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14. ABSTRACT

Impaired social behavior is a core symptom of autism that manifests in other psychiatric disorders and tends to be treatment-resistant. Selective serotonin reuptake inhibitors (SSRIs) such as Prozac enhance sociability in some patients, but their efficacy is diminished if 5-HT transporter (SERT) function is compromised. Thus, our goal was to characterize the effects of blocking ancillary transporters of 5-HT instead of SERT in inbred mouse strains differing in level of SERT function and sociability (BTBR, 129S, C57BL/6 and DBA1). These auxiliary 5-HT transporters, known as 'uptake 2', include organic cation (OCT) and plasma membrane monoamine transporters (PMAT) in the brain. Through synaptosomal uptake, radioligand binding and chronoamperometry we found that the pseudoisocyanine decinium-22 (D-22) improves sociability otherwise impaired in mice, blocks 5-HT uptake (Km=92±12 nM) but has negligible affinity for SERT (Ki > 3000 nM). Systemically administered D-22 (0.1 mg/kg, i.p.) slows 5-HT clearance in the brain. Chronic D-22 administration via osmotic minipumps produced similar effects to acute administration in BTBR mice, both treatments increased social sniffing and dwelling near strangers. This shows that uptake 2 blockade may be an effective approach to treating sociability impairments in autism.

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

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INTRODUCTION:

Impaired social behavior is a treatment-resistant core symptom of autism. Disrupted central 5-HT transmission may underlie such deficits in social behavior [1-4]. For this reason, selective 5-HT reuptake inhibitors (SSRIs) are prominent among frontline treatments for autism. However, impaired social behavior improves with SSRI treatment in only 20-30% of autistic patients [5-10]. This relatively poor therapeutic outcome has brought into question the utility of SSRIs as a treatment for autism [11]. SSRIs inhibit 5-HT uptake by blocking the high-affinity 5-HT transporter (SERT), the primary regulator of extracellular 5-HT in brain. However, ancillary low-affinity, high-capacity transporters for 5-HT, collectively referred to as 'uptake 2', include organic cation and plasma membrane monoamine transporters (OCTs and PMAT) that also play a significant role in regulating 5-HT transmission [12-16]. Recent studies show activity of these ancillary 5-HT transporters can dampen therapeutic effects of SSRIs in depression-related mouse behaviors [16,17]. This raises the possibility that their activity could underlie the poor efficacy of SSRIs to improve the impaired social behaviors of autism. Given this, we hypothesized that OCT and/or PMAT blockade may ameliorate social behaviors otherwise impaired. The pseudoisocyanine decynium-22 (D-22) is an effective blocker of uptake 2 transporters, so we use it as a tool to examine such effects on social behavior and serotonin uptake in four inbred mouse strains differing in social behavior and gene polymorphisms affecting SERT: BTBR T+Itpr3 tf/J (BTBR) and 129S1/SvImJ (129S), with impaired sociability and high SERT function, versus gregarious C57BL/6J (C57) and DBA1 mice with either impaired or high SERT function, respectively. Recently the antimicrobial herbal compound berberine was discovered to also be a substrate and blocker of OCT2 & OCT3, albeit with lower affinity than D-22 [18,19]. Therefore we will examine the properties of berberine to block D-22 binding, as a new tool to use in examining effects of 'uptake 2' transporter blockade.

KEYWORDS: autism, auxiliary monoamine transporters, corticosteroids, decynium-22, impulsivity, inbred mice, organic cation transporters, repetitive behavior, serotonin, social behavior, repetitive behavior

ACCOMPLISHMENTS:

Task 1: Regulatory Approvals

An Institutional Animal Care and Use (IACUC) progress report was submitted for review on July 24, 2014 and was approved on August 21, 2014. This means use of animals is approved through August 21, 2015, after which time an updated progress report will be required.

Task 2: Pharmacokinetic & Behavioral Characterization of D-22 in Mice

Aim 1a: Pharmacokinetics of D-22 in mouse brain and blood:

This year, measurements of D-22 injected at a dose of 1 mg/kg was performed in serum from BTBR and C57 mice sacrificed at 0, 15, 30, 45, 60 and 120 min after injection, as **Fig. 1** shows. Adult male BTBR mice were injected with D-22 at 1 mg/kg i.p. It had a half-life 35 min, based on post-injection sacrifice points at 5, 20 or 50 min. This 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 detectable in serum by HPLC beyond 2 hours. With the information that D-22 injected systemically at 10 mg/kg enters the brain in hand [16], in the last year we aim to obtain measures of brain concentrations of D-22 at the behaviorally active doses of 0.01 – 0.1 mg/kg. Due to initial struggles with detection limits for HPLC measurement of D-22 in brain samples, Dr. Javors' technical staff put much effort into optimizing the HPLC protocol than initially anticipated to obtain these results shown for BTBR and C57 mice (**Fig. 1**).

An alternative approach Dr. Javors suggested for brain samples is to instead measure D-22 content by gas chromatography/mass spectroscopy (GC/MS). We anticipate being able to commence these studies in the next few months.

Further, D-22 (1 mg/kg) has been injected into 129S and DBA1 mice, and tissue samples were collected for all of the 15 min to 2-hour time points. Since these samples were collected more recently, the serum has not been analyzed by HPLC or GC/MS. Finally, some (N= 1-3 per time point) 129S, BL6 and BTBR mice were injected with 0.1 mg/kg of D-22 and brain and serum and blood samples were collected and frozen for subsequent analysis. Initially we had anticipated completion of the majority of D-22 pharmacokinetics by the close of year 2. While the GC/MS approach will take a little longer to develop, we anticipate being able to commence measures in the next few months and have mice on hand from each strain to continue progress toward pharmacological characterization of D-22 in our third year of this project.

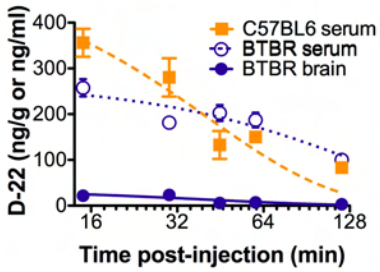

Fig. 1. D-22 clearance in adult mice. Half-life for clearance of D-22 (1 mg/kg) from serum was ≈29 min in C57, ≈90 min in BTBR mice. D-22 was detectable in BTBR mouse brain at 15 min, with half-life ≈28 min.

Aim 1 b & 3. Dose-response in three-chamber sociability tests for D-22 and drug combinations:

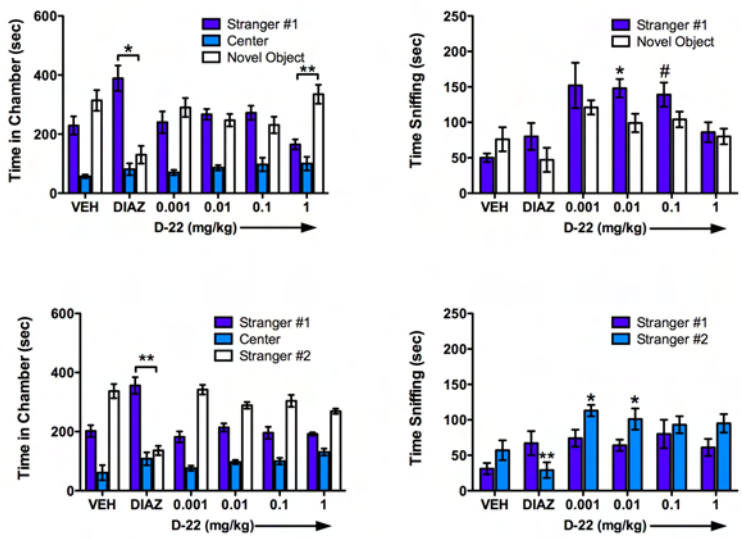

Fig. 2. D-22 Dose-Response in BTBR 3-Chamber Sociability Tests. In social interaction tests (top) only diazepine (DIAZ) increased dwelling by stranger mice (*p < 0.05), while 1 mg/kg of D-22 increased dwelling near novel objects. However, D-22 at 0.01 mg/kg (*p < 0.05) increased social sniffing, and tended to do so at 0.1 mg/kg (#p = 0.1). In social novelty tests (bottom) DIAZ significantly reduced preference for social novelty, both in dwelling and sniff time measures (**p < 0.05). In contrast, D-22 maintained the BTBR preference for social novelty [10], and increased time spent sniffing stranger mouse 2 (*p < 0.05).

We examined in BTBR mice effects of D-22 at a range of doses on social behavior preferences in comparison to diazepam (1 mg/kg), a known positive control to improve social behavior in BTBR mice [20]. D-22 and diazepam were dissolved in dimethylsulfoxide (DMSO) that was diluted to >10% of the vehicle administered to mice. Mice were injected (i.p.) with vehicle (saline + ≈10% DMSO) or D-22 (0.001 – 1 mg/kg), 30 min prior to arena acclimation. Mouse sociability tests were performed as in our study published last year [21]. As shown in **Fig. 2**, D-22 at doses ranging from 0.1–0.1 mg/kg (N=7-10) resulted in an increase in interaction preference that was evident in social sniffing. Further, there was no loss of social novelty preference typical of BTBR mice in the second stage of testing, while by contrast diazepam reduced the inherent social novelty preference of BTBR mice.

Tests of the acute effects of several doses (mainly 0.1 and 1 mg/kg) of D-22 in comparison to risperidone, fluoxetine or vehicle on social behavior testing are also underway in 129S mice. This year we've treated and tested 45 male 129S in 3-chamber sociability tests, providing sample sizes for each treatment group ranging from three to nine. So far the data from 129S mice are highly variable, and only social sniffing during the interaction phase is shown in **Fig. 3**. Given this it doesn't appear that any of the drug treatments have improved sociability in 129S mice. However in 129S mice we observed that a change in diet from Harlan Teklad cereal based chow to a purified diet dramatically improved their social behavior [22]. Further tests are planned for the next year in order to increase the sample sizes to resolve any differences in response to drug treatments by this measure in 129S mice. Also in DBA mice we have performed more experiments examining the effects of D-22 at 0.1 mg/kg and combined D-22 and fluoxetine treatment effects in 8 mice per treatment group to add to the data reported last year. These videos from DBA1 mice have not been analyzed yet. Further studies also need to be performed in C57BL/6 to resolve effects of D-22 at two doses, but so far it looks like D-22 does not alter C57BL/6 sociability at 0.001 or 0.01 mg/kg. We are poised to complete these studies.

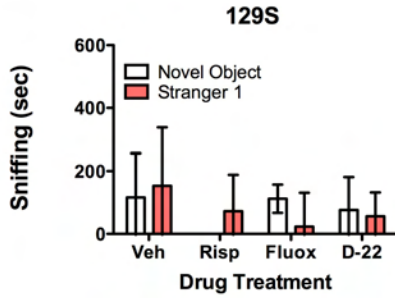

Fig. 3. Partial 129S data showing sniffing during social interaction tests. No preferences for social interaction are evident for any drug treatment due to high variability. More experimental data are needed to resolve any effects of these drug treatments, N =3-9.

Aim 1 c. Measures of serum corticosterone levels following behavior tests by EIA

As reported in our publication from last year [21], serum corticosterone levels consistently increase in all mouse strains following social behavior tests and marble burying tests. We so far have not seen strong effects of drug treatments in these measurements, but we will continue to collect serum to measure corticosterone levels. We will also perform time course experiments to determine corticosterone levels after each phase of sociability tests, including the social interaction test, social novelty test and marble burying.

Task 3: Serotonin Uptake Studies *in vitro* **and** *in vivo* **in mice**

Aim 2a. Effects of D-22 on [^{3}H] serotonin uptake in vitro

We have made good progress toward characterizing D-22's ability to block serotonin (5-HT) uptake in each of the four mouse strains, in both the frontal cortex and hippocampus, as shown in **Fig. 4**. Briefly, adult male mouse frontal cortex or hippocampal synaptosomes were isolated and used to examine competitive 5-HT uptake blocking properties of D-22 at 37°C for 5 min, as compared to fluoxetine, and was assessed along with fluoxetine or risperidone *in vitro*. We have found that fluoxetine blocks 5-HT uptake with a Km value of approximately 50 nM in BTBR DBA and 129S mice and with a Km value of approximately 70 in C57BL/6 mice (differences are likely due to the previously identified SERT gene polymorphism impairing SERT function in that strain [23]). We have sample sizes of 5-7 in each group and are planning to prepare a manuscript reporting these results and strain behavior responses to D-22 for peer review soon.

Aim 2b. [^{3}H] Histamine uptake and D-22's effects

We continue to optimize our histamine uptake saturation experiments using a protocol based on our serotonin uptake protocol except for a total of 10 min. This assay has continued to be challenging to develop, and we still need to modify our protocol to establish saturable binding. Synaptosomes have been isolated from the cerebellum, and we next plan to add more tissue to the assay and add SKF 91488 dihydrochloride (Tocris, Bristol, UK) to reduce enzymatic breakdown of HA prior to attempting to isolate synaptosome from astrocyte preparations.

a.

b.

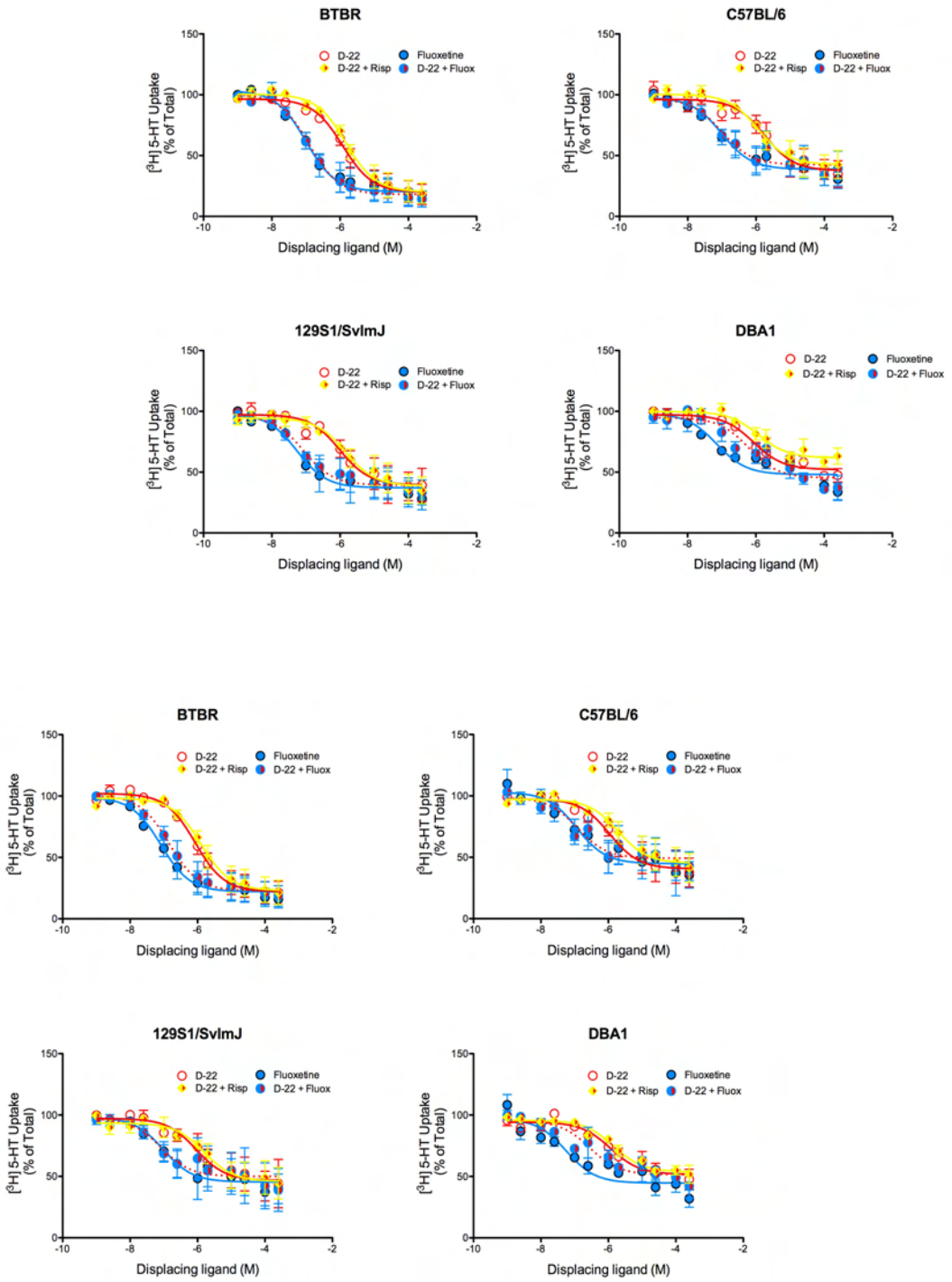

Fig 4. [^{3}H] 5-HT Synaptosomal Uptake Blockade by D-22 and Fluoxetine. D-22 is effective in vitro at μM concentrations, and seems to block 5-HT uptake via SERT independent sites in BTBR and wild-type C57BL/6 mice in frontal cortex (top) and hippocampus (bottom). Fluoxetine blocked 5-HT uptake at nM concentrations, but in absence of the SSRI, D-22 only blocked 5-HT uptake at μM concentrations. Among strains Km values were generally similar, with the exception of being slightly higher in C57BL/6 mice, N = 5-7.

Aim 2 c. In vivo chronoamperometry to measure the effect of D-22 on 5-HT uptake

In BTBR mice, the effects of systemic (i.p.) injection of D-22 on in vivo serotonin clearance were examined in the anterior cingulate cortex. The stereotaxic coordinates for recording were [from Bregma]: AP (+0.7mm); ML (+0.15mm); DV (from dura: (-0.75mm)). D-22 was injected after recording had begun, and serotonin was exogenously applied and recorded over four time intervals as shown in **Fig. 5.** The barrel concentration of serotonin was 200uM and the volume delivered ranged from 25-250nL. The amount ejected was 5-50 pmols, and mice were recorded from for roughly 2 hours. Further studies will be conducted to increase the sample size, and also will be performed in C57BL/6 mice to compare the magnitude of these effects between strains.

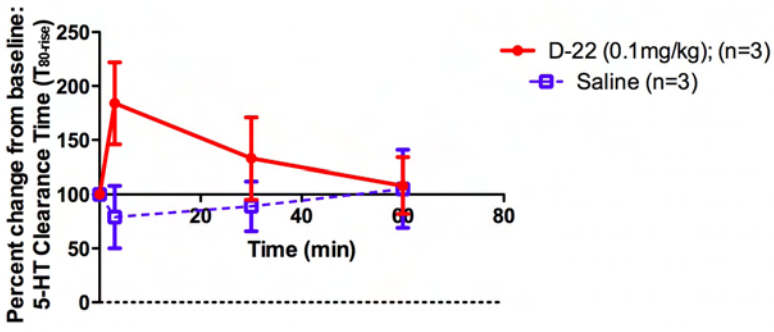

Fig. 5. Effects of systemic D-22 administration on serotonin clearance capacity in the anterior cingulate cortex. Systemically injected D-22 clearly slows the clearance of serotonin (5-HT) from brain extracellular fluid as compared to systemic saline injections.

Task 4: Measuring Effects of D-22 on social dominance, grooming and marble-burying

a. Dominance tube tests: We have used the tube test for social dominance to examine the effects of D-22 on impulsive social behavior. These tests are performed in a tube that is sufficiently narrow that mice can't turn around in them, nor crawl over or under one another. No pre-conditioning of subjects takes place prior to testing, and 129S1/SvImJ mice are used as stimulus 'competitors'. The test starts when mice meet in middle of the tube and the barrier is removed. The test ends when one mouse's back feet touch ground or after 3 minutes. Mice are scored as follows: Win = 1; Loss = 0; Draw = 0.5. For each mouse a minimum of 6 rounds of testing is performed, with a 10 min rest between each match. We were able to examine the behavior of C57BL/6 mice on D-22 or fluoxetine as compared to vehicle controls using the tube test, and as shown in **Fig. 6** we observed a dose-specific increase in impulsive behavior, as evidenced by an increased number of advances and wins in those treatment groups. In BTBR

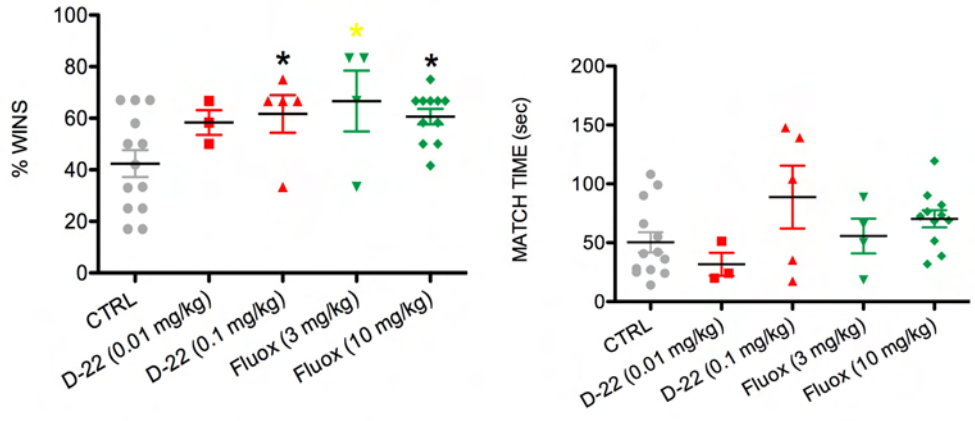

Fig. 6. Effects of systemic D-22 administration on social dominance in C57BL/6 mice. Systemically injected D-22 (0.1 mg/kg) increased wins in the social dominance tests in a similar manner to fluoxetine, without increasing the duration of the tube test matches.

and 129S mice we did not observe a parallel increase in social dominance with D-22 at 0.1 mg/kg and we have yet to examine the effects of SSRIs. In DBA1 mice there was no effect of fluoxetine on social dominance. Thus it appears that social dominance is more responsive to drug treatment in C57BL/6 mice than in these other strains. Further tube tests are underway to confirm these findings. However it seems that strains with more overtly dominant behavior such as 129S have less sensitivity to drug treatments in these tests.

b. Marble burying and self-Grooming During Sociability Tests

We have been measuring marble burying immediately after and self-grooming during three-chamber social interaction and novelty tests. In summary D-22 does not have any significant effects on marble burying, but it appears to reduce self grooming during testing.

c. Quantitative autoradiography of [^{3}H] D-22 at 'uptake 2' sites in the brain

Coronal sections (20 um) were taken in frontal cortex, hippocampus and amygdala regions of fresh frozen brains from 5 and 16 week old C57, BTBR and 129S mice. The sections mounted onto gelatin-coated slides, desiccated for 18-24 h at 4°C stored at -80°C. Prior to experiments sections were thawed for 1h at 4°C. They were then pre-incubated for 20 min in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl pH 7.4 at ~26°C. Incubation in a slide mailer (10 ml) for 1 hour at room temperature in buffer + 40 nM [^{3}H] D-22 (ARC, St. Louis, MO), plus 25 nM mazindol and 25 nM sertraline to block binding to uptake 1 monoamine transporters. Based on saturation binding in membrane homogenates, occupancy of binding sites at this concentration of [^{3}H] D-22 = 86%. Non-specific binding was defined by 100 uM D-22, and was 50% of total binding. Incubation was terminated by two 10 min washes in 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl pH 7.4 at 4°C, followed by a 5 sec dip in de-ionized water at 4°C. Slides were dried on a slide warmer for 1 hour. [^{3}H]D-22 labeled sections on slides exposed to Kodak MR film for 66 h, with 3H standards. Autoradiogram images on film were captured on a digital imaging system. In sum, as in **Fig. 7** we observed no significant differences among strains or ages in D-22 binding sites in the cortex, and measurements of 6 other brain regions are underway. Since non-specific binding was so high, we may need to refine this assay to reduce this measure. It may be that high concentrations of berberine can more effectively block D-22 binding to OCT2 and OCT3 uptake 2 sites effectively enough to accomplish this, and we will examine it in forthcoming experiments.

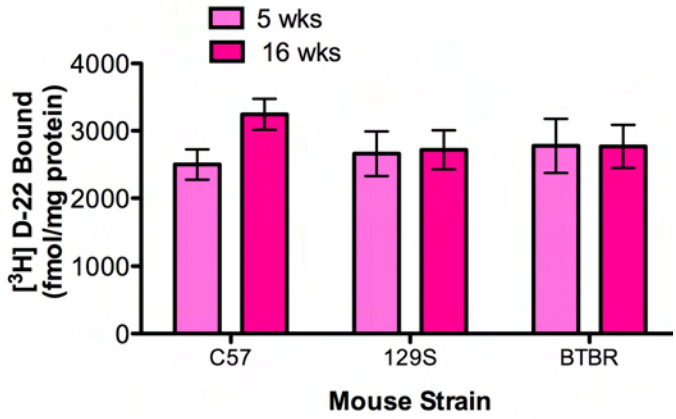

Fig. 7. [^{3}H] D-22 specific binding in 5 or 16 week old mouse cortex. We did not find significant differences between strains or ages, although values in 16 week old C57 mice tended to be elevated, and non-specific binding was 50% of total, N= 8 mice per treatment group.

Stated goals not met: Western blotting for uptake 2 and serotonin transporters in the brain is going to be complicated by the fact that no reliable antibodies are currently available for many of them to examine in the mouse brain. We will continue to try different antibodies until a reliable one is found. Also we have not been able to make much progress with [^{3}H] histamine uptake, but will continue to try to refine our use of that substrate as it is more selectively transported than alternative substrates like [^{3}H] MPP+.

Opportunities for training and professional development:

Georgianna Gould, PI, prepared an application for promotion to associate professor, research track that was submitted and is under review by UTHSCSA. Also Dr. Gould was interviewed by Baylor University for a tenure-track assistant professor position in the psychology department, and was the second choice candidate. Dr. Gould has an application under consideration for a tenure track position at the University of Texas at San Antonio in the Psychology Department. Finally Dr. Gould was invited to review abstracts for the INFAR autism meeting and to serve as an ad-hoc member of a committee reviewing grants for the National Science Foundation.

Corey Smolik, research assistant in the lab was taught to perform the uptake assays, and radioligand binding this year and performed these experiments proficiently. He subsequently took Emergency Medical Technician training, and in 7/2014 he got a job at the University Hospital as an emergency room technician. He also attended the Experimental Biology Meeting in 2014 to present this work. He plans to return to school to become a physician's assistant.

Wynne Q. Zhang, a freshman at Rice U. served for years as a high school and college level undergraduate student researcher in the lab for the past 3 years. This should provide helpful with her college applications and provide her with the confidence and experience she needs to successfully apply for graduate school.

Dissemination of Results:

Research Oral Presentations:

7/2014 Voelcker Scholars Research Presentation, UT Health Science Center, San Antonio, TX "Mouse Behavior Models in Autism Research"

12/2013 Dept. of Neurobiology and Psychology, Baylor University, Waco TX "A second look at serotonin uptake blockade to improve social behavior"

Posters at National Meetings:
Mitchell, N, Owens W, Horton R, Vitella M, Gould G, Koek W, Daws L. 2013. Mechanisms contributing to lack of antidepressant efficacy in juveniles and adolescents. Society for Neuroscience Meeting, San Diego, Poster # 227.12/E21.

Gould, GG 2013. Targeting serotonin uptake to ameliorate social behavior deficiencies in pre-clinical models. Society for Neuroscience Meeting, San Diego, Poster # 547.01/OO16.

Smolik, CM, Zhang WQ, Vitela M, Sanchez JJ, Javors MA, Koek W, Daws, LC, Gould, GG. 2014. Blockade of serotonin uptake by decynium-22 enhances social behavior. Experimental Biology 2014, San Diego, Poster #14-3827-EB.

Manuscripts:
Zhang WQ, Smolik CM, Barba-Escobedo, PA, Gamez M, Sanchez JJ, Javors MA, Daws LC, Gould GG. 2014. Acute Dietary Tryptophan Manipulation Differentially Alters Social Behavior, Brain Serotonin and Plasma Corticosterone in Three Inbred Mouse Strains. Neuropharmacology, in press.

Manuscripts under review (requiring revision and resubmission):

Sanchez A, Smolik CM, Pham TV, Lalani K, Gould GG. Sociability of Two C57BL/6 Mouse Substrains from Different US Suppliers. J Vet Behavior – minor revisions pending

Plan for Next Reporting Period:
1. Complete all social behavior studies involving 3-chamber tests and acute drug treatments.

2. Optimize and perform ^{3}H Histamine uptake, or utilize another positively charged amino acid.

3. Complete [^{3}H] serotonin uptake studies in vitro and in vivo.

4. Complete marble burying and grooming data collection and analysis for all groups.

5. Perform corticosterone enzyme immunoassays (EIAs) and complete 3H D-22 binding.

6. Optimize [^{3}H] D-22 binding protocols and perform binding studies.

7. Submit two new manuscripts for peer review and publication in journals based on studies described in this and previous progress reports.

8. Present research at society for Neuroscience meeting in Washington DC:

Zhang WQ, Barba-Escobedo P, Gamez M, Smolik CM, Daws LC, Gould, GG. 2014. Acute dietary tryptophan manipulation differentially alters social behavior and plasma corticosterone in inbred mice. Society for Neuroscience Meeting, Washington DC, Poster # 564.01/VV29.

Sanchez A, Smolik CM, Pham T, Lalani K, Gould GG. 2014. Berberine blocks 'uptake 2' and enhances mouse sociability. Society for Neuroscience Meeting, Washington DC, Poster # 564.06/VV34.

9. Present research at the American College of Neuropsychopharmacology Meeting

Gould, GG. 2014. Decynium-22 Enhances Social Behavior in Serotonin Transporter Knock-out Mice. Poster W188, Phoenix, AZ

10. Present research at the Experimental Biology Meeting in Boston

Zhang WQ, Smolik CM, Daws LC, Gould GG. 2015. Serotonin transporter density and social behavior: Is there any link? Experimental Biology, Boston

IMPACT:
The findings from this project and its publications highlight the importance of serotonin neurotransmission in the brain in shaping adult social behavior, and how it is sensitive to drug treatments targeting the serotonin system. This lends support to the approach of treating patients with autism with compounds that target the serotonergic system in the brain. These findings might also be extended to other fields within biomedical neuroscience such as schizophrenia or depression wherein impaired social behavior is prominent.

Technology Transfer:
Nothing to Report.

Commercial Technology
Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change:
We may need to use a ligand other than cold D-22 to examine its non-specific binding properties, and berberine may be the correct compound to address this need. We are also having difficulty obtaining good data from use of 3H-histamine in uptake assays. This may require our use of [3H] MPP in lieu of histamine uptake.

Problems: Unexpected low uptake with [^{3}H] histamine, we will work on optimizing the protocol to improve uptake conditions. A possible alternative approach will be to use [^{3}H] MPP+, as this substrate is mainly taken up by "uptake 2" transporters, and it can be proficiently performed by our research team.

Actual or anticipated problems or delays and actions or plans to resolve them
No major delays to report.

Deviations in reporting and IACUC
Nothing to Report

Significant changes in use of biohazards and/or select agents
Nothing to Report

PRODUCTS:

Journal publications (see Appendix A).
Zhang WQ, Smolik CM, Barba-Escobedo, PA, Gamez M, Sanchez JJ, Javors MA, Daws LC, Gould GG. 2014. Acute Dietary Tryptophan Manipulation Differentially Alters Social Behavior, Brain Serotonin and Plasma Corticosterone in Three Inbred Mouse Strains. Neuropharmacology, in press.

Manuscripts requiring revision and resubmission (See Appendix A):
Sanchez A, Smolik CM, Pham TV, Lalani K, Gould GG. Sociability of Two C57BL/6 Mouse Substrains from Different US Suppliers. J Vet Behavior – minor revisions, pending review

Books or other non-periodicals:
Nothing to Report.

Technologies or techniques:
Nothing to Report

Other Products:
Invited Oral Presentations:
7/2014 Voelcker Scholars Research Presentation, UT Health Science Center, San Antonio, TX "Mouse Behavior Models in Autism Research"

12/2013 Dept. of Neurobiology and Psychology, Baylor University, Waco TX "A second look at serotonin uptake blockade to improve social behavior"

Posters at National Meetings:
Mitchell, N, Owens W, Horton R, Vitella M, Gould G, Koek W, Daws L. 2013. Mechanisms contributing to lack of antidepressant efficacy in juveniles and adolescents. Society for Neuroscience Meeting, San Diego, Poster # 227.12/E21.

Gould, GG 2013. Targeting serotonin uptake to ameliorate social behavior deficiencies in pre-clinical models. Society for Neuroscience Meeting, San Diego, Poster # 547.01/OO16.

Smolik, CM, Zhang WQ, Vitela M, Sanchez JJ, Javors MA, Koek W, Daws, LC, Gould, GG. 2014. Blockade of serotonin uptake by decynium-22 enhances social behavior. Experimental Biology 2014, San Diego, Poster #14-3827-EB.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

1. Project Director/Principal Investigator
Name: **Georgianna Gould**
Project Role: Principal Investigator
Researcher Identifier: gouldg (ERA Commons)
Nearest person month worked: 5.8 mos/yr
Contribution to Project: Dr. Gould is the PI on the project and is responsible for performing or overseeing the performance of all aspects of the project.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD $125,000 Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Gould, Other Support:
Role: PI, 4.1 mos/yr
Lindow, Stevens & Treat Research Award, 2/1/2012-7/30/2015, $40,000/yr
Title: Neuroprotection from pesticide-induced sensitization via transporter blockade
Goals of project: To examine in zebrafish if up-regulation of uptake 2 transporters contributes to pesticide-induced sensitization to neurotoxins, and if their blockade is neuro-protective.

Role: Collaborator, 1.9 mos/yr
5R01MH093320-02 (Co-PIs Daws/Koek), 3/1/2012 – 11/30/2016, $350,000/yr
Title: *Organic cation transporters as targets for novel antidepressant drugs*
Goals of project: To examine the efficacy of OCT3 blockade as an antidepressant *in vitro* and *in vivo*.

2. Other Effort-Contributing Researchers

Name: **Corey Smolik**
Project Role: Research Assistant
Nearest person month worked: 6 mos/yr
Contribution to Project: Assisted Dr. Gould by maintaining mouse colonies, performing radioligand uptake and binding assays, performing ELISA assays for serum corticosterone, and collecting data from behavior videos.

Other Support: Dr. Gould's LST Research Award
Nearest person month worked: 6 mos/yr
Contribution to Project: Perfomed radioligand binding assays, repaired aquatic habitat

Name: **Wynne Q. Zhang**
Project Role: Undergraduate Research Assistant
Nearest person month worked: 2 mos/yr
Contribution to Project: Assisted Dr. Gould by measuring autoradiograms, performing behavior tests and collecting data from behavior videos.

Other Support: None

Name: **William Anthony Owens**
Project Role: Senior Research Associate
Nearest person month worked: 1.2 mos/yr
Contributions to Project: Performed in vivo chronoamperometry in BTBR mice.

Other Support: 5R01MH093320-02 (Co-PIs Daws/Koek)
Contribution to Project: In vivo chronoamperometric recordings.

Name: **Lynette C. Daws**
Project Role: Co-Investigator
Researcher Identifier: daws (ERA Commons)
Nearest person month worked: 1.2 mos/yr
Contribution to Project: Dr. Daws oversees performance of chronoamperometry and makes intellectual contributions to uptake assays she also makes her laboratory space and personnel available for these studies.

W81XWH-12-1-0506 (Gould, PD/PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD $125,000 Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Daws Other Support:
Role: Principal Investigator
Project #: R01 MH106978
Nearest person month worked: 3.6
Age-related differences in serotonin clearance: Novel targets for antidepressants
The goal of this project is to examine the relative roles of serotonin and uptake 2 transporters in juvenile and adolescent brains.
Role: Principal Investigator
Project #: R01 MH64489
Nearest person month worked: 3.6
Mechanisms regulating serotonin clearance in vivo: Studies using KO mice
Goal: studies involving monoamine transporter and ancillary transporter mechanisms using knock-out mice

Role: Principal Investigator
Project #: R21 DA038504

Nearest person month worked: 2.4
The dopamine transporter in eating disorders: Uncovering novel therapeutic targets
Goal: Given the well-established role of dopamine in reward and motivation, these studies will not only provide mechanistic insight into dysregulation of dopamine neurotransmission in eating disorders, but also other illnesses, including addiction and depression, which are often co-morbid with eating disorders.

Role: Principal Investigator
Project #: R56 MH64489
Nearest person month worked: 3.6
Mechanisms regulating serotonin clearance in vivo: studies using KO mice
Goal: The goal of these studies is to determine how mechanisms that control clearance of serotonin from extracellular fluid are altered in mice with genetic deficiencies of the serotonin transporter. How these adaptations influence the sensitivity of these mice to drugs that target these sites (i.e. antidepressants) is a focus of these studies.

Name: **Martin A. Javors**
Project Role: Collaborator
Researcher Identifier: javors (ERA Commons)
Nearest person month worked: 1.2 mos/yr
Contribution to Project: Dr. Javors measures tissue levels of decynium-22 and other drugs for pharmacokinetic studies, and also measures brain levels of monoamines by HPLC.

W81XWH-12-1-0506 (Gould, PD/PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD $125,000 Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Javors Other Active Support:
R01-AA022361 **co-PIs: Javors/ Dougherty** 9/2013 - 6/2017
NIAAA $200,000 *Role: Co-Principal Investigator (20%) – 2.4 months*
Title: Phosphatidylethanol and Other Ethanol Consumption Markers
This study is designed to characterize and validate phosphatidylethanol as a biomarker for alcohol consumption and then determine how it alone (and in combination with 3 other alcohol biomarkers) can be used to identify an individual's level and pattern of drinking.

P30AG013319-20 (PI: Javors) 6/1/2014 – 6/30/2015
NIA $65,000 *Role: Principal Investigator (3%)- 0.36 months*
Title: Bioanalytical Pharmacology Core, Nathan Shock Center, Barshop Institute
The purpose of the center is to provide analytical support to research groups for aging studies. Measurement of drug levels in dosage forms (usually food) and animal blood and tissues is provided in addition to assistance with study design is offered.

5UO1-AG022307-09 Strong (PI) 09/01/2014 - 08/31/19
NIA $1M *Role: Co-Investigator (30%)- 3.6 months*
Center for Testing Potential Anti-aging Interventions
The purpose of the center is to participate in a cooperative study to test interventions for which therapeutic targets have been identified that have been shown to control the aging process.

3R01-AA014988-11S2/NIH Dougherty (PI) 05/10/14 - 03/31/15
NIMH $400,000 *Role: Co-Investigator (8%)- 0.96 months*
Impulsivity and Biological Markers for Suicidality and Drug Use in Adolescents
This 5-year longitudinal study is designed to examine the interrelationships among impulsivity, 5-HT, stressful life events and the outcomes of drug use and suicidality in high-risk adolescents.

2R01-MH076929-06A1 Xin-Yun Lu (PI) 04/01/06 - 07/31/17
NIMH $225,000 *Role: Co-Investigator (5%)- 0.6 months*
Characterization of leptin's antidepressant activity
The goals of this renewal project are to determine the key components of glutamate neurotransmission that are responsive to leptin signaling and are responsible for the antidepressive effects of leptin.

1R21-MH097092-01 Bowden (PI) 04/25/12 - 03/31/14 (no cost extension)
NIMH $125,000 *Role: Co-Investigator (2%)- 0.24 months*
Calcium Study of Lymphoblasts in Bipolar Patients to Aid Diagnosis and Treatment
The overall goals of this project are to study the regulation of calcium activity in immortalized lymphocytes (LCLs) to develop a biological component as part of the diagnoses of bipolar disorder, resulting in more personalized, effective treatments and outcomes of bipolar disorder.

Name: **Wouter Koek**
Project Role: Collaborator
Researcher Identifier: koek (ERA Commons)
Nearest person month worked: 0.6 mos/yr
Contribution to Project: Dr. Koek provides space for animal behavior studies and statistical consultation and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD $125,000 Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Dr. Koek Other Active Support:
R01 MH093320 (Daws, Koek) 03/12 – 11/16
NIH/NIMH $357,000 – 3.6 cal mo
Organic cation transporters as targets for novel antidepressant drugs
Major depression is unsuccessfully treated in more than half the patient population, underlining the urgent need to identify new targets for antidepressant medications. OCT3 is emerging as one such target. By validating this target, the experiments proposed here will lay the foundation for the discovery of novel antidepressants with marked therapeutic potential, especially in treatment resistant patients.

R01 DA05018 (France) 02/10 – 01/15
NIH/NIDA $225,000 - 1.8 cal mo
Discriminative Stimulus Effects of Opioid Withdrawal

This grant examines interactions between morphine and serotonergic (e.g., fluoxetine) or cannabinoid (e.g., THC) drugs to determine whether the combination enhances their ability to alleviate pain without increasing, and possibly decreasing, their abuse and dependence.

R01 DA029254 (France) 07/10 – 06/15
NIH/NIDA $187,500 - 1.2 cal mo
Delay discounting: effects of drug dependence and withdrawal
The goal of this project is to examine the effects of chronic drug administration and its discontinuation (withdrawal) on delay discounting to determine how common drugs of abuse affect impulsivity.

Name: **Julie Hensler**
Project Role: Collaborator
Researcher Identifier: hensler (ERA Commons)
Nearest person month worked: 0.6 mos/yr
Contribution to Project: Dr. Hensler provides critical equipment for uptake studies, consultation for planning experiments and manuscripts, and other intellectual contributions as needed.

W81XWH-12-1-0506 (Gould, PI) 09/30/12 – 09/29/2015
Autism Idea Award AR11019 CDMRP/DOD $125,000 Annual Direct
Novel Therapeutic Targets to Treat Social Behavior Deficits in Autism and Related Disorders
The goal of this project is to investigate whether ancillary uptake 2 transporter activity, serotonin neurotransmission, and social behavior are linked, with the objective of providing new therapeutic targets for the treatment of the social impairments associated with autism.

Pending:
R01 (Hensler, PI) 04/01/2015-03/31/2020 NIH $292,967 Annual Direct Costs
2.4 calendar mos/yr
BDNF: a negative modulator of central neuroinflammation in stress-precipitated depression
Exposure to trauma or stress has been shown to be one of the main predisposing risk factors to major depression, which is often viewed as a manifestation of an inability to cope with stress. However, many individuals exposed to adversity maintain normal psychological functioning, and the factors underlying resistance to the deleterious effects of stress remain unknown. We propose that BDNF functions as a negative modulator of neuroinflammation, and that it is through this mechanism that BDNF confers resilience to stress.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
No changes affecting key personnel effort on this project have changed since the last reporting period. Research personnel (Corey Smolik and Wynne Zhang) have advanced in their careers as a result of this work and have taken new jobs. They will be replaced by students and other personnel now in training to work on this project: Melissa Vitela and Alicia Sanchez.

Partner Organizations
None to report

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APPENDICES:

A. Manuscripts recently published (Zhang et al., 2014) or under review (Sanchez et al.)

B. IACUC Approval Update from Progress Report

*Title page

Acute Dietary Tryptophan Manipulation Differentially Alters Social Behavior, Brain Serotonin and Plasma Corticosterone in Three Inbred Mouse Strains

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Running Head: Trp, 5-HT and Mouse Sociability

Corresponding Author: Georgianna G. Gould, Ph.D., The University of Texas Health Science Center at San Antonio, Department of Physiology, MS 7756, San Antonio, TX, USA 78229-3900. Phone: 210-567-4302, Fax 210-567-4410, e-mail: gouldg@uthscsa.edu

Key Words: 129S1/SvImJ; autism; BTBR; C57BL/6; grooming; marble burying; serotonin; social behavior, tryptophan

ABSTRACT:

Clinical evidence indicates brain serotonin (5-HT) stores and neurotransmission may be inadequate in subpopulations of individuals with autism, and this may contribute to characteristically impaired social behaviors. Findings that depletion of the 5-HT precursor tryptophan (TRP) worsens autism symptoms support this hypothesis. Yet dietetic studies show and parents report that many children with autism consume less TRP than peers. To measure the impact of dietary TRP content on social behavior, we administered either diets devoid of TRP, with standard TRP (0.2 gm%), or with 1% added TRP (1.2 gm%) overnight to three mouse strains. Of these, BTBRT^{+} *Itpr3*^{tf}/J and 129S1/SvImJ consistently exhibit low preference for social interaction relative to C57BL/6. We found that TRP depletion reduced C57BL/6 and 129S social interaction preference, while TRP enhancement improved BTBR sociability ($p < 0.05$; N= 8-10). Subsequent marble burying was similar regardless of grouping. After behavior tests, brain TRP levels and plasma corticosterone were higher in TRP enhanced C57BL/6 and BTBR, while 5-HT levels were reduced in all strains by TRP depletion ($p <0.05$; N= 4 -10). Relative hyperactivity of BTBR and hypoactivity of 129S, evident in self-grooming and chamber entries during sociability tests, were uninfluenced by dietary TRP. Our findings demonstrate mouse sociability and brain 5-HT turnover are reduced by acute TRP depletion, and can be enhanced by TRP supplementation. This outcome warrants further basic and/or clinical studies employing biomarker combinations such as TRP metabolism and 5-HT regulated hormones to characterize the conditions wherein TRP supplementation can best ameliorate sociability deficits.

*Manuscript
Click here to view linked References

1. INTRODUCTION

Sociability deficits, specifically interpersonal interaction impairments such as social anxiety, withdrawal, inattentiveness, or lack of social motivation are characteristic of autism spectrum disorders. Serotonin (5-HT) system dysfunctions are implicated in some forms of autism, and may contribute to characteristic social interaction impairments (Lam et al., 2006; Rubin et al., 2013; Yang et al., 2014). During fetal and juvenile brain development, 5-HT plays many critical roles (Daws and Gould, 2011). Clinical and basic research findings indicate that 5-HT-regulated brain developmental trajectories are disrupted in autism, either via deficient or excessive central 5-HT availability (Chandana et al., 2005; Azmitia et al., 2011; Madden and Zup, 2014; Yang et al., 2014).

Among individuals with autism, brain 5-HT availability and neurotransmission are variable, since a diverse range of genetic and environmental risk factors can manifest in common core behavioral deficits (Unwin et al., 2013; Whitehouse and Stanley, 2013). Yet subpopulations with distinct autism phenotypes can be identified, including a group with physiological markers and behavioral symptoms consistent with central hyposerotonemia (Brune et al., 2006; McNamara et al., 2008; Veenstra-VanderWeele et al., 2012). Such markers comprise reduced 5-HT transporter binding in frontal cortex (Makkonen et al., 2008; Nakamura et al., 2010), low oxytocin and low melatonin levels (Alabdali et al., 2014, Ruggeri et al., 2014). Selective 5-HT reuptake inhibitors (SSRIs) improve autism symptoms in some patients (West et al., 2009; Kumar et al., 2012; Hollander et al., 2012; Politte et al., 2014)[1], suggesting 5-HT neurotransmission may be reduced and/or brain 5-HT depleted.

[1] However, parallel benefits of SSRI treatment are frequently muted or absent in children with autism (Henry et al., 2009; Williams et al., 2013; Politte et al., 2014).

Tryptophan (TRP) is the essential amino acid 5-HT precursor. Acute TRP depletion can be used to assess the prognosis of patients with depression to benefit from 5-HT-based treatments (Delgado, 2006; Toker et al., 2010). With TRP depletion, depression symptoms worsen and cognitive functions decline in patients that responded favorably to SSRI treatments, or in individuals with high 5-HT turnover rates (Bell, 2001; Delgado, 2006; Feder et al., 2011). TRP depletion in individuals with autism likewise worsens core behavior symptoms, indicating heightened sensitivity to fluctuations in TRP and 5-HT availability (McDougle et al., 1993; 1996). On the other hand, increasing dietary TRP intake ameliorated autism symptoms in a case study (Beretich, 2009). This presents a paradox, in light of reports that many individuals with autism prefer foods with relatively low TRP content or have aversions to high dietary protein content (Kidd, 2002; Arnold et al., 2003; Herndon et al., 2009; Hyman et al., 2012; Johnson et al., 2014).

Given this, we tested the hypothesis that acute dietary TRP depletion should impair social behavior, while TRP enhancement might improve it. Inbred mice expressing the high-functioning TRP hydroxylase 2 (Tph2) enzyme isoform to convert TRP to 5-HT, with well-characterized sociability phenotypes (Carneiro et al., 2009; Moy et al., 2007) were used. These included socially deficient BTBR T+ Itpr3tf/J (BTBR) and 129S1/SvImJ (129S), and relatively gregarious C57BL/6J (C57) mice. Preferences for social interaction and novelty, chamber entries, self-grooming during sociability tests and subsequent marble burying were compared among strains and overnight TRP diet treatments. After behavior tests, brain TRP, 5-HT turnover and plasma corticosterone (CORT) -- since it can be suppressed by central 5-HT transmission (Gould et al., 2014) -- were measured in tissues collected from all strain x diet treatment groups to assess their central 5-HT status.

2. METHODS

2.1. Mice and Acute Dietary Tryptophan Manipulation

All procedures involving live mice were approved by the UTHSCSA Institutional Animal Care and Use Committee, and were in accordance with current NIH guidelines. Mice tested were fifth and sixth generation male offspring bred in the laboratory animal facilities at The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX. BTBR, C57 and 129S, founders came from Jackson Laboratory (Bar Harbor, ME, USA).

Mice were maintained at 22-25°C on 14:10 light dark cycles, with lights on at 0700 h, and *ad-libitum* access to Teklad LM-485 mouse/rat irradiated food pellets (#7912, Harlan, Madison, WI) and water in cages lined with wood-chip bedding that were changed bi-weekly. Mice were weaned at postnatal days 20-22 and were housed in same-sex groups of 3-5 per cage. Dietary TRP manipulations and behavior tests were conducted in 3-4 month-old males. For 24-30 hours prior to behavior testing (beginning 0900 or 1000 h), mice had *ad libitum* access to purified ingredient or "open source" standard diets with either a) control levels of TRP (2.1 g/kg or 0.2% = green pellets), b) diet devoid of TRP (-TRP, 0% = red), or c) diet with 1% added TRP (+TRP 12.6g/kg = yellow) from Research Diets Inc. (New Brunswick, NJ). Nutritional information for open-standard purified diets and the Teklad chow the mice were reared and maintained on are provided in Table 1.

2.2. Sociability Tests, Self Grooming During Tests and Subsequent Marble Burying

Preference tests for social interaction and social novelty were performed in three chambered testing arenas between 9:00 and 16:00 h CST, as described in prior studies (Gould et al., 2011; 2014; Silverman et al., 2010; Yang et al., 2011). Conditioning and sociability tests were conducted under low red lighting (16 lux). A daily experiment schedule is provided, and sociability-testing arena illustrated in the on-line supplement (Appx A.1.a

and b). Subject mice (4-6 tested in different arenas at the same time, 1 per treatment group) were brought from housing to the testing room 30 min prior to testing 24, 26 or 28 hours (typically 0900, 1100 or 1400h CST) after diets were administered to acclimate. Next, subjects acclimated to and explored the sociability arenas for 20 min. Then, subjects were confined to the center chamber for $\geq$1 min while pre-conditioned 'strangers' (novel male 129S mice, 8-10 weeks old) and novel objects (empty wire cages) were placed on either end chamber. Stimulus placement for testing \was randomized and counter-balanced among groups. Ten min tests were videorecorded for subsequent data collection. Preference for social interaction was tested in the first 10 min with a stranger mouse in a cup cage at one end and an empty cage at the other end chamber. Then subjects were re-confined in the center while new strangers (stranger #2) were placed under empty cages and old strangers (stranger #1) were re-positioned in the arena (Appendix A.1.b). Preference for social novelty was measured in the second 10 min phase. Between subjects strangers were returned to home cages, and arenas cleaned with 70% ethanol and dried with paper towels.

Data collected by treatment-blind observers from 10-11 min videorecordings of social interaction and novelty preference tests included time spent in chambers, sniffing and grooming. Chamber dwelling was tracked as subjects entered new chambers by recording the time and each chamber entered and into a spreadsheet, subtracting the exit times to determine each dwelling duration, and adding durations separately for each chamber and each test phase. Sniffing was recorded by timer when a subject directed its nose toward strangers or novel objects (cup cages) from a distance of < 1 cm, and ended when they turned their head or stepped away. Self-grooming was recorded by timer when a stationary subject

was observed to lick or use forepaws to smooth its head, body or tail and ended when they stopped moving its head and paws or stepped away from the site where grooming occurred.

2.3. Marble Burying

Immediately following each bought of social novelty testing, each subject was transferred to a 50 x 28 x 23 cm cage filled to a depth of 8 cm with bedding, on top of which was placed 15 blue flattened marbles spaced evenly apart in a 3 x 5 grid. Cages were covered with filter tops and mice had 30 min to bury marbles. For each mouse marbles $\geq$ 2/3 buried were tallied.

2.4 Whole Brain TRP Levels and 5-HT Turnover

After marble burying (at 1100, 1300 or 1600h CST) subject mice were sacrificed by decapitation, brains were harvested and frozen at -80°C, and trunk blood collected into tubes containing 25 µl of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO).

Levels of TRP, 5-HT and its metabolite 5-hyroxyindoleacetic acid (5-HIAA) were measured in whole brains by high performance liquid chromatography (HPLC) with electrochemical detection. HPLC was performed as in prior studies (Callaghan et al., 2007), with minor modifications (e.g. software and system updates). In summary a gradient mobile phase was used, and samples were analyzed using an ESA coulometric detector (Chelmsford, MA) and a C-18 column, on a Waters system (Milford, MA). Peak heights were converted to compound concentrations using BSA EZ Start software.

2.5 Plasma Corticosterone (CORT) Levels

Plasma isolated by centrifugation (≈3000 rpm) for 10 min at 4°C was frozen at -80°C until use. CORT levels were measured following the 'small sample protocol' for ELISA (#ADI-900-097, Enzo Life Sciences, Farmingdale, NY) on a plate reader (Molecular Devices, Sunnyvale, CA). A non-linear standard curve was generated and concentrations determined using Prism (GraphPad, San Diego, CA).

2.6. Statistical Analyses

Three-way (strain x test-phase x diet) repeated-measures multivariate analysis of variance (RM-MANOVA) comparisons of chamber-dwelling and sniffing data were performed to reveal any differences in preference between strains and among diets across social-interaction and social-novelty test-phases. Repeated measures results across strains are in Appendix (A.2 on-line supplement). Next, by strain, effects of test-phase (1. social interaction or 2. social novelty) x diet on chamber-dwelling and sniffing preferences were compared by two-way RM-ANOVA. Within each strain, diet and test-phase, significant chamber preference differences were resolved via two-tailed t-tests. Then, within each strain and test-phase, to compare effects of diet on each preference-related time variable, ANOVA was performed and significant differences were resolved by Fisher's least significant difference (LSD) test. Other variables such as chamber entry, self-grooming, marble burying, brain TRP, 5-HT, 5-HIAA and plasma corticosterone was compared by two-way MANOVA or ANOVA, correlation and Fisher's LSD post-hoc tests. Analyses were performed using Statistica (Statsoft, Tulsa, OK).

3. RESULTS

3.1.1. Effects of Dietary Tryptophan Manipulation on C57 Sociability

During social-interaction preference tests (phase 1), diet and chamber-dwelling interacted ($F_{2,24}$ = 5.93, $p < 0.01$) for C57 mice since only controls dwelled more with strangers than objects ($F_{2,24}$ = 3.68, $p < 0.05$, LSD $p < 0.05$, t_8 = 5.45, $p < 0.001$, Fig. 1a). By contrast, +TRP and -TRP C57 dwelled in novel-object chambers moreso than controls ($F_{2,24}$ = 11.2, $p < 0.001$, LSD $p < 0.001$, Fig. 1a). For C57 sniffing, diet x chamber-preference interacted ($F_{2,24}$ = 5.9, $p < 0.01$) since controls sniffed strangers more than objects ($F_{2,24}$ =

9.5, $p < 0.01$, $t_8 = 4.94$ $p < 0.01$, Fig. 1b), while + TRP or -TRP C57 mice sniffed novel objects more than controls ($F_{2,24} = 6.33$, $p < 0.01$, LSD $p < 0.001$, Fig. 1b).

In social-novelty preference tests (phase 2), C57 chamber preference differed among diets ($F_{1,24} = 10.71$, $p < 0.005$), as only -TRP C57 mice dwelled near stranger 2 moreso than stranger 1 ($t_8 = 2.47$, $p < 0.05$, Fig. 1c). However both -TRP and +TRP C57 mice sniffed stranger 2 more than stranger 1 ($F_{1,24} = 20.96$, $p < 0.001$, $t_8 = 3.63$ or 4.66, $p < 0.05$, Fig. 1d).

3.1.2. Effects of Dietary Tryptophan Manipulation on 129S Sociability

During social-interaction tests, diet x chambers interacted ($F_{2,23} = 5.6$, $p < 0.01$) for 129S mice. 129S controls were sociable ($F_{2,23} = 3.9$, $p < 0.05$, $t_9 = 2.42$, $p < 0.05$), while 129S on -TRP and +TRP diets had no preference for social-interaction by chamber-dwelling ($F_{2,23} = 11.95$, $p < 0.001$, LSD $p < 0.05$, Fig. 2a). –TRP 129S spent more time in the arena center than controls ($F_{2,23} = 3.9$, $p < 0.05$). For 129S sniffing, diet x chambers interacted ($F_{2,23} = 4.7$, $p < 0.05$), since controls sniffed strangers more than novel-objects ($t_9 = 2.63$ $p < 0.05$), and -TRP sniffed strangers less than controls ($F_{2,23} = 6.9$, $p < 0.01$; LSD $p < 0.01$ Fig. 2b).

In 129S social-novelty preference tests +TRP mice exhibited greater preference for the second novel stranger mouse (stranger 2) than stranger 1, both in chamber-dwelling ($F_{1,23} = 6.49$, $p < 0.01$, $t_7 = 2.75$, $p < 0.05$, Fig. 2c) and sniffing ($F_{1,23} = 7.83$, $p < 0.01$, $t_7 = 3.98$, $p < 0.01$, Fig. 2d), while 129S controls displayed no such preference for novelty.

3.1.3. Effects of Dietary Tryptophan Manipulation on BTBR Sociability

In social-interaction tests, BTBR mice given +TRP diet exhibited enhanced sociability in chamber-dwelling ($F_{2,26} = 6.2$, $p < 0.01$, $t_8 = 2.37$, $p < 0.05$ Fig. 3a), and sniffing ($F_{2,26} = 5.4$, $p < 0.01$, $t_8 = 2.31$, $p < 0.05$ Fig. 3b) while other groups did not. BTBR given +TRP spent less time in chambers with ($F_{2,26} = 5.44$, $p < 0.05$; LSD $p < 0.05$) or sniffing ($F_{2,26} = 4.03$, $p < 0.05$; LSD $p < 0.05$) novel objects.

BTBR mice failed to exhibit any preference for the second new stranger introduced in the social-novelty phase, as measured by chamber dwelling ($F_{2,26} = 1.0$, $p = 0.3$, Fig. 3c) or social sniffing ($F_{2,26} = 3.2$, $p = 0.08$, Fig. 3d). Also there was no difference among BTBR diets in lack of social-novelty preference (chambers $F_{2,26} = 1.4$, $p = 0.26$; sniff $F_{2,26} = 0.6$, $p = 0.54$).

3.2. Chamber Entries and Self Grooming During Sociability Tests

The number of chamber entries differed among strains in both social-interaction ($F_{2,73} = 26.38$, $p < 0.0001$) and social-novelty ($F_{2,73} = 13.82$, $p < 0.0001$) preference tests. 129S mice made fewer entries than C57 or BTBR mice during social interaction tests (LSD $p < 0.01$), while BTBR made more chamber entries than C57 or 129S mice (LSD $p < 0.0001$, Fig. 4a) during both test phases. BTBR on +TRP diet made more entries (66 ± 5) than BTBR controls (53 ± 8, $F_{2,73} = 3.49$, $p < 0.05$ LSD $p < 0.05$). There were no other effects or interactions of diet with strain for chamber entries during sociability testing.

Diet had no effect on self-grooming during sociability tests ($F_{2,73} = 1.61$, $p = 0.2$), but strain ($F_{2,73} = 5.20$, $p < 0.01$), test phase ($F_{2,73} = 17.96$, $p < 0.0001$) and their interaction ($F_{4,73} = 2.63$, $p < 0.05$) were significant. During social-interaction preference tests, 129S did less self grooming than BTBR or C57 mice ($F_{2,73} = 5.8$, $p<0.01$, LSD $p <0.005$, Fig. 4b). In social-novelty preference tests, BTBR self-groomed more than the other strains ($F_{2,73} = 3.9$, $p < 0.05$, Fig. 4b).

3.3. Marble Burying after Sociability Testing

Diet treatment ($F_{2,73} = 1.49$ $p = 0.23$) and strain ($F_{2,73} = 1.43$ $p = 0.25$) had no significant effects on marble burying, and they did not interact ($F_{4,73} = 0.1$, $p <0.98$). Mice in all groups buried to a similar extent on these diets, and their pooled mean was 9 ± 0.3 marbles buried.

3.4 Whole Brain TRP Levels and 5-HT Turnover Following Behavior Tests

Two-way MANOVA revealed significant interactions between strain and diet (Wilks' $\lambda_{16,70} = 0.18$, $p < 0.001$), with respect to whole brain wet tissue content of TRP ($F_{4,26} = 3.6$, $p < 0.05$), 5-HT ($F_{4,26} = 4.2$, $p < 0.01$), 5-HIAA ($F_{4,26} = 4.3$, $p < 0.01$) and 5-HT turnover (% 5-HIAA/5-HT $F_{4,26} = 3.2$, $p < 0.05$) in HPLC measurements of whole brains collected after behavior tests. C57 and BTBR mice had increased brain TRP content with either TRP enhancement or depletion relative to controls (Fig. 5 a, LSD $p < 0.05$). As shown in Fig. 5b and 5c, acute TRP depletion significantly reduced (LSD $p<0.05$) brain 5-HT and 5-HIAA content in all strains relative to controls. However TRP depletion in only C57 reduced 5-HT turnover, while TRP enhancement only in BTBR mice enhanced 5-HT turnover (Fig. 5d).

3.5 Plasma Corticosterone Levels Following Behavior Tests

There was a significant interaction between strain and diet treatment ($F_{4,49} = 3.3$, $p < 0.05$), in plasma CORT levels measured in representative samples following our behavior test battery, as shown in Fig. 6. Specifically both TRP depleted and TRP enhanced C57 mice had higher CORT than those on standard control diet (LSD $p <0.05$). Only TRP-enhanced BTBR mice had higher CORT levels than controls. By contrast, TRP-enhanced 129S had lower CORT than controls (LSD $p < 0.05$). For reference, baseline plasma CORT levels from aged-matched naïve mice were 20 ± 4 in C57, 32 ± 8 in 129S, and 28 ± 4 in BTBR (N = 3-5).

4. DISCUSSION

4.1. Summary of Key Experimental Findings

This study utilized 24-30 h of *ad libitum* feeding on purified diet to assess the impact of acute TRP manipulation on sociability of three inbred mouse strains. Indeed, dietary TRP depletion altered murine sociability in three-chamber tests, but in different ways dependent upon mouse strain. For example in typically sociable C57 mice, preference for social-

interaction was lost in -TRP mice, as they investigated novel objects more than controls (Fig. 1). Likewise, 129S on –TRP diet dwelled less with strangers, spending more time in central and novel-object chambers (Fig. 2). However in BTBR mice that usually exhibit sociability deficits, –TRP diet had little effect on social-interaction preference, while +TRP improved BTBR sociability (Fig. 3). Specifically +TRP BTBR paid less attention to novel objects, both in chamber-dwelling and sniff, and spent more time in the center (closer to strangers) relative to control or -TRP mice (Fig. 3). These findings support our hypothesis that deficits in social interaction preference can stem from reduced 5-HT availability, and parallel observations in patients with autism (McDougle et al., 1993; 1996, Beretich, 2009; Daly et al., 2012).

An unanticipated finding was that 129S mice on control diet were sociable. Previously, naïve 129S fed Teklad chow exhibited impaired sociability, in agreement with Moy et al. (2007), even as collinear (129S) strangers were used (Gould et al., 2014). However, distinct oxytocin-mediated responses to same vs. different mouse strains were found in other studies (Macbeth et al., 2009; Hattori et al., 2014). To assess whether collinear (129S) strangers influenced 129S sociability herein, we replicated overnight (24h) control-diet treatment and tested 129S sociability using C57 strangers instead (Appendix A.3, Fig. 7). Even with C57 strangers, 129S controls displayed preference for social-interaction. This enhanced 129S sociability may instead stem from some aspect of the control diet, such as its composition or novelty. Likewise, purified diets may have precipitated the loss of BTBR's characteristic social-novelty preference (Fig. 3), with little effect on C57 controls (Fig. 1).

The texture of the open standard diet was similar to Teklad chow (Table 1); it has similar fat content and was not oily, and like cereal chow it was compressed to prevent crumbling. However, open standard diets are made from purified ingredients, so due to their

refinement they may have a different aroma than Teklad chow (Ricci and Ulman, 2005). Hence introducing purified diets might have presented odor and flavor novelty. Yet mice in all groups consumed their diets, and feces color matched the group's diet color. While not empirically quantified, feeding did not appear to be suppressed, as comparable volume reductions to overnight feeding on Teklad diets were evident across groups.

However aside from variable TRP, other differences in macronutrient content between Teklad chow and open-standard diets were notable and may have enhanced social interaction preference in 129S controls or reduced social novelty preference in BTBR. Specifically, vitamin A, D, and E contents were greater in purified diets. Vitamin A deficiency has anxiogenic effects (Bonhomme et al., 2014), and E deficiency alters CORT release in rats (Terada et al., 2011). Vitamin D influences sniffing and following (Kalueff et al., 2006), and can modulate 5-HT synthesis (Patrick and Ames, 2014). Thne level of menadione, an oxidative stressor (Giustarini et al., 2006) was reduced in purified diets. Finally, consistent with National Research Council (1995) recommended 35 mg/kg, open standard diets had 37 mg/kg of iron, while Teklad chow contained 240 mg/kg iron.

Also 129S mice on +TRP diet exhibited social-novelty preference (Fig. 2c). We postulate this change may stem from confluence of the following factors: 1) novelty of diet, 2) +TRP in diet, 3) sociability test duration and phase (social novelty test after 30 min in arena), and 4) previously characterized contextual fear-extinction deficiencies in 129S that resemble post-traumatic stress disorder (Camp et al., 2012; Temme et al., 2014). Previously we found evidence for involvement of hippocampal 5-HT_{1A} receptors in the abnormal CORT responses of 129S mice, highlighting an important role for 5-HT in this process (Gould et al., 2014). In accord, the CORT response in +TRP 129S after behavior tests was relatively

blunted (** in Fig. 6). Otherwise, plasma CORT following behavior tests differed among strain such that in C57 higher CORT in +TRP and –TRP corresponded with loss of preference for interaction, whereas higher CORT in +TRP BTBR corresponded with reduced interest in novel objects. Overall correlations between % 5-HT turnover (Fig. 5d) and plasma CORT were r = -0.36 ($p = 0.27$) for C57, r = - 0.59 ($p < 0.05$) for 129S, and r = 0.66 ($p < 0.1$) for BTBR, more detailed correlations are presented in Appendix A4.

As with +TRP, BTBR sociability is also enhanced by treatment with the SSRI fluoxetine, indicating that deficient 5-HT neurotransmission may underlie its impaired sociability (Gould et al., 2011). Given this, it is interesting that CORT blocks ancillary transporters of 5-HT (i.e. "uptake 2") such as organic cation transporters (OCTs) from clearing extracellular 5-HT in the brain (Baganz et al., 2010; Hill et al., 2011). This lends support to the hypothesis that their blockade may be a useful strategy for treating sociability deficits, warranting further studies in this area.

4.2. Prior Findings with Acute or Chronic Dietary Tryptophan Depletion in Mice

In C57 mice Van Donkelaar et al. (2010) found that TRP depletion achieved by oral gavage of solutions with a high ratio of large neutral amino acids relative to TRP failed to alter serum TRP, central 5-HT levels, 5-HT metabolism or behavior in forced swim and zero mazes. Blood samples were taken 30 min prior to start, at gavage (t0) and 30, 60 and 120 min after, and brain samples were collected at 20, 40, 60 and 240 min after. The authors attributed this difference to slower TRP metabolism and higher baseline TRP in C57 mice. Rat responses to TRP manipulation also vary by strain, both when TRP-to-5-HT metabolism in the brain and behavior are considered (Jans et al., 2010). Biskup et al. (2012) compared C57 and BALB/c responses to a TRP-free nutrient solution with low methionine (Moja-De) on a schedule including post-gavage tissue measures from 90-330 min, and saw depression-

like behaviors were augmented by TRP depletion (Biskup et al. 2012). They also discovered both strains had less TRP and reduced 5-HT and 5-HIAA levels in brain (similar to Fig. 5 herein). However, when given a balanced amino acid mixture including more TRP (0.7g/10 kg) 5-HT synthesis was not enhanced (Biskup et al., 2012). Acute TRP depletions in mice by oral gavage are expeditious, but associated procedures such as food deprivation, restraint, forced-feeding, and repeated blood sampling, are stressful (van Donkelaar et al., 2010; 2011). Since stress-sensitive social behavior tests were our main interest, we opted instead to deplete TRP via *ad libitum* administration of purified diet overnight to achieve this end.

Modified pellet diets were employed in prior studies to examine the effects of chronic TRP depletion on rodent behaviors. In one study, dietary TRP depletion for 7 and 14 day increased immobility in forced swim, decreased TRP and 5-HT in brain, and increased serum CORT at both times in rats (Franklin et al., 2012). One-month dietary TRP depletion in C57 mice enhanced aggression, dominance and hyperactivity (Uchida et al. 2005). In C57 and BALB/c mice, Browne et al. (2012) examined chronic effects of dietary TRP manipulation (depleted, deficient, control and enhanced) in emotionality tests, and on TRP and 5-HT turnover. Pertinent to our finding of enhanced sociability in BTBR by +TRP diet (Fig. 3) was their discovery that TRP enhancement promoted nesting behavior (Browne et al., 2012).

With TRP depletion, plasma and brain TRP and 5-HT turnover was reduced and nesting and marble-burying were suppressed (Browne et al., 2012). Marble burying is typically sensitive to serotonergic manipulations (Deacon et al., 2006), and a limitation inherent in the present study is that marble-burying was measured after sociability tests, and associated CORT increases may have obscured effects of TRP manipulation. Yet we measured marble

burying after sociability tests before and found drug (SSRI)-induced changes (Gould et al., 2011). This indicates that dietary TRP effects may be less robust than SSRI effects.

4.3. Other Considerations: Tph Metabolic Capacity and Supplementation

Our observations that 5-HT availability can impact social BTBR social behavior are paralleled in other inbred (BALB/c) and transgenic (Tph2 knock-out) mice with impaired sociability (Flood et al., 2012; Kane et al., 2012). Indeed, metabolic TRP abnormalities due to enzymatic deficiencies may contribute to brain hyposerotonemia in some patients with autism (Boccuto et al., 2013; Schwartz, 2014). In case-control studies, children with autism had elevated levels of plasma-free TRP and blood 5-HT levels, suggestive of compromised Tph2 function in brain (Hoshino et al., 1984; 1986; Coon et al., 2005). Thus, low dietary TRP intake or compromised TRP to 5-HT conversion in brain can impair social behavior. In the latter, TRP supplementation may be more problematic than helpful. However if capacity to produce 5-HT is compromised, drug interventions prolonging presence of 5-HT in the synapse, as might be achieved by OCT3 blockade (Baganz et al., 2008; Horton et al., 2013), may be of great therapeutic benefit for the treatment of sociability impairments.

Developmental depletion of cortical 5-HT by 5,7-dihydroxytryptamine in mice had transient effects on 5-HT transporter and receptor expression, yet it produced persistent increases in anxiety in response to change (Hohmann et al., 2007). On the other hand monoamine oxygenase-A knock-out mice have reduced capacity to degrade 5-HT and are aggressive, antisocial and bury many marbles, effects that were blocked by the Tph blocker p-chloro-phenylalanine ((PCPA) Bortolato et al., 2013). PCPA treatment was found to

impair object recognition in mice (Alkam et al., 2011). Given this it will be of great interest learn of the effects of PCPA treatment on sociability in these strains in future studies.

4.4. Clinical Relevance: Dietary TRP Concerns in Autism and Social Behavior

'Picky eating' is commonly reported among children with autism, and manifests in a limited range of acceptable foods, high frequency intake of a single food type, or food refusal (Marí-Bauset et al., 2013). Selective avoidance of proteins rich in amino acids such as TRP may relate to the fact that about one-third of patients with autism exhibit "platelet hyperserotonemia" or higher blood 5-HT levels than normal (Anderson et al., 1990; Croonenberghs et al., 2005). This biomarker may result from excess intestinal 5-HT synthesis and release, or reduced hepatic 5-HT removal (Janušonis, 2008; Gabriele et al., 2014). So TRP avoidance by some children with autism might be a response to discomfort associated with elevated gut 5-HT (Aitken, 2008; Nakamura and Hasegawa, 2009).

To the limited extent that plasma amino acids and dietary intake in children with autism have been studied, evidence of protein and nutrient malnutrition has emerged, including relatively low levels of TRP, particularly in 4 and 8 year olds; and this is even more profound with casein/gluten restricted diets (Arnold et al., 2003; Herndon et al., 2009; Kałuzna-Czaplinska et al., 2010; Hyman et al., 2012; Tanoue et al., 2012; Naushad et al., 2013). While popular among alternative-approaches to manage autism symptoms, in general the effects of restricted diets have not been well-characterized (Marcason, 2009). However there is evidence that restrictions can worsen symptoms or hinder social development in children with central hyposerotonemia and autism (Christison and Ivany 2006; Hijej et al., 2008; Johnson et al., 2014). On the other hand, while TRP supplementation may ameliorate symptoms in some patients (Lakhan and Vieira, 2008), evidence for its effects in a broader

population of autism patients is lacking. Hence studies on developmental and long-term effects of manipulating TRP intake are also warranted.

4.5. Conclusions: Significance of Experimental Findings

In conclusion, we have shown that overnight *ad libitum* dietary TRP manipulation alters social behaviors in BTBR, C57 and 129S mice in different ways: C57 and 129S mice behaviors were sensitive to TRP depletion (reduced preference for social interaction) that corresponded with clinical responses in autistic patients (McDougle et al., 1993; 1996). Also enhanced social interaction preference in BTBR after TRP supplementation resembled the response of a similarly-treated autism patient (Beretich, 2009). More clinical studies examining effects of dietary TRP supplementation are needed to determine how prevalent such beneficial responses might be in the greater population of patients with autism. However, it appears that screening 5-HT regulated hormones such as cortisol, oxytocin or prolactin, could provide some insight into the functional status of 5-HT system in patients. Such screens may help to determine if TRP supplements or 5-HT-based therapeutic interventions would be beneficial on an individualized basis.

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FIGURE LEGENDS

Fig. 1. C57 mouse preference for social interaction or novelty is differentially altered by acute dietary tryptophan (TRP) manipulation. Bars represent means and lines show standard error. The symbol * indicates significant preference for interaction or novelty ($p < 0.05$), τ indicates trend ($p < 0.1$) toward novelty preference, and ** indicates a change (an increase) in attention toward novel objects ($p < 0.05$). TRP enhancement (+) or depletion (-) produced a loss of interaction preference in (a) chamber dwelling and (b) sniff time. However, preference for novelty in (c) chamber dwelling or (d) sniffing was enhanced by –TRP. N = 9 mice/group.

Fig. 2. 129S mouse preference for social interaction or novelty is differentially altered by dietary TRP manipulation. Graph legend is as for Fig. 1, plus # indicates significantly more time spent in the center arena ($p < 0.05$) and *** indicates significantly less time with stranger ($p < 0.05$). TRP depletion resulted in loss of social interaction preference for (a) chamber dwelling and (b) sniff time. TRP enhancement promoted preference for social novelty in (c) chamber dwelling and (d) sniff time. N = 8-10 mice/group.

Fig. 3. BTBR mouse preference for social interaction or novelty is differentially altered by dietary TRP manipulation. Graph legend is as for Fig. 2. TRP enhancement increased preference for social interaction in (a) chamber dwelling time and (b) social sniffing, through a reduction in the attention paid to novel objects (**). There were no significant differences in the lack of preference for social novelty displayed by all treatment groups for (c) chamber dwelling or (d) sniffing time. N = 8-12 mice/group.

Fig. 4. Chamber entry and grooming behaviors during sociability tests were enhanced in BTBR and reduced in 129S mice. Bars represent means and lines show standard error. The symbol * indicates greater than and ** indicates less than other strains ($p < 0.05$). (a) BTBR mice made more chamber entries and 129S made fewer during sociability tests. (b) 129S did less self-grooming during preference for interaction tests than the other strains, while BTBR did more self-grooming during preference for novelty tests. N = 8-12 mice/group.

Fig. 5. Whole brain TRP and 5-HT turnover after social interaction and marble burying test battery. (a) Dietary TRP enhancement (+TRP) or depletion (-TRP) significantly increased brain TRP levels in C57 and BTBR mice (**$p < 0.05$). Depletion of TRP reduced brain (b) 5-HT levels and (c) 5-HIAA levels after behavior tests (* $p < 0.05$) in all strains, but it reduced (d) 5-HT

turnover in C57 mice only. On the other hand TRP enhancement only increased 5-HT turnover in BTBR mice (d, **p <0.05). N = 4 mice/group (randomly selected).

Fig. 6. Plasma corticosterone after behavior tests varied with strain and dietary TRP. The symbol * indicates greater than and ** indicates less than other strains ($p < 0.05$). In C57 mice either supplementing (+TRP) or depleting (-TRP) resulted in higher CORT relative to controls. +TRP reduced CORT significantly in 129S while it increased CORT in BTBR mice. Serum was collected after 70 min of behavior tests. N = 4-9 mice/group (randomly selected).

Table(s)

Table 1. Comparison of nutrient content of sustaining mouse chow that was given prior to study to the experimental open standard diet that was acutely administered before testing.

Dietary Component	Teklad LM-485 (Irradiated 7012, Harlan)	Exp. Open Standard (A11022501, Research Diets)
Macronutrients (% kcal)		
Protein	25	18
Carbohydrates	58	66
Fat	17	16
Fiber (gm%)	14%	10%
Essential[2] L-Amino Acids (gm%)		
Arginine	1.2	0.6
Histidine	0.5	0.4
Isoleucine	0.8	0.7
Leucine	1.7	1.5
Lysine	1.0	1.3
Methionine	0.4	0.5
Phenylalanine	0.9	0.8
Threonine	0.8	0.7
Tryptophan	0.3	0.2 (-TRP 0, +TRP 1.2)
Valine	0.9	0.9
Vitamins (IU/g)		
A	30	4000
D	2.4	1000
E	0.2	50
Vitamins (mg/kg)		
Menadione (K3)	80	0.5
B-complex		
Thiamine (B1)	95	6
Riboflavin (B2)	14	6
Niacin (B3)	100	30
Pantothenate (B5)	87	16
Pyroxidine (B6)	17	7
Biotin (B7)	0.8	0.2
Folate (B9)	7	2
Cobaltamin (B12)	0.09	0.01
Minerals (gm%)		
Calcium	1.0	0.9
Phosphorus	0.7	0.3
Sodium	0.3	1.7
Potassium	0.8	0.6
Chloride	0.5	1.0
Magnesium	0.2	0.5
Minerals (mg/kg)		
Zinc	63.0	29.0
Manganese	93.0	59.0
Copper	23.0	6.0
Iodine	3.0	0.2
Iron	240.0	37.0

[2] For juvenile mice, per John and Bell, 1976.

Figure1

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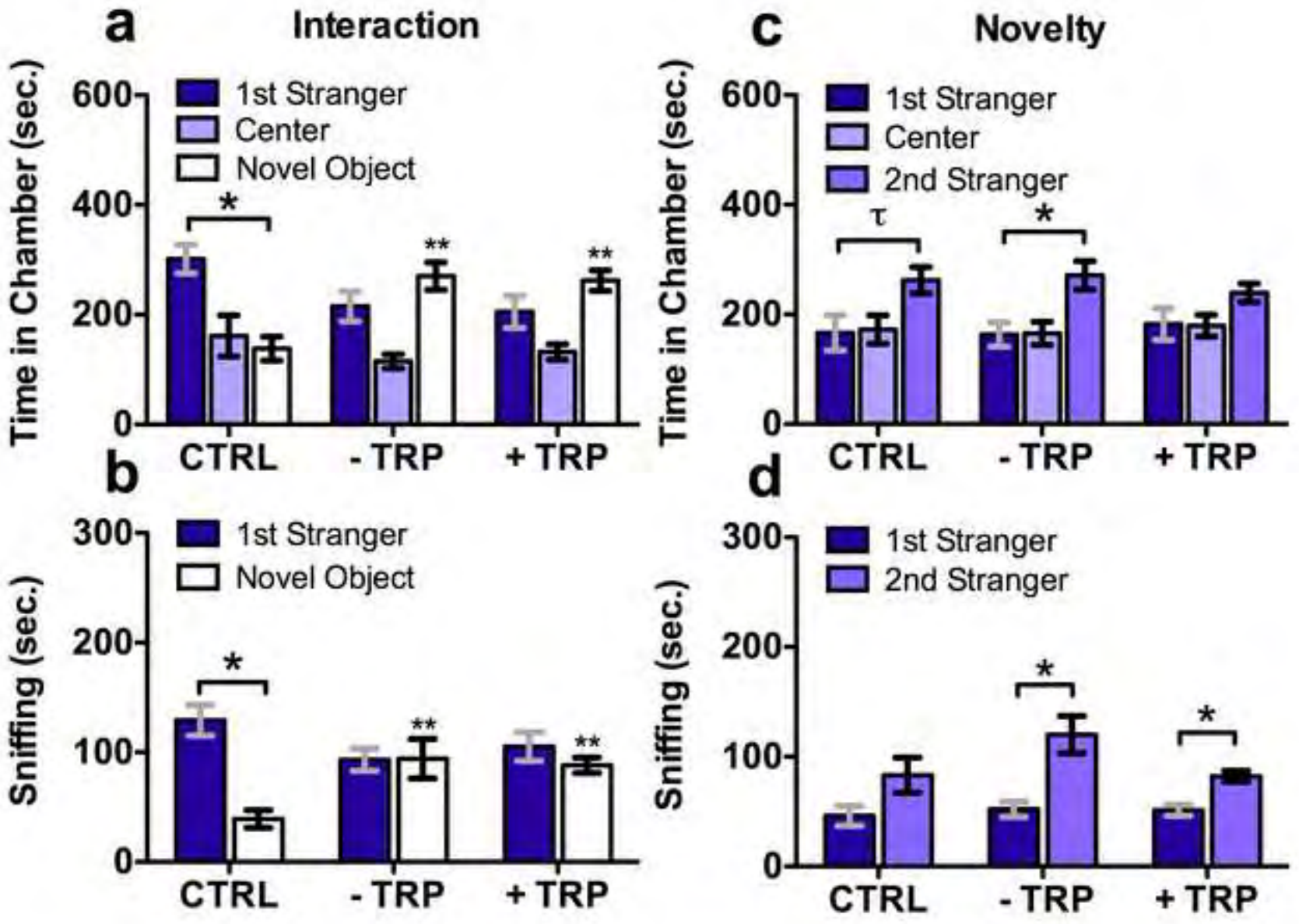

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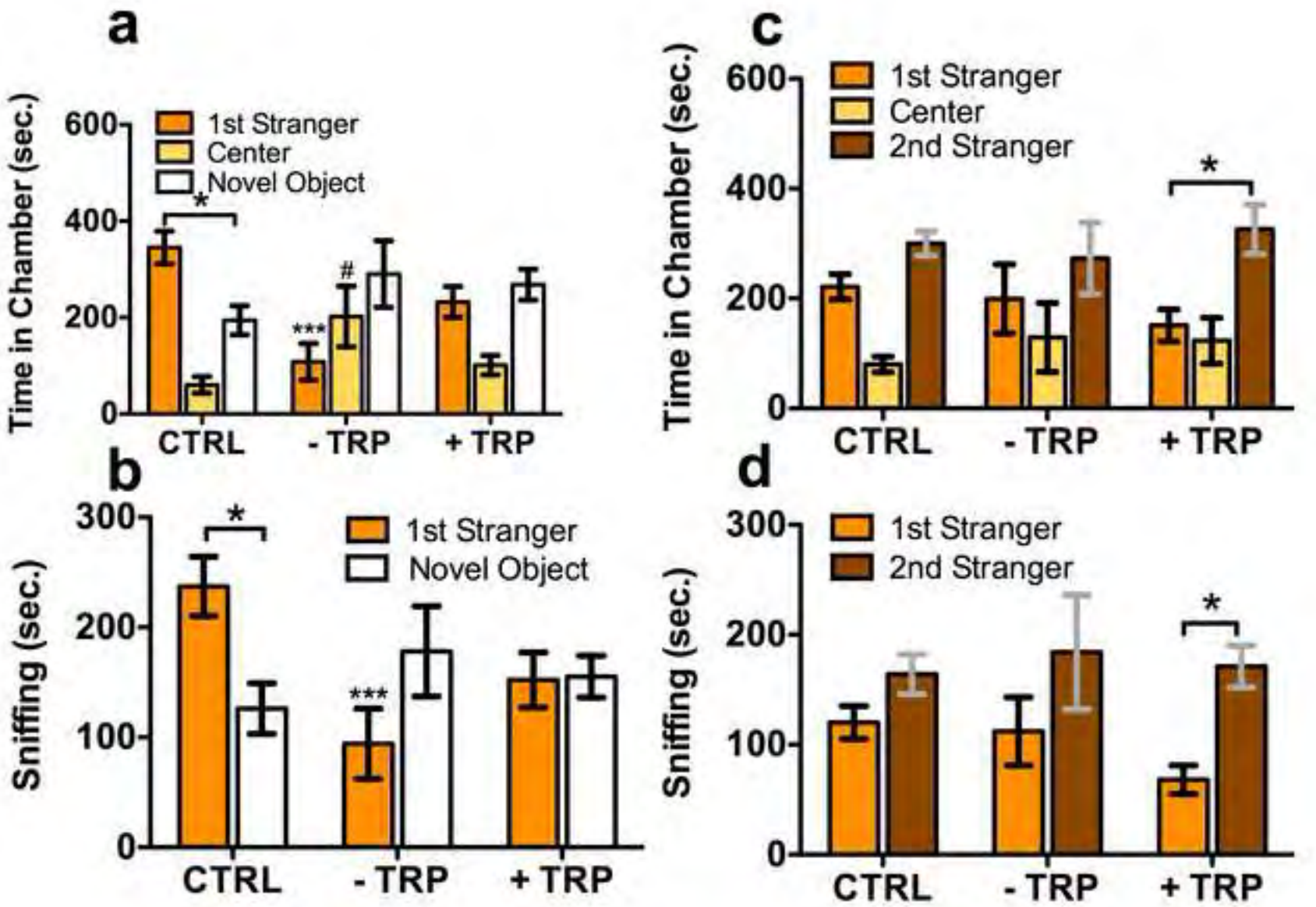

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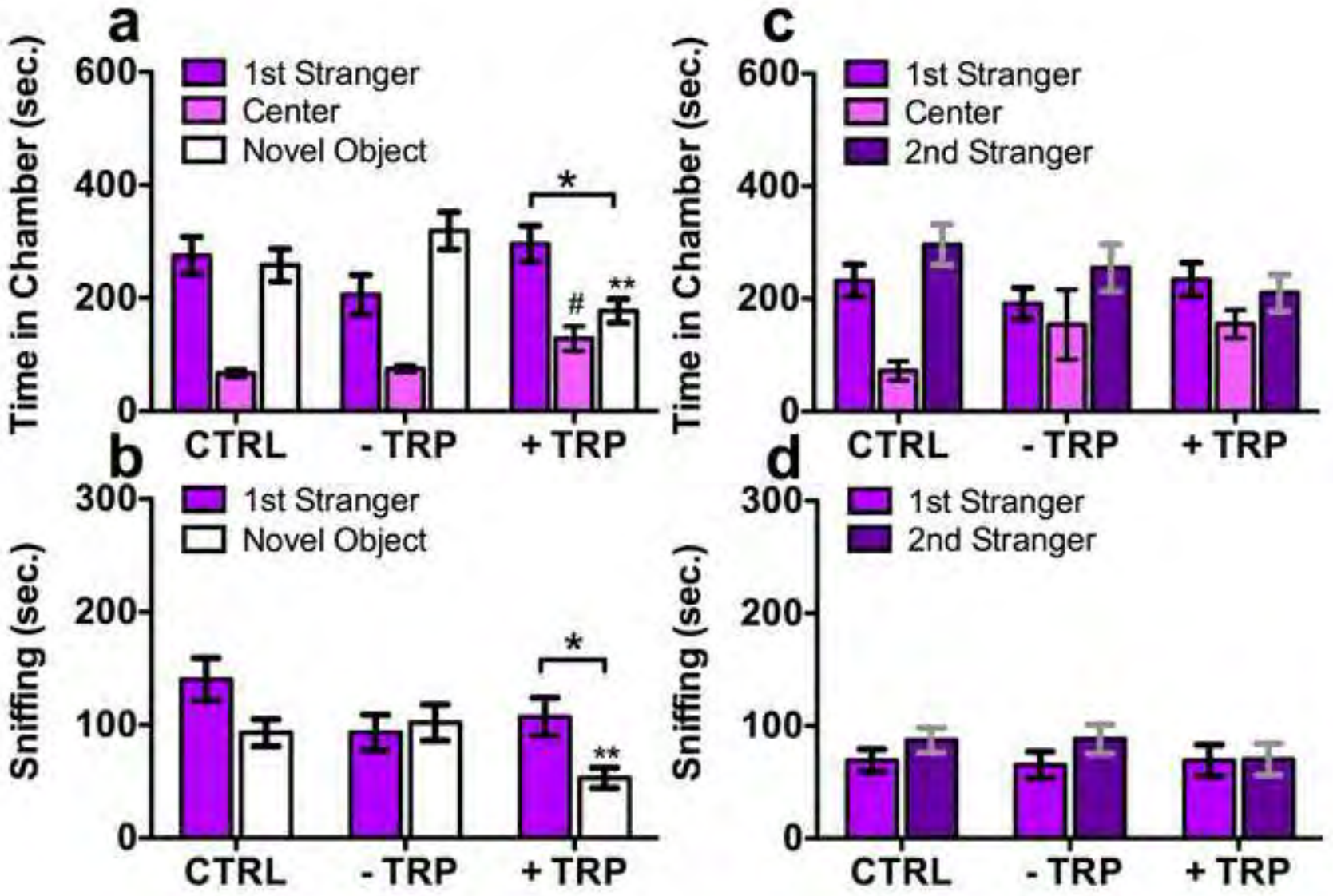

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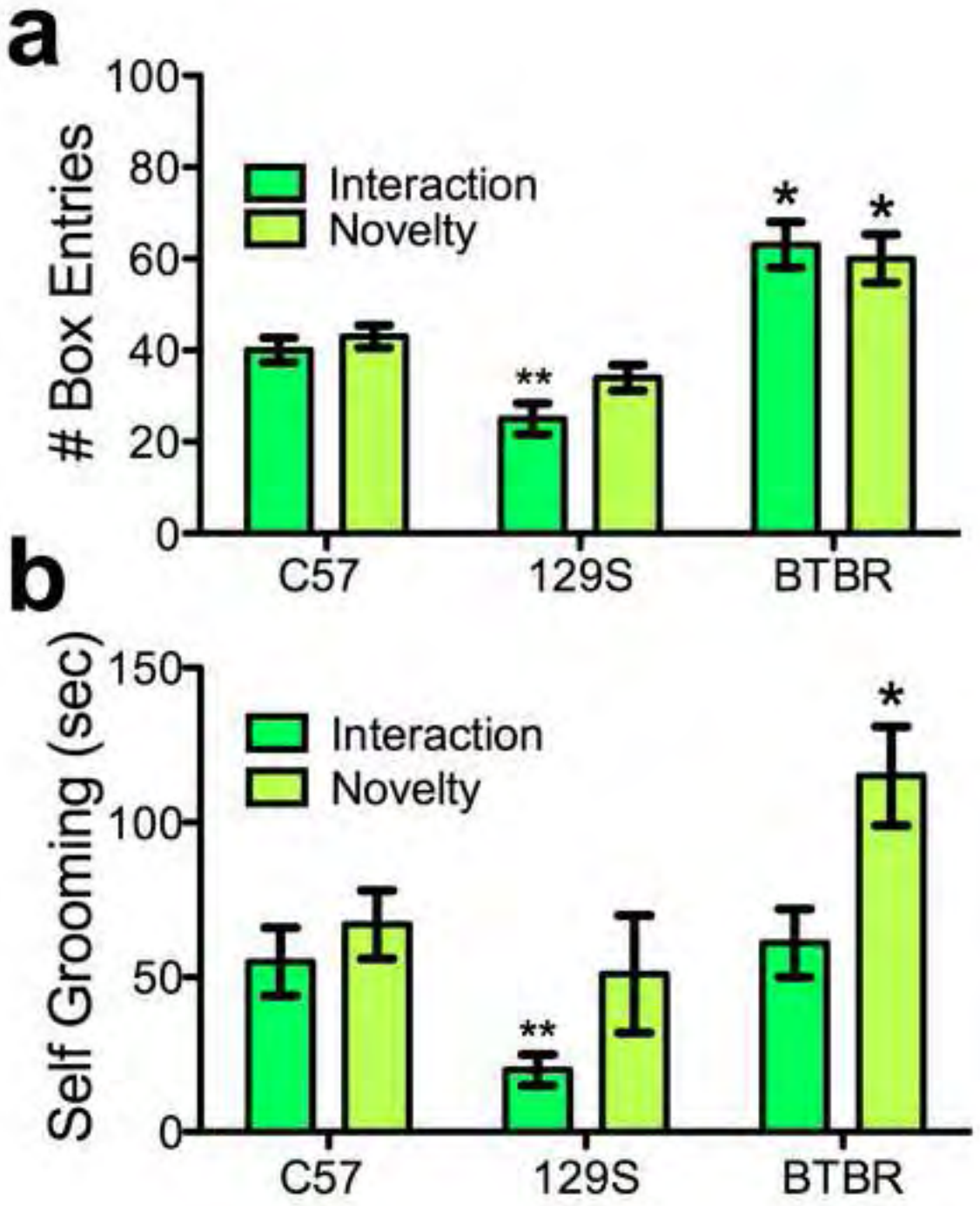

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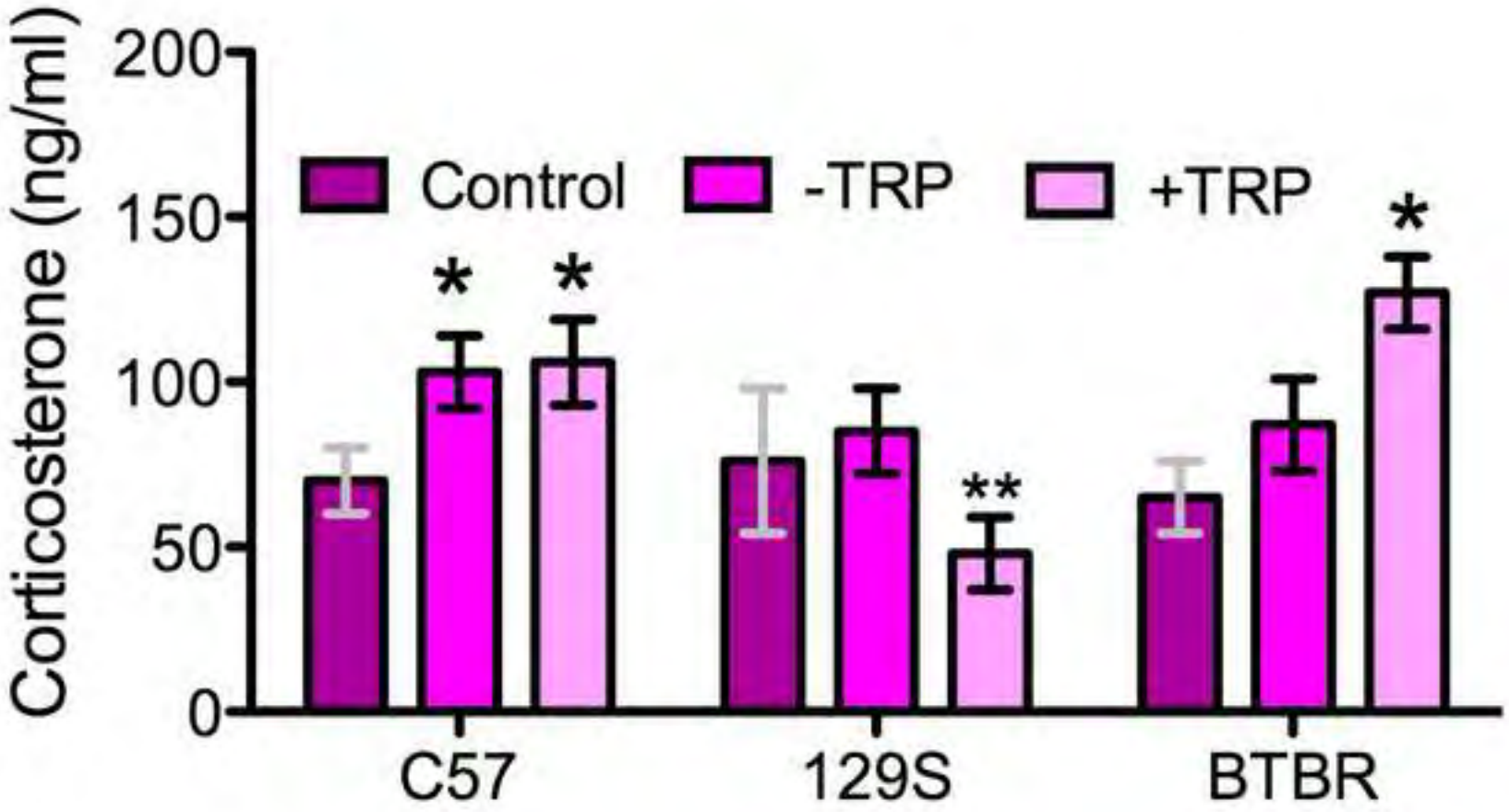

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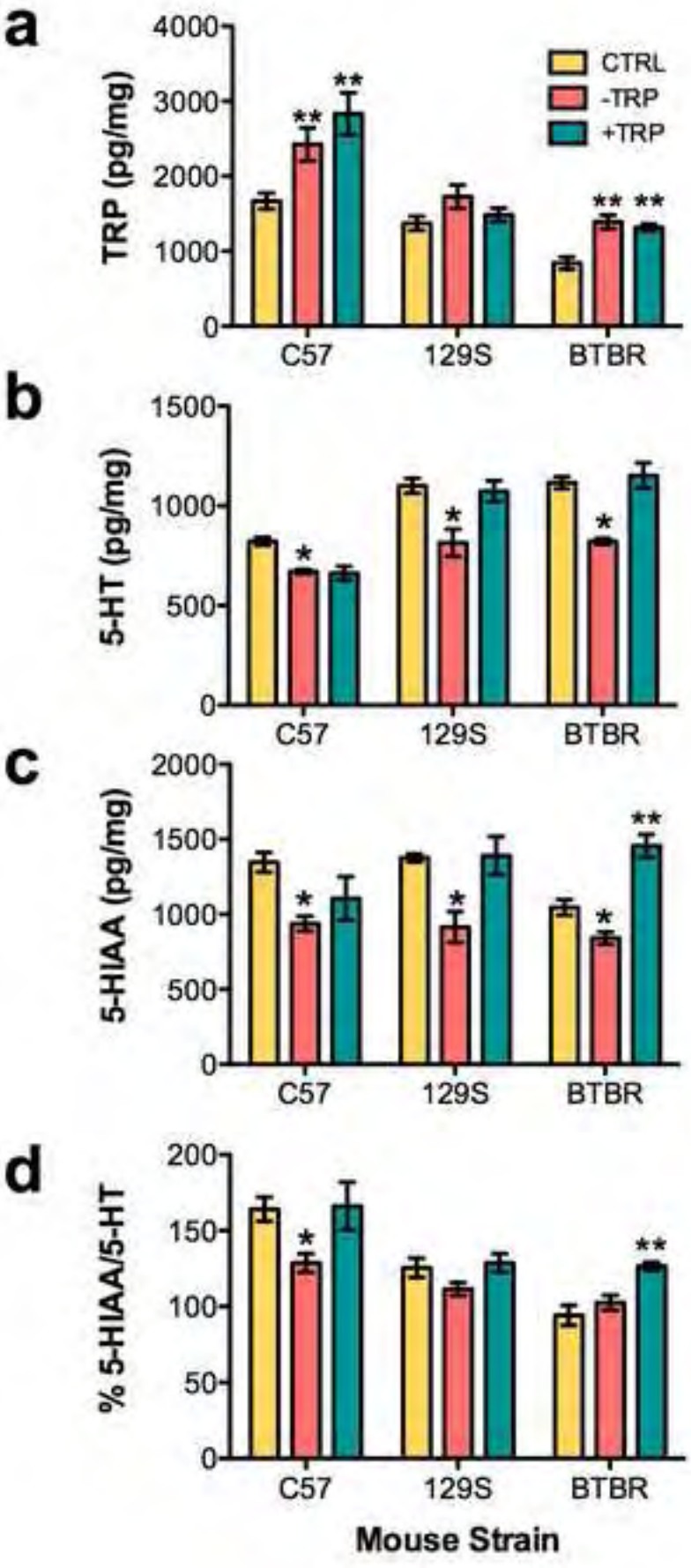

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Sociability of Two C57BL/6 Mouse Substrains from Different US Suppliers

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Abstract

Murine three-chamber sociability tests have been standardized by National Institutes of Health researchers for use in translational biomedical studies of social behavior. Performance in these tests has been compared among many inbred mouse strains, and preference for social interaction and novelty can vary greatly between them. We were concerned about behavioral divergence in isolated, inbred mouse populations or substrains from different suppliers, given that mutation and genetic drift occur more rapidly since mice have relatively short generation spans. Specifically we wanted to know if C57BL/6 mouse lines, separated since 1951 at two major laboratory animal suppliers in the United States (Harlan C57BL/6NHsd (NHsd) and Jackson C57BL6/J (BL6J)), would exhibit distinct social behaviors due to the previously described genetic divergence and different behavioral phenotypes found in other types of behavior tests. Thus we compared their preference for social interaction and novelty through chamber dwelling times and social sniffing, and also examined their marble burying behavior. While NHsd mice did significantly more social sniffing than BL6J, mice from both suppliers had similar preference for social interaction, and NHsd lacked preference for social novelty. Both buried similar numbers of marbles after sociability tests. Our study demonstrates that while it is possible that some test-phase specific differences in social behavior between NHsd and BL6J substrains may occur, C57BL/6 mice from either source can probably still be used comparably in sociability tests.

Introduction

Impaired social behavior is a treatment-resistant core symptom of autism that also occurs in other psychiatric disorders such as schizophrenia, bipolar disorder and depression. Sociability impairments have been modeled for translational research in inbred mouse strains using standardized three-chamber sociability preference tests for behavioral studies testing treatments for autism (Moy et al., 2004; 2007; 2008; Silverman et al., 2010; Yang et al., 2011). In these tests, C57BL/6J mice are often used as a control for other inbred strains, since they are relatively sociable and generally demonstrate preference for social interaction and social novelty in them.

C57BL/6 mouse lines from two United States distributors, Harlan, Indianapolis IN (C57BL/6NHsd, hereafter NHsd) and the Jackson Laboratory, Bar Harbor, ME (C57BL/6J hereafter BL6J) were separated 63 years ago in 1951 when Jackson Labs provided some to the National Institutes of Health (NIH). Both lines originated in 1921 from a mouse breeding project of Dr. Clarence C. Little, the founder of the Jackson Laboratory and graduate student of Dr. W.C. Castle at the Bussey Institute at Harvard University, Boston MA (Weir, 1994; Matsuo et al., 2010). Between 1974 and 1988 Harlan Laboratories acquired the C57BL/6N line from NIH.

Maintenance of isolated, inbred C57BL/6 lines at each facility for > 60 years resulted in independent mutations including some single nucleotide polymorphisms (SNPs) with functional consequences (Mekada et al., 2009; Zurita et al., 2011; Almodovar et al., 2013). This gave rise to the hypothesis that such mutations may have produced differences in their social behavior. Indeed, differences in behavior performance in several tests relevant to psychiatric disorders such as anxiety and depression were already discovered between BL6J and NHsd (Mayorga and Lucki, 2001; Matsuo et al., 2010). Hence to test this hypothesis, in the present study the social and repetitive behaviors of naïve adult male C57BL/6J and C57BL/6NHsd mice were compared.

Methods

Mice

All procedures involving live mice were approved by the UTHSCSA Institutional Animal Care and Use Committee, and were in accord with current NIH guidelines. The 16 mice used in this study were 10-12 week old adult male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, USA (BL6J) N = 8) or C57BL/6NHsd mice from Harlan (Houston, TX, USA (NHsd) N= 8). Mice were housed in groups of 3-5 at 22-25°C on 14:10 light dark cycles, with lights on at 0700 h, and ad-libitum access to Teklad LM-485 mouse/rat irradiated food pellets (#7912, Harlan, Madison, WI) and water in cages with wood-chip bedding that were changed bi-weekly.

Sociability Tests

Three chamber sociability (social interaction and social novelty preference) tests were performed between 0900 and 1600 h CST as described in Gould et al. 2011), employing methods consistent with previously established protocols (e.g. Silverman et al., 2010; Yang et al., 2011). Briefly sociability tests took place in a custom-made acrylic (4 mm) three-chamber testing arenas (60 cm long x 30 cm wide x 22 cm high) with opaque black sides subdivided by two transparent interior walls with doors (9 cm wide x 9 cm tall), under low red light (16 lux). The sociability testing protocol consists of three phases, 1) 20 min of pre-test acclimation, 2) 10 min of preference for social interaction testing, and 3) 10 min of preference for social novelty testing.

Pre-test acclimation of subject mice took place in an empty test arena. Mice were first confined in the center chamber for 10 min, and then permitted free exploration of the entire arena for 10 min. To test social interaction preference, subjects were confined to the center chamber, while a stranger mouse in a wire cup cage was placed in one end chamber and an empty wire cup cage was placed in the other end. Subjects then were released to explore the entire arena for 10

min while their behavior was videorecorded. Finally, subjects were confined to the center while new strangers (stranger #2) were placed in the empty cages and "old" strangers (stranger #1) were re-positioned in the arena. Preference for social novelty was then tested and recorded for 10 min. Between subjects, strangers were returned to home cages while arenas and cup cages were cleaned with 70% ethanol and paper towels. Stranger mice were 8-10 week old male 129S1/SvImJ that were pre conditioned prior to testing by confinement under cup cages 3 X for 30 min intervals on prior days. Stimulus (strangers or empty cages) placement at either arena end was randomized and balanced among groups. Tests were videorecorded for subsequent data collection by treatment-blind observers using digital cameras (R742 Photosmart, Hewlett Packard, Palo Alto, CA, USA) mounted on tripods (Targus, Anaheim, CA, USA). Observers measured time in chambers, chamber entries, time engaged in sniffing of stranger mice or objects (identified on videos by nose being in contact or close proximity to cup cages).

Marble Burying

After social novelty testing, each subject mouse was transferred to a plastic 50 x 28 x 23 cm rat housing cage filled 8 cm deep with wood chip bedding. Fifteen blue flattened marbles were spaced evenly apart on top of the bedding in a 3 x 5 grid pattern. Cages were covered with filter tops and mice were permitted 30 min to bury marbles. Marbles > 66% buried counted as buried.

Statistical Analysis

Repeated measures (mixed model) analysis of variance (RM-MANOVA) was used to compare social and novelty preference for time in chamber and sniffing data among C57BL/6 substrains. Significant differences were resolved by Fisher's least significant difference (LSD) or t-tests for dependent samples. Chamber entries and marbles buried were compared by Fisher's LSD. Analyses were performed using Statistica (Statsoft, Tulsa, OK).

Results

Both BL6J and NHsd displayed a significant preference for social interaction by spending more time in the chambers with the novel strangers than in those with novel objects ($F_{1,14} = 35.9$, $p < 0.0001$, $t_7 > 4$, $p < 0.01$, Figure 1a), and by sniffing strangers more than novel objects ($F_{1,14} = 51.8$, $p < 0.00001$, $t_7 > 3$, $p < 0.05$, Figure 1b). However NHsd mice spent significantly more time than BL6J sniffing stranger mice, as indicated by a significant substrain difference ($F_{1,14} = 14$, $p < 0.005$) and a significant substrain by chamber interaction ($F_{1,14} = 7.8$, $p < 0.05$, Figure 1b) for this parameter.

BL6J and NHsd both failed to display preference for social novelty based on time spent in chambers ($F_{1,14} = 0.9$, $p = 0.34$, Figure 1c). However BL6J exhibited a trend toward more social sniffing of the second (last introduced) stranger mouse than the first that did not reach significance in the t-tests ($F_{1,14} = 4.9$, $p < 0.04$, $t_7 = 2.02$ $p = 0.08$, Figure 1d).

The total number of chamber entries during the social interaction (mean ± standard error = 46 ± 4 vs. 36 ± 5) and social novelty (46 ± 7 vs. 49 ± 4) tests did not differ significantly among BL6J and NHsd mice (Fisher's LSD $p = 0.18$ and $p = 0.7$, respectively). Furthermore, the total number of marbles buried by BL6J (6.1 ± 1.3) vs. NHsd (4.8 ± 1.2) was not significantly different (Fisher's LSD $p = 0.44$).

Discussion

Social interaction, and social novelty tests for mice in three-chambered arenas have been extensively refined and validated to standardize the procedure and promote reproducibility among labs (Yang et al., 2008; Silverman et al., 2010). These robust sociability tests have been used to characterize over 20 inbred strains and also some transgenic mice (Moy et al., 2004; 2007; 2008; 2009). These tests are widely used to assess sociability, and are demonstrably sensitive to SSRIs or drugs with anxiolytic properties (Silverman et al., 2010; Gould et al., 2011; 2012; Chadman, 2011; Defensor et al., 2011).

We used these murine three-chamber tests to compare the social behaviors of naïve C57BL/6 mice from Jackson (BL6J) and Harlan (NHsd) laboratories. Our study revealed similar strong preferences for social interaction by measure of chamber dwelling, and enhanced social sniffing of stranger mice by NHsd mice during the social interaction preference test phase. These findings were consistent with those of other researchers testing BL6J social interaction preference in this manner (Moy et al., 2007; Chadman, 2011, Gould et al., 2011). Thus, NHsd appear to engage in more social sniffing than BL6J in the first phase of sociability testing.

If the relative time spent by mice in chambers and sniffing during social interaction tests is compared more broadly, BL6J are less sociable than other strains such as FVB/NJ or C3H/HeJ (Moy et al., 2007; Bolivar et al., 2007). Interestingly both highly sociable strains FVB/NJ or C3H/HeJ are homozygous for Pde6b^{rd1} allele that triggers early onset retinal degeneration and they are blind by weaning (Corrigan et al., 2002). NHsd and other C57BL6/N lines carry an Rd8 deletion in *Crb1* that results in retinal degeneration evidenced by spotty fundis lesions by 6-8 weeks of age; such lesions do not occur in BL6J that do not carry the Rd8 deletion (Mattapallil et al., 2012; Harlan, 2013). The NHsd we tested did more social sniffing than BL6J, as did

C3H/HeJ in the study by Moy et al., (2007). Hence blind or low vision mice may do more social sniffing during the social interaction phase than sighted ones in three-chamber sociability tests.

However, we also saw that both BL6J and NHsd failed to exhibit preference for social novelty by measurement of chamber dwelling. This outcome is at face inconsistent with prior findings of novelty preference in BL6J in three-chamber tests (Moy et al., 2007; Silverman et al., 2010; Chadman, 2011), but is in agreement with findings by Pearson et al. (2010). While BL6J generally tend to prefer social novelty in these tests, this preference is much less pronounced than that of other strains such as FBV or BTBR (Bolivar et al., 2007; Moy et al., 2007; Defensor et al., 2011). We did see a trend toward more stranger 2 sniffing by BL6J, however it was not significant. A limitation of our study is that it is small, as we only tested 8 mice from each substrain. It is possible that the absence of BL6J social novelty preference we observed was due to chance associated with random sampling. In this case, and since a trend was there if larger populations were compared preference for social novelty might become significant in BL6J, as it has in prior studies (Moy et al., 2007; Gould et al., 2011; Chadman, 2011).

Genetic drift is the change in allelic frequencies within a population due to random mutations, some of which come in the form of single-nucleotide polymorphisms (SNPs). SNPs are positions in the genome where there is a variation in a single base. Because of redundancy in the genetic code, SNPs rarely change phenotypes, but can be used to map strain relationships (Petkov et al., 2004). According to Jackson Labs website (http://jaxmice.jax.org/geneticheath/drift.html) genetic drift is probable when 20 or more generations of separation occur between inbred mouse populations. There is clear evidence that inbreeding of C57BL/6 mice has generated substrains with genetic and phenotype differences as a result of drift (Bryant et al., 2008; Mecada et al., 2009; Zurita et al. 2011; Almodovar et al., 2013). One such example involves hippocampal

mossy fiber terminal field length variations that pare with impaired learning, increased aggression and nest building behavior in the European C57BL/6JNmg substrain, for which the mutation(s) responsible remains elusive (Sluyter et al., 1998; Crusio and Schwegler, 2005).

Other specific behavior differences that have been recorded include a greater tendency by BL6J vs. C57BL/6N substrains to tail-climb during tail suspension tests (Mayorga and Lucki, 2001); to exhibit enhanced performance on rotorods (Bryant et al., 2008); blunted startle response, greater locomotor activity, longer center dwelling and open arm times in open field and light/dark box tests (Matsuo et al., 2010). When open field social interaction tests were performed, C57BL6/N (from Charles River) had fewer and more brief social contacts then BL6J (Matsuo et al., 2010). This finding inspired this study comparing NHsd and BL6J sociability.

In conclusion, our comparison of BL6J and NHsd performance in three-chamber sociability tests revealed generally similar preferences for interaction based on chamber dwelling. However these substrains were distinct in their sniffing behaviors. In social interaction tests NHsd (with Rd8 retinal degeneration gene) did more social sniffing, while in social novelty tests BL6J tended toward greater novelty preference in sniffing. While C57BL/6 from either source might be used interchangeably as background or control strains for three chamber sociability tests, blindness in NHsd is of potential concern. Hence our findings do not discount concerns about genetic drift confounding social behavior experiment outcomes.

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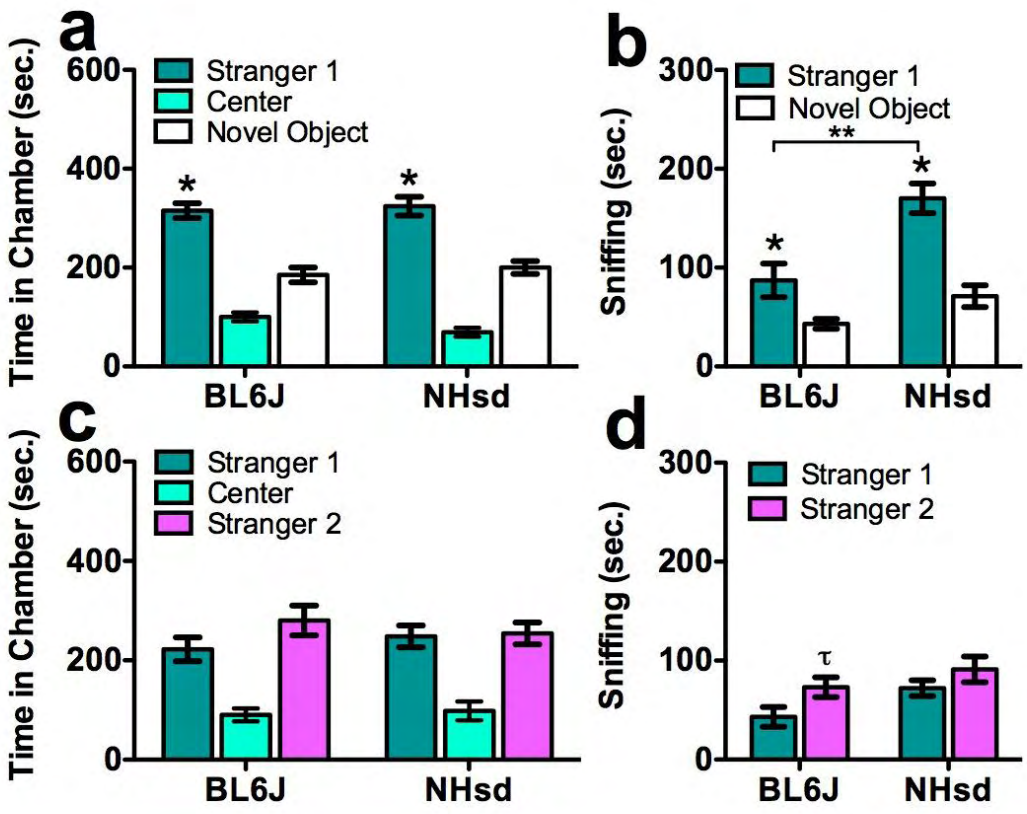
Figure 1. Social interaction and novelty preferences of C57BL/6J and C57BL6NHsd in three-chamber sociability tests. Both Jackson (BL6J) and Harlan (NHsd) mice exhibited preference (*p <0.05) for stranger 1 over a novel object in terms of (a) time in each chamber and (b) social sniffing, but NHsd engaged in more sniffing of stranger mice than did BL6J (**p <0.05). Tests for social novelty (c,d) did not reveal significant preferences for novel strangers (stranger 2) over mice encountered in the first test phase (stranger 1), although BL6J showed a trend toward preferential sniffing of stranger 2 (τ p = 0.08).