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Enhanced Novelty-Induced Corticosterone Spike and Upregulated Serotonin 5-HT_{1A} and Cannabinoid CB_1 Receptors in Adolescent BTBR Mice

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Abstract

Hypothalamic pituitary adrenal (HPA) axis responses to change and social challenges during adolescence can influence mental health and behavior into adulthood. To examine how HPA tone in adolescence may contribute to psychopathology, we challenged male adolescent (5 wk) and adult (16 wk) BTBR T^+tf/J (BTBR) and 129S1/SvImJ (129S) mice with novelty in sociability tests. In prior studies these strains had exaggerated or altered HPA stress responses and low sociability relative to C57BL/6J mice in adulthood. In adolescence these strains already exhibited similar or worse sociability deficits than adults or age-matched C57 mice. Yet BTBR adolescents were less hyperactive and buried fewer marbles than adults. Novelty-induced corticosterone (CORT) spikes in adolescent BTBR were double adult levels, and higher than 129S or C57 mice at either age. Due to their established role in HPA feedback, we hypothesized that hippocampal Gαi/o-coupled serotonin 5-HT_{1A} and cannabioid CB_1 receptor function might be upregulated in BTBR mice. Adolescent BTBR mice had higher hippocampal 5-HT_{1A} density as measured by [^{3}H]8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) binding than C57 mice, and adult BTBR 8-OH-DPAT-stimulated GTPγS binding was higher than in either C57 or 129S mice in this region. Further, BTBR hippocampal CB_1 density measured by [^{3}H]CP55,940 binding was 15-20% higher than in C57. CP55,940-stimulated GTPγS binding in adult BTBR dentate gyrus was 30% higher then 129S ($p<0.05$), but was not a product of greater neuronal or cell density defined by NeuN and DAPI staining. Hence hyperactive HPA responsiveness during adolescence may underlie 5-HT_{1A} and CB_1 receptor up-regulation and behavioral phenotype of BTBR mice.

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Keywords

129S1/SvImJ; adolescent; BTBR; C57BL; cannabinoid; GTPγS autoradiography; hippocampus; HPA feedback; novelty stress; serotonin; social behavior

1. INTRODUCTION

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

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

Corticosteroids bind to hippocampal GRs, the activation of which promotes serotonin transmission that in turn attenuates CORT release (Lanfumey et al., 2008; Pompili et al., 2010). CORT binding to GR also increases hippocampal endocannabinoid levels, and this likewise suppresses CORT release (Cota, 2008; McLaughlin et al., 2009; Atsak et al., 2012). This HPA axis suppression is likely mediated via inhibitory metabotrophic Gαi/o coupled serotonin 5-HT_{1A} and CB_1 receptors. When direct HPA axis feedback by circulating CORT via GRs is impaired, 5-HT_{1A} and CB_1 receptors may be up-regulated in compensation (Lanfumey et al., 2008; Pompili et al., 2010; Hensler et al., 2010). We hypothesized that this type of compensation may occur to a greater extent during adolescence, particularly if the HPA stress-response is exaggerated.

We have found that in adult BTBR mice hippocampal 5-HT_{1A} receptor function was enhanced relative to C57BL/10 mice (Gould et al., 2011). We hypothesized that exaggerated stress-evoked CORT release during adolescence could underlie this 5-HT_{1A} up-regulation, and may likewise up-regulate hippocampal CB_1 receptors. Since higher binding could stem from increases either in receptor expression or neuronal density, we compared hippocampal neuronal cell density in adults of each strain. Further, receptor up-regulation might be more pronounced in adolescent mice with HPA axis hyperactivity. Thus, we compared adolescent to adult 5-HT_{1A} and CB_1 receptor densities and agonist-stimulated G-protein coupling in these strains.

2. METHODS

2.1. Mouse Subjects

BTBR T+tf/J, 129S1/SvImJ, C57BL/10J and C57BL/6J colony founders were from Jackson Laboratory (Bar Harbor, ME, USA). The mice were second or greater generation offspring bred in the laboratory animal facilities at William Paterson University, Wayne, NJ for quantitative autoradiography, or at The University of Texas Health Science Center at San Antonio (UTHSCSA), San Antonio, TX for autoradiography, immunostaining, behavior and corticosterone measures. Mice at both facilities were maintained at 20–22°C on 12:12 light dark cycles, with lights on at 0700 h, and ad-libitum access to food and water in cages lined with wood chip bedding (at UTHSCSA) or shaved wood bedding (at William Patterson University) that was changed bi-weekly. Mice were weaned at around postnatal day 21 and were housed in same-sex littermate groups of 2–5 per cage. All procedures involving mice were approved by Institutional Animal Care and Use Committees, and were consistent with current NIH guidelines.

2.2. Exposure to Novelty in Sociability and Marble Burying Tests and Serum CORT Levels

Mice used for novelty exposures and corticosterone (CORT) measures were 5 or 16 week-old male C57BL/6, 129S and BTBR. Prior to trunk blood collection, half of the mice were subjected to three-chamber sociability tests (40 min), immediately followed by marble burying (30 min), as in Gould et al. (2011; 2012). Briefly, each mouse subject was individually placed in a novel three-chamber arena for sociability testing. First subjects explored their arena for 20 min of preconditioning, then an unfamiliar object (wire cup) and 'stranger 1' a 129S male mouse (4 – 5 weeks old, in wire cup) was introduced at either end for 10 min, and finally a second novel 'stranger 2' mouse under a wire cup was introduced for 10 min, replacing the empty cage. Self-grooming was only scored during the sociability test and was not scored in an independent task. Afterward each subject mouse was transferred for 30 min to a 51 × 28 × 23 cm box filled with 10 cm of wood-chip bedding topped with 15 blue marbles in a grid pattern. The untested group of mice remained in their home cages until sacrifice. All mice were humanely sacrificed by decapitation between 1500 and 1650 h CST, and trunk blood was collected into tubes containing 25 μl of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO).

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

2.3. Brain Tissue Preparation

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

2.4. Quantitative Autoradiography

Binding assays with [^{3}H] 8-OH-DPAT (2 nM) for serotonin 5-HT$_{1A}$, with 1 μM WAY 100,635 (Tocris, Ellisville, MO) to define non-specific binding, and [^{3}H] CP55,940 (5 nM) for cannabinoid CB$_1$ receptors, with 200 μM WIN,55-212-2 (Ascent Scientific, Princeton

NJ) for non-specific binding, were performed on brain sections as per Gould et al. (2012). [^{35}S] GTPγS binding in absence or presence of 1 μM of agonists 8-OH-DPAT or CP 55,940 was performed as described in Gould et al. (2011; 2012). Radioligands were from Perkin-Elmer (Boston, MA), and Kodak Biomak MR film (ThermoFisher, Waltham, MA) was used for all experiments.

Digital images were captured on a camera (1612M, Scion Corp., Frederick, MD) with a 60 mM lens onto a Macintosh (OS 10), with Image J software (NIH, Bethesda, MD) for density measures. Grey scale units were converted to fmol/mg protein or nCi/mg using calibration standards (American Radiolabeled Chem., St. Louis, MO), as per Gould et al. (2011). Brain regions measured included the hippocampus CA1, CA3 and dentate gyrus regions, frontal and parietal cortex, caudate putamen, nucleus acumbens (CB_1 only), amygdala and hypothalamus. The amygdala and hypothalamus were not measured for GTPγS due to high basal binding.

2.5. Immunohistochemical Labeling

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

2.6. Statistical Analysis and Sample Sizes

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

3. RESULTS

3.1. Effects of Mouse Strain, Age and Behavior Testing on Serum CORT

Corticosterone levels increased significantly after 70 min of novelty exposure in behavior tests ($F_{1,66} = 177$, $p < 0.0001$), and differed in magnitude among mouse strains ($F_{2,66} = 24$, $p < 0.0001$) and ages ($F_{1,66} = 17$, $p < 0.0001$), with significant interactions ($p < 0.017$). The post-novelty increase in CORT was two-fold greater in 5 week-old BTBR mice than other

strains, and was also greater than in adult BTBR mice ($p < 0.0001$) (Figure 1). Baseline CORT levels tended to be higher in adult BTBR than C57BL/6, and this difference was nearly significant ($p = 0.06$).

3.2. Comparison of Social and Repetitive Behaviors in Adolescent and Adult Male Mice

In global analysis of the three-chamber sociability tests, there was a significant interaction between mouse strain and age for time spent in either end chamber of the test arena ($F_{2,30} = 3.23$, $p < 0.05$). In the social interaction phase, C57 adults spent significantly more time by the stranger mouse vs. novel object ($t = -3.8$, $p < 0.05$), while C57 adolescents also tended to, this trend was not significant ($t = -2.14$, $p = 0.09$). Five week-old BTBR mice spent less time in chambers with stranger mice and more time in chambers with novel objects ($t = 3.2$, $p < 0.02$), and they differed in comparison to BTBR adults with respect to this measure ($F_{1,10} = 4.95$, $p<0.05$, Fisher's LSD $p < 0.05$, Figure 2a). In the second phase of the test, only C57BL/6 adult mice showed a significant preference for social novelty based on time spent in the chamber with the new stranger mouse ($p < 0.05$, Figure 2b).

A global repeated measures analysis of social sniff time during sociability tests revealed significant differences between ages ($F_{1,30} = 15$, $p < 0.001$), test phase ($F_{1, 30} = 25$, $p < 0.0001$), with significant interactions among all main effects. With the finding of a significant age effect in the repeated measures ANOVA ($F_{1,30} = 9.45$, $p < 0.01$), in post-hoc ANOVAs performed on the social interaction phase, there was a significant effect of age for the amount of time spent sniffing empty boxes ($F_{1,30} =15$, $p < 0.001$), and an interaction between strain and age ($F_{2,30} = 3.9$, $p < 0.32$). Specifically BTBR adolescents spent more time sniffing the empty cages than all other groups except 5 week-old 129S mice. There was no significant difference in social sniff of stranger mice between strain ($p = 0.4$), or age ($p = 0.7$), as shown in figure 2c. However only C57 adults and adolescents had a significant preference for stranger mice ($t > 3.4$, $p < 0.05$). In the social novelty phase, there was a significant effect of strain ($F_{1,30} = 15$, $p <0.0001$) and age ($F_{2,30} = 0.001$), with interactions among factors ($F_{2,30} = 12$, $p <0.0001$). This was due to an increased amount of sniffing by 129S adolescents of either stranger relative to all other strains (F2, $30 > 5$, $p <0.05$, Fisher's LSD $p <0.05$), and more sniffing of the new strangers in comparison to 129S adults (F1, 30 = 22, $p < 0.0001$, Fisher's LSD $p <0.05$), shown in figure 2d.

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

3.2. Serotonin 5-HT$_{1A}$ Receptor Density and 8-OH-DPAT-Stimulated GTPγS Binding

There were significant differences in [^{3}H] 8-OH-DPAT binding density among strains (Wilks' $\lambda_{16,70} = 0.17$, $p<0.0001$) and ages ($\lambda_{8,35} = 0.32$, $p<0.0001$), with interactions among these factors ($\lambda_{16,70} = 0.51$, $p = 0.05$) in the hippocampus and in other brain areas. Among 16 week-old mice, 129S [^{3}H] 8-OH-DPAT binding was higher in the CA1 of hippocampus (Figure 3b) and cingulate cortex (by roughly 30%) than either BTBR or C57BL/10 ($F_{2,42} > 8$, $p< 0.01$; Newman-Keuls $p < 0.05$). Further, 5-week old 129S and BTBR mice had higher [^{3}H] 8-OH-DPAT binding in the CA1 of hippocampus than C57BL/10 at either age ($F_{2,42} = 21$, $p<0.001$; $p < 0.01$, Figure 3b). Five-week old BTBR mice also had higher [^{3}H] 8-OH-DPAT binding in the CA3 of hippocampus (Figure 3c, $F_{2,42} =7.1$, $p < 0.002$; $p < 0.05$).

Also, 16-week old BTBR mice had lower [^{3}H] 8-OH-DPAT binding than 5-week olds in the basolateral amygdala (108 ± 14 *vs*. ≈ 165 fmol/mg protein) and ventromedial hypothalamus (129 ± 11 vs. ≈ 200 fmol/mg protein) ($F_{1,42}$ or $F_{2,42} > 3.15$, $p < 0.05$). Non-specific was 8–10% of total binding.

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

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

3.3. Cannabinoid CB_1 Receptor Density and CP55,940-Stimulated GTPγS Binding

There were differences in [^{3}H] CP55,940 binding density among strains ($\lambda_{18,68} = 0.1$, $p < 0.001$), specifically C57BL/10 had lower density than BTBR and/or 129S mice in dentate gyrus, CA1, CA3 of hippocampus and in the parietal cortex (175 vs. >202 fmol/mg protein) at 5 weeks, and in the dentate gyrus and hippocampal CA3 at 16 weeks of age ($F_{2,42} > 3$, $p < 0.05$, Figure 4a–c, left panels). While a significant age effect was detected ($\lambda_{9,34} = 0.29$, $p<0.001$), post-hoc ANOVA failed to reveal age differences in the regions measured ($F_{1,42} < 2$, $p > 0.15$), and there were no interactions ($\lambda_{18,68} = 0.5$, $p = 0.19$). Non-specific binding was 40–50% of total.

CB_1 receptor CP55,940-stimulated [^{35}S] GTPγS binding differed among strains ($\lambda_{10,76} = 0.55$, $p<0.01$) and ages ($\lambda_{5,38} = 0.65$, $p<0.01$) without interaction ($\lambda_{10,76} = 0.67$, $p = 0.1$). In all strains, CP55,940-stimulated binding increased with age in the hippocampus, as shown in the right panels of figure 4, and also in the cingulate cortex (18–40% greater in adults). In 16 week-old BTBR mice CP55,940-stimulated [^{35}S] GTPγS binding was higher in dentate gyrus than in the other strains ($F_{2,42} = 3.7$, $p < 0.05$), figure 4a, left. We also observed in the hippocampal CA1 and CA3 of 5 week-old 129S mice that CP55,940-stimulated [^{35}S] GTPγS binding was lower ($p <0.05$) than in C57BL/10 mice of the same age. This corresponded with modest increases in 129S CB_1 receptor density in the 5-week old hippocampus, as measured by [^{3}H] CP55,940, which may compensate for impaired CB1 receptor functionality in adolescent 129S mice.

3.4. C57BL/10 vs. C57BL/6 Binding Comparison

Because C57BL/6 mice are more commonly used than C57BL/10, we compared [^{3}H] 8-OH-DPAT, [^{3}H] CP55,940, and agonist stimulated [^{35}S] GTPγS binding density in them. We did not observe any significant differences in 5-HT_{1A} ($\lambda_{8,7} = 0.28$, $p = 0.15$) or CB_1 ($\lambda_{9,6} = 0.44$, $p = 0.61$) binding site density among these strains. Further, agonist-stimulated [^{35}S] GTPγS binding did not reveal any real differences among C57BL/6 and C57BL/10 adults ($\lambda_{5,2} = 0.05$, $p = 0.12$).

3.5. Immunostaining for Neuronal Density in the Dentate Gyrus

There were no differences in stain intensity for DAPI ($F_{2,19} = 2.8$, $p = 0.09$) or NeuN ($F_{2,19} = 1.0$, $p = 0.38$) in the dentate gyrus among adult male 129S, BTBR or C57BL/6 mice. NeuN stain intensity ranged from a mean ± S.E.M. of 43 ± 9 to 64 ± 11 optical density units, while DAPI ranged from 72 ± 7 to 92 ± 5 density units. Representative images are shown in Figure 5.

4. DISCUSSION

Herein we provide evidence of greater novelty-induced increases in serum CORT in adolescent male BTBR mice, accompanied by greater hippocampal serotonin 5-HT_{1A} and cannabinoid CB_1 receptor density relative to C57 mice. In BTBR adults, agonist-stimulated GTPγS binding to hippocampal 5-HT_{1A} receptors was augmented, but this was not accompanied by higher receptor, cellular or neuronal density. Enhanced hippocampal 5-HT_{1A} Gαi/o-coupled receptor activation capacity could facilitate inhibition of CORT release, and as such it may be a key compensatory response in adult BTBR HPA axis feedback. While hippocampal CB1 receptor density was greater in BTBR and 129S mice, CP55,940 stimulated GTPγS binding was no higher than, and in adolescents it was reduced relative to C57 mice. These factors may contribute to the age-dependent patterns in sociability deficits and hyperactivity we observed in BTBR males.

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

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

Based on prior studies, we predicted that CORT spikes in response to novel stimuli, at least in the adolescent C57BL/6 males, might be exaggerated compared to those in adults (Romeo et al., 2013), and that even higher levels might be found in BTBR mice (Benno et al., 2009). Indeed, we saw an exaggerated CORT response to novelty in adolescent BTBR after 70 min of sociability testing and marble burying. Our CORT level measures were also consistent with earlier reports of higher baseline and hyperactive HPA axis responsiveness in BTBR relative to C57BL/6 mice (Benno et al., 2009; Silverman et al., 2010). In contrast, CORT levels in 129S mice were no higher than C57BL/6 following novelty exposure, although restraint stress produced greater initial increases in them in a prior study (Camp et al., 2012). Since we did not measure CORT levels at earlier time points (e.g. at 30 min intervals) during our behavior tests, we suspect that we did not capture peak CORT levels induced by handling and novelty exposure. However, higher peak levels and/or extended release of CORT was nonetheless evident in BTBR adolescents after novelty exposure under our testing paradigm, and we also saw higher CORT levels in all strains and ages after behavior testing as compared to baseline values.

However, elevated CORT levels, either at baseline or in response to novelty, may not necessarily be indicative of a heightened anxiety state in BTBR mice. Five weeks is just prior to puberty onset at around postnatal day 40 for most male mice, including strains such as C57BL/6 and 129S (Wisniewski et al., 2005; Qiu et al., 2013). Male puberty has not been characterized in BTBR mice, so it could occur earlier, as in strains such as CD1 or C3H/HeJ

(Divall et al., 2010; Zhou et al., 2012), or later. Yet it is unlikely that any differences in puberty onset alone could have so distinguished the BTBR behavioral and neuroendocrine phenotype. Other endocrine abnormalities are more likely responsible, for example in BTBR mice serum levels of insulin, testosterone, and progesterone are also relatively high (Flowers et al., 2007, Frye and Llaneza et al., 2010). Taken together with their elevated baseline CORT levels and exaggerated response, there is evidence of pituitary over-activity, possibly due to feedback disruption in BTBR mice, and these conditions can adversely impact cognition and behavior (Stranahan et al., 2008).

4.2 Age-dependent patterns of 5-HT_{1A} receptor regulation in BTBR mice

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

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

4.3. Mounting evidence for possible GR dysfunction in BTBR mice

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

4.3 Up-regulation of CB_1 function in the adult male BTBR dentate gyrus

Social behavior in inbred mice is also sensitive to synthetic cannabinoid agonists and manipulation of endogenous cannabinoid levels in frontal cortex and other brain regions by acetaminophen (Umathe et al., 2009; Gould et al., 2012). Given this, we compared CB1

receptor density and G-protein coupling capacity among strains in adolescent and adult male mice. We saw that CP55,940 stimulated G-protein coupling was enhanced in the dentate gyrus of BTBR adults relative to 129S adults and adolescent mice in all strains. We investigated this finding further, since a survey of neuropathological markers in BTBR mice revealed that neurogenesis in their dentate gyrus and glial fiber growth was altered (Stephenson et al., 2011). We saw no differences in neuronal density as quantified by NeuN labeling, DAPI staining, or CB1 receptor expression as quantified by autoradiography with [^{3}H] CP55,940 in the adult BTBR dentate gyrus. However, enhancement of CP55940-stimulated G-protein coupling may yet be due to differences in neural vs. glial cell composition in this brain region (Lopez-Gallardo et al., 2012). More numerous glial cells in BTBR adults, and subsequent increases in the actions of Gαi/o coupled CB_2 receptors expressed on them (Brusco et al., 2008) could account for enhanced G-protein coupling capacity in the dentate gyrus of BTBR adults.

4.4 Relative down-regulation of CB_1 function in adolescent 129S hippocampi

Agonist-stimulated [^{35}S] GTPγS binding generally increased with age at hippocampal CB_1 receptors in all strains, while CB1 receptor density did not. 129S adolescents and BTBR mice had higher [^{3}H] CP55,940 binding density than C57 mice at both ages in the dentate gyrus and CA3 regions. Despite this, 129S adolescent mice had relatively lower CP55,940 stimulated GTPγS binding in all hippocampal areas than C57 or BTBR adolescents. This disconnect between CB1 density and function was not present in adult 129S mice, given our CP55,940 stimulated GTPγS binding data from all three hippocampal subregions, and earlier findings in the cingulate cortex (Gould et al., 2012).

Instead, it appears to be a distinct developmental strain difference in the dentate gyrus that may relate to stress reactivity impairments and fear-related memory impairments that have already been characterized in adult 129S1/SvImJ mice (Camp et al., 2012). Endocannabinoids, via CB_1 receptors can prevent the retention of inappropriate generalized fear responses in mice by interfering with hippocampal long-term potentiation and plasticity (Reich et al., 2008; Jacob et al., 2012), and 129S mice exhibit fear overgeneralization and motor activity impairments that have been attributed to elevated anxiety (Kalueff and Tuohimaa, 2004; Camp et al., 2012). It is also possible that the increased 5-HT_{1A} expression we found in the 129S hippocampus might compensate for functional CB_1 impairments, but incompletely at the level of G-protein coupling.

4.5. Age-dependent interactions among CB1 and 5-HT_{1A} in BTBR HPA axis regulation

Adolescent rodents might be less sensitive to cannabinoid-induced modulation of 5-HT_{1A} expression and function then adults (Zavitsanou et al., 2010). Hence while in the aggregate the functional G-protein binding response of both CB_1 and 5-HT_{1A} receptors is lower in BTBR adolescents than in adults, inhibitory feedback to the HPA axis may be muted to a greater extent. This could be why adolescent, but not adult CORT responses to novelty were exaggerated in BTBR mice. It is plausible that up-regulation of inhibitory coupling capacity at both 5-HT_{1A} and CB_1 receptors in these hippocampal regions is required to keep BTBR HPA axis activity in check and dampen the exaggerated adolescent novelty-induced CORT peak in BTBR adults. In turn, up-regulation of hippocampal 5-HT_{1A} and CB_1 receptor function or density in BTBR and 129S mice, while it may occurs for different reasons, may similarly contribute to the lack of preference for social interaction in these strains.

4.6 Implications for strain selection in studies of social behavior

Poor emotional resilience in response to stress is associated with impulsive or aggressive behaviors, and psychiatric disorders such as anxiety and depression (Cuomo et al., 2008; Stevens et al., 2009). Impaired HPA axis feedback in susceptible individuals may contribute

to this state (Sher et al., 2006; Spijker and van Rossum, 2012). Glucocorticoid tone may be particularly relevant to how the mature BTBR behavioral phenotype develops, since it shapes dendritic spine formation and pruning in the hippocampus (Liston and Gan, 2011, Gourley et al., 2013). This discovery is also potentially relevant for social interactions in teens with autism, since in controlled studies novel social stimuli raised cortisol levels higher in autistic than normotypic adolescents (Corbett et al., 2010; Schupp et al., 2013). In this sense, the BTBR mouse could provide critical mechanistic insight into how glucocorticoids shape adolescent social behavior. Given the variability in CORT response, 5-HT_{1A} and CB_1 receptor expression among BTBR, 129S and C57 mice, their use as a combined model system could help to clarify their effects on endpoints such as social behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of interest statement

The authors have no conflicts of interest to report.

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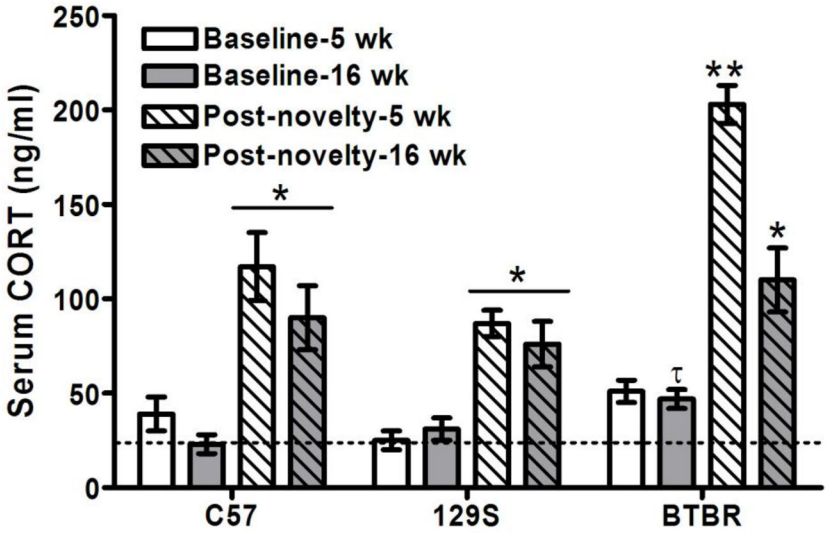

Figure 1. Novelty exposure raised serum CORT levels the most in adolescent BTBR mice

CORT levels increased above mean baseline levels in adolescent (5 wk) and adult (16 wk) male mice of C57BL/6, 129S1/SvImJ and BTBR strains after 70 min of novelty exposure in behavior tests (*$p<0.05$). However, CORT levels in novelty-exposed 5 week-old BTBR mice were far higher than all of the other strains and ages (**$p < 0.0001$). Baseline CORT levels tended to be significantly higher in BTBR than in C57BL/6 adults ($\tau p = 0.06$). The dashed horizontal line illustrates the mean CORT level found in adult C57BL/6 mice to facilitate visual reference.

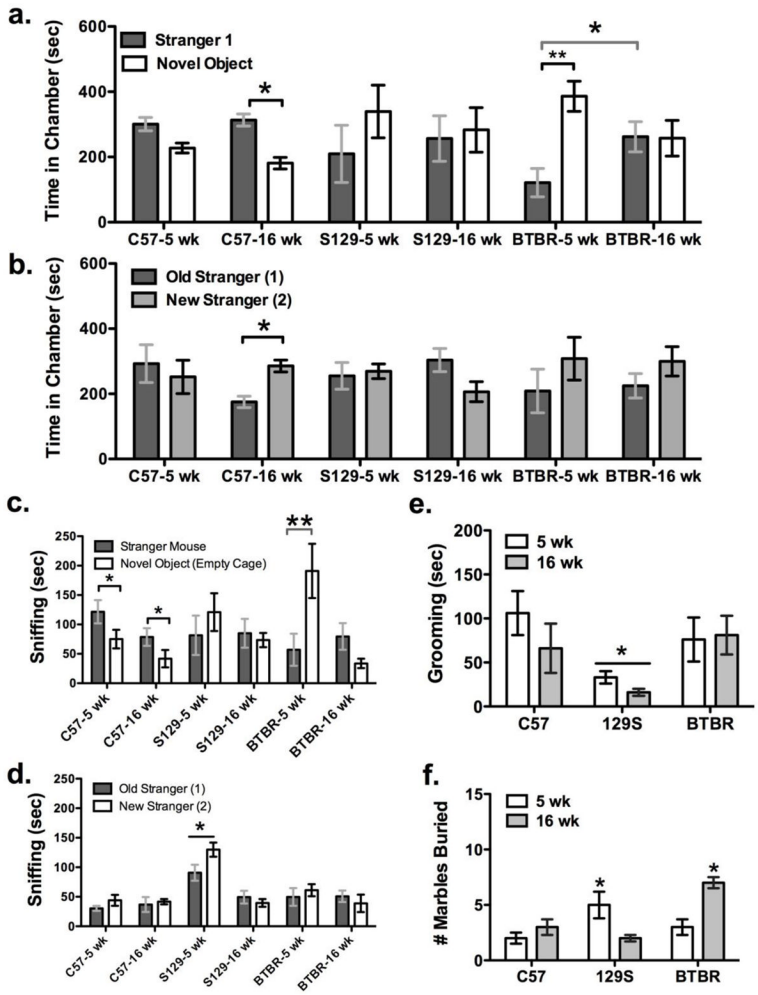

Figure 2. Behavior of adolescent and adult BTBR mice in sociability and repetitive tests

(a) A significant preference for social interaction was only found in C57 adults (*p < 0.05). Adolescent BTBR mice spent more time in chambers with novel objects than with stranger mice in the social interaction test (**p < 0.001), in contrast to adults (*p < 0.01). (b) Only adult C57 mice spent significantly more time in chambers with new strangers than with the 'old' original ones (*p<0.05). (c) Both C57 adolescent and adult mice spent more time sniffing stranger mice than novel objects (*p<0.05), however BTBR adolescents spent significantly more time sniffing novel objects than all other mice (**p<0.001). (d) Time spent sniffing new and old stranger mice during the social novelty test was greatest in 129S adolescents (*p <0.05). (e) Self-grooming among C57 and BTBR mice was comparable, but was lower in 129S mice during sociability testing (*p < 0.05). (f) BTBR adults and 129S adolescents buried more marbles over 30 min after sociability testing than C57BL/6 mice (*p < 0.05).

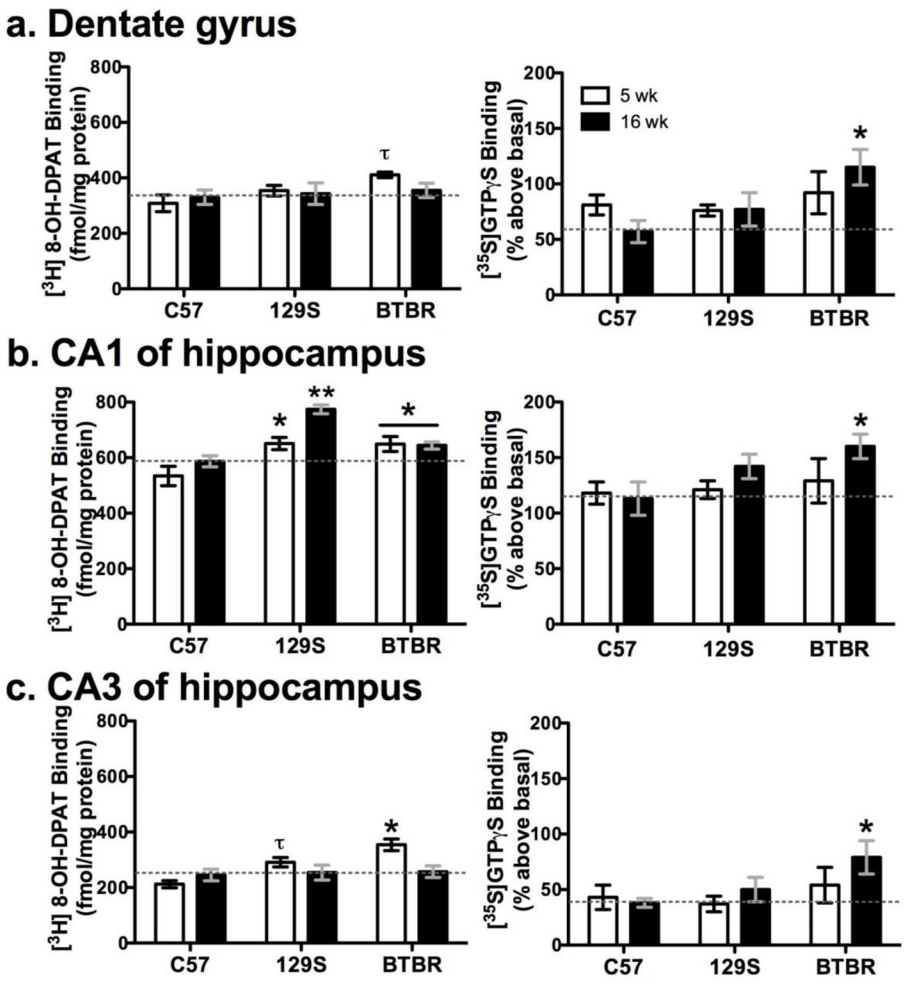

Figure 3. Specific [^{3}H] 8-OH-DPAT binding and 8-OH DPAT-stimulated [^{35}S] GTPγS binding to serotonin 5-HT$_{1A}$ receptors in the hippocampus of adolescent and adult mice

In the left-side panels [^{3}H] 8-OH-DPAT binding was higher in 5-week old BTBR and/or 129S mice in (b) the CA1 and (c) CA3 of hippocampus than age-matched C57 mice (*$p < 0.05$; ** $p < 0.01$; $\tau p = 0.067$). In (a) the dentate gyrus, there was also a non-significant trend toward BTBR mice at 5 weeks having greater [^{3}H] 8-OH-DPAT binding ($\tau p = 0.085$). In the right-side panels 8-OH-DPAT stimulated [^{35}S] GTPγS binding was enhanced in adult BTBR mice in (a) dentate gyrus (b) CA1 and (c) CA3 of hippocampus as compared to adult C57 (*$p \leq 0.05$). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus, in 5-week old BTBR mice hippocampal 5-HT$_{1A}$ density was high, whereas in adults 5-HT$_{1A}$ function was enhanced.

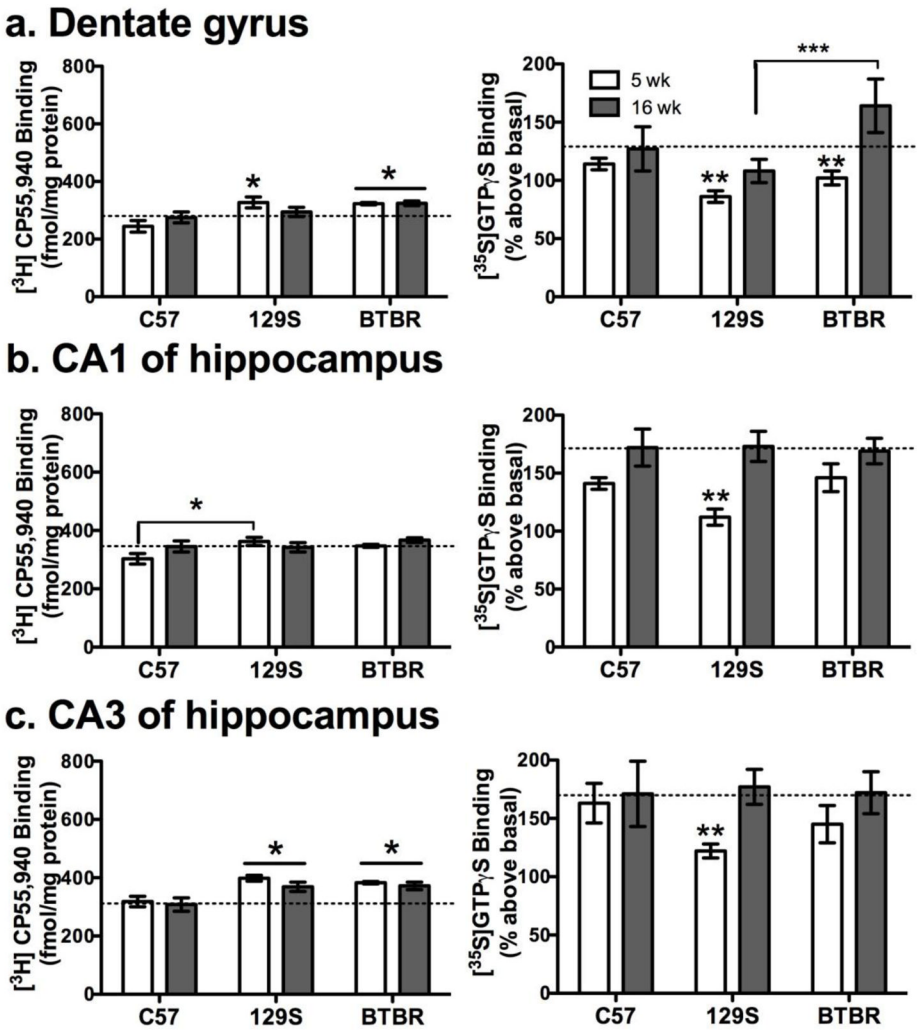

Figure 4. Specific [^{3}H] CP55,940 binding and CP55940-stimulated [^{35}S] GTPγS binding to hippocampal cannabinoid CB_1 receptors in adolescent and adult mice

As shown in the left-side panels [^{3}H] CP55,940 binding was greater in the (a) dentate gyrus and (c) CA3 of BTBR *vs.* C57 mice (*p<0.05). Adolescent 129S mice had significantly higher [^{3}H] CP55,940 binding in the (a) dentate gyrus, (b) CA1 and (c) CA3 than adolescent C57 mice (*p<0.05). On the other hand, CP55,940 stimulated [^{35}S] GTPγS binding generally increased with age, as shown in the panels to the right. However, hippocampal CP55,940 stimulated [^{35}S] GTPγS binding was consistently lower in 129S adolescents (**p<0.05, a–c). Also agonist stimulated GTPγS binding was higher in adult BTBR than in 129S mice (*** p< 0.05, a). Dashed horizontal lines are a reference marker for mean density in adult C57 mice. Thus CB1 receptor coupling capacity is reduced, despite elevated CB1 receptor density in the adolescent 129S hippocampus.

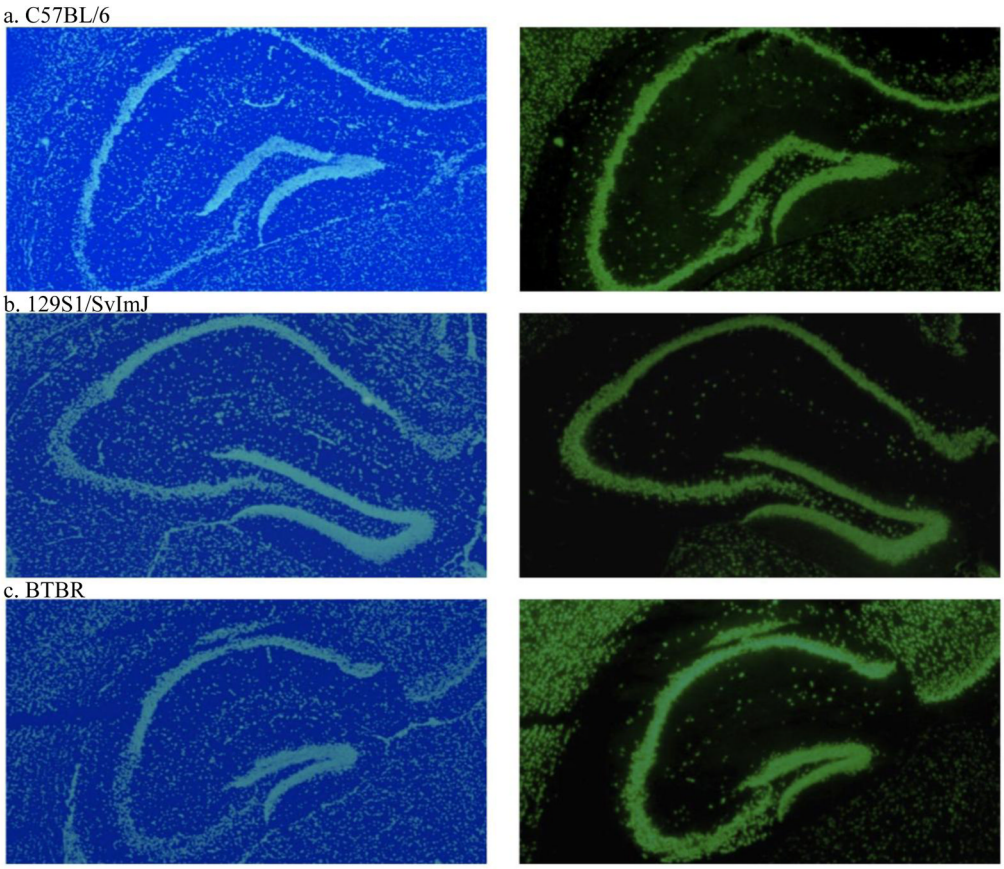

Figure 5. DAPI nuclear (left) and NeuN neuronal (right) staining in the adult mouse hippocampus

(a) C57BL/6, (b) 129S1/SvImJ and (c) BTBR mouse hippocampi had similar cell (in light blue) and neuron (in light green) density in the dentate gyrus. Representative sections from each strain are shown. For interpretation of the references to color in the figure legend, the reader is referenced to the web version of this article.