to inhibit 5-HT clearance in the CA3 region of hippocampus. This raises the possibility that D-22's augmentation of the antidepressant-like effect of fluvoxamine in the TST is mediated by brain regions other than, or in addition to the CA3 region of hippocampus, and/or by its activity at other sites, possibly other OCT subtypes or PMAT, where it may inhibit uptake of 5-HT as well as other biogenic amines considered important in antidepressant-like response, discussed below.

Implications for increasing the clinical effects of antidepressants

The lack of symptom relief in many patients treated with currently available antidepressants emphasizes the need for novel pharmacological approaches with improved efficacy to treat depression. Our findings support D-22-sensitive uptake mechanisms as additional targets for the discovery of novel antidepressant therapies to maximize blockade of 5-HT uptake in brain. However, in addition to 5-HT, D-22-sensitive OCTs and PMAT are also low-affinity, high-capacity transporters for norepinephrine (NE) and DA (Wu et al., 1998; Amphoux et al., 2006; Cui et al., 2009; Bacq et al., 2012). Thus, while SSRIs have been the focus of this study, our results may generalize to other classes of antidepressants, including selective NE reuptake inhibitors, which block NE uptake via the NE transporter (NET), dual 5-HT-NE reuptake inhibitors, and triple-uptake inhibitors, which block transport of 5-HT, NE, and DA via their high-affinity transporters, SERT, NET, and DAT. To date, antidepressants that block SERT, NET, and DAT are thought to have the greatest clinical efficacy (Sulzer and Edwards, 2005; Chen and Skolnick, 2007). This may be in part attributed to the promiscuity that exists among SERT, NET, and DAT, which are all capable of low-affinity transport of their non-native biogenic amines (Pacholczyk et al., 1991; Daws et al., 1998; Norrholm et al., 2007; Rice and Cragg, 2008; Daws, 2009), as well as to the likelihood that successful treatment of depression requires targeting multiple neurotransmitter systems. Regardless, our findings suggest that the presence of D-22-sensitive transporters might also limit therapeutic efficacy of antidepressants other than SSRIs, and that blockade of D-22-sensitive transporters might serve to enhance their therapeutic efficacy by enhancing their ability to inhibit uptake of NE and DA, as well as 5-HT. Consistent with this idea, Hagan et al. (2011), using rotating disk voltammetry to measure 5-HT uptake into whole brain synaptosomes, showed that complete blockade of 5-HT uptake could only be achieved after incubating synaptosomes with blockers of SERT, NET, and DAT together with D-22. Thus, in terms of elevating extracellular levels of 5-HT and other biogenic amines to those that effectively treat depression, several lines of evidence, including the present study, point to blockade of D-22-sensitive transporters as a promising strategy.

In addition to being a potent blocker of low-affinity, high-capacity 5-HT transporters, D-22 has α-1-adrenoceptor antagonist properties (Russ et al., 1996). α-1 antagonism is unlikely to

mediate the enhancement by D-22 of fluvoxamine-induced inhibition of 5-HT clearance and antidepressant-like effects, because the α-1-adrenoceptor antagonist prazosin reportedly produces effects that are unlike those observed here with D-22. For example, prazosin decreases SSRI-induced increases of 5-HT levels (Rea et al., 2010), whereas D-22 enhanced the fluvoxamine-induced increase of 5-HT levels. In addition, prazosin increases immobility in the TST (Stone and Quartermain, 1999), whereas D-22 was inactive in this test when given alone at doses that enhanced effects of fluvoxamine. Finally, prazosin can block behavioral actions of antidepressants (Kostowski, 1985), whereas D-22 enhanced the antidepressant-like effects of fluvoxamine. Thus, it appears unlikely that α-1 antagonist properties underlie the ability of D-22 to enhance effects of fluvoxamine. Recently, the D-22 congener disprocynium-24 (D-24) has been reported to have antagonist properties not only at α-1 receptors, but also at α-2 receptors (Amphoux et al., 2010). It is presently unknown whether D-22 shares α-2 antagonist properties with D-24. If it does, these properties are unlikely to account for the effects of D-22 observed here, because the α-2 antagonist yohimbine does not affect 5-HT clearance (Ansah et al., 2003), and yohimbine increases immobility time in the TST (Ferrari et al., 1991). Together, there is evidence that D-22 has α-1-adrenoceptor antagonist properties, and evidence that α-1 antagonism produces effects that are opposite to those observed here with D-22. Thus, analogs of D-22 that lack α-1 antagonist properties may enhance effects of SSRIs even more markedly than D-22.

Although the idea of low-affinity, high-capacity transporters for 5-HT is not new, heralding back to the 1960s and 1970s when several groups reported promiscuous uptake of 5-HT by the so-called "uptake-2" transporter (Bertler et al., 1964; Fuxe et al., 1967; Lichtensteiger et al., 1967; Shaskan and Snyder, 1970), the identity of the transporter(s) has remained under some debate (Daws, 2009). In 2004, Schildkraut and Mooney proposed the extraneuronal monoamine transporter (uptake-2) as a target for development of more rapidly acting antidepressants, but still, the precise identity of "uptake-2" remained controversial. Since that time, several reports have emerged pointing to D-22-sensitive OCTs and PMAT as being primary uptake-2 (i.e., low-affinity, high-capacity) transporters for the biogenic amines (for review, see Daws, 2009). The challenge now will be to determine which of these might be the best to target for the discovery of new antidepressant drugs. While this field is still in its infancy OCT3 and more recently, OCT2, are emerging as leading candidates (Kitaichi et al., 2005; Baganz et al., 2008; Bacq et al., 2012). The markedly reduced potency of D-22 to enhance the antidepressant-like effect of fluvoxamine in the TST in OCT3 KO mice provides the first direct evidence that OCT3 is important for this action of D-22. Our behavioral and neurochemical results encourage further research into the role of D-22-sensitive transporters in regulating biogenic amine neurotransmission and behavior. Selective targeting of different OCT subtypes or PMAT may be a valid strategy to discover treatments with improved antidepressant efficacy, either as a cotreatment with existing antidepressant drugs or as novel 5-HT reuptake inhibitors with additional OCT or PMAT blocking properties.

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Appendix B

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Enhanced novelty-induced corticosterone spike and upregulated serotonin 5-HT$_{1A}$ and cannabinoid CB$_1$ receptors in adolescent BTBR mice

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Summary Hypothalamic pituitary adrenal (HPA) axis responses to change and social challenges during adolescence can influence mental health and behavior into adulthood. To examine how HPA tone in adolescence may contribute to psychopathology, we challenged male adolescent (5 weeks) and adult (16 weeks) BTBR T+tf/J (BTBR) and 129S1/SvImJ (129S) mice with novelty in sociability tests. In prior studies these strains had exaggerated or altered HPA stress responses and low sociability relative to C57BL/6J mice in adulthood. In adolescence these strains already exhibited similar or worse sociability deficits than adults or age-matched C57 mice. Yet BTBR adolescents were less hyperactive and buried fewer marbles than adults. Novelty-induced corticosterone (CORT) spikes in adolescent BTBR were double adult levels, and higher than 129S or C57 mice at either age. Due to their established role in HPA feedback, we hypothesized that hippocampal Gαi/o-coupled serotonin 5-HT$_{1A}$ and cannabioid CB$_1$ receptor function might be upregulated in BTBR mice. Adolescent BTBR mice had higher hippocampal 5-HT$_{1A}$ density as measured by [^{3}H] 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) binding than C57 mice, and adult BTBR 8-OH-DPAT-stimulated GTPγS binding was higher than in either C57 or 129S mice in this region. Further, BTBR hippocampal CB$_1$ density measured by [^{3}H]CP55,940 binding was 15—20% higher than in C57.

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CP55,940-stimulated GTPγS binding in adult BTBR dentate gyrus was 30% higher then 129S ($p < 0.05$), but was not a product of greater neuronal or cell density defined by NeuN and DAPI staining. Hence hyperactive HPA responsiveness during adolescence may underlie 5-HT$_{1A}$ and CB$_1$ receptor up-regulation and behavioral phenotype of BTBR mice.

1. Introduction

Exposure to severe stressors during adolescence can persistently alter hypothalamic pituitary adrenal (HPA) axis response, perception, cognition and mood into adulthood (McCormick and Mathews, 2007; Stevens et al., 2009). For example, unstable childhood home environments are associated with exaggerated HPA responses to stress, particularly among males (Hackman et al., 2012; Brenner et al., 2013). However, teenage boys with autism, depression or other psychiatric disorders often exhibit exaggerated HPA responses (e.g. higher cortisol peaks) following social or novel stimuli than their 'normotypic' peers, paralleling individuals severely stressed in youth, even without a prior history of severe stress exposure (Lopez-Duran et al., 2009; Corbett et al., 2010; Spratt et al., 2012; Schupp et al., 2013). This low HPA axis resilience can have profound long-term impacts on social behavior, and may also increase the risk of suicide (Sher, 2006; Sunnqvist et al., 2008; Garlow et al., 2008).

Paralleling this, in male BTBR T+tf/J (BTBR) and 129S1/SvImJ (129S) mice exhibiting low social interaction (Gould et al., 2011; 2012), stressors induce exaggerated peak corticosterone (CORT) levels. For example, stressed BTBR released more CORT, yet BTBR have higher hippocampal glucocorticoid receptor (GR) mRNA levels than C57BL/6 mice (Benno et al., 2009; Silverman et al., 2010). 129S mice also had higher post-stress peak CORT, but had lower hippocampal GR mRNA than C57BL/6 mice (Camp et al., 2012). All mice in these studies were adults. Since murine stress reactivity appears to be exaggerated prior to puberty (Romeo et al., 2013), yet adolescent and adult hippocampal GR expression are similar (Pryce, 2008), adolescent HPA axis tone was of great interest in these strains. Hence we compared adolescent and adult CORT responses to mild stressors, specifically novel social interaction and novel object exposure.

Corticosteroids bind to hippocampal GRs, the activation of which promotes serotonin transmission that in turn attenuates CORT release (Lanfumey et al., 2008; Pompili et al., 2010). CORT binding to GR also increases hippocampal endocannabinoid levels, and this likewise suppresses CORT release (Cota, 2008; McLaughlin et al., 2009; Atsak et al., 2012). This HPA axis suppression is likely mediated via inhibitory metabotrophic Gαi/o coupled serotonin 5-HT$_{1A}$ and CB$_1$ receptors. When direct HPA axis feedback by circulating CORT via GRs is impaired, 5-HT$_{1A}$ and CB$_1$ receptors may be up-regulated in compensation (Lanfumey et al., 2008; Pompili et al., 2010; Hensler et al., 2010). We hypothesized that this type of compensation may occur to a greater extent during adolescence, particularly if the HPA stress-response is exaggerated.

We have found that in adult BTBR mice hippocampal 5-HT$_{1A}$ receptor function was enhanced relative to C57BL/10 mice (Gould et al., 2011). We hypothesized that exaggerated stress-evoked CORT release during adolescence could underlie this 5-HT$_{1A}$ up-regulation, and may likewise up-regulate hippocampal CB$_1$ receptors. Since higher binding could stem from increases either in receptor expression or neuronal density, we compared hippocampal neuronal cell density in adults of each strain. Further, receptor up-regulation might be more pronounced in adolescent mice with HPA axis hyperactivity. Thus, we compared adolescent to adult 5-HT$_{1A}$ and CB$_1$ receptor densities and agonist-stimulated G-protein coupling in these strains.

2. Methods

2.1. Mouse subjects

BTBR T+tf/J, 129S1/SvImJ, C57BL/10J and C57BL/6J colony founders were from Jackson Laboratory (Bar Harbor, ME, USA). The mice were second or greater generation offspring bred in the laboratory animal facilities at William Paterson University, Wayne, NJ for quantitative autoradiography, or at The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX for autoradiography, immunostaining, behavior and corticosterone measures. Mice at both facilities were maintained at 20–24 °C on 12:12 light dark cycles, with lights on at 0700 h, and ad libitum access to food and water in cages lined with wood chip bedding (at UTHSCSA) or shaved wood bedding (at William Paterson University) that was changed bi-weekly. Mice were weaned at around postnatal day 21 and were housed in same-sex littermate groups of 2–5 per cage. All procedures involving mice were approved by Institutional Animal Care and Use Committees, and were consistent with current NIH guidelines.

2.2. Novelty exposure and plasma CORT levels

Mice used for novelty exposures and corticosterone (CORT) measures were 5 or 16 week-old male C57BL/6, 129S and BTBR. Prior to trunk blood collection, half of the mice were subjected to three-chamber sociability tests (40 min), immediately followed by marble burying (30 min), as in Gould et al. (2011, 2012). Briefly, each mouse subject was individually placed in a novel three-chamber arena for sociability testing. First subjects explored their arena for 20 min of pre-conditioning, then an unfamiliar object (wire cup) and 'stranger 1' a 129S male mouse (4–5 weeks old, in wire cup) was introduced at either end for 10 min, and finally a second novel 'stranger 2' mouse under a wire cup was introduced for 10 min, replacing the empty cage. Self-grooming was only scored during the sociability test and was not scored in an independent task. Afterward each subject mouse was transferred for 30 min to a 51 cm × 28 cm × 23 cm box filled with

10 cm of wood-chip bedding topped with 15 blue marbles in a grid pattern. The untested group of mice remained in their home cages until sacrifice. All mice were humanely sacrificed by decapitation between 1500 and 1650 h CST, and trunk blood was collected into tubes containing 25 μl of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO).

Mouse blood was centrifuged at 4 °C for 10 min at 2600 rpm, and serum collected and frozen at −80 °C. Plasma CORT levels were measured colorometrically on a plate reader (Molecular Devices, Sunnyvale, CA) after using an enzyme immunoassay kit (#ADI-900-097, Enzo, Plymouth Meeting, PA), following the manufacturer's small sample volume protocol.

2.3. Brain tissue preparation

Mice used for quantitative autoradiography and immunostaining were 5 and/or 16 week old naïve males. The mice were sacrificed by decapitation and their brains were frozen on powdered dry ice and stored at −80 °C. Coronal sections (20 μm) were collected from 0.90 to 0.60 mm and −1.80 to −2.00 mm relative to Bregma on a cryostat (Leica, Buffalo Grove, IL), and were thaw mounted onto chilled gelatin coated microscope slides. The sections were desiccated for 12−24 h under vacuum at 4 °C and then stored at −80 °C until use in autoradiography or immunostaining.

2.4. Quantitative autoradiography

Binding assays with [^{3}H] 8-OH-DPAT (2 nM) for serotonin 5-HT$_{1A}$, with 1 μM WAY 100,635 (Tocris, Ellisville, MO) to define non-specific binding, and [^{3}H] CP55,940 (5 nM) for cannabinoid CB$_1$ receptors, with 200 μM WIN,55-212-2 (Ascent Scientific, Princeton NJ) for non-specific binding, were performed on brain sections as per Gould et al. (2012). [^{35}S] GTPγS binding in the absence or presence of 1 μM of agonists 8-OH-DPAT or CP 55,940 was performed as described in Gould et al. (2011, 2012). Radioligands were from Perkin-Elmer (Boston, MA), and Kodak Biomak MR film (ThermoFisher, Waltham, MA) was used for all experiments.

Digital images were captured on a camera (1612M, Scion Corp., Frederick, MD) with a 60 mM lens onto a Macintosh (OS 10), with Image J software (NIH, Bethesda, MD) for density measures. Gray scale units were converted to fmol/mg protein or nCi/mg using calibration standards (American Radiolabeled Chem., St. Louis, MO), as per Gould et al. (2011). Brain regions measured included the hippocampus CA1, CA3 and dentate gyrus regions, frontal and parietal cortex, caudate putamen, nucleus acumbens (CB$_1$ only), amygdala and hypothalamus. The amygdala and hypothalamus were not measured for GTPγS due to high basal binding.

2.5. Immunohistochemical labeling

Brain sections on slides were thawed, rinsed with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Grand Island, NY) and fixed with 4% paraformaldehyde at 4 °C for 1 h. They were rinsed in DPBS 3 times for 5 min each, and permeablized in 0.5% triton X-100 in DPBS at 26 °C for 1 h. The tissue was blocked for 1 h at 26 °C, anti-NeuN (rabbit polyclonal, Millipore, Billerica, MA) 1:500 was added for an overnight incubation at 4 °C. The sections were washed 3 times with DPBS for 5 min each, and Alexa Fluor 488 goat anti-rabbit (Millipore) 1:500 secondary antibody was added before incubating for 2 h at 26 °C. After a final round of 3, 5 min DPBS washes, sections were cover slipped with vectashield mounting medium containing 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Vector Labs, Burlingame, CA). Sections were digitally captured on an Eclipse TE-2000-E (T-HUBC) microscope with a digital camera (Model: DXM1200C, Nikon, Melville, NY). Staining intensity in the dentate gyrus of hippocampus was measured using Nikon NIS-Elements AR 3.0 software.

2.6. Statistical analysis and sample sizes

Three-way (strain × age × treatment) analysis of variance (ANOVA) was used to compare mean CORT levels, with Fisher's least significant difference (LSD) post hoc tests performed when significant main effects occurred. Repeated measures ANOVA was used to compare time in chamber and sniffing time in sociability tests, significant effects were further resolved by post hoc ANOVA and/or t-test comparisons. Two-way ANOVA was used to compare mean chamber entries, grooming times, and marbles buried, with Fisher's LSD post hoc tests performed to resolve significant effects. There were 6−9 mice per strain/age/treatment group. For autoradiography two-way (strain × age) multivariate (several brain regions measured) ANOVA was used to compare mean binding density for several different brain regions, with Newman Keuls post hoc tests performed, there were 8 mice per group. ANOVA was used to compare immunostaining intensity in the dentate gyrus, there were 7−8 mice per strain. All analyses were performed using Statistica software (StatSoft, Tulsa, OK).

3. Results

3.1. Effects of strain, age and behavior tests on plasma CORT

Corticosterone levels increased significantly after 70 min of novelty exposure in behavior tests ($F_{1,66}$ = 177, $p < 0.0001$), and differed in magnitude among mouse strains ($F_{2,66}$ = 24, $p < 0.0001$) and ages ($F_{1,66}$ = 17, $p < 0.0001$), with significant interactions ($p < 0.017$). The post-novelty increase in CORT was two-fold greater in 5 week-old BTBR mice than other strains, and was also greater than in adult BTBR mice ($p < 0.0001$) (Fig. 1). Baseline CORT levels tended to be higher in adult BTBR than C57BL/6, and this difference was nearly significant ($p = 0.06$).

3.2. Social and repetitive behaviors in adolescent and adult male mice

In global analysis of the three-chamber sociability tests, there was a significant interaction between mouse strain and age for time spent in either end chamber of the test arena ($F_{2,30}$ = 3.23, $p < 0.05$). In the social interaction phase, C57 adults spent significantly more time by the stranger mouse vs. novel object ($t = −3.8$, $p < 0.05$), while C57 adolescents also tended to, this trend was not significant
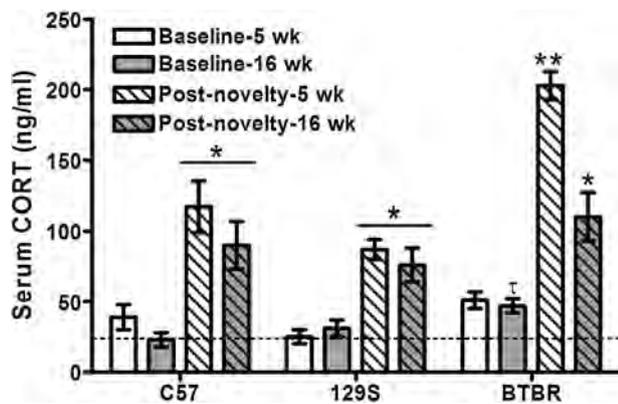
Figure 1 Novelty exposure raised plasma CORT levels the most in adolescent BTBR mice. CORT levels increased above mean baseline levels in adolescent (5 week) and adult (16 week) male mice of C57BL/6, 129S1/SvImJ and BTBR strains after 70 min of novelty exposure in behavior tests ($*p < 0.05$). However, CORT levels in novelty-exposed 5 week-old BTBR mice were far higher than all of the other strains and ages ($**p < 0.0001$). Baseline CORT levels tended to be significantly higher in BTBR than in C57BL/6 adults (τ $p = 0.06$). The dashed horizontal line illustrates the mean CORT level found in adult C57BL/6 mice to facilitate visual reference.

($t = -2.14$, $p = 0.09$). Five week-old BTBR mice spent less time in chambers with stranger mice and more time in chambers with novel objects ($t = 3.2$, $p < 0.02$), and they differed in comparison to BTBR adults with respect to this measure ($F_{1,10} = 4.95$, $p < 0.05$, Fisher's LSD $p < 0.05$, Fig. 2a). In the second phase of the test, only C57BL/6 adult mice showed a significant preference for social novelty based on time spent in the chamber with the new stranger mouse ($p < 0.05$, Fig. 2b).

A global repeated measures analysis of social sniff time during sociability tests revealed significant differences between ages ($F_{1,30} = 15$, $p < 0.001$), test phase ($F_{1,30} = 25$, $p < 0.0001$), with significant interactions among all main effects. With the finding of a significant age effect in the repeated measures ANOVA ($F_{1,30} = 9.45$, $p < 0.01$), in post hoc ANOVAs performed on the social interaction phase, there was a significant effect of age for the amount of time spent sniffing empty cages ($F_{1,30} = 15$, $p < 0.001$), and an interaction between strain and age ($F_{2,30} = 3.9$, $p < 0.32$). Specifically BTBR adolescents spent more time sniffing the empty cages than all other groups except 5 week-old 129S mice. There was no significant difference in social sniff of stranger mice between strain ($p = 0.4$), or age ($p = 0.7$), as shown in Fig. 2c. However only C57 adults and adolescents had a significant preference for stranger mice ($t > 3.4$, $p < 0.05$). In the social novelty phase, there was a significant effect of strain ($F_{1,30} = 15$, $p < 0.0001$) and age ($F_{2,30} = 0.001$), with interactions among factors ($F_{2,30} = 12$, $p < 0.0001$). This was due to an increased amount of sniffing by 129S adolescents of either stranger relative to all other strains ($F_{2,30} > 5$, $p < 0.05$, Fisher's LSD $p < 0.05$), and more sniffing of the new strangers in comparison to 129S adults ($F_{1,30} = 22$, $p < 0.0001$, Fisher's LSD $p < 0.05$), shown in Fig. 2d.

During social interaction tests, BTBR adults were hyperactive, making more chamber entries than other groups

($F_{2,30} = 4.7$, $p < 0.02$; Fisher's $p < 0.05$), yet their self-grooming duration across both sociability test phases combined was similar to other groups ($F_{2,30} = 0.87$, $p = 0.42$, Fig. 2e). In contrast, 129S mice spent significantly less time self-grooming than C57BL/6 mice during the sociability tests ($F_{2,30} = 5.1$, $p < 0.01$; $p < 0.05$, Fig. 2e), while C57BL/6 and BTBR self-grooming was similar. Immediately after sociability tests, adult BTBR and 5-week 129S mice buried more marbles than C57BL/6 mice ($F_{2,30} = 13$, $p < 0.001$, Fisher's LSD $p < 0.05$, Fig. 2f).

3.3. Serotonin 5-HT$_{1A}$ receptors

There were significant differences in [^{3}H] 8-OH-DPAT binding density among strains (Wilks' $\lambda_{16,70} = 0.17$, $p < 0.0001$) and ages ($\lambda_{8,35} = 0.32$, $p < 0.0001$), with interactions among these factors ($\lambda_{16,70} = 0.51$, $p = 0.05$) in the hippocampus and in other brain areas. Among 16 week-old mice, 129S [^{3}H] 8-OH-DPAT binding was higher in the CA1 of hippocampus (Fig. 3b) and cingulate cortex (by roughly 30%) than either BTBR or C57BL/10 ($F_{2,42} > 8$, $p < 0.01$; Newman–Keuls $p < 0.05$). Further, 5-week old 129S and BTBR mice had higher [^{3}H] 8-OH-DPAT binding in the CA1 of hippocampus than C57BL/10 at either age ($F_{2,42} = 21$, $p < 0.001$; $p < 0.01$, Fig. 3b). Five-week old BTBR mice also had higher [^{3}H] 8-OH-DPAT binding in the CA3 of hippocampus (Fig. 3c, $F_{2,42} = 7.1$, $p < 0.002$; $p < 0.05$). Also, 16-week old BTBR mice had lower [^{3}H] 8-OH-DPAT binding than 5-week olds in the basolateral amygdala (108 ± 14 vs. ≈ 165 fmol/mg protein) and ventromedial hypothalamus (129 ± 11 vs. ≈ 200 fmol/mg protein) ($F_{1,42}$ or $F_{2,42} > 3.15$, $p < 0.05$). Non-specific was 8–10% of total binding.

8-OH-DPAT-stimulated [^{35}S] GTPγS binding tended to differ among strains ($\lambda_{8,78} = 0.11$, $p = 0.067$), but not among ages ($\lambda_{5,38} = 0.87$, $p = 0.38$), with no interaction ($\lambda_{10,76} = 0.84$, $p > 0.7$). Specifically, agonist-stimulated 5-HT$_{1A}$ binding in 16 week-old BTBR mice was higher in all hippocampal regions measured ($F_{2,42} > 3$, $p \leq 0.05$), as shown in the plots on the right side in Fig. 3a–c. Basal binding ranged from a mean of 249 ± 14 to 435 ± 31 nCi/mg in the brain regions measured, and non specific binding was roughly 40% of basal and 10% of stimulated binding.

Taken together, the age-dependent density and functional relationship between 5-HT$_{1A}$ and 8-OH-DPAT stimulated GTPγS binding in the hippocampus of BTBR mice (Fig. 3a–c) is of particular interest, given this receptor's proposed modulatory role in HPA axis feedback. Specifically [^{3}H] 8-OH-DPAT binding density in 5-week old mice was higher in BTBR than in C57BL/10 mice in the CA1 ($p < 0.01$) and CA3 ($p < 0.005$) regions of the hippocampus, yet at 16 weeks of age, [^{35}S]GTPγS binding was significantly higher in BTBR mice than in C57BL/10 mice in the CA1 ($p = 0.05$) and dentate gyrus ($p < 0.05$) regions of the hippocampus.

3.4. Cannabinoid CB$_1$ receptors

There were differences in [^{3}H] CP55,940 binding density among strains ($\lambda_{18,68} = 0.1$, $p < 0.001$), specifically C57BL/10 had lower density than BTBR and/or 129S mice in dentate gyrus, CA1, CA3 of hippocampus and in the parietal cortex (175 vs. >202 fmol/mg protein) at 5 weeks, and in the dentate gyrus and hippocampal CA3 at 16 weeks of age
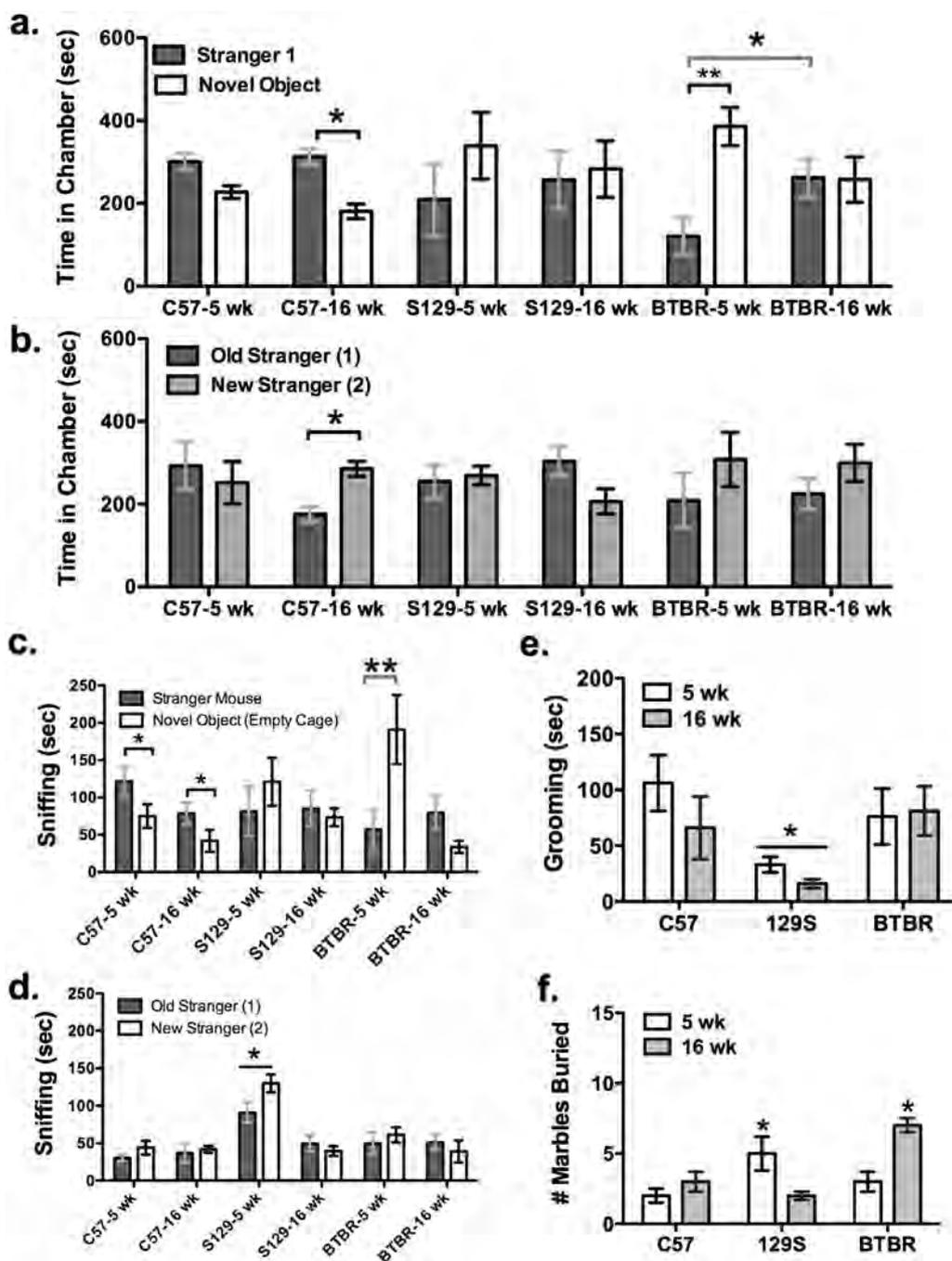
Figure 2 Behavior of adolescent and adult BTBR mice in sociability and repetitive tests. (a) A significant preference for social interaction was only found in C57 adults (*$p < 0.05$). Adolescent BTBR mice spent more time in chambers with novel objects than with stranger mice in the social interaction test (**$p < 0.001$), in contrast to adults (*$p < 0.01$). (b) Only adult C57 mice spent significantly more time in chambers with new strangers than with the 'old' original ones (*$p < 0.05$). (c) Both C57 adolescent and adult mice spent more time sniffing stranger mice than novel objects (*$p < 0.05$), however BTBR adolescents spent significantly more time sniffing novel objects than all other mice (**$p < 0.001$). (d) Time spent sniffing new and old stranger mice during the social novelty test was greatest in 129S adolescents (*$p < 0.05$). (e) Self-grooming among C57 and BTBR mice was comparable, but was lower in 129S mice during sociability testing (*$p < 0.05$). (f) BTBR adults and 129S adolescents buried more marbles over 30 min after sociability testing than C57BL/6 mice (*$p < 0.05$).

($F_{2,42} > 3$, $p < 0.05$, Fig. 4a–c, left panels). While a significant age effect was detected ($\lambda_{9,34} = 0.29$, $p < 0.001$), post hoc ANOVA failed to reveal age differences in the regions measured ($F_{1,42} < 2$, $p > 0.15$), and there were no interactions ($\lambda_{18,68} = 0.5$, $p = 0.19$). Non-specific binding was 40–50% of total.

CB$_1$ receptor CP55,940-stimulated [^{35}S] GTPγS binding differed among strains ($\lambda_{10,76} = 0.55$, $p < 0.01$) and ages ($\lambda_{5,38} = 0.65$, $p < 0.01$) without interaction ($\lambda_{10,76} = 0.67$, $p = 0.1$). In all strains, CP55,940-stimulated binding increased with age in the hippocampus, as shown in the right panels of Fig. 4, and also in the cingulate cortex (18–40%
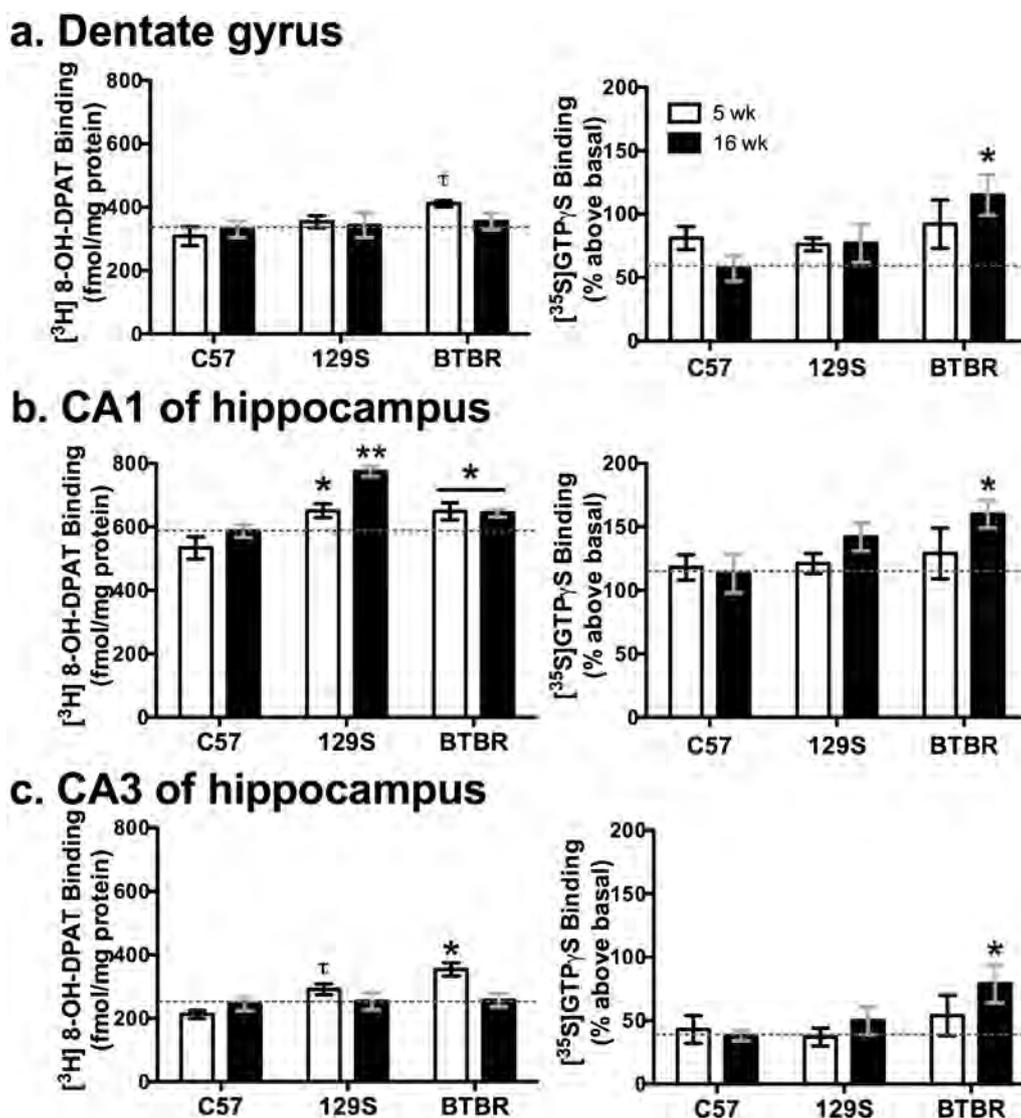
Figure 3 Specific [^{3}H] 8-OH-DPAT binding and 8-OH DPAT-stimulated [^{35}S] GTPγS binding to serotonin 5-HT$_{1A}$ receptors in the hippocampus of adolescent and adult mice. In the left-side panels [^{3}H] 8-OH-DPAT binding was higher in 5-week old BTBR and/or 129S mice in (b) the CA1 and (c) CA3 of hippocampus than age-matched C57 mice (*$p < 0.05$; **$p < 0.01$; τ $p = 0.067$). In (a) the dentate gyrus, there was also a non-significant trend toward BTBR mice at 5 weeks having greater [^{3}H] 8-OH-DPAT binding (τ $p = 0.085$). In the right-side panels 8-OH-DPAT stimulated [^{35}S] GTPγS binding was enhanced in adult BTBR mice in (a) dentate gyrus, (b) CA1 and (c) CA3 of hippocampus as compared to adult C57 (*$p \leq 0.05$). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus, in 5-week old BTBR mice hippocampal 5-HT$_{1A}$ density was high, whereas in adults 5-HT$_{1A}$ function was enhanced.

greater in adults). In 16 week-old BTBR mice CP55,940-stimulated [^{35}S] GTPγS binding was higher in dentate gyrus than in other strains ($F_{2,42} = 3.7$, $p < 0.05$, Fig. 4a, right). We also observed in the hippocampal CA1 and CA3 of 5 week-old 129S mice that CP55,940-stimulated [^{35}S] GTPγS binding was lower ($p < 0.05$) than in C57BL/10 mice of the same age. This corresponded with modest increases in 129S CB$_1$ receptor density in the 5-week old hippocampus, as measured by [^{3}H] CP55,940.

3.5. C57BL/10 vs. C57BL/6 binding comparison

Because C57BL/6 mice are more commonly used than C57BL/10, we compared [^{3}H] 8-OH-DPAT, [^{3}H] CP55,940, and agonist

stimulated [^{35}S] GTPγS binding density in them. We did not observe any significant differences in 5-HT$_{1A}$ ($\lambda_{8,7} = 0.28$, $p = 0.15$) or CB$_1$ ($\lambda_{9,6} = 0.44$, $p = 0.61$) binding site density among these strains. Further, agonist-stimulated [^{35}S] GTPγS binding did not reveal any real differences among C57BL/6 and C57BL/10 adults ($\lambda_{5,2} = 0.05$, $p = 0.12$).

3.6. Immunostaining in dentate gyrus

There were no differences in stain intensity for DAPI ($F_{2,19} = 2.8$, $p = 0.09$) or NeuN ($F_{2,19} = 1.0$, $p = 0.38$) in the dentate gyrus among adult male 129S, BTBR or C57BL/6 mice. NeuN stain intensity ranged from a mean ± S.E.M. of 43 ± 9
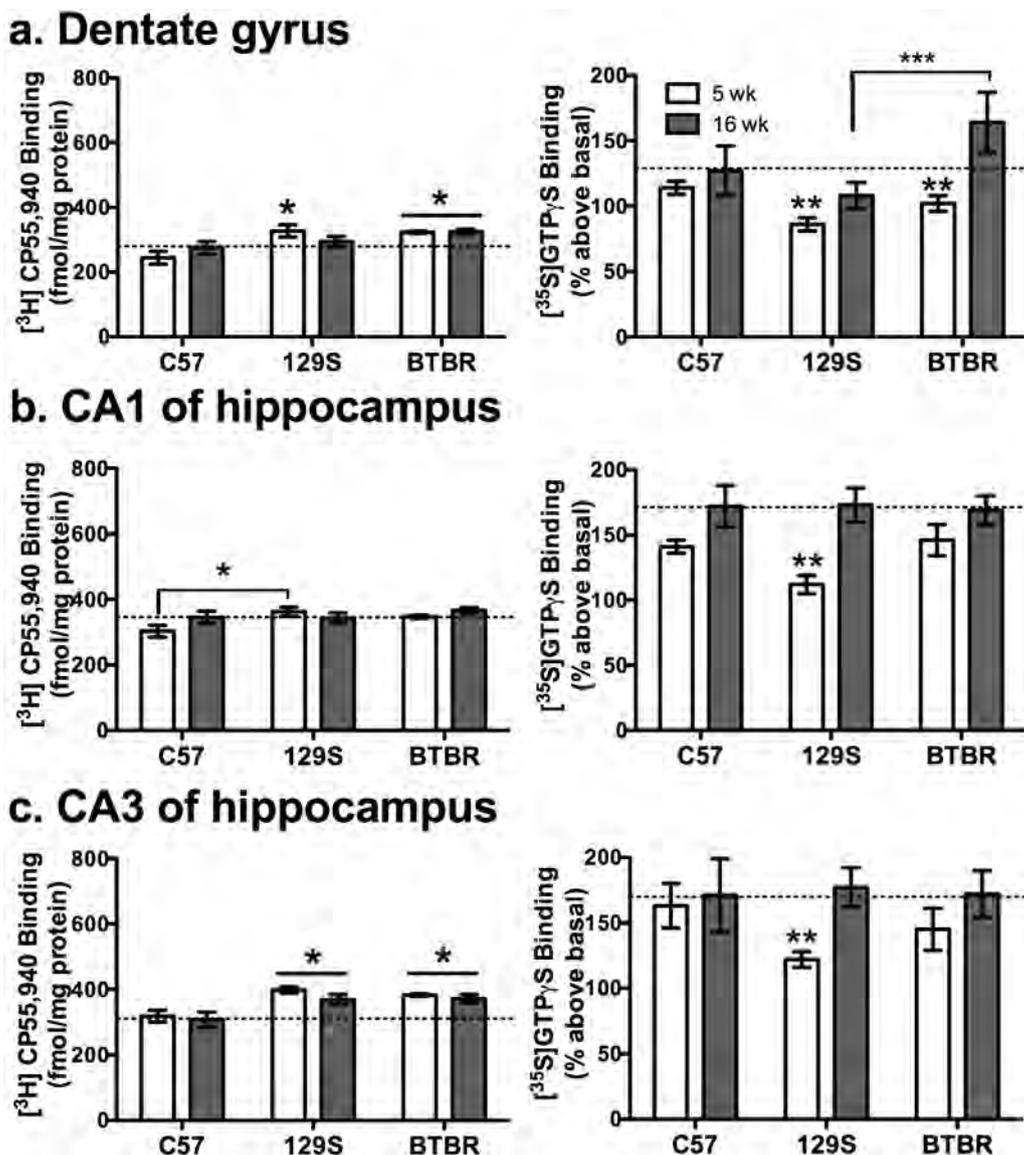
Figure 4 Specific [³H] CP55,940 binding and CP55940-stimulated [³⁵S] GTPγS binding to hippocampal cannabinoid CB₁ receptors in adolescent and adult mice. As shown in the left-side panels [³H] CP55,940 binding was greater in the (a) dentate gyrus and (c) CA3 of BTBR vs. C57 mice (*$p < 0.05$). Adolescent 129S mice had significantly higher [³H] CP55,940 binding in the (a) dentate gyrus, (b) CA1 and (c) CA3 than adolescent C57 mice (*$p < 0.05$). On the other hand, CP55,940 stimulated [³⁵S] GTPγS binding generally increased with age, as shown in the panels to the right. However, hippocampal CP55,940 stimulated [³⁵S] GTPγS binding was consistently lower in 129S adolescents (**$p < 0.05$, a–c). Also agonist stimulated GTPγS binding was higher in adult BTBR than in 129S mice (***$p < 0.05$, a). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus CB1 receptor coupling capacity is reduced, despite elevated CB1 receptor density in the adolescent 129S hippocampus.

to 64 ± 11 optical density units, while DAPI ranged from 72 ± 7 to 92 ± 5 density units. Representative images are shown in Fig. 5.

4. Discussion

Herein we provide evidence of greater novelty-induced increases in serum CORT in adolescent male BTBR mice, accompanied by greater hippocampal serotonin 5-HT₁ₐ and cannabinoid CB₁ receptor density relative to C57 mice. In BTBR adults, agonist-stimulated GTPγS binding to hippocam-

pal 5-HT₁ₐ receptors was augmented, but this was not accompanied by higher receptor, cellular or neuronal density. Enhanced hippocampal 5-HT₁ₐ Gαi/o-coupled receptor activation capacity could facilitate inhibition of CORT release, and as such it may be a key compensatory response in adult BTBR HPA axis feedback. While hippocampal CB1 receptor density was greater in BTBR and 129S mice, CP55,940 stimulated GTPγS binding in 129S was no higher than, and in adolescents it was reduced relative to C57 mice. These factors may contribute to the age-dependent patterns in sociability deficits and hyperactivity we observed in BTBR and 129S males.
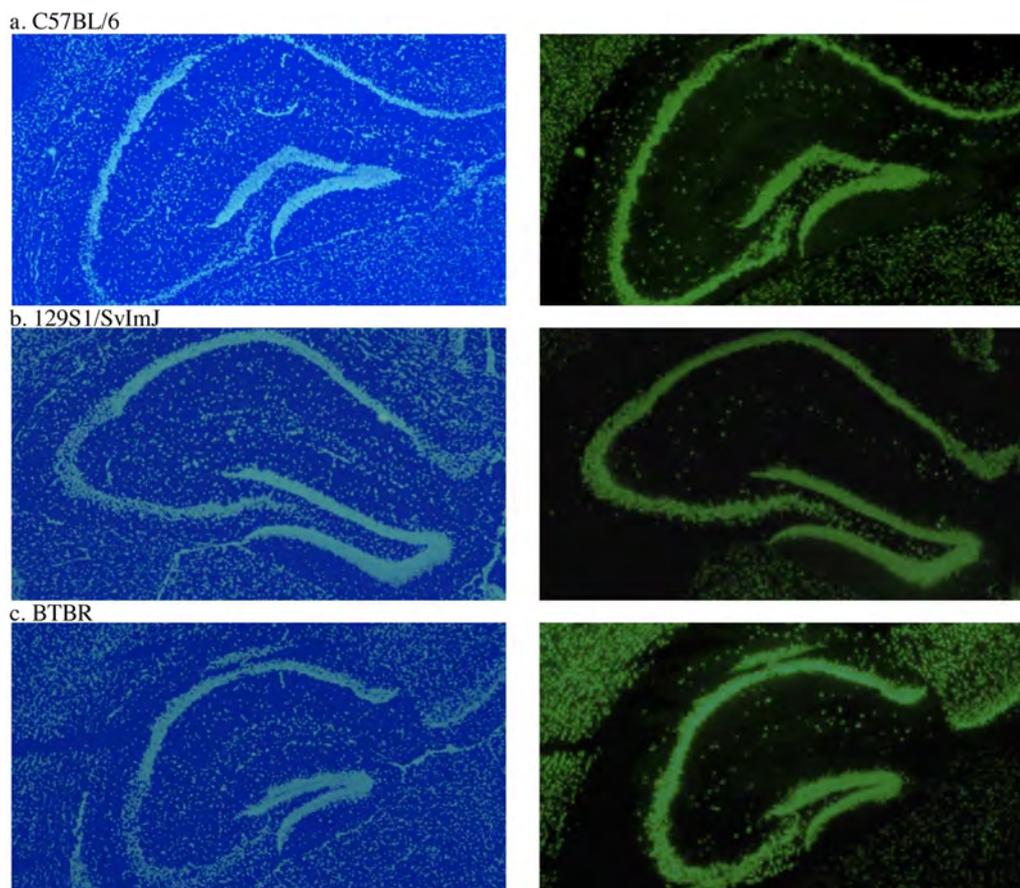
Figure 5 DAPI nuclear (left) and NeuN neuronal (right) staining in the adult mouse hippocampus. (a) C57BL/6, (b) 129S1/SvImJ and (c) BTBR mouse hippocampi had similar cell (in light blue) and neuron (in light green) density in the dentate gyrus.

4.1. Social behavior and CORT level comparisons across age and strains

A preference for chambers with novel objects over stranger mice — that might arguably be interpreted as social avoidance — was evident in adolescent male BTBR relative to C57BL/6 mice. This behavioral pattern was pronounced, even as compared to BTBR adults that generally lack preference for social interaction (Moy et al., 2007; Silverman et al., 2010). It also corresponded with higher levels of CORT in plasma after behavior testing. No similar age-dependent sociability patterns occurred in either C57 or 129S mice. Further, adult, but not adolescent BTBR mice entered the chambers in the sociability testing arena more frequently than other strains, consistent with earlier findings of hyperactivity in this strain (Silverman et al., 2010).

Based on prior studies, we predicted that CORT spikes in response to novel stimuli, at least in the adolescent C57BL/6 males, might be exaggerated compared to those in adults (Romeo et al., 2013), and that even higher levels might be found in BTBR mice (Benno et al., 2009). Indeed, we saw an exaggerated CORT response to novelty in adolescent BTBR after 70 min of sociability testing and marble burying. Our CORT level measures were also consistent with earlier reports of higher baseline and hyperactive HPA axis responsiveness in BTBR relative to C57BL/6 mice (Benno et al.,

2009; Silverman et al., 2010). In contrast, CORT levels in 129S mice were no higher than C57BL/6 following novelty exposure, although restraint stress produced greater initial increases in them in a prior study (Camp et al., 2012). Since we did not measure CORT levels at earlier time points (e.g. at 30 min intervals) during our behavior tests, we suspect that we did not capture peak CORT levels induced by handling and novelty exposure. However, higher peak levels and/or extended release of CORT was nonetheless evident in BTBR adolescents after novelty exposure under our testing paradigm, and we also saw higher CORT levels in all strains and ages after behavior testing as compared to baseline values.

However, elevated CORT levels, either at baseline or in response to novelty, may not necessarily be indicative of a heightened anxiety state in BTBR mice. Five weeks is just prior to puberty onset at around postnatal day 40 for most male mice, including strains such as C57BL/6 and 129S (Wisniewski et al., 2005; Qiu et al., 2013). Male puberty has not been characterized in BTBR mice, so it could occur earlier, as in strains such as CD1 or C3H/HeJ (Divall et al., 2010; Zhou et al., 2012), or later. Yet it is unlikely that any differences in puberty onset alone could have so distinguished the BTBR behavioral and neuroendocrine phenotype. Other endocrine abnormalities are more likely responsible, for example in BTBR mice serum levels of insulin, testosterone, and progesterone are also relatively high (Flowers et al.,

2007; Frye and Llaneza, 2010). Taken together with elevated baseline CORT levels and exaggerated stress response, there is evidence of pituitary over-activity, possibly due to feedback disruption in BTBR mice. These conditions can adversely impact cognition and behavior (Stranahan et al., 2008).

4.2. Age-dependent patterns of 5-HT$_{1A}$ receptor regulation in BTBR mice

5-HT$_{1A}$ receptors in the ventral and dorsal hippocampus shape social behavior, since 5-HT$_{1A}$ agonists and antagonists affecting anxiety state were found to bi-directionally alter social interactions in rodent open-field tests (File et al., 1996; File and Seth, 2003). Indeed in a prior study we found that a low dose of buspirone (2 mg/kg) improved sociability in adult male BTBR mice (Gould et al., 2011), while a higher dose (10 mg/kg) worsened it (unpublished data). We also saw that 8-OH-DPAT stimulated GTPγS binding in the CA1 of hippocampus was higher in BTBR than in C57BL/10 adult male mice. Hence we sought to determine if our finding of enhanced hippocampal agonist-stimulated 5-HT$_{1A}$ G-protein coupling would generalize to other socially impaired strains such as 129S, and if it was also evident in adolescent BTBR mice.

We found an age-dependent pattern of up-regulation at hippocampal 5-HT$_{1A}$ receptors in BTBR relative to C57 mice. Specifically, higher [^{3}H] 8-OH-DPAT binding density was found in adolescents, while greater 8-OH-DPAT stimulated G-protein coupling was evident in adult BTBR mice as compared to age-matched C57 males. Yet 5-HT$_{1A}$ receptor density did not differ between BTBR and C57 adults. The 129S mice also had higher [^{3}H] 8-OH-DPAT binding density, but only in the CA1 region of the hippocampus, and their 8-OH-DPAT stimulated GTPγS binding was similar to C57 mice.

4.3. Mounting evidence for possible GR dysfunction in BTBR mice

One way by which glucocorticoids modify behavior and HPA feedback response is via changes they generate at the neurotransmitter receptor level (e.g. Schutsky et al., 2011). However, the pattern of hippocampal 5-HT$_{1A}$ expression and function in BTBR mice, taken together with other findings in the literature, indicates that their GR functional integrity may be compromised. For example, adrenalectomized rodents have increased hippocampal 5-HT$_{1A}$ expression without changes in agonist-stimulated [^{35}S] GTPγS binding (Pompili et al., 2010), paralleling our findings in adolescent BTBR mice. Also, hippocampal GR are over-expressed in adult BTBR mice, yet baseline CORT levels are elevated (Silverman et al., 2010; Benno et al., 2009). Further, BTBR and 129S have relatively high thermal pain thresholds compared to C57BL/6 mice (Silverman et al., 2010; Hossain et al., 2004). Yet chronically elevated corticosteroids, as found in BTBR, typically increase pain intensity via GR-induced alterations in cellular signaling (Khasar et al., 2008; McEwen and Kalia, 2010; Tramullas et al., 2012). Finally, in GR+/− mice CORT administration enhanced 8-OH-DPAT-stimulated [^{3}H] GTPγS binding in the hippocampal CA1 (Hensler et al., 2010) to a similar extent to what we found in BTBR adults. Thus CORT levels may be higher due to compromised GR function in BTBR

mice consequentially leading to increased 5-HT$_{1A}$ function at the G-protein level.

4.4. Up-regulation of CB$_1$ function in the adult male BTBR dentate gyrus

Social behavior in inbred mice is also sensitive to synthetic cannabinoid agonists and manipulation of endogenous cannabinoid levels in frontal cortex and other brain regions by acetaminophen (Umathe et al., 2009; Gould et al., 2012). Given this, we compared CB1 receptor density and G-protein coupling capacity among strains in adolescent and adult male mice. We saw that CP55,940 stimulated G-protein coupling was enhanced in the dentate gyrus of BTBR adults relative to 129S adults and adolescent mice in all strains. We investigated this finding further, since a survey of neuropathological markers in BTBR mice revealed that neurogenesis in their dentate gyrus and glial fiber growth was altered (Stephenson et al., 2011). We saw no differences in neuronal density as quantified by NeuN labeling, DAPI staining, or CB1 receptor expression as quantified by autoradiography with [^{3}H] CP55,940 in the adult BTBR dentate gyrus. However, enhancement of CP55,940-stimulated G-protein coupling may yet be due to differences in neural vs. glial cell composition in this brain region (López-Gallardo et al., 2012). More numerous glial cells in BTBR adults, and subsequent increases in the actions of Gαi/o coupled CB$_2$ receptors expressed on them (Brusco et al., 2008) could account for enhanced G-protein coupling capacity in the dentate gyrus.

4.5. Relative down-regulation of CB$_1$ function in adolescent 129S hippocampi

Agonist-stimulated [^{35}S] GTPγS binding generally increased with age at hippocampal CB$_1$ receptors in all strains, while CB1 receptor density did not. 129S adolescents and BTBR mice had higher [^{3}H] CP55,940 binding density than C57 mice at both ages in the dentate gyrus and CA3 regions. Despite this, 129S adolescent mice had relatively lower CP55,940 stimulated GTPγS binding in all hippocampal areas than C57 or BTBR adolescents. This disconnect between CB1 density and function was not present in adult 129S mice, given our CP55,940 stimulated GTPγS binding data from all three hippocampal subregions, and earlier findings in the cingulate cortex (Gould et al., 2012).

Instead, it appears to be a distinct developmental strain difference in the dentate gyrus that may relate to stress reactivity impairments and fear-related memory impairments that have already been characterized in adult 129S1/SvImJ mice (Camp et al., 2012). Endocannabinoids, via CB$_1$ receptors can prevent the retention of inappropriate generalized fear responses in mice by interfering with hippocampal long-term potentiation and plasticity (Reich et al., 2008; Jacob et al., 2012), and 129S mice exhibit fear overgeneralization and motor activity impairments that have been attributed to elevated anxiety (Kalueff and Tuohimaa, 2004; Camp et al., 2012). It is also possible that the increased 5-HT$_{1A}$ expression we found in the 129S hippocampus might occur to compensate for functional CB$_1$ impairments to some extent.

4.6. Age-dependent interactions among CB1 and 5-HT$_{1A}$ in BTBR HPA axis regulation

Adolescent rodents might be less sensitive to cannabinoid-induced modulation of 5-HT$_{1A}$ expression and function then adults (Zavitsanou et al., 2010). Hence while in the aggregate the functional G-protein binding response of both CB$_1$ and 5-HT$_{1A}$ receptors is lower in BTBR adolescents than in adults, inhibitory feedback to the HPA axis may be muted. This could be why adolescent, but not adult CORT responses to novelty were exaggerated in BTBR mice. It is plausible that up-regulation of inhibitory coupling capacity at both 5-HT$_{1A}$ and CB$_1$ receptors in these hippocampal regions is required to keep BTBR HPA axis activity in check and dampen the exaggerated adolescent novelty-induced CORT peak in BTBR adults. In turn, up-regulation of hippocampal 5-HT$_{1A}$ and CB$_1$ receptor function or density in BTBR and 129S mice may similarly contribute to the lack of preference for social interaction in these strains.

4.7. Implications for strain selection in studies of social behavior

Poor emotional resilience in response to stress is associated with impulsive or aggressive behaviors, and psychiatric disorders such as anxiety and depression (Cuomo et al., 2008; Stevens et al., 2009). Impaired HPA axis feedback in susceptible individuals may contribute to this state (Sher, 2006; Spijker and van Rossum, 2012). Glucocorticoid tone may be particularly relevant to how the mature BTBR behavioral phenotype develops, since it shapes dendritic spine formation and pruning in the hippocampus (Liston and Gan, 2011; Gourley et al., 2013). This discovery is also potentially relevant for social interactions in teens with autism, since in controlled studies novel social stimuli raised cortisol levels higher in autistic than normotypic adolescents (Corbett et al., 2010; Schupp et al., 2013). In this sense, the BTBR mouse could provide critical mechanistic insight into how glucocorticoids shape adolescent social behavior. Given the variability in CORT response, 5-HT$_{1A}$ and CB$_1$ receptor expression among BTBR, 129S and C57 mice, their use as a combined model system could help to clarify their effects on endpoints such as social behavior.

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Conflict of interest statement

The authors have no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.09.003.

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Appendix C

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Neuroscience
2013

Presentation Abstract

Program#/Poster#:	547.01/OO16
Presentation Title:	Targeting serotonin uptake to ameliorate social behavior deficiencies in pre-clinical models
Location:	Halls B-H
Presentation time:	Tuesday, Nov 12, 2013, 8:00 AM - 9:00 AM
Topic:	++C.19.b. Monoamines and behavior: Serotonin and histamine

Authors: ***G. G. GOULD**;
Physiol., UT Hlth. Sci. Ctr, SA, San Antonio, TX

Abstract: Impaired social behavior is a symptom that occurs in several psychiatric disorders including autism, schizophrenia and depression. Sociability impairments manifest in several forms, including indifference to social engagement, anxiety and/or empathy deficits. These dimensions, especially indifference to engagement, have proven difficult to treat with available pharmaceuticals. Based on clinical findings and experiments in rodents, serotonin (5-HT) neurotransmission is often disrupted in the socially-impaired brain, and the frontal cortex may be the main brain region involved. Two drugs are commonly used to treat autism; risperidone curbs aggression but blunts other forms of social interaction, and fluoxetine improves social behavior somewhat but is uneffective in individuals with reduced 5-HT transporter (SERT) function (due to common or rare gene polymorphisms). We have utilized two mouse models of impaired social behavior, inbred BTBR mice and SERT knock-out mice on a C57BL/6 background, to examine the effects of blocking SERT and novel drug targets on social behavior. In three-chambered sociability tests, social behavior of adult male BTBR mice worsened with 24h dietary tryptophan depletion, but improved significantly with 24h 5% dietary tryptophan supplementation ($p < 0.05$). BTBR sociability also improved with acute fluoxetine ($p < 0.05$), but not with citalopram treatment at doses ranging from (0.5 - 50 mg/kg). These findings indicate that 5-HT neurotransmission may be relatively low in BTBR mice. Our goal is to characterize the effects of blocking

ancillary transporters of serotonin, with lower affinity but greater capacity than the SERT to remove 5-HT from extracellular fluid. These auxillary transporters, collectively known as 'uptake 2' include dopamine and norepinephrine transporters, as well as organic cation transporters and plasma membrane monoamine transporters found throughout the brain. The pseudoisocyanine decinium-22 (D-22) blocked 5-HT uptake in vitro (Km = 92 +/- 12 nM) but had negligible affinity for the SERT (Ki > 3000 nM) in hippocampal synaptosomes. In SERT -/- and BTBR mice, social behavior improved with acute D-22 treatment at doses of 0.01 - 0.1 mg/kg. This indicates that uptake 2 blockade may be an effective treatment for inpaired social behavior, and warrants further study. Our findings also support the idea that insufficient 5-HT neurotransmission may underlie impaired social behavior in autism and other psychiatric disorders.

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Keyword(s):	SEROTONIN TRANSPORTER
	AUTISM
	FRONTAL CORTEX
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