Spring 5-1-2020

Amygdala and Neocortical Structural Volume Analysis in the Shank3B Mutant Mouse Model of Autism Spectrum Disorder

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An Honors Thesis

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May 2020

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Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder that is characterized by abnormal social behavior, deficits in communication, and motor stereotypy. The *SHANK3* gene, responsible for the generation of a scaffolding protein that is integral for the development of synapses, has been identified as one of the primary candidate genes implicated in the disorder. *Shank3B* is the rodent homolog for this gene. Research has shown that when this gene is disrupted in rodent models (e.g., via knock-out (KO)), ASD-like behaviors result. These include deficits in social interaction, increased anxiety, and repetitive self-grooming. The current study aimed to identify a physiological marker for autism in *Shank3B* mutant mice. A neuroanatomical analysis of the volumes of the neocortex and amygdala, two of the primary brain structures implicated in ASD, was conducted in order to determine if a biomarker was present in the form of a volume difference in *Shank3b* KO mice. It was found that there was no significant volume difference between the gene knockout and control mice across both structures. However, the volume of the right cortex was found to be marginally decreased in the heterozygous mice compared to the control. This may be related to some of the memory deficits that are typical of ASD. Future research in this field should focus on analyzing some of the other brain structures that are functionally affected in the disorder, with the goal of finding a biological marker that may enable earlier diagnosis and intervention for ASD.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that affects 1 in 54 children (Maenner *et al*., 2020). The most common behavioral markers include deficits in social interaction, verbal and nonverbal communication, and stereotyped patterns of behavior (Amaral *et al.,* 2008). The symptoms of autism are present from early childhood and affect daily functioning. Over the past few decades, the number of children diagnosed with autism has

steadily increased, leading the emergence of ASD as a major public health concern in the United States (Kulage *et al*., 2014). However, it is unclear whether this increase is due to a higher incidence of children being affected or simply due to heightened awareness and broader diagnostic criteria. Prior to the publication of DSM-5 in 2013, three distinct autism spectrum disorders were defined: Autistic disorder, Asperger's disorder, and Pervasive developmental disorder-not otherwise specified. However, in 2013 these were all combined into one broad diagnosis of Autism Spectrum Disorder (DSM-5). The term "spectrum" refers to the wide range of symptoms, skills, and difficulties that can occur in people with ASD depending on the severity of their diagnosis. It is crucial to identify the signs of ASD as soon as possible in a child's life, and some symptoms may be present as early as toddlerhood. According to the National Institutes of Health, infants with ASD may become overly focused on certain objects, avoid eye contact, and fail to engage in babbling. In other cases, children may develop completely normally until two or three years of age, at which point symptoms begin to appear and they start to withdraw from social engagement (regression). Those children who display earlier signs are more likely to have a more severe and debilitating form of ASD, requiring support for their lifetime. In any case, indicative signs that a child may have ASD include social impairment, communication difficulties, and repetitive behaviors.

There is no singular known cause for ASD, but research has shown that both genetics and the environment play a role in pathogenesis. It is likely that ASD arises as a result of disruptions in brain growth very early in development. These disruptions in growth can be attributed to genes that are involved in the development of synapses and neuronal pruning, as well as environmental disturbances to growth such as premature birth. Families with a history of developmental disorders, mental retardation, or mental illness are at a higher risk of giving birth to a child with ASD. However, *de novo* gene mutations and copy number variants of

chromosomes have been identified in some individuals with ASD, making it possible for two perfectly healthy parents to give birth to an autistic child. *De novo* copy number variants were found in 7% of individuals with idiopathic autism (Marshall *et al*., 2008).

Although the exact neuropathology of ASD remains unknown, *postmortem* brain scans have identified the amygdala, frontal lobe, and cerebellum as pathological in autism (Amaral *et al*., 2008).The amygdala theory of autism, as suggested by Baron-Cohen *et al.* (2000), suggests that atypical development of the amygdala results in the social intelligence deficits seen in individuals with ASD. In experiments involving primates, lesions in the amygdala resulted in failed social interactions, characterized by less initiation of social interactions and less responding to social gestures. The lesioned subjects became socially isolated and left their social group (Bickart *et al*., 2010). This study demonstrated that there is indeed a positive correlation between amygdala volume and social group size, which is one indicator of social intelligence. In an fMRI study by Baron-Cohen *et al.* (2000), subjects with and without autism were shown pictures of people with different facial expressions and told to judge the emotion of the person in the picture. Those with autism did not show amygdala activity during the task while those without autism did show amygdala activation. In a similar study by Howard *et al.* (2000), it was also found that people with high functioning autism display impairments characteristic of amygdala damage, such as the inability to accurately recognize facial expressions of fear as well as impairment in facial recognition memory. It is likely that these neuronal defects are a result of incomplete neuronal pruning during early brain development. Research by Schumann *et al.* (2004) further supported this idea of incomplete neuronal pruning by finding evidence that the amygdalas of children with autism (7.5-12.5 years of age) were enlarged compared to the control, but there was no difference in amygdala volume between adolescents with autism (12.75-18.5 years of age) and the control group. This suggests that individuals with autism

display the opposite brain growth pattern of typically developing individuals, whose brains grow in size as they age. Rather, the amygdala in children with autism is initially larger, but it does not undergo the same age-dependent increase.

Many individuals with autism also exhibit poor fine and gross motor skills, pointing to possible dysfunction in the cerebellum. Several studies have found evidence of Purkinje cell death in the brains of autistic individuals postmortem (Fatemi *et al.*, 2002). Cross sectional areas of Purkinje cells were found to be 24% smaller in the brains of individuals with ASD when compared to brains of typical individuals. Additionally, deep cerebellar nuclei have been found to differ in size and number depending on the age of the individual affected. Autistic individuals over 21 possessed small, pale neurons significantly decreased in number compared to the control. However, autistic individuals aged 5-13 possessed large and plentiful neurons, very similar to unaffected people their age (Fatemi *et al*., 2012). This evidence points to the possibility that the etiopathogenesis of ASD is not just a prenatal process, but an ongoing postnatal process that occurs throughout one's lifetime. A study by Allen and Courchesne (2003) found evidence pointing to the role the cerebellum plays in attention deficits in autism due to cerebellar projections to higher order brain structures. The cerebellum's function in the pathology of ASD, not just with motor coordination but also with cognition, supports the fact that autism is a complex and multifaceted disorder.

Neuronal areas of the frontal lobe, particularly the prefrontal cortex, have also been found to be heavily implicated in ASD. The prefrontal cortex is involved in higher order emotional, social, and cognitive development. In a study by Courchesne *et al.* (2011), it was found that brain overgrowth in the prefrontal cortex during the early stages of development, as well as an excess of neurons, is characteristic of autism. In this study involving the analysis of postmortem brain tissue from autistic children aged 2 to 16 years, both the brain weight and neuron count was

significantly increased compared to the control group. Additionally, a significant increase in microglial density in the dorsolateral prefrontal cortex has been identified in male autistic subjects (Morgan *et al.*, 2010). They also identified an increase in microglial activation in the same brain areas via immunohistochemistry. This points to the possibility that microglial activation plays a key role in the pathogenesis of ASD, or alternatively that the activation may be representative of the brain's innate immune response to synaptic and neuronal network disturbances. Not only does the structure of the prefrontal cortex affect behavior, but behavior also affects structure. In an experiment conducted by Liu *et al*. (2012) where mice were socially isolated for a long period of time, it was found that as a result, oligodendrocytes in their prefrontal cortex were degraded and myelination was impaired. However once these mice were reintegrated back into the social group, these transcriptional effects were reversed and their behavior returned back to normal. It is evident that a clear age-dependent pattern has been found between the cortex and the pathophysiology of ASD.

There is not one standard treatment for ASD, but current research is focused on identifying key genes implicated in the disorder using animal models. ASD is a complex disorder with various causes and multiple genes of interest. The *SHANK3* gene has been identified as one of the most promising candidate genes for ASD. *SHANK3* encodes scaffolding proteins that are integral for the postsynaptic density of excitatory synapses. It also plays a role in neurotransmitter receptors, ion channels, and dendritic spine maturation (Dhamne *et al.*, 2017). Much of the current research is focused on the *SHANK3B* allelic mutant due to its more pronounced behavioral defects. It has been found that mice with deletions in the *Shank3B* gene exhibit ASD-like behaviors such as repetitive self-injurious grooming, anxiety, and deficits in social interaction. This was concluded through experiments in which it was shown that *Shank3B* knockout mice exhibit less rearing than WT in the open field test and less time in the open arms

of elevated plus maze (Peca *et al.,* 2011). The same study found that the *Shank3B* knockout mice preferred the empty cage and displayed less social novelty in the three chamber social task. Repetitive grooming behavior has also been reported in *Shank3B* mice, suggesting alterations in cortico-striatal functionality, as reported by Peca *et al*. (2011). Another study conducted by Rendall *et al.* (2018) observed atypical social behavior in *Shank3B* mutants, through increased dominance in the social dominance tube task. Although these results are typical of social aggression, this study concluded that they may reflect failure to properly process social cues when confronted when another mouse. These mice have decreased levels of scaffolding proteins and glutamate receptors in the striatum, providing overwhelming support for the connection between a disruption in the *Shank3B* gene and the development of ASD-like behaviors in mice (Balaan *et al.,* 2019).

The current experiment sought to contribute to the current body of knowledge by identifying a physiological marker of ASD that may be used to screen toddlers, allowing for earlier detection and diagnosis. According to the National Institutes of Health, early diagnosis and intervention for autism can have immense positive effects on the individual's skills later on in life. The only treatment currently available for ASD involves a combination of therapies; speech therapy, cognitive behavioral therapy, behavioral management therapy, social skills training, and physical therapy to name a few. Currently, the average age for ASD diagnosis in the United States is just over four years of age (Maenner *et al., 2020).* However, depending on the severity of the disorder, it can be possible to diagnose some children before they turn two. Early interventions prior to the preschool age, while a child's brain is still very much plastic, allow treatment to be more effective throughout the individual's life. Early intervention has the ability to greatly improve an individual's behavioral outcome. The primary goal of this experiment was to identify a biomarker for ASD, such as brain volume, that can allow medical

professionals to help diagnose children with autism as early as possible. Oftentimes children who display some of the less severe behavioral signs are misdiagnosed. Furthermore, many children do not display any symptoms until the age of four – at this age, improved outcomes through behavioral intervention can be negatively impacted . However, if a significant difference in neuronal volume can be identified in children with autism when compared with their peers of the same age, an ASD diagnosis can be made before atypical behaviors are noticed and intervention can be made available earlier than before.

The use of a mouse model was necessary for this study. Mutations in the *SHANK3B* gene are implicated in ASD in humans. When the *Shank3B* gene is targeted for disruption in mice, the rodent homolog of *SHANK3B*, they exhibit ASD-like behaviors such as self-injurious stereotypy, increased anxiety, and social deficits. The current study focuses on the neuroanatomical analysis of the structural volume of the neocortex and amygdala — two of the main brain structures implicated in ASD. Due to prior research that has been collected, it is evident that the amygdala is involved in social interaction and facial/emotional recognition, while the cortex is involved in higher order cognition and goal-directed behavior. An age-dependent pattern of reverse growth in autistic brains has been found for both the cortex and amygdala. While typically developing individuals start out with a smaller brain volume which then increases as they age, autistic individuals seem to have the opposite pattern of growth. They are born with an excess overgrowth in brain tissue compared to controls, and these differences level out after the adolescent years. Therefore, it was predicted that an increase in structural volume of both the amygdala and cortex would be observed in *Shank3B* knockout mice compared to heterozygous and wildtype.

Methods

Subjects

Heterozygous breeding pairs of B6.129-*Shank3*^{tm2Gfng} mutant mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). In this strain, exons 13-16 of the *Shank3* gene, which encodes the PDZ domain, were replaced with a neo cassette. This resulted in the absence of Shank3α and Shank3β isoform expression, and a reduction in the Shank3γ isoform (Peca *et al.*, 2011). Subjects for this experiment were generated from Het x Het breeding at the University of Connecticut. Genotypes of the offspring were determined by mouse ear punch DNA, using the following PCR primers: Common (GAGACTGATCAGCGCAGTTG), Wild Type Reverse (TGACATAATCGCTGGCAAAG), and Mutant Reverse (GCTATACGAAGTTATGTCGACTAGG) (Rendall *et al.*, 2018). 22 male subjects were chosen for this study through behavioral phenotyping and histological assessment. Of the total subjects, 7 were Wildtype (WT), 9 were Heterozygous (HT), and 6 were *Shank3B* knockouts (KO). Following breeding, all subjects were individually housed in standard mouse tubs, in 12 hour light/dark cycles with food and water available ad libitum. Data collection was carried out blind to genotype. All procedures were conducted in compliance with the National Institutes of Health and the University of Connecticut's Institutional Animal Care and Use Committee.

Brain Slicing and Nissl Staining

Following behavioral testing (see Rendall *et al.*, (2019) for the behavioral characterization of *Shank3B* KO mice), the adult mice were prepared to be euthanized at P200. The subjects were weighed and anesthetized with 100/15 mg/kg ketamine/xylazine. They were transcardially perfused with 0.9% saline and 10% formalin. Their brains were individually extracted and fixed in 10% formalin. The brains were serially sectioned on the coronal plane using a vibratome (Leica; VT 1000S). Sections were sliced 60 μm thick. Rather than mounting every section, every second section was mounted on gelatin-subbed slides. Nissl bodies were stained using cresyl violet.

Stereological Measures

All prepared brain slices were analyzed using *Stereo Investigator* (MBF Biosciences, Williston, VT, USA) in conjunction with a Zeiss Axio Imager A2 Microscope (Karl Zeiss, Thornwood, NY). The volumes of the cortex and amygdala of each subject were measured using the *Cavalieri Estimator* probe in *Stereo Investigator*. For the cortex, every fourth mounted slice was traced, accounting for every eighth brain section, since every other section was mounted. Tracing started at the very first mounted slice. About 8-10 sections per brain were traced for cortex analysis. Total number of sections differed by subject depending on total brain volume. For the amygdala, every mounted slice was traced, accounting for every other brain section. Rather than beginning at the very first mounted slice, tracing began around the eighth slice, since the amygdala is localized medially in the brain. Ten sections per brain were traced for amygdala analysis. Contours for volumetric measurements were drawn at 2.5x magnification, and a standard stereotaxic atlas was utilized to help determine the anatomical location of the cortex and amygdala.

Results

Data analysis was run using IBM SPSS Statistics, using a (one-way?) ANOVA test to determine if Genotype had a significant effect on the structural volumes of the cortex and amygdala. Results showed that there was no significant difference among the right amygdala, left amygdala, or total amygdala volume across all three genotypes (WT, HT, and KO). These results can be seen in Figure 1 ($p>0.05$). Preliminary analysis also found that there was no significant difference among the right cortex, left cortex, or total cortex volume across all three genotypes ($p>0.05$). However, recognizing that the right cortex volume of the HT treatment group seemed prominently less than that of the WT and KO groups (as seen in Figure 2), a repeated ANOVA was performed to compare the cortex volume of the HT subjects to the control

group. Only the HT and WT treatment groups were included in this ANOVA, and the results can be seen in Figure 3. This statistical analysis revealed that the volume of the right cortex was marginally decreased in the HT mice compared to the WT mice [$F(1,15)=3.274$, $p<0.10$].

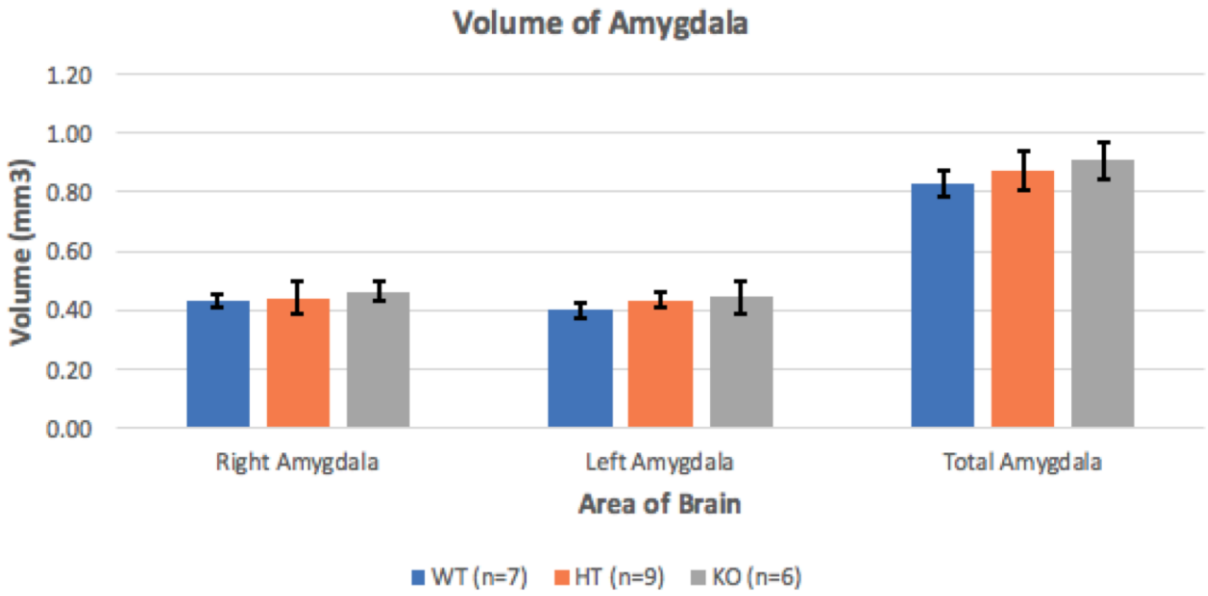

Figure 1. Comparison of amygdala volume across all genotypes. No significant difference was found.

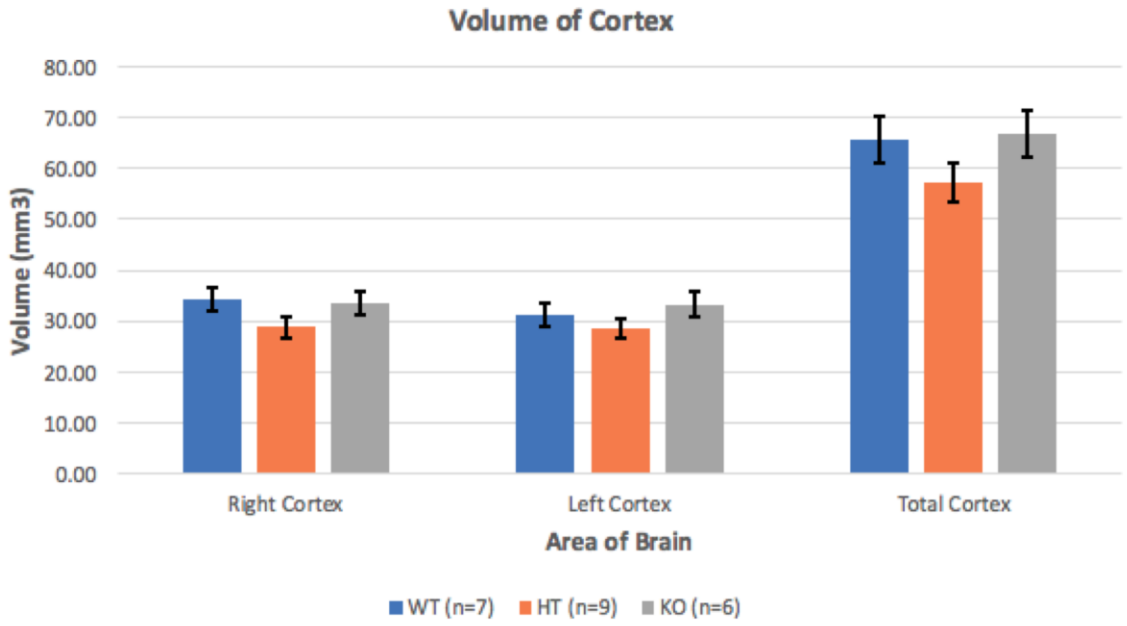

Figure 2. Comparison of cortex volume across all genotypes. No significant difference was found.

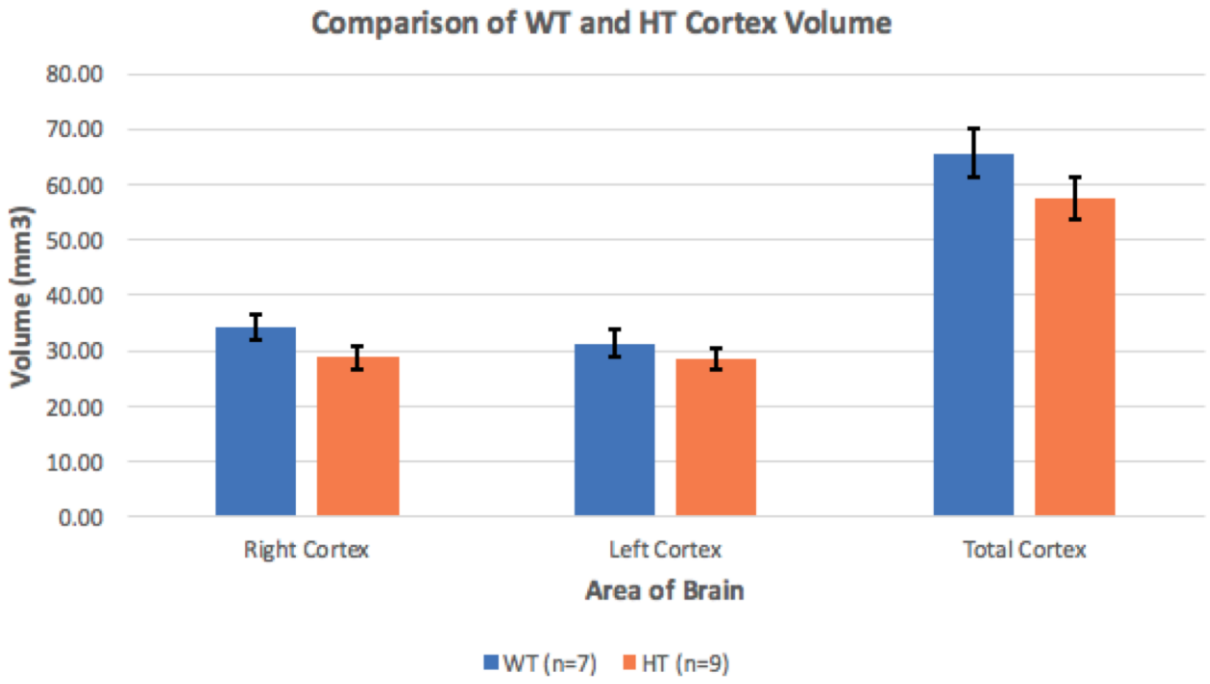

Figure 3. Comparison of cortex volume between WT and HT groups. The right cortex was found to be marginally significantly decreased in HT subjects compared to control ($p<0.10$). The average right cortex volume of WT was 34.32 mm^3, with a SEM of 2.24 mm^3, and the average right cortex volume of HT was 28.84 mm^3, with a SEM 2.02 mm^3.

Discussion

The lack of significance in the volumes of the cortex and amygdala between the *Shank3B* KO mice and WT mice may indicate something important. Despite the substantial dysfunctions in the neural networks and synapses of these brain areas, the total structural volume is not significantly different. From these data, it can be concluded that ASD is a disorder of neural abnormalities, as opposed to a disorder in which brain structures are diminished, as is common in many other neurodevelopmental disorders. Physiological differences in the neuroanatomy of these structures may be seen at a molecular level, or even at the level of a single cell. Replicating this study using live animals and functional imaging may help illuminate some of the cellular-

level differences in circuitry. However, at the gross level of the brain as a whole there was no statistical difference in the volume of the cortex and amygdala.

The data show a trend towards a larger amygdala volume in the HT and KO mice compared to the WT control. This seems to support the prior research and hypothesis. This trend may be indicative of the brain overgrowth that is characteristic of children with autism, according to human research that has been conducted. It is possible that if this study were repeated with mice of a younger age, or with a larger sample size, that a more significant increase in volume may be discovered.

The marginal significance ($0.05<p<0.10$) in the right cortex volume of the HT mice compared to WT mice presents novel information. The right cortex may play a significant role in the pathology of ASD. Compared to the left cortex, where a significant difference between volume was not found, the volume of the right cortex was marginally reduced in HT mice compared to WT. This warrants investigation into the distinct functions of the right and left cortex, and how these may play a role in the disorder. The left cortex, specifically the left inferior prefrontal cortex, mediates semantic memory and retrieval as seen in neuroimaging studies (Wagner *et al.*, 2001). Another study conducted by Badre & Wagner (2007) implicates the left ventrolateral prefrontal cortex in the cognitive control of semantic memory, in which memory is strategically accessed when it is relevant to current goals and actions. Based on substantial research, there is a clear connection between the left cortex and semantic memory.

The right cortex on the other hand, has been found to play a more distinct role in episodic memory. Research by Kennan *et al.* (2000) utilizing neuroimaging technology and split-brain individuals has found a link between the right frontotemporal prefrontal cortex and autobiographical memory. It appears that there is a self-recognition center localized in the right prefrontal cortex that is important in self evaluation and autobiographical retrieval. Additional

studies have also found that the right anterior prefrontal cortex is activated during episodic memory retrieval tasks (Allan *et al.,* 2000). In autobiographical memory studies with autistic individuals, it has been found that adults with ASD demonstrate an episodic memory deficit, while their semantic memory remains intact (Crane & Goddard, 2007). This may explain why anatomical analysis revealed a marginally significant decrease in the right cortex volume of HT subjects, but no such volume reduction was found in the left cortex.

These findings are likely related to the memory split that is characteristic of ASD. Episodic memory is composed of previous experiences and events in the context of their associated emotions. By contrast, semantic memory is related to information about oneself or others, such as words, concepts, and numbers. While semantic memory remains intact in individuals with ASD, episodic memory is negatively affected. The behavioral effects of this can be seen in studies such as those conducted by Baron-Cohen *et al.* (2000) and Howard *et al.* (2000), where autistic individuals failed to recognize emotion and demonstrated deficits in facial recognition memory, components of episodic memory. Further evidence supporting the right hemisphere laterality of emotional facial recognition can be seen in research by Suberi and McKeever (1977). They tested phenotypically normal females on how quickly they were able to discriminate familiar faces from non-familiar faces. Faster reaction times were obtained for images shown in the left visual field, compared to images shown in the right visual field. This supports right hemisphere superiority in facial recognition, an ability that is impaired in autistic individuals.

The right cortex also plays a key role in response inhibition, which is the suppression of actions that are inappropriate in a given context or that interfere with goal-driven behavior (Mostofsky & Simmonds, 2008). Human lesion-mapping supports the localization of the executive control of inhibition to the right inferior frontal cortex (Aron *et al.,* 2004). In response

inhibition tasks involving autistic individuals, it has been found that compared to the control, they have less brain activation in the neural areas involved in the inhibition network, but more brain activation in premotor brain areas (Kana *et al.,* 2007). These results indicate that the response inhibition circuitry is atypical in the brains of autistic individuals, and postulate an additional explanation for the abnormal volume reduction of the right cortex that was seen in this study.

Ultimately these findings in the *Shank3B* mutant mouse model of autism may not be perfectly generalizable to Autism Spectrum Disorder in humans. Though there has been a multitude of scientific evidence connecting the phenotypic behavior of *Shank3B* knockouts to autism-like behavior displayed in affected humans, there has yet to be unequivocal research on the physiological and anatomical similarities between the two species. While the *SHANK3* gene is one of the major genes implicated in the majority of ASD cases, autism in humans is a polygenic disorder. For this reason we cannot offer definitive conclusions, but further research into the neuroanatomical analysis of ASD is encouraged.

Future Directions

Confirmation and clarification of the results from this experiment would require a larger sample size as well as multiple experimenters to control for the inconsistency and human error that may arise from having a single experimenter conduct tracing. Future research geared toward accomplishing the goal of this experiment, which was to identify a clear physiological marker for Autism Spectrum Disorder, should focus on studying other brain structures that are implicated in the disorder. Such structures include the cerebellum and white matter tracts. Plentiful research has found evidence to support the role of the cerebellum in the pathology of ASD (Fatemi *et al.,* 2002; Fatemi *et al.,* 2012; Allen & Courchesne, 2003). There have also been studies that identified an increase in the volume of white matter tracts in certain areas of the brain. Herbert *et*

al. (2004) found that all white matter tracts in the outer radiate zone of the brain had been enlarged in the brains of individuals with high-functioning autism. Several studies have implicated that the enlargement and decrement of the structural integrity of white matter tracts persist into adulthood, demonstrating that the pathology of ASD is an ongoing postnatal process (Herbert *et al.,* 2004; Keller *et al.,* 2007). Furthermore, the lack of significant results from this study may be due to the age at which the mice were sacrificed. Adult mice were used in this study, however given the age dependent effects of volume seen in humans, it may be helpful to use younger mice when replicating this study. It would be seminal to take this research one step further and investigate if there is a biomarker of ASD present at birth, or from a very early age, in order to help enable earlier diagnosis and intervention for autism.

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