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The Social Brain in Williams Syndrome: Cellular Disturbances of the Orbitofrontal Cortex and the Amygdala in a Disorder of Social Cognition

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Anthropology
Specialization in Anthropogeny

by

Caroline Horton Lew

Committee in charge:

Professor Katerina Semendeferi, Chair
Professor Ursula Bellugi
Professor Andrea Chiba
Professor Steven Parish
Professor Cynthia M. Schumann
Professor Shirley Strum

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The Dissertation of Caroline Horton Lew is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2018

DEDICATION

In memory of those I’ve loved and lost during this journey: Dr. Lisa Stefanacci, brilliant scientist, supportive mentor, and kind friend, whose exceptional mind not only made much of this research possible, but also provided invaluable training, guidance and comfort even after her death; my grandmother Connie, beloved by all for her sharp wit, colorful sense of humor, and ‘imaginative’ retelling of events, whose battle with dementia in the last few years of her life taught me how precious the human mind is; and lastly, my one-of-a-kind dog Bentley, whose constant companionship (whether that be cuddling on my lap and staring lovingly into my eyes, or attacking my hand after I accidentally made a scratching sound against the fabric of the couch) throughout all but this last year of graduate school was an unforgettable source of much needed love and laughter. I miss you all.

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Chapter 2, in full, is an adaptation of a literature review previously published in the reference work *Evolution of Nervous Systems, 2nd Ed.* The dissertation author was the primary author of this paper. Citation: Lew CH**,** Semendeferi K. 2017. "115. Evolutionary specializations of the human limbic system". *In Evolution of Nervous Systems* 2 ed. Vol. 4. Kaas and Pruess. Elsevier. 277-291.

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ABSTRACT OF THE DISSERTATION

The Social Brain in Williams Syndrome: Cellular Disturbances of the Orbitofrontal Cortex and the Amygdala in a Disorder of Social Cognition

by

Caroline Horton Lew

Doctor of Philosophy in Anthropology with a Specialization in Anthropogeny

University of California, San Diego 2018

Professor Katerina Semendeferi, Chair

Sociality in humans is unparalleled by any other animal, and serves as an essential defining characteristic of our species. Human/non-human primate comparative neuroanatomical studies, in conjunction with evidence from the hominin fossil record, suggest that the human brain has undergone significant changes since the human-ape ancestral split, not only in size, but more significantly in microstructural reorganization and regional specialization. The prefrontal cortex, a region attributed to many of the higher-order functions involved in human cognition, and the amygdala, a subcortical structure involved in the

integration of perceptual information from the cortex and the elicitation of social and emotional responses, have both undergone recent, rapid and functionally significant change in human brain evolution. Connectivity and neuroimaging studies have shown that the amygdala and the orbitofrontal cortex (OFC), a subdivision of the prefrontal cortex implicated in social/emotional cognition, share an important functional relationship within social brain circuitry. Comparative neuroanatomical studies of human neural pathologies such as Williams syndrome (WS), a rare neurodevelopmental disorder caused by a discrete hemizygous deletion of ~26 consecutive genes and characterized by hyper-affiliative social drive and atypical social cognition, further illuminate substrates of neural architecture that are critical for normative function of the human social brain, and offer new insight into the evolutionary trajectory of the social brain in humans. Utilizing standard and advanced tissue staining techniques and quantitative stereology in postmortem human brains, this dissertation offers a cellular characterization of the orbitofrontal cortex and the amygdala in WS and typically developing individuals. Our key findings of altered microstructure in WS include decreased neuron density in the infragranular layers of the orbitofrontal cortex and relative sparing of unimodal cortical areas, increased neuron number in the lateral nucleus of the amygdala, an amygdaloid subdivision that has undergone significant reorganization in human brain evolution, and decreased amygdaloid serotonergic innervation in the basolateral nuclei, which underlie sociocognitive functions of the amygdala. These findings constitute evidence supporting disrupted social brain circuitry in WS, and are a critical first step towards identifying mechanisms underlying the atypical social phenotype. Additionally, these findings identify possible microstructural specializations that may contribute to uniquely human social cognition.

CHAPTER 1:

Introduction

What defines the human experience? We live in neighborhoods, cities, and countries. We form families, societies and governments, and identify ourselves as a lineage, as a member, as a citizen. We create works of art and storytelling, and invent incredible feats of technology, in a never-ending plight to strengthen our bonds, to engage more deeply, to understand each other more thoroughly. This drive to engage is so all-encompassing, that it has created a modern human experience that exists almost entirely in the abstract, such the primary method of survival in the physical world, of obtaining sustenance, shelter, and safety, is to participate in such social constructs as the workforce, monetary systems, and economies. The uniqueness of humankind, then, could be defined by our ability and desire to live almost entirely within social constructs. If a central purpose of biological anthropology is to identify the biological mechanisms underlying behaviors which we categorize as "uniquely human", then a fundamental step towards achieving that goal would be to examine the neural substrates behind the complex feats of our social cognition.

The question of what changed within the human brain during evolution has long captivated biological anthropologists. Endocasts from the hominin fossil record have demonstrated that a substantial and steady increase in overall brain size in *Homo* (Falk et al. 2000; Holloway et al. 2005), and this increase has been attributed by some to be the primary adaptation underlying *Homo sapiens* intelligence and social cognition (Jerison 1955; Dunbar 1998). However, comparative studies with nonhuman primates have demonstrated that size alone is not the answer- the human frontal lobe, functionally attributed to many of the feats of

human cognition, as a whole does not deviate in terms of relative size, or relative quantities of grey matter or white matter (Semendeferi et al. 1997; Semendeferi et al. 2002; Smaers et al. 2011). Instead, multiple lines of evidence suggest that the evolution of the human brain has undergone significant reorganization and specialization of multiple cortical regions (Semendeferi and Damasio 2000; Semendeferi et al. 1998, 2001, 2011; Schenker et al. 2008; Buxhoevden et al. 2001; Spocter et al. 2012; Buxhoeveden and Casanova 2002; Elston et al. 2006; Elston 2007; Elston et al. 2011; Allman et al. 2002, 2010; Hof et al. 2001), as well as subcortical regions previously thought to be relatively conserved in humans (Barger et al. 2007, 2012, 2014; Stephan 1983; Andy and Stephan 1968; Armstrong 1980,1981). It is likely that this reorganization and specialization is evidence of the formation of larger and more complex distributed networks in human brain evolution that underlie the abilities and behaviors characterized as uniquely human (Barger et al. 2012; Barton and Venditti 2013).

Contributions of Williams syndrome to Anthropology

While utilizing the comparative neuroanatomical method with human and non-human primates can help identify evolutionary changes from the last common ancestor, with this evidence alone it is not possible to parse out which cognitive and behavioral differences can be attributed to differences in neural phenotype, which can be attributed to a given species' strategy to the very different environments and ecological pressures each has faced in their divergent evolutionary histories, and which can be attributed to a messy combination of both. Many neurodevelopmental disorders and neurological diseases disrupt systems underlying complex cognitive processes characterized as uniquely human (Hanson, Hrvoj-Mihic, and Semendeferi 2014). Networks modulating normative human social cognition and behavior appear to be one of the most broadly affected within neuropathologies- disruptions in social

cognition and behavior characterize a wide variety of disorders and diseases, including Autism Spectrum Disorder, schizophrenia, mood disorders, Alzheimer's disease and frontotemporal dementia (for a review see Kennedy and Adolphs 2010). It has been hypothesized that recent neural specializations and increased connective complexity in the human brain may have also resulted in an increased vulnerability to neuropathologies (Burns 2004; Randall 1998; Crow 1997), and indeed, certain structures that have undergone significant reorganization in human evolution, such as the amygdala, appear to be particularly targeted in neurodevelopmental disorders (Schumann, Bauman and Amaral 2011). Therefore, one method to shed light on the functional significance of variation in neural structure and circuitry is through the examination of such variation in human neuropathologies.

WS is a neurodevelopmental disorder caused by a single deletion of 26 contiguous genes on chromosome 7. q11. 23 (Morris et al. 2010). The disorder is characterized by a suite of morphological and physiological abnormalities, including dysmorphic facial features, connective tissue irregularities, short stature, and elastin arteriopathy (Morris 2010), as well as significant deficits in visuospatial construction, varying degrees of intellectual disability ranging from severe mental delays to low-average intelligence, and relatively preserved language abilities marked by strong expressive language and extensive vocabularies (Mervis et al. 2000). WS is perhaps best known, however, for the distinct and consistent hypersocial personality that accompanies the disorder. Individuals with WS demonstrate an abnormally strong and indiscriminate drive for social engagement (Jarvinen-Pasley et al. 2010), increased attention-seeking behavior (Dykens and Rosner 1999) and increased displays of empathy (Klein-Tasman and Mervis 2003) compared to typically developing individuals. Furthermore, this atypical social drive appears early in development- WS infants demonstrate prolonged

facial gazing and preferred attention to human faces over other sensory stimuli that typically developing infants find more engaging (Jones et al. 2001). Despite this increase in social drive, individuals with WS have difficulty maintaining social relationships, likely due to their tendency to their hyperverbal speech patterns, which include excessive use of stereotyped phrases, over-familiar referents, and tendency to break into contextually irrelevant tangents of conversation (Mervis and John 2011). Furthermore, WS individuals lack social inhibition, demonstrating a great propensity for approaching and engaging with unfamiliar conspecifics (Doyle et al. 2004), and have difficulty processing angry facial expressions (Santos et al. 2010), which suggests that they are unable to detect negative social cues or perceive possible threat in a social context.

Given the discrete genetic etiology and distinct behavioral phenotype, WS is perhaps one of the best human models in which to examine how variation in neural substrates affects uniquely human social behavior. Intriguingly, chromosome 7, the location in which the WS deletion occurs, has undergone significant purifying selection in the hominoid lineage, and the genetic profile of many neurodevelopmental disorders includes deletions or duplications on chromosome 7, so this region appears particularly susceptible to genomic change in humans (Hanson et al. 2014).

The social brain

The social brain is composed of complex networks of multiple brain regions that allow individuals to quickly evaluate and flexibly adapt to the social environment (Brothers et al. 1992), an ability that is present in other social primates (e.g. baboons, Strum 1994), but particularly advanced in our species (Adolphs et al. 2009). In humans (and some non-human primates, to a lesser extent, see Hare et al. 2000), the neural substrates of the social brain

underlie socio-cognitive processing that enables an individual to make inferences about another person's internal perspective (Adolphs 2009). The orbitofrontal/ventromedial prefrontal-amygdala network is one such network underlying social cognition, and is implicated in the monitoring and regulation of one's own emotional states, as well as the ability to knowledgably predict the emotional feelings and reactive responses of others (Bachevalier and Loveland 2006). The orbitofrontal/ventromedial prefrontal cortices (for simplicity from here on referred to just as orbitofrontal cortex), are critically implicated in higher-order associative functions of social and emotional cognition (Dimitrov et al. 1999), while the amygdala, a subcortical structure deep within the temporal lobe, is involved in the detection and categorization of socially salient stimuli, and the cognitive and autonomic modulation of emotion, including fear and anxiety (Adolphs et al., 1994,1999; Adolphs 2001; Meunier et al. 1999; Emery et al. 2001; Bauman et al. 2006). The orbitofrontal cortex and the amygdala share extensive reciprocal connections with modulatory effects in both directions (Stefanacci and Amaral 2000, 2002), and it has been suggested that these connections are involved in the concomitance of emotional behaviors and autonomic responses to external environments (Barbas et al. 2011). In both humans and nonhuman primates, the amygdala and orbitofrontal cortex demonstrate significant coupling in response to socially salient stimuli and during socially relevant tasks (see Bickhart et al. 2014 for review) and this coupling is thought to be a mechanism of emotion regulation (Banks et al. 2007). Below, we briefly review the anatomy of these structures as regions of interest targeted in the present work.

Region of interest: the ventromedial/orbitofrontal cortex

The prefrontal cortex (PFC) consists of all cortex covering the frontal lobe anterior to the motor and premotor cortices. The PFC is the only portion of the neocortex that receives input from every sensory modality (Fuster 2001), which suggests that the PFC is an ideal intermediary for sensory input from the external environment and the homeostatic regulation of internal environment of the organism, and is well equipped for mediating behavioral flexibility in a dynamic social environment. In general, the PFC shares the organization of the mammalian neocortex, characterized by six functionally and architectonically distinct cortical layers. The outer-most layer, layer I, is mostly acellular; layers II and III, also called the supragranular layers, are involved in interhemispheric cortico-cortical connectivity; layer IV, also called the granular layer due to its distinct composition of small, dense cells, is the primary target of thalamo-cortical afferents and is also involved in intrahemispheric cortico-cortical connections; layers V and VI, the infragranular layers, are involved in connections between the cortex and subcortical structures (such as the amygdala). The neocortex in humans has been partitioned into approximately 52 cytoarchitectonically distinct functional areas (Brodmann areas, BA) differentiated by morphological properties such as cortical layer thickness, number and composition of cortical layers, and the size, shape, and density of cortical neurons (Brodmann 1909). The orbitofrontal/ventromedial regions of the PFC, which are implicated in socio-emotional cognition, include the gyrus rectus, the mesial portion of the orbital gyrus, and the rostrocaudal extent of the ventral portion of the medial surface of the PFC, and consists of BAs 10, 11, 12, 13, 25, and 32 (Barbas 1995; Bechara, Damasio and Damasio 2000). These regions show strong connectivity to structures involved in functional networks of the limbic system, including the insula, temporopolar cortex, parahippocampal cortex, striatum, nucleus basalis of Meynert, and the amygdala (Carmichael and Price 1995).

Region of interest: the amygdala

The amygdala lies in the anteromedial temporal lobe just rostral to the hippocampus, and is composed of 13 intra-connected yet functionally distinct nuclei, or clusters of cells, surrounded by white matter fiber tracts (. Four nuclei in particular, the lateral, basal, accessory basal, and central, are critically implicated in emotion. The lateral, basal, and accessory basal nuclei, collectively referred to as the "deep" or "basolateral" nuclei, demonstrate significant connectivity to the neocortex, while the central nucleus is the primary output of the amygdala to regions in the brainstem and hypothalamus, modulating autonomic functions (Price et al. 1987; Stefanacci and Amaral 2000, 2001). The flow of information through the amygdala is as follows: the lateral nucleus serves as a stimulus "gateway" of the amygdala, receiving the majority of sensory cortical input. The lateral nucleus then sends this information to the basal and accessory basal nuclei, which have bidirectional connections to the orbitofrontal cortex, thus allowing the basolateral nuclei to integrate sensory stimuli with context determined by cognitive association areas, and categorize the stimuli based on social/contextual saliency and emotional valence (Janak and Tye 2015). The basolateral nuclei also project to the central nucleus, which then transmits this information to the brainstem. Thus the basolateral nuclei are considered to play a major role in the cognitive functions of the amygdala, while the central nucleus is considered to be primarily involved in the physiological modulation and behavioral response of emotion (Janak and Tye 2015; Freese and Amaral 2009; Amaral et al. 1992; Davis 1992; Pitkanen et al. 1997; Schoenbaum, Chiba, and Gallagher 1992).

Outline of the dissertation

This dissertation examines the postmortem histology of two important structures of the social brain, the orbitofrontal cortex and the amygdala, in the brains of individuals diagnosed

with WS and typically developing brains, in order to better understand the relationship between neuroanatomical variation and uniquely human social behavior. Four stand-alone manuscripts serve as the body of this dissertation. While there is significant overlap in the postmortem sample studied in each manuscript, data sets are not identical due to differential availability of neural substrates for each individual, as well as differential success of scientific techniques used (i.e. immunohistochemical staining vs Nissl staining).

Chapter 2 provides theoretical background to the dissertation by reviewing specializations of the limbic system in human brain evolution, which includes neural networks underlying the social brain. It is was originally published in *Evolution of Nervous Systems, Second Addition, Volume 4,* (editors J. Kaas and T. Preuss) and coauthored with K. Semendeferi. The author of this dissertation is the primary author of the publication.

In chapter 3, We asked whether orbitofrontal cortical regions, which are involved in social brain networks, are differentially affected in WS compared to other cortical areas underlying functions that appear relatively preserved in the disorder. We used Nissl-stained sections to quantify neuron density in the infra- and supragranular layers of two areas of the orbitofrontal cortex, BA 10 and BA 11, as well as three unimodal cortical areas areas not associated with systems disrupted in WS, motor cortex BA 4, somatosensory cortex BA 3, and secondary visual cortex BA 18, in the postmortem brains of six WS subjects and six matched typically developing (TD) subjects. This chapter is published in the journal *Autism Research*. The author of this dissertation is the primary investigator and author of the publication, and C. Brown, U. Bellugi and K. Semendeferi are co-authors.

In chapter 4, we examined whether the structure and neuronal distribution of the basolateral nuclei, which subserve the neural circuitry implicated in the socio-cognitive

functions of the amygdala, and the central nucleus, which is primarily implicated in autonomic functions of the amygdala, are differentially affected in WS. We used Nissl-stained sections to quantify nucleus volume, neuron size, and neuron number in the lateral, basal, accessory basal, and central amygdaloid nuclei in a WS/matched TD postmortem sample composed of two early postnatal infant pairs and five adult pairs. This chapter is published in the journal *Brain Structure and Function*. The author of this dissertation is the primary investigator author, and K. Groeniger, L. Stefanacci, U. Bellugi, C. Schumann, and K. Semendeferi are co-authors.

In chapter 5, we examined whether the pattern of serotonergic innervation within the basolateral and central nuclei is different in WS and TD, given that the serotonin system is an important modulator of social behavior, and the amygdala is densely innervated by SERT axons. We utilized immunohistochemical techniques in order to stain and quantify the density of serotonin-transporter (SERT)-containing axons in the lateral, basal, accessory basal, and central amygdaloid nuclei in the postmortem adult tissue of four WS brains and three TD brains. A previous study (Stimpson et al. 2015) found significant differential SERT axon density of these same four amygdaloid nuclei in two closely related ape species with markedly different patterns of social behavior, chimpanzees and bonobos, suggesting a link between sociality and patterns of serotonergic innervation of the amygdala. This chapter is currently in preparation for submission to a scientific journal. The author of this dissertation is the primary investigator and author, and K. Groeniger, K. Hanson, D. Cuevas, D. Greiner, U. Bellugi, and K. Semendeferi are co-authors.

Chapter 6 serves as a general discussion and conclusion of the dissertation, examining the summation of our evidence for the disruption of the social brain in WS. This chapter also

includes next steps and future directions for further defining the neuroanatomical phenotype of WS in order to better understand how neuroanatomical variation influences human social cognition, and how evolutionary changes in neural substrates shaped sociality in the hominin lineage.

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CHAPTER 2:
Evolutionary Specializations of the Human Limbic System

ABSTRACT

The limbic system consists of multiple neural circuits, composed of select brain areas and structures, that underlie emotion processing and are implicated in many functions throughout the brain, including complex socio-cognitive abilities that are thought to be uniquely human. Here, we review the history of comparative primate limbic neuroanatomy in order to define and contextualize the limbic system in human evolution. We then identify cortical and subcortical regions and structures that demonstrate significant involvement in emotional processing, and review evidence from the human/non-human primate comparative literature that may indicate human-specific specializations of the limbic system.

Introduction

The concept of the limbic system refers collectively to multiple neural circuits, composed of select brain areas and structures, that underlie emotion processing and are implicated in a wide variety of functions throughout the brain, ranging from autonomic and endocrine processing to cognition and behavior (Heimer and Van Hoesen, 2006). Historical views of limbic structures as highly conserved have resulted in scant representation of these structures in human evolution research (Geschwind, 1965). However, later studies in lesion and human disorder/disease research have demonstrated the critical contribution of emotion processing structures in complex social cognition (Damasio and Van Hoesen, 1983; Bliss-Moreau, Moadab, Bauman, and Amaral, 2013; Adolphs, Tranel, Damasio, and Damasio,

1994; Mega and Cummings, 1994; Brady and Nauta, 1953). Given this paradigm shift in neuropsychology and the importance of complex social cognition in the emergence of modern humans, comparative primate studies of limbic structures are generating greater interest in human evolution research. Thus, more recent trends in evolutionary neuroscience have moved away from the conception of the brain as functionally and phylogenetically compartmentalized (Peterson and Sporns, 2015; Smaers and Soligo, 2013). Evidence now shows significant changes in older and more recently derived structures and neural systems, including subcortical limbic structures and the neocortex (Armstrong, 1990; Barger, Hanson, Teffer, Schenker-Ahmed, Semendeferi, 2014), giving strong support to the hypothesis that selective pressures resulted in adaptive changes in neural structures linked through complex and overlapping circuits (Finlay, Darlington, and Nicastro, 2001; Heimer and Van Hoesen 2006). Here, we review the history of comparative primate limbic neuroanatomy in order to define and contextualize the limbic system in human evolution. We then identify neural structures that demonstrate roles in emotional processing, and review the evidence from the human/non-human primate comparative literature that indicates human-specific specializations of the limbic system.

The limbic system in human brain evolution

The term "limbic", which means "hoop" in French, was first used by anatomist Paul Broca in 1878, to name the mesial surface of the brain, comprised mostly of the cingulate and parahippocampal gyri, the limbic "lobe" due to it's ovoid shape (Fulton, 1953; Roxo, Franceschini, Zubaran, Kleber, and Sander, 2011). Later studies found that this limbic lobe had extensive connectivity to several subcortical nuclei, including the amygdala, anterior thalamic nuclei, hypothalamus, and basal ganglia (Ramon y Cajal 1903), and together were

thought to be involved in phylogenetically ancient neural circuits involved in autonomic and behavioral output of olfactory processing, and were therefore highly conserved across mammals (Roxo, Franceschini, Zubaran, Kleber, Sander, 2011). A hierarchical organization of the brain, in relation to both topological location and function, was the predominant conception of the brain at the time. Structures located in the mesial surface of the cerebral hemisphere and in subcortical areas of the brain were associated with "primitive" functions, such as olfaction and autonomic processing, and were considered to be highly conserved across mammals, while structures located in more lateral areas of the brain (primarily neocortex) were associated with more recently evolved, higher functions, such as greater intelligence and more complex cognition. This conception colored the view of the limbic system for over a century (Pessoa and Hof, 2015). In 1937, James Papez analyzed a series of clinical studies documenting individuals suffering from cingulate gyrus lesions, and noted that abnormal emotive behavior (either loss of emotion or nonsensical/inappropriate emotional response) was a predominant symptom, leading him to suggest the cingulate gyrus as the seat of emotion in a neural circuit that also included the hypothalamus, hippocampus, and anterior thalamus and other limbic structures (i.e. amygdala, septal nuclei, parts of the basal ganglia; Papez, 1937). In 1948, Yakovlev added to this circuit to include the orbitofrontal cortex, the insula, and the temporal pole. Paul MacLean named this circuit the "visceral brain" and later the "limbic system", and suggested that it serves as a relay between autonomic function and behavior and cognition (MacLean, 1952). MacLean's conception that systems of interconnected structures within the "visceral brain", rather than a single structure, underlie emotion, has been regarded as his greatest contribution to neuroscience. Phylogenetic, structural, and functional hierarchies of the brain were a strong central thesis in his work.

MacLean noted that psychiatric patients, children, and modern hunter-gatherers, whom he called "primitives", all share a commonality of failing to discriminate between internal and external stimuli, suggesting that the visceral brain is less acted upon by the neocortical areas of the brain responsible for intelligent behavior and cognition, because the latter areas are less developed compared to "civilized" typical adults (MacLean, 1949). He later elaborated this into his theory of the "triune brain", which assumed a topographically and functionally nested hierarchy of the evolution of the vertebrate forebrain. MacLean proposed that the most ancient "reptilian brain" is composed of the basal ganglia, is involved in basic species-typical behaviors such as aggression, dominance, and ritualistic displays, and is present in all vertebrates. According to MacLean, the "paleomammalian/visceral brain" arose early in mammalian evolution, and is involved in emotion and motivational drive required for offspring care, reproduction, and feeding. Lastly, MacLean proposed that the "neomammalian brain", or the neocortex, which arose later in mammalian evolution, is more developed and complex in "higher" mammals, specifically primates and humans, and underlies higher order cognition and intelligence (MacLean, 1985).

Despite the advances MacLean's theory brought to neuroscience, central to his theory was the assumption that the emotional brain is a separate entity to the brain that supports reason and intelligence, an idea following the widely accepted Cartesian approach to the mind (Damasio, Grabowski, Frank, Galaburda, Damasio, 1994; Damasio, 1995). This conceptual dichotomy contributed to the viewing of the limbic system as highly conserved, and therefore, neural structures subserving limbic function have been largely ignored in the context of human brain evolution. However, neuropsychological studies have demonstrated that emotion plays an essential role in social cognition (Damasio, Grabowski, Frank, Galaburda,

Damasio,1994; Bechara, Damasio, and Damasio, 1990; Bar-On, Tranel, Denburg, Bechara, 2003; Rilling, 2008; Powell, Lewis, Dunbar, Garcia-Finana, Roberts, 2010), and it is therefore likely that alterations to the limbic system were crucial to human brain evolution Furthermore, it is now evident that evolutionary changes to the brain, and the adaptation of new circuits and specializations, do not occur in isolation but rather are embedded into ancestral systems (Pessoa and Hof 2015). As will be discussed in detail below, while few comparative primate studies have examined limbic structures, there is increasing evidence for human specialization.

Another possible reason human-specific changes to the limbic system are not often examined is that brain size has dominated human brain evolution research (Preuss 2011). This bias is likely due to the significant increase in absolute brain size that can be detected in the fossil record, and in particular, the 3-fold increase in absolute size of the brain in humans compared to our closest living relatives, the chimpanzees. Such emphasis on on overall size can mask small-scale changes that may be important to the niche-specific adaptation of a particular species (Teffer and Semendeferi 2012). Despite this bias, some early researchers recognized the importance of looking beyond absolute brain size, in light of advances of neurophysiology on model animals (Kaas, Nelson, Sur, Lin, Merzenich, 1979). In 1968, an extensive review of the comparative neuroanatomy was used to propose that brain evolution may entail neural reorganization within larger structures, defined as quantitative shifts between components or substructures of the brain that occur under natural selection in the evolutionary history of a specific species, and which may or may not ultimately alter the product of the larger structure (Holloway 1968). This work emphasized that increasing social complexity throughout human evolution likely necessitated reorganization of limbic areas

specialized for greater cortical control of emotional behavior. Such evidence for reorganization was found to be present in selected areas of the brain as discussed below, including the human frontal lobe, which is not disproportionately larger than a chimpanzee (Semendeferi, Damasio, Frank, and Van Hoesen, 1997; Semendeferi, Lu, Schenker, and Damasio, 2002), but instead linked to cortical reorganization of individual functional areas (Schenker, Buxhoeveden, Blackmon, et al. 2008; Semendeferi, Armstrong, Schleicher, Zilles, and Van Hoesen, 1998, 2001). Other evidence suggests that neural reorganization can also occur within interconnected regions forming a larger neural system; an extensive comparative analysis across 131 mammal species, including primates, found significant size covariance of most major brain regions (Barton and Harvey, 2000; Finlay, Darlington, and Nicastro, 2001).

The relative scarcity of evolutionary studies of the human limbic system may also be due in part to the complexity surrounding the anatomical definition of the limbic system. The most commonly agreed-upon core structures of the limbic system include the anterior cingulate cortex, the parahippocampal gyrus, the amygdala, and the hippocampus (Allen, 2009). It has been suggested that given the primary role of the limbic system as an intermediary between autonomic functions in the brainstem and cognition in the neocortex, one anatomical definition could be any structure with direct connectivity to the hypothalamus; however, the hypothalamus also has direct connectivity with structures that are not implicated in emotion (LeDoux, 1996). Furthermore, emotion is deeply imbedded in other functional networks, such as fear, memory, and cognition, and conversely, structures implicated in emotion also have roles in other neural circuits (LeDoux, 1993). Given these limitations and challenges, some have suggested that the limbic system is best considered a concept rather

than a discrete set of structures, and that emotional functions are better understood in terms of multiple overlapping neural pathways (Heimer and Van Hoesen, 2006).

While there are currently many unknowns regarding the full extent of these limbic pathways, several neural regions have been identified that demonstrate roles in emotional processing (Bush, Luu, Posner, 2000; Kurth, Zilles, Fox, Laird, and Eickhoff, 2010; Bechara, Damasio, and Damasio, 2000; Olsen, Plotzker, and Ezzyat, 2007; Blood 1999; Adolphs, Tranel, Hamann, et al. 1999; Moll and de Oliveira-Souza 2009; Ross, Freeman, Spiegel et al. 2009). Below, we will review these regions (see Figure 2.1), which include cortical limbic areas (anterior cingulate cortex, anterior insula, posterior orbitofrontal/ventromedial frontal cortex, temporo-polar cortex, parahippocampal cortex, and hippocampus), and subcortical structures (amygdala, septal nuclei, striatum, anterior nuclei of the thalamus, and hypothalamus/mammillary bodies), with particular consideration to areas with evidence that supports human specialization in the limbic system.

Limbic Cortex

Limbic cortical structures exhibit differences compared to the typical neocortical (ie isocortex) six-layer distribution of lamina, and are generally described as *proisocortex*, where layer IV is not present or poorly developed (i.e. anterior cingulate, anterior insula, posterior orbitofrontal and ventromedial prefrontal cortex, temporo-polar cortex), *periallocortex*, characterized by atypical, multi-layered cortex with some layers missing entirely (i.e. parahippocampal gyrus), or *allocortex,* composed of a thin cortex with many laminar components missing (i.e. hippocampus). Limbic cortical areas have strong reciprocal connections to each other and subcortical structures via large white matter pathways that include the cingulum, uncinate fasciculus, and performant path, as well as the fornix, which

connects the hippocampus with the mammillary bodies (Vilensky, Van Hoesen, Damasio, 1982; Catani, Dell'Acqua, de Schotten, 2010). Differences in volume and unique cell types found in limbic cortical regions reviewed below may reflect specialization in human evolution.

Anterior Cingulate Cortex

The anterior cingulate cortex (ACC) was one of the first structures to be identified as a key brain area in emotion (Papez 1937). The cingulate cortex lies on the mesial surface of the brain, and is involved in a number of different functions, including pain processing, visuospatial processing, and memory retrieval (Devinksy, Morrell, and Vogt, 1995). As part of the limbic system, the ACC is a site of integration of several circuits, including motivation, evaluation of error, and cognitive and emotional networks, and acts as a modulator in cognitive, endocrine, and visceral responses (Bush, 2000). Ventral regions of the ACC have strong connectivity with the basolateral division of the amygdala and the hypothalamus, and are likely more involved in appraisal and expression limbic functions, while more dorsal regions of the ACC have strong connections with the dorsolateral prefrontal cortex, and are likely more involved in top-down regulation of emotional behavior (Etkin, Egner, and Kalisch, 2011).

The human ACC demonstrates a higher density of spindle neurons (also known as Von Economo neurons), compared to other apes and any other animal in which they are present (Allman, Hakeem, and Watson, 2002). Spindle neurons are bipolar projection neurons characterized by a very large, elongate, tapered soma that can be discerned from pyramidal neurons not only by their shape, but also by their substantial size (they are largest in humans) and single basal dendrite (Figure 2.1). Spindle neurons have been reported in medical

literature over the past century and beyond (Betz 1881; Cajal 1899; Von Economo 1926), but were not well-studied until recently. Spindle neurons have restricted distribution, and are found only in layer Vb of the ACC and the anterior insula in humans (Nimchinsky, Vogt, Morrison, and Hof, 1995; Allman, Tetreault, Hakeem et al 2010; but also see Fajardo, Escobar, Buritica et al 2008 for possible spindle neurons in human BA 9), non-human apes (Nimchinsky, Gilissen, Allman et al 1999), elephants (Hakeem, Sherwood, Bonar *et al* 2009; some spindle neurons also found in the dorsolateral prefrontal cortex in elephants), and cetaceans (Butti, Sherwood, Hakeem, Allman, and Hof, 2009). The function of these neurons is not known, but it is thought to be related to socio-emotional intelligence, given the restricted provenience in neural areas associated with emotion and cognition, and presence only in highly intelligent mammals with complex social structures (Allman, Hakeem, Erwin, Nimchinsky, and Hof, 2001). In hominoids (human and non-human apes), the density of spindle cells in the ACC increases with decreasing phylogenetic distance to humans, and the volume of the spindle cell soma correlates positively with relative brain size, such that humans have the greatest number and volume of spindle neurons, followed by bonobos and chimpanzees (Allman, Hakeem, and Watson, 2002). While spindle neurons are present in late-term fetal brains of chimpanzees, they are not present until about four months after birth in the human brain, and it has been hypothesized that these neurons are involved in self-control, and in humans, are specialized to be influenced by social insight during development (Allman, Hakeem, and Watson, 2002). Spindle neurons are selectively targeted in individuals with fronto-polar dementia, a degenerative brain disease that causes deterioration of social and emotional self-awareness, moral reasoning, empathy, and theory of mind (Seely, Carlin, Allman et al 2006), further suggesting the role of these neurons in adaptive human social

behavior. However, the role of spindle neurons in the evolution of uniquely human or hominoid cognition remains highly contested- as stated previously, these neurons are found in other highly intelligent species with great phylogenetic distance to humans and non-human apes (cetaceans and elephants), and a recent study has found what appears to be a homolog of spindle neurons in macaques (Evrard, Forro, and Logothetis, 2012). Furthermore, while humans demonstrate the highest number of spindle neurons, the relative density of spindle neurons compared to other neurons is significantly lower in humans than in the rest of the non-human apes (Allman, Tetreault, Hakeem et al 2010).

Calretinin-containing pyramidal neurons are another, less-studied population of projection neurons that appear to be present in the ACC in human and non-human apes only (Hof, Nimchinsky, Perl, and Erwin, 2001). Calretinin is a calcium-binding protein that serves as a calcium ion buffer, and is typically expressed in distinct populations of inhibitory interneurons. A study examining calretinin expression in 13 primate species and several other mammalian species found a distinct population of calretinin-containing pyramidal neurons in layer V of the ACC in human and non-human apes only, with highest density in humans and lowest density in orangutans. While the function of these neurons is unknown, it has been suggested these neurons are evidence of possible adaptive modification in the ACC that emerged recently in primate evolution (Hof, Nimchinsky, Perl, and Erwin, 2001).

Anterior Insula

The insula is a deep cortical structure located within the lateral sulcus (see Figure 2.1) that serves as a region of integration of information from diverse functional systems related to autonomic regulation, perception, emotion and cognition (Kurth et al. 2010). Neuroimaging studies in humans revealed functionally distinct regions on the insula: sensorimotor tasks in

the posterior insula, olfacto-gustatory activity in the central insula, and cognitive and socio-emotional tasks in the anterior insula, although the anterior insula was also a site of overlap for many different types of stimuli processing, emphasizing the complex relationship between sensory, autonomic, emotional and cognitive processing (Craig 2009; Kurth et al. 2010). Cytoarchitectonic studies reveal a similar anatomical division, such that the posterior insula contains granular neocortex, which transitions to an intermediate zone of dysgranular cortex, while the anterior insula is agranular and more limbic-like (Mesulam and Mufson 1982). The anterior insula has significant connectivity to other limbic regions, including the amygdala, temporal pole, orbitofrontal cortex, and ACC (Mesulam and Mufson 1982; Uddin and Menon 2009), and in humans, has been implicated in the generation of feeling states in response to affective and environmental stimuli (Dolan 2002), pain in others (Frith and Singer 2008), emotion-recognition in faces (Phillips et al. 2003) and as a link between the mirror neuron system and emotion-processing that enables empathic theory of mind (Iacoboni and Dapretto 2006).

Spindle neurons are found in the fronto-insular cortex (FI) and ACC of humans, non-human apes, elephants, and cetaceans, with humans demonstrating the highest density of these neurons, possibly indicating human specialization in limbic circuitry (Nimchinsky et al. 1995; Hakeem et al. 2009; Butti et al. 2009; Allman et al. 2010). The FI, located in the inferior anterior insula, and the ACC receive differential afferents for somatosensory stimuli such as pain, itch, warming, cooling, and sensual touch that are important in the regulation of physiological homeostasis, and may be extended to include mechanisms evolved to maintain social homeostasis (Craig 2003, 2009; Allman, Tetrault, Hakeem et al. 2010). However, unlike the ACC, in which spindle neuron density in the non-human apes is highest in bonobos

and chimpanzees, spindle neuron density in the fronto-insular cortex of non-human apes is highest in gorillas (Allman, Tetrault, Hakeem et al. 2010). Given the proposed role of spindle neurons as an adaptation related to increasingly complex social behavior, these findings are of interest considering that compared to bonobos and chimpanzees, gorillas' smaller group size is thought to indicate decreased social complexity with weaker social ties (Maryanski 1987).

Some volumetric differences across species have been identified in the insula and its two major cytoarchitectonic divisions, the ventral anterior insula, containing the agranular insular cortex and the FI, and the posterodorsal insula, containing the granular and dysgranular insular cortex. A postmortem study examining 30 primate species, including humans and all species of non-human apes (Bauerfeind et al. 2013), found that total insula volume scales hyperallometrically (larger than expected based on absolute brain size) corroborating the findings of a previous MRI comparative study in which humans had a slightly larger insula than expected, Semendeferi and Damasio 2000) and that the ventral anterior agranular insula and FI increases at an even greater rate than the granular and dysgranular insula, such that the ventral anterior insula/FI is 10-36% larger in the larger-brained humans and great apes compared to other primates. Furthermore, the human left and right agranular insula, and the human left FI demonstrate a greater increase in absolute volume compared to chimpanzees than any other cortical region previous examined. While no species deviate from primate-wide allometric scaling patterns, and there is no correlation between volume of the subdivisions and social group size, the authors suggest that these differences in absolute volume of the anterior insula might underlie cognitive specializations related to complex social interactions that were amplified in the human lineage (Bauernfeind, de Sousa, Avasthi et al. 2013).

Posterior Orbitofrontal/ Ventromedial Prefrontal Cortex

Neuroimaging and lesion studies have implicated ventromedial/ posterior orbitofrontal cortical regions (from here on referred to as posterior OFC) in limbic circuits involved in emotion and social cognition (Bechara et al. 2000). This classification is further supported on neuroanatomical grounds; while more rostral/lateral areas of the orbitofrontal cortex demonstrate granularity typical of the prefrontal cortex, posterior OFC regions lack a clear granular layer, reflecting their limbic designation (Semendeferi et al. 1998). The posterior OFC has strong interconnectivity with several other limbic structures, including the insula, temporo-polar cortex, parahippocampal cortex, striatum, nucleus basalis of Meynert, and amygdala (Carmichael and Price 1995; Semendeferi et al. 1998).

Despite substantial evidence for the prominent role of the OFC in many functions related to emotion and social cognition (Bechara, Damasio, and Damasio 2000; Rolls 2004) that reflect uniquely human behaviors (Bachevalier and Loveland 2006; Beer, John, Scabini, and Knight, 2006), few neuroanatomical differences with non-human apes have been found that could account for these behavioral differences. An MRI study examining the volume of the frontal lobe and the three major divisions of the frontal lobe (dorsal, medial, and orbital) in humans, apes, and macaques found that the relative size of the three major subdivisions, including the orbital frontal lobe, is proportionate across all primates, including humans (Semendeferi, Damasio, Frank, and Van Hoesen, 1997), but human OFC is 11% larger than predicted for an ape brain of human brain size (Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014).

There is some evidence of smaller-scale differences that suggest reorganization of this region occurred in human brain evolution. Area 13 is a cytoarchitectonically distinct limbic

cortical region that makes up the core of the posterior OFC region (Figure 2.1). Area 13 is present in humans, non-human apes, and monkeys, and is distinguishable from non-limbic OFC by the presence of an incipient layer IV (more rostral OFC demonstrates a clearly defined granular layer IV; Semendeferi, Armstrong, Schleicher, Zilles, and Van Hoesen, 1998). A postmortem study (Semendeferi, Armstrong, Schleicher, Zilles, and Van Hoesen, 1998) of area 13 across primate species found that while area 13 is similar in structure and laminar distribution across human and non-human apes, the relative size of area 13 varies across the species. Humans and bonobos demonstrated the smallest relative volume of BA 13 out of the apes, as well as the smallest length of BA 13 relative to the rest of the OFC. In contrast, orangutans demonstrated the greatest relative length of BA 13, a finding attributed to the relatively short length of the orangutan OFC compared to other apes. While not limbic cortex, BA 10, which constitutes the frontal pole in humans and shares connections with limbic posterior OFC, demonstrates significant differences in humans, including relative expansion of the region (twice as large as expected for an ape brain of human size), as well as cellular differences related to increased neuropil space, especially in supragranular layers II/III that are involved in cortico-cortical connectivity (Semendeferi et al. 2001; 2010; Bianchi et al. 2012). It is also of note that neuron density is decreased throughout the cortex in humans compared to non-human apes, but that the prefrontal cortex, including BA 13 and BA 10, demonstrates an even greater decrease in density compared to non-human apes in other cortical areas (Semendeferi, Armstrong, Schleicher, Zilles, and Van Hoesen, 1998, 2001; Semendeferi, Teffer, Buxhoeveden et al. 2010; Schenker, Buxhoeveden, Blackmore et al. 2008; Buxhoevden, Switala, Roy, Litaker, and Casanova, 2001).

Temporo-Polar Cortex

The temporo-polar cortex consists of the cortex that covers the most-anterior end of the temporal lobe. The temporal pole has long been implicated in emotional circuitry (Yakovlev 1948), and has strong connections with the amygdala and OFC, efferents to the hypothalamus (Gloor, Olivier, Quesney, Andermann, and Horowitz, 1982) and afferents from the thalamic nuclei and the insula (Markowitsch, Emmans, Irle, Streicher, and Preilowski, 1985; Gower 1989). The temporal pole is also a site of convergence of highly processed inputs from sensory cortical areas (Olsen, Plotzker, and Ezzyat, 2007). In addition to the incipient layer IV that characterizes limbic cortex, the temporal pole is identified by its significant thickness compared to surrounding cortex and well-defined infragranular layers (Ding, Van Hoesen, Cassell, and Poremba, 2009; Von Economo 1929). While the precise function of the temporal pole is not clear, neuroimaging studies have demonstrated that it is involved in the processing of emotional and affective content, and in humans, is important in facial recognition (Olsen, Plotzker, and Ezzyat, 2007).

Despite evidence that the human temporal pole is an important region for human-specific socio-emotional behavior, no cross-species comparative studies yet exist. This may be in part due to the difficulty of defining the extent and boundaries of the temporo-polar cortex, particularly in humans, as it is the site of gradual transition to other temporal convolutions (Von Economo 1929). However, some evidence suggests the temporal lobes as a whole have undergone change in human evolution: MRI studies examining temporal lobe volume in humans and non-human apes found that the human temporal lobe is larger than expected (Semendeferi and Damasio 2000), and that the overall temporal lobe volume, temporal white matter volume, and temporal surface area are significantly increased in humans compared to apes (Rilling and Seligman 2002). Furthermore, endocast studies of

extinct hominids demonstrate an anterior expansion of the temporal pole in select species (*Australopithecus africanus*, early *Homo,* including *Homo floresiensis*) similar to anatomically modern humans, and not present in chimpanzees (Falk, Redmond Jr., Guyer, et al. 2000; Falk, Hildebolt, Smith, et al. 2005), so it is possible that the larger temporal lobe in humans is in part related to specializations of the limbic temporal pole.

Parahippocampal Gyrus

The parahippocampal gyrus lies along the ventromedial edge of the temporal lobe adjacent to the hippocampus, and was identified as a prominent structure of the limbic lobe early on in human neuroanatomical research (Broca 1878; Fulton 1953). The parahippocampal gyrus is made up of several cortical areas, including the primary olfactory cortex at the anterior pole and the entorhinal cortex just posterior to the anterior pole (Van Hoesen 1982). Given the close proximity and significant innervation to olfactory areas, the parahippocampal gyrus was initially thought to be involved in olfaction only, but studies in nonhuman animals and humans indicate that the structure is involved in complex emotive processes and has significant interconnectivity to other cortical limbic structures as well as the amygdala (Van Hoesen 1982; Blood 1999; Powell 2004). Furthermore, positron emission tomography studies in humans have demonstrated that the parahippocampal gyrus is significantly implicated in negative but not positive emotive response, suggesting that this structure is part of a highly specialized network for processing different types of emotional stimuli (Blood 1999; Lane 1997). No studies have yet investigated human/ non-human ape comparisons in the parahippocampal gyrus.

Hippocampus

The hippocampus is a component of the medial temporal lobe implicated in limbic circuitry, receiving input from limbic structures including the septal nuclei, the amygdala, and the thalamus (Witter 2007). The basic structure of the hippocampus is conserved across mammals, and can be divided into the dentate gyrus, the hippocampus proper (which includes CA 3 and CA 1; Figure 2.1) and the subiculum. The flow of information from the entorhinal cortex through the hippocampus is unidirectional: entorhinal cortex→ dentate gyrus → CA 3→ CA 1→ back to entorhinal cortex (LeDoux 1993). The entorhinal cortex (a component of the adjacent parahippocampal cortex) has significant connectivity to the hippocampus and is the primary interface between the hippocampus and the neocortex in a multitude of widespread neural circuits involved in memory function (Witter 2007). Functionally, the hippocampus is implicated in declarative memory, including emotional memory. The hippocampus likely plays a secondary role in emotion itself: hippocampal damage does not result in abnormalities in emotional behavior or response (Selden, Everitt, Jarrard, and Robbins, 1991), and electrical stimulation of the hippocampus modulates stimulation of the hypothalamus which can modulate emotional response, but stimulation of the hippocampus alone does not affect emotional response (LeDoux 1993). These findings suggest that the hippocampus is an important input to limbic networks, but is likely involved in non-emotional functions of these networks (LeDoux 1993).

While overall structure is conserved, changes in cytoarchitecture and size have been observed in primate evolution. The human hippocampus is 50% larger in humans than predicted for an ape of human hemisphere volume, with CA 1 demonstrating the greatest increase in volume compared to other regions of the hippocampus (Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014; Stephan, 1983). The cytoarchitecture of CA 1 also

demonstrates specialization in humans: in some insectivores, pyramidal neurons are contained to a thin narrow band with high neuronal density, whereas in primates the pyramidal layer is significantly more diffuse, with humans demonstrating the greatest dispersion of pyramidal neurons throughout CA 1, possibly indicating a shifting emphasis in patterns of connectivity (Stephan, 1983).

Subcortical limbic structures

Subcortical regions are comprised of nuclei and subnuclei formed from aggregations of neuronal bodies and lack the laminar organization seen in the cortex. Subcortical components of the limbic system have strong connections to cortical limbic areas and the hypothalamus, as well as connectivity with other subcortical limbic structures. (Vilensky, Van Hoesen, and Damasio, 1982). Given that subcortical structures are phylogenetically ancient relative to much of the cortex (but see Karten 1969 for more on topic), they were historically considered to be less subject to selective pressures, and therefore relatively unchanged and of little consequence to primate/human evolution (Pessoa and Hof 2015; Vilenski Van Hoesen, and Damasio,1982). This has likely contributed to a neglect of subcortical regions in comparative primate neuroanatomical studies. However, more recent research approaches demonstrating the integration of multiple neural systems across all levels of neural processing has generated a greater interest in subcortical structures (Petersen and Sporns 2015; Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014; Barton and Harvey 2000). While significant gaps are still present, a handful of studies, reviewed below, have identified some evidence for the presence of human specialization in subcortical limbic nuclei.

Amygdala

The amygdala, or amygdaloid complex, is a subcortical structure involved in the integration and mediation of the internal environment with stimuli in the external environment, and is a key structure in emotion and social cognition. The amygdala lies in the anteromedial temporal lobe and sits rostral to the hippocampus. The structure is composed of roughly 13 distinct nuclei, which are distinguished from each other by cytoarchitecture, histochemistry, and connectivity (Freese and Amaral 2009; Sah, Faber, Lopez de Armentia, and Power, 2003). The four nuclei that are most implicated in social and emotional behaviors are the lateral nucleus, the basal nucleus, and the accessory basal nucleus, which together make up the basolateral nuclei, and the central nucleus (Figure 2.1). The lateral nucleus is the primary source of sensory input from the thalamus and temporal cortex into the amygdala (Stefanacci and Amaral, 2000, 2002), while the basal and accessory basal nuclei receive intrinsic input from the lateral nucleus, as well as input from the OFC and ACC (Stefanacci and Amaral 2002; Janak and Tye 2015). The central nucleus receives little cortical input, but is a major output for downstream connections with the hypothalamic nuclei and brainstem (Gallagher and Holland 1994).

Amygdala function in mammals is well-studied, and there is a large body of research demonstrating the direct role of the structure in emotion and social cognition. Targeted chemical lesions of the amygdala in monkeys have shown a consistent pattern of socio-behavioral abnormalities, including a decrease in species-typical aggressive behaviors, an increase in affiliative behaviors, and a decrease in normative anxiety and fear in the face of potentially negative social interactions, and a generalized lack of awareness towards emotional stimuli (Meunier, Bachevalier, Murray, Malkova, and Mishkin, 1999; Adolphs 2001; Emery, Capitanio, Mason, et al. 2001; Bauman, Toscano, Mason, Lavenex, and

Amaral, 2006; Machado, Emery, Capitanio, et al. 2008). Lesions in the human amygdala have demonstrated significant impairments in human-specific socio-emotional functions. Lesioned individuals have difficulty interpreting negative facial expressions, including fear, anger, sadness, and disgust (Adolphs, Damasio, and Damasio, 1994; Young, Aggleton, Hellawell, et al. 1995; Adolphs, Tranel, Hamann, et al. 1999), using direction of gaze as a cue of emotional saliency (Cristinzio, N'Diaye, Seeck, Vuilleumeir, and Sander, 2010), show deficits in some theory of mind tasks (Stone, Baron-Cohen, Calder, Keane, and Young, 2003), and have increases in social approach behavior and difficulties adhering to social norms about interpersonal space (Kennedy, Glascher, Tyszka, and Adolphs, 2009). Functional neuroimaging studies in humans point to similar roles of the amygdala in the social brain. A number of studies have shown an elevated amygdala response to fearful or angry facial expressions (Blair, Morris, Frith, Perrett, and Dolan, 1999; Fusar-Poli, Placentino, Carletti, *et al.* 2009; Whalen, Shin, McInerney, et al. 2011; Fossati 2012) as well as a strong interaction between amygdala response and the screening and detection of changing facial emotions in others (Glascher, Tuscher, Weiller, and Buchel, 2004). In addition to decoding social cues, the amygdala is also involved in guiding behavior towards conspecifics in the socio-environment. One study found that a strong amygdala response was elicited when individuals experienced affective empathy (Cox, Uddin, Martino, et al. 2012), while another found amygdala activation when an individual chose not to conform to the social group (Berns, Chappelow, Zink, et al. 2005). Together, these studies suggest that the amygdala is critical in both the detection of the changing social environment and the behavioral response within the social group, and is thus a key component in social cognition.

The amygdala has been considered relatively conserved across mammals, and several features of the structure appear to be phylogenetically retained, such as topographic location, relationships with the olfactory, autonomic, and multimodal systems, the structure as the origin of important hypothalamic projections, a common embryological origin, and a number of local circuit neurons that are shared across most mammalian species (Pabba 2013). A homolog of the amygdala is even thought to be present in amphibians (Schumann, Bauman, and Amaral, 2011). However, recent studies suggest that the amygdala has also undergone specialization in human evolution. A stereological study of volume of the four major amygdaloid nuclei in humans and non-human apes revealed that the lateral nucleus in humans is relatively larger than expected in an ape brain of human size, and is the largest nucleus of the basolateral division, while the basal nucleus is largest in all other apes (Barger, Stefanacci, and Semendeferi, 2007). Furthermore, these differences in nuclei volume are accompanied by differences in neuron number: the lateral nucleus contains the greatest number of neurons in the human amygdala, containing 59% more neurons than expected for an ape brain of human size, while the basal nucleus contains the greatest number of neurons in the amygdala of all other apes (Barger, Stefanacci, Schumann, et al. 2012). In contrast, the basal nucleus and the central nucleus in humans are significantly smaller than expected for an ape brain of human size, with the central nucleus demonstrating a very large (-312%) decrease in volume (Barger, Stefanacci, and Semendeferi 2007). Neuron numbers in both nuclei are lower than expected, although not significant in either nucleus (Barger, Stefanacci, Schumann, et al. 2012).

These findings suggest that while many features of the amygdala as a whole may be conserved, subtle specializations of the structure, which is directly involved in the detection and processing of social stimuli, occurred over the course of human evolution and may have

been selected for as an adaptation to an increasingly nuanced and complex social environment. Primate studies have found a positive correlation between basolateral amygdala volume and social play frequency across species (Lewis and Barton 2006), and in humans, amygdala volume has been positively correlated with social network size (Bickart, Wright, Sautoff, Dickerson, and Feldman Barret, 2011; Kanai, Bahrami, Roylance, and Rees, 2011). Lastly, abnormalities of amygdala structure, including overall size, neuron number, neuron density, and volume of nuclei are a common feature of neurodevelopmental disorders in humans, and are associated with severe deficits in the social domain (Schumann, Hamstra, Goodlin-Jones, *et al.* 2004; Schumann and Amaral 2006; Levitt, Blanton, Caplan, et al. 2001; Welch et al. 2010; Mosconi, Cody-Hazlett, Poe, et al. 2009; Velakoulis, Wood, Wang, et al. 2010; Krecksmanski, Heinsen, Mantua, et al. 2007), demonstrating that even slight changes to the amgydala is detrimental to one's ability to navigate the human social environment.

Septal Nuclei

The septal nuclei are present in most vertebrates, and in primates they are located medially in the cerebral hemispheres inferior to the rostrum of the corpus callosum and anterior to the third ventricle (Figure 2.1; Mark, Daniels, Naidich, Hendrix, and Maas, 1994). The septal nuclei are implicated in several limbic networks and are connected to other limbic structures, including the hippocampus, amygdala, hypothalamus, anterior thalamic nuclei, and the cingulate gyrus (Swanson and Cowan 1979). Human and non-human studies have demonstrated that the septal nuclei are involved in reward circuits, and electrical stimulation of the region has been reported to result in sensations of pleasure (Heath 1958). Lesions of the septal nuclei in rats demonstrated a significant increase in emotional reactivity, demonstrating the involvement of the region in regulating affective behavioral response (Brady and Nauta

1953). In humans, the septal nuclei are activated in several socio-emotional behaviors, including cooperation and emotional attachment (Moll and de Oliveira-Souza 2009).

Like other subcortical structures, the septal nuclei were previously considered to be implicated in "primitive" neural functions, and were thought to be atrophied in humans compared to non-human primates (Shimazono 1912; Andy and Stephan 1968). Nevertheless, the septum was shown to be largest in humans, in both absolute and relative terms (Andy and Stephan 1968; Stephan 1981) and demonstrates a 19% increase in size compared to the anthropoid regression (Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014). While these findings should be approached with caution, as the data set available for analysis (Stephan 1981) included only a single human septum, they suggest that specialization of the septal nuclei may have occurred in human evolution.

Striatum

Found in all vertebrates, the striatum is a major component of the basal ganglia (forebrain nuclei) and is composed of the nucleus accumbens, the olfactory tubercle, and the caudate and putamen (Graybiel and Ragsdale Jr. 1978). Limbic associations of the striatum are contained primarily in the nucleus accumbens, a ventral subdivision of the striatum. The nucleus accumbens has interconnections to the amygdala, thalamus, hippocampus, and hypothalamus, and is thought to integrate information from limbic and prefrontal regions in order to regulate goal-directed behavior (Haber, Kim, Mailly, and Calzavara, 2006; Goto and Grace 2008). The ventral striatum is involved in several cognitive processes, including learning and memory (Grahn, Parkinson, and Owen, 2009), and is innervated by several neurotransmitters such as dopamine, involved in motivation reinforcement (Robbins and

Everitt 1992), and oxytocin, important in social attachment behaviors (Ross, Freeman, Spiegel, et al. 2009).

Few cross-species comparative studies have examined the striatum in the context of evolution. An early study examining the volume of brain structures in monkeys, prosimians, and insectivores found that the striatum demonstrated the second-largest increase in size relative to body weight in monkeys (neocortex demonstrated largest increase in size in monkeys; Stephan and Andy 1964). However, the more recent Barger and colleagues (2014) analysis of this data set along with an additional expanded hominoid data set found that the human striatum demonstrated a statistically significant decrease in size from the anthropoid regression (-54%; the only limbic structure to fall outside the predicted interval in the anthropoid data set), as well as a large, albeit not significant decrease in size from the hominoid regression (-27%; Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014). While these findings are surprising given the importance of the striatum in complex cognitive functions, it has been suggested that specializations have occurred in human evolution at the cellular level that are not directly tied to volume, such as changes in neurotransmitter systems (Barger, Hanson, Teffer, Schenker-Ahmed, and Semendeferi, 2014). Indeed, investigations in this direction are ongoing (Hanson and Semendeferi 2016).

Thalamic nuclei: anterior group and mediodorsal nucleus

The thalamus lies in the forebrain near the center of the brain in vertebrates, and is composed of multiple nuclei with distinct connectivity and function, ranging from sensory and motor function, to regulating consciousness, to limbic function (Hererro, Barcia, and Navarro, 2002). The anterior nuclei of the thalamus and the medial part of the mediodorsal (MD) nucleus of the thalamus (Figure 2.1) demonstrate significant connectivity to other

limbic structures. The anterior group receives input from the mammillary bodies, and sends efferent connections to the cingulate, and the dorsomedial nucleus receives input from the amygdala and temporal cortices, and sends efferent connections to the prefrontal cortex (Vertes, Linley, and Hoover, 2012; Robertson and Kaitz 1981; Powell 1973). Functionally, the limbic thalamic nuclei have demonstrated a role in learning and memory (Mitchell and Chakraborty 2013) and mediating stress and anxiety (Vertes, Linley, and Hoover, 2015), and likely serve as an important node for encoding information from subcortical limbic structures for goal-directed behaviors (Mair, Miller, Wormwood, et al. 2015).

A comparative postmortem study (Armstrong 1980) examining the thalamic nuclei of gibbon, chimpanzee, gorilla, and human subjects found that the human anterior and MD thalamic nuclei were much larger in absolute terms compared to the great apes, although the relative increase in volume was not significant; however, total neuron counts of the these thalamic nuclei in humans were significantly greater than expected for an ape brain of human size. In contrast, neuron numbers of other thalamic nuclei implicated in sensory and motor function were as expected for an ape brain on human size (Armstrong 1980, 1981). Interestingly, anthropoid species with single-male social systems have greater neuron numbers of the anterior and MD thalamic nuclei than multi-male social systems, suggesting a correlation between social structure and the limbic thalamus (Armstrong 1987). These findings suggest evidence of reorganization of the thalamus during hominoid evolution, with a greater emphasis on limbic roles of the thalamus.

Hypothalamus/mammillary bodies

The hypothalamus, which forms the wall and floor of the third ventricle, is made up of a collection of nuclei specialized to regulate homeostasis by regulating the endocrine,

autonomic nervous, and limbic systems (Waxman 2011). The hypothalamus is strongly connected to the limbic system via the amygdala and the septal nuclei, as well as bidirectional connectivity with cortical regions, including the prefrontal cortex (Rempel-Clower and Barbas 1998; Risold, Thompson, and Swanson, 1997).

The mammillary bodies are a pair of specialized nuclei (medial and lateral) in the posterior hypothalamus. Both nuclei receive significant projections from the hippocampus, and have efferent projections to the anterior thalamic nuclei (Rich 2011). The mammillary bodies, along with the hippocampus, anterior thalamic nuclei, and cingulate gyrus, compose the Papez circuit (Papez 1948; Armstrong 1986). Mammillary body lesions in rats have been shown to cause impairments in spatial memory (Vann 2010), although mammillary lesions in humans did not demonstrate a decline in any cognitive ability (Duprez, Serieh, and Raftopoulos, 2005). Dense connectivity to the anterior thalamic nuclei designate the mammillary bodies as limbic, although the role these nuclei play in emotion or affective state is unclear. Although it was expected that the mammillary bodies would demonstrate a similar increase in neuron number as the anterior thalamic nuclei in humans given the strong association between the two structures, the number of neurons in the mammillary bodies (medial mammillary) in humans fall within prediction intervals for an ape brain of human size (Armstrong 1986). These findings suggest the presence of mosaic evolution within this limbic circuit. Furthermore, the anterior thalamic nuclei represent a strong limbic-cortical connection, while the mammillary bodies have primarily subcortical projections. Therefore, these findings might be evidence of an emphasis in greater limbic-cortical connectivity in human brain evolution (Armstrong 1986).

Nucleus basalis of Meynert

The nucleus basalis of Meynert lies in the substantia innominate of the basal forebrain, and has been implicated in limbic circuitry given the numerous direct cortical afferents from orbitofrontal and temporal cortex (Mesulam and Mufson 1984), as well as inhibitory inputs from the amygdala and striatum (Carnes, Fuller, and Price, 1990), and cholinergic inputs to the PFC, thalamus, and hypothalamus (Wenk 1997; Williams, Marsh, Macdonald, *et al.* 2013). The precise function of the nucleus basalis of Meynert is unknown, but over 90% of the principal neurons in the nucleus basalis of Meynert are cholinergic, and this cholinergic system has been associated with several cognitive and other behavioral functions (Wenk 1997). Human-specific patterns of cholinergic innervation are found in the prefrontal cortex (Raghanti, Stimpson, Marcinkiewicz, et al. *2008*) suggesting alterations of the cholinergic system may have occurred over the course of human evolution. However, a study comparing cholinergic neuron numbers in the nucleus basalis of Meynert in New World Monkeys, Old World Monkeys, non-human apes, and humans found that human numbers fall within the anthropoid prediction interval (Raghanti, Simic, Watson, et al. 2011). While no current study has identified human-specific changes in the nucleus basalis of Meynert, it is possible that changes to the cholinergic system in this region have occurred that are not related to neuron number, such as modifications resulting in more diffuse neuromodulatory circuitry decoupled from neuron distribution (Raghanti, Simic, Watson, et al. 2011).

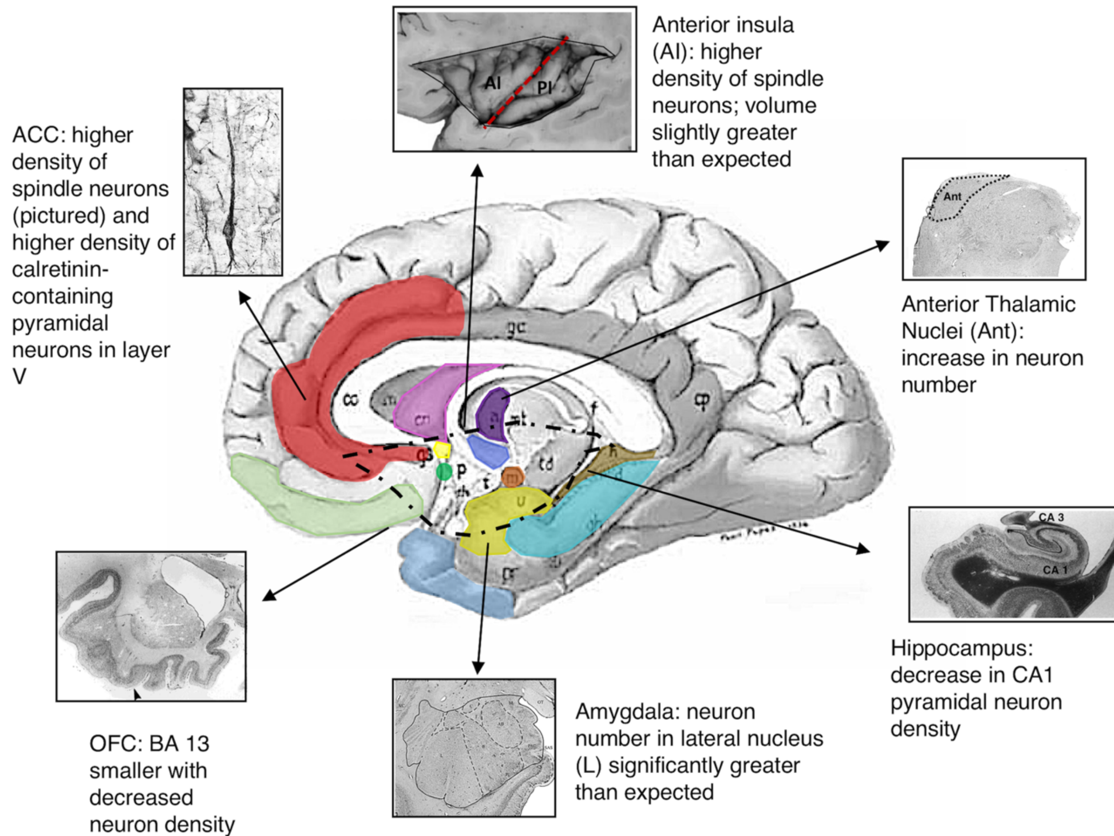
Conclusion

While few comparative human to non-human ape studies have examined limbic structures, those that have demonstrate some evidence of recently derived changes to the limbic system in human evolution (Figure 2.1, Figure 2.2). Human limbic structures

demonstrating significant increases in size relative to what is expected for an ape of human brain size include the OFC, the hippocampus, and the lateral nucleus of the amygdala, while structures demonstrating significant decreases in size relative to the hominoid regression include the basal and central nucleus of the amygdala. The human striatum also demonstrated a decrease in size, although the decrease was significant relative to the anthropoid regression only, suggesting change within the striatum may have occurred earlier in primate evolution. Some differences in neuron number of limbic structures have also been observed. The lateral nucleus of the amygdala and the anterior and MD thalamic nuclei in humans contain significantly more neurons than expected for an ape of human brain size, while non-limbic nuclei within these same structures do not demonstrate a deviation from predicted neuron number. Lastly, there is some evidence of cellular specialization in the human ACC, which contains higher densities of unique classes of specialized pyramidal neurons (spindle and calretinin-containing) in humans compared to apes. It is possible that similar specializations exist in other human limbic structures, and lack of evidence is due to either to shortage of tissue and/or methods that make such analysis currently unfeasible, or, simply, has not yet been a target of investigation. Furthermore, it is likely that human limbic regions contain many specializations not detectable at the above levels or with the specific parameters, and future studies utilizing other techniques, such as immunohistochemical staining to uncover interneuron subtypes (Hanson, Hrvoj-Mihic, and Semendeferi, 2014), neurotransmitter distribution (Raghanti, Stimpson, Marcinkiewicz, *et al.* 2008; Stimpson, Barger, Taglialatela, *et al.* 2015) or dendritic and axonal specializations (Hrvoj-Mihic, Bienvenu, Stefanacci, Muotri, and Semendeferi, 2013), may uncover additional human specific modifications in neural systems underlying some of the most crucial aspects of our species' behavior.

Chapter 2, in full, is an adaptation of a literature review previously published in the reference work *Evolution of Nervous Systems, 2nd Ed.* The dissertation author was the primary author of this paper. Citation: Lew CH**,** Semendeferi K. 2017. "115. Evolutionary specializations of the human limbic system". *In Evolution of Nervous Systems* 2 ed. Vol. 4. Kaas and Pruess. Elsevier. 277-291.

FIGURES AND TABLES



Structure Key: **Orbito-frontal cortex**; **Anterior Cingulate Cortex**; **Striatum**; **Septal Nuclei**; **Nucleus Basalis of Meynert**; **Anterior Thalamic Nuclei**; **Hypothalamus**; **Mammilary Body**; **Amygdala**; **Temporopolar Cortex**; **Hippocampus**; **Parahippocampal Gyrus**

Figure 2.1: Human specializations of the limbic system at the cellular level. ACC, amygdala and anterior thalamic nuclei, Anterior insula (AI, including fronto-insula, FI; insula not visible on mesial surface, photo of sagittal view of human brain with outer cortex removed to display insula), and BA 13 specialization is based on human to ape comparisons.

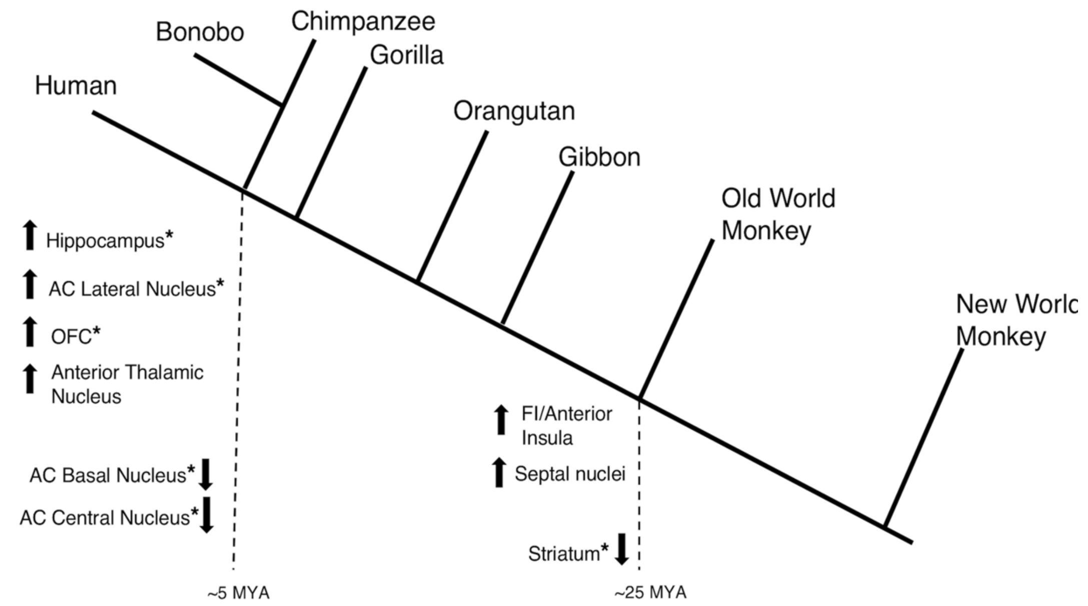


Figure 2.2: Volumetric changes to limbic structures in human evolution. *indicates statistical significance.

Table 2.1: The limbic system in human evolution: gross anatomical and cellular anatomy. *postmortem histological study; **MRI study

Limbic Region	Human-derived features compared to non-human apes	Human and non-human ape-derived features compared to monkeys; other notes	Reference
Anterior cingulate cortex	Higher density of spindle neurons		Allman et al. 2002*
	Higher density of calretinin-containing pyramidal neurons in layer V	No calretinin-containing pyramidal neurons found in monkeys	Hof et al. 2001*
Anterior Insula	Highest density of spindle neurons		Allman et al. 2010*
	Volume slightly greater than expected		Semendeferi et al. 2000**
Anterior insula/FI	Absolute volume of left FI greater	Absolute volume 10-36% larger in humans and apes compared to monkeys	Bauernfeind et al. 2013*
Posterior orbital/ventromedial PFC	Relative volume 11% larger than predicted for hominoids		Semendeferi et al. 1997**; Barger et al. 2014**
	Along with bonobos, have smallest volume and length of BA 13 relative to OFC		Semendeferi et al. 1998*
Temporo-polar cortex	Not yet examined, but expansion in overall temporal lobe in humans		Semendeferi and Damasio 2000**; Rilling and Seligman 2002**
Hippocampus	Relative volume 50% larger than predicted for hominoids	CA1 demonstrates region of greatest increase in hippocampus	Stephan et al. 1983*; Barger et al. 2014*
	Decrease in density of pyramidal neurons in CA1		Stephan et al. 1983*

Table 2.1 continued: The limbic system in human evolution: gross anatomical and cellular anatomy.*postmortem histological study; **MRI study

Amygdala	Relative volume of lateral nucleus larger than expected, and largest nucleus of basolateral division	In all other apes, basal nucleus has largest volume in basolateral division	Barger et al. 2007*
	Lateral nucleus contains greatest number of neurons in basolateral division, and 59% more neurons than expected	Basal nucleus contains greatest number of neurons in all other apes	Barger et al. 2012*
Septal nuclei	Largest absolute and relative volume (PM)	19% larger in humans than expected for anthropoids	Andy and Stephan 1968*; Barger et al. 2014*
Striatum	Relative volume 27% smaller than expected for hominoids	54% smaller in humans than expected for anthropoids	Barger et al. 2014*
Anterior thalamic nuclei	3-4-fold increase in absolute volume		Armstrong 1980*
	Significantly greater number of neurons than expected	Number of neurons in other thalamic nuclei similar in humans and apes	Armstrong 1980*, 1981*
Mammillary bodies	Neuron numbers similar to apes		Armstrong 1986*
Nucleus basalis of Meynert	number of cholinergic neurons as predicted/ similar to apes		Raghanti et al. 2011*

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CHAPTER 3:

Neuron density is decreased in the prefrontal cortex in Williams Syndrome

ABSTRACT

Williams Syndrome (WS) is a rare neurodevelopmental disorder associated with a hemi-deletion in chromosome 7, which manifests a distinct behavioral phenotype characterized by a hyper-affiliative social drive, in striking contrast to the social avoidance behaviors that are common in Autism Spectrum Disorder (ASD). MRI studies have observed structural and functional abnormalities in WS cortex, including the prefrontal cortex (PFC), a region implicated in social cognition. This study utilizes the Bellugi Williams Syndrome Brain Collection, a unique resource that comprises the largest WS postmortem brain collection in existence, and is the first to quantitatively examine WS PFC cytoarchitecture. We measured neuron density in layers II/III and V/VI of five cortical areas: PFC areas BA 10 and BA 11, primary motor BA 4, primary somatosensory BA 3, and visual area BA 18 in six matched pairs of WS and typically developing controls (TD). Neuron density in PFC was lower in WS relative to TD, with layers V /VI demonstrating the largest decrease in density, reaching statistical significance in BA 10. In contrast, BA 3 and BA 18 demonstrated a higher density in WS compared to TD, although this difference was not statistically significant. Neuron density in BA 4 was similar in WS and TD. While other cortical areas were altered in WS, prefrontal areas appeared to be most affected. Neuron density is also altered in the PFC of individuals with Autism Spectrum Disorder. Together these findings suggest that the PFC is targeted in neurodevelopmental disorders associated with socio-behavioral alterations.

Introduction

Alterations in social behavior are a key characteristic of both Autism Spectrum Disorders (ASD) and the significantly less common Williams Syndrome (WS), a rare neurodevelopmental disorder (1 in 7500 to 1 in 12,000 births; Stromme et al. 2002), caused by a hemizygous deletion of ~26 consecutive genes on chromosome 7.q11.23 (Morris et al 2010a). While ASD is characterized by behaviors of social avoidance, the WS phenotype is characterized by hyper-affiliative social behavior, including an abnormally strong desire for social engagement and exaggerated gregariousness and empathy for others, a propensity for approaching strangers, and atypical social judgment, particularly in negative or dangerous social situations (Morris et al. 2010b; Doyle et al. 2004; Santos et al. 2010; Jarvinen-Pasley et al. 2008; Jarvinen et al. 2013). Furthermore, there appears to be a genetic parallel between WS and some cases of ASD - one study identified the genotype of some ASD individuals to include a duplication of the region deleted in WS (Merla et al. 2010), and a case study described a WS individual with an atypical WS deletion and an ASD diagnosis/socio-behavioral phenotype (Edelmann et al 2007; Sakurai et al. 2011). These findings demonstrate that WS studies serve not only as a model in which to better understand the etiology of socio-behavioral alterations, but also may uncover similar and/or related mechanisms affected in ASD.

Little is known about the neuroanatomical phenotype of WS, in part because the rare occurrence of the disorder further limits availability of postmortem subjects for study. Neuroimaging studies examining living individuals with WS have found an overall reduction in cerebral volume (Reiss et al. 2000), as well as statistically significant reductions in occipital cortex volume in WS (Reiss et al. 2004), but increased volume in two regions of the

PFC (orbitofrontal and medial prefrontal cortex) that are implicated in emotional and social processing (Reiss et al. 2004). Abnormal patterns of neural activity in the PFC in WS during exposure to social stimuli have also been observed (Meyer-Lindenberg et al. 2005). So far, few observations have been made about the neuroanatomy and histology of the WS cortex, and PFC in particular (Galaburda et al. 1994, 2002; Galaburda and Bellugi 2000; Hollinger et al. 2005). Galaburda and Bellugi (2000) qualitatively examined select cortical areas in four WS brains and one TD, and found that neuron density is highly variable in layer VI in WS in contrast to TD. No such observation was made for layers II/III in WS, suggesting that neuron density in the infragranular layers may be more affected (Galaburda and Bellugi 2000).

Our research group curates the largest WS postmortem brain collection in existence, the Bellugi Williams Syndrome Brain Collection. With this unique collection, we can now explore the biological underpinnings of WS at the cellular and molecular levels (Chailangkarn et al., In Press; Horton et al. 2013, 2016). Here, we used stereological morphometric techniques to examine neuron density in layers II/III (supragranular) and V/VI (infragranular) in two prefrontal cortical areas implicated in socio-emotional behavior, BA 10 and BA 11 (Shamay-Tsoory et al. 2009; Pochon et al. 2002; Burgess et al. 2007), and three unimodal areas in the neocortex previously examined in human post-mortem studies (motor cortex BA 4, somatosensory cortex BA 3, and visual area BA 18; Jacobs et al. 1997; Jacobs et al. 2001; Jacobs et al. 2003) in the postmortem brains of six adult WS subjects and six matched TD subjects. Given that the gross structure of the PFC and behaviors associated with the PFC appear to be affected in WS, we predicted that neuron density in the PFC will be altered in WS compared to TD. Furthermore, we predicted that this difference in neuron density would

be driven by a greater decrease in neuron density in the infragranular (V/VI) layers compared to the supragranular (II/III) layers of the WS cortex compared to TD.

Methods

Brain Tissue

We examined cortical tissue from six WS subjects and six TD subjects. We targeted five cortical areas: prefrontal cortical area BA 10 (frontal pole), prefrontal area BA 11, primary motor (BA 4), primary somatosensory (BA 3), and visual area BA 18. The tissue analyzed included one hemisphere from each subject. TD subjects were matched with WS subjects for age, sex, and hemisphere, to control for cytoarchitectonic asymmetries and possible age and sex related differences (Zilles et al. 1997; Mohlberg et al. 1999; Amunts et al. 2000; Cykowski et al. 2008; Scheperjans et al. 2008; Renteria et al. 2012).

All subjects in the Bellugi Williams Syndrome Brain Collection are part of an ongoing donation-based program run by the Laboratory for Cognitive Neuroscience at the Salk Institute for Biological Studies and the Laboratory for Human Comparative Neuroanatomy at UCSD (La Jolla). See Table 3.1 for subject background information.

Regions of Interest

Our regions of interest were chosen because abnormalities of the PFC (which includes BA10 and BA 11) are linked to sociobehavioral and cognitive alterations that characterize ASD and WS (Carper et al. 2002; Carper and Courchesne 2000; Courchesne et al. 2003; Courchesne et al. 2011; Casanova et al. 2002a,b; Casanova et al. 2006; Jones et al. 2001; Morris et al. 2010a,b; Meyer-Lindenberg et al. 2005; Langdon et al. 2007), while the three unimodal regions of the cortex examined here (BA 4, BA3, and BA18) are not associated with functional alterations in WS, and have been the target of several postmortem studies in

typically developing human controls (Jacobs et al. 1997; Jacobs et al. 2001; Jacobs et al. 2003). We do not have information on hand dominance for our subjects, and hemispheric asymmetries related to handedness have been noted in BA 4 (Amunts et al. 2000) so it is possible that the inclusion of both hemispheres would yield different results. However, while each individual is only represented by one hemisphere, our sample represents both hemispheres collectively, as four matched pairs are right hemispheres and two matched pairs are left hemispheres.

The regions of interest were identified using strict anatomical landmarks, and cortical areas were identified and confirmed at the microscopic level using detailed and distinctive cytoarchitectonic characteristics (Fig. 3.1). A brief description of these characteristics are as follows: BA 10 occupies the frontal pole of the brain (sampled here from the tip of the pole), and all six cortical layers are easily distinguished; BA 11 occupies the rostral orbital surface of the PFC, and can be distinguished from the limbic cortices by the distinct presence of the granular layer IV, and from BA 10 by a comparatively thinner layer IV; BA 4 occupies the area of the dorsal precentral gyrus anterior to the central sulcus (sampled here from the motor hand region) in the posterior frontal cortex, and can be identified by its lack of layer IV and presence of giant Betz cells in layer V; BA 3 occupies the postcentral gyrus in the parietal cortex (sampled here from the post-central gyral region directly adjacent to the motor hand region), and can be identified by a very wide layer IV, lack of Betz cells in layer V, and presence of large, Betz-like cells in layer III; BA 18 occupies regions of the cuneus, lingual gyrus, and middle occipital gyrus in the occipital cortex, and can be identified by its high cell density, with a particularly dense layer IV, and large pyramidal cells in deep layer III and layer V (Amunts et al. 1995, 2000; Braak 1979; Geyer et al. 1995; Hof et al. 1995; Ongur et

al. 2003; Rivara et al. 2003; Semendeferi et al. 2001; Von Economo 1927; White et al. 1997).

Processing of tissue

Tissue was fixed in a solution of 10% buffered formalin and stored for a range of a few months to several years. Extracted blocks containing the brain areas of interest were immersed in a sucrose solution with 0.1M phosphate buffer until saturated to cryoprotect tissue for freezing. Frozen tissue was cut on a Leica SM 2010R sliding microtome into two series of alternating section thicknesses- 60 microns for thionin staining and 30 microns for a separate project.

The processed tissue was stained with thionin in order to visualize cell bodies. Mounted sections were dried in an oven and then dehydrated in a 1:1 chloroform ethanol solution overnight. These slides were stained with 0.25% thionin, re-hydrated, submerged in xylenes for 20 minutes after staining, and then cover-slipped with permount.

Unbiased, design-based stereology

Data collection was performed using Stereoinvestigator software (MBF Bioscience, Williston, VT) on a Dell workstation receiving live video feed from an Optronics MicroFire color video camera (East Muskogee, OK) attached to a Nikon Eclipse 80i microscope equipped with a Ludl MAC5000 stage (Hawthorn, NY) and a Heidenhain z-axis encoder (Plymouth, MN).

Given inter-subject variability of cortical area boundaries and the difficulty of mapping cytoarchitectonic boundaries of the cortex to gross topological location (Amunts et al. 1999, 2000; Eickhoff et al. 2007), we carefully examined and sampled tissue well within each area's cytoarchitectonic boundaries and thus report neuron density rather than neuron number, a standard approach that increases accuracy and consistency of measurements across

subjects in the cortex (Benes et al. 1986; Oblak et al. 2011; Smiley et al. 2011; Underwood et al. 2012). All data was collected by a single investigator (CHL) after establishing inter-rater reliability in neuron density estimations on a sample previously reported in the literature (Barger et al. 2012) to 95% concordance. Sections were coded prior to data collection to blind the rater. Neuron numbers in layers II/III and V/VI of Nissl-stained sections were estimated using the Stereoinvestigator optical fractionator probe in combination with fractionator sampling. We excluded layer IV from analysis, as this layer is not present in BA 4 and is difficult to distinguish with Nissl stain in some cortical areas. Two rectangular regions of interest per section, one bounding layers II/III and the other bounding layers V/VI, were drawn at a 1x magnification. Sections were analyzed using a 100x oil objective, with a grid size of 300x300 microns, a dissector height of 15 microns, and a counting frame of 50x50 microns. Section thicknesses were measured at each counting site. Neurons were counted by placing a mark on each neuron nucleolus visible (and not touching the line of exclusion) in the given counting frame. 12 sections per subject in each of the five cortical areas were analyzed in this way. Volumes of the regions of interest were estimated using the Stereoinvestigator Cavalieri probe. Neuron densities were calculated by dividing the neuron population estimation by the volume estimation of each region of interest separately (layers II/III and layers V/VI, respectively). Neuron densities of "all layers" (i.e. layers examined here, excluding layer IV; henceforth referred to only as "all layers") were calculated using the mean of layers II/III and V/VI in each cortical area.

Statistical Analysis

Standard two-tailed T-tests ($P<0.05$) were calculated by comparing WS and TD in each region of interest in each cortical area for supragranular neuron density, infragranular

neuron density, and neuron density of all layers combined. Percent change of TD neuron density compared to WS was calculated by dividing the mean of WS neuron density by the mean of TD neuron density in each region of interest in each cortical area. All data was run through a Grubbs' outlier test (GraphPad Outlier calculator) in order to determine whether any single value was a statistically significant outlier from the respective group (WS or TD).

Results

General findings

Some qualitative differences between WS and TD were noted. Layer I, which is mostly acellular in TD, contained visibly more cells in WS. Pyramidal neurons in WS appeared rounder compared to TD. Lastly, cortical layers were less distinct in WS compared to TD.

WS and TD means for neuron density in each cortical area are listed in Table 3.2. We found that the mean neuron density of all five cortical areas combined was similar between TD and WS ($p = 0.903$). There was more variation in neuron density between subjects in WS compared to TD in all cortical areas except BA 4 (See Table 3.2 and Figure 3.2). However, a Grubbs' test revealed that no single subject was a statistically significant outlier in any cortical area within a relative group (WS or TD).

Prefrontal cortical areas

Both PFC areas demonstrated a pattern of lower neuron density in WS compared to TD, and this decrease was largest in layers V/VI (see Table 3.3 and Fig. 3.3).

BA 10

Mean neuron density of all layers combined in BA 10 was significantly lower in WS compared to TD (P=0.033; Fig. 3.2A, 3.3). Neuron density in layers II/III was not significantly different between the two groups, (P=0.093; a 10% decrease in density in WS compared to TD), but neuron density in layers V/VI was significantly lower in WS (P= 0.038; an 18% decrease in density compared to TD, Table 3.3, Fig. 3.2A). Compared to their age-matched pairs, four WS subjects demonstrated a large decrease in neuron density compared to TD in the infragranular layers and in all layers combined, and only one of these individuals demonstrated a large decrease in neuron density in the supragranular layers. Two of the six cases were similar to TD, with modestly lower infragranular density (Fig.3.4A).

BA 11

Mean neuron density of all layers combined in BA 11 was lower in WS than in TD (Fig. 3.2B, 3.3), but the difference did not reach statistical significance (P=0.105). Mean neuron density was lower in both layers II/III and V/VI in WS compared to TD. The difference in neuron density between the two groups was not statistically significant in either set of layers (P= 0.183 and P= 0.207 in layers II/III and layers V/VI, respectively), although the percent decrease in mean neuron density in WS compared to TD was larger in the infragranular layers compared to the supragranular layers (Table 3.3). Compared to their age-matched pairs, five out of six WS individuals demonstrated lower density than TD in layers V/VI and all layers, and out of these individuals, only two demonstrated lower density in layers II/III compared to TD. One WS individual demonstrated neuron densities similar to TD (Fig 3.4B).

Other Cortical Areas

BA 4 demonstrated the greatest similarity between WS and TD, such that the WS motor cortex was the least affected cortical area in the five cortical regions investigated. Both BA 3 and BA 18 demonstrated a pattern of higher neuron density in WS, a reversal of what was observed in PFC, although statistical significance was not reached in either area.

BA 4

Mean neuron density of all layers combined in BA 4 was very similar between the two groups (Figure 3.2C, 3.3) and did not reach statistical significance (P=0.945).

Mean neuron density in BA 4 in layers II/III and V/VI was also very similar between the two groups. The difference in mean neuron density between the two groups was not significant in either layer (P=0.78; P=0.945, supragranular and infragranular, respectively), and the percent difference in mean neuron density in WS compared to TD was minimal (Table 3.3). As expected, all individual WS and TD subjects had lower density in layers V/VI compared to layers II/III (Fig. 3.4C).

BA 3

Mean neuron density of all layers combined in BA 3 was increased in WS compared to TD (Fig. 3.2D, 3.3). The difference between the two groups was not statistically significant (P=0.249). Within WS-TD matched pairs, four out of six WS subjects demonstrated higher neuron densities compared to TD, and two WS subjects demonstrated similar density (WS 9) and lower density (WS 10) (Fig. 3.4D).

Mean neuron density of BA 3 in WS layers II/III and layers V/VI was increased compared to TD (Fig. 3.2D, 3.3). The difference in neuron density between the two groups was not significant in either set of cortical layers (P=0.186; P=0.355, layers II/III and layers V/VI, respectively). The percent difference in average neuron density in WS compared to TD

was large in both cortical layer regions (Table 3.3), but this effect was partially driven by the very high neuron density of one subject, WS 8 (Fig. 3.4D; see individual variation section below).

BA 18

Mean neuron density of all layers combined in BA 18 was increased in WS compared to TD (Fig. 3.2E, 3.3). The difference between the two groups in mean total density was approaching significance (P=0.089) and between matched pairs, five out of six WS subjects had higher density than TD (Fig. 3.4E). The youngest WS subject, WS 10, was the only WS individual demonstrating a lower density than its TD matched pair.

All subjects had higher neuron density in layers II/III compared to layers V/VI, as expected. Mean neuron density in BA 18 in both the supragranular and infragranular layers was increased in WS compared to TD (Fig. 3.2E, 3.3). The difference in neuron density between the two groups was not significant in either set of cortical layers (P=0.188; P=0.0925, layers II/III and layers V/VI, respectively). The percent difference in mean neuron density in WS compared to TD was large in both cortical layers (Table 3.3).

Mean density patterns across the cortical areas

In both WS and TD, mean neuron density of all layers combined was highest in BA 18, lowest in BA 4 and BA 11, and intermediate in BA 10 and BA 3 (BA 4/BA 11>BA 3/BA10> BA 18; Fig. 3.3). Within this pattern, there was some variation between the WS and TD groups. In the PFC (BA 10 and BA 11), there was more variation in neuron density between individuals in the WS population compared to the TD population. While BA 18 demonstrated the highest neuron density of the cortical areas examined across both groups, neuron density in WS BA 18 was markedly higher compared to TD. Neuron density in BA 4

tended to be lower than all other cortical areas in TD, while in WS, neuron density in both BA 4 and BA 11 tended to be lower than the other cortical areas. These patterns were present in some of the individual TD and WS subjects, but there is individual variability as expected (Fig. 3.4; see below).

Patterns of differences in density between cortical layers

In both WS and TD, mean neuron densities in all cortical areas demonstrated a pattern of lower density in the infragranular layers (II/III) compared to the supragranular layers (V/VI) (Fig. 3.2 and 3.3; although one WS individual demonstrated a reversal of this pattern in BA 11; see below and Fig. 3.4B). Variability in neuron density was higher in WS compared to TD in general, with the infragranular layers demonstrating greater variability than the supragranular layers in all brain areas except BA 3 (see Table 3.3 standard deviations).

Individual variation in neuron density

A Grubbs' outlier test demonstrated that no single individual was a significant outlier from the respective population (WS or TD) in any cortical region. While no individual deviations from the population mean were statistically significant, individual variation in neuron density that deviates from the population pattern was present in TD, and to a much greater extent in WS (Fig. 3.4A-E). Only one subject, TD2, stood out in the typically developing group. This individual demonstrated the lowest neuron density in BA 3 (in all other TD subjects BA 4 demonstrated the lowest density). In contrast, four subjects in the WS group demonstrated deviations from the pattern of WS population mean density:

WS 10: WS 10 has an overall cortical density that is very low compared to the other subjects in this study (both WS and TD). WS 10 was the only WS subject in which neuron density was lower than its TD matched pair in BA 3 (Fig. 3.4D). WS 10 demonstrated slightly lower

neuron density to its TD matched pair in BA 18, while all other WS subjects demonstrated greater neuron density than their matched pairs in BA 18 (Fig. 3.4E). WS 10 was the youngest WS subject included in this study (17.5 years).

WS 9: WS 9 demonstrated differences from the population pattern of neuron density in BA 11 and BA 3. In BA 11, WS 9 stood out with respect to the following: a) It is the only WS individual in which total neuron density in BA 11 is higher compared to its TD matched pair- in all other matched pairs, total neuron density in TD was higher; b) neuron density in the infragranular layers was higher than the supragranular layers (a reversal of the pattern expected and seen all other individuals for BA 11) which seems to also drive the overall neuron density, and c) neuron density in the infragranular layers was higher in this individual compared to all other WS individuals and also all TD individuals (Fig. 3.4B). By excluding WS 9 BA 11 from the sample, we found that the difference between WS and TD in total neuron density and neuron density in layers V/VI reached statistical significance (P=0.023 and P=0.006, respectively), but the difference in neuron density in layers II/III did not (P=0.262). In BA 3, WS 9 demonstrated the lowest neuron density in all cortical areas examined (Fig. 3.4A-E), a deviation from the WS population pattern, in which BA 4 and BA 11 demonstrate the lowest neuron density (Fig. 3.3). Furthermore, mean neuron density in BA 3 tended to be higher in WS and TD, but neuron density in WS 9 BA 3 was similar to its TD matched pair (Fig. 3.4D). Excluding WS 9 BA 3 from analysis did not reveal any statistically significant differences in WS-TD comparisons. WS 9 is the only female in the WS group.

WS 6: Neuron density in BA 11 was higher than in BA 10 in WS 6 (Fig. 3.4A,B). In both WS and TD population means, total neuron density in BA 10 was higher than BA 11 (Fig. 3.3).

WS 8: Neuron density in BA 3 was much higher in WS 8 than in any other subject examined, and was the only individual in which BA 3 layers II/III demonstrated greater neuron density than BA 18 layers II/III (Fig. 3.4D,E). Excluding WS 8 BA 3 from the sample, the difference between WS and TD is still not statistically significant in either layer region (P=0.259 and P=0.491, infragranular and supragranular, respectively). However, while the WS-TD percent difference in mean neuron density is still high in the supragranular layers, excluding WS 8 results in minimal WS-TD percent difference in the infragranular layers (+15.0% increase in supragranular density in WS compared to TD; +8% increase in infragranular density in WS compared to TD).

Discussion

Summary

This study is the largest post-mortem analysis of the WS cortex, and the first stereological analysis in WS to examine the prefrontal cortex, which is implicated in the atypical socio-behavioral phenotype of the disorder. In addition, our study also utilizes modern stereological techniques to contribute TD neuron density measurements to the existing body of cortical literature (Benes et al. 1986; Braendgaard et al. 1990; Ketzler et al. 1990; Pakkenberg et al. 1993; Rajkowska et al. 1995; Selemon et al. 1995; Semendeferi 1994; Sholl 1959; Underwood et al. 2012). Our findings demonstrate variation in neuron density across cortical areas and between the cortical layers that is consistent with the expected mean population pattern based on these previous reports.

Here, we found that neuron density in the PFC was decreased in WS, with greatest decrease in the infragranular layers, and demonstrated the greatest difference in neuron density compared to TD of all the areas examined, while neuron density in BA 4 was similar

between WS and TD. Neuron density in BA 3 and BA 18 demonstrated increased density in WS, an opposing direction of change compared to changes in the WS PFC. While the difference in BA 11, BA 3 and BA 18 was not statistically significant here, this could be an effect of small sample size. Given that differences in WS and TD were approaching significance in two areas, BA 11 and BA 18, we performed a retrospective power analysis (JavaStat) and determined that the minimum sample size needed to increase the likelihood of reaching statistically significant differences in these regions is n=9. Unfortunately, we are limited to the current sample size due to the profound scarcity of WS tissue. Overall, these findings demonstrate that different functional areas of the cortex are differentially affected by the WS genetic deletion, with prefrontal areas, which are involved in socio-behavioral processing, appearing more affected than unimodal areas.

Defining the cytoarchitecture of neural substrates that underlies typical and atypical social behavior is an important component of ASD research, as socio-behavioral alterations are a core feature of ASD. WS also demonstrates discrete differences in the socio-behavioral domain, but unlike ASD, WS has a consistent and well-defined genotype. Examinations of the relationship between genotype, the brain, and behavior in WS may shed light on the more complex mechanisms involved in the atypical behavioral phenotype that is a core feature of ASD.

Previous observations of atypical neuroanatomy and cytoarchitecture in WS

Neuroimaging studies have found an overall reduction of the cerebrum in WS, with a preservation of cerebral grey matter volume and a significant reduction of cerebral white matter volume (Martens et al. 2008). Statistically significant reductions in the volume of the occipital cortex were also found (Reiss et al. 2004). In contrast, the orbitofrontal cortex

(OFC) and medial prefrontal cortex (MPFC), regions of the PFC that are implicated in emotional and social processing (Reiss et al. 2004) demonstrate increased volume in WS, indicating that structural abnormalities of the PFC may be linked to the behavioral phenotype of hypersociality and empathy that is characteristic of WS. Several functional neuroimaging studies have demonstrated abnormal patterns of neural activity in the WS PFC, particularly in the OFC, during exposure to social stimuli (Langdon et al. 2007; Meyer-Lindenberg et al. 2005; Porter et al. 2007), and it has been suggested that hypersocial drive in WS may be linked to poor response inhibition (Porter et al. 2007) an important idea already under investigation (Hanson et al. 2014; Hanson et al. 2015). The present finding of decreased neural density only in the two PFC/OFC areas examined suggests that the cortical abnormalities previously identified through imaging studies are indicative of area-specific cytoarchitectonic differences in WS.

Few postmortem observations of the WS cortex have been made in previous studies. A qualitative case study of a single postmortem WS brain found that overall size of the frontal lobe appeared in the normal range for typically developing subjects, but the parietal, occipital, and posterior temporal lobes were reduced in size (Galaburda et al. 1994). A later quantitative study examining two additional WS subjects found that WS BA17 (primary visual cortex) consistently had a higher density of small neurons in the granular and infragranular layers than TD in the left hemisphere only (Galaburda et al. 2002). Hollinger and colleagues (2005) examined the primary auditory cortex (BA 41) in the same subjects, and found that while WS lacked the cytoarchitectonic asymmetries seen in TD, there were no significant differences in cell density overall in WS compared to TD. These findings, in contrast to area 17, correlate with the clinical observations that auditory skills remain intact in WS, while visuo-spatial

skills are altered (Hollinger et. al. 2005). Similarly, the present findings of increased neuron density in visual area BA 18 in WS may also be related to visuospatial alterations, while the preserved cytoarchitecture of BA 4 reflects the more intact motor function in the disorder. The potential functional correlation to the increase in density in BA 3 is less clear, as no differences linked to the somatosensory cortex have been observed in WS (Pober 2010). However, gross postmortem anatomical observations made by Galaburda and colleagues (1994; reduced volume of the parietal and occipital lobe but not the frontal lobe in WS) may be linked to the current findings.

Abnormalities of the PFC are also a common feature of ASD

Similar to WS, neuroimaging and postmortem neuroanatomical studies have demonstrated that the PFC is structurally altered in ASD. MRI studies have demonstrated increased brain volume during infancy in ASD compared to TD, with the PFC demonstrating the most significant increase (Carper et al. 2002; Carper and Courchesne 2000; Courchesne et al. 2003). A developmental neuroimaging study found that the early period of rapid brain growth in ASD infants is followed by reduced growth compared to TD, and by age 8 brain volumes between ASD and TD children are mostly indistinguishable (Carper et al. 2002). There is also a slight trend between severity of ASD stereotypic behaviors and enlargement of frontal lobe white matter in early childhood. These results suggest that although the genetic and behavioral profile of ASD is highly variable, there appears to be a typical neurodevelopmental trajectory in ASD, with the frontal lobe most severely affected (Carper et al 2002). This corresponds well to the behavioral data, which has found that cognitive abilities related to the frontal lobe, such as social function, language and attention are altered, while

other abilities, such as motor function and visuospatial perception, are unimpaired (Ozonoff et al. 1991; Haas et al. 1996).

Atypical microstructure has also been observed in postmortem studies of the ASD cortex, with prefrontal cortical areas most significantly affected. The ASD PFC presents an overabundance of neurons compared to TD (Courchesne et al. 2011), as well as a decrease in neuropil space, reduced density and width of minicolumns, and overall higher neuron density (Casanova et al. 2002a,b; Casanova et al. 2006). Despite these differences, cortical thicknesses in ASD are similar to TD (Hutsler et al. 2010).

Possible mechanisms underlying atypical neuron density in WS and future directions

Here, we demonstrated a decrease in neuron density in the WS PFC that appears localized to the infragranular layers. Given that the formation of infragranular and supragranular layers is regulated by different genetic programs during development (Casanova and Trippe 2006), disruptions that alter developmental timing and/or target genetic programs underlying the infragranular layers may allow for the infragranular and supragranular layers to be differentially affected in Williams Syndrome. One possible mechanism may involve genetic alterations that result in atypical development and dysfunction of the serotonergic system. Serotonin is a neurotransmitter that is important in embryogenesis and postnatal neural development as well as mood and cognition (Sodhi and Sanders-Bush 2004). An animal study demonstrated that variants of different serotonin transporter knockouts resulted in differential thickness and cell density in the infragranular and supragranular layers (Altamera et al. 2007). Alterations in the serotonin system are implicated in a number of neurodevelopmental disorders, including ASD and schizophrenia, and there is some evidence of abnormal serotonergic metabolism in the WS PFC (Proulx et al.

2010). Furthermore, mice with genetic knockouts of GTF2IRD1, a gene within the WS hemizygous deletion, have demonstrated increased serotonin levels as well as reduced fear and aggression (Young et al. 2008). Other possible mechanisms underlying alterations of neuron density in WS may involve disturbances in molecular and genetic mechanisms during development that direct proliferation, cell cycle regulation, and apoptosis (Courchesne et al., 2011, Chailangkarn et al., In Press).

Additional studies are underway to explore atypical neuronal morphology that may be related to neuron density, such as dendritic branching (Hrvoj et al. 2013a; Hrvoj et al. 2013b ; Hrvoj-Mihic et al. 2014). Less complex dendritic branching (Hayes and Lewis 1993; Diamond et al. 1967; Jacobs et al. 1997) has been linked to smaller soma size and in ASD, such reductions have been linked to decreases in functional integrity (Jascot-Descombes et al. 2012). Interestingly, animal models of genetic knockouts in LIMK1, a gene within the WS hemizygous deletion, demonstrate abnormalities in dendritic spine morphology and function (Meng et al. 2002).

Conclusions

The findings of this study suggest that the disparate cortical abnormalities, some previously identified through imaging studies, such as in the WS PFC (increase in cortical volume), are indicative of area-specific cytoarchitectonic changes in the disorder, and may be related to separate disturbances during development. Given that neuron density in the PFC appears to be significantly altered in both WS and ASD, future hypotheses in WS should aim to examine possible mechanisms of altered neuron density in neural systems including the PFC.

The mechanisms underlying differences in neuron density are likely disorder-specific given the vastly different genetic etiologies and phenotypic characteristics associated with each. Furthermore, it is likely that the developmental trajectory of the two disorders is very different- some evidence suggests neuronal changes in ASD are not consistent across lifespan, such that there may be neuronal overgrowth during development, followed by decline/degeneration in adulthood (Courchesne et al. 2001; Carper et al. 2002; Schumann and Amaral 2006; Courchesne et al. 2011). The current study includes only adult subjects, so developmental comparisons between WS and ASD cannot yet be made. However, WS and ASD both demonstrate alterations in social behavior and cognition, as well as abnormal prefrontal cortical microstructure. A reoccurring challenge in ASD research is that the genetic mechanisms underlying ASD are complex and highly variable. WS has a clear genetic etiology that provides a unique model for identifying how a known genotype affects neural substrates that underlie atypical social behavior. WS studies have the potential to uncover the link between genes, the brain, and social behavior, and may lead to future research that identifies targets for novel therapeutics in ASD and WS.

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Chapter 3, in full, is an adaptation of a primary data paper previously published in the peer-reviewed scientific journal *Autism Research*. The dissertation author was the primary investigator and author of this paper. Citation: Lew CH, Brown C, Bellugi U, Semendeferi K. 2016. Neuron density is decreased in the prefrontal cortex in Williams Syndrome. *Autism Research* 10(1): 99-112.

TABLES AND FIGURES

Table 3.1: Subject Background Information. *BA 3 was unavailable for this specimen; TD 6 BA 3 (same age, sex, and hemisphere as TD 5) is used for analysis in results
**BA 10 was unavailable for this specimen; TD 5 BA 10 (same age, sex, and hemisphere as TD 6) is used for analysis in results
University of Maryland Brain and Tissue Repository codes for TD subjects:
TD1= 4916; TD2= C1114; TD3= C5552; TD4= 4598; TD5= A0248; TD6= 1590

Subjects	Age at Death	Sex	Diagnosis	Hemisphere	PMI (hours)	Cause of death
WS 10	18y	M	WMS	R	24	Cardiac complications
TD 1	19y	M	TD	R	5	Drowning
WS 1	31y	M	WMS	R	26	Cardiac complications
TD 2	31y	M	TD	R	15	Accident
WS 9	43y	F	WMS	R	12	Cardiac complications
TD 3	42y	F	TD	R	18	Heart disease
WS 12	45y	M	WMS	R	24	Cardiac complications
TD 4	45y	M	TD	R	6	Dilated myocardiopathy
WS 6	47y	M	WMS	L	<30	Cardiac complications
TD 5*	51y	M	TD	L	18	Accident
WS 8	48y	M	WMS	L	<30	Respiratory illness
TD 6**	51y	M	TD	L	30	Cardiac complications

Table 3.2: Mean neuron density (neurons/mm^3) and standard deviation in the five cortical areas. "All layers" refers to II/III and V/VI combined.

Cortical Area	BA 10			BA 11			BA 4			BA 3			BA 18		
Cortical layers	Layers II/III	Layers V/VI	All Layers	Layers II/III	Layers V/VI	All Layers	Layers II/III	Layers V/VI	All Layers	Layers II/III	Layers V/VI	All Layers	Layers II/III	Layers V/VI	All Layers
WS	34336±4198	28468±4963	31402±3926	31347±3759	24893±6149	28120±3712	31228±4137	24422±5052	27825±4875	43815±10506	31792±6698	37803±8365	55269±6099	45450±7624	50359±6667
TD	38373±1827	34650±1496	36512±1542	34323±2669	29109±2895	31716±2431	30443±4527	25578±3202	28001±3840	36136±4736	28365±3202	32260±3909	49680±6008	38289±2521	43984±2439

Table 3.3: Percent change in neuron density in WS compared to TD ([(WS/TD)-1]x100)

Cortical Area	BA 10	BA 11	BA 4	BA 3	BA 18
% Change Total Density	-14%	-11%	-1%	+17%	+15%
% Change II/III Density	-10%	-9%	+2%	+21%	+11%
% Change V/VI Density	-18%	-15%	-4%	+12%	+19%

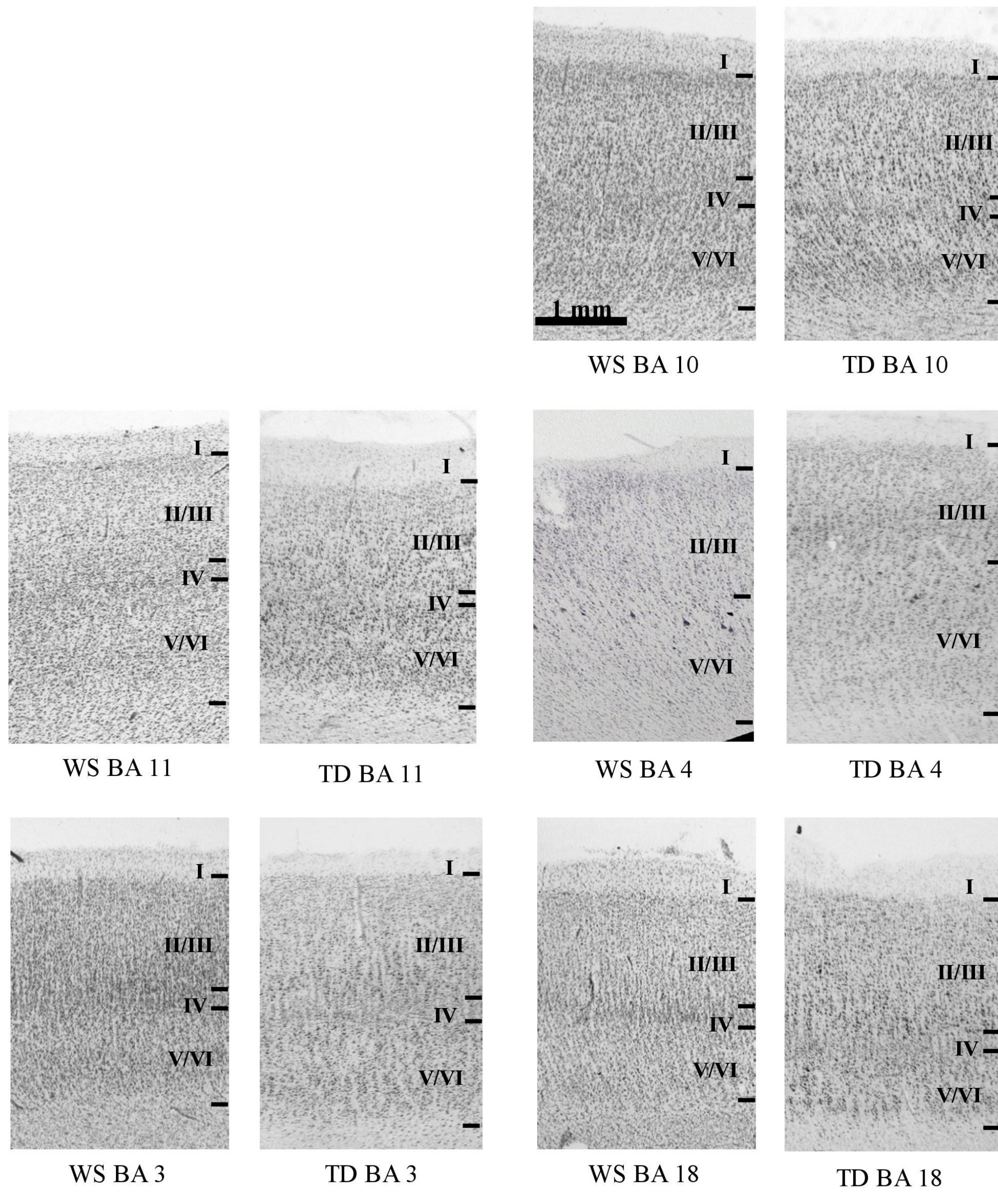

Figure 3.1: Microphotograph of each area examined in WS and TD. Photographed at 1x magnification.

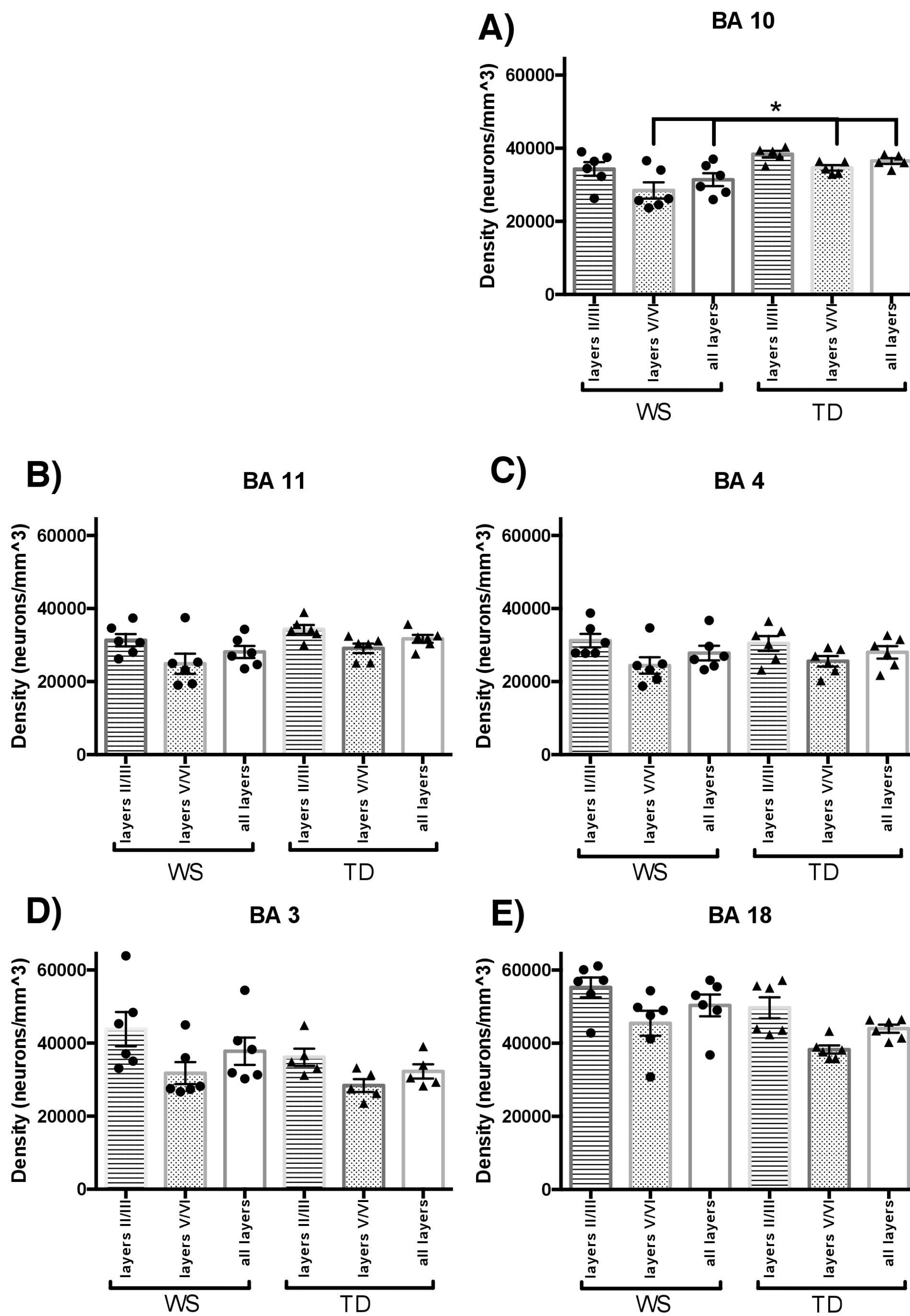

Figure 3.2 A-E: Mean neuron density in layers II/III, V/VI, and “all layers” (layers II/III and V/VI combined) in WS and TD in each cortical area, with individuals plotted.

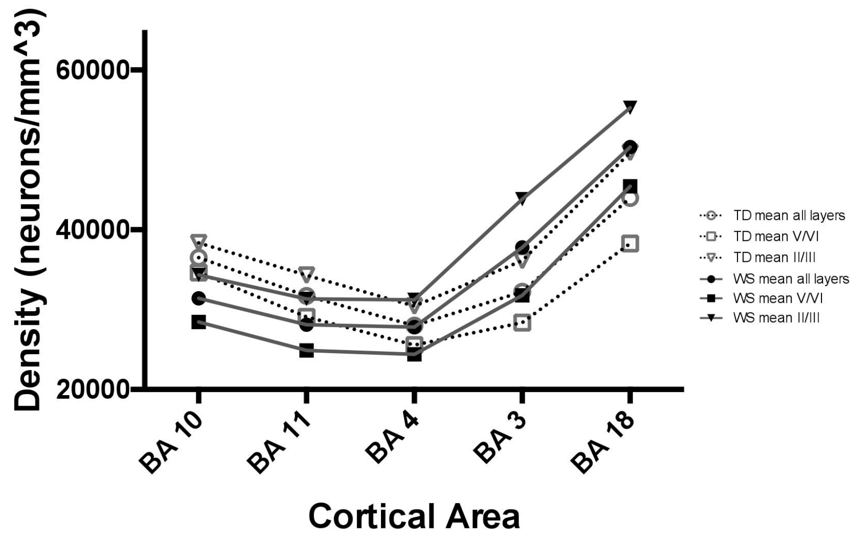

Figure 3.3: Line graph of neuron density means in WS and TD in each cortical area in layers II/III, V/VI, and “all layers” (layers II/III and V/VI combined).

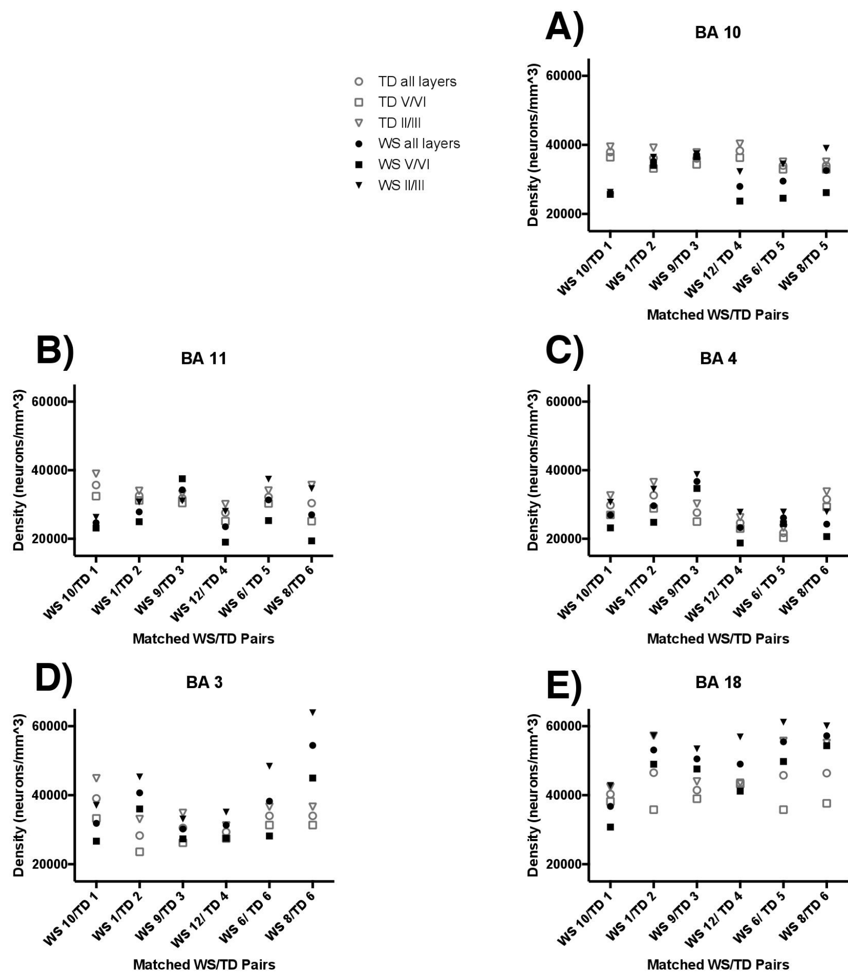

Figure 3.4A-E: Individual variation in neuron density patterns across cortical areas.

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CHAPTER 4:

A postmortem stereological study of the amygdala in Williams syndrome

ABSTRACT

Perturbations to the amygdala have been observed in neurological disorders characterized by abnormalities in social behavior, such as autism and schizophrenia. Here we quantitatively examined the amygdala in the postmortem human brains of male and female individuals diagnosed with Williams Syndrome (WS), a neurodevelopmental disorder caused by a well-defined deletion of ~26 genes, and accompanied by a consistent behavioral profile that includes profound hypersociability. Using unbiased stereological sampling, we estimated nucleus volume, number of neurons, neuron density, and neuron soma area in four major amygdaloid nuclei- the lateral nucleus, basal nucleus, accessory basal nucleus, and central nucleus- in a sample of five adult and two infant WS brains and seven age-, sex- and hemisphere matched typically developing control (TD) brains. Boundaries of the four nuclei examined were drawn on Nissl-stained coronal sections as four separate regions of interest for data collection. We found that the lateral nucleus contains significantly more neurons in WS compared to TD. WS and TD do not demonstrate significant differences in neuron number in the basal, accessory basal, or central nuclei, and there are no significant differences between WS and TD in nuclei volume, neuron density, and neuron soma area in any of the four nuclei. A similarly designed study reported a decrease in lateral nucleus neuron number in autism, mirroring the opposing extremes of the two disorders in the social domain. These results suggest that the number of neurons in the lateral nucleus may contribute to pathological disturbances in amygdala function and sociobehavioral phenotype.

Introduction

Williams Syndrome (WS) is a rare neurodevelopmental disorder (1 in 7,500 to 1 in 12,000 births; Stromme et al. 2002) caused by a hemizygous microdeletion of ~26 consecutive genes in chromosome band 7q11.23. WS is characterized by a consistent behavioral phenotype broadly described as a hyperaffiliative social drive, marked by an atypically strong desire for social engagement, exaggerated gregariousness, a lack of inhibition in approaching and interacting with unfamiliar conspecifics, and impaired social perceptual ability (Jarvinen-Pasley et al. 2010). While WS is rare, it has garnered broad interest in behavioral neurobiology- not only does the syndrome present a unique opportunity to study the relationship between genotype, neural structure, and social behavior, but WS also demonstrates strong links to the much more prevalent autism spectrum disorders (ASD). WS and ASD display behavioral phenotypes that include hypersociability and social avoidance, respectively, representing opposing extremes in the social domain (Jarvenin, Korenberg, and Bellugi 2012). Intriguingly, some ASD cases include a duplication of the WS deletion (Merla et al. 2010), and some WS cases with atypical deletions demonstrate behavioral phenotypes more fitting to an ASD diagnosis (Edelmann et al. 2007; Sakuri et al. 2011).

The amygdala has been critically implicated in social behavior and cognition (Adolphs et al., 1994, 1999; Adolphs 2001; Meunier et al. 1999; Emery et al. 2001; Bauman et al. 2006). Neuroimaging and postmortem histological studies have found significant alterations to the amygdala in ASD, including an increase in amygdala volume in childhood that is no longer detectable in adolescence, and reduced neuron numbers in the lateral nucleus of the amygdala across age groups (Schumann et al. 2004; Schumann and Amaral 2006; Mosconi et al. 2009). Neuroimaging studies in WS have demonstrated enlargement of the amygdala in

adolescence and adulthood (Reiss et al. 2004; Martens et al. 2009; Haas et al. 2014; but see Meyer-Lindenberg et al. 2004, Meda et al. 2012, which found no change), and reduced activation of the amygdala in response to negative social stimuli (Haas et al. 2009, 2010). However, little is known about WS at the histological level. A single case report on a postmortem adult WS brain noted that the lateral nucleus of the amygdala appeared smaller than expected relative to the other nuclei (Galaburda and Bellugi 2000). A stereological analysis of the cortex in six adult WS postmortem brains and six matched controls found a significant decrease in neuron density in the infragranular layers of the orbitofrontal cortex (OFC) in WS (Lew et al. 2017), and another study examining dendritic branching in the same cortical areas in two WS brains found that dendritic branching may be reduced in the WS OFC (Hrvoj-Mihic et al., 2017). The OFC demonstrates significant connections to subcortical structures including the amygdala (Barbas 1995), so such findings could indicate atypical downstream input.

Utilizing a sample of seven postmortem WS brains, comprising two early postnatal infants and five adults, and seven typically developing matched controls (TD), we measured volume, neuron number, neuron density, and neuron soma area of four major subdivisions of the amygdala: the lateral nucleus, basal nucleus, and accessory basal nucleus, most often implicated in the cognitive/sociobehavioral functions of the amygdala (Saddoris et al. 2005; Murray 2007; Seymour and Dolan 2008; Janak and Tye 2015; but see Goossens et al. 2009 for fMRI data demonstrating evidence of superficial amygdaloid nuclei involvement in human social behavior), and the central nucleus, involved in autonomic functions of the amygdala (Gallagher and Holland 1994). This is the first study to quantitatively examine the WS amygdala utilizing unbiased stereological methods, and to our knowledge, the first

postmortem stereological study to examine the human amygdala in early postnatal infants. Given findings from previous studies in ASD and WS, we expected to observe differences in neuron number in the lateral nucleus of the amygdala in WS, as well as differences in volume throughout the WS amygdala. While amygdala volume would be smaller in the infants than the adults, we expected the infant subjects to demonstrate neuron numbers similar to their respective adult diagnostic groups (WS or TD), given that most amygdala neurogenesis and migration is complete before birth (Chareyron et al. 2012; Kordower et al. 1992; but see Bernier et al. 2002 for evidence of some adult neurogenesis in the amygdala in nonhuman primates).

Materials and Methods

The materials of this study include amygdala tissue from seven WS subjects and seven TD subjects (Table 4.1). Only subjects free of seizures or other neurological disorders were used in this study. Fluorescence in-situ hybridization (FISH) probes for elastin, a gene consistently deleted in the WS hemideletion, were used to determine genetic diagnosis in the WS cases, and all WS subjects used in this study demonstrate the typical WS genetic deletion. TD and WS subjects were matched for age, sex, and hemisphere (right), with the exception of one pair, that was matched for age and sex only. Due to the rare occurrence of the disorder, our TD sample was matched to meet the availability of WS tissue, and comprises two early postnatal infant pairs and five adult pairs. While developmental differences between infant and adult cohorts will be present in the volume of the amygdala, neuron number should be similar in infants and adults, as neurogenesis of the amygdala is complete well before birth (Chareyron et al. 2012; Kordower et al. 1992). This study includes one hemisphere per subject (in all but a single case, right hemisphere), as both postmortem and neuroimaging studies

have found an absence of asymmetry in the human amygdala (Barger et al. 2007; Brierley et al. 2002).

All WS tissue was obtained from the Ursula Bellugi Williams Syndrome Brain Collection, an ongoing donation-based program run by the Laboratory for Human Comparative Neuroanatomy at UCSD (La Jolla). TD brain tissue was obtained through the NIH NeuroBioBank.

Tissue Preparation

All brains were immersed in 10% buffered formalin after autopsy (see Table 4.1 for PMI), and remained in formalin until processed for experimentation, with fixation time ranging from several months to over 20 years. For each subject, a 4cm block containing the entire rostro-caudal extent of the amygdala was removed from a single hemisphere. This large block was then bisected into 2-3 smaller blocks along the coronal plane in order to fit on the microtome stage (Schumann and Amaral 2005). These smaller blocks were cryoprotected until saturated in a gradient of sucrose and 0.1M phosphate buffer solutions (10%, 20%, 30%) in preparation for cutting. Tissue was frozen with dry ice and cut along the coronal plane on a Leica SM sliding microtome. Infant tissue was cut in five series of 80 micrometer (μm) sections, and adult tissue was cut in three series of 80 μm sections and four series of 40 μm sections, so that each series represented the entire rostro-caudal extent of the amygdala, and the distance between consecutive sections in each series was 400 μm. The number of sections per series ranged from 23-26 sections in adult subjects and 20-21 sections in infant subjects. One 80 μm series per individual was mounted on gelatin-coated glass slides and stained for Nissl substance with 0.25% thionin for analysis in this study, and additional series were stored in the freezer for future experiments.

Region of interest

We examined four amygdaloid nuclei: the lateral nucleus, basal nucleus, and accessory basal nucleus, which together make up the basolateral amygdala, and the central nucleus. We chose these nuclei for three primary reasons: 1) tracer studies in nonhuman primates have found that the basolateral nuclei are the primary site of amygdalar connectivity with higher-order cortical areas associated with social and emotional cognition (Aggleton et al. 1980; Leichnetz and Astruc 1976, 1977; Leichnetz et al. 1976; Carmichael and Price 1995; Stefanacci and Amaral 2000, 2002); 2) these nuclei demonstrate distinct anatomical boundaries in Nissl-stained tissue, and can therefore be identified with high accuracy, while boundaries of the individual superficial amygdaloid nuclei are less clear and thus less suitable for the purpose of the present study; 3) similarly designed postmortem human studies in typically developing brains and brains from individuals with ASD have examined the same nuclei (Schumann and Amaral 2005, 2006). Anatomical boundaries within the amygdala were defined cytoarchitectonically (Figure 4.1) and guidelines, briefly summarized below, were adapted from previous studies (Sorvari et al. 1995; Schumann and Amaral 2005 and Barger et al. 2012):

Lateral nucleus: The lateral boundary of the lateral nucleus is the border of the external capsule, while white matter forms the dorsal and ventral borders. The lateral medullary lamina marks the medial border of the lateral nucleus (and lateral border of the basal nucleus). In addition, the presence of smaller, more compact cells distinguish the lateral nucleus from the adjacent basal nucleus. In caudal sections, the larger cells in the dorsal region of the lateral nucleus distinguish it from the putamen.

Basal nucleus: The medial border is defined by the intermediate medullary lamina, which separates the basal nucleus from the accessory basal nucleus. The dorsomedial region of the basal nucleus can be distinguished from the accessory basal nucleus by the presence of large cells, which are absent in the accessory basal nucleus. The paralaminar nucleus, a narrow, densely packed band of small, darkly stained cells, forms the ventral border of the basal nucleus throughout most of the amygdala. The paralaminar nucleus is difficult to distinguish from the basal nucleus, and is therefore included here as part of the basal nucleus.

Accessory basal nucleus: In addition to being defined laterally by the intermediate medullary lamina, the accessory basal nucleus can be further identified by the absence of the large cells that are present in the adjacent basal nucleus. The medial medullary lamina forms the medial border, and separates the accessory basal nucleus from the superficial cortical nuclei. The ventral region of the accessory basal nucleus can be distinguished from the basal nucleus by smaller cell size.

Central nucleus: the central nucleus is only visible in more caudal sections, and lies dorsal to the basolateral nuclei. The central nucleus is distinguished from the rest of the amygdala by fiber tracts. A fiber tract also divides the central nucleus into two subdivisions: a lateral region, which has small, densely packed neurons that stain lightly, and a medial region that is stained more darkly and has more loosely packed neurons. The basal nucleus is ventrolateral to the central nucleus, and the accessory basal nucleus is ventromedial to the central nucleus. The dorsolateral border of the central nucleus forms part of the outer border of the amygdala.

Unbiased, design-based stereology

Data was collected by a single investigator (CHL) after establishing inter-rater reliability in neuron count estimations in the amygdala of a TD sample previously reported in

the literature (Schumann and Amaral 2005) to 95% concordance. Sections were coded prior to data collection to blind the investigator. Data collection was conducted using the Cavalieri, Optical Fractionator, and Nucleator probes in Stereoinvestigator software (MBF BioScience, Williston, VT) on a Dell workstation receiving live video feed from a Lumenera color video camera (Ottawa, Ontario) attached to an Eclipse 80i microscope equipped with a Ludl MAC5000 stage (Hawthorn, NY) and a Heiden z-axis encoder (Plymouth, MN). The entire rostro-caudal extent of the basolateral nuclei and central nucleus was defined on every 80 μm-thick section in which the nuclei were present in the series (see Table 4.2 for average number of sections per nucleus in infant and adult subjects) and the distance between each section was 0.4 mm. Cytoarchitectonic boundaries were drawn at 1x magnification. The Cavalieri method was used to determine the post-processing volume of each nucleus. The Cavalieri method (Garcia-Finata et al. 2003) operates by randomly overlaying a lattice of points (0.15 mm distance between each point) over each region of interest on each section, and counting the number of points within each region to calculate area. Volume is then calculated by multiplying the area by the average section thickness (measured at each counting site). Due to absolute volume differences of the nuclei (e.g. central nucleus is smallest, lateral nucleus is largest), different grid sizes were used for each nucleus (Schumann and Amaral 2006), as well as for the infant and adult subjects (Table 4.2).

Utilizing the same boundaries drawn for volumetric measurements, neuron number in each amygdaloid nucleus was estimated using the Optical Fractionator probe in combination with fractionator sampling. This quantitative method counts neurons using optical disectors (a three-dimensional probe) in a reference space, and is independent of volume measurements so that estimation of numbers of neurons is unaffected by tissue shrinkage (Gunderson and

Jensen 1987; West and Gunderson 1990). Tissue was viewed at high magnification (100x, 1.4 numerical aperture, oil lens). Similar to the set-up of the Cavalieri probe, different grid sizes were used for each nucleus and age cohort to account for differences in absolute volume. A disector height of 10 μm and a counting frame of 60 μm x 60 μm was used, and mounted section thickness was measured at each counting site. Neurons were counted by placing a marker on each neuron within the counting frame and not touching the red line of exclusion, for which the nucleolus was visible (Figure 4.2). We excluded cells with a diameter smaller than 5 μm, given the difficulty in distinguishing glia from some very small neurons in the amygdala. Neuron density was calculated by dividing neuron number by nucleus volume in each amygdaloid nucleus of each subject.

Neuron soma area for each amygdaloid nucleus was determined by using the Nucleator probe during neuron counts. For every tenth neuron counted with optical fractionator, a grid of four radially extending lines, centered on the nucleolus, overlaid the cell body, and the point of intersection with the edge of the neuron body was marked on each line. Neuron soma area is defined as the cross-sectional area of the cell body. We opted to report soma area rather than volume given that three-dimensional measures of soma volume and cell shape are not optimal in the stereological literature (Schumann and Amaral 2006; Gittins and Harrison 2011) due to the fixed orientation of the tissue sections.

Statistical analyses

Statistical analyses were performed using Graphpad Prism statistical software (v7b; La Jolla, CA). Two-tailed student's t-tests ($P<0.05$) were used to determine differences in volume, neuron number, neuron density, and neuron soma area in WS and TD. Correlation tests were run to identify any effects of age or sex. Given the age range of our sample, in the

case of a result reaching or approaching significance, an analysis of covariance was performed to determine whether the finding was still significant with age as a covariate. Additionally, t-tests were calculated with the infant subjects excluded from the sample to determine that including or excluding the infants did not affect the significance of findings. All data was run through a Grubbs test ($P<0.05$) to identify any outliers.

Results

Findings from comparisons between WS and TD groups in each nucleus (Figure 4.4) are discussed below. The Grubbs test revealed no significant outliers in our sample. Mean age at death was similar between WS and TD (Table 4.3). TD neuron number values found here were consistent with other TD neuron number values reported in the literature (Schumann and Amaral 2005, 2006). No effects of sex were observed in any of the parameters examined. Similar statistical significance findings were observed in all nuclei on all measures examined when omitting the infants from the sample, with the exception of neuron density in the lateral nucleus, which was approaching significance when calculated in the adult sample only (Table 4.4). There was no correlation between subject age at death and number of neurons in the lateral nucleus, accessory basal nucleus, or central nucleus in either WS or TD.

Lateral Nucleus

There was a significant difference in neuron number in the lateral nucleus, with WS demonstrating significantly more neurons compared to TD (P-val= 0.0012; Table 4.4; Figure 4.3). This difference remained significant even when age was included as a covariate (P-val= 0.0017). Lateral nucleus volume and neuron density were slightly greater in WS compared to TD (Table 4.3), although this difference was not significant. Neuron soma area in the lateral nucleus was similar between the two groups (Table 4.3, 4.4).

Basal Nucleus

All four measures in the basal nucleus- volume, neuron number, neuron density, and neuron soma area -were slightly greater in WS compared to TD (Table 4.3; Figure 4.3), although this difference was not significant (Table 4.4). In both WS and TD, there was a slight correlation between subject age and number of neurons in the basal nucleus ($r= 0.6276$; $p= 0.0163$).

Accessory Basal Nucleus

Accessory basal nucleus volume was slightly smaller in WS compared to TD, while neuron number and neuron density were slightly higher in WS compared to TD (Table 4.3; Figure 4.3). None of these differences reached significance (Table 4.4). Neuron soma area was similar between the two groups (Table 4.3, 4.4).

Central Nucleus

Central nucleus volume, neuron number, and neuron density were similar in WS and TD (Table 4.3, 4.4; Figure 4.3). Neuron soma area was slightly larger in WS compared to TD, although this difference was not significant (Table 4.3, 4.4).

Discussion

Our main finding was that the number of neurons is significantly increased in the lateral nucleus of the amygdala in WS compared to TD. No other measure demonstrated significant differences between the two groups. While the infant subjects were at the lower end of the range of values in some nuclei, the infant pairs followed adult trends of WS/TD differences, and excluding infants from the sample did not change the significance of the results. Furthermore, the increase in neuron number in the lateral nucleus in WS was still significant when age was included as a covariate. These findings demonstrate that the infant subjects are similar to the adult counterparts of their respective diagnostic group, and that

processes leading to atypical development of the amygdala in WS likely occur during fetal development. Interestingly, we found a slight correlation between age and neuron number in the basal nucleus that was present in both WS and TD, with infant subjects containing fewer neurons. Our boundaries of the basal nucleus include the paralaminar region, which contains a large pool of neurons that are immature at birth (Chareyron et al. 2012). Cells with a diameter smaller than 5μm were not counted during data collection (see Methods), so it is likely that small, immature neurons were present but excluded from analysis in the infant basal nucleus.

While not significant, some trends differentiated the two groups. Comparing group means, the WS lateral nucleus was larger and had a greater density of neurons compared to TD. The WS basal nucleus was smaller, with greater neuron number and density of neurons compared to TD. The WS accessory basal nucleus was smaller and had a greater neuron density compared to TD, although neuron number was similar between the two groups. In contrast, the WS and TD group means for measures in the central nucleus were highly similar, suggesting that the central nucleus is relatively unaffected in WS.

Postmortem human studies, and particularly those examining rare neurological disorders, are limited by small sample sizes and this study is no exception. The group differences in the basal and accessory basal nuclei could potentially reach significance with a larger sample size. Furthermore, developmental differences may become apparent with a more complete age spectrum- one neuroimaging study employing multiple age cohorts in ASD found evidence of atypical brain growth in two different developmental phases in infancy (Courchesne et al. 2003), and another found evidence of atypical amygdala growth in childhood that was not detectable in adolescence or adulthood in ASD (Schumann et al 2004). Future postmortem studies are needed to examine neuronal and glial subtype populations, as

well as neuronal dendritic branching, in order to further identify specific microstructural characteristics disrupted in individuals with an altered social phenotype.

A similarly designed study examined the amygdala in nine postmortem ASD and ten TD brains in a developmental sample (ages 10 years to 44 years) and found that neuron number and density are significantly decreased in the lateral nucleus in ASD, while all other measures examined are similar between the two groups (Schumann and Amaral 2006). Postmortem studies of the amygdala in schizophrenia found that the lateral nucleus demonstrates significant microstructural changes in that disorder as well, including a reduction in volume and a decrease in neuron number (Kreczmanski et al. 2007; Beretta et al. 2007). Neuroimaging studies have long demonstrated that alterations of the amygdala are a common occurrence in neurodevelopmental and neuropsychiatric disorders (Schumann, Bauman, and Amaral 2011), and it is possible that microstructural changes to the lateral nucleus underlie these findings.

The lateral nucleus is the primary recipient of multimodal sensory cortical input in the amygdala (Stefanacci and Amaral 2000, 2002). It is thought to be involved in the categorization of emotional saliency of sensory stimuli, as well as integration of this information with information about social context sent from orbitofrontal cortical projections in the basal and accessory basal nuclei (LeDoux et al. 1990; Barbas 1995; Stefanacci and Amaral 2002). Reduced activation of the amygdala in response to social stimuli has been observed in WS (Munoz et al. 2009), and increased activation of the amygdala in response to social stimuli has been observed in ASD (Aschwin et al. 2007). Behavioral studies suggest that both WS and ASD individuals struggle with determining saliency of conspecific stimuli in social interactions (Vivanti et al. 2017). Atypical function of the lateral nucleus as a result

of altered microstructural development may contribute to the associated cognitive and behavioral deficits observed in these disorders.

Our results yield two major questions that deserve examination in future studies: 1) What mechanisms underlie the increase in neuron number in the lateral nucleus of the amygdala in WS, and 2) What events in fetal development result in the individual amygdaloid nuclei to be differentially affected in WS? Regarding the first question, there are two possibilities that would result in an increase in neuron number in the lateral nucleus of the WS amygdala- either an atypical initial over-proliferation of neurons, or a later disruption of the programmed cell death critical to typical neural development, resulting in a greater number of neurons. Future postmortem studies examining neuronal markers for cell proliferation and apoptosis are critical, as no data currently exists that would enable us to support or reject either possibility. A study examining WS neural progenitor cells found higher rates of apoptosis in WS cortex compared to TD (Chailangkarn et al. 2016), which would suggest that an over-proliferation of neurons in the lateral nucleus is a more likely scenario.

The developmental processes in WS underlying the increase of neurons in the lateral nucleus, yet relative preservation of typical neuron number in the central nucleus, are also unclear. Our findings show that the early postnatal infant WS amygdala already demonstrates the same pathological changes to the lateral nucleus seen in the mature WS adult amygdala, indicating a prenatal origin. Distinct amygdaloid nuclei are first present in the human fetus at gestational week six (Muller and O'Rhahilly 2006), and are under differential timing in fetal development (Kordower, Piecinski, and Rakic 1992; Nikolic and Kostovic 1986; Muller and O'Rhahilly 2006). However, neurogenesis does not appear to respect neuroanatomical subdivisions in the amygdala, but rather occurs in a smooth medial to lateral gradient, such

that neurogenesis in the central nucleus begins earlier and is completed first, and neurogenesis in the lateral nucleus begins later and is completed last (Kordower, Piecinski, and Rakic 1992). Interestingly, the differences in mean neuron number in WS compared to TD reported here reflect this lateral to medial gradient: differences are greatest in the lateral nucleus, followed by the basal nucleus, then the accessory basal nucleus, and finally by the central nucleus, where values are most similar between the two groups. Therefore, it is possible that these differences are related to a dysfunction of developmental events along this gradient. A few possible genetic candidates in the WS deletion have been identified that may underlie our findings, such as transcription factor WBSCR14, which regulates tissue-specific gene expression controlling neurogenesis (de Luis, Valero, and Jurardo 2000; Meng et al. 1998), Gtf2i, involved in the regulation of several genes that are critical to embryonic neural development (Sakurai et al. 2011), FZD9, involved in timing of cell division and apoptosis (Chailangkarn et al. 2016), and PSD-95, demonstrated to play a role in differential cellular morphology in the basolateral nuclei, but not the central nucleus of the amygdala in PSD-95 mouse knockouts (Feyder et al. 2010).

This is the first postmortem study to provide quantitative evidence that the cellular microstructure of the amygdala is altered in WS. The current study follows our previous histological investigation of the cortex in WS, which found a decrease in neuron density in the infragranular layers of the OFC in WS (Lew et al. 2017), a region that shares significant connectivity with the basolateral amygdala (Barbas and Pandya 1989). Both studies found that the neuroanatomical regions affected in WS are associated with functional specificity in the socio-behavioral domain, while adjacent regions not implicated in social function remain relatively preserved, supporting the hypothesis of an impaired social brain network in WS.

The present finding that the lateral nucleus of the amygdala is specifically affected in WS is a feature shared with other neurological disorders that include alterations in social behavior, such as ASD and schizophrenia (Schumann, Bauman, and Amaral 2011; Beretta et al. 2007; Rubinow et al. 2016). It is of interest that the basolateral amygdala has undergone significant reorganization in human evolution, with changes in size and neuron number that indicate an increasing emphasis on the lateral nucleus in humans compared to nonhuman apes (Barger et al. 2007, 2012). Together, these findings may indicate a possible link between recent evolutionary change and susceptibility to dysfunction, and warrants further investigation.

This study is the first step towards defining the neural architecture of the amygdala in light of a disorder characterized by a distinct genetic and behavioral profile. Such studies can contribute to future research examining the cellular composition of the brain and its relationship to the genotype and behavior, an essential step in identifying targets of future therapeutics in disorders of social dysfunction.

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Chapter 4, in full, is an adaptation of a primary data paper currently in press to be published in the peer-reviewed scientific journal Brain Structure and Function. The author of this dissertation is the primary investigator and author of this paper. Citation: Lew CH, Groeniger KM, Bellugi U, Stefanacci L, Schumann CM, Semendeferi K (2017) A postmortem stereological study of the amygdala in Williams syndrome. Brain Struct Funct 0:0 Available at: http://link.springer.com/10.1007/s00429-017-1592-y.

TABLES AND FIGURES

Table 4.1: Subject background information.

Subject ID	Age at Death	Diagnosis	Gender	Hemisphere	Cause of Death	Postmortem Interval (hours)
WS 11	26 days	Williams syndrome	Male	Right	Unknown	34.5
TD 4353	34 days	Typically developing	Male	Right	SIDS	5
WS 7	114 days	Williams syndrome	Male	Right	Multiple organ failure	30
TD 5183	107 days	Typically developing	Male	Right	SIDS	13
WS 10	17 years	Williams syndrome	Male	Right	Cardiac complications	24
TD 4916	19 years	Typically developing	Male	Right	Drowning	5
WS 14	42 years	Williams syndrome	Female	Right	Cardiac complications	18
TD 5445	42 years	Typically developing	Female	Right	Pulmonary thromboembolism	10
WS 9	43 years	Williams syndrome	Female	Right	Cardiac complications	12
TD 5758	43 years	Typically developing	Female	Right	Sepsis	22
WS 4	46 years	Williams syndrome	Female	Right	Breast cancer	28
TD 4640	47 years	Typically developing	Female	Right	Pneumonia	5
WS 8	48 years	Williams syndrome	Male	Left	Respiratory illness	30
TD 4598	45 years	Typically developing	Male	Right	Unknown non-neurological	6

Table 4.2: Stereology parameters.

Amygdaloid nucleus	Mean no. of sections	Dissector grid area (mm^2)
Infant		
Lateral	20	4.00
Basal	21	2.25
Accessory Basal	20	1.00
Central	12	0.50
Adult		
Lateral	23	6.25
Basal	26	4.00
Accessory Basal	23	2.25
Central	15	1.00

Table 4.3: Mean stereological results in WS and TD. ** indicates statistically significant difference between WS and TD.

	WS, n= 7	TD, n= 7
Age (years)	28.13 ± 21.61	28.06 ± 21.21
Nucleus Volume (mm^3)		
Lateral nucleus	375.13 ± 128.30	362.00 ± 102.90
Basal nucleus	241.90 ± 93.71	266.30 ± 90.08
Accessory basal nucleus	102.00 ± 32.20	109.50 ± 30.96
Central nucleus	24.83 ± 8.32	25.12 ± 7.37
Neuron Number (x 10^6)		
Lateral nucleus**	5.16 ± 0.31	4.39 ± 0.37
Basal nucleus	3.79 ± 0.43	3.56 ± 0.42
Accessory basal nucleus	1.44 ± 0.26	1.45 ± 0.23
Central nucleus	0.36 ± 0.03	0.35 ± 0.04
Neuron density (neurons/cm^3)		
Lateral nucleus	15.53 ± 6.38	13.144 ± 4.23
Basal nucleus	17.63 ± 6.15	14.88 ± 5.33
Accessory basal nucleus	15.24 ± 4.60	14.20 ± 4.26
Central nucleus	16.23 ± 7.08	15.42 ± 6.06
Neuron Soma Area (μm^2)		
Lateral nucleus	214.00 ± 48.50	205.00 ± 61.40
Basal nucleus	223.30 ± 36.55	211.20 ± 34.86
Accessory basal nucleus	223.40 ± 52.58	226.70 ± 61.35
Central nucleus	183.10 ± 41.40	158.40 ± 47.50

Table 4.4: Statistical results of student's T-test comparing WS and TD. The first value listed represents the P-value of WS versus TD, all subjects included; the italicized value listed represents the P-value of WS versus TD adult subjects only, infant subjects excluded. Statistical significance indicated by **.

	Lateral nucleus	Basal nucleus	Accessory basal nucleus	Central nucleus
Nucleus Volume	0.8367 (*0.4911)*	0.6280 (*0.4026)*	0.6653 (*0.4833)*	0.9461 (0.9385)
Neuron number	0.0012**(*0.0012***)	0.3372 (*0.2018*)	0.8248 (*0.6707)*	0.9475 (*0.8102)*
Neuron density	0.4254 (*0.0952)*	0.3875 (*0.2066)*	0.6707 (*0.5636)*	0.8102 (*0.9814)*
Neuron soma area	0.7831 (*0.9994)*	0.5715 (*0.6022)*	0.9223 (*0.7716)*	0.3600 (*0.4851)*

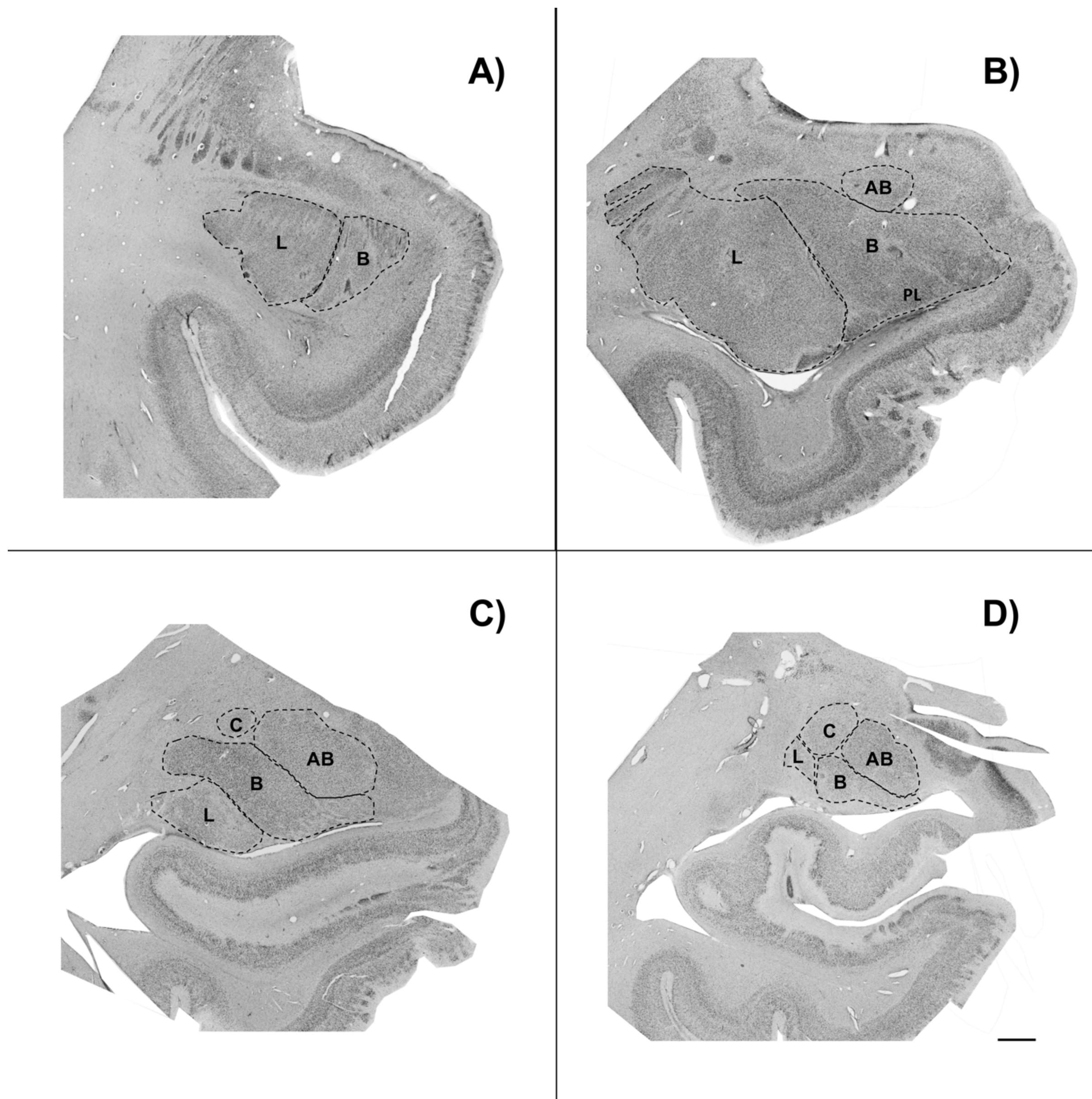

Figure 4.1: Brightfield photomicrograph of Nissl-stained coronal sections through rostral A), midrostro-caudal B) and C), and caudal D) levels of the amygdala from a typically developing subject, demonstrating nuclei boundaries used in data collection. L= lateral nucleus of the amygdala; B= basal nucleus of the amygdala; AB= accessory basal nucleus of the amygdala; C= central nucleus of the amygdala. Scale bar= 2 mm.

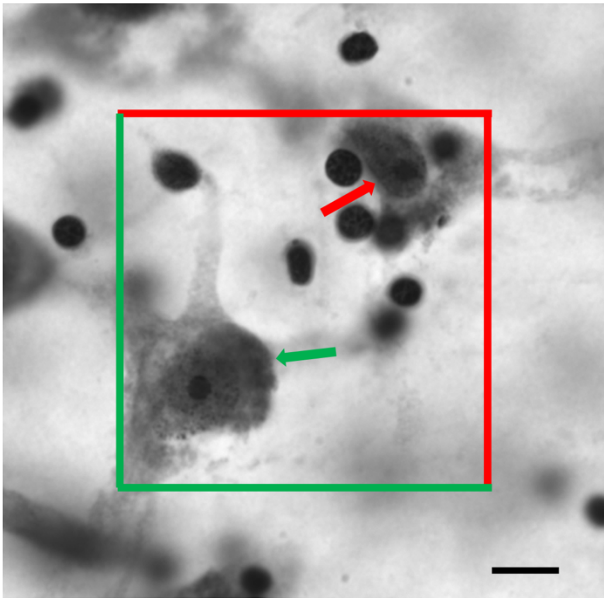

Figure 4.2: Photomicrograph of a Nissl-stained section illustrating the optical fractionator probe in the lateral nucleus of the amygdala at 100x from an individual with Williams syndrome. Neurons within the counting frame and not touching the red line of exclusion are counted (green arrow), while neurons outside the counting frame or touching the line of exclusion (red arrow) are not counted. Scale bar= 10 μm.

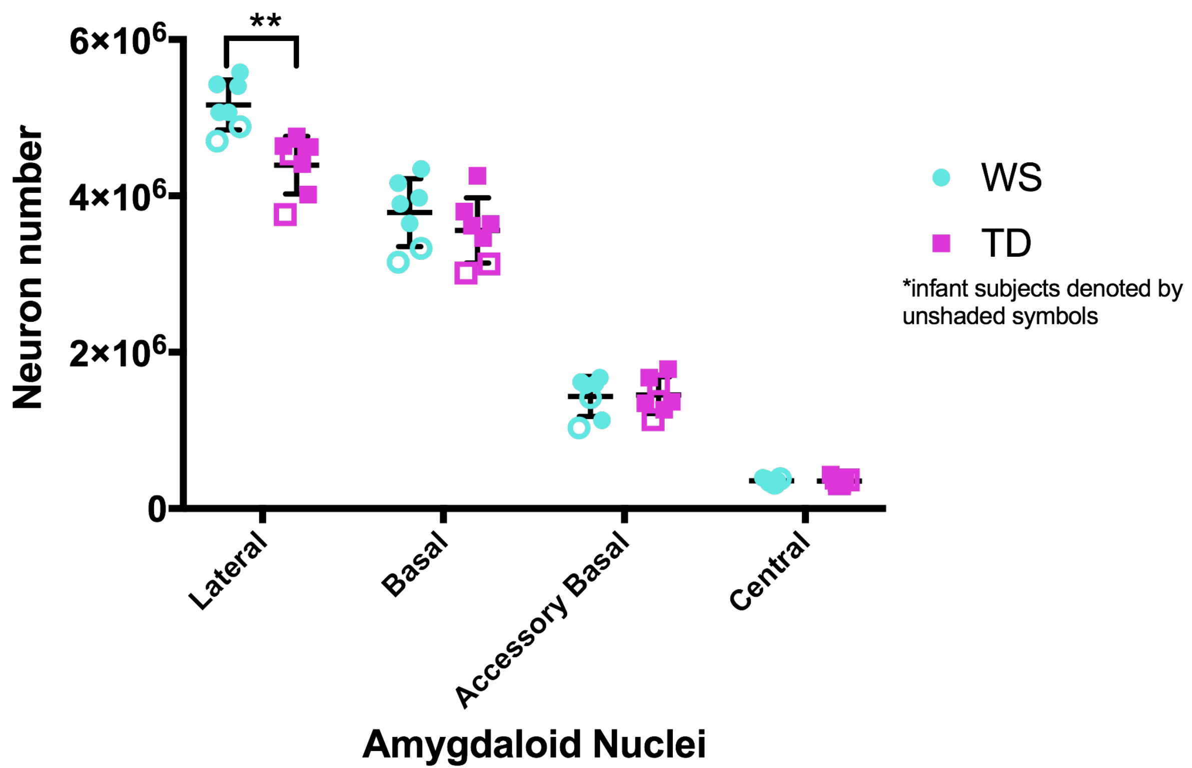

Figure 4.3: Number of neurons in the lateral, basal, accessory basal, and central nuclei of the amygdala in Williams syndrome (blue circle) and typically developing control (purple square) brains. Infant subjects are denoted by unshaded symbols. ** indicate significant difference (p-val= 0.0012) between Williams syndrome and TD controls in neuron number of the lateral nucleus.

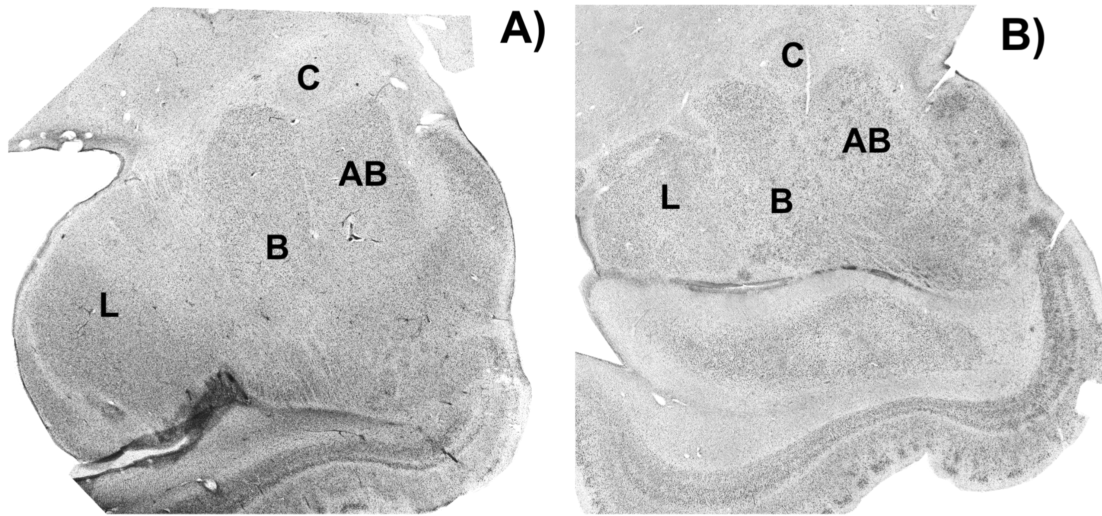

Figure 4.4: Brightfield photomicrograph of Nissl-stained coronal sections of the amygdala from a Williams Syndrome subject A), and a typically developing subject B) with all four amygdaloid nuclei present. L= lateral nucleus of the amygdala; B= basal nucleus of the amygdala; AB= accessory basal nucleus of the amygdala; C= central nucleus of the amygdala.

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CHAPTER 5:

Serotonergic innervation of the amygdala in Williams syndrome

ABSTRACT

Neuroimaging studies have demonstrated that the amygdala is functionally impaired in Williams syndrome (WS), and a recent postmortem study identified cellular disturbances in the basolateral nuclei of the WS amygdala, which are implicated in sociocognitive neural circuitry. The neurotransmitter serotonin has a prominent role in neurodevelopment, and within the mature brain is involved in the modulation of social behavior and emotion. The amygdala is heavily innervated by serotonergic axons, and it has been hypothesized that WS may be in part a disorder of serotonin disruption. Utilizing immunohisochemical staining techniques and unbiased stereological methods, we quantified serotonin-transporter-containing (SERT) axon density in the lateral, basal, accessory basal, and central nucleus of the amygdala in WS and TD postmortem brains. We found that SERT axon density was lower in the amygdala of most WS subjects compared to TD, with SERT axon density in the basolateral nuclei demonstrating the greatest difference between the two groups. These findings provide preliminary evidence for disrupted serotonergic innervation of the amygdala in WS, and particularly of the basolateral nuclei, which are critically seated in social brain circuitry and therefore may contribute to hypersocial behavioral phenotype characteristic of the disorder. Furthermore, these findings contribute to a growing body of literature demonstrating that the basolateral nuclei of the amygdala, which have undergone significant reorganization in human evolution, are targeted in neurodevelopmental disorders such as

Williams syndrome, schizophrenia, and autism, supporting the hypothesis that neural regions that have undergone recent evolutionary change are more susceptible to dysfunction.

Introduction

Williams syndrome (WS) is a rare neurodevelopmental disorder caused by a hemizygous deletion on chromosome band 7q11.23, and associated with a distinct socio-affective phenotype that includes an atypically strong drive for social engagement, an uninhibited propensity to approach and engage with strangers, impaired social perception, decreased social anxiety and increased general anxiety (Jarvenin-Pasley et al 2010). The amygdala, a subcortical structure composed of 13 structurally and functionally distinct nuclei, is critically implicated in socio-emotional behavior, and is involved in both the cognitive and autonomic processing and modulation of fear and anxiety (Adolphs et al., 1994,1999; Adolphs 2001; Meunier et al. 1999; Emery et al. 2001; Bauman et al. 2006). Neuroimaging studies have demonstrated structural and functional abnormalities in the WS amygdala (Reiss et al. 2000; Haas et al. 2009; Meyer-Lindenberg et al. 2005), and a recent postmortem study by our laboratory (Lew et al., 2017b) found that neuron number and density of the basolateral (lateral, basal, and accessory basal) nuclei, thought to underlie socio-affective cognitive functions of the amygdala, are increased in WS, while the central nucleus, implicated in autonomic functions of fear/stress response, appears to be relatively unaffected.

Serotonin, a monoamine, is produced by serotonergic neurons in the raphe nuclei of the brain stem; while mostly limited to the raphe nuclei, these neurons have axonal projections that innervate almost every region of the brain (Hensler 2006). Serotonergic functions within the nervous system are diverse, and include roles in neural development,

several physiological mechanisms, such as those involved in cardiovascular/respiration function, sleep, stress, fear and sexual activity, and numerous behaviors critically involved in social and emotional cognition (Murphy 1998; Murphy et al. 2008; Vallender et al. 2009; Whitaker-Azmitia 2001). The serotonin system is most active early in development- in humans, serotonin-containing cell bodies are first present in the raphe nuclei during gestation week 5, rapidly increase until gestation week 10, and display adult organization by week 15 (Whitaker-Azmitia 2001). Serotonin arrives to target neural regions early in development, and is involved in neural maturation through the regulation of several processes including neurogenesis, neuronal differentiation, neuropil formation, axon myelination, and synaptogenesis (Chubakov et al. 1986, 1993; Lauder and Krebs 1978; Lauder 1990; Whitaker-Azmitia 2001). Human metabolic studies have shown that serotonin levels steadily increase postnatally until 2 years of age, then decline until 5 years of age, when they reach adult levels (Hedner et al. 1986; Toth and Fekete et al. 1986). In the mature brain, serotonin has a significant influence on the modulation of information flow within several structures, including the amygdala (Asan et al. 2013). Animal studies in both rodents and primates have shown that the amygdala is densely innervated by serotonergic fibers (Xu and Pandey 2000; Bauman and Amaral 2005; Stimpson et al. 2015), and the serotonin system serves as an important neurotransmitter/neuromodulator mediating several socio-affective behaviors, such as anxiety, behavioral impulsivity, emotion and regulation of anxiety and fear, and social stimuli response (Murphy 1998; Murphy et al. 2008; Vallender et al. 2009; Whitaker-Azmitia 2001; Sodhi and Sanders-Bush 2004; Lesch et al. 2012).

Disruptions of the chemoarchitecture of structures innervated by the serotonergic system may contribute to neurodevelopmental pathologies underlying atypical socio-affective

behaviors. While it is not possible to directly measure serotonin in postmortem human brains due to the rapid decay of the monoamine, methods that label serotonin transporter (SERT) can give insight into the chemoarchitecture and anatomy of the serotonin system. Postmortem studies utilizing these methods have demonstrated an increase in SERT binding sites in the prefrontal cortex in schizophrenia (Murguruza et al. 2013), and a decrease in SERT axons in the cortex of depressed suicide victims (Rajkowska et al. 2017; Austin et al. 2002). A series of qualitative studies reported an increase in serotonergic innervation and the presence of dystrophic SERT axons in the globus pallidus, temporal cortex, and amygdala in individuals with autism (Azmita et al. 2011a,b), a disorder associated with social avoidance behaviors. Autism is often compared to WS, given the opposing extremes of the two disorders in the socio-behavioral domain. While no study has yet directly examined how the serotonergic system might be altered in WS, the effective use of selective serotonin uptake inhibitors (SSRIs) in patients with WS (Martens et al. 2012), as well as genetic evidence of altered serotonergic metabolism WS (Young et al. 2008; Proulx et al. 2008), suggests serotonin may contribute to the WS phenotype. Here, we utilized immunohistochemical techniques to determine the density of SERT immunoreactive (SERT-ir) fibers in the lateral, basal, accessory basal, and central nuclei of the amygdala in WS and TD in order to test the hypothesis that serotonergic chemoarchitecture of the amygdala is disrupted in WS.

Materials and Methods

The materials of this study include amygdala tissue from four WS subjects and three TD subjects (Table 5.1). Only subjects free of seizures or other neurological disorders were used in this study. Fluorescence in-situ hybridization (FISH) probes for elastin, a gene consistently deleted in the WS hemideletion, were used to determine genetic diagnosis in the

WS cases, and all WS subjects used in this study demonstrate the typical WS genetic deletion. Our original sample size consisted of six TD and six WS subjects, matched for age, sex, and hemisphere. However, despite multiple attempts, immunohistochemical staining of two WS subjects and 3 TD subjects was unsuccessful. The remaining sample examined here includes three TD and three WS subjects were matched for age, sex, and hemisphere (right), as well as an additional unmatched WS subject. This study includes one hemisphere per subject, as both postmortem and neuroimaging studies have found an absence of asymmetry in the human amygdala (Barger et al. 2007; Brierley et al. 2002).

All WS tissue was obtained from the Ursula Bellugi Williams Syndrome Brain Collection, an ongoing donation-based program run by the Laboratory for Human Comparative Neuroanatomy at UCSD (La Jolla). TD brain tissue was obtained through the NIH NeuroBioBank.

Tissue Processing

All brains were immersed in 10% buffered formalin after autopsy (see Table 5.1 for PMI), and remained in formalin until processed for experimentation, with fixation time ranging from several months to over 20 years. For each subject, a 4cm block containing the entire rostro-caudal extent of the amygdala was removed from a single hemisphere. This large block was then bisected into 2-3 smaller blocks along the coronal plane in order to fit on the microtome stage (Schumann and Amaral 2005). These smaller blocks were cryoprotected until saturated in a gradient of sucrose and 0.1M phosphate buffer solutions (10%, 20%, 30%) in preparation for cutting. Tissue was frozen with dry ice and cut along the coronal plane on a Leica SM sliding microtome. Tissue was cut in three series of 80 μm sections and four series of 40 μm sections, so that each series represented the entire rostro-caudal extent of the

amygdala. One 80 μm series per individual was immediately mounted and stained for Nissl substance, and the remaining series were placed in cryoprotectant solution and stored in a -20 °C freezer until use.

A 1-in-20 series of 80 micron-thick sections per subject were selected from the frozen tissue to be stained with mouse monoclonal antibody against SERT (MAB5618, EMD Millipore, Billerica, MA). Antibody specificity has been demonstrated at 60-70 kDa (Ramsey and DeFelice 2002; Serafeim et al. 2002), and two previous studies to date (Raghanti et al. 2008; Stimpson et al. 2015) have demonstrated the selectivity of this antibody in formalin-fixed human and non-human primate neural tissue using a heat-based antigen retrieval immunohistochemical staining protocol. An avidin-biotin peroxidase method for heavily fixed tissue (Raghanti et al. 2008), was used, with slight modifications based on results of several pilot studies in our laboratory. Briefly: free-floating sections were removed from cryoprotectant and rinsed in PBS, then pretreated for antigen retrieval by incubation in a 0.05% citraconic acid solution and submersed in a water bath set at 95°C for one hour. Sections were again rinsed with PBS and incubated for 20 minutes in a 75% methanol, 2.5% hydrogen peroxide solution to quench endogenous peroxidases. Sections were rinsed and incubated for one hour in a PBS dilution buffer of 4% normal horse serum, 5% bovine serum albumin, and 0.6% Triton-X detergent, rinsed again, then incubated in the primary antibody (1:20,000 dilution with PBS) for three days on an orbital shaker (24 h at room temperature, 48 h at 4 °C). After removal from primary antibody, sections were rinsed and then incubated in biotinylated anti-mouse secondary antibody (1:200 dilution, BA-2000, Vector Laboratories, Burlingame, CA) with 4% normal horse serum and PBS for one hour. Sections were again rinsed and treated with an Avidin-Peroxidase Complex kit (PK-4000, Vector Laboratories). A

DAB Chromogen kit (SK-4100, Vector Laboratories) was used to visualize immunoreactive SERT-positive fibers, and then rinsed in PBS to halt the reaction. Before mounting, free-floating stained sections were chilled in the refrigerator for 24-48 hours to reduce the likelihood of tissue tears during handling.

Data Collection

Stereologic sampling to determine length density of SERT-immunoreactive fibers was conducted using the Spaceballs probe in the Stereoinvestigator software suite (MBF BioScience, Williston, VT) on a Dell workstation receiving live video feed from a Lumenera color video camera (Ottawa, Ontario) attached to an Eclipse 80i microscope equipped with a Ludl MAC5000 stage (Hawthorn, NY) and a Heiden z-axis encoder (Plymouth, MN). For each section examined, boundaries of the amygdaloid nuclei were first traced in Stereoinvestigator at 1x magnification. For each SERT-stained section, a photo of an adjacent section from the Nissl-stained series processed for use in a prior study (Lew et al. 2017b) was utilized as a visual aid during tracing to ensure precision of boundaries (Fig 5.1 A,B). The cytoarchitectonic features used to identify these boundaries are described in detail in Lew et al. 2017. After boundaries were identified, Spaceballs analysis was performed using a 100x objective (1.4 numerical aperture, oil lens). Using systematic random sampling determined by a predefined grid size, Spaceballs employs a virtual isotropic hemisphere (radius of 7 μm and a 1 μm on the top and bottom of the section) that the investigator toggles through the section on the z-axis, marking the hemisphere each time a fiber crosses the probe's circumference (Fig. 5.2). Different grid sizes were used for each nucleus given the difference in nucleus size (1100μm x1100μm lateral nucleus, 1000μm x1000μm basal nucleus; 800μm x800μm accessory basal nucleus; 600μm x600μm central nucleus) so that an average of 100 sampling

sites per nucleus was achieved. Total axon length density was calculated by dividing the total axon length by the planimetric reference volume (Raghanti et al. 2008; Stimpson et al. 2015).

Statistical analyses were performed using Graphpad Prism statistical software (v7b; La Jolla, CA). Two-tailed student's t-tests ($P<0.05$) were used to determine differences in SERT-axon density in WS and TD. An analysis of covariance was performed to determine age and sex effects, and a Grubbs outlier test was used to identify any significant outliers.

Results

General Findings

Mean SERT-ir axon density was lower in WS compared to TD in all nuclei examined (Table 5.2, Fig. 5.3), but no significant differences in SERT-ir axon density were observed in WS and TD (Table 5.2). The difference in SERT-ir axon density between WS and TD was largest in the accessory basal nucleus, followed closely by the lateral nucleus, and the difference was smallest in the central nucleus (Table 5.2).

Patterns of innervation and individual variation in WS and TD

We found that in both WS and TD, the central nucleus contained the lowest density of axons and axon density in the basolateral nuclei were relatively homogenous within the respective diagnostic group (Fig. 5.3).

Variation in SERT-ir axon density between TD subjects was minimal across the basolateral nuclei, with the exception of one subject (5758), which demonstrated a higher density in the accessory basal nucleus compared to the other two TD in the data set (Fig. 5.5). Relative to TD, variation in SERT-ir axon density in the basolateral nuclei between WS subjects was much greater (Table 5.2, 5.3 Fig. 5.4,5.5). Compared to TD, two subjects, WS 10 and WS 9, demonstrated low SERT-ir axon density in all three basolateral nuclei, and one

subject, WS 8, demonstrated SERT-ir axon density in the basolateral nuclei that were within the low range of variation for TD (Fig. 5.4). In contrast, one subject, WS 14, demonstrated very high SERT-ir axon density in all three basolateral nuclei (Fig. 5.4,5.5). When WS 14 was excluded from the sample (Table 5.3), the difference in SERT-ir axon density between WS and TD was significant in the lateral (p-value= 0.031) and accessory basal (p-value= 0.014) nuclei, and approaching significance in the accessory basal nucleus (p-value= 0.068). WS 8 demonstrated SERT-ir axon density in the basolateral nuclei that were within the low range of variation for TD (Fig 5.4,5.5). Both WS and TD demonstrated minimal variation in SERT-ir axon density in the central nucleus (Fig 5.3,5.4,5.5). Despite the individual variation listed above, no significant outliers within the sample were detected by the Grubbs outlier test, and there was no correlation between SERT-ir axon density and age (p-value (WS)= 0.752; p-value (TD)= 0.872) or sex (p-value (WS)= 0.684; p-value (TD)= 0.516) in either diagnostic group.

Discussion

We tested the hypothesis that serotonergic innervation of the amygdala would be altered in WS compared to TD, given the importance of both the amygdala and the serotonin system in regulating socio-affective behavior. While we found no significant differences between the two groups, mean SERT axon density was lower in WS compared to TD, with the greatest difference in the accessory basal and lateral nuclei, while SERT axon density in the central nucleus was similar between the two groups. Additionally, there was considerably more individual variation in SERT axon density in the basolateral nuclei in WS compared to TD (both with WS 14 included and excluded, Table 5.2,5.3), while variance was minimal in the central nucleus for both WS and TD (Table 5.2,5.3). A previous study by our lab (Lew et

al., 2017b), which utilized a larger sample, including all of the brains in the current study, found that neuron number was increased in the lateral and basal nuclei of the amygdala in WS, including a significant increase in the lateral nucleus, and neuron density was increased in all three basolateral nuclei in WS. In contrast, neuron number and density in the central nucleus was similar in WS and TD. Together, these findings support the hypothesis that disturbance of the basolateral nuclei, yet relative preservation of the central nucleus, is a feature of atypical neurodevelopment in WS. The flow of information within the amygdala suggests that the basolateral nuclei and central nucleus are implicated in two highly overlapping, but somewhat distinct neural circuits: the cortico-amygdala circuit, which is implicated in socio-emotional cognitive processes, and the amygdala-brainstem circuit, which is implicated in autonomic response (Janak and Tye 2015). In the cortical-amygdala circuit, the lateral nucleus receives multimodal sensory input from sensory cortices, and is thought to be involved in the categorization of emotionally salient sensory stimuli. The lateral nucleus projects this information to the basal and accessory basal nuclei, which have strong bidirectional connectivity with the medial/orbitofrontal cortex, thus integrating socio-cognitive context. In contrast, the central nucleus receives no prefrontal projections, but instead receives information from all three basolateral nuclei, and is the major center of output from the amygdala to the brainstem (Janak and Tye 2015). We previously examined neuron density in the orbitofrontal, somatosensory, motor, and visual cortex in WS, and found that neuron density of the infragranular layers of the orbitofrontal cortex, which project to subcortical structures including the amygdala, are significantly decreased in WS (Lew et al. 2017a). Together, our findings of abnormal neuronal distribution in the prefrontal cortex and amygdala, and decreased serotonergic innervation of the amygdala, along with neuroimaging

studies, which have demonstrated atypical connectivity between the amygdala and orbitofrontal cortex in WS (Meyer-Lindenburg et al. 2005; Munoz et al. 2010), support the hypothesis that cortical-amygdala circuitry is under genetic control of the WS deletion, and may underlie the characteristic WS behavioral phenotype. Disruptions to amygdala-prefrontal connectivity have also been found in more prevalent neurological disorders such autism (Bachevalier et al. 2006; Mori et al. 2013; Zalla and Sperduti 2013) and schizophrenia (Williams et al. 2004). Future studies further examining the mechanisms of altered cortical-amygdala circuitry in WS may contribute to medical research for more common disorders with more complex genetic etiologies.

This is the first quantitative stereological study to examine serotonergic innervation of the amygdala in a neurodevelopmental disorder characterized by an altered socio-affective behavioral phenotype. A previous study examining serotonergic innervation throughout the whole brain in autism utilized immunohistochemistry paired with morphometric, non-stereological analysis (Azmitia et al. 2011a,b), and observed increased SERT axon density in multiple areas of the brain in autism, including the globus pallidus, amygdala, and temporal cortex. While Azmitia and colleagues did not examine SERT axon distribution within the individual amygdaloid nuclei, the finding of overall increased SERT axon density in the amygdala in autism is interesting in light of our findings of decreased SERT axon density in the WS amygdala. As discussed above, our lab previously found an increase in neuron number in the lateral nucleus of the amygdala in WS, while a similarly designed study (Schumann et al. 2006) found a decrease in neuron number in the lateral nucleus in autism. Given the role of serotonin in the regulation of several neurodevelopmental processes, including neurogenesis, neuronal differentiation, neuropil formation, axon myelination, and

synaptogenesis (Chubakov et al. 1986, 1993; Lauder and Krebs 1978; Lauder 1990; Whitaker-Azmitia 2001), perhaps the dichotomous amygdala pathologies observed in WS and autism in these two domains, neuron number and SERT axon density, could be related to the effect of different manifestations of serotonergic disruption to amygdala cellular development and seemingly opposing behavioral phenotypes (Haas and Reiss 2012).

Other lines of evidence suggest that the serotonergic system is affected in both disorders. One possible genetic link to serotonergic disruption of the amygdala is GTF2IRD1, a general transcription factor included in the WS deletion, linked to the characteristic WS behavioral phenotype (van Hagen et al. 2006), and also implicated as a common site of allelic variation in autism (Weiss et al. 2009). Genetically altered mice with a deletion of GTF2IRD1 demonstrate altered serotonergic metabolism in the amygdala and frontal cortex, as well as reduced fear and aggression compared to TD mice (Young et al. 2008; Proulx et al. 2010). Another possible mechanism could be related to genetic variation of serotonin transporter genes, which have been linked to cognitive and behavioral differences in primates (Jedema et al. 2010). A high concentration of serotonin in the blood, called hyperserotonemia, occurs in about one-third of autism cases (Campbell et al. 1974), while blood levels of serotonin are normal in most cases of WS (August and Realmuto 1989). Interestingly, however, two separate studies have reported on a total of four cases in which patients with the common WS genetic deletion display hyperserotonemia, along with social and communicative deficits diagnostic of autism rather than WS (Reiss et al. 1985; Tordjman et al. 2013). The researchers of the later study (Tordjman et al. 2013) also genotyped the serotonin transporter polymorphism (5-HTTLPR) for the two subjects they examined, and found both were homozygous for the short allele (5-HTTLPR *s*). Tordjman and colleagues suggest that that the

deviation from typical WS phenotype displayed by the two subjects in their study could be due to an interaction of WS genetic deletion with other genetic factors, such as the 5-HTTLPR polymorphism. The 5-HTTLPR polymorphism has been linked to socio-affective behavioral variation in humans and non-human primates (Lesch et al. 1997; Pezawas et al. 2005), and the 5-HTTLPR *s* allele is associated with heightened amygdala reactivity (Harrari et al. 2002; Bertolino et al. 2005) and stronger amygdala-prefrontal functional connectivity (Heinz et al. 2005) in healthy subjects. Furthermore, the 5-HTTLPR *s* allele is thought to be a genetic risk factor for neuropathologies associated with deficits in affect and social behaviors (Jedema et al. 2010; Murphy et al. 2013). It is possible that the 5-HTTLPR polymorphism could contribute to the present findings in the WS amygdala, as well as the WS behavioral phenotype. A future project aimed at genotyping the 5-HTTLPR polymorphism in the WS and TD subjects of this study is needed to shed light on the possible effects of this polymorphism to the WS phenotype.

Evolutionary studies examining serotonergic innervation of the amygdala in closely related species, while not directly comparable to human neuropathologies, could help shed light on how different patterns of serotonergic innervation might contribute to socio-affective behavior. A study by Stimpson and colleagues (2015) utilized similar methods to examine SERT axon density in the basolateral and central nuclei of the amygdala in chimpanzees and bonobos, two closely related species with distinct differences in social behavior. They found that SERT axon density was similar across all four nuclei in chimpanzees, while bonobos demonstrated higher SERT axon density in the basal and accessory basal nuclei compared to the lateral and central nuclei, as well as a statistically significant increase in SERT axon density in the basal and accessory basal nuclei compared to chimpanzees. The authors suggest

that the variation in serotonergic innervation between the two species results in differences in the modulation of amygdaloid output to cortical and autonomic projections, resulting in differential reactivity to social stimuli, and therefore different adaptive strategies to the social environment (Stimpson et al. 2015). The present study is the first quantitative stereological study to examine serotonergic innervation of the amygdala in humans. Previous studies have examined serotonergic distribution in the human amygdala utilizing autoradiographic methods, with conflicting results regarding serotonergic receptor and transporter density across the basolateral and central nuclei (Plenge et al. 1990; Duncan et al. 1992; Gurevich and Joyce 1996). This technique, which employs multiple radioactively-labeled ligands to visualize density of serotonin-uptake sites, has less selective binding than immunohistochemistry, and therefore offers significantly lower resolution and greater likelihood for inconsistencies (Beudet et al. 1998). Utilizing immunohistochemistry and stereological counting methods, we found relatively similar SERT axon densities throughout the basolateral nuclei, and comparatively low SERT axon density in the central nucleus. A future study directly comparing the present human data set with the chimpanzee and bonobo data set is needed to further examine how differences in serotonergic innervation within the amygdala might contribute to differences in social behavior.

The small sample size of this study is driven by the availability of tissue as well as the differential success of immunohistochemical staining, a process that does not always yield results in heavily fixed tissue, likely due to variables during autopsy and fixation that are beyond the control of the researcher. While the present data do not demonstrate any significant differences in serotonergic innervation in WS compared to TD, the group differences in SERT axon density in the basolateral nuclei could potentially reach significance

with the addition of a few more subjects. Given the frequent use of SSRIs in patients with WS, yet the relative lack of knowledge of the mechanisms involved (Martens et al. 2012), more studies that examine the role of serotonin in the etiology and phenotype of WS are needed to better inform treatment and identify targets of future, more effective therapeutics.

Chapter 5, in part, is currently being prepared for submission to a peer-reviewed scientific journal. The author of this dissertation is the primary investigator and author of this paper. Authorship: Lew, CH; Groeniger, KM; Hanson KL; Cuevas, D; Greiner, D; Hrvoj-Mihic, B; Bellugi, U; Semendeferi, K.

TABLES AND FIGURES

Table 5.1: Subject Background Information

Subject ID	Age at Death	Diagnosis	Gender	Hemisphere	Cause of Death	Postmortem Interval (hours)
WS 10	17 years	Williams syndrome	Male	Right	Cardiac complications	24
TD 4916	19 years	Typically developing	Male	Right	Drowning	5
WS 14	42 years	Williams syndrome	Female	Right	Cardiac complications	18
TD 5445	42 years	Typically developing	Female	Right	Pulmonary thromboembolism	10
WS 9	43 years	Williams syndrome	Female	Right	Cardiac complications	12
TD 5758	43 years	Typically developing	Female	Right	Sepsis	22
WS 8	48 years	Williams syndrome	Male	Left	Respiratory illness	30

Table 5.2: Results in each amygdaloid nucleus. Columns 2 and 3 lists mean SERT axon density and standard deviation in WS and TD and column 4 lists the percent difference in SERT axon density in WS compared to TD. Column 5 and 6 lists the p-value and t-ratio of the student's T-test comparing WS and TD.

Amygdaloid nucleus	WS mean SERT-ir axon density±SD ($\mu m/\mu m^3$)	TD mean SERT-ir axon density ±SD ($\mu m/\mu m^3$)	% Difference WS compared to TD	P-value	T-ratio
Lateral	0.0050±0.0030	0.0067±0.0005	-25%	0.3250	1.003
Basal	0.0056±0.0030	0.0064±0.0007	-30%	0.6335	0.4842
Accessory Basal	0.0054±0.0030	0.0072±0.0020	-25%	0.2975	1.0700
Central	0.0032±0.0010	0.0035±0.0005	-8%	0.8417	0.2023

Table 5.3: Results in each amygdaloid nucleus, with WS 14 excluded from analysis. Columns 2 and 3 lists mean SERT axon density and standard deviation in WS and TD and column 4 lists the percent difference in SERT axon density in WS compared to TD. Column 5 and 6 lists the p-value and t-ratio of the student's T-test comparing WS and TD.

Amygdaloid nucleus	WS mean SERT-ir axon density±SD ($\mu m/\mu m^3$)	TD mean SERT-ir axon density ±SD ($\mu m/\mu m^3$)	% Difference WS compared to TD	P-value	T-ratio
Lateral	0.0038±0.0010	0.0067±0.0005	-43%	0.0314	2.894
Basal	0.0041±0.0020	0.0064±0.0007	-36%	0.0679	2.3100
Accessory Basal	0.0038±0.0010	0.0072±0.0020	-47%	0.0144	3.406
Central	0.0031±0.0010	0.0035±0.0005	-12%	0.6770	0.4243

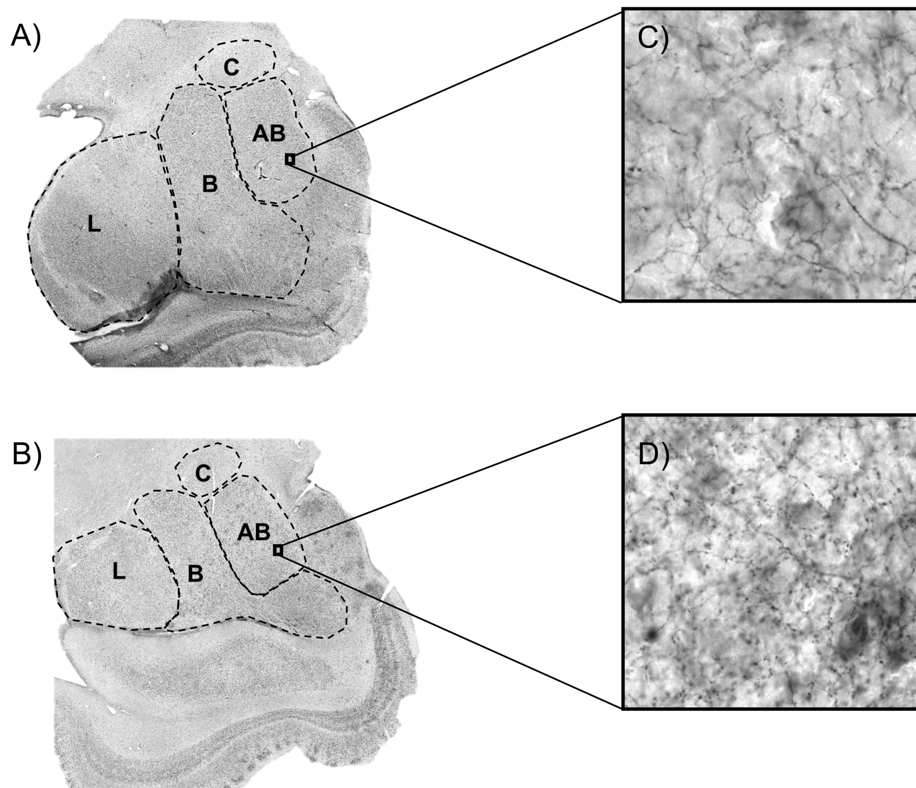

Figure 5.1: Nucleus boundaries in Nissl stained sections of WS (A) and TD (B) at 1x magnification, L= lateral nucleus, B= basal nucleus, AB= accessory basal nucleus, C= central nucleus; SERT-ir axons in the accessory basal nucleus of WS (C) and TD (D) at 60x magnification.

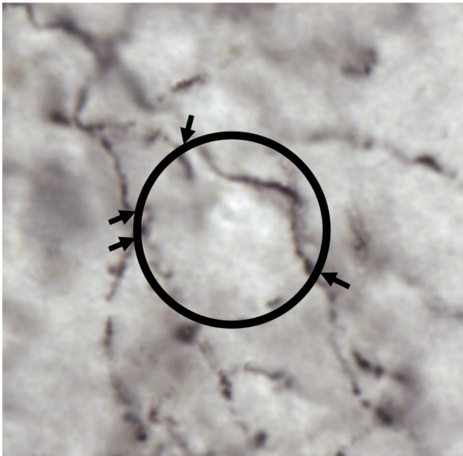

Figure 5.2: Photo demonstrating the Spaceballs probe used in tissue at 100x magnification. Black arrows indicate the point at which SERT axons cross the circumference of the probe.

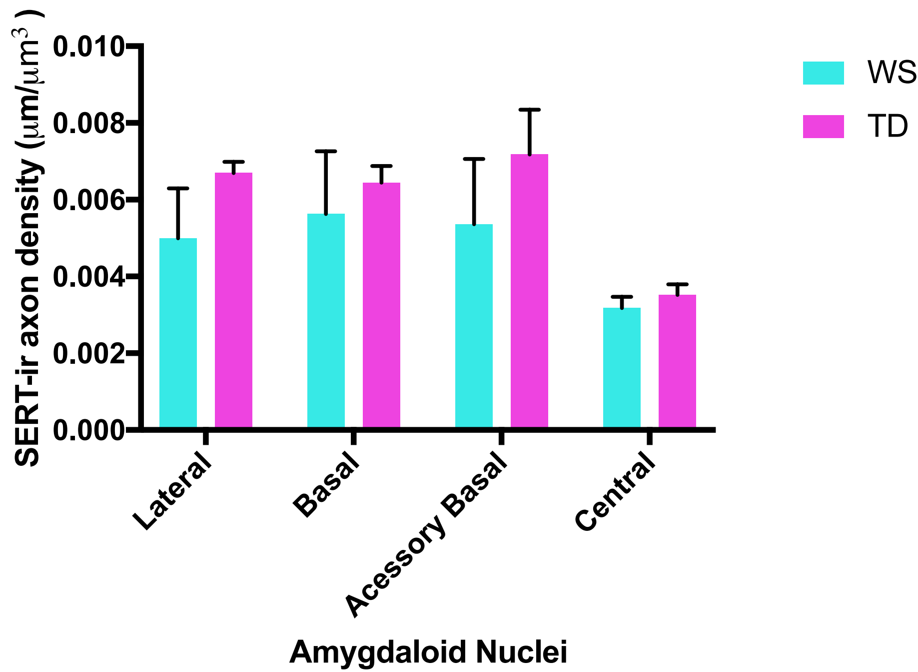

Figure 5.3: Graph of mean SERT axon density in the lateral, basal, accessory basal, and central nucleus in WS (turquoise) and TD (purple).

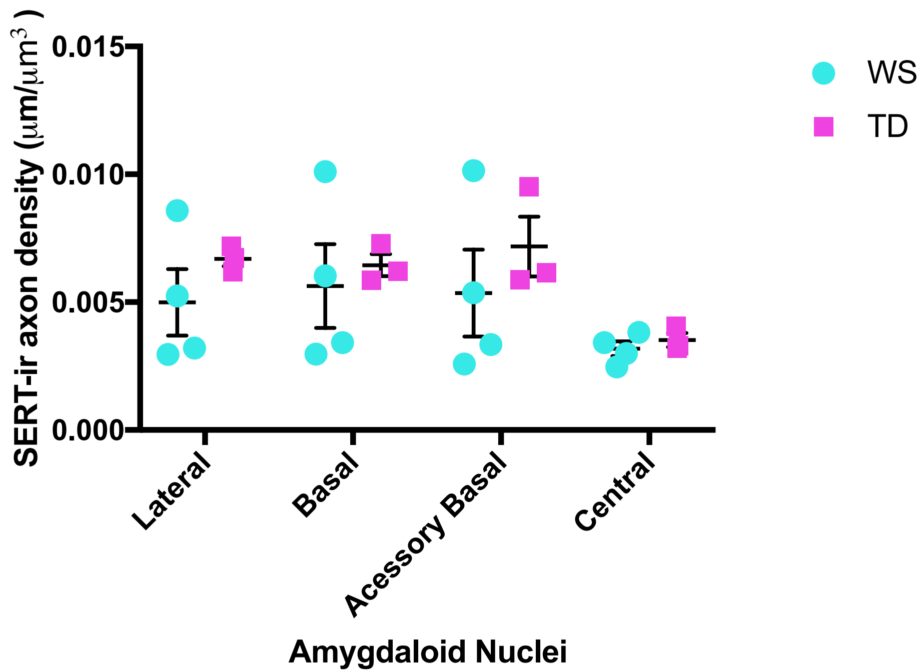

Figure 5.4: Graph of SERT axon density showing individual data points in the lateral, basal, accessory basal, and central nucleus in WS (turquoise circle) and TD (purple square).

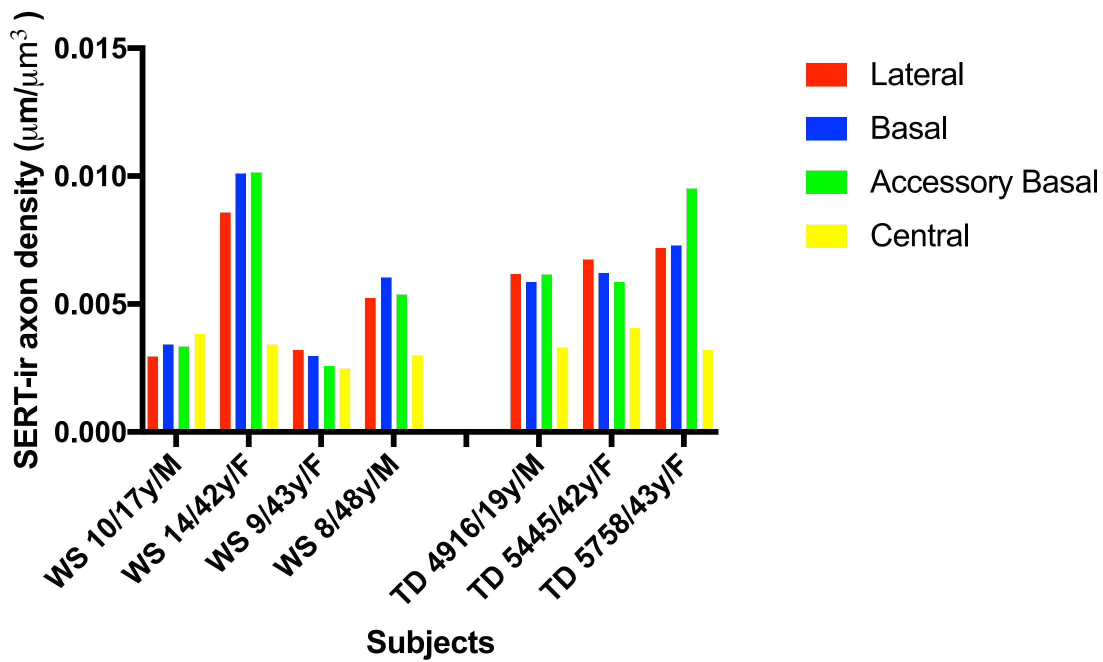

Figure 5.5: Graph of SERT axon density in each amygdaloid nucleus of each subject.

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Chapter 6:

Conclusions

Utilizing stereological techniques in tissue derived from a unique postmortem brain collection, this dissertation sought to identify microstructural changes underlying orbitofrontal-amygdala dysfunction in Williams syndrome (WS), a rare neurodevelopmental disorder with a discrete genotype and a distinct and consistent sociobehavioral phenotype, in order to examine how neuroanatomical variation may influence aspects of social cognition and behavior that appear to be fundamental to successful conspecific interaction and communication in our species. We targeted two closely interconnected structures with critical roles in social brain circuitry, the orbitofrontal cortex and the amygdala, in a postmortem sample of brains from individuals diagnosed with WS and typically developing controls (TD), performing stereological analyses to quantify neuron density in the supra- and infra-granular layers of two orbitofrontal cortical areas (BAs 10, 11) and three unimodal cortical areas (BAs 4,3,18), and nucleus volume, neuron size, neuron number, and serotonergic fiber density in the basolateral nuclei and central nucleus of the amygdala. Cyto- and chemoarchitectonic modifications of the orbitofrontal cortex and basolateral nuclei of the amygdala were observed in WS, which likely contribute to the unique socio-behavioral phenotype of the disorder.

Summary of Key Findings

Neuron density in the WS cortex. Neuron density was decreased in the orbitofrontal cortex in WS relative to TD, with the infragranular layers demonstrating the largest decrease in neuron density and reaching statistical significance in BA 10. Neuron density was slightly increased in the somatosensory cortex BA 3 and secondary visual cortex BA 18 in WS,

although this increase was not significant. There was no difference in neuron density of motor cortex BA 4 in WS and TD. These findings demonstrate that different functional areas of the cortex are differentially affected in WS, such that orbitofrontal cortical regions implicated in socio-cognitive processing, and specifically the infragranular layers which project to subcortical structures such as the amygdala, appear more affected than unimodal cortical areas. A study examining neural progenitor cells and cortical neurons derived from WS induced pluripotent stem cells (iPSCs) found that WS neural progenitor cells demonstrate an increase in time of apoptosis in neurodevelopment, the genetic mechanism of which was determined to be frizzled 9, a gene hemizygously deleted in WS (Chailagkarn et al. 2016). This same study also found that layer V/VI cortical neurons derived from the WS iPSCs had longer dendrites, increased spine and synapse number, and altered network connectivity compared to TD (Chailangkarn et al. 2016). Another study examining dendritic branching in postmortem WS cortex found less dendritic branching in BA 10 and 11 than expected relative to BA 4, 3, and 18 (Hrvoj-Mihic et al. 2017). The changes in neuron density and dendritic morphology in the orbitofrontal cortex in WS may be linked to alterations in apoptotic events caused by the hemideletion of frizzled 9.

Neuron number in the WS amygdala. Neuron number was increased in the lateral and basal nuclei of the amygdala in WS, reaching significance in the lateral nucleus. While neuron number of the accessory basal nucleus was similar in WS and TD, nucleus volume was lower and neuron density was higher in WS, although these differences were not significant. The central nucleus, in contrast, was very similar in WS and TD in all parameters examined. It is of interest that a similarly designed study examining the amygdala in autism, a disorder often characterized by behaviors of social avoidance, found a directly opposing pattern in the lateral

nucleus of significantly decreased number of neurons, but like our findings in WS found no significant differences in any other nuclei, and the central nucleus demonstrated high similarity in autism and TD (Schumann et al. 2006). Together these findings suggest that the lateral nucleus, and by association neural networks involving the lateral nucleus such as the orbitofrontal-amygdala network, are especially vulnerable to disruption in these neurodevelopmental disorders. One possible candidate gene within the WS hemideletion that may contribute to altered neuron number in the lateral nucleus is PSD-95, which has been shown to influence cellular morphology in the basolateral nuclei but not the central nucleus in genetic knockout mice (Feyder et al. 2010).

Serotonergic innervation of the WS amygdala. The density of serotonin-transporter-containing (SERT) axons was lower in the WS amygdala compared to TD, and this difference between the two groups was much larger in the basolateral nuclei (36-47% decrease in WS) than in the central nucleus (12% decrease in WS). While no other human postmortem study has quantified SERT axon density within individual amygdaloid nuclei, a qualitative study observed an increase in SERT axons in several regions including the amygdala in autism (Azmitia et al. 2011). These studies demonstrate that the serotonergic system is disrupted in both disorders, which could possibly contribute to the altered cellular phenotype of the amygdala in WS and autism, given the significant role of serotonin in the regulation of neural development (Whitaker-Azmitia 2001). A possible gene in the WS hemideletion that may contribute to serotonergic disruption is GTF2IRD1, which is also a common site of allelic variation in autism (Weiss et al. 2009). GTF2IRD1 has been linked to decreased serotonin, reduced fear and aggression and altered serotonergic metabolism in the amygdala as well as the prefrontal cortex in genetic knockout mice (Young et al. 2008; Proulx et al. 2010). The

serotonin transporter (5-HTTLPR) polymorphism, which has been linked to behavioral variation in the socio-emotional domain in humans and nonhuman primates (Lesch et al.1997; Pezawas et al. 2005), as well as variation in amygdala reactivity in healthy subjects (Harrari et al. 2002; Bertolino et al. 2005), may also interact with other genetic mechanisms of the WS hemideletion and contribute to alterations in serotonergic innervation of the amygdala in the disorder.

Evolutionary implications of serotonergic innervation of the human amygdala. In humans as a whole, SERT axon density was found to be relatively homogenous across the basolateral nuclei, while the central nucleus demonstrated relatively lower density. A similarly designed study examining SERT axon density in two closely related species, chimpanzees and bonobos, found that chimpanzees demonstrated relatively homogenous density in all four nuclei (lateral, basal, accessory basal, and central), while bonobos demonstrated high SERT axon density in the basal and accessory basal nuclei relative to lateral and central nuclei (Stimpson et al. 2015). Together these findings suggest that patterns of serotonergic innervation across the amygdaloid nuclei might reflect the functional specializations of these nuclei, and could contribute to differences in social tolerance between species.

Future Directions

This dissertation represents the first quantitative microstructural characterization of two interconnected regions that play a pivotal role in social brain circuitry, the orbitofrontal cortex and the amygdala, in Williams syndrome, a neurodevelopmental disorder with a unique etiology and phenotype that may help bridge the gap between genes and behavior. The findings from this dissertation provide fundamental information that sets the stage for future

important lines of research including: examining neuronal distribution of other regions with significant connectivity to the orbitofrontal-amygdala network in WS, such as the superior temporal sulcus, to further characterize the neuroanatomical phenotype of WS social brain circuitry; examining morphology and dendritic branching of amygdala pyramidal neurons in WS; examining SERT axon density in the orbitofrontal cortex in WS and TD to determine whether disruptions to the serotonergic system extend to other areas of the social brain beyond the amygdala; genotyping the serotonin-transporter polymorphism of the WS and TD subjects included in these studies to examine if/how this polymorphism contributes to the neuroanatomical phenotype of the social brain; directly comparing quantification of SERT axon density in the amygdala in our typically developing human data set with the chimpanzee and bonobo data (Stimpson et al. 2015) in order to better understand how serotonergic innervation within the amygdala might contribute to species-specific sociality.

These future directions of study aim to further characterize how the orbitofrontal-amygdala network may be disrupted in the WS, and will help shed light on the relationship between genotype, variation in social brain structure and circuitry, and variation in social behavior. Such insight, in addition to identifying possible targets for therapeutic research, may also characterize specializations of the human brain that underlie uniquely human social behavior and cognition.

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