SEROTONIN: FROM MOUSE MODELS TO HUMAN DISORDERS

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ABSTRACT

Autism is an increasingly common neurodevelopmental disorder characterized by deficits in social interactions, impaired communication, and repetitive and stereotyped behaviors. While the etiology of autism is unknown, several lines of evidence implicate dysfunction of the serotonin (5-HT) system in the anatomical and behavioral features of the disorder. Work reported in this series investigated components of the 5-HT system in animal models of autism as well as in post-mortem human brain tissue in an attempt to clarify the role of 5-HT in this disorder. The activity of the central 5-HT system was investigated in the BTBR T+Itpr3(−)/J (BTBR) mouse, a strain that displays behaviors consistent with all three diagnostic categories for autism. In comparison to the common C57BL/6J (B6) control strain, BTBR mice showed a pattern of behaviors characterized by reduced frontal orientations in social interactions and altered cortical and cerebellar concentrations of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). 5-HT concentrations may be regulated by the serotonin transporter (5-HTT) gene (SLC6A4) and variants in this gene are implicated in autism. Mice with targeted disruptions of SLC6A4 displayed increased stereotyped grooming that is consistent with one of the core symptoms of autism. However, social, aggressive, and communicative behaviors were not altered in SLC6A4 knockout mice. The research reported here also described altered structural and molecular composition in the subventricular zone (SVZ) of individuals with autism. 5-HTT immunoreactivity was significantly increased in the SVZ of autism-diagnosed (AD) individuals when compared to typically-developing controls. Furthermore, structural features of the SVZ in AD cases were suggestive of altered neurogenesis and cellular migration; consistent with the neurodevelopmental nature of this disorder. Collectively, these results provide strong support for the role of serotonergic system dysfunction in the anatomical and behavioral traits characteristic of autism.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>PREFACE</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Autism Spectrum Disorders</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Etiology of ASD</td>
<td>1</td>
</tr>
<tr>
<td>1.3. Neuropathology of Autism</td>
<td>2</td>
</tr>
<tr>
<td>1.4. Serotonin</td>
<td>5</td>
</tr>
<tr>
<td>1.5. Serotonin in Neurodevelopment</td>
<td>6</td>
</tr>
<tr>
<td>1.6. Serotonin in Autism</td>
<td>8</td>
</tr>
<tr>
<td>1.7. Dissertation Overview</td>
<td>11</td>
</tr>
<tr>
<td>1.8. References</td>
<td>13</td>
</tr>
<tr>
<td>CHAPTER 2. NEUROCHEMICAL CHARACTERIZATION OF THE BTBR Tpr3/J MOUSE MODEL FOR AUTISM</td>
<td>31</td>
</tr>
<tr>
<td>2.1. Abstract</td>
<td>31</td>
</tr>
<tr>
<td>2.2. Introduction</td>
<td>33</td>
</tr>
<tr>
<td>2.3. Materials and Methods</td>
<td>35</td>
</tr>
<tr>
<td>2.3.1. Subjects</td>
<td>35</td>
</tr>
<tr>
<td>2.3.2. Apparatus</td>
<td>35</td>
</tr>
<tr>
<td>2.3.3. Procedure</td>
<td>35</td>
</tr>
<tr>
<td>2.3.4. Statistical Analyses</td>
<td>38</td>
</tr>
<tr>
<td>2.4. Results</td>
<td>38</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 2.1. Regional baseline concentrations of monoamines, metabolites, and monoamine turnover for BTBR and B6 mice.........................................................40

Table 2.2. Regional concentrations of monoamines, metabolites and monoamine turnover collapsed across nonsocial and social conditions.................................44

Table 3.1. Statistical results for behaviors run in the VBS ..................................73

Table 4.1. Diagnostic characteristics of autism cases..............................................98

Table 4.2. Antibodies...............................................................................................101

Table 4.3. Postmortem information on autism-diagnosed and typically-developing controls .................................................................................................108
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure Number</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distribution of the major serotonergic pathways in the central nervous system</td>
<td>6</td>
</tr>
<tr>
<td>2.1</td>
<td>BTBR mice in the social proximity test</td>
<td>37</td>
</tr>
<tr>
<td>2.2</td>
<td>Frequency of behaviors in the social proximity test</td>
<td>41</td>
</tr>
<tr>
<td>2.3</td>
<td>Concentrations of NE in the medial prefrontal cortex in the proximity</td>
<td>45</td>
</tr>
<tr>
<td>2.4</td>
<td>Concentrations of MHPG in the cortex in the proximity conditions</td>
<td>45</td>
</tr>
<tr>
<td>2.5</td>
<td>Concentrations of DOPAC:DA in the hypothalamus in the proximity conditions</td>
<td>45</td>
</tr>
<tr>
<td>3.1</td>
<td>Frequency of social behaviors during the light phase of the VBS</td>
<td>74</td>
</tr>
<tr>
<td>3.2</td>
<td>Locomotion in the three chamber test</td>
<td>76</td>
</tr>
<tr>
<td>3.3</td>
<td>Time spent in each chamber in the three chamber test</td>
<td>76</td>
</tr>
<tr>
<td>3.4</td>
<td>Behaviors displayed in the autogrooming test</td>
<td>78</td>
</tr>
<tr>
<td>3.5</td>
<td>Behaviors displayed in the social proximity test</td>
<td>79</td>
</tr>
<tr>
<td>3.6</td>
<td>Behaviors displayed in the repetitive novel object contact test</td>
<td>81</td>
</tr>
<tr>
<td>3.7</td>
<td>Scent marking behavior</td>
<td>82</td>
</tr>
<tr>
<td>3.8</td>
<td>Behaviors displayed in the resident intruder test</td>
<td>83</td>
</tr>
<tr>
<td>4.1</td>
<td>Tissue acquisition and histology</td>
<td>99</td>
</tr>
<tr>
<td>4.2</td>
<td>Sample preparation for immunohistochemical staining</td>
<td>103</td>
</tr>
<tr>
<td>4.3</td>
<td>Layered cellular organization of the human SVZ</td>
<td>109</td>
</tr>
<tr>
<td>4.4</td>
<td>Hypocellular gap widths in the AD and TD SVZ</td>
<td>111</td>
</tr>
<tr>
<td>4.5</td>
<td>Hypocellular gap density in the AD and TD SVZ</td>
<td>112</td>
</tr>
<tr>
<td>4.6</td>
<td>Area Fraction of 5-HTT in the AD and TD SVZ</td>
<td>114</td>
</tr>
</tbody>
</table>
Figure 4.7. Area fraction of FGF-2 in the AD and TD SVZ .............................................116
Figure 4.8. Area fraction of GFAP in the AD and TD SVZ ............................................118
Figure 4.9. NeuN staining of the SVZ ..........................................................................120
Figure 4.10. IBA-1 staining of the corpus callosum in autism ................................ ....121
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HIAA</td>
<td>5-Hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin (5-hydroxytryptamine)</td>
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<tr>
<td>5-HTT/SERT</td>
<td>Serotonin transporter</td>
</tr>
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<td>5-HTTLPR</td>
<td>Serotonin transporter gene linked polymorphic region</td>
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<tr>
<td>AD</td>
<td>Autism-diagnosed</td>
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<td>ASD</td>
<td>Autism spectrum disorder</td>
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<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>BTBR</td>
<td>BTBR $T+Itrp3^{+/J}$</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>DA</td>
<td>Dopamine</td>
</tr>
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<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
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<tr>
<td>FGF-2</td>
<td>Fibroblast growth factor 2</td>
</tr>
<tr>
<td>HET</td>
<td>Heterozygous</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
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<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>MHPG</td>
<td>3-Methoxy-4-hydroxyphenylglycol</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural precursor cell</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
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<td>PC</td>
<td>Purkinje cell</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PMI</td>
<td>Postmortem interval</td>
</tr>
<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>Serotonin transporter gene</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TD</td>
<td>Typically-developing</td>
</tr>
<tr>
<td>VBS</td>
<td>Visible burrow system</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
PREFACE

This dissertation has been formatted to allow for the separate publication of Chapters 2, 3, and 4. Chapter 2 titled "Neurochemical characterization of the BTBR $T+I^{pr}3^{d}/J$ mouse model for autism" and Chapter 3 titled "Behavioral characterization of the serotonin transporter knockout mouse model for autism" have been prepared for submission to the journal *Behavioral Brain Research.*
1.1. Autism Spectrum Disorders

Autism Spectrum Disorders (ASD) are a group of complex and heterogeneous neurodevelopmental disorders that includes autism, Asperger's syndrome, Rhett syndrome, childhood disintegrative disorders, and pervasive developmental disorders not otherwise specified (American Psychiatric Association, 2013). Disorders within this spectrum are characterized by social deficits, impaired communication and stereotyped and repetitive behaviors. In addition to these core features, a number of associated conditions including enhanced anxiety and responsivity to sensory stimuli (Green and Ben-Sasson, 2010), as well as gastrointestinal and sleep dysfunction (Buckley et al., 2010; Mazurek et al., 2013) are frequently observed. These conditions are pervasive, life-long and carry significant interpersonal, societal and economic costs.

Autism Spectrum Disorders are reported in all ethnic and socioeconomic groups (Baio, 2012) and are estimated to afflict 1 in 50 children; making ASDs the most frequently diagnosed developmental disorders in the United States (Blumberg et al., 2013). Despite improved awareness and behavioral interventions, prognoses for ASD patients are highly variable. Significant functional deficits typically persist into adulthood (Howlin et al., 2004; Billstedt et al., 2005; Farley et al., 2009) and consequently, individuals with an ASD often require lifetime support (Hume et al., 2009). Families of children with an ASD report increased stress as well as decreased marital satisfaction and reduced income compared to families with typically developing children or children with other developmental disorders (Karst and Van Hecke, 2012). The lifetime costs per capita associated with autism, the most common ASD, exceed $3 million dollars in the U.S. alone (Ganz, 2007). The significant personal, societal and economic consequences of these disorders highlight the need for improved understanding of their causes, prevention and effective treatment.

1.2. Etiology of ASD

There is no known singular cause of ASD. Rather, these disorders are likely the consequence of complex interactions between a number of genetic, epigenetic, and environmental factors (Bale et al., 2010; Lord, 2011). ASDs are among the most genetically determined of all neuropsychiatric disorders with an estimated heritability of
over 90% (Bailey et al., 1995). Concordance rates range from 3%-14% in siblings (Constantino et al., 2010; Ozonoff et al., 2011) and are highest (75%-95%) among monozygotic twins (Hallmayer et al., 2011; Ronald and Hoekstra, 2011). Moreover, higher rates of autism relevant phenotypes have been reported in first-degree relatives of patients with autism (Piven et al., 1997b; Murphy et al., 2000). The high concordance of the disorders has been linked to mutations and variations in a number of candidate genes (International Molecular Genetic Study of Autism Consortium, 2001; Persico and Bourgeron, 2006; Geschwind and Levitt, 2007; Hussman et al., 2011). The X chromosome in particular, has received considerable attention given the 4:1 prevalence of ASDs in males compared to females (Baron-Cohen et al., 2011). However, less than 20% of all autism cases can be accounted for by these known genetic variants and mutations (Abrahams and Geschwind, 2008). Additionally, whole-genome scans suggest that interactions between a large number of genes (>10) likely contribute to the disorders (Muhle et al., 2004).

There is also evidence for environmental contributions to ASD diagnoses (Chaste and Leboyer, 2012). Several prenatal factors have been associated with these disorders, including maternal viral infection (Atladóttir et al., 2010; Patterson, 2011), advanced paternal age (Reichenberg A, 2006; Croen et al., 2007), and maternal medication use (Croen et al., 2011). Genetic susceptibility and environmental influences may interact to contribute to the heterogeneity of ASD phenotypes. Promising new research showed that possessing the low expressing version of the serotonin transporter gene (SLC6A4) interacted with maternal smoking during pregnancy, increasing problems in social interaction, and also interacted with low birth weight, increasing rigid behaviors in offspring (Nijmeijer et al., 2010).

1.3. Neuropathology of Autism

Despite the lack of a clear biological cause, a number of specific brain pathologies have been described in autism. Clinical, neuroimaging, neurochemical, and neuropathological studies consistently report findings suggestive of disrupted brain development and organization; such as accelerated brain growth (Courchesne et al., 2007), structural abnormalities (Bauman and Kemper, 2005; Amaral et al., 2008), and atypical structural and functional connectivity between brain regions (Amaral et al.,
3

2008; Ecker et al., 2013b). These dysfunctions are hypothesized to alter information processing in the brains of autistic individuals and contribute to the social, communicative, and motor symptoms associated with this disorder (Persico and Bourgeron, 2006; Hussman et al., 2011; Ecker et al., 2013b).

One of the most consistent findings in autism is altered brain growth. Children with autism frequently undergo a period of early postnatal brain overgrowth (Courchesne et al., 2001; Hazlett et al., 2005, 2012; Amaral et al., 2008) and subsequent arrest of growth (Courchesne et al., 2001, 2011a) coinciding with the onset of behavioral symptoms. Head circumference (Courchesne et al., 2003), brain volume (Courchesne et al., 2001; Palmen et al., 2004a) and brain mass (Palmen et al., 2004b; Redcay and Courchesne, 2005) are larger in 2-5 year-old autistic children when compared to typically developing controls of the same age. These volume differences diminish after age 5 and are negligible by adulthood (Courchesne et al., 2007). Larger than average brains have also been reported in first-degree relatives of autistic individuals (Woodhouse et al., 1996; Fidler et al., 2000). The molecular and cellular contributions to this overgrowth are unknown, however, excessive neurogenesis, disturbed migration, abnormal apoptosis, and disrupted synaptogenesis have been proposed as potential mechanisms (McCaffery and Deutsch, 2005; Persico and Bourgeron, 2006; Courchesne et al., 2007; Vaccarino et al., 2009). Consistent with these hypotheses, increased neurons (Courchesne et al., 2011b), cell density (Casanova et al., 2002, 2006), microglia-neuron clustering (Morgan et al., 2012), and neurogenesis (Wegiel et al., 2010; Kotagiri et al., 2013; Pearson et al., 2013) have all been reported in the postmortem brains of autistic individuals.

Structural abnormalities are consistently described for the cortex and cerebellum in autism; suggesting disturbances in neuronal migration and organization. In some patients cortical minicolumns are increased in number and narrower in width, with reduced neuropil space and smaller neuron bodies (Bailey et al., 1998; Casanova et al., 2002, 2006; Wegiel et al., 2010). These minicolumn disruptions are thought to contribute to the increased cortical gyrification (Wallace et al., 2013) and thickness (Ecker et al., 2013a) as well as the attentional and sensory-discrimination deficits in autism (Opris and Casanova, 2014). The most consistent neuropathologies of the cerebellum are hypoplasia and reduced cerebellar Purkinje cells (PC) (Piven et al., 1997c; Fatemi et al., 2002;
Palmen et al., 2004b; Wegiel et al., 2010, 2013). A recent study suggests that cerebellar
dysplasia and PC cell loss are associated with the atypical gaze that is a characteristic
feature of autism (Wegiel et al., 2013). Taken together, these observations suggest that
developmental disturbances in neural migration and organization may contribute to at
least some of the neuropathological and clinical features of autism.

In addition to the reported morphological abnormalities, there is evidence for
altered structural and functional connectivity in the brains of autistic individuals
(Belmonte et al., 2004a, 2004b; Courchesne and Pierce, 2005; Geschwind and Levitt,
2007; Rudie et al., 2012). Neuroimaging studies report structural differences in white-
matter volume (Boddaert et al., 2004; McAlonan et al., 2005), microstructural integrity
(Alexander et al., 2007; Catani et al., 2008), and atypical connectivity between brain
regions (Just et al., 2007; Pugliese et al., 2009). Consistent reports of reduced corpus
callosum volumes (Egaas et al., 1995; Piven et al., 1997a) are indicative of reduced
intra-hemispheric connectivity (Anderson et al., 2011) and are suggested to contribute to
behavioral features of autism. For example, smaller corpus callosum volumes have been
associated with lower intelligence quotient scores (Alexander et al., 2007) and greater
symptom severity (Hong et al., 2011) in individuals with autism.

Functional imaging of the brains of autistic individuals during tasks of working
memory (Koshino et al., 2005, 2008), executive function (Just et al., 2007), as well as
language (Just et al., 2004) and emotion processing (Kleinhans et al., 2008) reveal
abnormal patterns of regional activation and synchronization. In particular, dysfunction
of the cerebello-thalamo-cortical circuit is thought to contribute to the cognitive and
affective symptoms characteristic of autism (Hoppenbrouwers et al., 2008). Functional
magnetic resonance imaging (fMRI) of high functioning autistic children revealed
reduced cerebellar activation with increased cortical activation in a simple motor task
when compared to typically developing controls (Mostofsky et al., 2009). An additional
fMRI study found that cerebellar activation was reduced while thalamo-cortical
activation was increased in high functioning autistic individuals during a sensorimotor
control task (Takarae et al., 2007).

Several lines of research suggest that neuronal communication is disturbed in
autism. Genes known to regulate neurotransmitter function (Sutcliffe et al., 2005;
Coutinho et al., 2007), axonal and dendritic growth (Gilman et al., 2011), and synaptogenesis (Toro et al., 2010; Hussman et al., 2011) have been implicated in the disorder. Biomarker analyses (Cook and Leventhal, 1996), neuroimaging investigations (Chugani et al., 1997, 1999) and pharmacological manipulations (Volkmar, 2001) implicate abnormalities in several neurotransmitter systems. Furthermore, in rodent studies, early disruptions to monoamine neurotransmitter systems, especially serotonin (5-HT) and dopamine (DA), result in anatomical and behavioral features relevant to autism (Kahne et al., 2002).

Collectively, these findings highlight the neurodevelopmental nature of autism-associated pathology. Evidence of altered neuronal development (Wegiel et al., 2010), irregularities in brain cytoarchitecture (Piven et al., 1997c; Casanova et al., 2002), disordered interregional connectivity (Egaas et al., 1995; Boersma et al., 2013), and disturbed neurotransmission (Lam et al., 2006) suggest enduring changes in the organization, connectivity and communication of the autism brain (Belmonte et al., 2004b; Pardo and Eberhart, 2007). However, the cellular and molecular mechanisms that drive these anomalies remain unknown. Therefore, studies of the underlying mechanisms that contribute to altered neurodevelopment in autism are needed.

1.4. Serotonin

Serotonin (5-HT) is a phylogenetically ancient and highly conserved monoamine neurotransmitter. 5-HT is found in nearly every organ of the body and in virtually every living organism (Müller and Jacobs, 2010). In the central nervous system (CNS), 5-HT synthesizing cells originate in the raphe nucleus of the brainstem and provide a dense innervation to all regions of the brain (Jacobs and Azmitia, 1992) (Figure 1). 5-HT neurons also project into the walls of the ventricles, forming a dense supra- and subependymal plexus (Richards et al., 1981; Jahanshahi et al., 2011). 5-HT affects numerous biological processes including appetite, circadian rhythms, sensory processing, cognition, motor system function, emotion, and reward processing (Lucki, 1998). Disturbed 5-HT function has been implicated in a variety of neuropsychiatric conditions including depression, anxiety disorders, obsessive compulsive disorders, and autism (Cook and Leventhal, 1996; Elhwuegi, 2004; Hart et al., 2010).
1.5. Serotonin in Neurodevelopment

5-HT is one of the earliest developing and most widely distributed neurotransmitters in the mammalian CNS (Sodhi and Sanders-Bush, 2004). In the human brain, neurons expressing 5-HT appear by the fifth week of gestation (Sundström et al., 1993), grow rapidly until the 10th week (Shen et al., 1989; Kontur et al., 1993; Levallois et al., 1993) and assume the typical adult organization in the raphe nuclei by week 20 (Kinney et al., 2007). Neurons expressing 5-HT innervate the cortical subplate around 10 weeks post gestation (Verney et al., 2002), during a highly active period of neural proliferation and migration (de Graaf-Peters and Hadders-Algra, 2006). 5-HT synthesis,
receptors and activity increase significantly during the first two years after birth and then decline to adult levels after the age of five (Hedner et al., 1986; Tóth and Fekete, 1986; Chugani et al., 1999); corresponding with peak periods of neural development and synaptogenesis (Chugani et al., 1987, 1999; Chugani, 1998).

5-HT modulates important neurodevelopmental processes such as: cell proliferation and apoptosis, as well as neuronal migration, maturation, and synaptogenesis (Azmitia, 2001; Pino et al., 2004; Sodhi and Sanders-Bush, 2004; Daubert and Condron, 2010). Collectively, these processes determine brain cytoarchitecture and connectivity (Lesch and Waider, 2012). Consequently, disturbances to 5-HT during development may produce enduring changes in brain structure and function and contribute to the pathology of neurodevelopmental disorders such as autism.

5-HT acts as a mitogen on a number of cell types. Increasing 5-HT during fetal development stimulates the proliferation of neural precursor cells (NPC) (Peng et al., 2013) and glial cells (Tajuddin et al., 2003). Excess 5-HT also can also produce morphological changes similar to those seen in autism, such as increased cortical cell density and thickness (Altamura et al., 2007). Conversely, early depletion of 5-HT reduces neurogenesis (Lauder and Krebs, 1976), gliogenesis (Khozhai, 2006), and embryonic brain size (Holson et al., 1994). Neurogenesis persists in two discrete regions in the adult brain, the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) (Ming and Song, 2005). 5-HT stimulates neurogenesis in both the SVZ and SGZ (Banasr et al., 2004) and protects against cell death by reducing apoptosis in the developing (Persico et al., 2003; Wang et al., 2011) and adult (Liu et al., 2011) brain. Thus, 5-HT has been suggested to play a role in both the development and life-long plasticity of the brain.

During later stages of development 5-HT regulates the migration and differentiation of neurons. High concentrations of 5-HT decrease the migratory speed of various cortical neurons (Ricchio et al., 2008, 2011) and cause malformations of cortical minicolumns (Janušonis et al., 2004) similar to those reported in autism (Casanova et al., 2002, 2006). 5-HT activity is also critical for directing axonal development and dendritic growth; including the outgrowth of 5-HT producing neurons themselves. Excessive amounts of 5-HT disrupt the development of axons in the primary somatosensory cortex.
(Cases et al., 1996) and primary visual cortex (Upton et al., 1999, 2002). Excess extracellular 5-HT, as a consequence of serotonin transporter (5-HTT) ablation, increased the number of 5-HT projections from the median raphe to the medial prefrontal cortex and decreased the number of corpus callosum projection neurons (Witteveen et al., 2013). 5-HT activity also regulates the growth (Mazer et al., 1997), complexity (González-Burgos et al., 1996; Vitalis et al., 2007) and pruning (González et al., 2008) of dendrites. Specifically, 5-HT has been shown to modulate the development (Oostland et al., 2013) and arborization (Kondoh et al., 2004) of cerebellar PCs; which are reduced in the brains of autistic individuals (Palmen et al., 2004b; Bauman and Kemper, 2005; Wegiel et al., 2010, 2013).

1.6. Serotonin in Autism

Due to its involvement in the regulation of a diverse number of physiological and emotional functions, dysfunction of 5-HT neurotransmission has been implicated in a number of psychopathologies, including autism. One of the most consistently reported findings in the disorder is a 50-70% increase in platelet concentrations of 5-HT. This hyperserotonemia is observed in one third of people with autism (Cook and Leventhal, 1996; Lam et al., 2006) and their first degree relatives (Kuperman et al., 1987; Cook Jr. et al., 1988; Abramson et al., 1989; Leboyer et al., 1999). Urinary concentrations of 5-HT are also increased in the disorder (Barthelemy et al., 1988; Martineau et al., 1992). The significance of these peripheral measures however, remains unclear. Attempts to correlate blood concentrations of 5-HT to autism symptoms have yielded inconsistent findings (Kuperman et al., 1987; Mulder et al., 2004; Kolevzon et al., 2010).

A number of genes related to 5-HT homeostasis and function have been implicated in autism. 5-HT homeostasis is maintained by 5-HTT, encoded by the gene SLC6A4 (Kalueff et al., 2010). Polymorphisms of the promoter region for SLC6A4 result in altered 5-HTT expression and 5-HT concentrations (Lesch et al., 1996) and have been associated with autism susceptibility (Cook and Leventhal, 1996; Tordjman et al., 1997; Kim et al., 2002; Devlin et al., 2005; Sutcliffe et al., 2005). Furthermore, these polymorphisms are associated with increased cortical gray matter volume (Wassink et al., 2007), increased amygdala activity (Wiggins et al., 2013) more severe communication deficits (Brune et al., 2006) and hyperserotonemia (Coutinho et al., 2004, 2007) in some
individuals with autism. The gene TPH2 encodes for tryptophan hydroxylase-2 the rate limiting enzyme for 5-HT synthesis in the CNS (Müller and Jacobs, 2010). Single nucleotide polymorphisms in TPH2 are associated with autism in Caucasian (Coon et al., 2005) and Korean (Yang et al., 2012) populations. Variants in MAO A, the gene for monoamine oxidase A, an enzyme responsible for 5-HT metabolism (Müller and Jacobs, 2010), are associated with an increased risk of autism (Yoo et al., 2009; Cheng et al., 2010; Tassone et al., 2011), as well as more severe symptom expression in individuals with autism (Roohi et al., 2009; Cohen et al., 2011). A recent study reported reduced expression of TPH2 and MAO A in cultured cell lines from individuals with autism (Boccuto et al., 2013). Additionally, several 5-HT receptor genes have also been associated with autism (Zafeiriou et al., 2009).

Additional evidence for the involvement of the 5-HT system in autism comes from drug treatment and challenge studies. Drugs that increase 5-HT, such as selective serotonin reuptake inhibitors (SSRIs) effectively alleviate anxiety, aggression and stereotypies in adults with autism (Cook and Leventhal, 1996). However the efficacy of these drugs in the treatment of children is debated (Volkmar, 2001). In adults, reducing 5-HT by tryptophan depletion exacerbates autism symptoms (McDougle et al., 1996). Prenatal exposure to compounds that increase 5-HT levels such as antidepressants (Croen et al., 2011; Rai et al., 2013), cocaine (Davis et al., 1992) and alcohol (Nanson, 1992; Aronson et al., 1997) increase the risk for autism. These compounds can cross the placenta (Rampono et al., 2009) or be excreted in the breast milk (Sie et al., 2012) and alter fetal brain development (Mulder et al., 2011) and infant behavior (Casper et al., 2003; Oberlander et al., 2005).

Neuroimaging studies suggest that 5-HT synthesis, concentrations and binding activity are reduced in autism. Developmentally, whole brain concentrations of 5-HT synthesis are altered in autism (Chugani et al., 1999). A positron-emission tomography (PET) study showed that typically developing children synthesized high concentrations of 5-HT that peaked around age 5 and then declined to adult values. Autistic children did not show this pattern, but rather had lower 5-HT synthesis capacity which increased slightly with age. Additional studies by this group showed that a subset of children with autism had asymmetries in cortical, thalamic, and cerebellar 5-HT synthesis (Chugani et
that correlated with the degree of language impairment and handedness (Chandana et al., 2005). 5-HTT binding is also reportedly reduced in both children (Makkonen et al., 2008) and adults (Nakamura et al., 2010) with autism. Reduced 5-HT2 binding has been noted in high functioning autistic adults (Murphy et al., 2006; Beversdorf et al., 2012), and in the parents of children with autism (Goldberg et al., 2009). Collectively these neuroimaging studies provide strong support for altered serotonergic neurotransmission in individuals with autism, as well as their family members.

Postmortem analyses of 5-HT in the autistic brain have only recently been reported. Contrary to the imaging studies mentioned, investigations of neurons expressing 5-HT, labeled with 5-HTT antibodies, are reportedly thicker and denser in the brains from autistic donors compared to those from controls (Azmitia et al., 2011b; Wegiel et al., 2013). Regions of increased neurons expressing 5-HTT include the amygdala, hippocampus, cortex (Alzmitia et al., 2011a) and cerebellum (Wegiel et al., 2013) of autism donors. Reports of increased 5-HTT expression do not necessarily conflict with reports of reduced 5-HTT binding. Although still reportedly increased, Azmitia's group noted that starting as young as age 12, the neurons expressing 5-HTT in the brains of autistic individuals took on a degenerative morphology (2011a, 2011b). The authors suggest that these dysmorphic 5-HT neurons may reflect degenerating neurons and consequently a loss of transporter function. Therefore, it is possible that the 5-HT system imbalance observed in autism reflects developmental over stimulation followed by degeneration in adolescence.

5-HT plays a critical role in the development of the brain and regulates many of the processes thought to be altered in ASDs, including, neurogenesis, apoptosis, cell migration and synaptic plasticity (Pino et al., 2004). It has been suggested that developmental disruptions to the 5-HT system, such as early exposure to elevated 5-HT concentrations, may down-regulate central 5-HT circuitry and activity in the developing brain (Whitaker-Azmitia, 2005) and contribute to the anatomical, behavioral and emotional disturbances reported in autism (Zafeiriou et al., 2009). Pharmacologically or genetically increasing neonatal 5-HT in rodents produces morphological and behavioral pathologies consistent with autism (Kahne et al., 2002; McNamara et al., 2008; Moy et
al., 2009). For example, rodents exposed to high concentrations of 5-HT had thinner corpus callosums (Bortolato et al., 2013; van der Marel et al., 2013), increased dendritic arborization of prefrontal cortical neurons (Wellman et al., 2007; Bortolato et al., 2013), and reduced numbers of cerebellar PC cells (Bortolato et al., 2013) when compared to controls. Behaviorally, rodents exposed to high 5-HT concentrations showed reduced juvenile play (Homberg et al., 2007; Simpson et al., 2011), reduced distress vocalizations (Kahne et al., 2002), and reduced social interactions as adults (Overstreet et al., 2000; Moy et al., 2009; Bortolato et al., 2013). Collectively the available data provides strong evidence for a role in disruptions to 5-HT homeostasis in the neuropathology and behavioral deficits of autism.

1.7. Dissertation Overview:

Autism is a disorder of altered brain development. 5-HT is a critical regulator of brain development and dysfunctional 5-HT signaling is associated with many of the neuroanatomical and behavioral symptoms of autism. Therefore, the aim of the current studies was to investigate the role of 5-HT in experimental and clinical models of autism.

Chapter 2 quantifies brain monoamine neurotransmitter concentrations, especially 5-HT, in the BTBR Itpr3<sup>tf</sup>/J (BTBR) mouse, a strain which displays behaviors resembling all three diagnostic symptom clusters of autism. In comparison to the common C57BL/6J (B6) control strain, BTBR mice showed cortical and cerebellar changes in 5-HT and its metabolite concentrations. BTBR mice had increased concentrations of cortical 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) as well as reduced cerebellar 5-HT and 5-HIAA when exposed to novel environments. These findings are consistent with the hypothesis of impaired cerebellar-cerebral communication in autism, and suggest that disturbed 5-HT-signaling pathways may contribute to some of the autism relevant behaviors displayed by the BTBR.

Chapter 3 aims to identify the consequences of genetic modulation of the serotonergic system in a mouse model of autism. The serotonin transporter (5-HTT) gene (SLC6A4) regulates 5-HT concentrations and polymorphisms in this gene are associated with an increased risk for autism (Devlin et al., 2005; Huang and Santangelo, 2008). Furthermore, reduced 5-HTT function appears to contribute to the anatomical and behavioral features of the disorder (Sutcliffe et al., 2005; Brune et al., 2006; Wassink et
al., 2007). Chapter 3 assesses autism-relevant behaviors of mice deficient in the serotonin transporter gene (5-HTT KO). 5-HTT KO mice displayed increased stereotyped grooming when compared to wild-type littermates. These results suggest that mice lacking 5-HTT display some modest behavioral changes relevant to autism and may be a useful model for investigating molecular mechanisms which contribute to the symptoms of ASDs.

Chapters 2 and 3 provide evidence that altered 5-HT neurotransmission may underlie some of the autism-relevant behaviors displayed by the BTBR and 5-HTT mutant mouse models. Therefore, Chapter 4 investigates the distribution of 5-HTT in the postmortem human subventricular zone (SVZ), a brain region involved in neurodevelopment (Brazel et al., 2003; Ming and Song, 2005). The SVZ tissue from autism-diagnosed (AD) individuals displayed a number of structural and compositional differences when compared to typically-developing (TD) controls. 5-HTT and fibroblast growth factor-2 (FGF-2) immunoreactivity were significantly increased in AD cases. Coupled with findings of reduced hypocellular gaps and increased cell density, these findings support reports of aberrant neurogenesis and migration in the brains of AD individuals.
1.8. References


CHAPTER 2. NEUROCHEMICAL CHARACTERIZATION OF THE BTBR $T+Itpr3^{3/i}/J$ MOUSE MODEL FOR AUTISM.

2.1. Abstract

Rationale

Animal models of autism provide opportunities to evaluate potential disturbances in biological systems in the disorders. Several lines of research suggest that abnormalities of neurotransmitter systems, especially serotonergic, occur in autism. The inbred BTBR $T+Itpr3^{3/i}/J$ mouse strain (BTBR) displays several behaviors analogous to the core symptoms of autism. Thus, the BTBR mouse provides an excellent opportunity to explore neurotransmitter systems and potential disturbances that may be relevant to autism.

Objectives

Experiment 1 measured neurotransmitter concentrations of BTBR mice and C57BL/6J (B6) mice at baseline. Experiment 2 measured neurotransmitter concentrations of BTBR and B6 mice in a novel environment with a nonsocial stimulus and in a novel environment with a social stimulus that provided social proximity conditions. BTBR and B6 behavior was also assessed in the novel nonsocial and social proximity conditions.

Methods

Brain tissue concentrations of norepinephrine (NE), dopamine (DA), serotonin (5-HT) and their respective metabolites were measured using high performance liquid chromatography (HPLC) with electrochemical detection.

Results

In the social proximity test, BTBR mice displayed decreased facial contact and increased crawl over and crawl under behaviors. Strain differences in brain concentrations of NE, DA, 5-HT and their metabolites were observed at baseline and in response to novel environments. Of the regions measured, the cerebellum showed the most differences in neurotransmitter concentrations between BTBR and B6 mice and the occurrence and direction of these differences were consistent in experiments 1 and 2.
Conclusions

These data provide the first documentation for altered neurotransmitter systems in BTBR mice. Neurotransmitter disturbances were especially prevalent in the cortex and cerebellum; consistent with the theory of dysfunctional cerebellar-cortical connectivity and communication in autism.
2.2. Introduction

Autism is a complex neurodevelopmental disorder that appears at an early age and remains throughout adulthood. There are currently no consistent biological markers or screening tests (Lord, 2011); therefore, diagnosis relies on the presence of core behavioral features, which can vary greatly in their severity and manifestation (American Psychiatric Association, 2013). Despite the lack of consistent biomarkers, growing evidence suggests altered anatomical and functional connectivity in the autism brain (Courchesne and Pierce, 2005; Geschwind and Levitt, 2007; Nair et al., 2013). Recent findings specifically implicate impairments of cerebral-cerebellar connectivity in the motor and affective symptoms of autism (Rogers et al., 2011; Heck and Howell, 2013). Additionally, a role for neurochemical dysfunction in autism has been suggested (McDougle et al., 2005; Lam et al., 2006); especially for the serotonergic (Cook and Leventhal, 1996; Zafeiriou et al., 2009) and dopaminergic systems (Walker, 2008; Kałuzna-Czaplinska et al., 2010).

Autism frequently occurs in conjunction with other conditions such as reduced attention and hyperactivity (Hofvander et al., 2009), hyper-responsivity to sensory stimuli (Green and Ben-Sasson, 2010), and mood disorders (Mazefsky et al., 2008; van Steensel et al., 2011). Norepinephrine (NE) mediates arousal and attention (Iversen et al., 2008; Ettinger, 2010) and has been implicated in the pathophysiology of depression and anxiety (Ressler and Nemeroff, 2001; Aston-Jones, Gary, 2002). Additionally, drugs that act on the adrenergic system have provided moderate improvements for hyperactivity and aggression in individuals with autism (Volkmar, 2001; Lam et al., 2006). Consequently, NE has been suggested to play a role in mediating these frequently co-morbid symptoms.

Dopamine (DA) is associated with a variety of social and affiliative behaviors such as pair bonding, reproduction, maternal care, and aggression (Insel, 2003; Young et al., 2008). Therefore, the DA system is of particular interest in understanding the deficits in social interactions observed in people with autism. Interest in DA has also been stimulated by evidence that DA antagonists such as the antipsychotic haloperidol reduce motor stereotypies, aggression and hyperactivity (Buitelaar and Willemsen-Swinkels, 2000; McDougle et al., 2005) in people with autism. Hyperactivity and stereotypy,
frequently observed in autism, can be induced in animals by agonizing central DA functioning (Fog, 1969).

5-HT is widely distributed in the CNS and has been implicated in diverse physiological states and behaviors (Iversen et al., 2008). The 5-HT system is one of the earliest to develop in all species and is implicated in regulating brain development and maturation (Whitaker-Azmitia, 2001; Zafeiriou et al., 2009), which may be especially relevant to developmental disorders such as autism. In rodents, serotonergic system disruptions produce autism-relevant behavioral phenotypes (Whitaker-Azmitia, 2005; Borue et al., 2007; Boylan et al., 2007; Martin et al., 2012). Furthermore, pharmacological interventions that enhance 5-HT activity reduce aggression and stereotyped movements (Cook and Leventhal, 1996; Tsai, 1999; Buitelaar and Willemsen-Swinkels, 2000) in autism. Treatments that reduce 5-HT availability, such as acute tryptophan depletion, exacerbate these symptoms (McDougle et al., 1996). These findings suggest that at least some symptoms of autism such as stereotypy, repetitive behaviors, and anxiety may be related to reduced 5-HT function.

Mouse models of autism provide opportunities to evaluate potential disturbances in biological systems in the disorder. In the absence of a clear biological marker, mouse models displaying autism-relevant behaviors may be useful for assessing genetic, environmental and biochemical contributions to the manifestation of symptoms (Bolivar et al., 2007; Moy et al., 2007; McFarlane et al., 2008; Silverman et al., 2010a). The BTBR inbred mouse strain presents a robust autism-relevant behavioral phenotype that includes parallels to all three diagnostic symptom clusters in autism (Bolivar et al., 2007; McFarlane et al., 2008; Blanchard et al., 2011; Meyza et al., 2012). When compared to C57BL/6J (B6) mice, previous studies have shown that BTBR mice display low levels of social interaction in a three-chambered test for social approach (Yang et al., 2007; McFarlane et al., 2008) and in a visible burrow system providing ethologically relevant social conditions (Pobbe et al., 2010). Scent marking and ultrasonic vocalizations, two modes of communication in mice, are also impaired in the BTBR strain (Scattoni et al., 2008, 2011; Roullet et al., 2011; Wöhr et al., 2011). In addition, BTBR mice display high levels of spontaneous repetitive self-grooming (Silverman et al., 2010a; Pearson et al., 2011). Thus, the BTBR mouse provides an excellent opportunity to explore potential
neurotransmitter systems and disturbances that may be relevant to autism. In the present experiment regional neurotransmitter concentrations were measured in BTBR and B6 mice at baseline after single housing, and when exposed to a novel nonsocial stimulus or to a novel social stimulus in a social proximity condition.

2.3. Materials and Methods

2.3.1. Subjects

Subjects were 60 adult male C57BL/6J (B6) and BTBR T+ Iipr3/3 (BTBR) mice (64-131 days of age) bred from stock originally obtained from the Jackson Laboratory (Bar Harbor, ME). At weaning all subjects were housed in same-strain, same-sex groups of 4 to 6 and maintained on a 12-h light/dark cycle (lights on at 06:00 am). Food and water were provided *ad libitum*. All procedures were conducted in accordance with protocols approved by the University of Hawaii Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines.

2.3.2. Apparatus

The social proximity chamber was a clear rectangular acrylic chamber (7 x 14 x 30 cm H).

2.3.3. Procedure

*Experiment 1: Baseline*

To assess baseline neurotransmitter concentrations, BTBR mice and B6 mice (n=10/strain), were singly housed 24 hours prior to the experiment. On the day of testing, subjects were transported to a room separate from the colony room, rapidly decapitated and their brains were removed for dissection.

*Experiment 2: Nonsocial and Social Proximity*

To assess neurotransmitter concentrations when in social proximity, subjects were singly housed 24 hours prior to the experiment and randomly assigned to either the nonsocial condition or the social condition. Subjects assigned to the nonsocial condition (n=10/strain) were placed in the proximity chamber with an upright 50 mL conical tube filled with 35 g of clean bedding to prevent it from falling over onto the mouse. This nonsocial object was included to provide a novel nonsocial stimulus to investigate and to restrict the space in the chamber to an extent similar to that in the social condition. In the nonsocial proximity condition several behaviors were measured including: sniffing,
rearing on and contacting the tube, as well as grooming, general rearing/upright and jump escapes. Subjects assigned to the social condition (n=10/strain) were paired with an unfamiliar, sex and strain-matched conspecific inside the proximity chamber (Figure 2.1). In the social proximity condition the frequencies and duration of the following behaviors were manually quantified:

- **Nose tip-to-Nose tip (NT):** subject's nose tip and or/vibrissae contact the nose tip and/or vibrissae of the other mouse.
- **Nose-to-Head (NH):** subject's nose tip contacts the head of the stimulus mouse.
- **Nose-to-Anogenital (NA):** subject's nose tip contacts the base of the tail or anogenital region of the other mouse.
- **Crawl Over (CO):** subject's forelimbs cross the midline of the dorsal surface of the other mouse.
- **Crawl Under (CU):** subject's head goes under the ventral surface of the other mouse to a depth of at least the ears of the subject animal crossing the midline of the other mouse's body.
- **Jump Escape (JE):** subject makes a vertical leap with all feet leaving the ground.

The apparatus was cleaned with 20% ethanol between each exposure session. All testing was conducted under dim red light during the light phase of the light/dark cycle, as previously described (Defensor et al., 2011). Social or nonsocial exposures lasted 20 minutes. Immediately following the exposure session mice were moved to a separate room, rapidly decapitated and their brains were removed.
37

Figure 2.1. BTBR mice in the social proximity test. The side walls of the chamber are tinted a false gray to emphasize the area of confinement. (Defensor et al., 2011)

Tissue preparation

Dissections of the medial prefrontal cortex, striatum, hippocampus, hypothalamus, brain stem, cerebellum, and the remaining cortex were collected for neurochemical analysis. All tissues were promptly weighed in tared 1.5 mL Eppendorf tubes and frozen on dry ice. The tissue concentrations of monoamines and metabolites were quantified by high performance liquid chromatography (HPLC) with electrochemical detection, using a slight modification of a previously described method (Dunn, 1993).

HPLC

Brain samples were homogenized by ultrasonic disruption in ice cold 0.1M HClO₄ containing 1 mM EDTA and an internal standard of N-methyl-dopamine (NMDA). Samples were centrifuged at 20,000xg for 20 minutes at 4°C. The supernatant was removed and 100 µL was injected into the chromatographic system at a flow rate of 1.1 ml/min. The HPLC system consisted of a liquid chromatograph pump (LC-20 AD; Shimadzu Co., Kyoto, Japan), a refrigerated Shimadzu autosampler (20AC), a 5 µm ODS1 reverse-phase column (Spherosorb 4.6 x 250 mm; Waters Corp., Milford, MA, USA) maintained at a constant temperature in the range of 35-42°C by a BioAnalytical Systems (BAS) column heater (LC22A; BioAnalytical Systems, West Lafayette, IN), a BAS dual channel detector (LC-4B) and collected by a data acquisition system (LC Solutions, Shimadzu). Electrode potentials were set at 0.78 and 0.95 V with respect to a
Ag-AgCl reference electrode. The mobile phase contained 0.1M NaH$_2$PO$_4$, 0.1mM EDTA (pH 2.8-3.1), 0.3mM 1-octanesulfonic acid and 4% (v/v) acetonitrile.

All tissue samples were analyzed for norepinephrine (NE) and its major metabolite 3-methoxy-4-hydroxyphenylethylenglycol (MHPG), dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), tryptophan (Trp), 5-hydroxytryptamine (serotonin, 5HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA). Concentrations of the monoamines and their metabolites within each sample were calculated with reference to known standards. Corrections were made relative to these standards and for brain tissue weight. Standard solutions were injected every day before each region and after every 5 samples. Monoamine turnover was calculated by determining the ratio of the metabolite to its monoamine as described previously (Dunn, 1988). Estimates of monoamine turnover are commonly used measures of the rate of utilization of a neurotransmitter; which are thought to directly reflect the activity of the neurons that release it (Commissiong, 1985; Jones et al., 1996). Data are expressed as nanograms of compound per milligram of brain tissue.

2.3.4. Statistical analyses

The neurochemical data from experiment 1 and behavioral data from the social proximity test of experiment 2 were analyzed using a two-tailed Student’s t-test. The neurochemical data for experiment 2 was analyzed with separate two-way ANOVAs (strain x condition) conducted for each brain region and each analyte. Some samples were lost during HPLC preparation resulting in different degrees of freedom across brain regions. The critical value for significance was set at $p < 0.05$ for all analyses. Fisher’s LSD post hoc tests were performed on statistically significant main effects.

2.4. Results

2.4.1. Experiment 1 - Baseline

Strain differences in baseline concentrations of the monoamines, metabolites and utilization ratios are shown in Table 2.1. BTBR mice had significantly less NE in the cortex [$t(18) = 3.81, p < 0.01$]; and significantly more NE in the hippocampus [$t(18) = 3.39, p < 0.01$] and hypothalamus [$t(18) = 2.45, p < 0.05$] as compared to B6 mice.

BTBR mice had significantly less of the NE metabolite MHPG in the cerebellum [$t(18) = 4.79, p < 0.001$] and brainstem [$t(18) = 2.16, p < 0.05$] compared to B6 mice. BTBR
mice had significantly increased MHPG:NE ratios in the cortex \[t(18) = 3.17, p < 0.01\], but decreased MHPG:NE ratios in the hippocampus \[t(18) = 2.67, p < 0.05\], and brainstem \[t(18) = 2.84, p < 0.05\].

BTBR mice had significantly more DA in the cortex \[t(18) = 2.19, p < 0.05\], but significantly less DA \[t(18) = 2.81, p < 0.05\] and DOPAC \[t(18) = 3.90, p < 0.001\] in the cerebellum compared to B6 mice.

BTBR mice had less 5-HT \[t(18) = 4.97, p < 0.001\] and 5-HIAA \[t(18) = 5.45, p < 0.001\] in the cerebellum when compared to B6 mice. There were no significant differences in baseline concentrations of tryptophan or 5-HIAA:5-HT ratios.
Table 2.1. Regional baseline concentrations of monoamines, metabolites, and monoamine turnover for BTBR and B6 mice.

<table>
<thead>
<tr>
<th>Strain</th>
<th>mPFC Mean (SEM)</th>
<th>Cortex Mean (SEM)</th>
<th>Striatum Mean (SEM)</th>
<th>Hippocampus Mean (SEM)</th>
<th>Hypothalamus Mean (SEM)</th>
<th>Cerebellum Mean (SEM)</th>
<th>Brainstem Mean (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE B6</td>
<td>0.957 (0.046)</td>
<td>0.651 (0.068)</td>
<td>1.614 (0.283)</td>
<td>2.803 (0.062)</td>
<td>5.529 (0.379)</td>
<td>0.870 (0.256)</td>
<td>1.536 (0.163)</td>
</tr>
<tr>
<td>BTBR</td>
<td>1.013 (0.066)</td>
<td>0.361 (0.034)**</td>
<td>1.432 (0.266)</td>
<td>3.165 (0.087)**</td>
<td>6.739 (0.317)*</td>
<td>0.686 (0.123)</td>
<td>2.210 (0.672)</td>
</tr>
<tr>
<td>MHPG B6</td>
<td>0.288 (0.094)</td>
<td>0.158 (0.010)</td>
<td>0.322 (0.041)</td>
<td>0.070 (0.004)</td>
<td>0.147 (0.012)</td>
<td>0.046 (0.003)</td>
<td>0.077 (0.006)</td>
</tr>
<tr>
<td>BTBR</td>
<td>0.174 (0.044)</td>
<td>0.173 (0.018)</td>
<td>0.336 (0.067)</td>
<td>0.062 (0.006)</td>
<td>0.157 (0.028)</td>
<td>0.030 (0.002)**</td>
<td>0.053 (0.009)</td>
</tr>
<tr>
<td>MHPG:NEB6</td>
<td>0.281 (0.082)</td>
<td>0.270 (0.032)</td>
<td>0.290 (0.061)</td>
<td>0.025 (0.001)</td>
<td>0.027 (0.002)</td>
<td>0.381 (0.298)</td>
<td>0.053 (0.005)</td>
</tr>
<tr>
<td>BTBR</td>
<td>0.193 (0.057)</td>
<td>0.527 (0.074)**</td>
<td>0.294 (0.068)</td>
<td>0.020 (0.002)*</td>
<td>0.023 (0.003)</td>
<td>0.063 (0.014)</td>
<td>0.031 (0.006)</td>
</tr>
<tr>
<td>DA B6</td>
<td>4.491 (2.698)</td>
<td>3.376 (0.218)</td>
<td>9.210 (1.902)</td>
<td>0.161 (0.035)</td>
<td>1.505 (0.157)</td>
<td>0.074 (0.004)</td>
<td>0.159 (0.005)</td>
</tr>
<tr>
<td>BTBR</td>
<td>2.346 (1.146)</td>
<td>4.409 (0.419)*</td>
<td>11.845 (2.450)</td>
<td>0.178 (0.053)</td>
<td>1.346 (0.166)</td>
<td>0.054 (0.006)*</td>
<td>0.160 (0.006)</td>
</tr>
<tr>
<td>DOPAC B6</td>
<td>0.375 (0.136)</td>
<td>0.140 (0.008)</td>
<td>0.440 (0.077)</td>
<td>0.036 (0.003)</td>
<td>0.172 (0.026)</td>
<td>0.020 (0.001)</td>
<td>0.034 (0.004)</td>
</tr>
<tr>
<td>BTBR</td>
<td>0.190 (0.051)</td>
<td>0.153 (0.011)</td>
<td>0.422 (0.078)</td>
<td>0.017 (0.006)</td>
<td>0.156 (0.020)</td>
<td>0.016 (0.001)**</td>
<td>0.029 (0.003)</td>
</tr>
<tr>
<td>DOPAC:DA B6</td>
<td>0.446 (0.261)</td>
<td>0.043 (0.003)</td>
<td>0.063 (0.011)</td>
<td>0.278 (0.032)</td>
<td>0.113 (0.007)</td>
<td>0.280 (0.013)</td>
<td>0.214 (0.023)</td>
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<tr>
<td>BTBR</td>
<td>0.234 (0.074)</td>
<td>0.037 (0.003)</td>
<td>0.053 (0.017)</td>
<td>0.094 (0.030)</td>
<td>0.118 (0.008)</td>
<td>0.311 (0.025)</td>
<td>0.182 (0.017)</td>
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<tr>
<td>5-HT B6</td>
<td>2.655 (0.325)</td>
<td>2.434 (0.105)</td>
<td>1.185 (0.234)</td>
<td>3.164 (0.180)</td>
<td>7.779 (0.546)</td>
<td>1.083 (0.086)</td>
<td>3.093 (0.228)</td>
</tr>
<tr>
<td>BTBR</td>
<td>2.396 (0.146)</td>
<td>2.584 (0.069)</td>
<td>1.700 (0.264)</td>
<td>3.269 (0.180)</td>
<td>7.357 (0.679)</td>
<td>0.588 (0.051)**</td>
<td>2.763 (0.231)</td>
</tr>
<tr>
<td>5-HIAA B6</td>
<td>2.329 (0.353)</td>
<td>1.508 (0.093)</td>
<td>2.040 (0.257)</td>
<td>3.205 (0.165)</td>
<td>7.729 (1.069)</td>
<td>0.998 (0.065)</td>
<td>3.947 (0.538)</td>
</tr>
<tr>
<td>BTBR</td>
<td>1.955 (0.175)</td>
<td>1.469 (0.060)</td>
<td>1.900 (0.115)</td>
<td>3.074 (0.147)</td>
<td>6.593 (0.663)</td>
<td>0.614 (0.028)**</td>
<td>2.952 (0.334)</td>
</tr>
<tr>
<td>TRP B6</td>
<td>3.779 (0.539)</td>
<td>1.528 (0.141)</td>
<td>4.657 (0.901)</td>
<td>3.790 (0.353)</td>
<td>6.274 (0.634)</td>
<td>2.298 (0.281)</td>
<td>3.693 (0.531)</td>
</tr>
<tr>
<td>BTBR</td>
<td>2.945 (0.170)</td>
<td>1.341 (0.049)</td>
<td>3.873 (0.487)</td>
<td>3.426 (0.108)</td>
<td>5.392 (0.408)</td>
<td>2.137 (0.179)</td>
<td>3.375 (0.076)</td>
</tr>
<tr>
<td>5-HIAA:5-HT B6</td>
<td>0.896 (0.091)</td>
<td>0.626 (0.062)</td>
<td>2.438 (0.507)</td>
<td>1.036 (0.074)</td>
<td>0.989 (0.097)</td>
<td>0.957 (0.070)</td>
<td>1.237 (0.105)</td>
</tr>
<tr>
<td>BTBR</td>
<td>0.817 (0.056)</td>
<td>0.569 (0.019)</td>
<td>1.433 (0.293)</td>
<td>0.953 (0.045)</td>
<td>0.896 (0.044)</td>
<td>1.082 (0.061)</td>
<td>1.065 (0.082)</td>
</tr>
</tbody>
</table>

Means are presented in ng/mg tissue. mPFC, medial prefrontal cortex; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylethylenglycol; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; TRP, tryptophan. Significant differences between BTBR and B6 mice are noted with *p < 0.05, **p < 0.01, and ***p < 0.001.
2.4.2. Experiment 2 - Nonsocial and Social Proximity

Behaviors in the nonsocial and social proximity conditions

No significant differences between the strains were detected for any of the behaviors in the nonsocial proximity condition (Figure 2.2A). There were clear strain differences in the frequency of behaviors observed in the social proximity chamber (Figure 2.2B). Consistent with the results of Defensor et al. (2011), BTBR mice made significantly fewer nose tip-to-nose tip investigations \(t(18) = 3.64, p < 0.01\) and significantly more nose-to-anus investigations \(t(18) = 3.03, p < 0.01\). Additionally, BTBR mice displayed reliably increased crawl over behaviors \(t(18) = 3.94, p < 0.001\). There was also a trend for BTBR mice to demonstrate increased crawl under behaviors, but this failed to reach statistical significance \(t(18) = 1.92, p = 0.07\).

Figure 2.2. Frequency of behaviors in the nonsocial (A) and social proximity (B) conditions. Data are expressed as mean (+ SEM). NT, nose tip-to-nose tip; NH, nose-to-head; NA, nose-to-anogenital; CO, crawl over, CU, crawl under; JE, jump escape. Significant differences between BTBR and B6 are indicated by * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).

Neurochemistry

Main effects of strain and condition

Significant main effects of strain indicated differences between BTBR and B6 mice when collapsed across the social and nonsocial conditions. Main effects of strain for brain NE, MHPG and MHPG:NE ratios are presented in Table 2.2a. BTBR mice had
higher concentrations of NE in the medial prefrontal cortex \( F(1,33) = 4.58, p < 0.05 \) and hippocampus compared to B6 mice \( F(1,34) = 20.62, p < 0.001 \). BTBR mice had less MHPG in the hippocampus \( F(1,34) = 20.56, p < 0.001 \) and in the cerebellum \( F(1,36) = 42.45, p < 0.001 \). BTBR mice also had lower MHPG:NE ratios in the hippocampus \( F(1,34) = 60.94, p < 0.001 \).

Main effects of strain for brain DA, DOPAC and DOPAC:DA are presented in Table 2.2b. BTBR mice had more DA \( F(1,36) = 7.87, p < 0.01 \) and DOPAC \( F(1,36) = 15.05, p < 0.001 \) in the cortex and less DA \( F(1,36) = 13.03, p < 0.001 \) and DOPAC \( F(1,36) = 31.42, p < 0.001 \) in the cerebellum as compared to B6 mice.

Main effects of strain for brain 5-HT, 5-HIAA and 5-HIAA:5-HT ratios are presented in Table 2.2c. BTBR mice had more 5-HT \( F(1,36) = 7.17, p < 0.05 \) and 5-HIAA \( F(1,36) = 9.21, p < 0.01 \) in the cortex, less 5-HT \( F(1,36) = 28.80, p < 0.001 \) and 5-HIAA \( F(1,36) = 18.06, p < 0.001 \) in the cerebellum, and less 5-HT \( F(1,36) = 8.28, p < 0.01 \) and 5-HIAA \( F(1,36) = 8.70, p < 0.01 \) in the brainstem when compared to B6 mice. BTBR mice had higher 5-HIAA:5-HT ratios \( F(1,36) = 19.85, p < 0.001 \) in the cerebellum.

A significant main effect of condition indicated a difference between the social and nonsocial conditions when collapsed across strains. A significant main effect of condition indicated that the DOPAC:DA ratios in the cerebellum \( F(1,36) = 8.79, p < 0.01 \) were higher in the nonsocial condition when compared to the social proximity condition.

**Interactions between strain and condition**

Post-hoc analysis following a significant interaction between strain and condition for NE in the medial prefrontal cortex \( F(1,33) = 5.17, p < 0.05 \) showed that BTBR mice in the social condition had more NE in the medial prefrontal cortex than B6 mice in the social condition (Fig. 2.3). In addition, B6 mice had less NE in the social condition when compared to B6 mice in the nonsocial condition.

Post-hoc analysis following a significant interaction between strain and condition for cortical MHPG concentrations \( F(1,36) = 6.06, p < 0.05 \) showed that BTBR mice in the social condition had more MHPG in the cortex than B6 mice in the social condition.
(Fig. 2.4). In addition, B6 mice had less MHPG in the social condition compared to their nonsocial counterparts.

Post-hoc analysis following a significant interaction between strain and condition for DOPAC:DA ratios \([F(1,36) = 6.08, p < 0.05]\) showed that BTBR mice in the social condition had higher DOPAC:DA ratios in the hypothalamus than B6 mice in the social condition (Fig. 2.5).

There were no significant strain by condition interactions for 5-HT, 5-HIAA, or 5-HIAA:5-HT ratios.
Table 2.2. Regional concentrations of monoamines, metabolites, and monoamine turnover collapsed across nonsocial and social conditions.

<table>
<thead>
<tr>
<th></th>
<th>mPFC</th>
<th>Cortex</th>
<th>Striatum</th>
<th>Hippocampus</th>
<th>Hypothalamus</th>
<th>Cerebellum</th>
<th>Brainstem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td>a. NE</td>
<td>B6</td>
<td>1.081 0.071</td>
<td>0.506 0.057</td>
<td>1.982 0.243</td>
<td>3.205 0.079</td>
<td>5.970 0.272</td>
<td>0.760 0.147</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>1.266 0.058*</td>
<td>0.454 0.038</td>
<td>2.133 0.238</td>
<td>3.690 0.072***</td>
<td>5.637 0.299</td>
<td>0.627 0.081</td>
</tr>
<tr>
<td>MHPG</td>
<td>B6</td>
<td>0.188 0.028</td>
<td>0.101 0.008</td>
<td>0.388 0.078</td>
<td>0.079 0.002</td>
<td>0.205 0.014</td>
<td>0.061 0.003</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>0.228 0.056</td>
<td>0.103 0.011</td>
<td>0.501 0.096</td>
<td>0.061 0.004***</td>
<td>0.214 0.016</td>
<td>0.038 0.001***</td>
</tr>
<tr>
<td>MHPG:NE</td>
<td>B6</td>
<td>0.194 0.041</td>
<td>0.250 0.031</td>
<td>0.291 0.079</td>
<td>0.025 0.001</td>
<td>0.035 0.003</td>
<td>0.384 0.180</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>0.193 0.051</td>
<td>0.267 0.033</td>
<td>0.358 0.088</td>
<td>0.016 0.001***</td>
<td>0.039 0.002</td>
<td>0.071 0.005</td>
</tr>
<tr>
<td>b. DA</td>
<td>B6</td>
<td>2.220 0.741</td>
<td>2.489 0.163</td>
<td>6.217 1.248</td>
<td>0.188 0.027</td>
<td>1.654 0.081</td>
<td>0.082 0.004</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>3.751 1.158</td>
<td>3.167 0.197**</td>
<td>9.503 1.787</td>
<td>0.150 0.026</td>
<td>1.498 0.083</td>
<td>0.064 0.003***</td>
</tr>
<tr>
<td>DOPAC</td>
<td>B6</td>
<td>0.164 0.029</td>
<td>0.108 0.005</td>
<td>0.342 0.059</td>
<td>0.048 0.004</td>
<td>0.200 0.013</td>
<td>0.026 0.001</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>0.228 0.051</td>
<td>0.140 0.007***</td>
<td>0.456 0.056</td>
<td>0.026 0.006</td>
<td>0.189 0.012</td>
<td>0.019 0.001***</td>
</tr>
<tr>
<td>DOPAC:DA</td>
<td>B6</td>
<td>0.183 0.038</td>
<td>0.045 0.002</td>
<td>0.067 0.006</td>
<td>0.299 0.035</td>
<td>0.123 0.006</td>
<td>0.322 0.014</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>0.160 0.031</td>
<td>0.046 0.002</td>
<td>0.068 0.007</td>
<td>0.196 0.043</td>
<td>0.127 0.005</td>
<td>0.311 0.018</td>
</tr>
<tr>
<td>c. 5-HT</td>
<td>B6</td>
<td>2.237 0.103</td>
<td>1.551 0.048</td>
<td>1.032 0.143</td>
<td>3.395 0.104</td>
<td>7.732 0.452</td>
<td>1.091 0.106</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>2.125 0.121</td>
<td>1.782 0.079*</td>
<td>1.151 0.168</td>
<td>3.246 0.135</td>
<td>7.049 0.450</td>
<td>0.499 0.023***</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>B6</td>
<td>2.521 0.178</td>
<td>1.193 0.028</td>
<td>2.131 0.123</td>
<td>4.026 0.123</td>
<td>8.220 0.516</td>
<td>1.118 0.106</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>2.481 0.140</td>
<td>1.337 0.041**</td>
<td>1.987 0.133</td>
<td>4.051 0.133</td>
<td>7.332 0.467</td>
<td>0.650 0.021***</td>
</tr>
<tr>
<td>TRP</td>
<td>B6</td>
<td>5.243 0.436</td>
<td>1.036 0.085</td>
<td>6.506 0.549</td>
<td>5.233 0.217</td>
<td>8.306 0.502</td>
<td>3.051 0.230</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>4.247 0.204</td>
<td>1.137 0.093</td>
<td>6.315 0.663</td>
<td>4.925 0.164</td>
<td>7.986 0.346</td>
<td>2.654 0.113</td>
</tr>
<tr>
<td>5-HIAA:5-HT</td>
<td>B6</td>
<td>1.117 0.049</td>
<td>0.776 0.021</td>
<td>2.793 0.295</td>
<td>1.192 0.030</td>
<td>1.062 0.029</td>
<td>1.049 0.045</td>
</tr>
<tr>
<td></td>
<td>BTBR</td>
<td>1.176 0.040</td>
<td>0.766 0.026</td>
<td>2.451 0.282</td>
<td>1.259 0.022</td>
<td>1.053 0.039</td>
<td>1.326 0.041***</td>
</tr>
</tbody>
</table>

Means are presented as ng/mg tissue. mPFC, medial prefrontal cortex; NE, norepinephrine; MHPG, 3-methoxy-4-hydroxyphenylethanol; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; TRP, tryptophan. Significant differences between BTBR and B6 mice are noted with * p < 0.05, ** p < 0.01, and *** p < 0.001.
Figure 2.3. Concentrations of NE in the mPFC in the proximity conditions. Data are expressed as mean (± SEM). * indicates difference, $p < 0.05$.

Figure 2.4. Concentrations of MHPG in the cortex in the proximity conditions. Data are expressed as mean (± SEM). * indicates difference, $p < 0.05$.

Figure 2.5. Concentrations of DOPAC:DA in the hypothalamus in the proximity conditions. Data are expressed as mean (± SEM). * indicates difference, $p < 0.05$. 
2.5. Discussion

Social Proximity

In the current study BTBR mice showed social avoidance in the social proximity test. These findings are consistent with a previous report of BTBR social deficits in social proximity (Defensor et al., 2011). Here BTBR mice showed reduced nose tip-to-nose tip contacts and increased crawl over behaviors (Figure 2.2B), suggesting active avoidance of frontal orientations. We have previously suggested a parallel between this unique behavioral pattern and the gaze aversion frequently observed in autism (Defensor et al., 2011). In support of this hypothesis, the current results revealed no strain differences in motor or investigative behaviors in the nonsocial condition (Figure 2.2A). In addition, no aggressive contact was displayed by either strain in the social proximity condition. Taken together, this suggests that the observed behavioral differences are not due to altered motor, exploratory or aggressive behaviors. The current results compliment previous findings of reduced frontal approaches (Yang et al., 2007; Pobbe et al., 2010) and nose sniffing (McFarlane et al., 2008) in BTBR mice and are consistent with an interpretation of active social avoidance in the BTBR in social proximity.

Norepinephrine

The noradrenergic system of the BTBR mouse showed disturbances in more of the brain regions examined than any other neurotransmitter system measured in this study. At baseline, BTBR mice had significantly different concentrations of NE, MHPG, and MHPG:NE ratios in cortical, limbic, and hindbrain regions, suggesting widespread regional differences in activity of NE systems in BTBR vs. B6 mice. Compared to exposure to a novel nonsocial situation, the social proximity condition reduced concentrations of NE in the mPFC and MHPG in the cortex for the B6 strain. Although turnover rates (MHPG:NE ratios) were not significantly different for either of these areas, these data suggest that regional activation of NE systems may differ in social vs. nonsocial conditions for the B6 mice. However, the social vs. nonsocial proximity situations produced no significant differences for any noradrenergic system parameters in the BTBR mice; suggesting that BTBR mice fail to show a NE-based differentiation between social and nonsocial conditions.
The central noradrenergic system originates in the brain stem and has diffuse projections throughout the limbic system and cortex (Iversen et al., 2008; Ettinger, 2010). Norepinephrine regulates several behaviors including attention, anxiety, and stress response (Iversen et al., 2008; Ettinger, 2010) which are frequently disturbed in autism. Drugs that reduce NE neurotransmission reduce aggression and impulsivity in individuals with autism (Volkmar, 2001; Bauman and Kemper, 2006; Lam et al., 2006), suggesting that disturbed noradrenergic functioning may contribute to certain behaviors observed in autism.

Tests of anxiety in response to stress have been inconsistent in the BTBR strain; with reports of no difference (Moy et al., 2007; Yang et al., 2009; Silverman et al., 2010b), reduced (McFarlane et al., 2008; Pobbe et al., 2011), or enhanced (Benno et al., 2009) anxiety; or anxiety that is dependent on context/stimulus (Pobbe et al., 2011). Here, to novel stimuli, B6 mice showed changes in medial prefrontal cortex, and remaining cortex consonant with those typically associated with stress, such as reductions in NE, increased MHPG:NE ratios or increased tryptophan (Bliss et al., 1968; Thierry et al., 1968; Dunn, 1988; Shanks et al., 1990). BTBR failed to show any of these, suggesting that these particular novelty situations were not highly salient or stressful for these mice; a view that is hard to evaluate against previous literature given the great variability in results of behavioral studies of stress responsivity in this strain. However, given the critical role of NE in the stress response, it is possible that the variable anxiety behaviors seen in the BTBR are linked to the numerous area-based disturbances in the NE system shown here under basal conditions.

**Dopamine**

At baseline and in response to novel situations, BTBR mice had higher concentrations of DA in the cortex and lower concentrations of DA and DOPAC in the cerebellum, as well as higher DOPAC in cortex in the novel situation. Recent findings indicate that stimulation of the cerebellum evokes DA release in the medial prefrontal cortex through glutamatergic inputs (Mittleman et al., 2008; Rogers et al., 2011, 2013). The current findings of differences in both the cerebellum and cortex for BTBR mice are compatible with a view of an impaired cerebellar-cortical pathway in the BTBR mouse, when compared to B6 mice.
Clinically, dysfunction of cerebello-thalamo-cortical circuits has been implicated in the symptom expression of autism (Catani et al., 2008; Hoppenbrouwers et al., 2008; Sivaswamy et al., 2010). Cerebellar abnormalities, especially reduced Purkinje cells, have been proposed to disrupt cerebello-thalamo-cortical communication through disinhibition of the deep cerebellar nuclei, resulting in increased excitatory output in the thalamus and cortex (Belmonte et al., 2004a, 2004b). Disturbances to the cerebellum, which are commonly observed in autism (Palmen et al., 2004; Wegiel et al., 2013), could result in a loss of functional connectivity between the cerebellum and cortex, which is manifested as abnormal DA activity in the cortex (Rogers et al., 2011).

Aberrant DA transmission, in the cerebellum and elsewhere, has been implicated in stereotypic motor behaviors. Rates of stereotyped behaviors were negatively correlated with the area of cerebellum vermis lobules VI-VIII in a study of children with autism (Pierce and Courchesne, 2001), while compounds that antagonize central DA activity, such as haloperidol, are effective in alleviating stereotypies and hyperactivity (Tsai, 1999; Buitelaar and Willemsen-Swinkels, 2000; Volkmar, 2001). Taken together, these studies suggest that reduced DA availability and activity in the cerebellum may be involved in the repetitive behaviors observed in autism, in addition to potentially contributing to cortical changes associated with the disorder. That such DA changes are also characteristic of the BTBR mouse adds to a view that these mice may provide a biologically meaningful animal model of autism.

Serotonin

Results from the current study suggest only moderate changes in regional 5-HT and 5-HIAA concentrations in the BTBR under baseline conditions, with differences (reductions) restricted to the cerebellum. However, in response to novel situations and social stimuli, 5-HT and 5-HIAA show a number of additional differences, with reductions in the cerebellum and brainstem, and with increases in both, in the cortex. This pattern, similar to the baseline DA differences in the cerebellum and cortex outlined above, is in agreement with a view that anterior-posterior connections may be disturbed in the BTBR mouse. In the forebrain, 5-HT is distributed largely by fibers originating in the superior raphe nuclei (Jacobs and Azmitia, 1992), while the inferior raphe nuclei project largely in a caudal direction, distributing 5-HT to the cerebellum and brainstem.
suggesting some differential involvement of projections originating in the superior and inferior raphe, for the BTBR mouse. In support of this hypothesis, recent research showed that 5-HTT expression during development regulates the guidance of 5-HT projections from the dorsal raphe to their cortical targets (Witteveen et al., 2013). A lack of developmental 5-HTT resulted in increased 5-HT projections from the median raphe to the medial prefrontal cortex. Given the reports of reduced 5-HTT in the BTBR brain (Gould et al., 2011), it is likely that the characteristics and projection patterns of the raphe neurons may be altered in the BTBR.

The serotonergic system regulates a variety of complex behaviors and affective states (Lucki, 1998) and is critical to CNS development and maturation (Whitaker-Azmitia, 2001). Disturbances to this system during development produce anatomical and behavioral features similar to those observed in autism (Palmen et al., 2004; Whitaker-Azmitia, 2005). One well documented feature of autism is reduced cerebellar Purkinje cells (Palmen et al., 2004; Bauman and Kemper, 2005), and 5-HT may be involved in this reduction. Serotonin innervates all areas of the cerebellum (Ottersen, 1993), modulates Purkinje cell firing (Dieudonné, 2001) and regulates the development (Oostland et al., 2013) and arborization (Kondoh et al., 2004) of Purkinje cells through various receptors. Taken together, these results suggest that altered cerebellar serotonin may be related to the reduced Purkinje cells observed in autism.

More general support for a role for 5-HT functioning in autism-relevant behaviors is evident from both genetic and pharmacological research. Aberrant social behaviors have been noted in 5-HT$_{3A}$ receptor knockout (Smit-Rigter et al., 2010) and serotonin transporter (5-HTT) knockout mice (Moy et al., 2009). Reduced 5-HTT binding has been reported in both children (Makkonen et al., 2008) and adults with autism and was negatively correlated with repetitive behaviors (Nakamura et al., 2010). Consistent with the clinical data, Gould et al., (2011) found reduced 5-HTT binding in the BTBR brain. Additionally, BTBR social deficits can be ameliorated by treatment with 5-HT agonists such as the selective 5-HT reuptake inhibitor (SSRI) fluoxetine (Chadman, 2011; Gould et al., 2011) or the 5-HT$_{1A}$ receptor agonist Buspirone (Gould et al., 2011). Several gain-of-function 5-HTT coding variants have also been reported in children with autism (Glatt et al., 2001; Sutcliffe et al., 2005). Mice with alterations in one of these, 5-HTT Ala56,
show enhanced 5-HT clearance rates and hyperserotonemia, with reduced basal firing of raphe 5-HT neurons, as well as 5-HT$_{1A}$ and 5-HT$_{2A}$ receptor hypersensitivity. They also display alterations in social function, communication, and repetitive behavior (Veenstra-Vanderweele et al., 2012).

These findings suggest that serotonin systems may have multiple roles in the development of an autism-relevant behavior phenotype. The present findings are consonant with a view that serotonin systems in BTBR mice show differences from those of B6 mice that are exacerbated in response to novel situations and social stimuli.

**Area Focus**

In this study, the cerebellum was the brain region that showed the greatest number of differences in neurotransmitter concentrations for BTBR vs. B6 mice, and these differences were consistent across Experiments 1 and 2 (Tables 2.1 and 2.2). While the absolute values of the monoamines in the cerebellum are low in comparison to other brain regions, there is considerable evidence of noradrenergic (Gordon Shepherd, 1990; Ottersen, 1993), dopaminergic (Chrapusta et al., 1994; Delis et al., 2008) and serotonergic (Gordon Shepherd, 1990; Ottersen, 1993) afferents to various layers of the cerebellum, and they may modulate the activity of the cerebellum through their interactions with various cerebellar neurons, including Purkinje cells (Gordon Shepherd, 1990; Ottersen, 1993).

Cerebellar disturbances are frequently reported in autism (Fatemi et al., 2012). Cerebellar volume differences are reported as either increased proportionally with total brain volume (Piven et al., 1997; Sparks et al., 2002; Herbert et al., 2003), or when corrected for total brain volume (Hardan et al., 2001). Reduced numbers of Purkinje cells are consistently reported in the disorder regardless of age, sex, or cognitive ability (Ritvo et al., 1986; Courchesne, 1991; Bailey et al., 1998; Fatemi et al., 2002). Recent investigations revealed cerebellar alteration in a signaling pathway (Ras/Raf/ERK1/2) involved in apoptosis of neural cells in BTBR mice (Zou et al., 2011) and in the brains of individuals with autism (Yang et al., 2011).

The cerebellum displays functional relevance to the behaviors that occur in autism. Gaze behavior, which has been associated with the cerebellum (Brettler et al., 2003; Fuchs et al., 2010; Kheradmand and Zee, 2011), is frequently abnormal in autism.
and has been used as a predictor for early detection of the disorder (Baron-Cohen et al., 1996; Clifford et al., 2007). The BTBR mouse also displays gaze aversion-like behavior (Defensor et al., 2011). In addition, oculomotor deficits, consistent with disturbances in the cerebellar vermis, have been detected in autism (Takarae et al., 2004; Nowinski et al., 2005; Wegiel et al., 2013). The cerebellum plays a critical role in the coordination of motor functions, which are frequently impaired in patients with the disorder (Fournier et al., 2010; Behere et al., 2012). In addition, impairments in communication are common following trauma to the posterior fossa, which includes the cerebellum and brainstem (Gudrunardottir et al., 2011), and reduced sociability has been reported in patients with lesions to the cerebellar vermis (Riva and Giorgi, 2000).

The cerebellum receives a fine plexus of serotonergic fibers through which 5-HT is released via volume transmission (Trouillas and Fuxe, 1993). 5-HT concentrations in the cerebellum are positively correlated to an organism's level of motor activity (Mendlin et al., 1996) and disturbances to these concentrations could have a number of deleterious effects. Reduced 5-HT in the cerebellum could contribute to reduced Purkinje cell complexity and firing (Oostland and van Hooft, 2013). As the primary cellular output for the cerebellum, Purkinje cells contribute significantly to cerebellar-thalamo-cortical communication. Therefore, Purkinje cell dysfunction as a consequence of deficient 5-HT signaling, may contribute not only to the gaze-aversion like behavior displayed by the BTBR but may also have long-ranging implications for autism brain connectivity (Hoppenbrouwers et al., 2008).

Summary

In summary, BTBR differences in regional monoamine concentrations and utilization, compared to those of B6 mice, suggest disturbances in anterior-posterior connections related to monoamine distribution. These results support and compliment findings of disturbed long-range connectivity in autism (Khan et al., 2013). The area most consistently involved in these differences was the cerebellum, a structure that is both the recipient of monoaminergic inputs and a modulator of monoaminergic projections to the forebrain. Numerous similarities between the profiles of BTBR differences compared to the B6 mouse, and findings from autism-diagnosed individuals, provide additional support for the BTBR mouse as a model of autism. Future analyses
may benefit from the inclusion of additional strains with documented autism relevant phenotypes and the use of in vivo techniques such as microdialysis or electrophysiology.
2.6. References


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Khan S, Gramfort A, Shetty NR, Kitzbichler MG, Ganesan S, Moran JM, Lee SM, Gabrieli JDE, Tager-Flusberg HB, Joseph RM, Herbert MR, Hamalainen MS,


CHAPTER 3. BEHAVIORAL CHARACTERIZATION OF THE SEROTONIN TRANSPORTER KNOCKOUT MOUSE MODEL FOR AUTISM.

3.1. Abstract

Rationale

Autism is a behaviorally defined disorder with a strong genetic component. Behavioral phenotyping of mice with targeted mutations in autism candidate genes are an important tool for discovering mechanisms that underlie the disorder. The serotonin transporter (5-HTT) gene (SLC6A4) regulates 5-HT concentrations and has been implicated in autism susceptibility. 5-HTT knockout (KO, 5-HTT -/-) mice show changes in 5-HT concentrations, neuroanatomy and behavior; however, they have not been extensively studied in the context of autism.

Objectives

The purpose of the current study was to examine the consequences of life-long 5-HTT inactivation on rodent behaviors relevant to the core symptoms of autism.

Methods

Comprehensive behavioral phenotyping was conducted on 5-HTT wild-type (WT, 5-HTT +/+), heterozygous (HET, 5-HTT +/-) and knock-out (KO, 5-HTT -/-) mice on measures of social interactions, communication, and repetitive behaviors. Social interactions between genotypes were assessed in the visible burrow system (VBS), three-chamber social approach and social proximity tests. Communication deficits were assessed by the quantification of scent marking to a conspecific. Stereotyped and repetitive behaviors were assessed by analysis of grooming patterns and spontaneous investigation of novel objects. Additionally, aggression, a potential motivating factor in social interactions, was examined in the resident intruder test.

Results

Results from the behavioral battery indicated that adult 5-HTT KO mice were similar to their WT and HET littermates in tests of social interaction and communication. However, 5-HTT KO mice displayed modest reductions in initial investigatory behaviors in the VBS. 5-HTT KO mice also displayed increased stereotyped and repetitive grooming patterns. Contrary to reports of reduced aggression in the 5-HTT KO, in the
VBS and resident intruder test, 5-HTT KO mice displayed similar levels of aggression to their WT littermates.

Conclusions

These data suggest that dysfunctional 5-HTT activity may contribute to the stereotyped and repetitive behaviors associated with autism.
3.2. Introduction

Autism is a behaviorally defined disorder characterized by a broad spectrum of symptoms with varying degrees of severity. The underlying cause of the disorder is unknown and there are no consistent biological markers available. However, a range of findings suggest a role for reduced serotonin transporter (5-HTT) function in autism (Devlin et al., 2005; Rose’meyer, 2013). Functional variants in the 5-HTT gene (SLC6A4) that result in reduced 5-HTT expression have been associated with susceptibility to autism (Cook and Leventhal, 1996; Kim et al., 2002; Devlin et al., 2005; Sutcliffe et al., 2005). Imaging studies indicate that 5-HTT binding is reduced in children (Makkonen et al., 2008) and adults (Nakamura et al., 2010; Oblak et al., 2013) with autism. Furthermore, selective serotonin transporter inhibitors (SSRIs), which bind to 5-HTT, effectively reduce aggression and stereotyped behaviors in adults with autism (Cook and Leventhal, 1996; Tsai, 1999; Buitelaar and Willemsen-Swinkels, 2000).

5-HTT acts as a key regulator of 5-HT neurotransmission and homeostasis by removing 5-HT from the synaptic cleft (Murphy et al., 2008). Thus 5-HTT determines the magnitude and duration of 5-HT activity on post-synaptic sites (Canli and Lesch, 2007). The transcriptional activity of SLC6A4 is modulated by a length variation in the promoter region of the gene (5-HTTLPR) (Heils et al., 1996). The short variant of this polymorphism results in reduced 5-HTT expression and therefore reduced 5-HT uptake from the synaptic cleft (Lesch et al., 1996; Murphy et al., 2008). The low expressing 5-HTTLPR short variant has been associated with a number of neuropsychiatric disorders, including autism (Devlin et al., 2005; Huang and Santangelo, 2008). Autistic individuals with one or two copies of the short allele reportedly have increased cortical gray matter volume (Wassink et al., 2007), altered amygdala activity in response to faces (Wiggins et al., 2013), reduced neurochemical metabolites (Endo et al., 2010), hyperserotonemia (Coutinho et al., 2004, 2007), and more severe impairments in social and communication domains (Tordjman et al., 2001; Brune et al., 2006).

To explore the neurobiological implications of reduced 5-HTT, mice with a partial or complete inactivation of 5-HTT function have been created on a variety of backgrounds (Bengel et al., 1998). Homozygous knockouts (KO) completely lack 5-HTT and consequently have increased 5-HT synthesis (Kim et al., 2005), extracellular 5HT
concentrations (Mathews et al., 2004), and turnover (Kim et al., 2005). Tissue concentrations of 5-HT are reduced in these mice (Fox et al., 2008). Heterozygous mice (HET) have approximately 50% fewer 5-HTT binding sites, elevated extracellular 5-HT but unchanged 5-HT tissue concentrations (Mathews et al., 2004; Kim et al., 2005). Behaviorally, the loss of 5-HTT results in a number of phenotypes including increased anxiety (Holmes et al., 2003; Kalueff et al., 2007), increased behavioral despair (Zhao et al., 2006; Wellman et al., 2007) and impaired fear extinction (Wellman et al., 2007).

Although these mice have been studied extensively, little is known regarding autism-relevant phenotypes in this strain. Given the considerable evidence of disturbed 5-HTT functioning in ASD, comprehensive phenotyping of mice with targeted 5-HTT disruptions may clarify the mechanisms underlying autism-relevant behaviors.

The purpose of the current study was to examine the consequences of 5-HTT inactivation on autism-relevant behaviors in adult male mice. Deficits in social interactions, a core feature of autism, were assessed in a semi-natural visible burrow system (VBS), three-chamber social approach and social proximity tests. Aggression was examined in the resident intruder test. Deficits in communication, another core symptom of autism, were assessed by the analysis of scent marking to a conspecific. Lastly, the presence of stereotyped and repetitive behaviors was assessed by analysis of grooming patterns and spontaneous investigation of novel objects.

3.3. Materials and Methods

3.3.1. Subjects

Subjects were male homozygous knockout (KO), heterozygous knockout (HET) and wild type (WT) littermates (n=12 per genotype) bred in the University of Hawaii Laboratory Animal facilities. The original breeding stock was obtained from The Jackson Laboratory (Bar Harbor, ME), where the 5-HTT KO mice were generated and backcrossed on a C57BL/6J (B6) strain. Mouse genotypes were determined using purified DNA from tail biopsy after weaning at postnatal day 21. Genotypes were identified by gel electrophoresis of DNA fragments of either 318 bp (WT), 210 bp (KO) or both (HET). Subjects were marked with commercial hair dye (Jerome Russel hair bleach) and ear tags for individual identification. Stimulus mice used in the social behavior tests were adult male C57/BL6J (B6) mice again bred in-house from stock.
obtained from The Jackson Laboratory (Bar Harbor, ME). Before and after weaning, mice were maintained on a 12-hour light-dark cycle (lights on at 6:00am) with ambient temperature at 22±2°C. After weaning, mice were housed with same-sex siblings in groups of 2-6 in ventilated cages (35.5 W x 20 L x 13 cm H) until behavioral testing. Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals and approved by the University of Hawaii Laboratory Animal Service Institutional Care and Use Committee.

3.3.2. Apparatus

*Visible Burrow System*

The visible burrow system (VBS) procedure was conducted in a large, rectangular, galvanized metal bin (86 x 61 x 26 cm H) lined with sawdust bedding as previously described (Pobbe et al., 2010). Briefly, a black Plexiglas wall divided the VBS into an open "surface" area and a "burrow" consisting of three clear Plexiglas chambers (12 x 7 x 6 cm H). Two of the chambers were connected to each other and to the surface by clear Plexiglas tubes, while the third chamber was connected only to the surface by a single clear Plexiglas tube. The tubes were fitted into the black dividing wall permitting free access between the chambers and the surface. Food and water were freely available on the surface.

*Three-Chamber*

The three-chamber apparatus was constructed of black Plexiglas (47 x 70 x 28 cm H) as previously described (Moy et al., 2004). The enclosure was divided into three equal sized compartments connected by manually operated doors. The outer compartments each contained an inverted wire cup (Galaxy Cup, Kitchen plus) weighed down by an empty glass jar. Clear Plexiglas inserts in the front wall of the apparatus allowed recording of interactive behaviors.

*Autogrooming*

Autogrooming behavior was assessed in a rectangular, clear Plexiglas chamber (7 x 14 x 30 cm H). A wire mesh lid on top of the chamber allowed air circulation and prevented escape.
Social Proximity

Social proximity testing was conducted in the same rectangular, clear Plexiglas chamber (7 x 14 x 30 cm H) used in the Autogrooming test.

Repetitive Novel Object Contact Task

The repetitive novel object contact task was conducted in a clean standard laboratory mouse cage (35.5 x 20 x 13 cm H) with approximately 1 cm of bedding on the floor. An inverted Plexiglas mouse cage was placed as a lid for the test chamber which permitted recording of the subject from above. The lid was divided into four equally sized quadrants using laboratory tape to measure general locomotion.

Urinary Scent Marking

The scent marking arena was an inverted rat cage (43 x 22 x 20 cm H) bisected by a wire mesh barrier which allowed the exchange of olfactory, visual, and auditory cues, while preventing direct physical contact between the subject and stimulus. The arena was lined with a 45 x 61 cm piece of drawing paper as substrate.

Resident Intruder

The resident intruder test was conducted in the home cage of the subject mouse (35.5 x 20 x 13 cm H) with the food and water hopper removed.

3.3.3. Procedure

Behavioral Testing

Behavioral testing of subjects took place between postnatal days 70-100. All subjects were exposed to the same series of behavioral procedures in the following order: visible burrow system, three-chamber social approach, autogrooming, social proximity, repetitive novel object contact, urinary scent marking, and resident intruder tests. Tests were separated by 24 hours, except in the case of scent marking: Immediately after the repetitive novel object contact test mice were singly housed for 15 days to encourage territorial scent marking (Arakawa et al., 2009). All behavioral tests were performed under ambient fluorescent lighting (120 lx) during the light phase of the light/dark cycle, between 0900h and 1700h. Temperature (22±2°C) and humidity (70%) were controlled in the experimental room. All experimental and stimulus mice were habituated to the experimental room at least 30 minutes prior to testing. To eliminate odor cues, each apparatus was thoroughly cleaned with 20% ethanol and dried with paper towels between
animals. All procedures were recorded for subsequent scoring by a trained observer blind to the genotype of the subjects. Behavior was analyzed using Observer software (Noldus Information Technology, Wageningen, The Netherlands) and J Watcher software (Blumstein and Daniel, 2007).

Visible burrow system

Testing for social behaviors in the VBS was conducted as previously described (Pobbe et al., 2010). Three days prior to colony formation, subjects were briefly anesthetized and given a unique identifying mark with commercial hair dye (Jerome Russel hair bleach). Colonies were formed by placing three male, non-littermate mice from the same genotype into the apparatus at the beginning of the first dark period (1800h). Colonies were maintained for 3 dark and 3 light periods. Video recordings were collected for four hours during each light (0600-1100h) and dark (1700h-2200h) period. The frequencies of behaviors for each mouse were analyzed by time sampling for 30 seconds, every 10 minutes. The following behaviors were counted: approaches to the front of another mouse, approaches to the back of another mouse, huddling, alone, allogrooming, self-grooming, chase, flight, follow, and vigorous contact (Arakawa et al., 2007b). The mean frequencies of each behavior were summarized for each genotype and for each light and dark phase during the three successive days and nights of the VBS period.

Three-chamber social approach test

Social approach behavior was assessed as previously described (Moy et al., 2004). Initially the subject mouse was placed into the center compartment and allowed to explore the apparatus for 10 minutes. At the end of this habituation period, the subject was returned to the center chamber, and an unfamiliar, same-sex, B6 stimulus mouse was placed into one of the wire cups in the outer chambers, while the other wire cup remained empty. The side containing the stimulus mouse was counterbalanced within groups. The subject mouse was allowed to explore the entire chamber for an additional 10 minutes. The frequency and duration of time spent in each of the chambers during the habituation and testing period were recorded. Additionally, during the test phase, the frequency and duration of investigative behaviors including: rear, self-grooming, contact with the cup,
sniff, stretch-attend, quick-withdraw, and nose-to-nose with the stimulus animal were recorded.

**Autogrooming**

Grooming behavior was assessed as previously described (Pearson et al., 2011). Mice were placed into the grooming apparatus for 20 minutes. The frequency and duration of grooming episodes involving the paw, head, body, leg, or tail/genital areas were scored. The frequencies of grooming bouts, interrupted bouts, transitions, incorrect transitions, correct transitions, the percent of interrupted bouts and of incorrect transitions were also measured. A grooming bout was an uninterrupted occurrence of grooming followed by at least 6 seconds of non-grooming. An interrupted bout was a bout of grooming that was interrupted by less than 6 seconds. The percentage of interrupted bouts was calculated by dividing the number of interrupted bouts by the total number of bouts. Transitions were identified as a change from one grooming site to another.

Correct transitions included transitions of grooming that followed the typical rostral-caudal progression (paw/head/body/tail) (Kalueff and Tuohimaa, 2004). Incorrect transitions were characterized by changes in grooming sites that did not follow the typical rostral-caudal pattern. The percentage of incorrect transitions was calculated by dividing the number of incorrect transitions by the total number of transitions.

**Social proximity**

Social proximity testing was conducted as previously described (Defensor et al., 2011). The subject mouse was placed into the apparatus with an age and weight matched, unfamiliar male B6 mouse for 10 minutes. The frequency and duration of the following behaviors were quantified:

- **Nose tip-to-Nose tip (NT):** the subject animal’s nose tip and/or vibrissae contacts the nose tip and/or vibrissae of the stimulus animal.
- **Nose-to-Head (NH):** the subject animal's nose tip and/or vibrissae contacts the head of the stimulus animal.
- **Nose-to-Anogenital (NA):** the subject animal's nose tip contacts the anogenital region of the stimulus animal.
Crawl Over (CO): the subject crawls over the stimulus animal, crossing over at least half of the stimulus animal's body.

Crawl Under (CU): the subject pushes under or crawls under the stimulus animal, crossing under at least half of the stimulus animal.

Allogroom (AG): the subject grooms the stimulus animal.

Self Groom (SG): the subject animal grooms its own body.

Repetitive novel object contact task

Investigation of novel objects was conducted as previously described (Pearson et al., 2011). Subjects were first habituated to a clean mouse cage containing fresh sawdust bedding. A clear, Plexiglas mouse cage was inverted and marked with four equal sized quadrants using laboratory tape. This was then placed on top of the cage to permit video recording from above. Mice were allowed to explore the apparatus for 20 minutes. The number of quadrant entries was recorded for baseline locomotor activity. Twenty-four hours later subjects were again placed into a similar clean cage containing fresh bedding. A small novel plastic toy object was placed in each of the four corners, approximately 2 cm from the wall. The toys included: a Lego piece, a jack, a die, and a bowling pin. The mice were allowed to explore the apparatus and the novel toys for 10 minutes. The number of visits to each toy, the sequence of toy visits, and the frequency of repetitive contacts with 3, or with 4 toys were scored. In order to determine if there was a genotype effect on the tendency to prefer specific toys, the frequency of contacts with each of the toys were ranked in decreasing order from maximum to minimum preference for each subject, averaged by genotype and compared.

Scent Marking

Testing for scent making was conducted as previously described (Arakawa et al., 2007a). Following the toy test, subjects were individually housed for 15 days. Twenty-four hours prior to the scent marking test, subjects were placed in one compartment of the apparatus for a 20 minute habituation period. At the same time the next day, the subject was again placed into one side of the chamber and an unfamiliar male B6 mouse was placed in the opposite compartment, separated by the wire mesh. Following the trial the
paper substrate was removed and fixed with a 6% Ninhydrin solution (Fisher) that binds to proteins in the urine making them visible. After drying for 24 hours the number of urinary scent marks was manually counted by placing a transparent 1 x 1 cm square grid sheet on top of the marked sheet and counting the number of squares containing scent marks.

**Resident-Intruder**

The resident-intruder test was conducted as previously described (Pearson et al., 2012). A sex, age and weight matched B6 intruder was placed into the resident's home cage for 10 minutes. The latency of the resident to attack was recorded. Additionally, the frequency and duration of investigative behaviors including: sniff face, sniff body, sniff rear, sniff tail, vigorous allogroom, attack, follow, upright, and tail rattle were recorded (Pearson et al., 2012).

### 3.3.4. Statistical Analyses

Each of the behaviors recorded in the VBS was analyzed by two-way repeated measures analysis of variance (ANOVA), with genotype as the between-subjects factor and period as the repeated-measures within-subject factor. Separate comparisons were conducted for the dark and light periods. Data from the three-chamber test were analyzed by two-tailed Student's t-test to compare the time spent in the empty chamber with time spent in the stimulus chamber for each genotype. One-way ANOVAs were used to compare the frequencies and durations of behaviors in the autogrooming task, social proximity test, and resident-intruder test. Scent marking data were analyzed by two-way repeated-measures ANOVA, with genotype as the between-subjects factor and condition (habituation vs. test) as the repeated-measures within-subjects factor. Post-hoc analyses, when appropriate were conducted using Newman-Keul’s. All statistical analyses were carried out using Statistica and significance was set at $p < 0.05$ for all tests.

### 3.4. Results

#### 3.4.1. Visible Burrow System

Repeated measures ANOVA revealed that the frequency of social investigation for all genotypes decreased across dark periods, suggesting habituation to the social environment (Table 3.1). This main effect of dark period was observed for approach front [$F(2,6) = 11.61, p < 0.001$], approach back [$F(2,66) = 10.04, p < 0.001$], vigorous
contact \( F(2,66) = 6.82, p < 0.01 \), huddle \( F(2,66) = 36.51, p < 0.001 \) and allogrooming \( F(2,66) = 4.69, p < 0.01 \). There was no effect of genotype, or any genotype x period interactions for any of behaviors measured during the dark periods. A main effect of genotype on vigorous contact during the dark periods approached but did not reach significance \( (p < 0.07) \). The average number of vigorous behaviors for each genotype suggests that both WT and KO had more vigorous contacts then HETs (WT =1.16, HET = 0.25, KO = 0.61). During the light periods, neither a main effect of genotype nor period was observed for any of the behaviors measured (Table 3.1). Approaches to the back and vigorous contacts were nearly absent in all genotypes during the light period and were not able to be analyzed. Several genotype x light period interactions were significant. For 5-HTT KO mice, following \( F(4,66) = 2.80, p < 0.05; \) Figure 3.1A] was increased during light period 3 when compared to light periods 1 and 2. Huddling behavior also increased in the 5-HTT KO mice during light periods 2 and 3 when compared to light period 1\( F(4,66) = 4.71, p < 0.01; \) Figure 3.1B]. Similarly, the frequency of being alone decreased in 5-HTT KO mice during light period 2 and 3 when compared to light period 1\( F(4,66) = 4.31, p < 0.01, \) Figure 3.1C].
## Table 3.1. Statistical results for behaviors run in the VBS.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Main Effects</th>
<th>Genotype</th>
<th>Period</th>
<th>Strain x Period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Approach Front</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dark</td>
<td>F(2,33)=117.32, p=0.25, n.s.</td>
<td>F(2,33)=11.61, p&lt;0.0001</td>
<td>F(2,33)=0.91, p=0.41, n.s.</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=1.90, p=0.17, n.s.</td>
<td>F(2,33)=2.89, p=0.06, n.s.</td>
<td>F(2,33)=0.91, p=0.41, n.s.</td>
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<tr>
<td><strong>Approach Back</strong></td>
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<td></td>
</tr>
<tr>
<td>Dark</td>
<td>F(2,33)=0.91, p=0.41, n.s.</td>
<td>F(2,33)=10.04, p&lt;0.0001</td>
<td>F(2,33)=0.91, p=0.41, n.s.</td>
<td></td>
</tr>
<tr>
<td>Light</td>
<td>N.A.</td>
<td>F(2,33)=10.04, p&lt;0.0001</td>
<td>F(2,33)=0.91, p=0.41, n.s.</td>
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<tr>
<td><strong>Flight</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=0.54, p=0.58, n.s.</td>
<td>F(2,33)=2.08, p=0.13, n.s.</td>
<td>F(2,33)=1.90, p=0.17, n.s.</td>
<td>F(2,33)=0.54, p=0.58, n.s.</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=0.07, p=0.92, n.s.</td>
<td>F(2,33)=0.59, p=0.56, n.s.</td>
<td>F(2,33)=0.07, p=0.92, n.s.</td>
<td>F(2,33)=0.07, p=0.92, n.s.</td>
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<tr>
<td><strong>Chase</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=0.41, p=0.66, n.s.</td>
<td>F(2,33)=1.59, p=0.21, n.s.</td>
<td>F(2,33)=0.41, p=0.66, n.s.</td>
<td>F(2,33)=1.59, p=0.21, n.s.</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=0.31, p=0.74, n.s.</td>
<td>F(2,33)=0.89, p=0.42, n.s.</td>
<td>F(2,33)=0.31, p=0.74, n.s.</td>
<td>F(2,33)=0.89, p=0.42, n.s.</td>
</tr>
<tr>
<td><strong>Follow</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=2.03, p=0.15, n.s.</td>
<td>F(2,33)=0.27, p=0.77, n.s.</td>
<td>F(2,33)=2.03, p=0.15, n.s.</td>
<td>F(2,33)=0.27, p=0.77, n.s.</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=1.91, p=0.16, n.s.</td>
<td>F(2,33)=2.49, p=0.09, n.s.</td>
<td>F(2,33)=1.91, p=0.16, n.s.</td>
<td>F(2,33)=2.49, p=0.09, n.s.</td>
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<tr>
<td><strong>Vigorous</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=2.92, p=0.07, n.s.</td>
<td>F(2,33)=6.82, p&lt;0.002</td>
<td>F(2,33)=2.92, p=0.07, n.s.</td>
<td>F(2,33)=6.82, p&lt;0.002</td>
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<tr>
<td>Light</td>
<td>N.A.</td>
<td>F(2,33)=6.82, p&lt;0.002</td>
<td>F(2,33)=2.92, p=0.07, n.s.</td>
<td>F(2,33)=6.82, p&lt;0.002</td>
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<tr>
<td><strong>Huddle</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=2.58, p=0.09, n.s.</td>
<td>F(2,33)=36.51, p&lt;0.0001</td>
<td>F(2,33)=2.58, p=0.09, n.s.</td>
<td>F(2,33)=36.51, p&lt;0.0001</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=2.09, p=0.14, n.s.</td>
<td>F(2,33)=1.97, p=0.15, n.s.</td>
<td>F(2,33)=2.09, p=0.14, n.s.</td>
<td>F(2,33)=1.97, p=0.15, n.s.</td>
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<tr>
<td><strong>Alone</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=1.80, p=0.18, n.s.</td>
<td>F(2,33)=1.34, p=0.27, n.s.</td>
<td>F(2,33)=1.80, p=0.18, n.s.</td>
<td>F(2,33)=1.34, p=0.27, n.s.</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=1.21, p=0.31, n.s.</td>
<td>F(2,33)=1.34, p=0.27, n.s.</td>
<td>F(2,33)=1.21, p=0.31, n.s.</td>
<td>F(2,33)=1.34, p=0.27, n.s.</td>
</tr>
<tr>
<td><strong>Allogroom</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=1.32, p=0.28, n.s.</td>
<td>F(2,33)=4.68, p&lt;0.05</td>
<td>F(2,33)=1.32, p=0.28, n.s.</td>
<td>F(2,33)=4.68, p&lt;0.05</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=0.04, p=0.96, n.s.</td>
<td>F(2,33)=2.77, p=0.07, n.s.</td>
<td>F(2,33)=0.04, p=0.96, n.s.</td>
<td>F(2,33)=2.77, p=0.07, n.s.</td>
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<tr>
<td><strong>Selfgroom</strong></td>
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<tr>
<td>Dark</td>
<td>F(2,33)=2.31, p=0.11, n.s.</td>
<td>F(2,33)=0.35, p=0.71, n.s.</td>
<td>F(2,33)=2.31, p=0.11, n.s.</td>
<td>F(2,33)=0.35, p=0.71, n.s.</td>
</tr>
<tr>
<td>Light</td>
<td>F(2,33)=0.98, p=0.39, n.s.</td>
<td>F(2,33)=0.12, p=0.89, n.s.</td>
<td>F(2,33)=0.98, p=0.39, n.s.</td>
<td>F(2,33)=0.12, p=0.89, n.s.</td>
</tr>
</tbody>
</table>

N.A. not able to be analyzed.
Figure 3.1. Frequency of social behaviors during the light phases in the VBS.
Frequency of following (A), huddling (B), and alone (C) behaviors in the VBS. Values are displayed as mean (± SEM) for each genotype. # p < 0.05, ## p < 0.01, within-group comparison, different from light period 1. & p < 0.05, within-group comparison, different from light period 2.
3.4.2. Three Chamber

During the 10 minute habituation phase, no differences in locomotor activity [$F(2,33) = 0.53, p > 0.05$, data not shown] were observed between genotypes. Similarly, no side biases were found during the habituation phase (WT: $t(22) = -0.20, p > 0.05$; HET: $t(22) = -1.08, p > 0.05$; KO: $t(22) = -1.23, p > 0.05$, data not shown). During the testing phase, 5-HTT KO mice displayed reduced locomotion when compared to HET littermates. KOs made fewer total compartment entries [$F(2,33) = 3.46, p < 0.05$, Figure 3.2A], fewer social compartment entries [$F(2,33) = 3.81, p < 0.05$] and fewer center compartment entries [$F(2,33) = 3.52, p < 0.05$] when compared to HET mice (Figure 3.2B). Figure 3.3 displays the amount of time each genotype spent in each of the compartments of the apparatus during the testing phase. T-tests indicated that all genotypes displayed a social preference, indicated by spending more time in the compartment containing the unfamiliar mouse when compared to the empty compartment (WT: $t(22) = 5.75, p < 0.001$; HET: $t(22) = 3.76, p < 0.001$; KO: $t(22) = 2.76, p < 0.05$). The genotypes did not differ in the frequencies of social behaviors recorded during the testing phase (data not shown).
Figure 3.2. Locomotion in the three chamber test.
Frequency of total (A) and individual (B) chamber entries in the three chamber test. Values are displayed as mean ± SEM. * p < 0.05, between-group comparison, different from the same measure in HET mice.

Figure 3.3. Time spent in each chamber in the three chamber test.
Values are displayed as mean ± SEM. # p < 0.05, ## p < 0.001, ### p < 0.001, within-group comparison, different from empty chamber.
3.4.3. Autogrooming

In the autogrooming test, KO mice had significantly increased frequencies of paw grooming \([F(2,33) = 7.13, p < 0.01; \text{Figure 3.4A}]\) when compared to WT and HET mice. KO mice also showed increased duration of paw \([F(2,33) = 8.35, p < 0.01]\) and head grooming \([F(2,33) = 4.30, p < 0.05]\) (Figure 3.4B) compared to WT and HET littermates. With regards to the pattern of grooming, KO mice had more completed bout sequences \([F(2,33) = 11.60, p < 0.001; \text{Figure 3.4C}]\) and correct transitions \([F(2,33) = 6.84, p < 0.01; \text{Figure 4D}]\) compared to WT and HET littermates. No significant differences were found for the number of bouts, interrupted bouts, proportion of interrupted bouts, transitions, incorrect transitions, or proportion of incorrect transitions.
Figure 3.4. Behaviors displayed in the autogrooming test.
Frequency (A) and duration (B) of body site grooming. Frequency of grooming bouts (C) and transitions (D). Values are displayed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, compared to WT and HET mice.
3.4.4. Social Proximity

In the social proximity chamber, HET mice displayed higher frequencies \( F(2,33) = 5.00, p < 0.05; \) Figure 3.5A] and longer durations of allogrooming \( F(2,33) = 4.06, p < 0.05; \) Figure 3.5B] when compared to WT and KO mice. No other significant differences between genotypes were found.

Figure 3.5. Behaviors displayed in the social proximity test. Values are displayed as mean ± SEM. NT, nose-to-nose; NH, nose-to-head; NA, nose-to-anogenital; CU, crawl under; CO, crawl over; AG, allogroom; SG, selfgroom. * \( p < 0.05, \) compared to WT and KO mice.
3.4.5. Repetitive Novel Object Contact

During the habituation phase of the repetitive novel object contact test, no significant differences were found between any of the genotypes in the number of transitions between quadrants [$F(2,33) = 3.01, p > 0.05$, Figure 3.6A]. There was a trend for KO mice to make fewer line crosses during the habituation phase, but this failed to reach statistical significance (WT = 155, HET = 144, KO = 122, p < 0.06). During testing, the genotypes did not differ in the frequency of visits to any of the four novel objects (Dice: [$F(2,33) = 0.60, p > 0.05$]; jax: [$F(2,33) = 0.34, p > 0.05$]; Lego: [$F(2,33) = 0.89, p > 0.05$]; bowling pin: [$F(2,33) = 0.78, p > 0.05$]; Figure 3.6B). Similarly, no effect of genotype was observed for the tendency to display a preference for a particular object ($F(2,33) = 0.56, p > 0.05$, Figure 3.6C). There were no differences in the number of visits to identical sequences of three [$F(2,33) = 2.48, p > 0.05$] or four [$F(2,33) = 2.48, p > 0.05$] objects (Figure 3.6D).
Figure 3.6. Behaviors displayed in the repetitive novel object task.
Number of transitions during habituation (A). Rate of novel object investigation (B).
Object preference ratings (C). Frequency of sequenced toy visits (D). Values are displayed as mean ± SEM.
3.4.6. Scent Marking

Repeated measures ANOVA revealed a significant main effect of genotype for the number of scent marks deposited. In total, HET mice deposited more scent marks than both WT and KO mice across both conditions [$F(2,33) = 8.69, p < 0.001$, Figure 3.7]. No effect of condition [$F(1,33) = 1.93, p > 0.05$] was observed for any of the genotypes. Further, no significant genotype x condition interactions were found [$F(2,33) = 0.68, p > 0.05$].

![Figure 3.7. Scent marking behavior.](image)

Number of squares containing scent marks during habituation and social conditions. Values are displayed as mean ± SEM. *** $p < 0.001$; main effect of genotype when compared to WT and KO mice.
3.4.7. Resident Intruder

No differences in latency to attack were observed between any of the genotypes \(F(2,33) = 0.56, p > 0.05, \) Figure 3.8A). There were no differences in the frequency (Figure 3.8B) or duration (Figure 3.8C) of any of the behaviors measured in the resident intruder test.

Figure 3.8. Behaviors displayed in the Resident Intruder Test.
Latency to attack (A), frequency (B) and duration (C) of aggressive behaviors in the resident intruder test. Values are expressed as mean ± SEM. SF, sniff face; SB, sniff body; SR, sniff rear; ST, sniff tail; VG, vigorous groom; AT, attack; FW, follow; UP, upright; TR, tail rattle.
3.5. Discussion

Autism is a behaviorally defined disorder with no known biological markers or screening tests (American Psychiatric Association, 2013). Although the causes of autism are still unclear, several lines of evidence suggest that the 5-HT system may play an important role (Cook and Leventhal, 1996). Variants in the 5-HTT gene are associated with an increased risk for autism (International Molecular Genetic Study of Autism Consortium, 2001; Devlin et al., 2005). Reduced 5-HTT binding has also been reported in people with autism (Makkonen et al., 2008; Nakamura et al., 2010; Oblak et al., 2013) and in rodent models of the disorder (Gould et al., 2011). In laboratory rodents, genetic and pharmacological inactivation of 5-HTT produces physiological and behavioral alterations relevant to conditions associated with autism, such as: gastrointestinal dysfunction (Coates et al., 2006), exaggerated neuroendocrine and sympathoadrenal responses to stress (Tjurmina et al., 2002; Jiang et al., 2009), and heightened anxiety (Holmes et al., 2003).

In the current study sociability was not significantly influenced by 5-HTT genotype. Social behaviors were assessed in three tests: the VBS, three-chamber social approach, and social proximity. There were no effects of genotype for any of the behaviors measured during the dark/active periods in the VBS. During the light periods, following and huddling increased over the course of testing while being alone decreased only in 5-HTT KO mice. Notably, Lewejohann et al., (2010) also failed to find reductions in social behaviors in 5-HTT KO mice on a B6 background, housed in enriched environments with same-genotype conspecifics. 5-HTT deficiency failed to alter social approach or social interaction in the three-chamber and social proximity tests, respectively. A previous study reported reduced sociability in these 5-HTT KO mice in the three-chamber social approach test (Moy et al., 2009). However, both the current study and the Moy et al., (2009) report, failed to find genotype differences in the amount of social exploration (sniffing) of the social stimulus. Taken together, these findings suggest that the very minimal reductions in social interactions in 5-HTT KO mice likely reflect the anxious and hypoactive phenotype of this strain (Kalueff et al., 2007), rather than a deficit in social motivation.
Aggression is an additional component of many rodent social interactions (Blanchard and Blanchard, 1989). A previous study reported longer attack latencies and reduced aggression in 5-HTT KO mice in the resident intruder paradigm (Holmes et al., 2002). However, in the current study, we found no differences between genotypes in the latency to attack or in the number of aggressive behaviors displayed in the resident intruder test. In line with our results, two recent studies reported no differences in aggression between 5-HTT KO and WT mice confronted with an intruding conspecific in their own territory (Jansen et al., 2011; Kloke et al., 2011). Given that aggressive behavior depends significantly on the type of opponent used (Brain et al., 1981; Miczek et al., 2001), these conflicting results are likely due to differences in the aggressiveness of the intruder used in each study. Holmes et al. used the more aggressive DBA/2J mouse, in comparison to the more docile C3H mice used by Kloke et al. and Jansen et al., and the B6 used in the current study (Jones and Brain, 1987; Guillot and Chapouthier, 1996).

Conspecific aggression may be modulated by a variety of factors including the behavior of the opponent (Brain et al., 1981; Miczek et al., 2001), sexual experience of the aggressor (O’Donnell et al., 1981), previous social experiences (Flannelly et al., 1984), and the territory where it takes place (Teskey and Kavaliers, 1987). In the current study, all genotypes showed comparable levels of aggression when housed in same-genotype colonies in the VBS. Similarly, a previous study (Lewejohann et al., 2010) found that 5-HTT KO mice were able to establish and maintain dominance hierarchies through aggression when housed in same-genotype groups. However, when the 5-HTT KO mice were placed in mixed genotype groups, the KOs were less likely to establish a dominant position and were more likely to be injured by the other mice (Lewejohann et al., 2010). Jansen and colleagues (2011) suggested that the aggression displayed by the 5-HTT KO mice in the resident-intruder paradigm was influenced by the opponent's behavior and the environmental situation (own territory, opponent's territory or neutral area) whereas 5-HTT wildtypes showed similar levels of aggression regardless of the opponent or environment. Similarly, Kloke et al. (2011) found that previous experiences as a winner or loser in agnostic encounters influenced aggressive behavior in the 5-HTT mutant mice, while 5-HTT genotype did not.
Scent marking is a primary method of communication for mice and is used to convey information about social status, territory boundaries, and reproductive abilities (Arakawa et al., 2007a). In the current study, there were no differences between 5-HTT WT and KO mice in scent marking to a male stimulus. This is consistent with our findings of normal territorial aggression in these mice in the resident intruder test.

Stereotyped and repetitive behaviors represent a core feature of autism. Clinically, mutations in the 5-HTT gene have been associated with repetitive and compulsive behaviors (Sutcliffe et al., 2005; Voyiaziakis et al., 2011). Here, 5-HTT KO mice displayed higher frequencies and durations of grooming. Additionally, KO mice had more correct transitions (transitions following the typical cephalo-caudal progression) and more completed bouts of grooming (paw/head/body/tail), suggesting adherence to a more rigid and fixed pattern of grooming. Although stress has been shown to increase grooming in rodents (Kalueff and Tuohimaa, 2004; Hart et al., 2010), it is unlikely that the current results reflect a heightened anxiety response in this test. Stress induced grooming is characterized by more incorrect transitions and interrupted bouts (Kalueff and Tuohimaa, 2004); neither of which were displayed by the KO in this test.

No differences were found between genotypes in the novel object contact test, designed to assess restricted interests. KO and WT mice explored the items with similar frequency, displayed similar preferences for objects, and did not differ on the number of sequenced visits to 3 or 4 toys. These data suggest that reduced 5-HTT function may contribute to stereotyped motor behaviors, but provide no evidence for 5-HTT deficiency in regulating restricted interest. Our findings are in line with clinical data that report a negative correlation between 5-HTT binding and repetitive behaviors in adults with autism (Nakamura et al., 2010).

This study provided a wide ranging evaluation of the effects of life-long 5-HTT deficiency on rodent behaviors designed to parallel the core symptoms of ASD. Although variants in the 5-HTT gene have been implicated in autism (Cook and Leventhal, 1996; Sutcliffe et al., 2005), this extensive phenotyping of the 5-HTT KO mouse revealed only modest and limited disturbances in sociability and repetitive behaviors. This relative lack of overt behavioral differences between genotypes may
reflect compensatory molecular mechanisms of the 5-HT system in these mice. For example, gene expression for an organic cation transporter with low affinity for 5-HT is up-regulated in the brain of 5-HTT KO mice (Schmitt et al., 2003). Additionally, 5-HTT KO mice also display decreased 5-HT$_{1A}$ autoreceptor mRNA, protein levels and binding sites (Kalueff et al., 2010). These alterations likely represent mechanisms to limit the adverse effects of continually elevated extracellular 5-HT concentrations.

Furthermore, 5-HTT deficiency may increase one's susceptibility to environmental challenges or toxins. Reduced 5-HTT gene expression is associated with increased susceptibility to stress (Kendler et al., 1999; Canli and Lesch, 2007) and environmental influences such as stressful life events may further impinge on 5-HTT expression via transcriptional and post-transcriptional mechanisms to contribute to autism pathology. Given that autism likely results from a complex interaction between genetic susceptibility and environmental insults (Trottier et al., 1999), it is possible that additional environmental or epigenetic factors may be important in the manifestation of an autism-relevant behavioral phenotype in the 5-HTT deficient mice.
3.6. References


CHAPTER 4. CHARACTERIZATION OF THE SEROTONERGIC PROFILE OF THE SUBVENTRICULAR ZONE (SVZ) IN INDIVIDUALS WITH AUTISM AND TYPICALLY-DEVELOPING CONTROLS.

4.1. Abstract

Rationale

Autism is a neurodevelopmental disorder associated with alterations in early brain growth (Courchesne et al., 2007). The SVZ neurogenic niche is a unique microenvironment critical for supporting the development and plasticity of the brain (Brazel et al., 2003; Quiñones-Hinojosa et al., 2006, 2007; Gage et al., 2008; Kazanis et al., 2008). Neurons expressing 5-HT innervate the SVZ and regulate its activity (Brezun and Daszuta, 1999; Banasr et al., 2004; Jahanshahi et al., 2011). The 5-HT system is involved in brain development (Daubert and Condron, 2010) and is implicated in autism (Zafeiriou et al., 2009). Therefore, disturbances in the serotonergic composition and regulation of the SVZ could play a critical role in autism pathogenesis. However, no studies to date have investigated the 5-HT innervation of the SVZ in autism.

Objectives

The purpose of the current experiment was to characterize the cellular organization and the distribution of 5-HT transporters (5-HTT) in the human SVZ. Furthermore, a primary goal of these studies was to compare the distribution patterns and densities of 5-HTT in autism-diagnosed (AD) and typically-developing (TD) individuals across the lifespan.

Methods

5-HTT protein expression in the SVZ from 4 pairs of age and sex matched AD and TD cases was confirmed by Western Blot. The cellular organization and composition of the SVZ was assessed using immunohistochemistry techniques.

Results

The current results confirmed and expanded on findings of altered SVZ structure and molecular composition in AD and TD cases. The hypocellular gap was significantly smaller and more densely packed with displaced cells in AD cases when compared to TD
controls. 5-HTT immunoreactivity was significantly increased in the SVZ of the youngest AD cases compared to the matched TD control. FGF-2, a growth factor responsive to 5-HT, was also increased in the younger AD cases. Evidence of occasional neuronal and microglia staining was observed in all cases.

Conclusions

These results are the first known demonstration of altered 5-HTT density in AD SVZ tissue. Altered composition of molecular factors in the SVZ may contribute to the altered brain growth trajectory reported in the disorder. Enhanced understanding of the composition and activity of this brain neurogenic niche could contribute to improved treatment for autism and similar neurodevelopmental disorders.
4.2. Introduction

Autism is a widespread, heterogeneous developmental disorder that affects 1 in 50 children in the United States (Blumberg et al., 2013). The disorder is characterized by impairments in sociability and communication and the presence of stereotyped behaviors (American Psychiatric Association, 2013). Further complicating research efforts is the fact that no unifying pathology exists (Palmen et al., 2004b) and there are no consistently effective treatments (Canitano and Scandurra, 2011).

While the neurobiological basis for autism remains largely unknown, evidence suggests that fundamental neurodevelopmental mechanisms are altered in the disorder (Persico and Bourgeron, 2006). Developmental changes such as altered cellular growth and migration, disrupted structural organization and connectivity, as well as altered cellular communication (Persico and Bourgeron, 2006; Ecker et al., 2013) are reported in the brains of autistic individuals. These findings underscore the importance of investigating cellular and molecular processes involved in brain development in autism.

The subventricular zone of the lateral ventricle (SVZ) is one of only two areas in the human brain, the other being the hippocampus, that generates new neurons throughout life (Quiñones-Hinojosa et al., 2006, 2007). The SVZ is visible around 7-8 gestational weeks in humans (Zecevic et al., 2005), undergoes extreme prenatal growth (Brazel et al., 2003), and generates neurons and glia that populate the prefrontal cortex and various subcortical structures (Brazel et al., 2003; Zecevic et al., 2005; Bayatti et al., 2008; Sanai et al., 2011). Later in development the SVZ shrinks in size but retains its neurogenic capabilities, producing new neurons and astrocytes into adulthood (Eriksson et al., 1998; Sanai et al., 2004, 2011).

The SVZ is composed of specific cell-cell contacts, extracellular matrix molecules as well as paracrine signals from growth factors and neurotransmitters (Decimo et al., 2012). This specialized microenvironment or “neurogenic niche” is critical for supporting and controlling the proliferation, migration, differentiation, and survival of neural stem cells (NSC) (Scadden, 2006). Neurons expressing 5-HT from the dorsal raphe provide a dense innervation of the SVZ, referred to as the supra- and subependymal 5-HT complex (Richards et al., 1981; Moore and Speh, 2004). 5-HT released from these neurons increases ependymal cell metabolism (Prothmann et al., 2001) and
ciliary beat frequency (Nguyen et al., 2001). These neurons expressing 5-HT may serve as a feedback loop, monitoring and regulating the flow and composition of cerebrospinal fluid (CSF) near the SVZ as well as throughout the brain (Mikkelsen et al., 1997).

Furthermore, infusion of 5-HT into the lateral ventricles increased NSC proliferation in the SVZ of rodents (Hitoshi et al., 2007) while 5-HT depletion reduced NSC proliferation (Brezun and Daszuta, 1999). In animal models, the administration of 5-HT agonists has been shown to enhance cell proliferation in the SVZ (Banasr et al., 2004; Hitoshi et al., 2007; Lau et al., 2007). These findings suggest an important role for 5-HT in modulating the microenvironment of the SVZ niche and the proliferation of NSC across the lifespan.

Neural proliferation is markedly increased in the SVZ of postmortem brains from autistic individuals (Kotagiri et al., 2013; Pearson et al., 2013). Components of the 5-HT system are also disturbed in various brain regions in the disorder (Cook and Leventhal, 1996). However, the role of the SVZ 5-HT subependymal plexus in autism has received little attention. Given the critical role of the SVZ in brain development and neuroplasticity, disturbed 5-HT release in this region could contribute to dysregulated neurogenesis, aberrant brain growth, and disturbed cerebral functioning. This study examined the SVZ in autism-diagnosed (AD) and typically-developing (TD) postmortem brains at different ages. Improved understanding of the composition and function of SVZ regulatory signals, such as 5-HT, may clarify the mechanisms underlying the neurodevelopmental origins of this disorder.

4.3. Materials and methods

4.3.1. Postmortem Brain Tissue Samples

Frozen postmortem tissue samples were obtained from the Harvard Brain Tissue Resource Center and the National Institute of Child Health and Human Development (NICHD) Brain and Tissue Bank for Developmental Disorders through the Autism Tissue Program of the Autism Speaks organization. Brain tissue was obtained from four male autism cases, confirmed by the Autism Diagnostic Interview Revised (ADI-R), and four control subjects matched for sex and age (within 1 year).

Postmortem donors' clinical records are available online from the Autism Tissue Program Data Portal (atpportal.org). All diagnoses of autism were based on responses to
the ADI-R by a parent or legal guardian of the deceased. An autism diagnosis is indicated when scores in all three behavioral domains meet or exceed the minimum cut off score (Lord et al., 1994). All cases met or exceeded the cutoffs for a diagnosis of autism using the ADI-R scale (Table 4.1). Control cases were determined to be free of neurological disorders including autism, based on information gathered at the time of death.

**Table 4.1. Diagnostic characteristics of autism cases**

<table>
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<th>ADI-Communication b</th>
<th>ADI-Restrictive &amp; Repetitive c</th>
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<td>22</td>
<td>14</td>
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ADI-R, Autism Diagnostic Interview-Revised

a Qualitative abnormalities in reciprocal social interaction (cutoff, 10; maximum, 30).
b Qualitative abnormalities in communication (cutoff, 7; maximum nonverbal communication, 14)
c Restricted, repetitive, and stereotyped patterns of interest (cutoff, 3; maximum, 12).

Donor brains were harvested and processed at either the Harvard or NICHD brain bank (Figure 4.1). Briefly, one hemisphere was fresh frozen and the other was formalin fixed. Coronal sections from the frozen hemisphere were used for analyses in these studies. Blocks of tissue were dissected from each coronal section along the dorsal-lateral ventricle wall including the anterior horn and body of the lateral ventricle. Blocks also included portions of the medial caudate nucleus and body of the corpus callosum. Coronal sections were obtained from three anterior-posterior sections for each case. Tissue blocks (2.0 cm$^3$) from each case were sectioned using a freezing microtome (Leica, CM1850UV) perpendicular to and containing the ependymal, subependymal and parenchymal zones in the coronal plane at 10 μM. Tissue sections (3-5 sections per slide) were mounted on Poly-L-lysine (1:5 Sigma Aldrich) coated glass microscope slides (VWR superfrost) and stored at -20°C until processing. Immunostaining was performed on 6-10 tissue sections per individual for each of the experiments described below. All tissue sections were coded and investigators were blind to the diagnosis.
Figure 4.1. Tissue acquisition and histology. Postmortem tissue processing protocol. (A) Donor brains were collected and processed at the brain bank. Hemispheres were either fresh frozen or fixed in formalin. (B) Frozen coronal sections were taken from one hemisphere. (C) Dissections of the subventricular zone were collected from three anterior-posterior coronal sections for each case. (D) Blocks of tissue were sectioned in our laboratory on a freezing microtome.
4.3.2. Procedure

*Immunofluorescent labeling*

Immunostaining of the serotonergic system in human post-mortem tissue is complicated by the fact that 5-HT is unstable and undergoes rapid degradation during the postmortem period (Azmitia and Nixon, 2008). For this reason studies of the morphology of the human 5-HT neurons must utilize antibodies generated against the more robust 5-HT-receptor proteins (Türk et al., 1992). In the current study, the membrane bound 5-HTT was used as a marker of serotonergic neurons. 5-HTT is a membrane bound protein which resides primarily in the axons and terminal buttons of serotonergic neurons (Azmitia and Nixon, 2008). Antibodies to 5-HTT provide patterns of staining that appear identical to those obtained with 5-HT antibodies in rodents; suggesting that 5-HTT is a faithful marker of 5-HT neurons in the brain (Zhou et al., 1996).

Prior to the study, careful pilot testing was done to determine optimal antibody concentrations, specificity and cross-reactivity (Table 4.2). The primary antibody concentrations were calibrated using dilutions of 1:50, 1:100, 1:200, and 1:400. Antibody specificity was determined by performing the standard incubations in the absence of the primary or secondary antibody. Tissue sections in which the primary antibody was omitted exhibited no immunostaining (data not shown). Control experiments were also conducted to ensure that antibodies did not cross-react with each other. To detect cross reactivity in double labeling experiments, each primary antibody in the pair was incubated with both secondary antibodies to be used in the double label. The control experiments showed that the secondary antibodies do not cross-react with each other (data not shown).
**Table 4.2. Antibodies**

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The selected tissue sections were processed using a standard immunohistochemistry staining procedure (Figure 4.2). Randomly selected slides from each case were fixed in -20°C acetone for two minutes and rinsed in phosphate buffered saline (PBS) for 5 minutes (Figure 4.2). Fixation is necessary to preserve tissue structure and morphology while immobilizing the antigens of interest. Wells were created on each slide with a hydrophobic barrier pen (Vector Labs, H-4000). In order to detect intracellular antigens, tissue sections were permeabilized with a 0.5% Triton-X-100 solution for 15 minutes. The sections were then blocked with a PBS/gelatin blocking solution (0.2%) for 10 min to suppress non-specific binding. Primary antibodies were incubated for 48 hours at 4°C. The slides were subsequently rinsed in PBS for 5 min followed by PBS/gelatin for 10 min and incubated for 40 minutes on a moving platform at room temperature with species-specific fluorescently labeled secondary antibodies (Table 4.2). The secondary antibodies were washed off (4 x 5 min) in PBS and once in water. Lipofuscin is a highly fluorescent pigment produced in aging cells that can result in background autofluorescence (Terman & Brunk, 2004). To reduce signal interference from lipofuscin all sections were incubated in a 1% Sudan Black solution for 5 minutes followed by a second incubation in a 3% Sudan Black solution for an additional 5 minutes. Sections were air-dried, mounted with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) nuclear marker (Vector Labs, H-1200), cover slipped and sealed.

Double immunofluorescence was performed on 8 tissue sections per sample. Selected tissues were first stained with either anti-5-HTT or anti-FGF-2 antibodies followed by incubations with the corresponding fluorescently labeled secondary antibody. Following incubation with the first set of antibodies the sections were then stained with anti-GFAP followed by incubations with the appropriate secondary antibody. Sections were air-dried, mounted with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) nuclear marker (Vector Labs, H-1200), cover slipped and sealed.
Figure 4.2. Sample preparation for immunohistochemical staining.

(A) Fixation of tissue in 20% acetone, followed by baths in PBS. (B) Drawing of wells around the tissue using a hydrophobic pen. (C) Incubation of slides in Triton X-100, PBS, and PBS/gelatin. (D) Incubation of slides in primary antibody in a moistened chamber. (E) Incubation of slides in secondary antibody in a moistened chamber. (F) Sudan Black solution washes for quenching of autofluorescence. (G) Mounting of slides with DAPI+ nuclear stain. (H) Sealing of slides with clear nail polish. (I) Mounted and sealed slides ready for microscopy.
Immunoperoxidase labeling

Additional tissue sections were prepared for anti-neuron and anti-microglial immunostaining using the highly sensitive and long lasting avidin-biotin method. Sections were fixed in a brief acetone bath (-20°C, 2 min) and rinsed in PBS. The sections were treated with a blocking solution (0.3% hydrogen peroxide in 0.3% normal goat serum, 5 min), incubated in the appropriate primary antibody (Table 4.2, 48 hrs), and processed using goat anti-mouse biotinylated secondary antibodies (1:200; 6 h), and the Vectastain ABC Elite reagent kit (1:100 dilution, 1 h; Vector Laboratories). The immunoperoxidase reaction was visualized by using 0.05% dianaminobenzidine (DAB; Sigma) and 0.015% hydrogen peroxide in PBS, which results in a brown reaction product. The reaction was terminated by rinsing in cold water. Sections were counterstained with haematoxylin, dried, mounted with Permount mounting medium (Fisher), coverslipped and sealed with clear nail polish.

Western Blot

5-HTT protein expression in the SVZ was confirmed by Western Blot. Approximately 12 tissue slices from each case were scraped off of the glass slides and collected in 1mL of lysis buffer. The absorbance at 280 nanometers (nm) was used to quantify the protein content of each sample. From each sample, 80 μg of protein was loaded into a 10% polyacrylamide gel and run in an electrophoresis chamber for approximately one hour at 120V at room temperature. The proteins were then transferred from the gel onto a low fluorescence PVD filter membrane (Bio-Rad) for one hour at 15V using the semi-dry transfer method. Membranes were blocked against nonspecific binding for one hour in Tris-buffered saline with Tween 20 (TBST) containing 5% bovine serum albumin (BSA). The membranes were then incubated with the primary antibody overnight at 4°C on a rotating platform. The following day the membranes were washed, blocked and incubated with fluorescently labeled secondary antibodies for one hour.

Data Acquisition

Images of immunofluorescent staining were obtained on an Olympus Fluoview FV1000 laser scanning microscope mounted on an Olympus IX81 inverted microscope at
the Biological Electron Microscope Facility of the University of Hawaii. DAPI, AlexaFluor 488 and AlexaFluor 546 were excited at 405 nm, 488 nm, and 546 nm respectively, with a blue diode, an Ar ion set at 488 nm and a red HeNe lasers. All sections were scanned using identical instrument settings. Emission from each fluorescent molecule was recorded through spectral filtering. Images were acquired using Fluoview Viewer software. The SVZ is composed of four cellular layers (Quinones-Hinojosa et al., 2006). These layers were located on the immunostained slides by viewing DAPI staining at low magnification (10x). Slides in which the SVZ was not visible were excluded from all analyses. On each immunolabeled section at least one photomicrograph was systematically and randomly captured using a 20x UplanApo (0.70) optical objective. Microscopy images were exported as digital TIFF image files (1024 x 1024 pixels). High magnification photomicrographs of GFAP immunoreactivity were captured using a 20x UplanApo (0.70) optical objective with a 2.0x digital zoom and were exported as digital TIFF image files (1600 x 1600 pixels). No further processing was performed on the images before analysis.

Images of immunoperoxidase stained slides were obtained on an Olympus BX-51 upright compound microscope using bright-field illumination. Photomicrographs of each slide were captured with an Optronics MacroFire digital camera mounted onto the microscope. Images were captured on each section at 4x, 10x, and 20x zoom. All images were saved and exported as digital TIFF image files (1200 x 1200 pixels). Peroxidase stained images were used to determine the location of labeled cells in relation to the anatomical landmarks under study.

Imaging of the Western Blot was performed using a Typhoon 9410 (Amersham Pharmacia Biotech) system. Images were converted to TIFF files using the ImageQuant software (Amersham Pharmacia Biotech) and exported to ImageJ.

Quantification

For immunofluorescence quantification five to ten tissue slices were processed per case and per antigen of interest. At least one image was acquired from a randomly selected location on each immunolabeled tissue slice. All images were processed using Image J/Fiji Software (NIH) (Schindelin et al., 2012). The quantification of fluorescently
labeled slides was carried out by measuring the area fraction for each of the molecules of interest in two regions of interest (ROI). Area fraction is a measure of the number of pixels of a defined color relative to the total area of the tissue under study (Kaczmarek et al., 2004; Diniz, 2010) and is commonly used as a method of immunohistochemical quantification (Pham et al., 2007). The two regions of interest were the SVZ (layers I-IV) and the hypocellular gap (layer II). The SVZ was defined as the region beginning at and extending 300μm from the ependymal wall. This standardized region was selected as it encompasses a range of variable SVZ widths that have been previously described (van den Berg, et al., 2010). The hypocellular gap was defined as the cell-sparse region between the ependymal wall and the beginning of the astrocytic ribbon (Quinones-Hinojosa et al., 2007).

The width of the hypocellular zone was assessed by measuring the distance from the ependymal wall to the astrocytic ribbon layer at five equidistant points along the SVZ and averaged. The cell density in the hypocellular gap was calculated by manually counting the number of DAPI+ cell nuclei in the hypocellular gap and dividing this number by the hypocellular gap area to determine the number of cells per millimeter. The width of the astrocytic ribbon was quantified by taking the average of five measures of the GFAP+ area.

The optical density of the Western Blot bands was quantified using the gel analysis feature in Image J (http://rsb.info.nih.gov/ij/docs/menus/analyze.html#gels).

4.3.3. Statistical analysis

Data are presented as mean ± standard error of the mean. The stained area fraction for each molecule of interest and the hypocellular gap width were compared with two-way analyses of variance (ANOVA) with condition (AD vs. TD) and Age as main factors. When main effects or interactions were found, Bonferroni post hoc comparisons were performed to assess significant differences between conditions at each age group. T-tests were used to compare demographic variables between AD and TD cases. The relationship between 5-HTT immunolabeling and protein expression was determined by Pearson product-moment correlation analyses. In all studies a p-value of < 0.05 was considered statistically significant.
4.4. Results

4.4.1. Clinical Characteristics

All of the autistic cases received for this study met or exceeded the criteria for autism diagnosis on the ADI-R assessment (Table 4.1). Control cases did not have any known neurological disorders. All of the individuals in the autism group were the products of full term, normal pregnancies. Each of the four autism cases revealed a family history of neurological disorders including attention-deficit-hyperactivity disorder (ADHD), pervasive developmental disorder (PDD), Manic Depression and Alzheimer's. The 60 year old in the autism group had a history of seizures.

4.4.2. Postmortem and Neuropathological Characteristics

Donor characteristics are presented in Table 4.3. The average age of the AD cases at death was 26.53 (range 5-60.1 years). The average age of control cases at death was 32.23 (range 6.9-60 years). The average postmortem interval for AD cases was 22.4 hours and 20.1 hours for controls. There were no statistical differences between groups in age at death or postmortem interval (data not shown). Consistent with reports of enlarged head size in autism, the brain from the 5 year old AD case was approximately 15% larger than the normative brain weight reported in the literature (Redcay and Courchesne, 2005) and nearly 18% larger than the brain of the 6 year old TD case.
Table 4.3. Postmortem Information on Autism-Diagnosed and Typically-Developing Controls.

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<th>PMI (h)</th>
<th>Cause of death</th>
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<td>1210</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>ID</th>
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<th>PMI (h)</th>
<th>Cause of death</th>
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<td>60</td>
<td>24.23</td>
<td>Heart attack</td>
<td>1340</td>
</tr>
</tbody>
</table>

*post mortem interval

4.4.3. Morphology of the SVZ

The SVZ in both cohorts was comprised of an ependymal layer, visualized as a single layer of tightly packed DAPI+ cells bordering the lateral ventricle (Layer I; Figure 4.3). Immediately adjacent to the ependymal layer was a hypocellular layer largely devoid of DAPI+ cells (Layer II; Figure 4.3). Both AD and TD cases displayed a small number of displaced DAPI+ cells within this region. Adjacent to the hypocellular gap was a layer of homogeneously dispersed DAPI+ cells (Layer III; Figure 4.3). Previous research has described Layer III as a dense ribbon of astrocytes (Quiñones-Hinojosa et al., 2006). DAPI+ cells were also observed in Layer IV, which has been described as a transitional zone between the astrocytic ribbon and the brain parenchyma (Quiñones-Hinojosa et al., 2006).
Figure 4.3. Layered cellular organization of the human SVZ.
Representative photomicrograph of the major layers of the SVZ. LV = lateral ventricle, I = ependymal wall, II = hypocellular gap, III = astrocytic ribbon, IV = transitional zone.

4.4.4. Hypocellular gap

The hypocellular gap width was smaller in AD cases than in TD cases \([F(1, 231) = 76.50, p < 0.001; \text{Figure 4.4A}]\). Across cohorts, the width of the hypocellular gap changed as a function of age \([F(3, 231) = 26.87, p < 0.001; \text{Figure 4.4B}]\). The youngest individuals (5-21 year olds) had the smallest hypocellular gaps while the oldest individuals (39-60 year olds) had the largest \([t(237) = -2.03, p < 0.05; \text{Figure 4.4C}]\).

Post-hoc analysis following significant interactions between condition and age showed that in the AD cases the hypocellular gap remained similar in size between the young ages (5-20 year olds) but increased in size with increasing age (Figure 4.4B). TD cases showed the opposite pattern. In TD cases, the hypocellular gap increased in size between the youngest ages (6-21) followed by a decrease in size at 41, which remained unchanged at 60. This is likely due to the large hypocellular gap in the 21 year old TD case.
The hypocellular gap contains a small number of displaced ependymal cells (Sanai et al., 2004; Quiñones-Hinojosa et al., 2006), neuroblasts (Wang et al., 2011) and astrocytes (Kotagiri et al., 2013). Both TD and AD cases had numerous DAPI+ cells in the hypocellular gap, however, the AD cases had a significantly greater number of DAPI+ cells/mm² when compared to TD controls \( F(1,76) = 42.67, \ p < 0.001 \). The young cases (5-21 year olds) had a significantly greater cell density in layer II than the old cases (39-60 year olds) \( F(1,76) = 23.99, \ p < 0.001 \). Post-hoc analyses following significant interactions between condition and age showed that the young AD cases had a greater hypocellular gap density when compared to young TD cases and old AD cases \( F(1,76) = 14.11, \ p < 0.001 \; \text{Figure 4.5} \).
Figure 4.4. Hypocellular gap widths in the AD and TD SVZ.

(A) Average hypocellular gap width measurements for AD and TD cases. (B) Hypocellular gap widths for AD and TD cases across ages. (C) Hypocellular gap widths for young versus old cases. * p < 0.05, ** p < 0.01, *** p < 0.001, between-group comparisons. # p < 0.05, ## p < 0.01, ### p < 0.001, within-group comparisons, different from all other AD ages. & p < 0.05, && p < 0.01, &&& p < 0.001, within-group comparison, different from all other TD ages.
Figure 4.5. Hypocellular gap density in the AD and TD SVZ.
Average hypocellular gap density measurements for AD and TD cases. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between-group comparisons. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, within-group comparisons, different from all other AD ages.

4.4.5. Localization and quantification of 5-HTT in the SVZ

Western blot analysis of tissue sections containing the SVZ confirmed the presence of 5-HTT in both AD and TD cases with a band measured at ~75kDa (data not shown). A comparison between 5-HTT values obtained by Western Blot and area fraction immunoreactivity revealed a highly significant correlation $r(8) = 0.95$, $p < 0.001$.

5-HTT immunoreactivity was observed throughout all layers of the SVZ with the exception of Layer II (Figure 4.6C). 5-HTT immunoreactivity could be seen as punctate staining in both AD and TD cases. 5-HTT+ staining was consistently found to be the most intense in Layer I of the SVZ (Figure 4.6C). Layers III and IV revealed moderate 5-HTT+ labeling that appeared to localize with DAPI+ nuclear staining.
The area fraction of 5-HTT staining in the SVZ was significantly increased in AD cases compared with typically-developing controls \([F(1,88) = 20.57, p < 0.001, \text{Figure 4.6A}]\). The area fraction of 5-HTT staining also differed as a function of age across both conditions \([F(3,88) = 16.88, p < 0.001, \text{data not shown}]\). 5-HTT staining in the SVZ was higher in the youngest cases (5-6 year olds) when compared to the tissues from the young adults (20-21 years old) and the 60 year olds. These main effects of condition and age are likely due to the high area fraction of 5-HTT+ staining seen in the young AD (5 year old) tissue.

Post-hoc analyses following significant interactions between condition and age revealed different age-related patterns of 5-HTT immunoreactivity between AD and TD tissues. The 5 year old autism case had the highest levels of 5-HTT+ staining when compared to every other AD and TD case \([F(3,88) = 26.80, p < 0.001; \text{Figure 4.6B}]\). In contrast the area fraction of 5-HTT+ staining did not differ across ages in the TD tissue.
Figure 4.6. Area fraction of 5-HTT in the AD and TD SVZ.

(A) Average area fraction of 5-HTT for AD and TD cases. (B) Area fraction of 5-HTT for AD and TD cases across ages. (C) 5-HTT immunoreactivity in the SVZ of the human brain. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between-group comparisons. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, within-group comparisons, different from all other AD cases.
4.4.6. Localization and quantification of FGF-2 in the SVZ

FGF-2 immunoreactivity was found almost exclusively in the hypocellular gap (Layer II) of both AD and TD cases. FGF-2+ staining was only observed outside of this region in connection with large blood vessels and capillaries. FGF-2 immunoreactivity was observed as patchy staining in the hypocellular gap of both TD and AD cases (Figure 4.7C).

The area fraction of FGF-2+ staining in the hypocellular gap was significantly increased in AD cases when compared to TD controls \( F(1,128) = 46.23, p < 0.001; \) Figure 4.7A]. FGF-2 immunoreactivity changed as a function of age across both conditions \( F(3,128) = 25.86, p < 0.001; \) data not shown. The youngest individuals (5-6 year olds) had the greatest area fraction of FGF-2+ staining compared to every other age group.

Post-hoc analyses following significant interactions between condition and age showed that the area fraction of FGF-2+ staining was significantly increased in the 5 and 20 year old AD cases when compared to their respective TD controls \( F(3,128) = 10.60, p < 0.001; \) Figure 4.5B]. Additionally, FGF-2 immunoreactivity was significantly increased in the 5 and 20 year old AD cases when compared to the 39 and 60 year old AD cases (Figure 4.5B). In TD tissue FGF-2+ staining was significantly increased in the 6 year old only when compared to the 21 year old (Figure 4.5B).
Figure 4.7. Area fraction of FGF-2 in the AD and TD SVZ.

(A) Average area fraction of FGF-2 for AD and TD cases. (B) Area fraction of FGF-2 for AD and TD cases across ages. (C) FGF-2 immunoreactivity in the SVZ of the human brain. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, between group comparison. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ within-group comparison, different from the young (5 year old) AD case. ^ $p < 0.05$, ^^ $p < 0.01$, ^^^ $p < 0.001$ within-group comparison, different from the young adult (20 year old) AD case. & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ within-group comparison, different from the young (6 year old) TD case.
4.4.7. Localization and quantification of GFAP in the SVZ

Both TD and AD cases contained a highly dense network of GFAP positive fibers (Figure 4.8B). Consistent with previously reported descriptions (Sanai et al., 2004) there was a particularly strong band of immunoreactivity in Layer III directly adjacent to the hypocellular gap. Diffuse and fibrous GFAP+ projections could also be seen innervating all layers of the SVZ. The width of the immunoreactive band, which averaged between 240-600 μm, did not differ between AD and TD cases. The area fraction of GFAP+ immunoreactivity did not differ between AD and TD controls (Figure 4.8A). There were no significant age by condition interactions in GFAP+ staining (data not shown).

Double stained slides for 5-HTT/GFAP revealed occasional cell processes that exhibited both 5-HTT and GFAP immunoreactivity (data not shown). The few processes found positive for 5-HTT and GFAP were localized to Layer III of the SVZ. Contrary to previous reports (Gómez-Pinilla et al., 1992), slides double-stained for FGF-2/GFAP immunoreactivity did not display co-localization (data not shown). The lack of clear and robust co-localization, which has been reported in other studies, was likely a result of the sequential staining method used in the current study (Christensen and Winther, 2009).
Figure 4.8. Area fraction of GFAP in the AD and TD SVZ.

(A) Average area fraction measurements of GFAP+ staining in AD and TD cases.
(B) Representative GFAP immunoreactivity in the SVZ of the human brain (20x zoom).
4.4.8. Cellular characterization of the SVZ

In order to determine additional cell types present in the SVZ, several slides were stained with either an anti-neuronal (NeuN) or microglial (IBA-1) marker. Anti-NeuN is a widely used and reliable antibody against postmitotic neurons. Neun immunostaining was observed in both AD and TD cases. Neun immunoreactivity could be seen as darkly stained, small, round or tear-drop shaped somata (~15 μm in diameter; Figure 4.9A). At higher magnification (40x) Neun staining also revealed darkly stained proximal processes (Figure 4.9B). Neun staining was primarily seen in layer IV of the SVZ (Figure 4.9A). SVZ neuronal staining was considerably more diffuse in comparison to the adjoining parenchyma (Figure 4.9B).

IBA-1 is a protein specifically expressed by brain microglia. In all cases a few lightly stained cells were detected in layers II, III and IV. In the 60 year old autism tissue, IBA-1 immunostaining revealed a dense population of darkly stained somata in the corpus callosum (Figure 4.10).
Figure 4.9. NeuN staining of the SVZ.

(A) Sparse NeuN immunoreactivity in the SVZ of the human brain at 20x magnification. (B) Denser immunoreactivity for NeuN in the adjacent parenchyma at 40x magnification.
4.5. Discussion

The current experiments were aimed at investigating the structural and molecular components of the SVZ in AD and TD brains. In addition to expanding on previous reports of SVZ architecture and cellular composition in neuropsychiatric disorders (Wegiel et al., 2010; Kotagiri et al., 2013; Pearson et al., 2013), this is the first study to describe the expression of 5-HTT in the SVZ in autism. Consistent with the existing literature on the neurodevelopmental nature of autism (DiCicco-Bloom et al., 2006), our results revealed a number of differences in the tissue from the young AD case.

Morphological examination of the SVZ confirmed that in both TD and AD cases the SVZ is organized into four distinct layers (Figure 4.3). The SVZ was separated from the underlying ventricular CSF by a single layer of ependymal cells (I). A hypocellular region (II) devoid of cell bodies was observed between the ependymal layer (I) and the
ribbon of GFAP+ astrocytes (III). Astrocytic processes could be observed extending into this gap region. In addition, the occasional DAPI+ cell were present. The exact function of this gap is unknown; however, it is thought to reflect a void left over from migrating stem cells during development (Quiñones-Hinojosa et al., 2006).

Consistent with previous reports, the hypocellular gap width increased as a function of age in both groups (Figure 4.4C) (Sanai et al., 2004). The width of the hypocellular gap was significantly smaller in AD cases when compared to TD controls (Figure 4.4A), possibly reflecting increased cellular proliferation in this region. This hypothesis supports findings of increased neurogenesis in the SVZ of post-mortem brains from autistic individuals (Kotagiri et al., 2013; Pearson et al., 2013). Furthermore, a number of displaced astrocytes and ependymal cells have also been observed in this gap (Quiñones-Hinojosa et al., 2006) and here we report increased hypocellular density in the young AD cases compared to controls (Figure 4.5). Taken together, the current findings of reduced hypocellular gap widths and increased cell density lend further support to findings of increased cellular proliferation, accelerated brain growth and disturbed cellular migration characteristic of this disorder (Wegiel et al., 2010).

Alternatively, ventricular expansion could augment the structure and size of the SVZ. Enlarged cerebral ventricles are frequently seen in the brains of autistic patients (Piven et al., 1995, 1996; Hardan et al., 2001) and ventricular enlargement in low-birth weight infants reportedly increases autism risk nearly seven fold (Movsas et al., 2013). The structural abnormalities found here could reflect damage to this region as a consequence of autism-related ventricular enlargement. However, we cannot confirm ventricular enlargement in these individuals as only a small region of the SVZ was received for analysis.

This study confirmed the existence of 5-HTT+ positive staining in the SVZ of human brains (Figure 4.6C); documenting increased 5-HTT immunostaining in the SVZ of young individuals with autism (Figure 4.6B). These findings compliment and expand on previous reports of increased 5-HTT immunoreactivity in the cortex (Azmitia et al., 2011a, 2011b) and cerebellum (Wegiel et al., 2013) of autism cases. Collectively these
findings provide strong evidence for altered 5-HT function in the autistic brain which may contribute to the disturbances in neurodevelopment characteristic of the disorder.

Here we report intense 5-HTT staining in the ependymal wall, particularly in the young autism case. Ependymal cells regulate the flow and molecular composition of the CSF, and serve as a critical barrier between the CSF and adjacent parenchyma (Bruni, 1998). The activity of ependymal cells is modulated by the release of 5-HT from the supra- and subependymal plexus. 5-HT increases ependymal cell metabolism (Prothmann et al., 2001) and ciliary beat frequency (Nguyen et al., 2001). High concentrations of 5-HT are likely to occur given the extensive plexus of 5-HTT expression in the SVZ (Porlan et al., 2013). Given that signals generated in distant locations may diffuse to the SVZ via the CSF, and vice versa, excessive 5-HT release in this region may have long ranging consequences for overall brain development and function.

Preclinical studies in rodents exposed to high concentrations of 5-HT during development show striking parallels in the physiological and behavioral symptoms of children with autism. Elevated central 5-HT concentrations during development produce increased cortical thickness (Altamura et al., 2007), disrupted cortical minicolumns (Janušonis et al., 2004) and deficits in the somatosensory cortex connections (Kinast et al., 2013). Additionally, increased central 5-HT concentrations permanently reduce novelty investigation behavior, increase anxiety and depression like behaviors, and induce sleep abnormalities (Ansorge et al., 2004, 2008; Popa et al., 2008). In the current study, the presence of dense 5-HTT+ staining in this neurogenic region and the well described role of 5-HT release on neurogenesis suggests that enhanced 5-HT release in this region may contribute to the reports of increased cellular proliferation in the SVZ of AD brains (Kotagiri et al., 2013; Pearson et al., 2013).

One mechanism by which excess 5-HT could alter SVZ activity and consequently brain development in autism is through the stimulation of growth factors. FGF-2 is a well documented neurotrophin that regulates the proliferation, self renewal and differentiation of neural precursors in the SVZ (Wagner et al., 1999; García-González et al., 2010; Werry et al., 2010). Disturbances to FGF-2 activity produce a range of
developmental defects (Itoh and Ornitz, 2004). Over expression of FGF-2 results in increased cortical size (McCaffery and Deutsch, 2005) and cortical gyrification (Rash et al., 2013). Similarly, increased brain size (Palmen et al., 2004a) and gyrification (Wallace et al., 2013) have been reported in autism.

Antidepressants that increase the synaptic concentration of 5-HT increase the expression of FGF-2 (Mallei et al., 2002; Maragnoli et al., 2004). In turn, FGF-2 may act upon FGF receptors located on multipotent stem cells (Type B-cells) and proliferating transient amplifying cells (Type C-cells) resulting in increased NSC proliferation (Mudò et al., 2007). In the current study the young autism cases had increased 5-HTT and FGF-2 immunostaining in the SVZ. This suggests that increased 5-HT release in the SVZ by 5-HTT+ neurons may increase FGF-2 concentrations in the developing brain and contribute to the well documented brain overgrowth reported in autism (Palmen et al., 2004a). Support for this hypothesis comes from additional studies that report an increase in BDNF, a well documented neurotrophin and 5-HT tropic factor (Ricci et al., 2013) coupled with increased neurogenesis (Kotagiri et al., 2013; Pearson et al., 2013) in autism.

This study confirmed previous reports of GFAP immunoreactivity in Layer III of the SVZ. GFAP is an intermediate filament protein that is expressed by mature astrocytes (Waldvogel et al., 2006). Astrocytes are involved in immune function, which is suspected to be involved in autism pathology (Patterson, 2011). Reportedly, GFAP immunoreactivity (Vargas et al., 2005) and protein content (Laurence and Fatemi, 2005) are increased in the cortex and cerebellum of individuals with autism. However, in the current study there were no differences in GFAP immunoreactivity in the SVZ between AD and TD cases. Our results are consistent with a report that failed to find GFAP changes in schizophrenic patients versus controls (Fatemi et al., 2004).

The lack of differences in gross astrocyte morphology in this sample does not preclude disturbances in astrocyte function in this disorder. Astrocytes provide structural, metabolic and tropic support for the components of the SVZ (Lim and Alvarez-Buylla, 1999). For example, astrocytes help regulate synaptic 5-HT content by uptake via 5-HTT (Kubota et al., 2001) and express FGF-2 and their respective receptors
(Porlan et al., 2013). Thus, although quantification of GFAP+ staining was not different between the groups, astrocytic regulation of 5-HT and/or FGF-2 could be impaired in this disorder. Astrocytes contribute to synaptic 5-HT content by taking up, releasing and responding to 5-HT and 5-HT modulating drugs (Inazu et al., 2001). Glutamate has recently been shown to block the ability of astrocytes to respond to 5-HT (Schipke et al., 2011). Glutamate is increased in autism (Purcell et al., 2001; Shinohe et al., 2006), and may therefore, impair astrocytic function in autism by inhibiting regulation of 5-HT.

The tissue from the 60 year old autism case in the current study displayed dense IBA-1 staining especially in the corpus callosum. This observation supports other studies that suggest a role for microglial pathology in autism (Morgan et al., 2012) and in the BTBR animal model of the disorder (Heo et al., 2011). In the current study IBA-1 labeling was most clearly observed in the 60 year old autism sample containing the corpus callosum. The limited number of samples with adjoining corpus callosum sections precluded any group comparisons in this study. However, given the consistent reports of corpus callosum abnormalities in autism, the current findings present an interesting avenue of investigation for future research.

4.6. Limitations:

Immunohistochemical examination of post-mortem tissue is a valuable tool for studying the chemical and structural anatomy of the brain in healthy and diseased states. However, these techniques are not without limitations. In general post-mortem human brain research is restricted by the availability of appropriate tissue samples. Pediatric samples, which are critical for neurodevelopmental disorder research, are especially rare (Abbott, 2011). The relatively small sample size in the current study reflected all of the available young autism samples at the time and was consequently unavoidable. Replication with a larger number of samples in addition to parallel investigations of these systems in animal models may lend support to the current findings.

An additional limitation of human post-mortem tissue research relates to the unpredictability of immunolabeling when compared to animal tissues. Various pre- and post-mortem factors that may interfere with molecular preservation can be rigorously
controlled in animal studies but will vary considerably in human samples. In the current study a consorted effort was made to match samples with regard to age, gender and post-mortem interval. Possible effects of agonal state, mode of death, or time in storage were not examined. However, the confirmation of 5-HTT protein expression in the current study by multiple methods (Western Blot and immunostaining) strengthens the validity of these findings. Given the scarcity of autistic brain tissue, the agreement of the current results with other published studies (Azmitia et al., 2011a, 2011b; Wegiel et al., 2013) suggest that these findings may provide valuable and valid biomarkers of this condition.

4.7. Conclusions

The studies described here were undertaken with the goal of determining structural and molecular differences in the SVZ of AD brains. Improved understanding of the composition, activity and functions of the SVZ may clarify the mechanisms underlying autism neuropathology and provide novel therapeutic targets. Although post-mortem brain research is hampered by the scarcity of appropriately-preserved tissue as well as an array of technical challenges associated with such tissues, we have shown here that serotonergic and growth factor proteins have expression patterns that parallel known cellular aberrations in autism. The increased 5-HTT and FGF-2 density in the autism SVZ are consistent with the neurodevelopmental overgrowth hypothesis of autism. Our results provide further evidence that serotonin plays a role in the pathogenesis of autism and provide strong support for assessing 5-HT specific marker expression in the SVZ as a biomarker for autism.
4.8. References


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CHAPTER 5. GENERAL DISCUSSION

5.1. Autism & Serotonin

Autism is a behaviorally defined neurodevelopmental disorder associated with pathological disturbances in neural growth and organization (Pardo and Eberhart, 2007). While the etiology of autism remains poorly defined, a combination of clinical, neuroimaging, neurochemical and neuropathological studies implicate the serotonergic system in the disorder (Zafeiriou et al., 2009). Hyperserotonemia is among the most consistently documented candidate biomarkers for the disorder (Cook and Leventhal, 1996). Furthermore, 5-HT regulates many of the behaviors that are altered in autism (Lucki, 1998) and increasing 5-HT activity often improves the behavioral deficits (Tsai, 1999), while reducing 5-HT can worsen these behaviors (McDougle et al., 1996). Finally, a number of genes that regulate 5-HT function have been implicated in autism (Zafeiriou et al., 2009).

5-HT is a critical developmental signal (Whitaker-Azmitia, 2001) and disruptions to this system produce physiological and behavioral symptoms associated with autism (Whitaker-Azmitia, 2005; Borue et al., 2007; Croen et al., 2011; Rai et al., 2013). Furthermore, neuroimaging studies suggest that the development of the 5-HT system is blunted in autism (Chugani et al., 1997, 1999). Given the critical role of 5-HT in neural growth and behavioral regulation, abnormalities of 5-HT development, activity, and the subsequent effects on downstream circuitry may influence the manifestation of autistic symptoms.

The studies described here were undertaken to investigate the role of serotonergic disruptions in autism using a multidimensional approach. Chapter 2 used an endophenotype method to investigate suspected 5-HT disturbances in an animal model that displays an autism-relevant phenotype. Chapter 3 used a reverse-genetics approach to assess the effects of a targeted 5-HTT mutation on mouse behaviors relevant to the disorder. Chapter 4 was designed as a neuropathological study aimed at identifying potential serotonergic disruptions that may directly or indirectly contribute to autism neuropathology. The rodent models reviewed in Chapter 2 and 3 parallel commonly reported clinical findings such as reduced 5-HT activity, and suggest additional candidate pathways for further research. Chapter 4 supports findings of increased 5-HTT
expressing neurons in post-mortem brains from autistic individuals and provides a potential novel biomarker and therapeutic target for the disorder.

5.2. Serotonin alterations in the BTBR mouse model for autism

The experiments described in Chapter 2 extend upon previous findings of reduced serotonergic functioning in the BTBR mouse (Gould et al., 2011) by showing reduced concentrations of 5-HT in the BTBR cerebellum; a structure consistently implicated in autism (Allen, 2005). In addition, the behavioral aspects of the experiments confirm previous reports that BTBR mice display an autism-relevant phenotype, including a mouse analog of gaze aversion, when confined with another mouse in social proximity (Defensor et al., 2011). Interestingly, the cerebellar flocculus, which is involved in eye gaze behavior and is reported to be disturbed in autism (Wegiel et al., 2013), is located in a 5-HT impaired brain region in this strain expressing gaze aversion. The cerebellum receives a fine plexus of serotonergic fibers through which 5-HT is released via volume transmission (Trouillas and Fuxe, 1993). 5-HT concentrations in the cerebellum are positively correlated to the level of motor activity (Mendlin et al., 1996). Taken together, the current results suggest that reduced 5-HT activity in the BTBR cerebellum may be related to the gaze aversion-like behavior displayed by this strain in the social proximity test.

Reduced concentrations of cerebellar 5-HT may contribute to disturbed oculomotor activity in the BTBR through the loss of Purkinje Cells (PC). PCs are the primary output cells of the cerebellum (Llinas and Walton, 1990) and are involved in oculomotor control (Wegiel et al., 2013). 5-HT activity in the cerebellum modulates PC development (Oostland et al., 2013), arborization (Kondoh et al., 2004) and firing (Dieudonné, 2001). Namely, a reduction in 5-HT activity in the cerebellum leads to a loss of PC complexity and firing (Oostland and van Hooft, 2013). One of the most consistent cerebellar abnormalities reported in autism is reduced numbers of PCs (Fatemi et al., 2002). Therefore, the reduced cerebellar 5-HT observed here in the BTBR could result in reduced PC activity and consequently, contribute to the gaze-like aversion displayed by this strain.

In addition to oculomotor control, the cerebellum is important for cognitive function, language, emotion regulation and social interactions (Riva and Giorgi, 2000;
Schmahmann, 2004). Dysfunction of the cerebello-thalamo-cortical circuit is thought to underlie the cognitive and affective symptoms associated with autism (Hoppenbrouwers et al., 2008). Reduced cerebellar 5-HT activity and consequent reductions in PC firing could result in a disinhibition of the deep cerebellar nuclei which would in turn lead to increased excitatory output to the thalamus and subsequently the cortex. Consistent with these hypotheses, imaging studies revealed altered cerebellar activation (Allen, 2005) and increased thalamo-cortical output in autism (Mizuno et al., 2006). Similarly, in Chapter 2 we report reduced 5-HT and 5-HIAA in the BTBR cerebellum as well as increased concentrations of 5-HT, 5-HIAA, DA and DOPAC in the BTBR cortex when exposed to novel environments. These neurochemical findings in the BTBR are consistent with the hypothesis of altered cerebral-cortical feedback in autism, which may be modulated through 5-HT signaling.

5.3. Enhanced stereotypy in 5-HTT knock-out mice

The experiments described in Chapter 3 aimed to understand the behavioral consequences of life-long 5-HTT deficiency. 5-HTT is a modulator of circulating 5-HT concentrations (Jacobs and Azmitia, 1992) and 5-HTT gene mutations have been implicated in autism (Zafeiriou et al., 2009). In the current study, the lack of 5-HTT produced modest enhancement of stereotyped and repetitive behaviors but did not alter social, aggressive, or communicative behaviors. Given the multifaceted etiology of the disorder and the diversity in symptom expression, it is not surprising that a single genetic knock-out failed to display the entire spectrum of behaviors typical of the disorder. Nevertheless, animal models are critical for investigating important aspects of behavior, physiology, and anatomy, and will continue to further clarify the underlying neurobiology of autism.

In addition to the enhanced stereotypy described here, additional studies of 5-HTT KO rodents indicate many of the physiological and behavioral features associated with autism. 5-HTT KO rodents show a reduction in corpus callosum connectivity (van der Marel et al., 2013), consistent with findings of reduced corpus callosum density in autism (Hong et al., 2011). In line with reports of gastrointestinal dysfunction (Mazurek et al., 2013) and rapid eye movement (REM) sleep deficiencies in autistic patients (Buckley et al., 2010), 5-HTT KO rodents also have increased gut motility and disturbed REM sleep.
Additionally, 5-HTT KO rodents show increased anxiety (Holmes et al., 2003; Kalueff et al., 2007) and depression-related behaviors (Murphy and Lesch, 2008; Kinast et al., 2013), which are frequently co-morbid with autism (van Steensel et al., 2011). 5-HTT regulates nearly every physiological and behavior domain that is disturbed in autism. Therefore, the 5-HTT KO mouse may continue to be a useful model to study the functional interactions between molecular and neurochemical systems that may contribute to the associated symptoms of autism.

The 5-HTT KO mouse has no detectable 5-HTT protein (Bengel et al., 1998) while the BTBR mouse described in Chapter 2 has 20-30% fewer 5-HTT binding sites compared to B6 mice (Gould et al., 2011). Both strains display autism-associated pathophysiology such as reduced tissue concentrations of 5-HT (Bengel et al., 1998; Kim et al., 2005), reduced sensory function reflected by blunted responses to mildly painful thermal stimuli (Vogel et al., 2003; Silverman et al., 2010) in addition to mild insulin resistance and obesity (Flowers et al., 2007; Murphy and Lesch, 2008). These findings are consistent with reports of reduced 5-HTT binding (Makkonen et al., 2008), hypo- and hyper-sensory responses (Horder et al., 2013) and increased prevalence of obesity (Curtin et al., 2010) in individuals with autism. Both strains also display autism-relevant phenotypes such as increased self-grooming (Kalueff et al., 2007; McFarlane et al., 2008; Pearson et al., 2011) and enhanced responsivity to stress (Tjurmina et al., 2002; Holmes et al., 2003; Benno et al., 2009; Pobbe et al., 2011). However, the two strains differed significantly in their manifestation of the three core autism features. BTBR mice display behaviors relevant to each of the three core symptom domains (Meyza et al., 2012), while the 5-HTT KO mouse only showed increased stereotypy. Taken together, the results from the current studies suggest a contributory role for 5-HTT function in the anatomical and behavioral features of autism. However, given the complex and diverse symptom expression in autism, other molecular and environmental factors likely contribute to the manifestation of the disorder.

Autism is recognized as a multi-factorial disorder with both genetic and environmental contributions (Persico and Bourgeron, 2006). Therefore, variations in 5-HTT genotype in conjunction with additional genetic and environmental factors could interact to contribute to autism pathology. For instance, reduced expression of the 5-HTT
gene contributes to increased stress sensitivity (Bartolomucci et al., 2010; Caspi et al., 2010) and a number of species show increased anxiety and depression behaviors after stressful life events in those individuals with reduced 5-HTT expression (Caspi et al., 2010). Further support for a 5-HTT gene x environment interaction in autism comes from a recent study that found that reduced expression of the 5-HTT gene interacted with maternal smoking during pregnancy and low birth weight to increase autism-relevant symptoms such as social interaction deficits and rigid behaviors in children with attention deficit hyperactivity disorder (ADHD) (Nijmeijer et al., 2010). Furthermore, many of the environmental risk factors associated with autism risk also interact and/or interfere with the serotonin system. Prenatal factors such as the use of serotonergic agonists during pregnancy (Croen et al., 2011), stress (Ronald et al., 2010), infection (Patterson, 2011), and a history of maternal depression (Rai et al., 2013) are recognized as risk factors for autism and are known to modulate serotonergic activity (Dunn et al., 1999). On the basis of these observations, it is possible that a combination of genetic vulnerability and an accumulation of environmental factors could interact to further disrupt the 5-HT system and increase the risk for autism. Overall, the findings from the two animal models described here support the general hypothesis that 5-HTT deficiency underlies complex and diverse phenotypes with implications for a number of psychiatric disorders, including autism.

5.4. Increased mitogenic factors in the SVZ of young autism brains

In contrast to the animal models described thus far, the final study revealed significantly increased 5-HTT immunostaining in the SVZ tissue from the 5 year old autism donor. These results compliment and extend on reports of increased 5-HTT immunoreactivity in the cerebellum (Wegiel et al., 2013), median forebrain bundle, ansa lenticularis, stria terminalis, and superior temporal cortex (Azmitia et al., 2011, 2011) in post-mortem brains of autistic individuals. Collectively these results suggest a global increase in all serotonergic pathways in the brains of autistic individuals when compared to TD controls.

5-HT is a well documented modulator of brain development (Whitaker-Azmitia, 2001) and the SVZ is critically involved in neural development and life-long plasticity (Brazel et al., 2003). Increased 5-HTT immunostaining in the SVZ likely reflects
increased 5-HT release in this region (Porlan et al., 2013). Increased concentrations of 5-HT in the SVZ could produce a number of neurodevelopmental effects including increased neurogenesis (Banasr et al., 2004), reduced apoptosis (Persico et al., 2003), inhibition of cellular differentiation (Menegola et al., 2004) and decreased cellular migration (Riccio et al., 2008). These cellular disruptions are consistent with reports of autism pathology (Palmen et al., 2004), suggesting a critical role for 5-HT signaling in the SVZ as a mediator of altered neurodevelopment in this disorder.

Excessive 5-HT signaling in this region may act through an increase in the secretion of growth factors. Compounds that increase 5-HT concentrations also increase FGF-2 expression (Maragnoli et al., 2004; Bachis et al., 2008). Moreover, astrocytes, which are abundant in this region, express 5-HTT (Kubota et al., 2001) and can secrete FGF-2 (Gómez-Pinilla et al., 1992); providing a possible mechanism for 5-HT action.

Here we show increased 5-HTT and FGF-2 immunoreactivity in the brains of young AD individuals. FGF-2 promotes neurogenesis and neural migration (García-González et al., 2010), and is responsible for regulating cerebral cortex size, neuron number and gyrification (Vaccarino et al., 2009; Rash et al., 2013). The increased brain weight observed in the 5 year old autism case could reflect serotonergic stimulation of FGF-2 signaling. Taken together, the findings of increased 5-HTT and FGF-2 immunoreactivity in the young autism samples support findings of accelerated brain growth in young autism individuals (Courchesne et al., 2001). Furthermore, these findings propose a potential mechanism for this brain overgrowth through 5-HT regulation of SVZ activity.

5.5. Conclusions, constraints & future directions

The studies described here represent an attempt to systematically examine the 5-HT system in autism using a variety of neurochemical, behavioral, and neuropathological techniques. The animal models described in Chapter 2 and 3 investigated neurochemical and genetic contributions to autism-relevant behaviors. In each of the mouse models, reduced brain tissue concentrations of 5-HT were associated with behaviors relevant to the symptoms of autism. In contrast, the neuropathological findings of Chapter 4 suggest increased 5-HT activity in the brain from the young autistic individual, as revealed by increased 5-HTT staining in the SVZ. Increased 5-HT in this mitogenic region may contribute to the altered brain growth trajectories observed in autism. One
Implication of the current findings is that optimal 5-HT activity may be confined to a narrow range, such that reduced or elevated function may contribute to autism vulnerability and to the wide range of behavioral phenotypes observed in the disorder (Veenstra-VanderWeele et al., 2009).

The current studies support and extend on the existing literature describing altered serotonergic function in autism (Cook and Leventhal, 1996) and suggest that 5-HT changes may correlate with the anatomical and behavioral features of the disorder. The use of animal models has many advantages for studying the suspected neurobiological underpinnings of this disorder. Thanks in large part to the mapping of the mouse genome, mice with genetic mutations relevant to human disorders can be easily created (Guénet, 2005). In comparison to humans, mice have a relatively short life-span (2-3 years), allowing for quick and convenient longitudinal studies. Furthermore, a number of experimental factors can be easily controlled and/or manipulated with animal models, which is not possible in studies with humans. However, as autism is a uniquely human disorder, animal models do not recapitulate all of the behavioral, physiological and neuroanatomical features of the disorder. Nevertheless, large-scale efforts are underway to improve behavioral phenotyping assays (Defensor et al., 2011; Pearson et al., 2011; Pobbe et al., 2011; Wöhr and Scattoni, 2013) and create gene x environment models (Moy and Nadler, 2007) to better examine the neurobiological, genetic, endophenotypic and pathogenic factors contributing to autism.

Given the uniquely human nature of autism, examination of the affected brain tissue is critical to understanding the underlying biological mechanisms that cannot be modeled through animal models or cell lines. Tissue studies are limited by the availability of donated samples. Brain samples from young autistic children are especially rare (Abbott, 2011), likely due to the fact that autism is a non fatal disorder. Individuals with autism often live into adulthood and while the adult brain may retain remnants of the neurodevelopmental disturbances of autism, investigation of brains at the age of symptom expression (2-3 years old) may yield more clues as to the causative factors of the disorder. Similarly, post-mortem tissue allows us to examine an individual only at a single time point; precluding longitudinal studies in autistic individuals. As with other research using human subjects, postmortem tissue research is confounded by
the inherent genetic, environmental and behavioral heterogeneity of human populations. Additionally, a number of factors that have no relationship to the disease pathology may produce variability in protein-expression analyses. Factors such as postmortem interval, length of tissue storage and agonal state are known to modulate gene and protein expression analyses of postmortem tissue (González-Maeso et al., 2002; Lewis, 2002).

Autism research will undoubtedly progress more quickly with the adoption of a multi-disciplinary approach that utilizes parallel investigations in animal models and human subjects. Furthermore, additional efforts should be made to publicly highlight the importance of brain tissue donation. The devastating loss of over 50 autism brain samples in 2012 (Weintraub, 2013), underscores the need for efficient allocation of samples to programs using varied and technologically advanced methods including high resolution microscopy as well as microarray and methylation expression analyses. Improved identification of biomarkers may further contribute to the therapeutic and preventative progress made through animal modeling.

The studies described here suggest a fine-tuning mechanism for 5-HT activity in regulating autism relevant pathology. Reduced 5-HTT expression and 5-HT tissue concentrations in the BTBR and 5-HTT KO mouse models produced autism relevant analogs of gaze aversion and stereotypy, respectively. In contrast, increased 5-HTT immunostaining, suggestive of increased 5-HT activity (Azmitia et al., 2011; Porlan et al., 2013), was seen in the SVZ of the young individual with autism. Given the diverse range of physiological and behavioral features mediated by 5-HT, these studies suggest that alterations in 5-HT activity, in either direction, may result in anatomical and behavioral features associated with autism. Continued research with the addition of more cases, autism-relevant brain regions, and complimentary methodologies may clarify the mechanisms that prompt 5-HT dysfunction in autism and lead to developments in the disorder's diagnosis, treatment, and prevention.
5.6. References


