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Serotonin Neuron Abnormalities in the BTBR Mouse Model of Autism

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Abstract

The inbred mouse strain BTBR $T^+ Itpr3^{tf}/J$ (BTBR) is studied as a model of idiopathic autism because they are less social and more resistant to change than other strains. Forebrain serotonin receptors and the response to serotonin drugs are altered in BTBR mice, yet it remains unknown if serotonin neurons themselves are abnormal. In this study, we found that serotonin tissue content and the density of serotonin axons is reduced in the hippocampus of BTBR mice in comparison to C57BL/6J (C57) mice. This was accompanied by possible compensatory changes in serotonin neurons that were most pronounced in regions known to provide innervation to the hippocampus: the caudal dorsal raphe (B6) and the median raphe. These changes included increased numbers of serotonin neurons and hyperactivation of Fos expression. Metrics of serotonin neurons in the rostral 2/3 of the dorsal raphe and serotonin content of the prefrontal cortex were less impacted. Thus, serotonin neurons exhibit region-dependent abnormalities in the BTBR mouse that may contribute to their altered behavioral profile.

Keywords

dorsal raphe; median raphe; hippocampus; monoamine; norepinephrine; SERT; swim

Introduction

Autism Spectrum Disorder (ASD) is an increasingly prevalent neurodevelopmental disorder associated with altered social behavior, impaired communication skills, and increased restricted and repetitive behaviors. The exact cause or causes of ASD are unknown and

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The neurotransmitter serotonin has been implicated in Autism Spectrum Disorders (ASDs). Yet, little is known about exactly how serotonin neurons in the brain might go wrong to contribute to ASD behavior. To understand this better, we studied a particular breed of mouse that is less social than most other mice and also exhibits some repetitive behavior. We measured the amount of serotonin in the mouse's brain and found lower levels in the hippocampus. Perhaps as a compensatory change, there was an increase in the number of serotonin neurons located in the area that projects to the hippocampus. While the hippocampus is best known for its key function in forming memories, it also plays an important role in regulating the response to stress. Therefore, our results raise the possibility that the specific serotonin circuit that influences hippocampal function could be dysfunctional in ASD. The results could support studies in humans to see if similar serotonin circuits could be abnormal, and they could justify studies to examine if manipulating these circuits to be helpful for treatment.

multiple genetic and environmental factors may be involved. Serotonin is one of several neurotransmitters that could underlie some of the behavioral impairments characteristic of ASD. Interest in serotonin originated over 50 years ago with the discovery that elevated blood serotonin levels was common in autistic patients [Schain and Freedman, 1961] (reviewed by Muller, Anacker, and Veenstra-VanderWeele [2016]). However, hyperserotonimia does not consistently correlate with any clinical feature of autism [Mulder et al., 2004], and the presence and nature of a correlative brain serotonin abnormality are still poorly understood.

Mutations in genes that influence monoamine neurotransmission appear sufficient to generate behavioral alterations relevant to ASD [Brunner, Nelen, Breakefield, Ropers, and van Oost, 1993; Piton et al., 2014]. Some evidence suggests that certain rare human variants of the serotonin transporter (SERT) gene *SLC6A4* may be associated with ASD and in particular with the presence of rigid-compulsive behaviors and tactile hypersensitivity [Sutcliffe et al., 2005]. Mice bearing the rare human Ala56 *SLC6A4* allele display altered behavioral performance in social and repetitive behavior, although the severity of these deficits depends on background strain [Kerr et al., 2013; Veenstra-VanderWeele et al., 2012]. Mice lacking the rate-limiting enzyme in brain serotonin synthesis, tryptophan hydroxylase (TPH) 2, have deficits in measures of sociability and cognitive flexibility [Kane et al., 2012; Mosienko et al., 2015]. These observations support the possibility that serotonin could be part of a common neural basis for ASD.

BTBR $T^+ Itpr^{3fl/J}$ (BTBR) mice may be useful tool to understand the neural circuit aberrations that underlie ASD due to their unique behavioral profile. This inbred mouse strain exhibits reduced play and social approach behavior compared to other strains [Bolivar, Walters, & Phoenix, 2007; McFarlane et al., 2008; Moy et al., 2007; Pobbe et al., 2010; Scattoni, Ricceri, & Crawley, 2011; Yang, Zhodzishsky, & Crawley, 2007]. In addition, BTBR mice have evidence of restricted-repetitive behaviors, such as excessive self-grooming and they persevere with active coping strategy in the forced swim test [Onaivi et al., 2011; Silverman et al., 2010]. A previous study examined the potential for brain abnormalities in the serotonin system in BTBR mice and found evidence for reduced SERT, increased 5-HT_{1A} receptor signaling capacity, and altered behavioral responses to 5-HT_{1A} receptor ligands [Gould et al., 2011, 2014]. Serotonergic drugs including fluoxetine and the 5-HT_{1A} partial agonist busperone enhance social interaction in these mice. In addition, risperidone and the selective 5-HT_{2A} antagonist M100907 normalize reversal learning in BTBR mice [Amodeo, Jones, Sweeney, & Ragozzino, 2014].

While the forebrain response to serotonergic ligands and some measures of the endogenous serotonin system are altered in the BTBR mouse, it remains poorly understood if there are developmental, functional, or morphometric aberrations within serotonin neurons themselves. Identification of such abnormalities could provide clues to what could be a common serotonergic circuit deficit present in ASD when generated by different causes. In this study, we asked if serotonin neurons themselves are different in BTBR mice or if deficits are restricted to the forebrain response to serotonin.

Methods and Materials

Mice

Procedures using animals were approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital. Breeding pairs of BTBR and C57BL/6J (C57) mice were obtained from the Jackson Laboratory (stock numbers 002282 and 000664, respectively). Progeny born at Boston Children's Hospital were used as study subjects at postnatal day 28 (± 1 day). Since autism is a disorder that manifests in childhood, we reasoned that this younger age is at least equally relevant to understanding the neural mechanisms of the disorder as older ages, if not more relevant. Postnatal day 28 is thought to represent an early adolescent state in rodent development [Laviola, Macri, Morley-Fletcher, & Adriani, 2003] and major postnatal developmental changes of the serotonin system have largely been completed by this age [Lidov and Molliver, 1982; Liu and Wong-Riley, 2010], although there are likely further refinements of the system into adulthood. For High-Performance Liquid Chromatography (HPLC), there were ten individuals per group. For serotonin neuron number estimates, there were six individuals per group (a subset of the animals used for swim and Fos analysis). For serotonin axon terminal analysis, there were eight individuals per group (a subset of the animals used for swim and Fos). For swim behavior and Fos analyses, there were 16–19 individuals per group. Swim behavior and Fos were analyzed first to determine if there was an effect of sex. Males and females, however, had similar behavioral performance and Fos expression (no significant main effect of sex or sex $\times$ strain interaction in an ANOVA). Therefore, the remaining analyses were sex balanced, but were underpowered for further analysis of sex effects (i.e., for HPLC four to six of each group were male).

HPLC Analysis

To measure the content of monoamines and their metabolites, whole brains were rapidly extracted and snap-frozen in isopentane cooled with dry ice ($n = 10$ per genotype). The prefrontal cortex, dorsal striatum (both medial and lateral), dorsal hippocampus (primarily the dentate gyrus and CA1 subfields), and dorsal raphe (DR) were punched out of serial 300- μm slices cut on a cryostat with a 2 mm diameter punch and analyzed by the Neurochemistry Core Facility at Vanderbilt University. These forebrain areas were selected because they represent termination fields with distinct areas of origin within the raphe nuclei, they were implicated as areas where serotonin neurotransmission may be compromised in BTBR mice, and they could be sampled reproducibly from animal to animal [Amodeo, Jones, Sweeney, & Ragozzino, 2012; Commons, in press; Gould et al., 2011]. For the HPLC, frozen brain tissue samples were homogenized by using a tissue dismembrator (Misonix XL-2000; Qsonica) in a solution containing 100 mM TCA, 10 mM NaC₂H₃O₂, 100 μM EDTA, 5 ng/mL isoproterenol (an internal standard), and 7.5% (vol/vol) methanol (pH 3.8). Using a Waters 2707 autosampler, 10 μL of supernatant from each sample was injected onto a Phenomenex Kintex (2.6 μm , 100 Å) C18 HPLC column (100 $\times$ 4.6 mm). Biogenic amines were eluted with a mobile phase [100 mM TCA, 10 mM NaC₂H₃O₂, 100 μM EDTA, and 7.5% methanol (pH 3.8)] delivered at 0.6 mL/min by using a Waters 515 HPLC pump. Analytes were detected by using an Antec Decade II (oxidation: 0.4) electrochemical detector operated at 33 °C. Empower software was used to manage

HPLC instrument control and data acquisition. Data were analyzed using ANOVA with factors for region, neurochemical, and strain.

Tissue from the same individuals was used for three analyses using immunofluorescence: quantification of serotonin axon density, serotonin neuron number, and Fos expression within serotonin neurons. Since serotonin neurons tend to have very low levels of Fos expression in naïve conditions, these mice were exposed to an acute stress, swim, prior to collecting their brains. Swim is known to activate serotonin neurons and modify serotonin release in the forebrain, which influences the behavior exhibited; thus, it is a stimulus well characterized with respect to serotonin involvement [Cryan, Valentino, & Lucki, 2005]. In addition, autism is associated with poor response to stress and difficulty adapting to change [Spratt et al., 2012] suggesting that the swim test may be a relevant behavioral challenge in autism models. The acute swim was not expected to have an effect on other endpoints (serotonin axon terminal ramification pattern or serotonin neuron number). As Fos was not compared under naïve conditions, there is no assumption that differences between strains would be necessarily contingent on the swim. However, this experimental design resulted in the potential to score swim behavior.

For the swim, mice were placed in a cylindrical glass tank (46 cm high × 20 cm diameter) filled with water (25 ± 1 °C) to a depth of 30 cm for 15 min and swim behavior video-recorded for subsequent behavioral scoring. Mice were perfused 120 min after the start of the acute stress event to allow for the expression and accumulation of the Fos protein. Swim behavior was scored as active (defined by lateral movement from quadrant to quadrant) or inactive (defined by minimal movement) coping every 5 sec throughout the swim test. Immediately after the test, the mice were removed from the tank, dried off, and returned to their home cage.

Mice were perfused transcardially through the ascending aorta with 30 mL 4% paraformaldehyde in 0.1 M phosphate buffer (4 °C, pH 7.4) after they were anesthetized with an intraperitoneal injection of a lethal dose of sodium pentobarbital (100 mg/kg). Brains were removed and stored in the same fixative solution for 48 hr and then equilibrated in a solution of 30% sucrose plus sodium azide in 0.1 M phosphate buffer. Brains were frozen and coronally sectioned at 40 μ m thickness. Sections were processed while floating.

SERT Axon Density Analysis

Immunofluorescence detection of SERT was used to quantify serotonin axon terminal ramification pattern. While some nonserotonin neurons express SERT earlier in development [Gaspar, Cases, and Maroteaux, 2003], after the third week of life, immunolabeling for SERT detects serotonin neuron axons with high sensitivity and specificity [Boylan, Bennett-Clarke, Chiaia, & Rhoades, 2000; Nielsen, Brask, Knudsen, & Aznar, 2006]. To account for potential technical variation in immunofluorescence detection efficiency with different experimental days, tissue from individuals of each genotype were processed side-by-side and analyzed in a matched pair design. Sections were incubated in primary antisera raised in rabbit (SERT, Calbiochem, catalog PC177L) diluted 1:1000 in 0.1 M phosphate-buffered saline (PBS) with 0.1% bovine serum albumin, 0.3% Triton X-100, and 0.01% sodium azide (PBS-BSA-T-A) at 4 °C for 2–3 days. All immunoreactivity was detected using fluorescent

secondary antisera raised in donkey and diluted in PBS-BSA-T-A at room temperature for 90 min.

Images of tissue from each pair of individuals processed together were photographed at the same exposure times and displayed with equivalent saturation curves. Using NIHs Image J software, an equivalent threshold was applied to matched pair images to define axons, and the number of objects defined was measured using Analyze-Measure. Four sections were sampled per individual mouse, and results were averaged to generate a mean density of labeled objects per individual. Values between eight sets of pairs were compared using a paired *t*-test. A paired *t*-test was consistent with our matched pair design, which was used to control for variability in immunofluorescence between technical runs.

Serotonin Neuron Number Estimates

To estimate the number of serotonin neurons, sections throughout the raphe were photographed to detect immunolabeling for TPH (Millipore, AB1541 raised in sheep) and a nuclear stain 4',6-diamidino-2-phenylindole (DAPI) applied in the coverslipping medium (Molecular Probes). Immunolabeling for TPH was completed in a similar fashion to SERT. The raphe nuclei were systematically sampled by analyzing every third section where they were present. Sections were photographed using an Olympus fluorescence microscope with a 10 × objective, a Hamamatsu Orca ER camera, and SlideBook computer software. Three rostrocaudal divisions of the DR were made: rostral was −4.16 to −4.24 mm, middle was −4.36 to −4.84 mm, and caudal was −4.96 to −5.20 mm relative to Bregma. These three divisions were divided in half into dorsal and ventral subregions. The lateral wing of the middle DR was pooled with the dorsal middle DR subregion to the extent to which they appeared in the same images. Images of the median raphe (MR) were sampled in sections that also contained the middle DR.

Using NIH Image J Software, images containing DAPI labeling for nuclei and TPH labeling for serotonin neurons were analyzed. Images were split into separate color channels. For each channel, the background was subtracted and a threshold was applied to define labeled areas. A selection area generated by the area immunolabeled for TPH was applied to the cognate image of DAPI-defined nuclei. The DAPI-defined nuclei within the TPH selection area were counted using Analyze-Measure command. The mean number of DAPI cells per section per region was determined for each mouse strain. Group means and standard error of the means were calculated for each region and analyzed for significant effect of mouse strain using ANOVA. Posthoc comparisons between the two genotypes were completed using a two-tailed Student's *t*-test with alpha set at $P < 0.05$. In areas where a positive result was found, the size of TPH neurons was analyzed by measuring the area of an oval that best fit over the cell body to determine if differences between groups could have been related to differences in the sizes of neurons (larger neurons would be sampled more often, generating the appearance of more neurons). For this analysis, measures were averaged from eight arbitrarily selected neurons/mouse and nine mice were sampled in each group.

Fos immunoreactivity was detected by incubating sections in rabbit anti-Fos sera (EMD Chemicals, PC38) diluted 1:10,000. 5-HT neurons were detected using an antiserum raised against TPH diluted 1:1,000 raised in sheep (Millipore, AB1541). Secondary antisera raised

in donkey with minimal cross reactivity to other species and were conjugated to CY3 or Alexa-488 diluted 1:100 were used to detect immunolabeling.

To quantify Fos labeling, individual mice were coded to conduct the analysis blind to the mouse strain. Within each subregion sampled as described for serotonin neuron number estimates, TPH cells with Fos expression were manually enumerated by visualizing each channel independently and together. Every third section through the DR was processed such that two to three sections were sampled for each subregion/mouse. The number of dually immunolabeled cells was normalized per section for each subregion for each individual mouse within a group. Then, by averaging numbers across mice, group means and standard error of the means were calculated for each subregion. An ANOVA was used to determine if there was a significant effect of strain and subregion. Posthoc determination of differences between the two strains was completed using a two-tailed Student's *t*-test (alpha set at $P < 0.05$).

Results

HPLC

Monoamine content of prefrontal cortex, dorsal striatum, dorsal hippocampus, and DR were assayed. Serotonin levels were significantly reduced in the hippocampus (about a 24% decrease $P = 0.004$), but undistinguishable from C57 in frontal cortex, striatum, and DR (Fig. 1). The major serotonin metabolite, 5-hydroxyindoleacetic acid (5-HIAA), trended lower but nonsignificantly so ($P = 0.564, 0.065, 0.076$, and 0.142 in cortex, striatum, hippocampus and DR, respectively). The ratio of 5-HT to its metabolite was significantly reduced only in the striatum ($P = 0.006$), as a consequence of both slightly higher absolute serotonin and lower 5-HIAA levels. In addition, in BTBR, norepinephrine (NE) was found significantly reduced in the cortex, striatum, and hippocampus (Fig. 1) ($P = 3 \times 10^{-5}, 0.04$, and 3×10^{-10} , respectively). However, in all regions, there was no significant effect of strain on dopamine or any of its metabolites except for 3-methoxytyramine (3-MT) (Fig. 2). 3-MT was elevated significantly in the striatum and DR ($P = 0.005$ and 0.01 , respectively) and nonsignificantly elevated in the hippocampus. While changes in 3-MT might suggest altered function of catechol-o-methyltransferase or monoamine oxidase, other products of these enzymes (homovanillic acid (HVA) and 5-HIAA, respectively) did not show similar changes.

To investigate if there were also changes in serotonin axon ramification in the hippocampus, we analyzed the density of axons immunolabeled for SERT in the CA1 subfield (Fig. 3). Immunofluorescent labeling of SERT axons revealed about a 16% reduction ($P = 0.0007$) in the density of innervation to the hippocampus compared to C57 mice, suggesting at least in part the reduction of serotonin may be caused by fewer axons overall. The possibility that there may also be less serotonin and/or SERT per individual axon was not ruled out.

To evaluate serotonin neurons themselves, we estimated serotonin neuron number in six subregions of the DR and MR. This analysis showed that the caudal ventral region and caudal dorsal region indicated on the Paxinos atlas as “interfascicular DR” and “DR caudal” both of which are also called “B6,” contained more serotonin neurons in BTBR compared to

C57 (Figs. 4 and 5) ($P = 0.0003$ for caudal ventral and $P = 0.01$ for caudal dorsal). In addition, estimates of serotonin neurons in the MR were found elevated in BTBR mice ($P = 0.01$). Meanwhile, serotonin neuron number trended higher in the remaining areas of the DR but was not significantly different from C57 mice. Serotonin neuron size was measured in B6 to confirm that the change in measured density more likely reflected increased neuron number, rather than larger neurons that could be sampled more frequently. This analysis showed that the average size of cell bodies in this region was equivalent to that in C57 mice (cell body area for C57 was $1211 \mu\text{m}^2 \pm 87$ (S.E.M.) vs. $1224 \mu\text{m}^2 \pm 69$ (S.E.M.) for BTBR, $P = 0.87$).

To evaluate if serotonin neurons showed differential activity state in BTBR mice compared to C57, we exposed mice of each genotype to an acute stress, swim, since this stimulus is known to increase the level of activation of the serotonin system. Subsequently, Fos expression specifically within serotonin neurons was measured (Figs. 6 and 7). Consistent with previous observations, we observed that BTBR mice exhibited excessive active coping in the swim compared to C57 [Onaivi et al., 2011; Silverman et al., 2010]. Specifically, over 15 min, the active coping scores (the number of 5-sec epochs rated as active) of BTBR averaged 154 ± 7 (S.E.M.) versus 103 ± 8 (S.E.M.) for C57 ($P = 0.0001$). Analysis of Fos showed that BTBR mice had excessive expression particularly within the same regions that had more serotonin neurons overall including the ventral caudal region of the DR ($P = 3 \times 10^{-7}$), dorsal caudal ($P = 0.02$), and in the MR ($P = 1 \times 10^{-5}$). In the area with the greatest magnitude differences (ventral caudal DR), there was less than a twofold increase in estimates of serotonin neuron number while the increase in Fos was fourfold; suggesting that the increased Fos was both a consequence of the presence of more neurons combined with higher activity state. Significantly higher Fos was also detected in the ventral middle ($P = 0.002$) area.

Discussion

This study extends the findings showing altered forebrain expression of serotonin receptors and response to serotonin ligands [Gould et al., 2011] by providing evidence that BTBR mice have altered serotonin tissue content, axon, and cell body organization, as well as altered activity as measured by Fos expression. Thus, by multiple measures, the endogenous serotonin system in BTBR mice is distinct from C57 mice. Given the potential of serotonin neurotransmission to influence autism-related phenotypic measures, these differences could contribute to altered behavior in this mouse line.

There were increases in the estimates of serotonin neuron number and Fos expression in BTBR mice, particularly in the caudal pole of the DR (B6) as well as in the MR. Previous studies have provided evidence that B6 and MR serotonin neurons may be disturbed in autism models and further that these could contribute to an autism-relevant phenotype. Specifically, another gene associated with autism, *Met*, is highly expressed in B6 and MR neurons in the adult [Okaty et al., 2015; Wu and Levitt, 2013]. Conditional knockout of *Met* within these serotonin neurons results in sociability deficits [Okaty et al., 2015]. Models of autism produced by embryonic exposure to valproic acid (VPA) and thalidomide result in an increase in number of serotonin neurons in B6 [Narita et al., 2002] while maternal viral

exposure results in increased number of serotonin neurons in the entire rostral raphe [Ohkawara, Katsuyama, Ida-Eto, Narita, & Narita, 2015]. These observations converge to raise the possibility that altered function of B6 and MR serotonin neurons may be a common feature of mouse models of autism when generated by (at least this subset) diverse causes.

The B6 region and MR share some features of connectivity and developmental origin (reviewed by Commons [in press]). One target of both these regions is the hippocampus, where serotonin abnormalities were evident in BTBR mice. However, in contrast to the increases in serotonin metrics in B6 and MR, the hippocampus had decreased serotonin content and a trend for altered ratio between serotonin and its metabolite. In addition, there was reduced density of SERT-containing axons. The opposing valence of changes in cell bodies versus terminal fields raises the possibility that they could be compensatory. A reduction in hippocampal serotonin coupled with increased serotonin neuron number is also shared with the VPA and maternal-viral infection models of autism [Dufour-Rainfray et al., 2010; Ohkawara et al., 2015]. Regardless of the origin, the current results confirm and extend the reports of reduced SERT binding in the hippocampus as measured by autoradiography [Gould et al., 2011] and indicate a reduction in axon density contributes to this observation and that both are associated with an overall reduction in serotonin content in BTBR mice.

Several other hippocampal abnormalities have been reported in the BTBR mouse including: age-dependent changes in 5-HT_{1A} receptors [Gould et al., 2014], severely reduced hippocampal commissure [Wahlsten, Metten, & Crabbe, 2003], reduced hippocampal neurogenesis, reduced hippocampal BDNF expression [Stephenson et al., 2011] several altered features of synaptic plasticity [MacPherson, McGaffigan, Wahlsten, & Nguyen, 2008; Seese, Maske, Lynch, & Gall, 2014; Scattoni, Martire, Cartocci, Ferrante, & Ricceri, 2013], and marked transcriptional changes [Daimon et al., 2015]. In fact, the reduction of BDNF could be a causal factor for the loss of serotonin innervation: BDNF is a known trophic factor for serotonin axons and reduction in BDNF expression is associated with loss of hippocampal serotonin innervation [Lyons et al., 1999; Mamounas et al., 2000]. Serotonin feeds back to regulate BDNF expression, likely through 5-HT_{2C} receptors [Hill et al., 2011; Miglierini, Pacini, Pelosi, Lunardi, & Pasqualetti, 2013] although the status of this receptor has not been investigated yet in BTBR mice.

BTBR mice showed altered behavioral coping strategies when faced with an acute stress, swim, replicating previous reports [Onaivi et al., 2011; Silverman et al., 2010]. The swim test is known to change the activity state of serotonin neurons [Commons, 2008] producing region-dependent changes in serotonin release in the forebrain [Kirby and Lucki, 1997] and there is an extensive literature on serotonin pharmacology in swim (reviewed by Cryan et al. [2005]) making the swim test a relevant approach to gain insight into the serotonin system. Interpretation of this behavioral performance with respect to depression in mouse models of autism is not trivial however. In humans, the diagnosis of depression in autism can be complicated and its prevalence is not agreed upon (reviewed by Chandrasekhar and Sikich [2015]). In rodents, the factors (emotional, motivational, and cognitive) that drive coping strategies under stress are unknown and they could be complex. Other behavioral features present in autism, and indeed present in BTBR mice, could be expected to influence stress-

extracellular NE in the BTBR mice [Stapley et al., 2013]. In this feature, BTBR mice resemble the Engrailed-2 knock-out (EN2KO) mice, another model of autism, that likewise have deficits in NE in some fore-brain areas (frontal cortex and hippocampus), as well as deficits in fear conditioning that are restored by a NE reuptake inhibitor [Briellmaier et al., 2014]. However, EN2KO and BTBR differ in several other respects including regional patterns of monoamine alterations and behavioral performance in the swim [Briellmaier et al., 2014; Genestine et al., 2015].

Conclusion

This study finds evidence for alterations intrinsic to serotonin neurons in BTBR mice, an inbred strain with behavioral deficits relevant to ASD. Some of these changes are similar to those that occur in other animal models of ASD, which suggest dysfunction of specific subset of serotonin circuits could be common to a subset of ASD cases. Specifically, neuronal function of the caudal pole of the DR (B6) and the MR as well as their targets including the hippocampus may be of interest.

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Figure 1.

HPLC analysis of serotonin (5-hydroxytryptamine, 5-HT), the serotonin metabolite 5-HIAA, their ratio, and NE in frontal cortex, dorsal striatum (STR), the hippocampal formation (HF), and the dorsal raphe (DR) in C57 (grey) and BTBR (black) mice. **(A)** BTBR mice have significantly less serotonin in the HF $p = 0.004$, **(B)** nonsignificant trends for lower 5-HIAA in several regions, and **(C)** a reduced ratio of metabolite to transmitter in the striatum ($P = 0.006$). **(D)** In addition, BTBR mice have significantly reduced NE content in all the forebrain areas examined ($P = 3 \times 10^{-5}$, 0.04, and 3×10^{-10} , respectively). $N = 10$ per group.

Figure 2.

(A) Dopamine (DA) and its metabolites, (B) 3,4-dihydroxyphenylacetic acid and (C) HVA were not significantly different between C57 (grey) and BTBR mice (black) in any area. (D) However, in BTBR mice, there was significantly greater 3-methoxytyramine (3-MT) both in the STR and DR ($P = 0.005$ and 0.01 , respectively). $N = 10$ per group.

Figure 3.

Comparison of axons containing the serotonin reuptake transporter (SERT) in C57 (**A**) and BTBR (**B**) mice. Images are of comparable fields in the CA1 subfield of the hippocampus including strata pyramidale (P) radiatum (R) and lacunosum-moleculare (L-M). (**C**) SERT axon density in BTBR mice is about 16% less than in pair-matched controls, ($P = 0.0007$) $N = 8$ per group. Bar = 150 μm .

Figure 4.

Per section estimates of serotonin (5-HT) neurons identified by immunolabeling for TPH showed BTBR mice have an increased number of neurons in the MR ($P = 0.01$) and caudal third of the DR, both ventrally and dorsally in the areas also known as B6 ($P = 0.0003$ for caudal ventral and $p = 0.01$ for caudal dorsal). $N = 6$ per group.

Figure 5.

Comparison of the morphology of B6 in C57 and BTBR. **A1-4** shows the same level from four different C57 mice and matched below (**B1-4**) are comparable images from 4 BTBR mice. Ventrally, the difference in cell number can be detected visually in that C57 mice have regions where neurons are sparse (**A** row, arrows) whereas in BTBR two paramedial columns of 5-HT neurons (identified by immunolabeling for TPH) are consistently visible (**B** row, arrows). Bar = 100 μ m.

Figure 6.

Measured after swim, BTBR mice have significantly more 5-HT neurons with activated expression of Fos. The largest magnitude and most statistically significant effects occurring in the MR and caudal ventral part of the DR. 5-HT neurons here identified by immunolabeling for TPH. ** $P < 0.0005$. Significant increases are also detected caudal dorsal and in middle levels both dorsal and ventral (* $P < 0.05$). $N = 16-19$ per group.

Figure 7.

Example images of the caudal ventral DR showing BTBR mice had more 5-HT neurons with Fos-immunolabeled nuclei. Same sections as in Figure 5. **(A1-4)** Same level for four different C57 mice showing 5-HT neurons (green; identified by immunolabeling for TPH) and Fos (magenta) immunolabeling together. **(B1-4)** Comparable images from four different BTBR mice. **(C1-4)** Same images as in A1-4 showing Fos-immunolabeling alone, arrows point to examples of positive Fos immunolabeling within the nucleus a serotonin neuron. **(D1-4)** Same images as in B1-4 showing Fos only. Some of the dually labeled neurons are indicated with arrows, and these are much more prevalent in BTBR than in C57 (quantification in Fig. 6). All panels same scale; bar in A1 = 100 μm .