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DECREASED DENSITY OF SEROTONIN TRANSPORTING FIBERS IN THE INFANT
WILLIAMS SYNDROME AMYGDALA

A thesis submitted in partial satisfaction of the requirements

for the degree of Masters of Science

in

Biology

by

Kimberly Mieko Groeniger

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2018

The thesis of Kimberly Mieko Groeniger is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

DEDICATION

I dedicate this thesis to my mother, Gayle, for providing me with continual love, guidance, and humor throughout my academic career. Her strength and constancy has been absolutely invaluable, and I am forever grateful for her support. I further dedicate this thesis to my wonderfully supportive and incredibly talented lab mates, without whom I would not be as confident in my skills or as humble in my pursuit of future endeavors.

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ABSTRACT OF THE THESIS

Decreased Density of Serotonin Transporting Fibers in the Infant Williams Syndrome Amygdala

by

Kimberly Mieko Groeniger

Master of Science in Biology

University of California San Diego, 2018

Professor Katerina Semendeferi, Chair
Professor Nicholas Spitzer, Co-Chair

Williams Syndrome (WS) is a rare neurodevelopmental disorder characterized by a known genetic profile, hypersociability, and increased attention. This unique embodiment of social atypicalities allows for distinctions to be drawn between WS and other neurodevelopmental disorders, such as Autism Spectrum Disorder (ASD), as well as emotional disorders such as Major Depressive Disorder (MDD), Bipolar Disorder (BD), and Schizophrenia (SZ). While these disorders are not the focus of this study, the breadth of research performed with regards to these disorders involving neuroanatomy and its relation to social and behavioral

profiles has provided a solid foundation for this study. Aspects of the WS social and behavioral phenotype have been extensively studied, however there has been less examination of the neuroanatomical characteristics which correlate with observed behaviors. The amygdala has been associated with the processing of socially salient information and coordination of responses to social stimuli, and within this structure serotonin is an active neurotransmitter responsible for modulating mood and emotional responses. To ascertain the role which serotonin plays within the infant WS amygdala, I examined the density of immunoreactive serotonin transporting (SERT-ir) fibers in the primary amygdaloid nuclei (lateral, basal, accessory basal, and central) of infants with WS compared to those of typically developing (TD) controls. Density in all four nuclei was decreased in WS as compared to TD, and in both WS and TD the central nucleus contained the highest density of SERT- ir fibers. This study contributes preliminary findings for future examinations of the serotonergic system in the WS amygdala.

INTRODUCTION

Williams Syndrome (WS) is a rare neurodevelopmental disorder characterized by a known etiology and distinctive social phenotype (Williams et al., 1961). This unique disorder embodies aspects of linguistic, spatial, and cognitive skills in a way that is different from other forms of mental retardation, such as Down's Syndrome and Autism Spectrum Disorders. Individuals with WS show a hemizygous deletion of 26 consecutive genes on chromosome 7.q11.23 (Morris, 2010). This known genetic component is partnered with a unique combination of physical characteristics, linguistic and lexical abnormalities, and socio- communicative behavior. The physical manifestations of this disorder include cardiac abnormalities, distinctive facial features, infantile hypercalcemia, and persistent growth failure or reduced stature (Jones & Smith, 1975; Pober, 2010; Preus, 1984). A compelling and frequently studied aspect of WS is its social phenotype, which characterizes the disorder as one of increased attention to faces (Mervis et al., 2011), overimitation (Vivanti et al., 2017) hypersociability and gregariousness (Järvinen et al., 2013), and "pro-social compulsion" (Frigerio et al., 2006; Martens et al., 2009). Additionally, this unique embodiment of social atypicalities allows for distinctions to be drawn between WS and other neurodevelopmental disorders, such as Autism Spectrum Disorder (ASD), as well as emotional disorders such as Major Depressive Disorder (MDD), Bipolar Disorder (BD), and Schizophrenia (SZ). While these disorders are not the focus of this study, their more heavily studied amygdala neuroanatomy and its relation to social and behavioral profiles provide a platform from which hypotheses of the WS amygdala can be formulated.

Autism Spectrum Disorders are associated with a variety of genetic (Baron-Cohen et al., 2000; Feyder et al., 2010) and phenotypic (Lombroso et al., 2017; Nordahl et al., 2012) characteristic profiles, all of which contribute to the wealth of knowledge regarding this

spectrum of neurodevelopmental disorders, but dearth of concrete information regarding their cause (Cynthia M Schumann & Amaral, 2006). The key element from which contrasts can be drawn between WS and ASD lies in the social manifestations of each disorder. Individuals with ASD have been found to have impairments in social interactions and communication (Cynthia M Schumann & Amaral, 2006) as well as a lack of overimitation (Marsh et al., 2013). Some profiles of ASD include decreased face gaze as determined by eye- tracking techniques (Riby & Hancock, 2008) and atypicalities in visual attention (Burack, 1994; Edelmann et al., 2007). In an effort to further elucidate the entire etiology of WS, studies focusing on the contrasting social phenotypes between the two (Grice et al., 2001; Riby & Hancock, 2008; Vivanti et al., 2017) have helped create a more comprehensive understanding of the WS behavioral phenotype. Physical manifestations of each disorder in key neural regions involved in the processing of social information as well as the fear response have been examined with the purpose of developing more informed characterizations.

In discussion of the social phenotype of WS, neural territories including the caudate, putamen, nucleus accumbens (Hanson et al., 2017), cortical regions (Caroline Horton Lew, Brown, Bellugi, & Semendeferi, 2017; Meyer-Lindenberg et al., 2005), and the amygdala (Feyder et al., 2010; Haas et al., 2009, 2010; Haas et al., 2014; Martens et al., 2009) have been examined. The results of these studies, in combination with research performed on the behavioral and cognitive profile of WS (Järvinen et al., 2013; Martens et al., 2009; Porter et al., 2007; Riby & Hancock, 2008) have directed interest to the role played by the amygdala. Functionally, connections maintained by this neural structure have been shown to support cortical input of socially salient information (Adolphs et al., 1998; LeDoux, 2007; Joseph L. Price, 1999), and processing of visual input with regards to facial expressions and related emotional recognition

(Adolphs et al., 1994; Davis et al., 2010; Schiller et al., 2009). These earlier findings combined with studies of cortical inputs (Stefanacci & Amaral, 2000, 2002) and serotonergic innervation directed to the amygdala in rats (Linley et al., 2017) and of the amygdala itself in non-human primates (Stimpson et al., 2016), as well as in individuals with ASD (Azmitia et al., 2011) ultimately prompted the examination of serotonergic innervation in the developmental WS amygdala as discussed in this paper.

The established and documented importance of the relationship between the amygdala and serotonin in the modulation of social behavior allows for the further examination of this relationship in humans, particularly during early post-natal developmental stages. The goal of this paper is to investigate the density of serotonin transporting fibers within the primary nuclei of the amygdala in infants (ages 26-114 days) with WS using immunohistochemical staining techniques and stereological analysis. Ultimately, this discussion will allow for further information to be gleaned on the impact which WS has on the developmental trajectory of the amygdala, particularly in relation to the establishment and maintenance of serotonergic fibers.

This paper consists of six chapters in which I utilize extensive review of the literature to support the findings of my original research project discussed in chapters IV and V. In Chapter I, I provide a comprehensive background on Williams Syndrome. This discussion includes both the unique genetic and physical characteristics of Williams Syndrome, as well as an examination of the social phenotype associated with this disorder. In Chapter II, I delve into the importance of the amygdala in discussion of emotional and social behaviors, including a presentation of the structure and function of the primate amygdala in conjunction with the cortical and subcortical connectivity of the major amygdaloid nuclei. Discussion of the amygdala then continues with an examination of varying types of damage and pathology in both human and non-human primates

which have allowed for major insights into the functioning of this neural structure, including bilateral lesions and Bipolar Disorder. In Chapter III, I discuss first the broad role of serotonin in the mammalian nervous system, followed by the specific importance of this neurotransmitter's presence in the modulation of social behavior in rats and non- human primates. The investigation of serotonin's role in relation to the regulation of social behavior also includes a discussion of serotonin presence in individuals with ASD which, as previously noted, is a disorder characterized by a social phenotype often described as antithetical to that of WS. Lastly, preliminary research done on the degree of serotonergic innervation in the amygdala of WS adults is discussed as a reference point from which my hypothesis was developed. Discussions performed in Chapters I-III culminate in my hypothesis that the post-mortem density of serotonin transporting fibers in the amygdala of infants with WS will be decreased relative to that of controls.

Chapter IV expands upon the subjects used in this study as well as the methods used to identify amygdaloid nuclei in Nissl stained sections, stain SERT-ir fibers using a detailed immunohistochemical technique, and fiber density quantification. The results of this experiment are enumerated in Chapter V, followed by Chapter VI which discusses in detail the implications of these findings with regards to protocol efficacy as well as the WS infant amygdala.

CHAPTER I: WILLIAMS SYNDROME

Genotype and Phenotype

Williams Syndrome as a disorder was first characterized in 1961 as including supravalvular aortic stenosis (SVAS), mental retardation, and distinctive facies (Williams et al., 1961). In the early years of its discussion in medicine, the diagnosis of WS included individuals with (Bejar et al., 1970) and without hypercalcemia (Dupont et al., 1970) or aortic defects (Jones & Smith, 1975), leading the syndrome to encompass a broad set of pathological characterizations. It was discovered that rats exposed to high levels of vitamin D prenatally presented with shortened craniofacies and aortic lesions (Friedman & Roberts, 1966), leading some to connect vitamin D overexposure to WS. However, it was later found that high levels of prenatal vitamin D can inhibit the synthesis of the elastin gene, a key gene which when deleted, as in WS, has been connected with SVAS (Curran et al., 1993), leading this to be the most probable connection between WS and vitamin D dosage (Morris, 2010).

As time progressed and the study of WS intensified, efforts to pare down the diagnosis and shape a more concise etiology included the categorization of facial and morphological features thought to be indicative of WS (Preus, 1984), as well as studies examining the genetic basis of the disorder. It was determined that individuals with WS shared a hemizygous microdeletion within chromosome band 7q11.23 of about 28-26 genes including the gene for elastin (ELN) (Perez Jurado et al., 1996; Robinson et al., 1996). Upon further examination, it was found that the majority of WS-region deletions (~67%) have been shown to occur due to unbalanced recombination during meiosis, or interchromosomal rearrangement, with a minority arising from defects in intrachromosomal rearrangement (Baumer et al., 1998; Urban et al., 1996). The deleted region itself has been found to include 26-28 genes, and is interestingly

duplicated in 7q11.23 Duplication Syndrome, an Autism Spectrum Disorder which presents with, but is not limited to, cardiovascular disease, abnormal gait, childhood apraxia, and behavioral pathologies including anxiety disorders (Mervis et al., 1993). A comparative study using mouse models has shown that a deletion of mouse Dlg4, which causes synaptopathies of electrical synapses, produces a hyper social phenotype contrary to the authors' expected ASD-like decrease in sociability (Feyder et al., 2010). Using human induced pluripotent stem cells (iPSCs), major electrophysiological defects were found in voltage gated potassium channels, causing repolarization of neurons to be longer and slower (Khattak et al., 2015). The proposed cause for this channelopathy is the downregulation of one or a combination of several potassium channel genes, ultimately impacting neuronal signaling (Fatemi et al., 2008; Khattak et al., 2015).

Social and Behavioral Profile

In conjunction with physical abnormalities, the behavioral phenotype of WS began to take shape as one encompassing loquacity, attention, hyper sociality, and anxiety (Beuren et al., 1964; von Arnim & Engel, 1964). Several investigative studies have been conducted with WS individuals of varying ages attempting to elucidate the neurobehavioral and social profile of adults, adolescents, and children with this disorder. These include, but are certainly not limited to, Piagetian tests of conservation and serial skill examining early cognitive ability (Bellugi et al., 1988), measures of length of face gaze in both WS and ASD (Riby & Hancock, 2008), tests of overimitation and its correlation to an over attribution of causality (Vivanti et al., 2017), and measures of attention during triadic interactions (Mervis et al., 2011). The social and behavioral profile of WS is complex in its embodiment of language atypicalities which differ in comparison

to other neurodevelopmental disorders such as ASD and Down's Syndrome combined with a high level of attention placed on both the faces and the implied intentions of others.

Individuals with WS between the ages of 8 and 28 years have shown increased length of time looking at the faces of others presented in images, and decreased time looking at background scenes in which those faces were placed compared to individuals aged 6- 17 years with ASD (Riby & Hancock, 2008). Interestingly, these individuals discovered hidden faces with the same speed as those with ASD, implying that there is no heightened face-finding ability in WS in combination with increased attention (Riby & Hancock, 2008). This suggests that individuals with WS use facial cues more continuously when interacting in a social environment, perhaps to extract information in more detail of others' behaviors (Riby & Hancock, 2008). It has been argued that the increased length of face gaze in WS can be related to the over attribution of causal relevance, particularly seen in a test of imitation from which comparisons between individuals with WS and ASD as well as controls can be drawn (Vivanti et al., 2017). This study sought to determine whether overimitation in preschoolers with WS and ASD stemmed from social-motivational (motivation to engage socially with a modeled action) or social-cognitive processes (understanding the causal structure of actions), and found that children with WS not only imitated a model action more than those with ASD (22% of 18 WS children compared to 1% of 19 ASD children), but also showed increased attention to the face of the demonstrator (Vivanti et al., 2017). Increased attention, measured as face gaze maintenance for 1 to 2 minutes, was also found in 92% of 25 toddlers aged 8- 43 months with WS (Mervis et al., 2011), supporting this aspect of the WS social and behavioral phenotype. The importance of these attentional characteristics can only be elucidated with further study of the neural pathways

and structures in which the processing of social and visual stimuli take place, and how these have been found to be altered in WS.

Discussions of the atypical social phenotype of WS have necessarily included the amygdala, an almond shaped structure located in the medial temporal lobe which plays an integral role in the appraisal of social situations and facial expressions, as well as the processing of social and emotional behaviors often related to fear and aggression (Adolphs et al., 1994; LeDoux, 2007; Price, 1999). Increased gray matter volume and density compared to typically developing (TD) controls has been found in the WS amygdala, however this study did not focus on the volumes of individual amygdaloid nuclei of which this structure is composed, leaving room for further study of volume and density differences within the structure itself (Reiss et al., 2004). Functional neuroimaging has shown that while abnormal amygdala reactivity in thirteen WS individuals was not found to be caused by functional impairment of the structure itself, the regulation of the amygdala via the orbital prefrontal cortex (OPFC) was found to be abnormally decreased, particularly when individuals were shown threatening faces (Meyer-Lindenberg et al., 2005). Further confirmation of this finding regarding abnormal regulation of the OPFC in addition to increased reactivity in the amygdala to non-socially relevant aversive stimuli irrespective of cognitive demand was provided by a fMRI study with 13 normal IQ-WS individuals and age/sex matched controls (Muñoz et al., 2010). The fMRI results showed little to no activation of the OPFC during both matching and labelling tasks of fearful images of non-human animals regardless of cognitive load, correlating this region which has consistently been associated with the processing of social stimuli (Adolphs, 2001) to abnormal regulation of the amygdala in WS leading to the social disinhibition characteristic of the disorder (Muñoz et al., 2010). Reactions to negative or aversive stimuli in social and non-social contexts have been

studied in depth in WS, leading to a host of data from which some occasionally inconsistent conclusion have been drawn.

The strong pro-social compulsion affiliated with WS has been characterized in approachability tests in which individuals with WS were asked to rate unfamiliar faces on levels of approachability (Bellugi et al., 1999) resulting in unfamiliar, negatively valanced faces being rated as more approachable by WS individuals than controls (Bellugi et al., 1999; Martens et al., 2009), indicating a tendency toward prosocial compulsions and an ability to empathize with others (Jones et al., 2000). Conflicting results of studies presenting emotionally valanced faces to WS individuals and TD controls highlight the importance of the context in which those faces are presented and how WS individuals process facial cues. For example, Frigerio et al., 2006 examined the perception of approachability in WS by presenting 21 WS individuals and age/sex matched controls with pictures of faces showing distinct emotions and lacking hair, ears, and background. The study found that WS individuals were not more likely to rate negatively valanced faces as being more approachable, as previously thought. A study by Porter et al., (2007) additionally found that WS individuals rated both "happy" and "sad" or "angry" faces similarly to the control group, further questioning the accuracy of the belief that individuals with WS are less able to distinguish positive and negatively valanced faces based on approachability than controls. However, in a subsequent study with 27 WS individuals and age/sex matched controls, WS individuals showed an increased tendency to rate negatively valanced faces as approachable and a positive correlation was found between right amygdala volume and approachability ratings, particularly of negative faces (Martens et al., 2009). Martens et al. postulated that the source of difference between the aforementioned results and those found by Frigerio et al. (2006) and Porter et al. (2007) may lie in whether or not the images included the

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whole head and more general facial cues, and the number of times the facial stimuli were viewed by the participants.

It has been found that the gaze of WS individuals focuses strongly on the eyes and mouth as key features for judgement of expression and facial identification (Riby et al., 2008). However, peripheral features such as hair, chin, and ears which are typically used by TD children between the ages of 5 and 9 (Want et al., 2003) are also used as focal points by WS individuals significantly more so than controls when perceiving affective facial expressions (Martens et al., 2009), linking presentation modes of test facial stimuli to the disparity in collected results regarding approachability ratings. Additionally, the argument has been made that the number of times a facial stimulus is presented to an individual may influence their rating of approachability, meaning that increased examination and processing may occur if a stimulus is presented more than once before the individuals are asked to rate their approachability (Martens et al., 2009; Porter et al., 2007).

CHAPTER II: THE AMYGDALA

Structure and Function

The amygdala, as previously discussed, has been traditionally described as an almond shaped structure located in the medial temporal lobe adjacent to the hippocampus (LeDoux, 2007; Price, 1999). The structure itself is divided into several nuclei on the basis of connectivity, with the four major nuclei moving laterally to medially being the lateral, basal, accessory basal, and central (LeDoux, 2007). As a key circuitry element used in the processing of social-emotional behaviors and aversive stimuli (Adolphs et al., 1994), the amygdala has been widely examined in the context of navigating the dynamic social sphere in a range of species from rodents to humans. As a result, the components and connectivity of this neural structure have been studied with the purpose of understanding how conveyance and processing of information from visuospatial sources occurs, as well as the implications for responsive behaviors. The function of the amygdala has been described in the context of evaluation of objects or individuals prior to interacting with them, and according to Amaral, 2002, these evaluations then determine a species-typical response coordinated by the amygdala. Following this hypothesis that the amygdala is designed to detect and avoid danger, it can be understood that information for the assessment of a threatening stimulus would enter the lateral nucleus via its connections to the temporal cortex, undergo threat assessment in an as yet unknown process, and leave via the basal and accessory basal nuclei to cortical areas involved in selective attention to the stimulus, or leave the central nucleus travelling to the parabrachial nuclei and vagus to initiate visceral and autonomic responses to the stimulus (Amaral, 2002).

Cortical and Subcortical Connectivity

A host of protocols have been employed to elucidate the role of the amygdala in the larger complex of the brain, including connections to both areas of the cortex as well as other subcortical structures. Some of these methods are able to map out connections emanating from and projecting to specific nuclei of the amygdala, such as retrograde and anterograde tracing to be discussed in the following sections, while others demonstrate more broad connections associated with the amygdala as a whole. For example, the use of diffusion tensor imaging (DTI), a method which measures the rate and manner of water molecule movement within neural tissue, allows for the observation of connections between the amygdala as a whole and areas such as the prefrontal cortex (Kim et al., 2011). By combining data acquired via fMRI with that of DTI in 20 psychiatrically healthy subjects, a pathway of white matter connecting the amygdala and the ventromedial prefrontal cortex was detected (Kim & Whalen, 2009). This study, which focused on the changes to this connection as a result of anxiety, did not examine the specific amygdaloid nuclei with which this pathway connected. However, it nonetheless provides a method in addition to anterograde and retrograde tracing which establishes a connection between the amygdala and the prefrontal cortex.

Lateral Nucleus

The nuclei of the amygdala are divided based upon their connectivity, with the principal location receiving input being the lateral nucleus. This nucleus receives informational input from visual, auditory, somatosensory, and olfactory systems with primary input stemming from the superior temporal gyrus and granular regions of the insula (Aggleton et al., 1980; LeDoux, 2007). A tracing study of cortical connections to the lateral nucleus of *Macaca mulatta* which involved the injection of fluorescent retrograde tracers into its dorsal, dorsal intermediate, ventral, and ventral intermediate subdivisions found there to be moderate to high input arising in

the agranular and dysgranular regions of the insula and the most prominent unimodal sensory input arising from the visual association area TE (Stefanacci & Amaral, 2000). On average, 65% of all retrogradely labeled cells were found in various regions of the temporal lobe, and 42% of all retrogradely labeled cells were seen in the perirhinal cortex areas 36d and 36r, an area which has been shown to receive sensory information from all sensory systems in the rat (Burwell & Amaral, 1998; Stefanacci & Amaral, 2000). Input from the perirhinal cortex was also found in a retrograde tracing study in *Macaca fasicularis* using both non-selective and selective tracers injected into the lateral nucleus (Amaral & Insausti, 1992). These cortical sensory inputs arise from associated areas and not the sensory systems themselves, however this array of sensory input received by the lateral amygdala has led this nucleus to be referred to as the gatekeeper of the structure (Price, 1999). This study was then corroborated by one using fluorescent anterograde tracers injected into cortical and subcortical regions of the macaque brain which found the lateral nucleus to be innervated largely by the superior temporal gyrus, temporal areas TE and TEO, and the granular regions of the insula (Stefanacci & Amaral, 2002). Outputs from the lateral amygdala extend to the other three major nuclei, with the basal and accessory basal receiving the majority of input.

Lew and colleagues explored the stereology of the amygdala in both adults and infants with Williams Syndrome, providing integral information regarding neuron number and density in the primary amygdaloid nuclei (lateral, basal, accessory basal, central) compared to those of typically developing individuals (Lew et al., 2017). The authors found a significant difference in the number of neurons in the lateral nucleus in WS subjects than TD, specifically that the WS subject showed increased neuron number. No statistically significant differences in neuron number or density were found in the basal, accessory basal, or central nuclei (Lew et al., 2017).

An increased number of neurons in the primary nucleus receiving input, coupled with anterograde and retrograde tracing studies of cortical- subcortical connectivity (Stefanacci & Amaral, 2000, 2002), provide insight into the potential increased connectivity between the lateral nucleus and the pre-frontal cortex in WS individuals. This increase in connectivity may be related to the heightened level of sociality experienced by individuals with WS, and perhaps to the degree to which social and emotional behavior is regulated in this disorder.

Basal Nucleus

It is important to note that an abundance of input to the lateral nucleus does not equate to a dearth in input to the other major nuclei. The basal nucleus has been found to be innervated by the orbito-frontal cortex, temporal pole, and anterior cingulate regions using intracellular horseradish peroxidase injections in rhesus monkeys (Aggleton et al., 1980). To further understand the relationship between amygdaloid connectivity and cellular communication, injections of D-[^{3}H]- aspartate in the amygdala of *Macaca fasicularis* were used as selective retrograde tracers for excitatory amino acid projections and paired with non-selective retrograde tracer injections of WGA-HRP in the same areas to determine which amygdaloid inputs use an excitatory amino acid as a neurotransmitter (Amaral & Insausti, 1992). Injections of both D-[^{3}H]- aspartate and WGA-HRP focused in the intermediate region of the basal nucleus resulted in labeling of medial aspects of the lateral nucleus as well as lateral regions of the accessory basal nucleus, highlighting the interconnectivity of this nucleus with its two closest neighbors. Injections into the basal nucleus showed D-[^{3}H]- aspartate labelling in areas 13 and 14 in the frontal cortex, and distinctive retrograde labelling via WGA-HRP in ventral portions of areas 46, 10 and 11. In the temporal lobe, basal injections of D-[^{3}H]- aspartate showed labelling in the parahippocampal cortex, and the subcortical basal nucleus of Meynert showed retrograde WGA-

HRP labelling and very little D-[^{3}H]- aspartate (Amaral & Insausti, 1992). Taken collectively, the findings of this study report that most of the cortical fields which provide input to the basal nucleus also transport excitatory amino acid neurotransmitters. Conversely, while subcortical inputs were often strongly labeled by non-selective WGA-HRP, indicating substantial input to the basal nucleus from the basal nucleus of Meynert, labelling of D-[^{3}H]- aspartate cells was little to non-existent (Amaral & Insausti, 1992). Additionally, the parvicellular subdivision of the basal nucleus showed dense inputs stemming from the agranular and dysgranular regions of the insula in *Macaca mulatta* in the anterograde tracing done by Stefanacci and Amaral, 2002. Primary output from the basal nucleus extends to the central nucleus as well as the striatum, which is involved in the control of voluntary action (Mcdonald, 1991; Price & Amaral, 1981).

Accessory Basal Nucleus

More recent retrograde and anterograde tracing studies of the *Macaca mulatta* and *Macaca fasicularis* amygdala sought to confirm the inputs to the basal nucleus as described by Amaral and Insausti, 1992 and describe the connections received by the accessory basal nucleus. Injections of horseradish peroxidase (HRP) into the accessory basal nucleus of the *Macaca mulatta* amygdala showed approximately 50% of retrograde axonal transport arose from the temporal pole, a region thought to be involved in the assignation of meaning to objects (Aggleton et al., 1980; Tsapkini et al., 2011). Additionally, these injections showed dense labelling in the lateral portion of the lateral nucleus of the amygdala, further emphasizing the input which the accessory basal receives from the lateral nucleus. Labelling was also noted in the claustrum when both the basal and accessory basal nuclei were injected, and in the parainsular cortex when only the accessory basal received the injection (Aggleton et al., 1980). The anterograde tracer study in *Macaca fasicularis* conducted by Stefanacci and Amaral, 2002 supports the results

found by Aggleton et al., 1980 of a strong connection between the accessory basal nucleus and the temporal pole as well as the superior temporal gyrus, which the authors found also provides input to the lateral nucleus.

Central Nucleus

The central nucleus is the most medial of the four major nuclei, and much of the input it receives is from the previously discussed basolateral nuclei. It has been speculated that the general process by which the amygdala functions with regards to receipt of input and the regulation of informational output is as follows: input enters the structure via the lateral nucleus, from which it will be forwarded on to the basal and accessory basal nuclei which receive additional input from the frontal and parahippocampal cortices as well as the temporal pole and parainsula. This information is then sent to the central nucleus, at which point input from various autonomic regions assist in coding emotional responses to visual stimuli which are then communicated to the brainstem (Stefanacci & Amaral, 2002). The anterograde tracing study of the *Macaca mulatta* amygdala found dense innervation of the central nucleus originating in the agranular and dysgranular regions of the insula, similar to the lateral and basal nuclei. The termination of this input was the most defined out of any cortical inputs to the central nucleus, supporting previous findings that innervation from the orbito-frontal cortex to the central nucleus arises solely in the insula (Carmichael & Price, 1995; Stefanacci & Amaral, 2002). However, the central nucleus is the primary source of efferent projections from the amygdala, therefor its outputs have been studied in depth in relation to the overall functional importance of the amygdala within the socio-emotional network. Using anterograde labelling via injections of ^{3}H-amino- acids into the central nucleus of *Macaca fasicularis,* labelling in the forebrain was observed in the bed nuclei of the stria terminalis, the basal nucleus of Meynert, and the nucleus

of the horizontal limb of the diagonal band (Price & Amaral, 1981). After injection, the animals were allowed to survive for 2 weeks which allowed the fiber pathways leading from the injection site to be well labeled, indicating the two primary pathways by which the central nucleus efferents travel are the ventral amygdalofugal pathway or the stria terminalis. The termination of the ventral amygdalofugal pathway was found most caudally in the substantia nigra, with inputs from the central nucleus occurring along the ventral amygdalofugal pathway in the preoptic area, lateral hypothalamus, and most prominently in the parabrachial region of the pons (Price & Amaral, 1981). These efferent connections combined with the inputs received by the major amygdaloid nuclei have been shown to be crucially involved in mediating a fight or flight response to stimulation in cats (Kaada, 1972), eliciting hissing, pupillary dilation, and elevated blood pressure among other autonomic responses. The innervation of the major amygdaloid nuclei by both varying and overlapping cortical and subcortical regions in conjunction with the array of regions to which the structure projects underscores the key role which the amygdala as a whole plays in the processing and conveyance of sensory and emotional information.

Amygdala Damage

Because the amygdala is an integral structure in the processing and control of behaviors in response to social and aversive stimuli, it is necessary to discuss the effects which damage and pathologies of the amygdala have on social emotional behavior in both humans and non- human primates. The rarity of human patients with selective amygdala lesions has necessarily made non-human primates the focus of study of amygdala damage, with an exception found in Adolphs et al., 1994. The authors studied subject S.M., a 30 year old woman with normal IQ, no diagnosis of depression, and Urbach- Wiethe syndrome (Adolphs et al., 1994). Urbach- Wiethe syndrome is a rare, autosomal recessive disease which causes bilateral calcification in the

anterior medial temporal lobes and affects the amygdala in 50-75% of cases (Siebert et al., 2003; Staut & Naidich, 1998). Adolphs et al., 1994 were able to use this unique opportunity to study S.M. and the effects which her amygdala damage had on her ability to recognize fear in the faces of others, multiple emotions in one facial expression, and the personal identity of faces. When presented with facial expressions of six basic emotions (happiness, surprise, fear, anger, disgust), S.M. rated expression of fear, anger, and surprise as less intense than control subjects of similar IQ and showed a significant recognition impairment in her ratings of fearful faces (Adolphs et al., 1994). Interestingly, her perception of multiple emotions in one face is interrupted, but not limited to only fearful or negative faces; when asked to describe how much disgust there was in sad expressions and how much surprise was in happy expressions she was only able to identify the prototypical emotion on each face. With regards to personal identity of faces, however, S.M. was able to correctly identify 19/19 pictures of individuals from her past, leading the authors to speculate that there must be a dissociation between the processing of facial identity and facial affect (Adolphs et al., 1994).

This study was then elaborated upon by a second which included S.M. as well as two additional individuals with bilateral amygdala damage and seven with unilateral amygdala damage. All individuals were able to discriminate faces, leading the authors to believe that discrepancies in judgements of approachability of faces would not be due to impaired visuospatial mechanisms (Adolphs, Tranel, 1998). Most notably, the subjects with bilateral amygdala damage tended to rate all faces more positively than controls and showed the largest deviation from control ratings when rating the most negatively valanced faces (Adolphs et al., 1998). To determine whether the subjects with bilateral damage gave these ratings based only on certain facial features or on the face as a whole, individual facial features of 109 pictures were

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manipulated (gaze direction, expression of the mouth, visibility of the eyes, etc.) and presented

alongside their un-doctored versions to S.M. and the controls. When asked which face they

would be more likely to approach, S.M.'s responses did not differ from controls in any of the

manipulations according to logistic linear analysis, implying that insensitivity to individual facial

features is not the likely cause for the abnormal judgement of approachability exhibited by S.M.

and the other bilateral damage subjects (Adolphs et al., 1998).

The previous studies provide an interesting glimpse into how human amygdala damage

can affect the processing of static social stimuli, such as pictures of faces. To understand how

similar damage to the amygdala affects interaction with conspecifics in a social structure

determined by the interpretation of dominance cues, it is necessary to turn to studies done in non-

human primates. In mature male *Macaca mulatta* monkeys with specific ibotenic acid lesions of

the amygdala, increased vocalizations and object exploration were noted (Emery et al., 2001),

leading others to examine the effects which amygdala damage in macaques have on their fear

response to threatening stimuli as well as their behavioral interactions with conspecifics. When

presented with a grape, lesioned macaques exhibited no period of evaluation compared to that

seen in normal macaques; even more interesting, however, is the observation that introduction of

a rubber snake also did not elicit the normal approach behavior and the period of evaluation was

also eliminated (Amaral, 2002). When normal macaques who are not familiar with each other are

introduced, a long investigatory period ensues mostly in an effort to prevent aggressive

interaction. However, lesioned macaques not only skip the initial familiarization phase, but the

their gregarious actions tend to initiate more affiliative behavior towards the lesioned animals

than toward control animals (Amaral, 2002). Interestingly, the lesioned macaques also showed

only modest or nonsignificant elevation in cortisol levels after these social interactions, but their

cortisol levels were increased on par with control macaques when they were physically restrained (Amaral, 2002), suggesting that the lesions impacted the perception of social threats, but did not impact the perception of physical stressors.

It is noteworthy to mention that the life stage at which amygdala lesions are performed in monkeys may have an impact on the social profile. Two- week- old macaque monkeys were lesioned and returned to their mothers for rearing, resulting in decreased fear of novel objects including rubber snakes when they reached 6-8 months of age (Prather et al., 2001). Interestingly, these same individuals showed a heightened fear response when being introduced to unfamiliar conspecifics, suggesting that the mechanism which facilitates fearful responses to social interaction is different from that for inanimate objects. These subjects showed no autism-like social behavior and expressed the normal range of social behaviors for their age, adding to the dynamic characterization of what exactly the role of the amygdala is in the mediation of social and non-social stimuli (Prather et al., 2001). As noted by Prather et al., 2001 and Amaral, 2002, this pattern of decreased fear of novel objects and increased fear of novel conspecifics is not seen in macaques lesioned when mature, indicating that there may be a developmental mechanism crucial to the mediation of a social fear response which is not manifested in subjects lesioned in infancy.

Amygdala Pathology

Major Depressive Disorder

Major Depressive Disorder (MDD) is a disorder related to emotions including sadness and dejection, however these emotions do not recede when an external cause of the emotions is removed (Belmaker & Agam, 2008). The established role of the amygdala in the perception and

processing of emotions has led to the exploration of its involvement, if any, in MDD. In a study

of 20 individuals with a history of recurrent major depression, an overall decrease in the volume

of the core amygdaloid nuclei (later, basal, and accessory basal as defined by the authors) was

found in both the left and right amygdalae compared to age/sex matched controls (Sheline et al.,

1998). The total cerebral volumes of those with depression did not differ from controls,

indicating that the reduction in amygdala volume was not due to overall brain atrophy. Similarly,

the overall volume of the hippocampus in individuals with major depressive disorder was also

found to be decreased relative to controls, which may suggest a correlation between the effect of

depression on the amygdala and its closest neighboring structure (Sheline et al., 1998). PET

scans have shown increased resting blood flow to the amygdala in patients with depression

(Drevets et al., 1992), prompting the question of what purpose this increased blood flow may

serve. In a study of 16 adults diagnosed with major depressive disorder, it was found that

exposure of subjects to positive stimuli (happy facial expressions) during a depressive episode

correlated with increased amygdala reactivity as measured by fMRI scans. This study went on to

note that individuals whose amygdalae were more responsive to positive stimuli than in baseline

scans were more likely to show greater symptom improvement 8 months later (Canli et al.,

2005). Interestingly, it has been found that both glial density and glia to neuron ratio in the MDD

amygdala are substantially decreased compared to controls, while no difference in neuron

number was found (Bowley et al., 2002). Taking these studies together, the role of the amygdala

in MDD may be one in which the structure still maintains its ability to process information in a

social context, such as the stimulus of facial expressions, but may also be reduced somehow in

its capacity or connectivity as suggested by Sheline et al., 1998.

MDD has also been examined with respect to the amygdala in children and adolescents, and the results differ with regards to whether or not early-onset MDD in children is normally accompanied by a decrease in the overall amygdala volumes. In an fMRI study of 20 children and adolescents with MDD and an average age of onset of 12.8 years compared with 24 controls, the MDD subjects were found to have an average decrease in volume of 12% of both the left and right amygdalae (Rosso et al., 2005). The authors speculate that this reduction in amygdalar volume may predispose children to the development of MDD and other mood disorders later in life, and suggest that there may be an important socioemotional role which the reduced amygdala of MDD subjects may play (Rosso et al., 2005), but more research must be done relative to the exact functional consequences of MDD on the amygdala. However, a more recent study of 32 psychotropic-naïve children with MDD as well as familial MDD (individuals have at least one first degree relative diagnosed with MDD) aged 8-21 years did not support the findings of Rosso et al., 2005 in that no significant difference in amygdala volume between MDD subjects and controls was measured (MacMaster et al., 2008). The authors state that this finding could be due to a small sample size (MacMaster et al., 2008), but in general there is a call for greater study of whether there are morphological and functional abnormalities in the amygdala of children, and if there are what possible significance they might have in predicting and treating adult MDD.

Bipolar Disorder

Bipolar disorder(BD) is a neurological disorder described by periodic shifts in mood and energy levels, often oscillating between emotional highs and lows. The connection between this disorder and the modulation of mood has necessarily called into question its relation to the amygdala, which has been examined morphometrically and functionally in order to better characterize this pathology. In a study utilizing post mortem tissue of 10 individuals diagnosed

with bipolar disorder and 12 control donors, the amygdalae of individuals with BD were found to have a decreased number of neurons, density, and volume in the lateral nucleus compared with controls (Berretta et al., 2007). Additionally, decreased density was also found in the accessory basal nucleus while no significant decreases were found in the neuron number, density, or volume of the basal nucleus (Berretta et al., 2007). In conjunction with findings of no statistically significant decrease in glial density in the amygdalae of individuals with BD (Bowley et al., 2002; Hamidi et al., 2004), Berretta et al., 2007 suggest that several subpopulations of neurons within the lateral nucleus may be affected by the disease, such as projection neurons, which in turn leads to the impairment of emotion processing experienced with BD. A later study using volumetric MRI techniques found that total volume of both the left and right amygdalae is increased in individuals with BD relative to those with Schizophrenia, but not compared to controls (Arnone et al., 2009). While this does not necessarily support the findings of Berretta et al., with regards to the size and density of individual nuclei within the amygdala, it also does not refute their supposition that BD may differentially affect subpopulations of neurons within the various nuclei, perhaps leading to an overall volume which does not diverge significantly from that of controls.

Functional observations of the amygdala in individuals with BD have found an increase in amygdala reactivity in response to fearful facial stimuli, noting specifically a decrease in the activation of the right dorsolateral prefrontal cortex and an increase in left amygdalar functionality (Yurgelun-Todd et al., 2000). These findings support previous studies of the connectivity between the amygdala and pre frontal cortical structures in macaque monkeys (Stefanacci & Amaral, 2002) and in humans (Damasio, 1998; Ketter et al., 1996), but do not shed more light on the pathological amygdala in BD other than the fact that there are as yet

unknown disruptions occurring in the prefrontal-amygdala network. A more recent study of functional connectivity between the amygdala and left ventrolateral prefrontal cortex (VLPFC) in 22 youths with BD found significantly positive functional connectivity compared with 23 age matched controls in a block-design emotional dynamic faces task evaluating the implicit processing of emotional stimuli (Hafeman et al., 2017). Individuals with BD consistently showed positive functional connectivity between the left VLFPC and amygdala in response to negative faces, indicating an increase in communication between these areas and providing additional support for the findings of Yurgelun-Todd et al., 2000 that left amygdala activity is increased in BD.

While childhood and adolescent early-onset bipolar disorder is more rare and its diagnosis can be a point of contention, there have been studies examining the morphological characteristics of the adolescent BD brain in an attempt to relate this manifestation of the disorder with that seen in adults. In a volumetric MRI study of 20 BD individuals with a mean age of 14.6 years and 20 control subjects with a mean age of 14.1 years found that individuals with BD had significantly smaller left and right amygdalae as well as decreased gray matter compared with controls (Chang et al., 2005). While the cause of this decrease in volume is most likely not attributed to a reduction in glia (Bowley et al., 2002; Hamidi et al., 2004), it may be due to abnormal pruning of neuronal branches during development combined perhaps with a decreased number of neurons in select nuclei (Berretta et al., 2007; Chang et al., 2005). Collectively, the structural imaging studies done with respect to childhood and adolescent bipolar disorder have shown distinct structural pathology of the amygdala which may differ from that seen in adults diagnosed with BD (Schumann et al., 2011), however further examination of

the source of these abnormalities in volume and gray matter are necessary to formulate a more comprehensive understanding of this neurological pathology.

Schizophrenia

Schizophrenia is often referred to as a psychiatric disorder with neurodevelopmental origins (Lewis & Levitt, 2002), and is described by a combination of disorganized thought and misperception of reality which can result in abnormal social functioning (American Psychiatric Association, 1994). The cause(s) of this disorder have yet to be identified, and avenues of genetic variation, environmental influences, and neurochemical imbalances have all been explored with little consensus reached. The idea that mature features of Schizophrenia derive from neurodevelopmental sources has been discussed over decades of research, and in this regard, can be related to WS when examining the relationship Schizophrenia may have with the amygdala. Morphologically, there have been conflicting studies with regards to whether or not the overall volume of the amygdala is reduced in Schizophrenia, as seen with in a 1998 finding of volumetric decreases of the amygdala in Schizophrenia using structural imaging techniques (Lawrie & Abukmeil, 1998). However, a more recent study of postmortem tissue stained for Nissl substrate from 16 adult individuals diagnosed with Schizophrenia found a modest decrease in neuron number in the lateral nucleus along with negligible differences in neuron number and neuron size in the basal and accessory basal nuclei (Berretta et al., 2007). Relationships between neuron numbers and history of antipsychotic use were explored, and the authors not only found no correlation between antipsychotic exposure and cell size, but a negative correlation between use of antipsychotic drugs in the last 6 months of life and neuron number (Berretta et al., 2007), indicating that the effects of antipsychotic medication on the reported neuron number were negligible. In contrast to these data, another postmortem histological study was performed using

tissue from 13 subjects with Schizophrenia aged 22-64 years which found a reduced mean volume and total neuron number in the lateral nucleus, once again characterizing Schizophrenia as a disorder of decreased amygdalar neuron number, at least in the lateral nucleus (Kreczmanski et al., 2007). Some of the subjects studied by Kreczmanski et al., 2007 also had seizure disorder and in some cases had long-term treatment with neuroleptics, the effects of which on the amygdala are not fully known (Cynthia M Schumann et al., 2011).

Functional studies of the amygdala in individuals with Schizophrenia report a similar pattern of incongruous results with some studies citing a decrease in amygdala activation in response to fearful faces/ negative affect experiments, and some finding an increase in similar tasks. In a study of 13 Schizophrenic individuals with flat affect (FA+) and 11 with no flat affect (FA-), the FA- individuals showed increased amygdala activation when shown emotionally negative images while the FA+ individuals showed no activation of the amygdala, leading the authors to speculate that an amygdaloid malfunction present in Schizophrenic individuals with flat affect disables them from perceiving the emotional meaning of the images (Fahim et al., 2005). In a subsequent fMRI study of both the functional connectivity of the amygdala- orbital prefrontal cortex and the amygdala itself, 12 adult subjects with Schizophrenia showed a reduction in amygdala and left medial orbital prefrontal cortex activation found when assigning levels of trustworthiness to faces in conjunction with an increase in left amygdala activity alone when viewing neutral or trustworthy faces (Baas et al., 2007).

Autism Spectrum Disorders

Autism Spectrum Disorders are neurodevelopmental in character and can be embodied in a variety of genotypic and phenotypic ways (Baron-Cohen et al., 2000; Feyder et al., 2010; Lombroso et al., 2017). With regards to the amygdala, there have been neuroanatomical studies

in both adult and developing ASD. A stereological analysis of the amygdalar volume and neuron number in adults with ASD found there to be no difference in overall or nuclear volumes in ASD compared with age matched controls. However, the number of neurons in the autistic amygdala overall and particularly in the lateral nucleus was found to be significantly decreased (Cynthia M Schumann & Amaral, 2006). The use of MRI has previously found that the autistic amygdala initially undergoes a rapid increase in volume postnatally followed by a lack of age related development normally seen in typically developing children (Schumann et al., 2004). When total cerebral enlargement (TCV) was controlled for in a structural MRI study of infants with a mean age of 37 months, amygdalar enlargement remained significant compared to age/ sex matched controls (Nordahl et al., 2012). This atypical development of the ASD amygdala implies critical alterations are made to this neural structure at differing developmental time points, leading to the possibility of altered social and behavioral profiles.

Because ASD is a spectrum, there are several manners in which social and behavioral abnormalities manifest within the disorder. However, there are aspects of autistic social behavior which have been found to be characteristic of many individuals included in this spectrum. Selective attention was measured in autistic individuals with a mean age of 20 yrs using a forced-choice reaction time test and incorporating varying numbers of distractors. When the number of distractors increased in the room the performance of ASD individuals was highly impaired, leading the authors to postulate that autistic individuals possessed a deficit in filtering stimuli and, more specifically, an inefficient attentional lens (Burack, 1994). More recently, a study focused on eye tracking movements and attention in subjects with ASD (mean age 12yrs 4mos) found that those with ASD took significantly longer than a control group to find a face hidden in a picture, and spent less time looking at said face once it was located. The authors proposed that

these significantly shorter face fixation times are related not to increased proficiency in interpreting facial information, but to in inattention and higher levels of disinterest in those with ASD (Riby & Hancock, 2008). In conjunction with this finding, a study of over imitation in children (mean age 9.4yrs) with ASD to be significantly decreased compared with typically developing controls. The children watched a demonstrator remove a toy from a box, including two necessary actions (opening box, removing toy) and one unnecessary action (tapping box). The authors postulate that the failure of ASD children to copy unnecessary actions may be due to reduced social motivation combined with a decreased desire to affiliate with the perceived norm (Marsh et al., 2013). Socially, the behavior of individuals with ASD encompasses a range including a lack of overimitation, impaired communication, and decreased attention. This profile provides insight into that of WS, which embodies a notably oppositional social phenotype. Examinations of physical manifestations of each disorder in key neural regions involved in the processing of social information may be crucial in developing more informed characterizations of the neuroanatomical profile of WS.

CHAPTER III: SEROTONIN

Serotonin is an amino acid neurotransmitter derived from tryptophan and is often referred to as 5-hydroxy tryptamine (5HT). Of key importance to this discussion and my original project is the serotonin transporter (SERT) which removes serotonin from the synaptic cleft and transports it back into the presynaptic terminal to be packaged into small, clear core vesicles (Purves, 2012). Because of its role in modulating the concentration of serotonin present in the synaptic terminal after release, SERT is crucial to regulating the degree of post-synaptic 5HT receptor activation (Munafò et al., 2008). Immunohistochemical techniques which employ primary and secondary antibody conjugations to stain for serotonin, serotonin synthetic enzymes such as tryptophan 5-hydroxylase, and/ or SERT have been used to ascertain the degree of serotonergic innervation in varying regions of the brain, including synthetic enzyme presence in the brainstem (Gardner et al., 2009; Walther & Bader, 2003), and SERT-ir (immunoreactive) fiber projections to the forebrain of rats (Smith & Porrino, 2008), in the amygdala of the rhesus monkey (Smith et al., 1999), and in the amygdala of bonobos, chimpanzees (Stimpson et al., 2016), and macaques (Bauman & Amaral, 2005). Presence of SERT-ir fibers in the cortex of macaques, chimpanzees, and humans (Raghanti et al., 2007) has also been examined, while other studies have involved the genes which code for the SERT in humans (Jasinska et al., 2012) and macaques (Watson et al., 2009).

This collection of information regarding the neurotransmitter and its associated molecules provides interesting insight into the role serotonin plays in a variety of brain locations in a range of species. The investigation of serotonin metabolites and SERT-ir fibers in the amygdalae of mouse and rat models has highlighted the impact which serotonin has on the control of social and emotional behavior. Additionally, the close genetic relationship coupled with the disparate social

phenotypes of chimpanzees and bonobos have provided a unique opportunity to examine whether differential serotonergic innervation of the amygdala can be responsible for the modulation of social behavior. Finally, when discussing serotonin and the human social brain, the inclusion of genetic research as well as SERT-ir fiber density in cortical and subcortical areas is essential to investigate the role of serotonin in both typically developing individuals and those with neurodevelopmental disorders possessing distinct social phenotypes such as ASD and WS.

Serotonin in Rodent Models

Animal models have been used to characterize both the pattern of serotonergic axon distribution within the rat amygdala as well as the impact that transcription factors have upon serotonin metabolism in the mouse amygdala. With regards to fiber distribution, the use of immunohistochemical staining for SERT found that the basolateral nuclei of the rat amygdala appeared to hold a significantly greater concentration of SERT-ir fibers compared to the central nucleus, however the concentration/ density of the labelled fibers was not quantitatively provided (Linley et al., 2017), leaving the amount of innervation undefined. A more direct correlate to WS in humans can be seen in examination of the deletion of GTF2IRD1, a general transcription factor which is deleted in WS and has been connected to the abnormal social phenotype associated with this disorder (Young et al., 2008). Mice with either a heterozygous or a homozygous deletion of this transcription factor showed decreased fear and aggression in social situations, but a more compelling result found that these mice also had increased levels of serotonin metabolites in the amygdala in addition to mild growth retardation. This result, obtained using liquid chromatography analysis, would normally correlate to increased levels of serotonin present in this structure in response to the deletion of GTF2IRD1, however the levels of serotonin found in the amygdala were not significantly increased. This suggests an alteration

in the turnover of serotonin, but not necessarily an increase in the neurotransmitter itself (Young et al., 2008). However, this model only examines the effect of one transcription factor being deleted in a rat model, meaning a direct connection to humans with WS cannot necessarily be drawn using only this information. Additionally, the concentrations of metabolites were not quantified in each nucleus of the structure, preventing more direct parallels with studies of specific nuclei innervation in humans and non-human primates.

Genetic variations regarding the expression levels of the gene encoding SERT have been found to have emotional and social implications in both macaque monkeys and humans. Expression levels of the short allele of the polymorphic SERT gene 5-HTTLPR were found to play a role in social aversion, gaze, and risk taking. Monkeys with one copy of the short allele and one of long allele showed decreased risk taking, increased deference to those of high higher status, and decreased likelihood of face gaze maintenance with conspecifics (Watson et al., 2009). While this study is not specific to the amygdala, variations in the 5-HTTLPR gene in the macaque is analogous to that in humans and may shape social phenotypes such as that seen in WS. A study of these allelic variants in typically developing humans has shown a similar result, correlating the presence of the short allele with increased fear and anxiety- related behaviors (Hariri et al., 2002). Further study of these gene variants possessed by those with WS could provide insight into the modulation of their specific suite of social behaviors.

Serotonin Innervation in the Non- Human Primate Amygdala

Examinations of serotonergic innervation in the amygdalae of non-human primates may add an additional layer of information regarding that present in humans. A study using polyclonal antibodies for serotonin itself in the amygdalae of three male *Macaca fasicularis* subjects found the highest level of serotonin immunoreactive fibers to be in the central nucleus,

which, as previously discussed, serves as the relay station from which information leaves the amygdala (Bauman & Amaral, 2005). The basal and lateral nuclei were found to have moderate innervation while the accessory basal nucleus possessed the lowest fiber densities. This study uses what appears to be a relative measurement scale, rating fiber densities in nuclei from low to moderate to high, providing qualitative measurements for between- nuclei comparisons of macaques but not for those to be made between species. A compelling case for the role of serotonin in the amygdala and its connections to social behaviors is found in a comparison of this innervation between chimpanzees and bonobos. As the two closest great ape relatives to humans, these species provide a remarkable platform upon which neurological causes of social variation can be studied, owing primarily to their differing responses to social and emotional stimuli. The amygdalae of seven bonobos and seven chimpanzees were subjected to immunohistochemical staining for the SERT containing fibers, and the whole amygdalae of the bonobos contained a higher density of fibers than that in chimpanzees (Stimpson et al., 2016). Serotonergic innervation was significantly increased in the central and basal nuclei of the bonobo amygdala, indicating that the modulation of both informational integration and output from the structure may be heightened and thus related to the increased attention bonobos have in response to social stimuli (Stimpson et al., 2016). While this may correlate with differences in human processing and response to social stimuli as seen in WS, a direct observation can only be made when the human amygdala is examined.

Serotonin and Autism Spectrum Disorder

A key experiment regarding this relationship between serotonin and the amygdala in humans has been conducted with regards to ASD. As previously discussed, ASD is a neurodevelopmental disorder which is often described as including impairments in social

interaction and engagement. Because the amygdala has been established as a structure involved in the social brain network, a crucial study of serotonergic innervation to the amygdala was conducted by Azmitia et al., 2011 in an effort to resolve conflicting evidence of the presence of this neurotransmitter. While a previous study found a reduced serotonin system in the cortex and midbrain of children (mean age 8yrs 8mo) with ASD using single photon emission computed tomography (Makkonen et al., 2008), this post mortem immunocytochemical examination of SERT-ir fiber tracts in young ASD individuals (ages 2.8-29 yrs) found an increase in density in the ansa lenticularis which sends fibers to the ventro-amygdalo- fugal pathway compared to that in TD controls (Azmitia et al., 2011). The authors connect this increased presence of serotonin in young ASD individuals with an increase in prenatal stress and use this finding to promote a reduction in the prescription of selective serotonin reuptake inhibitors (SSRIs) to individuals with ASD. Though this study does not examine the density of SERT-ir fibers within the amygdala itself, it provides a platform from which hypotheses regarding SERT-ir fiber density in the WS amygdala can be drawn.

Hypothesis

Preliminary research on the serotonergic innervation of the adult WS amygdala (Lew et al., in preparation) has found there to be an overall decrease in the density of SERT-ir fibers within the major amygdaloid nuclei in WS compared with controls. Examination of the serotonergic system in the infant amygdala may serve to further characterize the WS amygdala at different life stages. A recent functional MRI study of human infants as young as 160 days found amygdala circuits with connections to cortical and subcortical areas to be both intact and functional, implying the possibility of an amygdalar serotonergic system at early postnatal developmental stages (Gabard-Durnam et al., submitted). Based on this finding and in

conjunction with the findings of Lew et al., in prep., I hypothesize that the post-mortem density of serotonin transporting fibers within the amygdala of infants with WS will be decreased relative to that of controls, supporting the trend of decreased SERT-immunoreactive fiber density observed in adults with WS. The direct impact of this decrease will be an accumulation of serotonin in the synaptic cleft, ultimately causing an elevation in mood and greater expression of gregarious behavior. This study will examine a currently unexplored aspect of human developmental neuroanatomy by investigating the density of serotonin transporting fibers within the primary nuclei of the amygdala in human infants with WS (age 26-114 days) using immunohistochemical staining techniques and stereological analysis. Additionally, I observed the impact which variations in the immunohistochemical protocol, specifically the use of mounted or floating sections, had on both tissue integrity and penetrance of stain. Through comparisons with typically developing controls, this study allows for further information to be gleaned on the impact which WS has on the developmental trajectory of the human amygdala in relation to the establishment of serotonergic fibers.

CHAPTER IV: MATERIALS AND METHODS

(Initials Key for Research Scientists and Research Assistants: KS= Dr. Katerina Semendeferi, CL= Dr. Caroline Lew, HO= Hailee Orfant, VJ= Valerie Judd, DC= Deion Cuevas, DG= Demi Greiner, KG= Kimberly Groeniger)

Subjects

The materials utilized in this study consist of two WS infant subjects aged 1 month (WS 11) and 3 months (WS 7), and two TD age, sex, and hemisphere matched controls (Table 1). Fluorescent in-situ hybridization (FISH) probes for the elastin gene which is normally deleted in individuals with WS were used to confirm the diagnosis of WS in both infant subjects. The WS tissue was obtained from the Ursula Bellugi Williams Syndrome Brain Collection in continual partnership with the Laboratory for Human Comparative Neuroanatomy. Tissue from both TD subjects was obtained through the NIH NeuroBioBank.

Tissue Processing

Following autopsy, all brains were immersed in 10% buffered formalin for varying post mortem intervals (Table 1) and remained in formalin until processing occurred. For each subject, a 4-cm block was obtained from one hemisphere including the rostro- caudal extent of the amygdala by KS; in some cases, blocks were then segmented coronally into two smaller sections to fit the microtome stage. These smaller blocks were then cryoprotected until they were saturated in a gradient of sucrose and phosphate buffer solutions (10%, 20%, 30%) before being frozen with dry ice and cut coronally on a Leica SM sliding Microtome by CL, HO, VJ, and KG. All subjects used in this study were cut in five series of 80 micrometer (μM) sections with one series mounted onto gelatin coated slides and stained by HO, VJ, and KG for Nissl substance using .25% thionin for use in identifying the boundaries of the major nuclei for comparisons to sections stained for SERT-ir fibers.

Immunohistochemistry

The following protocols were performed by KG with assistance from CL, DC, and DG. A series of 1 in 20 sections spanning the rostral- caudal extent of the amygdala from two WS infants and their corresponding TD controls underwent immunohistochemical staining to quantitate the density of fibers carrying serotonin transporter within the four major nuclei of the amygdala. Both floating and mounted sections of WS and TD control infants were stained using a modified avidin- biotin-peroxidase method as described by Raghanti et al., 2008 in their comparison of serotonergic innervation of the cortex in humans, chimpanzees, and macaque monkeys.

Sections were removed from frozen storage in tissue collecting solution (TCS), and prior to staining for SERT-ir fibers, sections were washed six times for five minutes each in a phosphate buffered solution with a pH of 7.4. After washing, some sections were immediately mounted on gelatin coated slides and dried to ensure tissue adherence. Mounting of sections prior to staining was done to minimize the damage done to the tissue during antigen retrieval and solution transfers. All sections then underwent antigen retrieval in a sodium citrate buffered solution (pH = 7.4) at 86°C for 60 minutes followed by 20 minutes of cooling at room temperature. Sections were then rinsed three times five minutes each, followed by incubation in pretreatment solution of 75% methanol, 2.5% hydrogen peroxide (30%), and 22.5% ddh2O to quench endogenous peroxidase for 20 minutes at room temperature. Following another round of washes, sections were then preblocked in a dilution buffer consisting of 4% normal horse serum, 0.6% Triton X-100, 90.7% PBS-B, and 5% BSA for 60 minutes at room temperature. After another 30 minutes of washes, the sections were then incubated in mouse anti-SERT-ir primary antibody diluted to 1: 10,000 in buffer solution for 24 hours at room temperature followed by 48

hours at 4 degrees C in the refrigerator. Following incubation, the sections were washed with phosphate buffered solution and incubated in a solution of 0.5% secondary mouse antibody, 2% normal horse serum, and 97.5% PBS-B for 60 minutes at room temperature during which time the secondary antibody bound to the primary antibody. Sections were then incubated in avidin-biotin- peroxidase complex (PK-6100, Vector Laboratories, Burlingame, CA) for one hour at room temperature followed by an additional 30 minutes of washes and incubation in a solution of 3,3"-diaminobenzidine-peroxidase with nickel solution used as the chromogen (SK-4100, Vector Laboratories) until the reaction was followed to completion. Sections were then immersed in phosphate buffered solution to halt the reaction and stored at 4 degrees C until mounting on gelatin coated slides occurred (for floating sections).

Fiber Density Quantification

All stereological analyses were performed by KG. Density of SERT-ir fibers in all four major nuclei was determined using the Stereoinvestigator Spaceballs probe (MBF Bioscience, Williston, VT). As detailed by Lew et al., 2017, all analyses were performed using a Dell workstation receiving live video feed from an Optronics MicroFire color video camera (East Muskogee, OK) which is attached to a Nikon Eclipse 80i microscope equipped with a Ludl MAC5000 stage (Hawthorn, NY) and a Heidenhain z-axis encoder (Plymouth, MN). Nuclei were identified on corresponding Nissl stained sections and borders were drawn using those outlined in Raghanti et al., 2016 for reference. The borders of each nucleus (lateral, basal, accessory basal, and central) were drawn at 1x magnification and the nuclei were examined using a 100x oil lens. Only sections which contained intact nuclei were examined. The nuclei were examined using the following grid sizes: 1400μm x 1400μm (lateral), 1200μm x 1200μm (basal), 800μm x 800μm (accessory basal), and 600μm x 600μm (central). For all nuclei section

cut thickness was 80μm, radius of the probe was set to 7.0μm, and guard zone distance was 1.0μm. Section thicknesses were measured at each site; mean thickness measured varied from 12.6μm to 13.05μm. Eight sections from WS 7 and nine sections from 5183 spanning the rostral-caudal extent of the amygdala were used for quantification and spaced to the best of the researcher's ability depending on the integrity of the tissue, presence of all four nuclei, and presence of stained SERT-ir fibers (generally a 1 in 20 series). The length of a fiber was determined when it crossed the diameter of the Spaceballs probe circle and each point of contact with the circle was counted. Fibers moving through the Z plane of the sections were also counted when in contact with the circle (Figure VI.1). Density of fibers in each subject was then calculated by dividing the estimated length of fibers in each nucleus (μm) by the planimetric volume (μm³).

CHAPTER V: RESULTS

In order to ascertain the density of serotonin containing fibers in the amygdalae of human infants with Williams Syndrome (WS) and typically developing controls (TD), we performed immunohistochemical staining techniques on four sets (two WS and two TD) of 1 in 20 series of 80μm cut sections of amygdaloid tissue. These amygdala sections were stained using a modified avidin- biotin-peroxidase method as described by Raghanti et al., 2008 in their comparison of serotonergic innervation of the cortex in humans, chimpanzees, and macaque monkeys. Following antigen retrieval and pretreatment with a methanol peroxidase, sections were incubated in a primary mouse antibody for SERT-immunoreactive fibers and later a secondary mouse antibody which reacted with 3,3"-diaminobenzidine-peroxidase, thus staining the SERT-ir fibers with a dark brown color. After eleven trials were used to modify the immunohistochemical protocol for delicate infant tissue, only WS 7 and its control 5183 (both 3 months old) stained successfully and remained intact; WS 11 and its control 4353 (both 1 month old) had no visible stained SERT-ir fibers, preventing data collection on these subjects.

Following examination using a 100x magnification lens and a Stereoinvestigator software probe, we found the density of SERT-ir fibers to be decreased across the four major amygdaloid nuclei in WS 7 compared to that in its age/ sex/ hemisphere matched control 5183. The most pronounced difference was in the central (8.97 x 10^{-4} μm/μm^3) and basal (7.04 x $^{10-}$4 μm/μm^3) nuclei, while the lateral nucleus was decreased moderately (3.07 x 10^{-4} μm/μm^3) and the accessory basal showed the least amount of variance in density of SERT- ir fibers (4.33 x 10^{-5} μm/μm^3). Statistical examinations could not be performed on the sample because the density of amygdalae of only two individuals were compared. Comparisons among the nuclei densities within 5183 indicate that the central nucleus had the greatest density of fibers (3.71x 10^{-3}

μm/μm^3) and the lateral nucleus had the lowest (2.18x 10^{-3} μm/μm^3). In WS 7, the accessory

basal density (2.86x 10^{-3} μm/μm^3) was only slightly higher than the central (2.80x 10^{-3} μm/μm^3),

and the lateral nucleus had the lowest density (1.87x 10^{-3} μm/μm^3).

CHAPTER VI: CONCLUSIONS

Fiber Density

The density of SERT-ir fibers in the major amygdaloid nuclei of WS 7 was decreased compared to that in its age/sex/hemisphere matched control, 5183. While the accessory basal nucleus showed very little difference in SERT-ir density, the central and basal nuclei had a pronounced decrease in fiber density with the central nucleus showing the largest difference between WS 7 and 5183. In 5183, the central nucleus possessed the greatest density of SERT-ir fibers, a characteristic which is opposite of what has been previously noted in the TD adult amygdala (Lew et al., in preparation). The amygdala develops medially to laterally with the central nucleus undergoing gestational development before the basolateral complex (Kordower et al., 1992). Therefore, the increase in density in the central nucleus compared to the lower density in this nucleus seen in TD adults could be due to the serotonergic system in the central nucleus developing before the more lateral nuclei postnatally. If this explanation is correct, it may also account for the increased central and accessory basal density noted in WS 7 compared to the more laterally situated basal and lateral nuclei. However, this experiment examined two individuals and each cannot necessarily be taken as representative of their respective groups.

The overall decrease in SERT-ir fiber density seen in WS 7 compared to 5183 indicates a modified serotonergic system in the WS infant amygdala. However, this decrease in fiber density does not necessarily imply that the functionality of this system is in some way impaired. This experiment stained for the post-mortem presence of SERT-ir fibers, but the *in vivo* activity of the serotonergic system may indicate an increase in serotonin present in amygdala. It is possible that if the WS infant amygdala contains less SERT, meaning there are less transporters moving serotonin back into the presynaptic terminal, this would allow for more serotonin to accumulate

within synaptic clefts. With increased serotonin available to bind to post-synaptic receptors, this decrease in SERT may function similarly to SSRIs in that the increased accumulation of serotonin would then translate to elevation in mood and attenuation of anxiety. However, while anxiety has been documented in some adult individuals with WS (Davies et al., 1998; Reiss et al., 2004), it is possible that the origins of this anxiety may be in other areas of the brain. Further research examining the *in vivo* activity of the serotonergic system in both adults and infants with WS may help to further characterize the role and amount of serotonin in the amygdala of this disorder. Additional post-mortem studies may also be done using immunohistochemical techniques staining for serotonin itself, providing the PMIs of the subjects are conducive to the prevention of serotonin degradation, or for its synthetic enzyme. These investigations would assist in removing uncertainty regarding the presence of serotonin in amygdaloid axons, and should be done to fully elucidate not only the nature of the serotonergic system in the infant and adult WS amygdalae, but the impacts of this system on the social and behavioral profile of this rare neurodevelopmental disorder.

Protocol Variations

I began the experiment with two pairs of two subjects: WS 7 & 5183 and WS 11 & 4353. However, after eight trials in which we varied elements of the immunohistochemical protocol (Table 2), only WS 7 and 5183 produced sections which contained the intact major amygdaloid nuclei as well as clearly stained SERT-ir fibers. Both WS 11 and 4353 proved to be too delicate to withstand the first step of antigen retrieval when floating sections were used, despite modifying the temperature of the water bath. I then mounted sections prior to immunostaining and although the tissue of both WS 11 and 4353 remained relatively intact throughout the process, no SERT-ir fibers were visible in either upon examination using a 100x oil lens. While

darker, dot-like structures were visible, they were more diffuse and lighter than those seen in WS 7 and 5183 (Figure VI.3) and were therefore not counted as fibers moving through the sections in the Z plane.

To test whether the use of floating or mounted sections had an impact on the penetrance of the stain, I ran the protocol using both floating and mounted sections of WS 7 and 5183 to compare with those previously done with sections of WS 11 and 4353 (Table 2). The floating sections of WS 11 and 4353 were, as previously stated, severely degraded and microscopy was unable to be performed; floating sections of WS 7 and 5183 remained intact and data collected from this trial is included in Figure VI.2. The mounted sections of all four subjects stained very lightly and upon examination none of the individuals possessed stained fibers (Figure VI.3), indicating the protocol must be altered further in order for maximum penetrance to be achieved when using mounted sections. I did not perform further modified trials using floating or mounted sections of WS 11 and 4353 due to the fragility of the tissue and in an effort to preserve the remaining, precious sections.

Two possible conclusions can be drawn from the comparison of these floating and mounted sections. First, WS 11 and 4353 possessed no stained fibers perhaps because the subjects are at an age when the serotonergic system has yet to fully develop in the amygdala. It may be that at one month of postnatal development elements of this system have yet to be established. This experiment focused on the presence of SERT and not tryptophan 5-hydroxylase or serotonin itself, hence the possibility remains that perhaps SERT may not be present while other elements of the serotonergic system are, at least in an early developmental capacity. However, with a dearth of information known regarding the developmental trajectory

of the serotonergic system in the human amygdala, this cannot be known for certain without further experimentation.

The second possible conclusion would be that the use of mounted sections hindered the protocol and prevented full penetrance of the stain; this could have occurred at various and/or multiple points in the protocol including antigen retrieval, conjugation of the primary and secondary antibodies, and reaction with DAB. This is supported by the lack of fibers seen in mounted sections of WS 7 and 5183, both of which proved to have successful staining when floating sections were used. If this explanation is correct, further experimentation and modification of the protocol, such as increasing time for antigen retrieval or increasing concentrations of primary and secondary antibody, must be done in order to achieve the maximum penetrance possible in mounted sections. Another avenue would be to maximize preservation when using floating sections of younger subjects. This approach may be more difficult as the balance between preservation of the tissue and penetrance of the stain must be carefully navigated, however manipulation of incubation time or temperature of the water bath during antigen retrieval may be able to keep the more fragile tissue intact. Transferring sections from vessel to vessel for different steps of the protocol also has an impact on the tissue integrity; perhaps more delicate means of transfer can be used to decrease the physical damage to sections throughout the protocol.

Future directions for this research include the expansion of the sample size, experimentation on the serotonergic system using human iPSC technology, and post-mortem IHC staining for tryptophan 5- hydroxylase. While the expansion of the sample remains difficult when using post-mortem human brain tissue, in vitro studies may be used with human iPSCs created from WS skin cells. These cells can be induced to become similar to serotonergic

neurons present within the amygdala and may provide insight into the serotonergic system of WS. Additionally, the use of IHC staining for serotonin's synthetic enzyme tryptophan 5-hydroxylase may make it possible to determine if the mechanism underlying the production of serotonin is also altered in individuals with WS, or if only the serotonin transporter (SERT) is in some way downregulated, as shown by this experiment.

APPENDIX

Table VI.1. Subject Information. Four individuals were initially examined in this experiment; all were males and all amygdalae obtained were from the right hemisphere. The post-mortem interval (PMI) ranged from 5- 34.5 hours. TD control tissue was obtained from the NIH NeuroBioBank; WS tissue was obtained from the Ursula Bellugi Williams Syndrome Brain Collection.

Subject ID	Age at Death	Diagnosis	Sex	Hemisphere	Cause of death	Post-mortem interval (hrs)
WS 7	114 days	Williams Syndrome	Male	Right	Multiple Organ Failure	30
TD 5183	107 days	Typically Developing	Male	Right	SIDS	13
WS 11	26 days	Williams Syndrome	Male	Right	Unknown	34.5
TD 4353	34 days	Typically Developing	Male	Right	SIDS	5

Trial	Subjects Included	Number of Sections Tested	Floating or Mounted	Amygdala Intact	Amygdala Stained
1	WS 7	1	Floating	✘	N/A
2	4353, 5183	2, 2	Floating	✘	N/A
3	WS 7	4	Floating	✘	N/A
4	WS 7, 5183	10, 10	Floating	✔	✔
5	WS 11	10	Floating	✘	N/A
6	4353	3	Mounted	✔	✘
7	WS 11, 4353	5, 5	Mounted	✔	✘
8	WS 7, 5183	8, 8	Both (4 of each mounted, 4 floating)	✔ Floating ✔ Mounted	✔ Floating ✘ Mounted

Table VI.2. Qualitative successes of immunohistochemical trials staining for SERT-ir fibers in the amygdala of infant WS and TD controls. Generally, WS 7 and 5183 were more robust with respect to amygdalae fragility than WS 11 and 4353. Often sections stained while floating were degraded and some or all of the nuclei were missing, disallowing data collection. In the case of mounted sections, comparison between WS 7 & 5183 and WS 11 & 4353 revealed no successful staining of fibers.
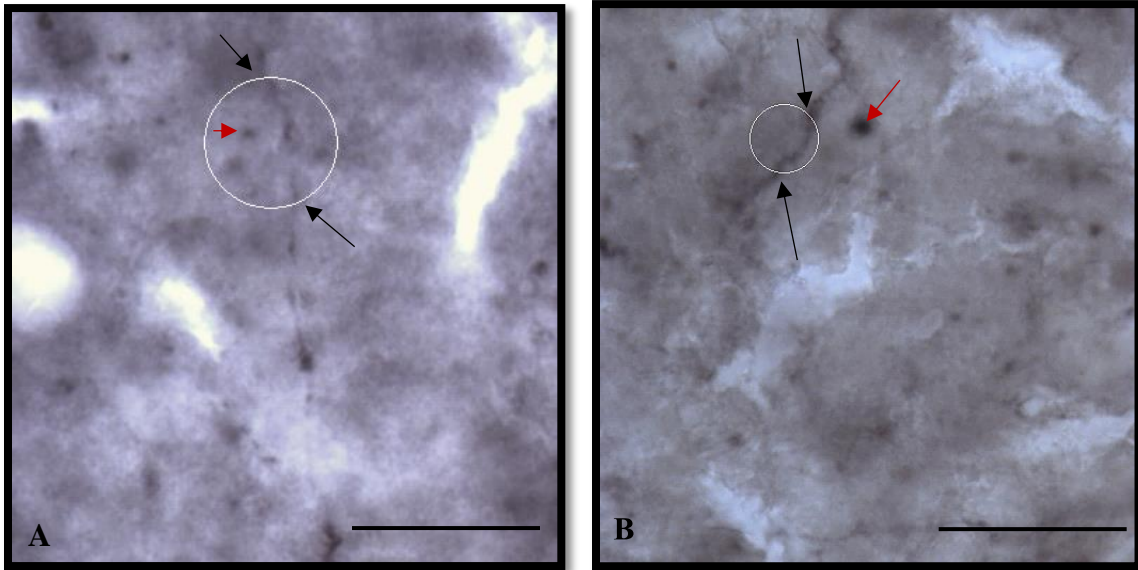
Figure VI.1. Brightfield photomicrograph images of SERT-ir stained fibers in rostral-medial sections of the WS 7 (**A**) and 5183 (**B**) central nucleus at 100x magnification. Fiber length measurements were obtained using the Stereoinvestigator Spaceballs Workflow; the radius of the circle increased as the lens was focused through the section. Fibers in the XY-plane were marked at points of intersection with the sphere (black arrows). Fibers in the Z-plane were marked if they touched the edge of the circle at any radius (red arrows). Scale bar = 20μm.
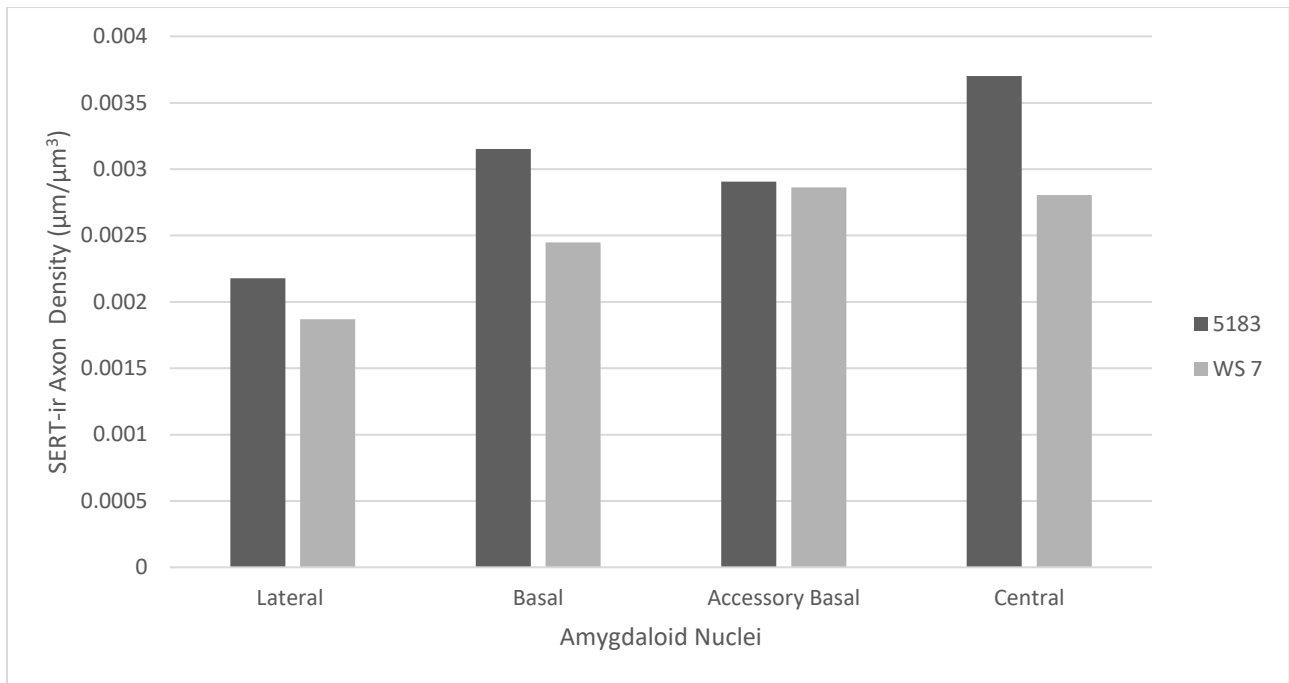
Figure VI.2. Density of SERT-immunoreactive fibers in the major amygdaloid nuclei of WS 7 is decreased compared to its typically developing control 5183. Density of fibers, measured in $\mu m/\mu m^3$, was notably decreased in the central (8.97×10^{-4} $\mu m/\mu m^3$) and basal (7.04×10^{-4} $\mu m/\mu m^3$) nuclei of WS 7 compared to in 5183. Less pronounced decreases in density were found in the lateral (3.07×10^{-4} $\mu m/\mu m^3$) and accessory basal (4.33×10^{-5} $\mu m/\mu m^3$) nuclei of WS 7. No statistical analyses were able to be used because only 1 WS subject and 1 control were used. Number of sections used (N)=8 for WS 7; N=9 for 5183.
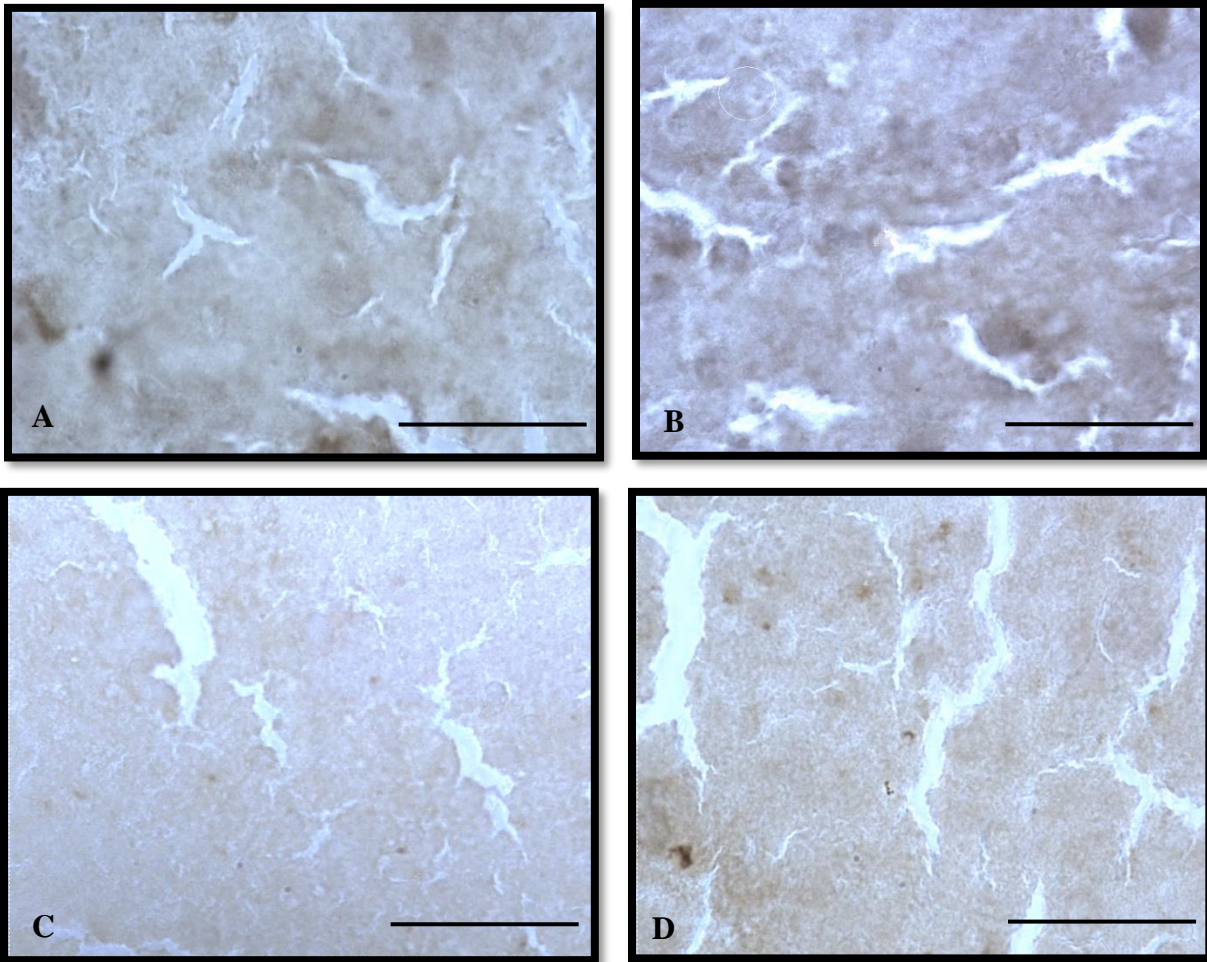
Figure VI.3. Brightfield photomicrograph images of sections stained while mounted. WS 7 (**A**), 5183 (**B**), WS 11 (**C**), and 4353 (**D**) all show an absence of defined fibers at 100 X magnification as pictured in Figure VI.1. All images depict rostral sections of the central nucleus of each subject. Scale bar = 20µm.

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