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Social cognition in Williams syndrome: insights from the organization of prefrontal microcircuitry

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

in

Anthropology

by

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The Dissertation of Branka Hrvoj Mihic is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter I, in full, is a reprint of the material as it appears in Hrvoj-Mihic B, Bienvenu T, Stefanacci L, Muotri AR and Semendeferi K (2013) Evolution, development, and plasticity of the human brain: from molecules to bones. *Frontiers in human neurosciences 7:*707. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Social cognition in Williams syndrome: insights from the organization of prefrontal microcircuitry

by

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Williams syndrome is a neurodevelopmental disorder characterized by a set of compromised and preserved features in social and general cognition. Some behavioral manifestations of the disorder – especially hypersociality and inadequate responses to social interactions – imply the involvement of prefrontal cortical (PFC) areas underlying higher-order social and emotional processing. Using Golgi and immunohistochemical techniques, I have examined dendritic branching and distribution of neurofilament protein expressing neurons in two prefrontal areas: BA 11 in the orbital frontal cortex, largely responsible for emotional processing, and BA 10 in the frontopolar cortex, an area

underlying executive cognitive functions.

The results suggest that the morphology of basal dendrites on the pyramidal neurons is altered in the cortex of WS, with differences that are layer-specific, prominent in both PFC areas, and display an overall pattern of dendritic organization that differentiates WS from other disorders. In particular, and unlike what was expected based on typically developing brains, basal dendrites in the two PFC areas did not display longer and more branched dendrites compared to motor, sensory and visual areas. Further analysis of layer III in BA 10 suggests a decrease in density of neurofilament protein expressing neurons (SMI-32) underlying long distance connections between PFC and other cortical areas.

This dissertation contributes to our understanding of the relationship between the structure and function of cortical areas underlying social cognition, and provides additional insights into the range of variation in PFC areas in developmental and psychiatric disorders.

INTRODUCTION

Little is known about evolution of the human brain. In some respects, our brain follows the general pattern observed in other primates, characterized by a relative increase in the overall brain size, expansion of the neocortex, and elaboration of the areas devoted to visual processing and reduction in those processing olfaction (Stephan and Andy, 1964; Stephan, 1972; Stephan et al., 1981). In others, it displays selective enlargement of structures underlying some of our behavioral adaptations, such as the lateral nucleus of the amygdala implicated in the processing of social behavior (Barger et al., 2007). At the same time, evolutionary changes in structures underlying important behavioral and cognitive aspects of our species, including problem-solving and the integration of multiple cognitive rules, and their processing in the prefrontal cortex (PFC), remain only partly explored.

The PFC consists of several areas differing in cellular organization, connectivity, and function, united by their overarching role in higher order cognitive and emotional processing (Petrides and Pandya, 2004). During the course of human evolution, PFC underwent a reorganization, characterized by a selective enlargement of some areas and a decrease in others (Semendeferi et al., 1998; 2001), changes in the number of minicolumns and the neuronal density in some of its areas (Semendeferi et al., 2011) and in distribution of specific subclasses of neurons (Hof et al., 1995). However, the extent of variation in organization of these areas in humans, as well as behavioral and cognitive correlates of a specific organization, are still a matter of speculation.

Of particular importance in elucidating the relationship between the structure and function of the human brain are neurodevelopmental and psychiatric disorders (Hanson et

al., 2014), especially those with distinct behavioral manifestations and the compromises in specific parts of the circuitry underlying the behaviors under study. The analyses of brain organization in disorders contribute to our understanding of the relationship between brain structure and function at two levels: (1) by offering the link between a structure and its functional implications in neurotypical subjects (NT) and individuals affected with the disorder, and (2) by providing insights into the range of variation in our species found in typical functioning.

This dissertation will explore microstructural aspects of organization in two PFC areas -- the frontopolar cortex (Brodmann's area 10; BA 10) and an area in the orbital frontal cortex (BA 11) – in Williams syndrome (WS). WS represents a neurodevelopmental disorder with a clear set of compromised abilities in social and general cognition (Bellugi et al., 1994; 2000) which suggest involvement of prefrontal circuitry.

Prefrontal cortex: its structure and evolution

PFC occupies the most frontal portion of the human cerebrum, anterior to the motor and supplementary motor areas (Fuster, 1997; Goldman-Rakic, 1995). In neuroanatomical literature, it has been variously defined based on several criteria. The most basic and most widely used is the definition based on the cytoarchitectonic criteria, which defines PFC as the granular part of the frontal lobe, characterized by a distinct (granular) layer IV (e.g. Brodmann 1909; Preuss 1995; Barbas 1995; Barbas and Zikopoulos, 2007). Perceived that way, PFC is part of the cortex unique to primates and absent in other mammals (Brodmann, 1909; Preuss, 1995). In terms of connectivity, PFC can be defined as the part

of the frontal lobe that receive inputs from the mediodorsal nucleus of the thalamus (Rose and Woolsey, 1948; Barbas et al., 1991; Fuster, 1997), which opens the possibility that the PFC – or at least some of its subdivisions – may be present in rodents and mammals other than primates (Uylings et al., 2003). Finally, from the neurophysiological perspective, the PFC represents 'silent' cortex, i.e. the cortex whose stimulation does not yield visible physical responses (Preuss and Goldman-Rakic, 1991). Regardless of the criteria employed, the PFC always encompasses a range of functionally distinct areas in the frontal lobe involved in higher-order emotional and cognitive processes.

Processing within the PFC can be broadly divided into two subdivisions with distinct – albeit overlapping – functions. The orbital frontal areas (BA 10, BA 11, BA 12, and BA 13) are typically involved into higher-order emotional and social processing (Beer et al., 2003; Fellows, 2007a,b; Habib et al., 1996; Stone et al.,1998). The 'executive functions,' comprising of working memory, preparatory set, and inhibitory control (Fuster, 1997) are mostly processed in its dorsolateral areas (BA 9, BA 10, and BA 46; Baddeley, 1992; Fuster,2000a; Jurado and Rosselli, 2007). Both dorsolateral and orbital frontal PFC areas form important parts of the network processing social stimuli (Adolphs, 2001; Burns, 2006), including perception of emotional information from the conspecifics and regulation of mood, "theory of mind", self-reference, and working memory (Grady and Keightley, 2002). In addition, orbital frontal areas are implicated in emotion-related decision making (Bechara et al., 2000; Rolls, 2000), thus forming part of the network emphasizing emotional processing. Dorsomedial PFC is important in tasks that require self-referencing (Craik et al., 1999), and dorsolateral PFC is implicated in general executive functions

which require intact working memory and inhibitory control (d'Esposio et al., 1995; Garavan et al., 1995). The role of behaviors processed in various PFC areas has been emphasized during the evolution of primates (Lew and Semendeferi, 2017) and increase in the PFC relative to the rest of the neocortex represents an evolutionary trend across primates (Bush and Allman, 2004). However, across different primate species, several lines of evidence suggest that PFC evolution is characterized by a structural reorganization, reflecting the selective advantage of behaviors processed in different PFC areas across species.

The general trend observed in primates is an increase in the number of dorsolateral PFC areas. More specifically, the dorsolateral part of PFC in rhesus macaques *(Macaca mulatta*) consists of twice as many areas as PFC of the galago (*Galago crassicaudatus* and *G. garnetti*), whereas the number of areas in the orbital and mesial PFC remains comparable between the species (Preuss and Goldman-Rakic, 1991). Another trend, observed in the great apes and humans, and possibly present in other primate species, is selective expansion of some areas and a decrease in size in others. In humans, the frontal pole (BA 10) underwent an expansion (Semendeferi et al., 2001), whereas parts of the orbitofrontal cortex, such as BA 13, decreased in size relative to the overall brain volume (Semendeferi et al., 1998). The pattern of relative decrease in BA 13 is shared between humans and bonobos (*Pan paniscus*) and contrasts with the pattern seen in orangutans (*Pongo pygmaeus*), the species that displays larger than expected BA 13 (Semendeferi et al., 1998). In summary, relative size of different PFC components displays differences across primates, suggesting that selective pressures acting on behaviors processed in

specific parts of the PFC may have resulted in different organization of the structure across species.

Further analyses, examining the distribution and organization of neurons, revealed that changes in size are accompanied by microstructural reorganization. In addition to its enlargement, BA 10 in humans is characterized by a higher number of minicolumns and greater spacing distance between the neurons compared to BA 10 of great apes (Semendeferi et al., 2010). Humans also display differences in the density of specific subclasses of neurons – such as neurofilament protein expressing neurons -- compared to macaques (Hof et al., 1995; Carmichael and Price, 1994). Further focus on one specific neuronal subtype – the excitatory pyramidal neurons – similarly revealed differences in the length, branching, and number of dendritic spines on the dendrites across functionally distinct areas within PFC, as well as across species. Morphology of dendrites, analyzed in the way that allows for comparisons across studies, has been examined in three primate species: macaques, chimpanzees, and humans. In all three species, pyramidal neurons vary across cortical areas in the length of dendrites, branching complexity, and in number and density of dendritic spines (Cupp and Uemura, 1980; Jacobs et al., 1997, 2001; Elston, 2007; Bianchi et al., 2012). In all three species, dendrites on pyramidal neurons in PFC are longer, more branched, and with more dendritic spines compared to the primary sensory areas, suggesting that an integrative role of the PFC represents a trait shared by macaques, great apes, and humans. However, comparison of dendritic organization across species suggests that humans displayed the highest complexity of dendrites in the PFC (Elson, 2000; Elston et al., 2006; Jacobs et al., 2001; Bianchi et al., 2012). Changes in the PFC

across species are, therefore, observed at several levels, each carrying different explanatory power for understanding the function and evolution of PFC organization in a species.

Prefrontal cortex: disorders of social cognition

Given the diversity of behaviors processed in the PFC, it is difficult to propose a clear link between the structure of a particular PFC area and specific behavioral manifestations. In humans, an important body of evidence comes from psychiatric disorders with clear behavioral manifestations and compromises in the structure of the PFC, which suggests involvement of prefrontal microcircuitry. Typical anatomy of PFC areas is often compromised in neuropsychiatric disorders, including schizophrenia, bipolar disorder, autism spectrum disorder (ASD), Rett syndrome (RTT) and Williams syndrome (WS).

Despite diverse symptoms, all of these disorders can essentially be considered disorders of social cognition (Burns, 2006), since each encompasses a compromised ability to interpret and adequately respond to behaviors of others, limiting the person's ability to function in complex social environments (Baron-Cohen et al., 2000). Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies have suggested that differences in the activity of social brain areas – including the PFC – are a typical finding in all of these disorders (Grady and Kneightley, 2002; Glahn et al., 2005; Mobbs et al., 2004; 2007). Even in the cases with a lack of observable structural differences between the patients and controls – such as in the case of ASD – the subjects with ASD perform worse on tasks involving dorsolateral PFC (Griebling et al., 2010). At the microscopic level, the disorders are associated with compromised organization of

various PFC areas. However, the effects appear to vary across disorders, with each disorder displaying a distinct 'signature' on PFC. Furthermore, the effects of observed pathologies are often more pronounced – or even limited -- to a specific cortical layer, suggesting involvement of specific circuits in which a particular PFC area participates (Mega and Cummings, 2004).

In schizophrenia, the lower layer III of dorsolateral PFC appears particularly affected. It is characterized by a lower proportion of magnopyramdial neurons compared to controls (Pierri et al., 2001) and a decrease in dendritic spine number (Glantz and Lewis, 2000). In ASD, on the other hand, the number of neurons is increased in PFC relative to controls with differences more prominent in dorsolateral PFC compared to mesial PFC areas (Courchesne et al., 2011). Unlike schizophrenia, dendritic morphology in ASD is manifested by increased density of dendritic spines and restricted to layer III of dorsolateral PFC (Hutsler and Zhang, 2010). In comparison, higher density of spines in the parietal cortex can be seen in both layers II/III and layers V/VI (Hutsler and Zhang, 2010), suggesting that the pathologies in dendritic organization do not affect all of the cortical areas equally. Similar effect on the dendrites in layers II/III are observed in BA 10 of RTT patients – affecting dendritic length and branching – whereas reduction in the number of spines can be observed across both supra- and infra-granular layers (Belichenko et al., 1994).

Williams syndrome and social brain areas

Williams syndrome (WS) is a disorder associated with a hemizygous deletion of ~ 25 genes on chromosome 7 (Schubert, 2009) and a distinct behavioral and cognitive profile. Some aspects of language and face processing are typically preserved in WS,

whereas social behavior – manifested as an increased desire for social interaction coupled with inadequate responses to social situations – displays differences with neurotypical individuals (Bellugi et al., 1999). Individuals with WS display differences in brain activity during social tasks and reduced activation of dorsolateral PFC in Go/NoGo tasks (Mobbs et al., 2007). In WS, the anterior brain areas are typically more active during face and gaze processing tasks, whereas primary and secondary visual areas display reduced activity compared to controls (Mobbs et al., 2004). Given that PFC areas are important in higher-order processing of socially relevant cues (Grady and Keightley, 2002), the distinct social behavior in WS can be linked with the differences in the activity of PFC areas compared to controls.

Although MRI studies did not reveal differences in the volume of frontal lobes in WS, some parts of PFC – notably the orbitofrontal cortex -- display larger gray matter volumes compared to controls (Reiss et al., 2004; Fan et al., 2017).

Analyses examining the density of neurons suggest that, both in BA 10 and BA 11, subjects with WS display a decreased density of neurons (Lew et al., 2017). The decrease is especially prominent in infragranular layers of BA 10. In contrast, in the somatosensory cortex (BA 3-1-2) and in the secondary visual area (BA 18) individuals with WS display higher density of neurons compared to control subjects (Lew et al., 2017), suggesting a different effect on the PFC compared to unimodal areas. An increase in neuronal density is also observed in the lateral nucleus of the amygdala (Lew et al., 2018) and an increased density of glia was reported in dorsal and medial caudate nuclei of the striatum in WS (Hanson et al., 2018). Both the amygdala and dorsal and medial caudate are important

structures in processing of social and emotional behavior, and they form important afferent sources into PFC (Amaral and Price, 1984; Haber, 2003). The existing research, therefore, suggests that WS is characterized by specific deficiencies in processing of socially relevant stimuli, which affect PFC areas and the connecting subcortical structures.

Summary of the present analysis

The purpose of the research included in this dissertation is to examine dendritic morphology and the density of neurofilament expressing neurons in two functionally distinct PFC areas in individuals with WS: the orbitofrontal cortex (BA 11) and the frontal pole (BA 10). Since existing research suggests that the behavioral phenotype in WS is underlined by deficiencies in inhibitory control, it is expected that differences between WS and controls will be more prominent in BA 10 compared to BA 11.

Chapter I examines existing research on evolutionary changes in brain morphology occurring in the human lineage, highlighting the gaps in our knowledge about brain evolution that can be filled by analyzing brain modifications in neuropsychiatric disorders. Chapters II and III examine neuroanatomical organization of PFC in WS. The focus of Chapter II is dendritic morphology of pyramidal neurons; the analysis of distribution of neurofilament protein expressing neurons in BA 10 is presented in Chapter III.

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CHAPTER I: EVOLUTION, DEVELOPMENT, AND PLASTICITY OF THE HUMAN BRAIN – FROM MOLECULES TO BONES

Abstract

Neuroanatomical, molecular, and paleontological evidence is examined in light of human brain evolution. The brain of extant humans differs from the brains of other primates in its overall size and organization, and differences in size and organization of specific cortical areas and subcortical structures implicated into complex cognition and social and emotional processing. The human brain is also characterized by functional lateralizations, reflecting specializations of the cerebral hemispheres in humans for different types of processing, facilitating fast and reliable communication between neural cells in an enlarged brain. The features observed in the adult brain reflect human-specific patterns of brain development. Compared to the brains of other primates, the human brain takes longer to mature, promoting an extended period for establishing cortical microcircuitry and its modifications. Together, these features may underlie the prolonged period of learning and acquisition of technical and social skills necessary for survival, creating a unique cognitive and behavioral niche typical of our species. The neuroanatomical findings are in concordance with molecular analyses, which suggest a trend toward heterochrony in the expression of genes implicated in different functions. These include synaptogenesis, neuronal maturation, and plasticity in humans, mutations in genes implicated in neurite outgrowth and plasticity, and an increased role of regulatory mechanisms, potentially promoting fast modification of neuronal morphologies in response to new computational demands. At the same time, endocranial casts of fossil hominins

provide an insight into the timing of the emergence of uniquely human features in the course of evolution. We conclude by proposing several ways of combining comparative neuroanatomy, molecular biology and insights gained from fossil endocasts in future research.

Introduction

The search for the evolutionary emergence of neural features underlying human cognitive and behavioral specializations represents a persistent field of inquiry spanning several disciplines. From comparative neuroanatomy through molecular biology and paleoanthropological reconstructions, years of research have yielded numerous insights into features unique to the human brain, their morphological correlates, evolutionary pathways, and context of their appearance. Compared to other primates, extant humans are unique in the nature of their sociality, ecological adaptations, and, most importantly, in a complete reliance on culture as the extrasomatic, transgenerationally transmitted behavioral adaptation (Alexander, 1989; Kaplan et al., 2000; Hill et al., 2009). Throughout the evolution of the genus *Homo*, the fossil record demonstrates an increase in brain size and appearance of cortical asymmetries suggestive of functional lateralization (Falk, 1987; Holloway et al., 2004). At the same time, comparative neuroanatomical studies suggest that, in addition to an increase in size, human brain evolution was characterized by selective enlargement and reorganization of specific cortical areas (Semendeferi and Damasio, 2000; Semendeferi et al., 2001, 2011) and subcortical structures (Barger et al., 2007, 2012), potentially promoting information processing unique to our species. In parallel, human life history is characterized by an extended period of offspring dependency compared to chimpanzees, delayed onset of reproductive maturation, and long post-

reproductive life-span (Bogin and Smith, 1996; Flinn, 2005; Hawkes, 2006), enabling prolonged cognitive maturation, acquisition of skills necessary for survival, and their transmission across generations.

The importance of complex morphological structures and flexible behaviors – allowing for novel responses to newly encountered selective pressures – was proposed as the key adaptation of the hominin lineage (Potts, 1998). In this sense, variability selection approached human evolution from a perspective different from fluctuating selection and developmental plasticity; it emphasized the evolutionary emergence of traits capable of providing selective advantage to hominins in unstable conditions, without invoking changes in the reaction norm or the need for genetic polymorphisms (Potts, 1998). Among these traits, expansion of the brain and behavioral complexity emerged as the key features carrying a selective advantage during the course of human evolution.

Behavioral variability, together with a more general cognitive complexity, has been typically considered in the context of overall encephalization. However, the relationship between the brain size of fossil hominins and their behavioral complexity inferred from the archaeological remains is neither simple nor straightforward (McBrearty and Brooks, 2000; Teyssandier, 2008). Whereas the first wave of increase in brain size early in the Pleistocene coincides with the appearance of first bifacial tools, the relationship becomes less clear later in human evolution, especially when assessing cognitive capacities of early modern *H. sapiens*. Although it has been proposed that novel tool technologies, new food procurement strategies, and the emergence of representational art appeared suddenly and concurrently at 50–40 kya (Klein, 2000; Bar-Yosef, 2002), recent reports provide evidence

that aspects of behavioral modernity may have already been present much earlier than that (McBrearty and Brooks, 2000; Brown et al., 2012). At the same time, anatomically modern humans were characterized by only a modest increase in the brain size compared to their predecessors (Ruff et al., 1997) leading some to suggest that the emergence of behavioral modernity may have been accompanied by subtle changes in cortical organization that cannot be inferred from the fossil record (Klein, 2000). The debate on the origin of behavioral modernity aside, changes in brain size are accompanied by numerous modifications in organization and connectivity. In the case of the neocortex, an expansion in cortical size tends to be accompanied by changes including absolute or relative size of cortical fields, enlargement of areas devoted to processing relevant sensory inputs, and changes in the amount of areas devoted to processing specific types of stimuli (Krubitzer and Kaas, 2005). Cortical expansion is often accompanied by an increase in modularity and a reduction in long axonal projections, thus decreasing the distance between neurons subserving the same set of information processing (Kaas, 2000).

A growing body of research suggests that neocortical pyramidal neurons – the basic units of cortical microcircuitry (DeFelipe et al., 2002) – display variations in homologous areas across primates, possibly underlying differences in cognitive potentials across taxa (Elston et al., 2006). As such, natural selection may have acted specifically on the morphology and organization of neurons, favoring a particular type of information processing in a given species (Kaas, 2000). When compared across primates, pyramidal neurons in humans tend to display more complex morphologies (Elston, 2003) that are capable of sampling from larger inputs and of participating in more extensive cortical

networks (Jacobs and Scheibel, 2002). In all primates examined to date, pyramidal neurons are characterized by extensive morphological changes during post-natal maturation and remodeling throughout life, potentially underlying flexible behavioral responses typical of all primates. Pyramidal neurons in the human neocortex display a prolonged period of development compared to other primates (Cupp and Uemura, 1980; Petanjek et al., 2008, 2011), especially in the cortical areas characterized by expansion during human evolution, including selected areas in the prefronal cortex (PFC). Similar developmental differences can be observed in gene expression studies, with delayed peak activity of genes involved in synaptogenesis and neuronal plasticity in humans compared to chimpanzees and macaques (Liu et al., 2012). At the same time, certain genes implicated in neuronal plasticity display mutations unique to humans (Lu et al., 2007, 2009), potentially suggesting differences in regulation of these processes between humans and non-human primates.

Even though insights into the microstructure of the cortex gained from comparative neuroanatomical studies cannot be directly compared with the fossil crania, certain features of human brain development and cortical organization allow for a synthesis of paleontological, neuroanatomical, and molecular evidence in reconstructing human brain evolution. In this review, we will combine these lines of research to examine plasticity in the human brain from an evolutionary perspective. We will specifically address maturation, cortical asymmetries, and lifelong changes in human neocortical pyramidal neurons, molecular aspects underlying neocortical plasticity, and a potential time-frame for the evolution of increased plasticity in the human brain based on the insights gained from fossil endocasts. Where possible, we will refer to the evolution of subcortical structures,

especially in relation to social and ecological adaptations unique to our species. Several specificities of the human brain, including its size, development, and hemispheric dominance can be examined in extant primates, traced through the course of human evolution, considered in the context of developmental patterns unique to the human brain, and supplemented by insights from molecular studies.

Human Brain Evolution: Insights from the Neuronal Phenotypes

During the course of human evolution, the brain underwent an increase in its overall size (Falk et al., 2000; Holloway et al., 2004), in the relative size of some of its gross components (Finlay and Darlington, 1995; Semendeferi and Damasio, 2000), and a selective enlargement of specific cortical areas and subcortical nuclei (Semendeferi et al., 2001; Barger et al., 2007). Along with changes in size came subtle modifications in organization, indicating possibly significant alterations in microcircuitry at the cellular level (Semendeferi et al., 2011; Barger et al., 2012). From an anatomical perspective, morphological characteristics of a particular cortical region reflect the number, size, and distribution of neurons within that region (DeFelipe et al., 2002). Thus, an analysis of properties and organization of neurons in homologous areas across species forms the basis for examining cortical organization from an evolutionary point of view (Kaas, 2000). Increasingly there is interest in the level of individual neurons and how they vary across functionally different cortical areas, across species, and how they change across the lifetime (Jacobs et al., 2001; Sherwood et al., 2003a; Bianchi et al., 2012). Analyses at the neuronal level enable the development of testable hypotheses linking the morphology of information processing units and their function. They can also provide insights into plastic

responses to environmental circumstances across different cortical areas, the limits of the plasticity, and possible differences in the nature or extent of plasticity across species.

At the cellular level, the neocortex consists of excitatory pyramidal and spiny stellate neurons, and of various classes of inhibitory neurons (Nieuwenhuys, 1994; Hof and Sherwood, 2007; DeFelipe et al., 2013). Despite this cellular diversity, neocortical pyramidal neurons constitute the principal class of neurons in the cortex, accounting for 70–85% of all cortical neurons (DeFelipe and Fariñas, 1992) and have been the target of a considerable number of developmental, comparative, and evolutionary studies. Pyramidal neurons form the basic units of cortical microcircuitry, determining the pattern of inputs and outputs into a particular cortical area (DeFelipe et al., 2002). In this review, we focus specifically on this morphological class of neurons. Pyramidal neurons are typically characterized by a pyramidal- or ovoid-shaped soma, the presence of one apical dendrite directed toward the pial surface, several basal dendrites emerging from sides of the soma, an axon emerging from the base of the cell body or from the proximal parts of basal dendrites, and the presence of spines representing sites of excitatory inputs onto dendrites (Figure 1.1; DeFelipe and Fariñas, 1992; Nieuwenhuys, 1994; Spruston, 2008).

In the cortex of adult primates – more specifically, macaques, chimpanzees, and humans – pyramidal neurons vary across cortical areas in the length of dendrites, branching complexity, and in number and density of dendritic spines (Cupp and Uemura, 1980; Jacobs et al., 1997, 2001; Elston, 2007; Bianchi et al., 2012). Pyramidal neurons in the primate neocortex also tend to display two trends: an increase in complexity in relatively larger cortical regions, and an increase in complexity from primary to higher-order sensory

processing areas (Elston, 2003; Elston et al., 2006). In all three species, pyramidal neurons in the prefrontal cortex (PFC) tend to be longer, more branched, and more spinous compared to primary sensory areas. Across species, pyramidal neurons in the human cortex typically emerge as morphologically the most complex when compared to homologous areas of other primates, with the difference being particularly prominent in PFC pyramidal neurons (Elston, 2000; Elston et al., 2006; Bianchi et al., 2012). The prefrontal cortex comprises several cytoarchitectonically defined areas, and many of them, especially the ones within the dorsolateral PFC, are involved in complex cognitive tasks and executive functions in primates (Goldman-Rakic, 1987; Barbas, 1995). During the evolution of the human lineage, parts of the prefrontal regions, notably the frontopolar part, underwent an increase in size (Semendeferi et al., 2001) and changes in neuronal organization (Semendeferi et al., 2011), potentially indicating localized microanatomical changes related to cognitive complexity typical to humans. Analyses of pyramidal neurons in macaque, chimpanzee, and human cortex suggest that an increased complexity of PFC neurons in all species may reflect a trend toward emphasis on executive functions shared by Old World monkeys, apes, and humans (Elston, 2000; Elston et al., 2009; Bianchi et al., 2012), while the integrative role of PFC and its complex behaviors became even further emphasized in humans.

Reorganization observed in the human neocortex has been argued to parallel reorganization in some subcortical structures (Barton and Harvey, 2000). Among those, the amygdala emerges as critical in mediating social and emotional behavior in both human and non-human primates. While subcortical structures are generally considered to be

conserved during primate evolution, the amygdala is anatomically connected with many neural systems that are differentially expanded in humans, such as parts of the prefrontal cortex and the temporal lobe (Stefanacci et al., 1996; Semendeferi and Damasio, 2000; Semendeferi et al., 2001; Stefanacci and Amaral, 2002). Amygdala connections with the prefrontal cortex are an important component of the social brain circuitry. Between 85 and 95% of neurons in the basal nucleus of the amygdala that project to the prefrontal cortex are pyramidal cells immunoreactive for the excitatory amino acids glutamate or aspartate (McDonald, 1996), suggesting the excitatory nature of amygdaloid inputs into the PFC.

When compared to the other members of the family Hominidae, namely chimpanzees, gorillas, bonobos, and orangutans, the amygdala in humans displays disproportional enlargement in the lateral nucleus (Barger et al., 2007, 2012) – both in terms of volume and number of neurons – suggesting a reorganization of the amygdaloid complex and an emphasis on functions processed in the lateral nucleus. This may reflect the primary connective relationship between the lateral nucleus and the temporal lobe (Stefanacci et al., 1996; Stefanacci and Amaral, 2002), which has also expanded over the course of evolution (Semendeferi and Damasio, 2000). The lateral nucleus also receives the majority of cortical sensory information directed to the amygdala (Stefanacci and Amaral, 2000, 2002; Ghashghaei and Barbas, 2002; Barbas et al., 2011), and it has been suggested that its expansion in humans may represent a heightened need to process more expansive and complex social stimuli and interactions (Barger et al., 2012). In addition, it has been argued that several features that set human cultures apart from behavioral traditions of non-human primates include socially shared regulation of behavior and

emotional reinforcement of cultural rules (Hill et al., 2009), both of which may emphasize processing in central executive cortical regions as well as in the amygdala.

The neurons in the amygdala are morphologically suited to provide the foundation for their functional connectivity with numerous other brain regions. The morphology of neurons in the adult amygdala was described through Golgi studies dating back to 1928 (Gurdjian, studies in the rat). Spiny, pyramidal-like neurons and spine-sparse stellate neurons were first described by Hall (1972) in the cat and Braak and Braak (1983) carried out the first Golgi study in the human amygdala. The morphology of neurons in the basolateral complex (lateral, basal, and accessory basal nuclei) has been especially well described. In the adult amygdala, spiny, pyramidal-type neurons, and spine-sparse or aspiny stellate neurons have been identified in the basolateral complex of all species studied to date, including rats, cats, monkeys, and humans (for review see McDonald, 1992). These neurons are very similar to their counterparts in the cerebral cortex. Each of the other amygdaloid nuclei also contain at least one type of projection neuron that is spine dense and one type of spine-sparse neuron that appears to be a local circuit neuron (McDonald, 1992).

Most of the spiny neurons in the basolateral complex have a pyramid-shaped soma with a main dendrite that is longer than the other basal processes, like cortical pyramidal neurons. Unlike cortical pyramidal neurons, however, the basolateral neurons do not exhibit a preferential orientation. The soma and proximal part of the dendrites are smooth while more distal regions are characterized by pedunculated spines. The dendrites generally do not extend beyond nuclear boundaries or into the adjacent white matter, but

axons have been observed to cross nuclear boundaries to join fiber bundles. This suggests that these represent projection neurons. An effective marker that can be used to identify pyramidal neurons in the basolateral complex is calcium/calmodulin-dependent protein kinase II (CaMKII), which has a critical role in long-term potentiation. When CaMKII was analyzed for neuronal localization in the basolateral nucleus of rats, virtually every pyramidal neuron appeared to be CaMKII-positive while non-pyramidal neurons were unstained (McDonald et al., 2002). Indeed, decades of studies in rats have demonstrated the importance of long-term potentiation in the amygdala for emotional learning and memory (Clugnet and LeDoux, 1990; Maren, 1999). Thus, the neurons in the basolateral complex of the amygdala are equipped to mediate the need for behavioral modifications encountered throughout life.

Dendritic Asymmetries in the Human Cortex

Cerebral hemispheres in humans, more so than the hemispheres of other primates, are specialized for different types of information processing (Gazzaniga, 2000; Sun and Walsh, 2006). Although communication between the hemispheres still remains important in humans (Gazzaniga, 2000), certain functions are preferentially processed in one hemisphere over the other. In processing of spatial and face recognition, the right hemisphere exerts dominance over the left hemisphere, whereas language processing tends to be subserved by the areas located in the left hemisphere (Geschwind, 1978; Geschwind and Miller, 2001). Asymmetries observed at the gross level in the human cortex represent structural correlates of functional lateralization: adult humans display right frontal/left occipital asymmetries (Geschwind and Miller, 2001) forming an example of predictable,

species-level cortical organization unique to humans that can be traced in the hominin lineage, as documented in the fossil record (see discussion below).

An important feature of cortical asymmetries is that they represent essentially a developmental phenomenon. Asymmetries can be observed in perisylvian regions and the planum temporale prenatally (30 gestational weeks; Chi et al., 1977a), and differences in gene expression between the two hemispheres are observed even earlier in the development (12–14 gestational weeks; Sun et al., 2005). During development, the right hemisphere may exhibit a faster tempo of development compared to the left hemisphere (Chi et al., 1977b; Sun et al., 2005) and the pattern of asymmetries seen in adults is either absent or reversed in infants and children. The typical adult-like pattern of asymmetry emerges during adolescence (Shaw et al., 2009). At the same time, structural asymmetries are either absent or reversed in several disorders – including dyslexia (Geschwind and Galaburda, 1985), autism, and developmental language disorder (Herbert et al., 2005). Changes in functional hemispheric dominance were reported in individuals with brain injuries (Joseph, 1986) and following corpus callosotomy (Gazzaniga et al., 1984). Taken together, these observations suggest that although development of asymmetries tends to be predictable in humans and may be primarily under genetic control, environment processing demands appear to influence the establishment of proper functional circuitry underlying functional lateralizations in humans.

Analyses of morphology of pyramidal neurons in cortical areas associated with lateralized behaviors suggest that the lateralization observed in gross anatomical studies find their equivalent at the cellular level. In language areas, the so-called “dendritic

laterality" has been reported in Broca's area, Wernicke's area, and Rolandic motor areas (Scheibel et al., 1985; Jacobs and Scheibel, 1993). The Wernicke's area equivalent in the right hemisphere was characterized by less neurophil, greater overlap among columns, and greater variability in orientation of pyramidal neurons. In the dominant (left) hemisphere, layer III pyramidal neurons were longer, more branched, and more spinous compared to the neurons in the right hemisphere. The hemispheric pattern changed with aging; in individuals older than 50 years, pyramidal neurons in the left hemisphere became more prone to degradation compared to the ones in the right hemisphere, resulting in the reversal of the dominance pattern. Unlike in younger individuals, the pyramidal cells in the left hemisphere of older individuals were shorter and less spinous than the cells in the right hemisphere (Jacobs and Scheibel, 1993). Pyramidal neurons in the language areas in the frontal lobe display a less clear pattern of hemispheric dominance. Scheibel et al. (1985) reported that the total dendritic length in Broca's area was comparable to the length of dendrites in the homologous area on the right hemisphere; the same pattern holds for Rolandic areas. The differences, however, were noted at more subtle elements of neuronal structure: pyramidal neurons in the left hemisphere were more branched and displayed greater number of high-order segments, i.e., fourth, fifth, and sixth order segments from the cell body. In the right hemisphere, pyramidal neurons in both areas displayed more lower order segments (first, second, third order) compared to the neurons in the left hemisphere. The pattern was consistent in right-handed subjects, and the hemispheric specificities was reversed in left-handed subjects (Scheibel et al., 1985). The authors

suggested that the observed pattern, namely different modification of segments relative to the proximity to the cell body, reflected segment-specific developmental timing.

The segments closer to the cell body are formed during development prior to the higher-order segments, thus before the emergence of complex, lateralized behaviors. The appearance of more branched higher order segments coincides with functional maturation of the left hemisphere as the dominant hemisphere. Alternatively, as the authors suggested, higher order segments may be more plastic, and greater branching of high order segments in the left hemisphere might represent a response to higher demands of the behaviors processed in the left hemisphere (Scheibel et al., 1985).

The study by Scheibel et al. (1985) highlights an important point in examining the variability of pyramidal neurons in humans: in their adult phenotype, pyramidal neurons reflect cell-autonomous influences, as well as computational responses imposed upon them based on the area they occupy. Different parts of a pyramidal neuron may not respond in the same way to environmental influences: the parts of pyramidal neurons maturing at the time of environmental input may be more responsive in modifying their morphology, while developmentally earlier parts may remain more stable.

Developmental Plasticity in Pyramidal Neurons

The emergence of pyramidal neurons and their differentiation and establishment of proper synaptic connections represents the first step in the formation of cortical connectivity. In primates, cortical neurogenesis is limited to the first half of gestation. At embryonic day 40 (E40) in macaques and E43 in humans (Rakic, 1982), neuronal progenitor cells exit the cell cycle and migrate along radial glia toward their position in the developing cortical plate. Earlier born neurons are destined to occupy subgranular cortical

layers (layers V/VI), whereas later born neurons migrate into supragranular layers (layers II/III; Rakic, 1982). In humans at 17 gestational weeks (gw), a set of neurons in the cortical plate starts displaying morphology typical of pyramidal neurons – large somata, three to five basal dendrites with developed secondary branches, and a distinct apical dendrite directed toward the marginal zone (Mrzljak et al., 1988). With the appearance of lamination in the cortical plate, it becomes possible to distinguish pyramidal neurons in the developing layer III from those in layer V: pyramidal cells in the developing supragranular layers appear less branched and less spinous compared to their layer V counterparts, displaying overall less mature morphology (Mrzljak et al., 1988). Despite being based on a small sample of prenatal human tissue, these studies show that already at this developmental stage layer III neurons are marked by variations – the neurons in the upper part of the layer III are less branched and shorter than their counterparts in the deeper portions of layer III (Marin-Padilla, 1970; Mrzljak et al., 1988). The differences in the morphology of pyramidal neurons based on their laminar affiliations will persist throughout development and into adulthood (Petanjek et al., 2008). Layer-specific developmental differences appear particularly prominent during the perinatal period, that is, the period marked by initial neuronal response to direct environmental stimuli (Bourgeois, 1997).

It is of particular interest that layer III pyramidal neurons in human PFC, i.e., the subset of neurons characterized by the most elaborate dendritic morphology and highest number of synaptic inputs in adulthood, are the least developed neurons at birth (Petanjek et al., 2008). The early post-natal period is marked by their extensive elaboration; by the

end of the first year of life, layer III pyramidal neurons in PFC appear as developed as layer V pyramidal cells, and by the end of third year of life, they emerge as most complex neurons in the human cortex (Petanjek et al., 2008). The morphological development of pyramidal neurons tends to parallel cognitive maturation, with an increase in language abilities, working memory, and symbolic thought in human infants during the same period (Goldman-Rakic, 1987). Interestingly, further elaboration in the morphology of pyramidal neurons, although at a smaller scale, continues into adulthood (Petanjek et al., 2008), thus spanning the period of continued cognitive and behavioral maturation in humans. As environment plays a crucial role in establishing proper cortical circuitry, the immaturity of layer III pyramidal cells at birth, rapid modification in the first few post-natal years, coupled with a continued modification until adulthood, allows for establishment of basic circuitry while enabling further individuation (sensu Bourgeois, 2001), depending on individual experiences and the needs of a particular social environment.

Significant changes during the post-natal period in the developing amygdala suggest that environmental inputs play an important role in specifying its morphology. It has been demonstrated both in humans (Joseph, 1999) and macaques (Harlow and Harlow, 1969) that lack of interaction with conspecifics and the inability to form attachments during the first year of life results in social and emotional abnormalities that persist throughout adulthood, possibly underlined by improper initial inputs into the amygdala from the social surrounding of an infant. As an example, humans infants suffering from neglect soon after birth tend to develop severe emotional non-responsiveness and fear of strangers, whereas those deprived of care after 6 months of age display increased need for

attention, but remain unable to develop proper social adhesion (Joseph, 1999). In macaques, changes in social behavior and increased anxiety in adults are related to early life stress such as maternal separation. In turn, neonatal amygdala dysfunction has been shown to underlie non-adaptive responses to environmental and social stimuli. This suggests that alterations in amygdala development are linked with external changes in the environment. Monkeys with neonatal lesions demonstrate increased fear behavior in social interactions compared to control monkeys (Thompson et al., 1969; Prather et al., 2001). In contrast, monkeys with lesions produced in adulthood engage in greater amounts of affiliative social interactions than controls, suggesting a lack of social fear (Emery et al., 2001).

Structurally, the amygdala primodium first appears during the embryonic period in humans as a thickening in the wall of the interventricular foramen at the time that the hemispheres begin to evaginate. It is contiguous with the hippocampus and closely related to the striatum. The amygdala nuclei form by the migration of neuroblasts from the germinal layer of the striatal ridge, or ventricular eminence (also referred to as ganglionic eminence, Humphrey, 1968; Ulfig et al., 2003; Muller and O'Rahilly, 2006). At first, three main subdivisions emerge: the anterior amygdaloid area, the corticomedial complex, and the basolateral complex. The anterior amygdaloid area is identifiable first, followed shortly by the corticomedial complex (the cortical, medial, and central nuclei) and then the basolateral complex. Before the end of the embryonic period fiber connections develop between the amygdaloid nuclei and the septal, hippocampal, and diencephalic regions (Muller and O'Rahilly, 2006).

In the fifth gestational month in humans, aggregations of cell columns extend from the ventricular eminence into the basolateral complex. The presence of radial glia (demonstrated by vimentin immunoreactivity) between the columns suggests that these aggregations represent early migratory systems. In the sixth and seventh gestational months the cell columns begin to lose their connections with the ventricular eminence and fibers are no longer found between the cell columns. Finally, in the eighth and ninth month the aggregates of cell columns are no longer present and the lateral nucleus appears distinctly separate from the ventricular eminence (Ulfig et al., 2003). In parallel with this development, punctate immunolabeling of GAP-43, which is correlated with synaptogenesis (McGuire et al., 1988), appears in the fifth gestational month in the corticomedial complex and in the seventh month in the basolateral complex. By the ninth month there is no longer evidence of GAP-43 in the amygdala (Ulfig et al., 2003).

The amygdala in primates is immature at birth and its development thus depends on incoming stimuli from the environment. Differentiation of individual amygdala nuclei continues from the embryonic period through the fetal period and on into the post-natal period. Many nuclei exhibit distinct developmental profiles. For example, post-natally in macaque monkeys, the nuclei of the basolateral complex demonstrate a dramatic enlargement in volume between birth and 3 months of age, with slower growth continuing beyond 1 year. In contrast, the medial nucleus is near adult size at birth, while the volume of the central nucleus is half the adult value at birth and exhibits slow but significant growth even after 1 year of age (Chareyron et al., 2012). At a cellular level, early pyramidal neurons can be distinguished in the human amygdala by the eighth and ninth

gestational months. Similarly to the pyramidal neurons in the neocortex, these early pyramidal neurons are characterized by medium diameter dendrites that emerge from pyramidal-shaped soma, a stout branching dendrite emerging from opposite pole of the soma, and an axon emerging from the base of the pyramids. The onset of synaptogenesis is delayed in the basolateral complex relative to the corticomedial complex (Ulfig et al., 2003). Since the lateral nucleus is characterized as derived in its organization in humans (Barger et al., 2007, 2012) and functions as an important part of the network processing of social and emotional stimuli, it remains possible that a prolonged period of maturation enables establishment of social and emotional bonds extending beyond the mother; a feature in particular important in humans species, where sharing offspring care represents an evolutionary strategy for increasing reproductive success (Hrdy, 2005). Compared to humans, infant care is less extensively shared among group members in great apes and most Old World monkeys, and the nature of alloparenting thus differs between humans and other primates.

Among the Efé of Central Africa, for example, by 18 weeks of age infants spend more than half a day with caregivers other than their mothers, averaging about 14 caretakers including both related and unrelated individuals (Hrdy, 2005). In comparison, a systematic study of alloparental episodes among the chimpanzees in Mahale Mountains, Tanzania, suggests that only certain members of the troop (e.g., nulliparous females) tend to display interest into handling infants, whereas parous females remain indifferent to the offspring of other females (Nishida, 1983). A similar pattern was observed among Japanese macaques (*Macaca fuscata*; Hiraiwa, 1981). Even among the species where

infant sharing is quite common, such as Barbary macaques (*M. sylvanus*; Small, 1990), the mother remains the primary caretaker of the infant, and alloparenting never reaches the extent seen in humans. Similarly, the development of 'stranger distress' is delayed in human infants compared to other primates, appearing at approximately 7 months in humans, 4 months in chimpanzees, and 3 months in macaques (reviewed in LaFreniere, 2005). Although the appearance of fear reaction to strangers doubtlessly depends on other cognitive (e.g., development of the concept of the caregiver; LaFreniere, 2005) and neural changes (e.g., neocortical maturation; Goldman-Rakic, 1987), developmental changes in the amygdala nevertheless underlie the emergent fear response in primates during the first year of life.

Epigenetic and Molecular Aspects of Human Brain Evolution

It has been proposed that the environment mediates the establishment of neuronal morphology by two mechanisms of plasticity: experience-expectant plasticity, preparing neuronal circuits for ubiquitous environmental inputs, and experience-dependent plasticity, responsive to the circumstances unique to each individual (Greenough et al., 1987). Experience-expectant plasticity likely reflects evolutionary mechanisms emphasizing a particular type of sensory processing shared by all members of a species (Greenough et al., 1987). This is manifested by overproduction of synapses during the perinatal period in cortical areas subserving the sensory system in question, followed by a rapid pruning of synapses at the end of the period. Experience-dependent plasticity, on the other hand, is less predictable, characterized either by prolonging the period of synapse overproduction or delaying the offset of synaptic pruning (Bourgeois, 1997). Synaptogenesis in the primate visual cortex represents a typical example of experience-expectant plasticity. In

rhesus macaques, rapid production of synapses in primary visual cortex (V1) begins 2 months before term, becomes intensified around birth, and ends at post-natal day 61 (P61; Bourgeois, 1997). The rate of synapse production remains stable even if the monkeys are delivered before term – thus exposed to light prematurely compared to the full-term controls – although the maturation rate of synapses appears to proceed faster in pre-term macaques (Bourgeois et al., 1989). It has been proposed (Joseph, 1999) that development of the amygdala and associated cortical regions involved in processing emotional and social stimuli represent another example of experience-expectant maturation (Harlow and Harlow, 1969; Joseph, 1999).

Experience-expectant plasticity is often associated with critical periods in development (Greenough et al., 1987) and it is in particular prominent in the maturation of sensory systems. In contrast, the basic premise of experience-dependent plasticity proposes that the opportunity to acquire complex behaviors varies across individuals and that the nature of the acquired information will differ from one animal to the next (Greenough et al., 1987). This type of plasticity underlies acquisition of multifaceted behaviors, including navigating one's social and ecological surroundings, language acquisition, and ability to acquire new technical and behavioral skills. Rather than providing a developmental window in which stimuli are necessary to establish functional circuitry, experience-dependent modifications are possible in late-maturing regions, depending on individual circumstances (Greenough et al., 1987). In macaques, rapid development of synapses proceeds uniformly in both V1 and PFC, although the two areas harbor two rudimentary different types of processing (Bourgeois et al., 1994). In humans, on the other hand,

development of synaptic densities is postponed in PFC compared to other cortical regions (Huttenlocher and Dabholkar, 1997), suggesting that maturation of executive control in humans may be postponed compared to macaques, allowing for a prolonged period of modifications. Dendritic systems of pyramidal neurons in human PFC continue to mature longer than PFC neurons in macaques (Cupp and Uemura, 1980; Petanjek et al., 2008), with elaboration of dendritic branching continuing until adolescence (Petanjek et al., 2008) and maturation of spines proceeding until the third decade of life (Petanjek et al., 2011). The prolonged period of maturation of cortical microcircuitry in PFC thus encompasses two developmental stages unique to humans: childhood and adolescence (Bogin and Smith, 1996; Bogin, 1997). The additional period of cognitive plasticity in humans enables the acquisition of baseline skills necessary for successfully navigating social and ecological environments (Leigh and Park, 1998; Flinn, 2005), forming the basis for their elaboration in later life (Geary, 2005). It is important to note, however, that modifications in cortical microcircuitry continue throughout life, even without obvious pathologies or physical traumas (Jacobs and Scheibel, 2002), enabling modifications of behavioral responses to newly encountered circumstances.

A discussion about plasticity inevitably introduces the question of cell-intrinsic and epigenetic influences on the development, and the relative importance of each in influencing a particular aspect of neuronal morphology. The development of new comparative genomics, epigenetic analyses, and gene expression tools has catapulted interest in the molecular aspects of human brain evolution. Variability selection posits the importance of regulatory mechanisms of gene expression in lineages subjected to

variability selection (Potts, 1998), with the activity especially prominent during development; comparative studies across primates have suggested differences in timing, increased importance of non-coding sequences, and accelerated rates of evolution of development-related genes in humans (Dorus et al., 2004; Prabhakar et al., 2006; Liu et al., 2012).

At the genomic level, several reported molecular events illustrate the complexity of human evolution. On one side, humans can acquire new genetic information. For example, KLK8 (also known as neuropsin) is a secreted-type serine protease that is involved in synaptogenesis, neurite outgrowth, and plasticity in the hippocampus and the neocortex (Mitsui et al., 1999). A human-specific point mutation gave rise to a novel functional isoform (type II) that is only expressed in humans during development in the embryo brain, suggesting a potential role in early CNS formation (Lu et al., 2007, 2009). On the other side, a loss of function is observed in the human genome, affecting a specific biochemical pathway. For example, the human deficiency of Neu5Gc is explained by the fixations of an inactivating mutation in the gene encoding CMP-N-acetylneuraminic acid hydroxylase, the rate-limiting enzyme in generating Neu5Gc in cells of other mammals. The mutation occurred after the split from our last common ancestor (Chou et al., 2002). Fixation in the ancestral population occurred at an unknown time thereafter and happens to be one of the first known genetic differences between humans and other hominids with an obvious biochemical readout. Together, these data are consistent with the presence of human-specific genomic alterations.

Alteration in gene expression is a common mode of evolutionary change and can result from multiple changes in the genome, affecting regulatory regions such as promoters and enhancers. These alterations may affect gene dosage, timing and localization. Some studies suggested several differences that seem human specific: the majority of genes showing expression differences between humans and chimpanzees are upregulated in the human cortex (Cáceres et al., 2003) and show a species-specific pattern of expression (Enard et al., 2002). Gene expressions in regions involved in complex cognitive tasks tend to resemble one another, differing from the expression profiles in primary processing areas (Khaitovich et al., 2004). At the same time, comparative studies of gene expression between humans and chimpanzees suggest that the overall pattern of gene activity during the post-natal period is shared between these two species. However, compared to chimpanzees, about half of genes specific to a particular developmental stage are expressed at different levels in humans. Moreover, the difference between the two species increases over time, with the greatest difference occurring at 10 years of age (Somel et al., 2009). Several functional groups of genes involved into synaptogenesis and neuronal function display prolonged expression in humans compared to chimpanzees and macaques; in humans, their levels remain high during the first 5 years of life whereas in chimpanzees their levels decline early in the post-natal period. As a comparison, the same set of genes is elevated prenatally in macaques (Liu et al., 2012). Overall, the comparative molecular analyses of brain development suggest a tendency toward heterochrony – with a prolonged period of expression in humans compared to other primates – an increased role of

regulatory mechanisms, and regional differences in gene expression across distinct brain regions.

Throughout the life of an individual, the brain faces two opposable needs: on one side, maintenance of the established functional circuitry and on the other, remodeling of the circuits in response to newly imposed computational needs (Abrous et al., 2005). Different parts of the brain may have solved this dilemma differently: regions characterized by continuous neurogenesis (e.g., hippocampus) through the addition of new neurons and the establishment of new circuitry (van Praag et al., 2002), while the non-neurogenic regions (e.g., the neocortex) through modifications in morphology of the existing neurons (Abrous et al., 2005). Morphological changes of pyramidal neurons – length, branching, and the number and distribution of dendritic spines – have been reported in the cortex of human subjects following physical (Jacobs et al., 2003) and chemical (Glantz and Lewis, 2000) changes, or behavioral manipulations in laboratory animals (Bock et al., 2005; Cerqueira et al., 2007). In a study of macaques raised in a cage without enrichment and with only visual contact with conspecifics, Bryan and Riesen (1989) reported decrease in density of spines on apical dendrites in V1 pyramidal neurons, but no reduction in their overall branching complexity. The same conditions resulted in decreased length, arborization, and density of spines on apical dendrites in primary motor cortex (M1; Bryan and Riesen, 1989), suggesting that the effects of deprivation affected neurons in different cortical regions differently, and that some parts of pyramidal morphology (e.g., spines) appear more prone to environmental influence than the others. These findings tend to be supported by gene expression analyses: expression of the immediate early genes

(IEGs) in the cortex has been associated with learning and memory (Kaufmann and Worley, 1999), and electrical activity in neurons appears to mediate the effects of brain-derived neurotrophic factors (BDNF) in the developing cortex (McAllister et al., 1996). Expression of some of IEGs seems to be focused specifically on dendrites (McAllister et al., 1996) and on dendritic spines (Schratt et al., 2006), facilitating rapid morphological modifications of the neurons.

An example of changes in neuronal morphology reported by Jacobs et al. (2003) suggests that the human cortex may respond to the same stressor differently than the cortex of other mammals. Several decades after undergoing corpus callosotomy, pyramidal neurons in layer III developed unusually long, branched, and spinous basal dendrites, which descended deep into subgranular layers. These 'tap root' dendrites were in particular common in Broca's area (Jacobs et al., 2003), which shares connections with its homolog in the right hemisphere and receives numerous interhemispheric afferents from the right inferior temporal cortex (Di Virgilio and Clarke, 1997). The unusually developed basal dendrite, as the authors suggested, may represent an attempt by the neurons to maintain their function after losing cross-callosal inputs by increasing the area available for connections within the same hemisphere. In rabbits, callosotomy resulted in the decrease of spine number on oblique branches of apical dendrites in the parietal cortex, while at the same time the morphology of basal dendrites remained largely unaffected (Globus and Scheibel, 1967). These findings suggest that several factors – including the highly lateralized function of Broca's area and an increased reliance on regulatory mechanisms modulating the relationship between cell structure and neuronal activity – may underline

the observed differences in the modifications of neuronal morphology between the two species. The study thus reinforces conclusions implicit to numerous comparative studies – that the cortex of each species is a product of its evolutionary history, favoring a particular way of processing or, in morphological terms, a particular pattern of cortical connectivity that is layer-, area-, and likely species-specific. While it is reasonable to expect that the neurons with the same biophysical properties will respond to the stimulus in a similar way, regardless of the species or the area they occupy, functional demands imposed upon the neurons likely differ, and their morphology will change in response to the epigenetic factors differently, depending on nature of the network they form.

The Direct Evidence of Human Brain Evolution: The Fossil Record

Fossil hominin endocasts can provide important clues to identify modifications of the human brain during evolution. An endocranial cast, or endocast, is a cast of the inner table of the cranial bones. Fossil endocasts are either naturally formed via filling and consolidation of sediment inside the braincase during the fossilization process, or artificially human-made. Endocasts of fossil specimens are the only available remnants of the morphology of their brains; as such, fossil hominin endocasts represent the only direct evidence of human brain evolution.

Endocasts preserve only some gross morphological characteristics of the brain's outer surface, as pia mater, arachoid tisue, and dura mater form a buffer preventing the brain from leaving imprints in the inner cranium. Typically, estimates of cranial capacity can be reliably extrapolated based on the endocasts, whereas finer aspects of cerebral organization, such as gyral and sulcal pattern, remain more problematic and debatable (Holloway et al., 2004). Correlating microanatomical information with endocasts is a

multistep process bridging microanatomy obtained from post-mortem histological sections with gross brain anatomy obtained from MRI. Such attempts have been made recently (e.g., Schenker et al., 2010; Annese, 2012; Yang et al., 2012), opening a promising field for future research. The second step is to evaluate the relationships between gross external neuroanatomy and endocranial morphology. Complex interactions throughout head ontogeny involve the brain, meninges, cranial vault, basicranium, face, mandible, and masticatory muscles (e.g., Moss and Young, 1960; Moss, 1968; Lieberman et al., 2000; Bastir et al., 2004; Bruner, 2004; Richtsmeier et al., 2006; Mitteroecker and Bookstein, 2008; Neubauer et al., 2009). Despite these interactions the shape of the cranial inner table (i.e., the shape of the endocast) reflects the shape of the brain until brain growth completion and throughout adulthood until incipience of brain tissue shrinkage (Courchesne et al., 2000; Resnick et al., 2003; Scahill et al., 2003; Kruggel, 2006; Sherwood et al., 2011; Ventrice, 2011). For this reason, endocranial volume and shape are used as proxies for brain size and shape.

The endocranial fossil record has been extensively reviewed (e.g., Bruner, 2003; Holloway et al., 2004; Falk, 2007, 2012). The ongoing study of the virtually reconstructed endocast of *Sahelanthropus tchadensis* (Brunet et al., 2002; Bienvenu et al., 2013), dated to 7 Ma (Mega Annum, a period of one million years) will open a unique window on the earliest stages of hominin brain evolution. Indeed, apart from this specimen, the earliest known hominin endocasts belong to australopiths dated around 3 Ma from South Africa and East Africa. They are formally separated into gracile (genus *Australopithecus*) and robust (genus *Paranthropus*) forms. Origins of the genus *Homo* are thought to be nested

within genus *Australopithecus*, while robust australopiths are generally considered as side branches. The earliest *Homo* endocasts come from East Africa and date to less than 2 Ma. *Homo erectus sensu lato* is the earliest species known out of Africa around 1.8 Ma, found in Caucasus and Indonesia. *H. heidelbergensis* encompasses African and European fossils from the middle Pleistocene (between about 0.8 and 0.1 Ma). African *H. heidelbergensis* specimens may be ancestral to *H. sapiens*, while European specimens may be ancestral to *H. neanderthalensis*, Eurasian late archaic *Homo* ranging in age from about 0.2 Ma to 30,000 years ago. Australopiths are characterized by great ape-sized brains. When brain size began to increase in hominins is debated: increase in brain size began either gradually from around 3 Ma (Falk et al., 2000) or suddenly from around 2 Ma (Carlson et al., 2011; Table 1.1).

The evolution of hominin brain ontogeny is attracting increasing interest (Zollikofer and Ponce de León, 2010, 2013; Leigh, 2012; Neubauer and Hublin, 2012) and deserves special attention here. Ontogeny includes growth (increase in size with age) and development (modifications in shape with age). From the growth perspective, the brain of modern humans is already bigger at birth compared to newborn chimpanzees (400 versus 145 cc; Zollikofer and Ponce de León, 2013) and it experiences a growth spurt during the first two post-natal years. This rapid initial growth does not occur in chimpanzees (Sakai et al., 2013) and it may account for our large adult brains, three to four times bigger than the brains of chimpanzees (1350 versus 385 cc; Zollikofer and Ponce de León, 2013). Brain growth slows down after the growth spurt, and brain size approaches that of adults after eruption of the first molar. From the developmental perspective, endocasts of humans and

chimpanzees already have distinct shapes at birth, reflecting different prenatal ontogenies: notably, human neonates have squared-off frontal lobes (Zollikofer and Ponce de León, 2013). During early post-natal development, the human brain undergoes an extensive period of growth and there are modifications of the endocranium, including expansion in the parietal area and widening of the post-erior temporal parts (Neubauer et al., 2010). This change results in a more globular shape of the human cranium compared to both chimpanzees and late archaic Homo (i.e., H. heidelbergensis and Neanderthals; Lieberman et al., 2002; Neubauer et al., 2010; Gunz et al., 2012, but see also Ponce de León et al., 2013 for shared patterns among hominids). Although each extant ape species evolved its own ontogenetic trajectory, as exemplified by the differences between chimpanzees and bonobos (Lieberman et al., 2007; Durrleman et al., 2012), the early post-natal growth spurt and the associated "globularization phase" appear to be developmental features unique to anatomically modern humans and are either absent, or undetectable, in the developing great ape crania.

An important topic in paleoneurological studies is dating the transition from a more ape-like pattern of brain growth and development to a modern human pattern. There is some support for the idea that fossil hominin maternal pelvic dimensions can be used as an indirect source of information for neonatal brain size as in modern humans (Tague and Lovejoy, 1986), but it has also been argued that australopith female pelvic dimensions are larger than neonatal neurocranial dimensions, and obstetrical constraints were absent in australopiths as in extant great apes (Leutenegger, 1987). Moreover, taxonomic attribution of some important pelvic remains is also debated (Simpson et al., 2008; Ruff, 2010). For

these reasons, we will only review the evidence coming directly from the endocasts of juvenile fossil hominins, in a chronological order.

Australopith brain ontogeny is documented mainly by the endocasts from Dikika and Taung. The Dikika child (*Australopithecus afarensis*), dated to 3.3 Ma, has an estimated age at death of approximately 3 years and an estimated endocranial volume between 275 and 330 cc (Alemseged et al., 2006). The Taung child (*A. africanus*; Dart, 1925), dated to 2.6–2.8 Ma (McKee, 1993), has an estimated age at death between 3.5 and 4 years (Lacruz et al., 2005) and an estimated endocranial volume of 405 cc (Neubauer et al., 2012). Brain ontogeny in early *H. erectus* is documented by one specimen, the 1-year-old Mojokerto child, dated to 1.8 Ma and with an estimated endocranial volume of 663 cc (Coqueugniot et al., 2004). In *H. neanderthalensis*, one specimen of special interest is the 1 to 2-week-old infant from Mezmaiskaya, Russia (Golovanova et al., 1999), dated to 0.073–0.063 Ma, with an endocranial volume estimated between 414 and 436 cc (Ponce de León et al., 2008; Gunz et al., 2012). *H. neanderthalensis* is probably the best known fossil hominin species concerning brain ontogeny, the whole range of individual ages being sampled, from the neonate of Mezmaiskaya to the "old man" of La Chapelle-aux-Saints.

The endocranial volume of a juvenile fossil can be compared to the endocranial volume of humans and apes of the same age in absolute terms, as a proportion of the estimated adult brain size, or as a proportion of the estimated neonatal brain size (Zollikofer and Ponce de León, 2010). For a fossil hominin species, estimated adult brain size is calculated as the average of the endocranial volumes of the conspecific adult specimens of the same sex. Estimated neonatal brain size is predicted from the regression

of adult brain size versus neonate brain size in extant anthropoids (DeSilva and Lesnik, 2008). These three modes of comparison (absolute brain size, percentage of adult brain size, percentage of neonate brain size) may lead to different conclusions (Figure 1.2). Absolute brain growth curve and growth trajectory expressed as a percentage of neonatal brain size prove to be more discriminatory and reveal whether a species experiences a brain growth spurt or not, independently from adult brain size.

The Dikika endocast has the expected volume for a chimpanzee of the same age. The average estimated endocranial volume for adult female *A. afarensis* is 375–425 cc (Alemseged et al., 2006). The endocranial volume of the Dikika child expressed as a percentage of this expected adult endocranial volume is in the overlapping ranges of chimpanzees, gorillas, and humans. As a proportion of its estimated neonatal brain size, the Dikika endocast falls within the variability range of chimpanzees (Zollikofer and Ponce de León, 2013). The Taung child is within the chimpanzee range of variation concerning the percentage of adult endocranial volume and neonatal endocranial volume (Zollikofer and Ponce de León, 2013). However, its absolute endocranial volume is slightly greater than expected for a chimpanzee of similar age (Zollikofer and Ponce de León, 2013). Estimates of australopith neonate brain size are slightly larger than for chimpanzees (180 cc versus 150 cc; DeSilva and Lesnik, 2008), implying that chimpanzees and australopiths displayed different prenatal growths. The partially fused metopic suture observed in the Taung endocast highlights this potential difference with chimpanzees (Falk et al., 2012). The Taung metopic suture may be correlated with an enlarged neonate brain size, rapid early post-natal brain growth, and squaring-off of the frontal lobes.

With *H. erectus*, the ontogenetic trajectory approaches the one for modern humans. The Mojokerto child has an estimated endocranial volume which falls at the lower end of the modern human range (Zollikofer and Ponce de León, 2013). The average adult endocranial volume in H. erectus is lower than in modern humans; consequently, the Mojokerto child has reached a high proportion of its expected adult brain size as is the case in chimpanzees (Figure 1.2A), which led Coqueugniot and colleagues (2004) to the conclusion that the growth pattern of H. erectus was similar to that of chimpanzees. However, the estimated neonatal brain size of *H. erectus* is clearly larger than that of chimpanzees, probably about twice as large (Leigh, 2006; DeSilva and Lesnik, 2008; Zollikofer and Ponce de León, 2013). When expressed as a percentage of the estimated neonatal endocranial volume, which yields better discrimination among taxa (Zollikofer and Ponce de León, 2010), the Mojokerto child falls well within the modern human range and out of the chimpanzee range (Figure 1.2B). From this, it appears that *H. erectus* experienced an early post-natal brain growth spurt, although for a shorter period than modern humans, which led to smaller adult brain sizes.

As evidenced by the Mezmaiskaya specimen, the neonate endocranial volume in Neanderthals was similar to modern humans, around 400 cc (Hüppi et al., 1998; Ponce de León et al., 2008; but see Coqueugniot and Hublin, 2012). The pattern of brain growth as a proportion of adult endocranial volume is similar in *H. neanderthalensis* and modern humans. As *H. neanderthalensis* reach a higher adult endocranial volume than modern humans, they express differences in absolute brain growth and in the pattern of brain growth as a percentage of neonate endocranial volume. Higher values are reached because

of a more sustained post-natal brain growth spurt. The growth pattern of *H. neanderthalensis* may indeed be similar to that for ancient fossil *H. sapiens*, as a decrease in brain size has been reported in modern humans since about 0.03 Ma (Henneberg, 1998). While *H. neanderthalensis* and *H. sapiens* have similar endocranial shapes at birth (Gunz et al., 2012; but see Ponce de León et al., 2008; Zollikofer and Ponce de León, 2013), their adult endocasts have different shapes, and a recent study suggested differences in their brain organization (Pearce et al., 2013). Each species appears to reach similar brain size via distinct developmental pathways: the globularization phase occurring during the brain growth spurt is an autapomorphy (uniquely derived character state) of *H. sapiens* absent in Neanderthals (Lieberman et al., 2002; Gunz et al., 2012), which retain a similar developmental pattern to *H. erectus* (Bruner et al., 2003; but see also Ponce de León et al., 2013 for patterns present in great apes). Overall, the fossil record of juvenile endocasts suggests that the modern human brain growth pattern became established gradually from about 2 Ma in genus *Homo* (growth spurt), or even already in australopiths between 2 and 3 Ma (larger neonatal brain size). Conversely, the globularization phase typical of modern human brain development has so far not been established in the archaic *Homo*.

As discussed earlier, human cerebral hemispheres are highly specialized for different types of information processing (Gazzaniga, 2000), and this functional lateralization has its structural correlates at a gross level. Petalias, the differential expansion of one of the frontal or occipital lobe compared to its contralateral homologous, leave an impression and can be traced on the inner surface of the cranium. Fronto-occipital petalias occur together with a distortion of the midsagittal plane known as Yakovlevian

torque, in which right frontal and left occipital lobe protrude across the midline, changing the position of the interhemispheric fissure (Toga and Thompson, 2003). Most pre-adolescent humans are characterized by a left frontal-right occipital petalial pattern (Ventrice, 2011), which reverses at adolescence, so that the most widespread adult human pattern is an association of a right frontal petalia and left occipital petalia (LeMay, 1976), in correlation with right-handedness (Galaburda et al., 1978). This pattern is also dominant in great apes, but to a lesser degree (Balzeau and Gilissen, 2010; Balzeau et al., 2012). No australopith petalial pattern approaches the pronounced right frontal-left occipital petalias observed in modern humans. Such marked petalias appear in early *Homo* around 1.8–1.9 Ma ago (Table 1.1). Taken together, the insights from the fossil endocasts suggest that structural lateralization typical of our species first appeared with the emergence of the earliest Homo. The petalias observed in fossil *Homo* may reflect the emphasis on preferential processing of certain tasks in one hemisphere over another, supporting the view that cerebra of the early members of our genus, in addition to an increase in size, were characterized by changes in organization and in the patterns of information processing compared to australopiths.

Challenges for the Future

Bringing together information on the structure of the human brain, its evolution, and development from endocasts through neural systems, neuronal morphology, and epigenetic control of cortical development is a multistep task. It involves the study of the relationship between endocranial morphology and gross external neuroanatomy (Figure 1.3), as well as the relationship between gross external neuroanatomy and microanatomy (Figure 1.1). This task also goes beyond developmental influences on the establishment of

adult morphology and encompasses instead the full spectrum of the human condition, including aging, cortical modifications in cognitive and neurodegenerative disorders, and comparison with closely related species. With respect to the fossil record, analyses of endocast to brain relationships remain scarce (Connolly, 1950; Fournier et al., 2011; Ventrice, 2011). From a methodological point of view, more of such studies are needed, as they are crucial in forming inferences about brain anatomy of fossil hominids based from the imprints they left on the endocranium. Notably in the context of brain aging the brain tissue shrinks from adolescence onward in humans, while the volume occupied by cerebrospinal fluid and ventricles increases (Courchesne et al., 2000; Wanifuchi et al., 2002; Resnick et al., 2003; Scahill et al., 2003; Kruggel, 2006; Sherwood et al., 2011; Ventrice, 2011). Endocranial volume reaches a plateau at brain growth completion and, contrary to the brain, it is not significantly modified with aging (Courchesne et al., 2000; Scahill et al., 2003; Kruggel, 2006; but see Royle et al., 2013). It is reasonable to assume that neural tissue shrinkage within the solid, non-shrinking neurocranium, results in an increased gap between the brain and its case, filled with cerebrospinal fluid. This increase in the distance between the pial and endocranial surface with aging may explain why the endocranial impressions left by the growing brain become smoother in aging human individuals (Connolly, 1950; Grgurević et al., 2004; Zollikofer and Ponce de León, 2013). In addition, aged brain shrinkage is accompanied by a thickening of the inner cranial table (Royle et al., 2013), likely resulting in the osteoblastic filling of the endocranial gyral impressions (Tobias, 2006). The brain does not shrink significantly in aging chimpanzees (Sherwood et al., 2011) or in rhesus monkeys (Herndon et al., 1998), except in the most

geriatric specimens (Herndon et al., 1999; Shamy et al., 2011). The smoothing of endocranial imprints from young adulthood in apes (Connolly, 1950) is more likely due to the continued expansion of the endocranial cavity after the completion of brain growth (Zollikofer and Ponce de León, 2013). The increased magnitude of brain shrinkage in humans may be a consequence of an extended lifespan (Sherwood et al., 2011) as increased longevity is a recent acquisition of modern humans (Caspari and Lee, 2004; Trinkaus, 2011). In this context, a study of the correlation between the level of endocranial gyral and sulcal details and age across hominin species would enable us to assess whether brain shrinkage only occurs in modern humans, or also happened in extinct human species with shorter lifespans.

Beyond endocasts, the study of the relationship between gross external neuroanatomy and microanatomy of the brain tissue is of special importance to the field of human neuroscience as a whole (e.g., Amunts et al., 1999; Schenker et al., 2010; Annese, 2012; Yang et al., 2012), and we expect that as such information becomes increasingly available, it will also assist in the meaningful interpretation of hominin endocasts in the years to come. Bridging different levels of analysis is a challenge and one good example of the types of complexities involved is provided by attempts to reconstruct the evolution of Broca's area.

Broca's area is defined cytoarchitectonically as the combination of Brodmann's areas (BA) 44 and 45. Macroanatomically, Broca's area roughly corresponds to a region in the inferior frontal lobe including the pars opercularis and the pars triangularis, bounded by specific sulci. However, the correspondence between sulcal pattern and cytoarchitectonic

areas is loose in humans (Amunts et al., 1999). Broca's area is larger on the left hemisphere than its contralateral homologous area in modern humans, according to both macroanatomical MRI-based studies (Foundas et al., 1998) and histological analyses (Uylings et al., 2006). These asymmetries are reflected in human endocasts, and lateralizations in the anterior language area were traditionally scored based on the appearance of Broca's cap, i.e., the lateral and inferior bulging on the third inferior frontal convolution on the left hemisphere which corresponds to the anterior portions of Broca's area (BA 45 and BA 47; Falk, 1987; Holloway et al., 2004). The presence of the asymmetries is typically determined by comparing the measurements for width of the left and the right frontal lobe measured at the level of the cap. Even subtle differences in the measurements, coupled with qualitative observations, are indicative of differences in the extent of Broca's cap between the hemispheres (e.g., Broadfield et al., 2001). Broca's cap appears in early *Homo* around 1.8–1.9 Ma ago (Table 1.1) and great ape and australopith endocasts do not have a Broca's cap as modern humans do (Falk, 1987). Even though Broca's cap is absent in apes, an MRI-based quantification of the macroanatomical features of Broca's area homolog in African ape brains shows a significant leftward asymmetry based on ape typical sulcal patterns for the inferior frontal lobe (Cantalupo and Hopkins, 2001; but see also Sherwood et al., 2003b). At the same time, even though Broca's area can be cytoarchitectonically defined in both humans and chimpanzees (Schenker et al., 2008), cytoarchitectonic asymmetry appears to be uniquely human (Schenker et al., 2010), suggesting that the insights gained from the three levels of evidence – endocasts, soft tissue analyses, and cytoarchitectonics – are still in need of

better integration. Future studies should investigate possible asymmetries in the morphology of pyramidal neurons between the two hemispheres in additional species in primates, and ultimately asymmetric expression of genes. As discussed previously (Scheibel et al., 1985), the differences in dendritic morphology of pyramidal neurons between two hemispheres are often subtle and it remains to be seen whether morphological analysis of neurons in other hominids will shed additional light at the discrepancy between macroscopic (Cantalupo and Hopkins, 2001) and cytoarchitectonic (Schenker et al., 2010) findings. Moreover, a major challenge will be to disentangle the functional attributes of these different structural levels. Finally, a comprehensive understanding of Broca's area structure and function also needs increased sample sizes, boundaries of regions of interest consistently defined across levels to allow comparisons among different studies, and developmental insights.

Reconstructing the evolutionary emergence of the neurobiological phenotype that underlies the unique human cognitive and behavioral specializations in development and adulthood is a multistep, multifield endeavor that requires contributions from molecular, neuroanatomical, and paleontological perspectives. Although some of our focus here has been on neocortical pyramidal neurons, we attempted to demonstrate how the insights gained from different fields can be combined to construct an evolutionary history of the human brain at several levels. We focused specifically on three aspects of human brain anatomy – asymmetries, development, and age-related changes – as those provide a fertile ground for combining different perspectives in creating testable scenarios about human brain evolution. Compared to other primates, the human brain displays specificities in the

morphology of excitatory neurons in the neocortex, differences in macroscopic organization, unique patterns of post-natal development, and responds to the same environmental influences differently compared to the brains of other mammals. All of these features may have been facilitated by an expanded period for establishing cortical circuitry in humans. At the same time, rapid modifications can be achieved throughout lifetime, thus providing a neural substrate for behavioral and cognitive capacities unique to our species.

Over recent decades, the number of fossil specimens has greatly expanded, and so has our knowledge of the genetic and molecular variations across primates. Long-term studies in the field have yielded additional insights into behavioral variations, adaptations, and cognitive potentials of non-human primates. The analyses of post-mortem brain material have begun to examine variation across primates – including the great apes – focusing on the organization of the brain typical of each species in the context of its behavioral, ecological, and cognitive adaptations. To understand the evolutionary history of the human brain, human behavioral specificities and the neural circuitry enabling their appearance must be placed within the larger context of similar behaviors and structures in other primates. At the same time, these characteristics must also be placed within the context of other human adaptations, exemplified by social and cognitive aspects unique to our species. While it is challenging to fully integrate the three lines of evidence discussed in this paper into a comprehensive analysis of human brain evolution, we hope to have opened a discussion across disciplines and to have provided opportunities for further studies surpassing the limitations of each individual field.

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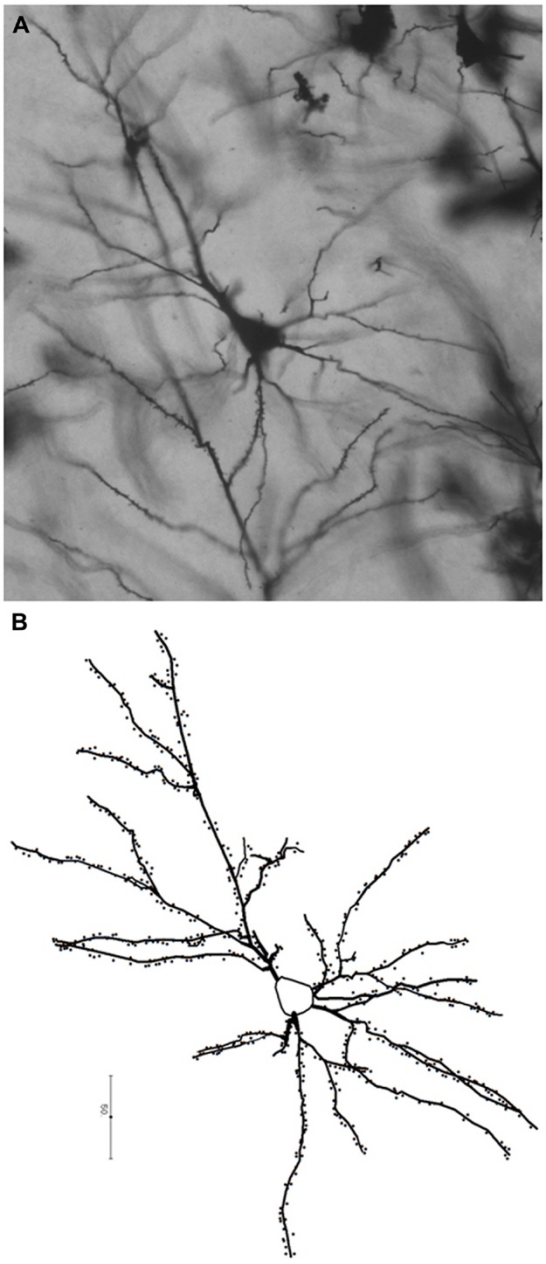

Figure 1.1. Photomicrograph (A) and a schematic representation (B) of a pyramidal neuron from the human prefrontal cortex (BA 10) processed with the Golgi–Kopsch method. Scalebar in (B) is in microns.

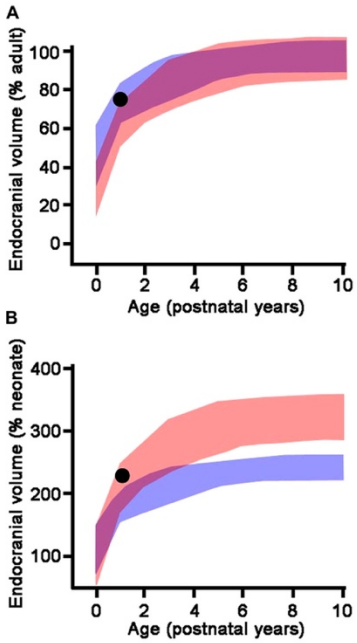

Figure 1.2. Endocranial growth trajectories as a proportion of adult endocranial volume and neonatal endocranial volume. Red areas represent modern human growth trajectories (mean ± 1 standard deviation). Blue areas represent growth trajectories for chimpanzees (mean ± 1 standard deviation). Purple areas represent overlap between human and chimpanzee growth trajectories. Black dot: *Homo erectus* (Mojokerto) infant dated at 1.8 Ma (average values for estimated age, expected adult endocranial volume, and predicted neonatal endocranial volume). As a percentage of its expected adult endocranial volume, the *Homo erectus* child follows a growth trajectory similar to chimpanzees, while as a percentage of its predicted neonatal endocranial volume, he falls within the modern human range of variation. This particular pattern accounts for the lower endocranial volume of *Homo erectus* compared to modern humans (high percentage of adult endocranial volume reached early in ontogeny), associated with an early postnatal brain growth spurt. Adapted from Zollikofer and Ponce de León (2010).

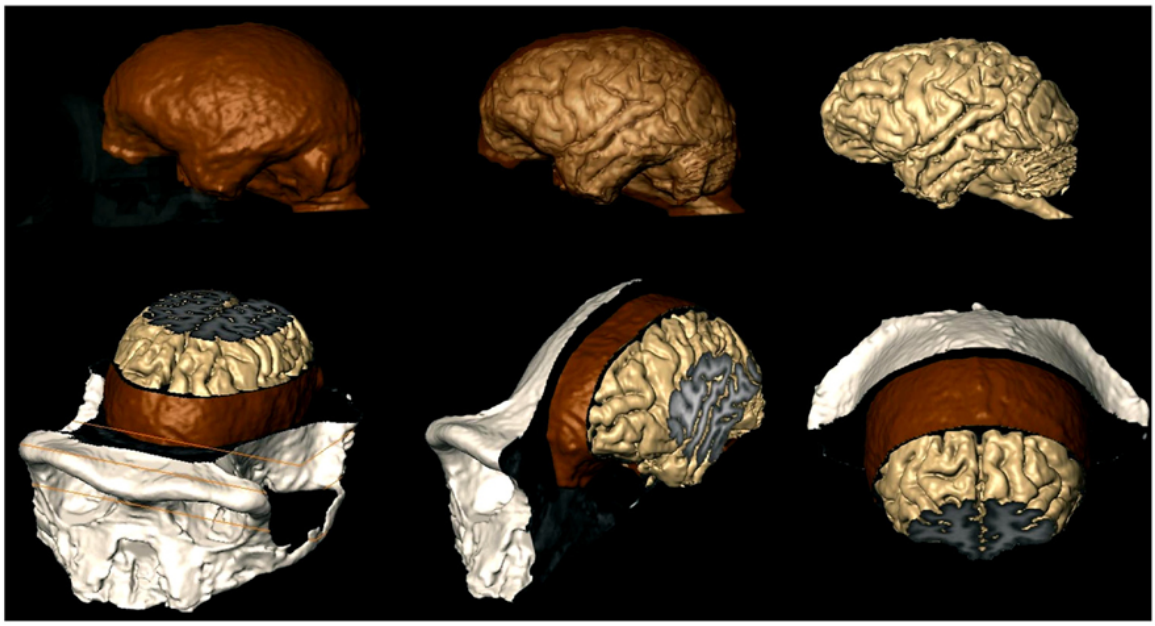

Figure 1.3 MRI techniques used to study the brains of chimpanzees (Semendeferi et al., 2002), can now investigate the relationship between the brain and the endocast as shown here. Brain is beige; endocast is brown; exocranium is white. Top row: exocranium (left) and endocast (middle) are shown transparent. Bottom row: MRI slices reveal internal structures of the brain, meninges, and bone.

Table 1.1 Endocranial asymmetries in selected fossil hominins.

Specimen	Species	Age	Location	Petalias	Broca's cap
Sterkfontein type 2	*Australopithecus africanus*	2.5 Ma	South Africa	No frontal petalia, occipital not preserved	Nascent?
MH1	*Australopithecus sediba*	2 Ma	South Africa	Right frontal	Nascent?
KNM-WT 17000	*Paranthropus aethiopicus*	2.5 Ma	East Africa	Right frontal-left occipital	Absent
OH 5	*Paranthropus boisei*	1.8 Ma	East Africa	Right frontal-left occipital?	Not preserved
SK 1585	*Paranthropus robustus*	1.5 Ma	South Africa	Left occipital	Absent
KNM-ER 1813	*Homo habilis*	1.8–1.9 Ma	East Africa	?*	Nascent?
KNM-ER 1470	*Homo rudolfensis*	1.8–1.9 Ma	East Africa	Pronounced right frontal-left occipital	Present
Any	Subsequent *Homo*	from 1.8 Ma	Africa, Eurasia	Pronounced right frontal-left occipital**	Present

Sources: Holloway and de la Coste-Lareymondie (1982); Holloway et al. (2004), Falk (2007), Grimaud-Hervé and Lordkipanidze (2010), Carlson et al. (2011), and Balzeau et al. (2012).
**Not scored consistently throughout the literature.*
***Most common pattern.*

CHAPTER II: BASAL DENDRITIC MORPHOLOGY OF CORTICAL PYRMIDAL NEURONS IN WILLIAMS SYNDROME – PREFRONTAL CORTEX AND BEYOND

Abstract

Williams syndrome (WS) is a unique neurodevelopmental disorder with a specific behavioral and cognitive profile, which includes hyperaffiliative behavior, poor social judgment, and lack of social inhibition. Here we examined the morphology of basal dendrites on pyramidal neurons in the cortex of two rare adult subjects with WS. Specifically, we examined two areas in the prefrontal cortex (PFC)—the frontal pole (Brodmann area 10) and the orbitofrontal cortex (Brodmann area 11)—and three areas in the motor, sensory, and visual cortex (BA 4, BA 3-1-2, BA 18). The findings suggest that the morphology of basal dendrites on the pyramidal neurons is altered in the cortex of WS, with differences that were layer-specific, more prominent in PFC areas, and displayed an overall pattern of dendritic organization that differentiates WS from other disorders. In particular, and unlike what was expected based on typically developing brains, basal dendrites in the two PFC areas did not display longer and more branched dendrites compared to motor, sensory and visual areas. Moreover, dendritic branching, dendritic length, and the number of dendritic spines differed little within PFC and between the central executive region (BA 10) and BA 11 that is part of the orbitofrontal region involved into emotional processing. In contrast, the relationship between the degree of neuronal branching in supra- versus infra-granular layers was spared in WS. Although this study utilized tissue held in formalin for a prolonged period of time and the number of neurons available for analysis was limited, our findings indicate that WS cortex, similar to that in other neurodevelopmental disorders such as Down syndrome, Rett syndrome,

Fragile X, and idiopathic autism, has altered morphology of basal dendrites on pyramidal neurons, which appears more prominent in selected areas of the PFC. Results were examined from developmental perspectives and discussed in the context of other neurodevelopmental disorders. We have proposed hypotheses for further investigations of morphological changes on basal dendrites in WS, a syndrome of particular interest given its unique social and cognitive phenotype.

Introduction

The prefrontal cortex (PFC) consists of a number of distinct cytoarchitectonic areas that are part of neural systems subserving higher order cognitive and emotional functions. One aspect of microstructural neuroanatomy that has received attention in PFC and in other cortical areas is the dendritic morphology of pyramidal neurons –specifically, the relationship with other areas outside PFC, variation between distinct PFC areas underlying different aspects of information processing, and variation across cortical layers within the same cytoarchitectonically defined PFC area. Investigations of pyramidal neurons, as the most common neuronal morphotype in the cortex (DeFelipe and Fariñas, 1992; DeFelipe et al., 2002), provide insights into the units forming the basis of cortical microcircuitry within a functional area, their response to various environmental inputs (i.e., plasticity; reviewed by Hanson et al., 2014), and illuminate the neuroanatomical substrates underlying various disorders in an evolutionary and developmental perspective.

Studies of pyramidal neurons in typically developed controls (TD) suggest that the length, number of branches, and number of dendritic spines on basal dendrites of cortical pyramidal neurons differ across functionally distinct cortical areas in adults (Jacobs et al., 2001). Dendrites on pyramidal neurons in high-integration areas, such as BA 10, are

typically longer, more branched, and have more dendritic spines than neurons in cortical areas devoted to a specific modality, like areas in the motor, sensory or visual cortex. Basal dendrites in BA 11 neurons are shorter, less branched and less spinous than in BA 10, differing only slightly from the primary somatosensory cortex (BA 3-1-2; Jacobs et al., 2001), the area with the least complex basal dendritic morphology. Primary motor cortex (BA 4) and secondary visual area (BA 18) are intermediate between BA 3-1-2 and BA 10, aligning more closely with the primary processing areas than with the high-integration ones (Jacobs et al., 1997, 2001). Basal dendritic morphology has also been shown to vary across layers within the same area—in PFC (BA 9), studies comparing layers II/III to V/VI in TD identify the basal dendrites of neurons in layer III as longer than those observed in infragranular layers (Petanjek et al., 2008), although some variation is present.

Developmentally, PFC is distinct from the rest of the cortex, with some aspects of its anatomy (e.g., dendritic spines) not reaching maturity until well into the third decade of life (Petanjek et al., 2011). Patterns similar to those described in neuroanatomical studies have been supported by gene expression analyses of the developing brain, suggesting prolonged activity of genes involved into maturation of synapses in PFC (Somel et al., 2009; Liu et al., 2012). This prolonged period of plasticity could potentially make PFC more prone to modifications in neurological disorders (Harris et al., 2009; Penzes et al., 2011). In various neurological disorders—ranging from schizophrenia, autism spectrum disorder (ASD), to chromosomal aberrations (Armstrong et al., 1998; Garey et al., 1998; Glantz and Lewis, 2000; Hutsler and Zhang, 2010)—PFC areas tend to display differences between the affected individuals and TDs. In neurological disorders, the organization of

basal dendrites on pyramidal neurons—dendritic length, branching, number and organization of dendritic spines—is often compromised compared to TD subjects. Of particular interest is dendritic morphology in neurodevelopmental disorders characterized by an early onset of symptoms, specific cognitive impairments, and associated with specific genetic etiology, because they allow for the examination of the interplay between development, neuroanatomy, and genetics. Examples include trisomies (Down syndrome, Patau syndrome, Edwards syndrome), Rett syndrome (RTT), and ASD. In each of the above disorders, differences with TD subjects appear postnatally (Marin-Padilla, 1972, 1976; Jay et al., 1990) and dendritic morphology varies according to cortical area and cortical layers, suggesting that distinct aspects of dendritic morphology may be compromised in each disorder (but see also Kaufmann and Moser, 2000 for consistent findings of dendritic spine anomalies across disorders). In each of the disorders where PFC is included in the analysis, PFC areas typically exhibit compromised dendritic morphology, with differences appearing to be area- and layer-specific.

Williams syndrome (WS) is a rare disorder caused by the hemideletion of ~25 genes on chromosome 7 and characterized by an unusual sociability and preservation of certain linguistic aspects, coupled with a compromised spatial and general cognition (Bellugi et al., 1999, 2000). WS phenotype can thus be contrasted with ASD, especially since duplication on the WS deleted region on chromosome 7 has been implicated in some cases of ASD (Berg et al., 2007; Sanders et al., 2011). Unlike ASD, WS has a very specific set of affected genes and related phenotypic expression, which makes this disorder most suitable for investigations of the relationship among genes, brain, and behavior. Given the

importance of PFC in social behavior, studying WS neuroanatomy allows to examine the possibility that changes in human social behavior can be traced to compromised PFC cortical microcircuitry. Here, we examined pyramidal neuron morphology in rare postmortem tissue from two WS individuals. We focused on the morphology of basal dendrites in supra- and infragranular layers in areas BA 10 and BA 11 in PFC and selected motor, somatosensory, and visual (MSV) areas—the primary motor (BA 4), primary somatosensory (BA 3-1-2), and secondary visual cortex (BA 18)—to examine whether PFC is differentially affected in comparison to other cortical regions.

More specifically, we addressed three questions: (1) does the morphology of basal dendrites in layers II/III reveal more branching in PFC than in MSV; (2) are there differences in the morphology of basal dendrites in layers II/III within PFC, between BA 10 and BA 11; and (3) are the basal dendrites of supragranular pyramidal neurons more complex than the basal dendrites in the infragranular pyramidal neurons? Although the present sample is small, due to a combination of the scarcity of postmortem tissue and the capriciousness of the Golgi technique, insights into the neural phenotype of this rare disorder can assist with the exploration of possible mechanisms leading to neurodevelopmental disorders (Chailangkarn et al., 2016) and can provide a platform for questions pertaining to the neural underpinning of human sociality and the evolution of the brain, specifically the PFC.

Materials and Methods

Subjects

The morphology of pyramidal neurons was examined in the postmortem brain tissue of two adults with Williams Syndrome (WS). WS1 was a 31-year-old male, and WS

6 was a 47 year-old-male. Both died of cardiorespiratory arrest. WS diagnosis was established based on the Diagnostic Score Sheet (DSS) for WS subjects and, for WS 6, the diagnosis was further confirmed with fluorescent in situ hybridization (FISH) probes for elastin (ELN), which revealed hemizygous deletion of the elastin gene. The subjects were not diagnosed with any other conditions besides WS, although WS 6 was reported to have had an ischemic stroke, and to have suffered from aphasia and some agraphia. Both brain specimens were harvested within a postmortem interval of 18–30 h and had been kept in 10% formalin for up to 20 years. Given the long fixation time of our specimens, we have utilized the Golgi-Kopsch method (see below) suitable for specimens kept in formalin for 15 months or longer (Rosoklija et al., 2003). This method has been previously successfully used for analysis of dendritic morphology in human samples (Jacobs et al., 2003). Special attention was paid to factors influencing the outcome of methods involving silver crystals, such as temperature, exposure to light, and agitation (Rosoklija et al., 2003), which were kept constant in all of our specimens.

Anatomic Delineations of Regions of Interest

Tissue was sampled from the following cortical areas in the left hemisphere in a manner consistent to previous studies on TDs: frontal pole (BA 10), orbitofronal cortex (BA 11), primary somatosensory cortex (BA 3-1-2), primary motor cortex (BA 4), and secondary visual area (BA 18; both hemispheres to allow for adequate number of Golgi-impregnated neurons). Blocks from PFC were removed from the rostral part of the frontopolar gyrus in case of BA 10, and from the most rostral portion of the lateral orbital gyrus for BA 11. BA 3-1-2 and BA 4 were sampled from adjacent regions of the post- and pre-central gyri, representing the arm/hand region. For BA 18, the sample was located ~1.4

cm superior to the inferior surface of the occipital lobe and 2 cm from the midline. From each region of interest (ROI), a cortical block of 5 mm3 was used for Golgi processing.

Tissue Processing and Morphological Analysis of Neurons

Cortical samples were processed using a modified Golgi-Kopsch technique (Jacobs et al., 2003), which appears effective for tissue stored in formalin for a prolonged period (Riley, 1979). The blocks sampled from each ROI were immersed in a 3% potassium-dichromate, 0.5% formalin solution and kept at 28°C for 8 days. The blocks were then transferred into 0.75% silver nitrate for 2 days and sectioned on a vibratome at a thickness of 120 μm. The sections were cut in 100% ethyl alcohol and transferred briefly into methyl salicylate, followed by toluene, mounted onto glass slides and cover-slipped.

In order to enable identification of the position within cortical layers for each traced neuron, adjacent blocks from each ROI were sectioned at 60 μm and stained for Nissl. These sections were used for a cytoarchitectonic analysis of the cortex in WS (Lew et al., 2017), and allowed for measurements of cortical laminar boundaries relative to the pial surface. In the Golgi stained sections, we measured the depth of the cell body relative to the pial surface and thus were able to distinguish neurons from supra- (layers II/III) and infra-granular (layers V/VI) layers.

Analysis of dendritic morphology was conducted only on neurons that displayed fully impregnated somata and three or more basal dendrites with at least third order dendritic branching (cf. Jacobs and Scheibel, 1993; Jacobs et al., 1997; Figure 2.1). The Golgi Kopsch method gave adequate staining mostly on basal dendrites; for this reason, and following Jacobs et al. (2001) in their analysis of basal dendritic morphology in typical subjects, we have limited our analysis to basal dendrites. Some of the analyzed dendrites

displayed incomplete endings. They were included if they otherwise displayed the criteria for inclusion as outlined above (as recommended by Uylings et al., 1986), since including only neurons with dendritic arbors entirely contained within 120 μm thick sections biases the sample toward smaller neurons. All neurons included were oriented with the apical dendrite perpendicular to the pial surface; inverted and horizontal pyramidal cells were not analyzed. The analysis was conducted on basal dendrites in a total of 61 neurons from supragranular layers (between 1 and 10 neurons/ROI; Table 2.1). An additional 28 neurons from infragranular layers were successfully Golgi-impregnated and analyzed for the comparison of basal dendritic morphology between layers. Based on the success of the Golgi-Kopsch method, we were able to obtain sufficient number of neurons in the supragranular layers to statistically analyze basal dendritic morphology across cortical areas, and to compare infragranular layer neurons in all MSV areas combined to both PFC areas combined.

Neuronal morphology was quantified along x-, y-, and z-coordinates using "Live Image" option on Neurolucida v.10 software (MBF Bioscience, Williston, VT) connected to Nikon Eclipse 80i microscope, with 40x(0.75) Plan Fluor dry objective. As the application of Sholl's concentric spheres or Eayrs' concentric circles for the analysis of neuronal morphology is not recommended when analyzing in three-dimensions (Uylings et al., 1986), we conducted dendritic tree analysis and included to following variables (cf. Jacobs and Scheibel, 1993; Jacobs et al., 2001): (1) total dendritic length (TDL)—summed length of all basal dendrites/neuron; (2) dendritic segment count (DSC)—total number of basal dendritic segments/neuron; (3) dendritic spine number (DSN)—total number of

dendritic spines/neuron; (4) mean segment length (MSL)—mean length of basal dendrite/neuron (calculated as TDL/DSC); (5) mean segment count (MSC)—mean number of segments/100 μm of basal dendritic length.

All tracings were conducted by the same investigator (Branka Hrvoj-Mihic), blind to the diagnosis and ROI. Intrarater reliability was assessed by having the tracer trace the same neuron after a period of time. The average coefficient of variation between the results of retraced neurons was 2% for TDL and DSC, and 3% for DSN. The accuracy of the tracings was further checked by having three raters (Branka Hrvoj-Mihic, Bob Jacobs, Lisa Stefanacci) trace the same neuron.

Statistical Analyses

Statistical analyses were conducted to address three lines of investigation: (1) variation in the morphology of basal dendrites across cortical areas in supragranular layers in each subject (WS 1, WS 6); (2) differences in the morphology of basal dendrites between PFC (BA 10 and BA 11) and MSV (BA 4, BA 3-1-2, BA 18) in each subject; and (3) comparison of dendritic morphology between supra- and infra-granular layers in high-integration (PFC; BA 10 and BA 11) versus MSV (BA 4, BA 3-1-2, BA 18) cortical areas in each subject.

Variation in basal dendritic morphology across cortical areas was analyzed using single-factor ANOVA. For the analysis of dendritic morphology between supra- and infragranular layers, as well as between combined PFC and MSV areas, the data were analyzed using unpaired Student's t-test with Welch correction or, in cases where the data did not display a normal distribution, using Mann-Whitney test. All analyses were performed on Prism v.7 (GraphPad Software, Inc.). Since the Golgi modification used in

this study differed from the modifications used in studies examining morphology of dendrites in TDs (Golgi-Kopsch versus rapid/Golgi Cox; Jacobs et al., 2001; Petanjek et al., 2008), we were not able to directly compare absolute values across studies. Instead, our analysis focused on relative differences in basal dendritic length and branching within different cortical areas and layers within each WS subject.

Results

Morphology of Basal Dendrites in Supragranular Cortical Layers in WS

Comparison of basal dendritic morphology in supragranular pyramidal neurons revealed no statistically significant difference in dendritic/spine measures for any of the individual cortical areas, either in WS 1 or WS 6 (Figures 2.2, 2.3). When the values for the two PFC areas—BA 10 and BA 11—were grouped together and analyzed against MSV areas (BA 4, BA 3-1-2, BA 18) combined, the difference between PFC and MSV reached statistically significant difference in WS 6. Specifically in WS 6, MSL values were significantly higher for MSV areas compared to the two PFC areas combined ($P = 0.02$) and the reverse was the case for MSC, with values higher in PFC ($P = 0.03$; Figures 2.5D,E). The other measures did not reveal any statistically significant differences (Figures 2.4, 5A–C).

Comparison of Basal Dendrites between Supra- and Infragranular Layers

We also examined pyramidal neurons in infragranular cortical layers in order to compare the basal dendritic morphology in supra- versus infragranular layers for PFC (BA 10 and BA 11) versus the three MSV areas (BA 4, BA 3-1-2, BA 18) combined. In PFC of WS 6, TDL and DSC values were higher in supragranular than in infragranular layers, reaching statistical significance (TDL $P = 0.02$ and DSC $P = 0.007$; Figures 2.7A,B). No statistically significant differences were observed in WS 1 PFC, but all variables analyzed

(except for DSN) pointed in both subjects either in the same direction with higher values in supra- relative to infragranular layers, or showed little difference between the layers.

In the MSV areas of both subjects, TDL was higher in infragranular than in supragranular layers, with a similar trend toward significance (P = 0.08 in both WS 1 and WS 6; Figures 2.6A, 7A), and DSN was significantly higher in infragranular layers (WS 1 P = 0.05 Figure 2.6C; WS 6 P = 0.02 Figure 2.7C). Also in the MSV areas, values of all variables, even if not reaching statistical significance, were higher in infragranular versus supragranular layers (Figures 2.6, 2.7). The only exception was MSC with supragranular layers higher than infragranular layers in both subjects, reaching statistical significance in WS1 (P = 0.01; Figure 2.6E). It is important to note that the absolute values for MSC differed little between unimodal areas of WS 1 and WS 6; however, only in WS 1 the difference reached statistical significance.

Discussion

Analysis of WS pyramidal neurons revealed differences in the organization of basal dendrites in WS when compared to TDs (Jacobs et al., 1997, 2001) or to other pathologies with compromised social functioning, including ASD and RTT (Belichenko et al., 1994; Hutsler and Zhang, 2010; see below). The differences were layer-specific, more prominent in PFC areas, and displayed an overall pattern of dendritic organization that sets WS apart from other disorders.

Organization of Basal Dendrites in Supragranular Layers Across the Cortex and within PFC

In analyzing supragranular cortical layers, we focused specifically on the difference in dendritic length (TDL, MSL) and branching (DSC, MSC) in PFC areas compared to MSV (BA 4, BA 3-1-2, BA 18). Our question was whether the morphology of basal

dendrites in supragranular layers would reveal more branching in PFC than in MSV areas in WS.

Based on prior reports of TD data (Jacobs et al., 2001), we expected that basal dendrites in supragranular layers would be longer, exhibit more dendritic spines, and increase in complexity from cortical areas BA 3-1-2, through BA 4 and BA 18, and be the most complex in the two PFC areas (BA 11 and BA 10). Nevertheless, our results revealed no statistically significant difference in dendritic length (TDL, MSL) or dendritic branching (DSC, MSC) of basal dendrites between each of the cortical areas examined, or even a general pattern pointing in such a direction, in either WS 1 or WS 6 (Figures 2.2, 2.3). For most variables in one or both subjects, PFC values were actually either below or about the same as MSV values, for each of BA 3-1-2, BA 4, and BA 18. This lack of dendritic variation between PFC and rest of cortical areas in supragranular layers represents another finding that has not been reported for most other disorders, with the exception of a similar pattern in RTT (Belichenko et al., 1994), another disorder with compromised social functioning.

In each WS subject, basal dendrites in one or more of the MSV areas emerged as having the longest and most branched basal dendrites relative to the rest of the cortical areas examined. In WS 1 for example, this was seen in BA 3-1-2 (Figures 2.2A,B), whereas in WS 6, it was seen in both BA 3-1-2 and BA 4 (Figure 2.3A). However, this pattern of longest and most branched dendrites in one MSV area disappeared when the mean values of segment length (MSL) and segment count (MSC) were taken into account (Figures 2.2D,E, 2.3D,E). Despite the lack of statistical significance, these findings are of

interest given that they were found in both subjects in our sample, while a similar pattern has not been reported in TD or in other disorders.

The functional implications of our observations are difficult to interpret, but it can be suggested that processing of one modality may be emphasized in WS, at the expense of stimulus evaluation and response choice in high-integration areas of the PFC. Partial support for the view that multi-modal integration is compromised in WS comes from fMRI research, which suggests that WS subjects display less activation in high-integration areas in the temporal lobe (superior and middle temporal gyri, and superior temporal sulcus) and increased activation in subcortical structures (Levitin et al., 2003) during music processing. Thus, it is possible that compromised integration and evaluation of different stimuli may be a general feature of cortex in WS.

With respect to whether there is variation within PFC, we expected based on TD findings (Jacobs et al., 1997, 2001) that BA 10 would display longer, more branched, and more spinous basal dendrites compared to BA 11. Nevertheless, we did not observe this relationship for TDL, DSN, MSL, and MSC. Instead, in each of our WS subjects, BA 10 and BA 11 differed little from one another (Figures 2.2A,B,D,E, 2.3A–E). Most studies examining neurodevelopmental disorders in PFC target a single cortical area (e.g., Vukšić et al., 2002; Table 2.1) and the ways in which different areas within the PFC may be affected in various disorders remain not well researched. It is intriguing that we did not find differences in basal dendritic complexity between BA 10 and BA 11 and that both areas displayed values equal or lesser than in MSV areas. Thus, compromised dendritic length and branching may not be limited to a single PFC area, but could instead represent a

shared feature of neural systems involving the PFC as a whole. It is important to note that the frontal pole (BA 10) and the orbitofrontal cortex (OFC; for example, BA 11) are part of distinct neural systems (Barbas, 2007), with BA 11 being part of socioemotional circuitry, while BA 10 is implicated in higher-order cognitive tasks (Stuss and Benson, 1984). Existing MRI studies report the presence of structural differences in frontal lobes between WS and TD (Reiss et al., 2000). More specifically, OFC has been previously reported as structurally different in WS (Meyer-Lindenberg et al., 2004) and, functionally, not activated in the same social situations (e.g., threatening faces) compared to TD, unlike the dorsolateral and mesial PFC (Meyer-Lindenberg et al., 2005). Given that the present study focused on two distinct areas of PFC at a resolution not possible for macroscopic studies and found no differences in the organization of basal dendrites between BA 10 and BA 11, it is possible that compromised social functioning specific to WS—characterized by lack of social inhibition, heightened desire to interact with strangers, and failure to follow the complex norms guiding social behavior (Karmiloff-Smith et al., 1995)—is not limited to OFC but, instead, is underlined by compromised morphology in a number of areas within PFC.

Comparison of Basal Dendritic Branching between Supra- and Infragranular Layers in PFC and MSV in WS Cortex

With respect to our last question, the aim was to determine whether WS neurons display differences in branching and length of basal dendrites between supra- and infragranular layers as reported for the PFC in TDs (Petanjek et al., 2008), with dendrites of the neurons in supragranular layers being longer than those on the neurons in infragranular layers. The results indicated that dendritic length and branching across layers

in PFC of WS were similar to TDs (Petanjek et al., 2008). As with TDs, there is individual variation, with differences being significant for some of the parameters in one subject (WS 6; Figures 2.7A,B), while a trend toward longer and more branched basal dendrites was present in the other subject (WS 1; Figures 2.6A,B). Although the TD study sampled exclusively magnopyramidal neurons from lower layer III (Petanjek et al., 2008), and in contrast, the present study was based on neurons throughout the depth of layer III, a similar pattern was observed here for WS. These findings suggested that the relative degree of branching as defined by the length (TDL) and segment number (DSC) of basal dendrites in supra- and infragranular layers of PFC may have been preserved in WS.

It is of interest that neuronal body density in layer V/VI of PFC (specifically BA 10) is decreased in WS compared to TDs, whereas no significant differences in density were observed in layer II/III (Lew et al., 2017). Based on this finding, there may be an increase in soma size or glia numbers and/or increased length and branching of pyramidal neurons in infragranular layers of BA 10 in WS. The present findings on PFC provided partial argument against the latter possibility, namely that a decrease in neuronal density is accompanied by an increase in dendritic length and branching in layers V/VI of BA 10. It is also of interest that, when the morphology of basal and apical dendrites of layers V/VI WS neurons in MSV areas was directly compared to TDs (Chailangkarn et al., 2016), dendrites in WS emerged as longer and more branched. Given that neuronal soma density is also increased—although not statistically significant—in BA 4, BA 3-1-2, and BA 18 in WS (Lew et al., 2017) it can be preliminary suggested that the networks subserving processing of motor, sensory, and visual stimuli through the infragranular layers are

emphasized in WS. The conclusion is further supported by the findings from the analysis of dendritic branching, especially with the results related to the increased length of basal dendrites compared to supra-granular layers (Figures 2.6, 2.7), more complex morphology of apical and basal dendrites compared to TD in layer V/VI (Chailangkarn et al., 2016), and longer and more branched dendrites in layer V/VI of MSV compared to layer V/VI in PFC (Figures 2.7A,B).

The current findings suggest that the pattern of basal dendritic complexity in WS differs from that described in TDs. It has been suggested that in TD, an increase in dendritic length tends to correspond with higher values for dendritic segments (i.e., branching Jacobs et al., 2001; Petanjek et al., 2008). In WS, this pattern seems to be reversed, and to differ across cortical areas, with PFC neurons being on average shorter and more branched, and the basal dendrites in MSV areas longer, but less branched. Although this finding is difficult to interpret, it may reflect regional developmental differences between PFC and MSV areas. Namely, pyramidal cell dendritic systems in primary processing areas appear to mature earlier than in the PFC (Marin-Padilla, 1970; Mrzljak et al., 1990; Koenderink et al., 1994; Travis et al., 2005; Petanjek et al., 2008). During postnatal maturation, basal dendrites undergo an extensive period of growth, branching, and spinogenesis. Analyses of maturation of the layer III magnopyramidal neurons in PFC suggest that the majority of dendritic maturation occurs during the first two and a half years, characterized by two growth spurts: from birth to 2.5 months postnatal, and from 16 months until 2.5 years (Petanjek et al., 2008). Whereas dendritic length increases in both of these periods, the first maturation period is characterized by an

increase in dendritic segments, whereas the increase in length observed during the second period results mostly from increase in length of dendritic segments, not by an increase in the number of segments (Petanjek et al., 2008). If the factors underlying the pathologies on basal dendrites in WS do not occur immediately after birth—the period when dendrites in primary processing areas are undergoing increases in length and PFC dendrites mostly in branching—the aspects of the morphology developing perinatally would be spared. This would result in longer dendrites in primary processing (MSV) areas and more branched basal dendrites in the PFC. This is the pattern observed here in the cortex of WS subjects. Additional studies analyzing the timing of the activity of genes deleted in WS could help evaluate this hypothesis, and the role of specific developmental time-periods, which may be crucial for the appearance of dendritic morphology in WS.

Alternatively, since the morphology of dendrites is influenced by synaptic activity (Rajan and Cline, 1998), it has been suggested that dendritic pathologies associated with various disorders may represent a neuron's attempt to supplement a lack of inputs by increasing the number of connections, inferred based on an increase in dendritic length, dendritic branching, or in the number of dendritic spines (reviewed by Fiala et al., 2002; Srinivasan et al., 2014). In WS, areas with varying integrative abilities may have solved this problem differently, by increasing dendritic length in the case of MSV areas, or increasing the number of branches in PFC neurons. In the early-maturing MSV regions, the increase in connectivity may have been achieved by increasing the basal dendritic length, whereas the same task may be achieved in later-maturing PFC by increasing the branching capacity.

It has been proposed that specific developmental features of the PFC may make the dendrites on the pyramidal neurons in PFC more prone to modifications in neurodevelopmental disorders (Penzes et al., 2011). As previously mentioned, in the disorders studied pre- and post-natally, differences in the morphology of basal dendrites were found during the perinatal period (Marin-Padilla, 1972, 1976; Jay et al., 1990), suggesting that they could result from neurons' inadequate response to received inputs. A similar hypothesis was previously proposed for abnormalities in dendritic spines in neurological disorders (Fiala et al., 2002), which interpreted findings on the spine number and morphology in disorders as the improper establishment of synaptic inputs. However, since inputs into a neuron are determined by the length of dendrites, the extent of dendritic branching, and the number and density of dendritic spines—all of which influence integrative properties of dendrites and response to the inputs (Poirazi and Mel, 2001; Srinivasan and Stevens, 2011)—it is important to take all these aspect of dendritic morphology into account when drawing conclusions about functional implications or potential causes behind the morphology of dendrites in developmental disorders.

Findings from WS and Implications for Understanding Unique Features of the Human Brain

Among the areas we examined in WS, PFC has received special interest from an evolutionary perspective. In human evolution, the PFC has been reorganized with some areas having become enlarged (BA 10) and others having decreased in size disproportionately (BA 13; Semendeferi et al., 1998, 2001). Specific PFC areas, such as BA 10, have also undergone a microstructural reorganization, including changes in the number and spatial distribution of neurons (Semendeferi et al., 2001, 2011), organization

of basal dendrites (Bianchi et al., 2012), and maturation (Sakai et al., 2011). These differences in neuronal organization between humans and great apes seem to be especially prominent in cortical layer III (Semendeferi et al., 2011), suggesting an emphasis on information processing in BA 10 and the functional demands placed specifically on layer III.In addition to humans (Jacobs et al., 2001), dendritic branching has been examined in the cortex of other primate species: the galago (*Otolemur garnetti*), owl monkey (*Aotus trivirgatus*), vervet monkey (*Cercopithecus pygerythrus*), marmoset (*Callithrix jacchus*), and baboon (*Papio ursinus*; Elston et al., 2006 and the references therein) and the common chimpanzee (*Pan troglodytes*; Bianchi et al., 2012). In all of these species, pyramidal neuron basal dendrites in PFC displayed more complex morphologies—defined as either higher values for basal dendritic field area (Elston et al., 2006) or higher TDL and DSC (Jacobs et al., 2001; Bianchi et al., 2012)—compared to primary processing and unimodal areas. The number of dendritic spines is also higher in the PFC (Jacobs et al., 2001; Elston et al., 2006; Bianchi et al., 2012) and, at least in humans and monkey species, the increase in DSN tended to be associated with the absolute size of the cortical regions, and not the size of a basal dendritic area (Elston et al., 2006). These comparative cross species studies suggest that an increase in complexity of basal dendrites in PFC may represent a feature shared by all primates (but see also data for the African elephant; Jacobs et al., 2011) and that organization of basal dendrites (basal dendritic field area or TDL and DSC) and the number of dendritic spines represent two aspects of pyramidal cell anatomy that respond differently to the computational demands of the cortical circuitry they form. It has been suggested that the need to navigate complex and hierarchical social environments

represented selective pressures influencing changes in the human brain during the evolution (Humphrey, 1976; Byrne and Whiten, 1989; Dunbar, 1998). Studies examining these areas in neurological disorders can further our understanding on the connection between anatomy, function, and specialization of cortical areas in humans.

Conclusions

In the present study, we explored the morphology of basal dendrites in WS—a disorder with distinct genetic and behavioral manifestations—with attention to PFC and the variation across cortical layers and cortical areas. We found that pathologies in the organization of basal dendrites in WS depend on the cortical area and layers examined, with most of the differences with TD found in supragranular layers and in the PFC. Thus, it can be concluded that the behavioral phenotype seen in WS may, at least in part, be influenced by alterations in the morphology of basal dendrites. It would be important in the future to combine the present findings with additional analyses of neuron density in the cortex, as well as with analyses of dendritic branching in the subcortical areas projecting to different areas in PFC. Such studies will provide further insight into organization of dendrites across several cortical areas in WS, a unique disorder that can offer valuable insights into the interplay between genes, brain and behavior.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter II, in full, is a reprint of the material as it appears in Hrvoj-Mihic, B., Hanson, K. L., Lew, C. H., Stefanacci, L., Jacobs, B., Bellugi, U., and Semendeferi, K. (2017). Basal dendritic morphology of cortical pyramidal neurons in Williams syndrome:

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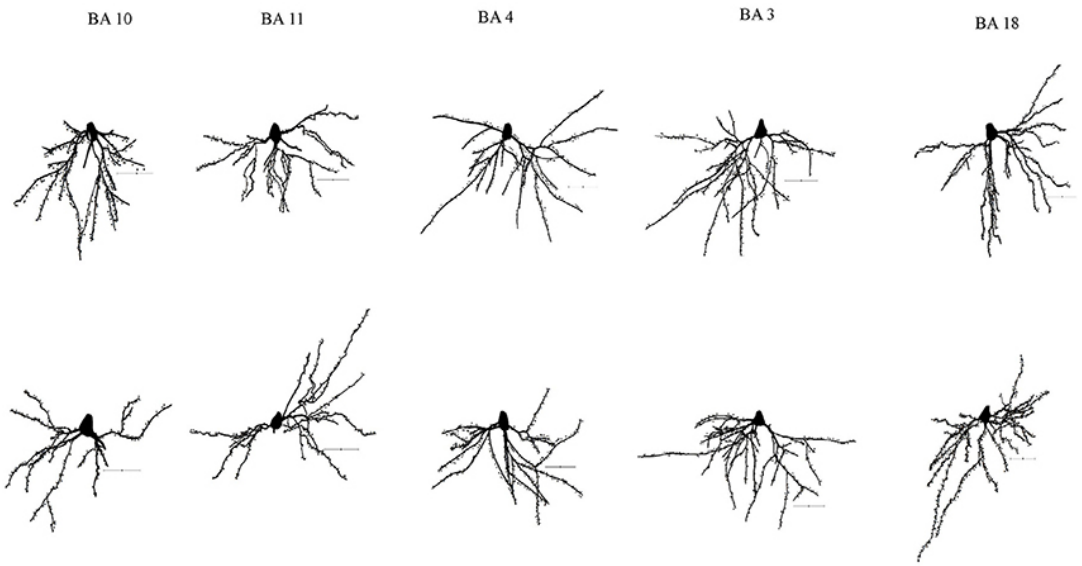

Figure 2.1 Representative tracings of cortical neurons from one subject (WS1) included in the present study. Scale bar: 50 μm.

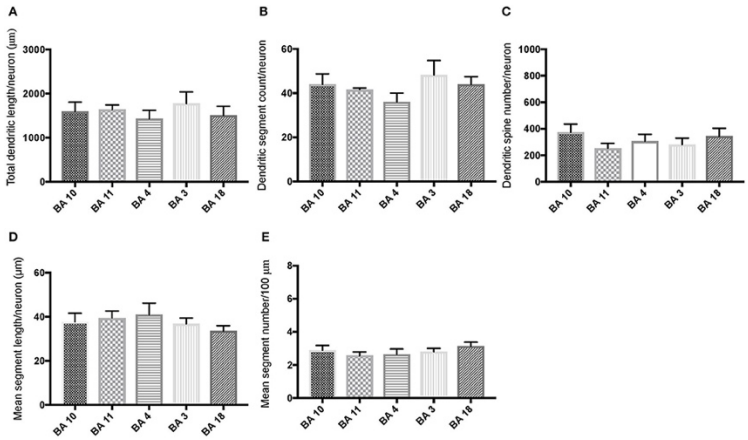

Figure 2.2 Bar graphs representing basal dendritic variables in supragranular layers (layers II/III) across cortical areas in subject WS 1 (31-year-old, male): (A) total dendritic length/neuron (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM.

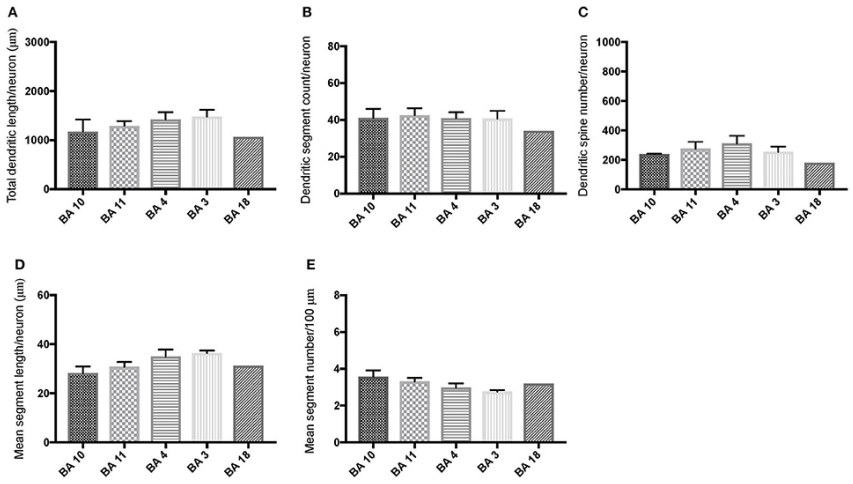

Figure 2.3 Bar graphs representing basal dendritic variables in supragranular layers (layers II/III) across cortical areas in subject WS 6 (47-year-old, male): (A) total dendritic length (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM.

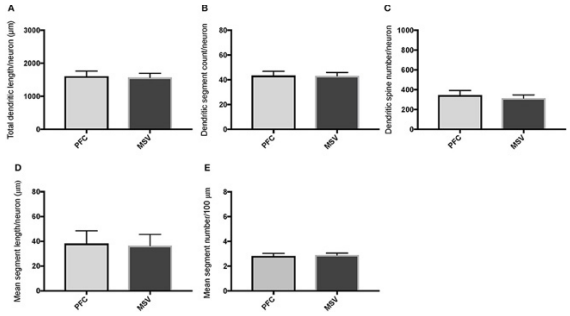

Figure 2.4 Bar graphs comparing basal dendritic variables in supragranular layers (layers II/III) of the prefrontal cortex (BA 10, BA 11) and motor-sensory-visual areas examined (MSV; BA 4, BA 3, BA 18) in subject WS 1 (31 year-old, male): (A) total dendritic length (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM.

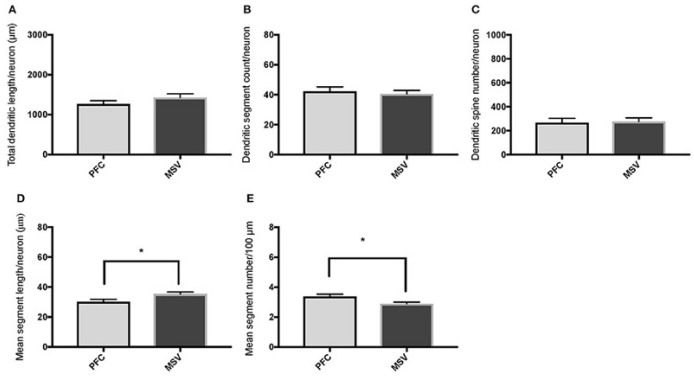

Figure 2.5 B ar graphs comparing basal dendritic variables in supragranular layers (layers II/III) of the prefrontal cortex (BA 10, BA 11) and the motor-sensory-visual areas examined (MSV; BA 4, BA 3, BA 18) in subject WS 6 (47 year-old, male): (A) total dendritic length (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM. *P ≤ 0.05.

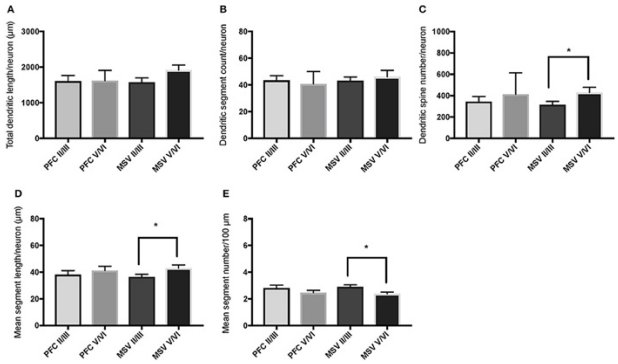

Figure 2.6 Bar graphs illustrating the relationship in basal dendritic morphology between supra- (II/III) and infra-granular layers (V/VI) in PFC and motor-sensory-visual areas (MSV; BA 4, BA 3, BA 18) in the subject WS 1 (31 year-old, male): (A) total dendritic length (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM; *P ≤ 0.05.

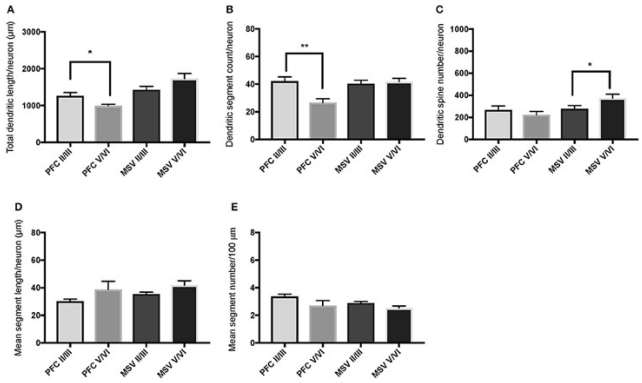

Figure 2.7 Bar graphs illustrating the relationship in basal dendritic morphology between supra- (II/III) and infra-granular layers (V/VI) in PFC and motor-sensory-visual areas (MSV; BA 4, BA 3, BA 18) in the subject WS 6 (47 year-old, male): (A) total dendritic length (TDL, µm); (B) dendritic segment count (DSC); (C) number of dendritic spines (DSN); (D) mean segment length (MSL, µm), and (E) mean number of dendritic segments (MSC). Data are presented as mean ± SEM; *P ≤ 0.05, **P ≤ 0.01.

Table 2.1 Number of neurons included in this study (*includes both hemispheres).

Subject	BA 10	BA 11	BA 4	BA 3	BA 18	Total
WS 1 (layersII/III)	9	3	6	7	10*	35
WS 1 (layers V/VI)	1	2	5	1	3*	12
WS 6 (layers II/III)	2	7	8	8	1	26
WS 6 (layers V/VI)	0	3	3	8	2	16

Table 2.2 List of selected postmortem human studies examining morphological changes on dendrites of cortical pyramidal neuron including PFC.

Areas examined	Pathology	Age	Subject numbers, diagnosis	References
Frontal cortex Parietal cortex Temporal cortex	ASD	12, 27 years	4 ASD	Williams et al., 1980
Prefrontal cortex (BA 9) Temporal cortex (BA 21) Parietal cortex (BA 7)	ASD	10–45 years	10 ASD 15 controls	Hutsler and Zhang, 2010
Prefrontal (BA 10) Frontal (BA 4) Temporal (BA 21)	RTT	16–24 years	2 RTT 11 TRPE 6 controls	Belichenko et al., 1994
Frontal cortex Temporal cortex Occipital cortex	RTT/DS	6–35 years	16 RTT 6 DS 9 controls	Armstrong et al., 1998
Prefrontal (BA 9)	DS	Newborn, 2.5 month	2 DS 2 controls	Vukšić et al., 2002
Temporal pole (BA 38) Temporal cortex (BA 22/21)	Schizophrenia	Adult	13 schizophrenia 1 schizophrenia-like psychosis 11 controls	Garey et al., 1998
Frontal cortex (BA 46) Occipital cortex (BA 17)	Schizophrenia	Adult	15 schizophrenia 15 non-schizophrenia with psychiatric illness 15 controls	Glantz and Lewis, 2000
Mesial frontal lobe Temporal lobe	Epilepsy	Not reported	14	Vaquero et al., 1982
Motor cortex (BA 4) PFC (BA 10) Broca's area (BA 44)	Callosotomy	Adult	2 callosotomy	Jacobs et al., 2003

ASD, autism spectrum disorder; RTT, Rett syndrome; DS, Down syndrome; TRPE, therapy resistant partial epilepsy.

CHAPTER III: DISTRIBUTION OF NEUROFILAMENT IMMUNOREACTIVE NEURONS (SMI-32) IN THE PREFRONTAL CORTEX OF WILLIAMS SYNDROME

Abstract

Williams syndrome (WS) is a neurodevelopmental disorder characterized by a set of compromised features in social behavior, including an increased desire to interact socially with strangers and inadequate responses to social situations. Several regions of the prefrontal cortex involved with processing of socially salient information - including the frontal pole and the orbitofrontal cortex -- display abnormalities in WS: decreased density of neurons compared to controls, and deficiencies in length and branching of dendrites, and the number of dendritic spines in the prefrontal cortex compared to unimodal cortical areas. In order to examine if a particular subclass of neurons within the prefrontal cortex is affected in WS, we used an antibody against non-phosphorylated epitope of neurofilament protein (SMI-32) to examine distribution of SMI-32ir neurons in the frontal pole of WS and neurotypical controls.

SMI-32 immunoreactive (SMI-32ir) neurons represent important parts of cortical circuitry, underlying long cortico-cortical connections. Our analysis revealed that SMI-32ir neurons in WS occupied the same relative position within layer III as in neurotypical controls, being restricted to the lower layer III of the frontal pole, but with important differences between WS and controls. More specifically, we found that density of SMI-32ir neurons was lower in WS and the neurons were characterized by smaller cell bodies. These findings imply that lower layer III magnopyramidal neurons in the prefrontal cortex may form a particularly vulnerable population in WS, suggesting deficiencies in cortico-cortical connectivity between the frontal pole and other cortical regions in the disorder.

Introduction

WS is a neurodevelopmental disorder underlined by a hemizygous deletion of ~25 genes on chromosome 7 (WS deleted region; Schubert, 2009) and characterized by a unique set of preserved and compromised behavioral and cognitive features: preservation of some aspects of language and face processing, compromised spatial cognition and lower overall intelligence, and an increased desire to interact socially and to approach strangers (Bellugi et al., 1999). Functional imaging has suggested differences in brain activation during social tasks in WS individuals compared to controls, especially in the frontal cortical areas. Individuals with WS display decreased activation of dorsolateral PFC, dorsal anterior cingulate, and the striatum in Go/NoGo tasks (Mobbs et al., 2007), and an overall higher activity of anterior brain regions at the expense of primary and secondary visual areas in face and gaze processing tasks (Mobbs et al., 2004). The behavioral phenotype in WS, therefore, can be at least in part be linked with the differences in higher-order processing of socially relevant cues, which involve distinct activity of PFC areas.

Despite the behavioral profile of WS, which would suggest anomalies in the organization of brain areas involved in processing of social behavior, frontal areas underlying higher-level processing of socially relevant stimuli appear to be relatively spared in WS. Based on MRI studies, volume of the frontal lobes appears comparable between WS and non-affected controls, despite the overall reduction of the brain volume in WS (Reiss et al., 2004). Dorsal and medial subdivisions of PFC similarly remain spared in WS (Fan et al., 2017). At the same time, certain subdivisions of the frontal lobe show differences between WS and controls. The differences are especially prominent in the orbitofrontal cortex and dorsal part of the anterior cingulate cortex (Reiss et al., 2004; Fan

et al., 2017) with WS individuals displaying higher gray matter volumes than controls. These findings are paralleled by the findings in two subcortical structures – the amygdala (Reiss et al., 2014) and the putamen/nucleus accumbens region of the striatum (Fan et al., 2017) -- both of which share reciprocal connections with PFC and are important parts of the circuits underlying social behaviors.

Further analyses focusing on the cellular organization and morphology of dendrites on pyramidal neurons revealed more specific patterns of organization of cortex in WS, with a set of well-defined features that differ between WS and control subject. In the prefrontal cortical areas, subjects with WS display decreased neuronal density, with patterns that appear layer- and area-specific. Both in the frontal pole (BA 10) and in the orbitofrontal cortex, WS subjects have decreased density of neurons relative to controls (Lew et al., 2017). The decrease is especially prominent in infragranular layers of BA 10, which contrasts with the pattern observed in the somatosensory cortex (BA 3-1-2) and secondary visual area (BA 18), where the density of neurons is higher in WS compared to control subjects (Lew et al., 2017). Both BA 10 and BA 11 in WS displaye pathologies in the length, branching complexity, and the number of spines on dendrites of pyramidal neurons, lacking the typical increase in dendritic complexity relative to primary processing areas, as seen in non-pathological individuals (Hrvoj-Mihic et al., 2017). In subcortical structures, an increase in neuronal density was observed in the lateral nucleus of the amygdala (Lew et al., 2018) and an increased density of glia was reported in dorsal and medial caudate nucleus of the striatum (Hanson et al., 2018). Both the amygdala and dorsal and medial caudate participate in processing of social behavior, emotional

responses, and cognitive control, and represent important sources of subcortical inputs into medial and orbital regions of PFC (Amaral and Price, 1984; Haber, 2003). In summary, existing findings suggest that the differences between WS and controls can be observed at the cellular level and that they are more prominent in some cytoarchitectonically defined PFC areas – and some laminae within these areas -- compared to others. Given that pyramidal neurons encompass a variety of functionally distinct subclasses (deFelipe et al., 2002), existing findings suggest that, instead of assuming general pathologies affecting all pyramidal neurons within a certain area or a cortical layer, it would be meaningful to examine specific subsets of neurons that may be driving the observed difference between WS and non-affected controls.

The present work focused specifically on one subpopulation of pyramidal neurons in BA 10 of WS: SMI-32 immunoreactive (SMI-32ir) magnopyramidal neurons in the lower layer III (layer IIIC). It has been shown previously (Lee et al., 1988) that monoclonal antibody SMI-32 labels nonphosphorylated epitope on human NF-M and NF-H subunits present in a distinct subpopulation of neurons in the cortex of primates (Campbell and Morisson, 1989; Hof et al., 1995a, 1995b; Zeba et al., 2008). The distribution of SMI-32ir neurons displays striking variation across different cortical areas and, in humans, SMI-32ir neurons appear to be especially abundant in the lower layer III (Campbell and Morisson, 1989). Within the layer III, neurofilament reactivity seems to be restricted to the neurons involved into long ipsilateral cortico-cortical connections (Hof et al., 1995b), important for proper connectivity across cortical areas. Lower (magnopyramidal) layer IIIC neurons in the PFC also display a distinct dendritic developmental pattern, characterized by two rapid

periods of growth, branching, and spinogenesis, separated by a year-long period of stasis (Petanjek et al., 2008; 2019) that has not been observed in upper layer III neurons. Unlike the pyramidal cells in the upper layer III, large pyramids in the layer IIIC do not display variation in dendritic morphology across functionally distinct cortical areas (Zeba et al., 2008). The maturation of magnopyramidal cell layer in PFC corresponds to important cognitive landmarks in early childhood, possibly reflecting maturation of cortical circuitry underlying higher-order integration of information from various sensory stimuli. This subset of neurons is selectively affected in several neurological disorders, including schizophrenia (Glantz and Lewis, 2000) and appears especially prone to degeneration in Alzheimer's disease (Hof et al., 1990). Given the importance of supragranular layers for proper inter-cortical connectivity, and since previous research that suggests differences in supragranular layers between WS and controls, we expect that the pathologies may be restricted to long-projecting, magnopyramidal neurons in lower layer III, influencing the manifestation of behavioral traits that suggest lack of adequate connectivity within the cortex.

Materials and Methods

Subjects

Distribution of SMI-32ir neurons was examined in BA 10 of two subjects with WS and one non-affected control. WS 1 was previously used in our study of dendritic morphology (Chailangkarn et al., 2016; Hrvoj-Mihic et al., 2017) and WS 9 was included in the study of density of neurons in the cortex and in the amygdala of WS (Lew et al., 2017; 2018). Both subjects were analyzed in the study of striatum in WS (Hanson et al., 2018). WS1 was a 31-year-old male and WS 9 was a 43-year-old female. Both subjects

died of cardiorespiratory arrest. WS subjects were diagnosed based on the Diagnostic Score Sheet (DSS) for WS subjects and, for WS 9, the diagnosis was further confirmed with fluorescent in situ hybridization (FISH) probes for elastin (ELN), which revealed hemizygous deletion of the elastin gene. The subjects were not diagnosed with any other conditions besides WS. The non-affected control subject (5552) was a 46 years-old female that died of heart disease. All brain specimens were harvested within a postmortem interval of 18–30 h and had been kept in 10% formalin for up to 20 years.

Tissue processing and data collection

Tissue blocks corresponding to BA 10 were cryoprotected in successive concentrations of 10%, 20%, and 30% buffered sucrose solutions, and sectioned at 30 um on a Leica SM2010 freezing microtome. Immunohistochemical staining was performed on free-floating sections, starting with an antigen retrieval step that consisted of heating the sections for 30 mins at 90□C in citrate buffer (pH 2.5). Following the pretreatment in 3% hydrogen peroxide and 0.2 Triton-X, sections were blocked in 5% bovine serum albumin with Triton-X, and placed into SMI-32 antibody (Biolegend, San Diego, CA) in a 1:1,000 dilution. The sections were then processed using the secondary anti-mouse antibody in a 1:200 concentration, followed by avidin-biotin method with Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Staining was visualized with the application of 3,3′-Diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA).

Qualitative observations

In the control subject, SMI-32 staining gave the appearance of two distinctly stained bands, separating the cortex into supra- and infra-granular layers divided by the unstained layer IV (Figure 3.1a). In supragranular layers, density of SMI-32ir neurons

increased toward deeper parts of layer III and the cell bodies of stained neurons became larger and more darkly stained toward the border with layer IV (Figure 3.1b). In infragranular layers, SMI-32 again revealed two bands of stained neurons: densely packed neurons in the layer V, immediately below the unstained layer IV and, toward the white matter, another band of dense, but lightly stained, neurons (Figure 3.1c). A similar pattern has been previously reported in human PFC (Hof et al., 1995a).

In the cortex of WS subject, SMI-32 staining also revealed two distinctly stained bands, corresponding to supra- and infra-granular layers, although the unstained layer IV was less distinct than in controls. In WS subject, SMI-32ir neurons were less densely packed and had more darkly stained and larger cell bodies (Figure 3.1d). In supragranular layers, there appeared to be less SMI-32ir neurons than in the controls. SMI-32ir also displayed a distinct organization, with areas containing only a few, sparsely distributed neurons with very dark cell bodies and areas with densely packed neurons (Figure 3.1e). In the parts of supragranular layers where only a few neurons were positive for SMI-32, those were found almost entirely in the 300 μm band above layer IV, corresponding to the lower layer III (layer IIIC), although an occasional SMI-32ir neuron could be found in upper parts of supragranular layers (Figure 3.1e).

Data collection and analysis

The number of SMI-32ir neurons was estimated using the Optical Fractionator probe on Stereoinvestigator software (MBFBioscience, Williston, VT) attached to a Nikon Eclipse 80i microscope. The region of interested (ROI) was delineated on lower layer III, which was distinguished by neurons with large and darkly stained somata located immediately above the unstained layer IV. Sapling sites were located within a 10 μm area

within each section, located between guard zones at the top and the bottom of each section. The neurons were counted in 6 sections for the unaffected control (5552), 7 sections for WS 9, and 3 sections for WS 1. During the data collection, every 8th neuron was sampled for soma area measurements using the Nucleator probe on StreoInvestigator.

Results

When density of SMI-32ir neurons was compared between the control subject and the two subjects with WS (WS1 and WS9), both WS subjects displayed lower density of SMI-32ir neurons than the control (Figure 3.2a). For the control, the density of SMI-32ir neurons was 9781 neurons/ mm^3 and values were 47% lower in WS 1 (6639 neurons/ mm^3) and 38% lower in WS 9 (7065 neurons/ mm^3). WS subjects also displayed lower average soma volume: 1284 +/- for WS1 and 1204 +/- 467 mm^3, compared to the volume of 1731 mm mm^{3}for the control (Figure 3.2b). Given the low number of subjects in the present study, it was not possible to conduct statistical analyses but both WS subjects showed a trend toward lower SMI-32ir neuron density and smaller cell bodies of SMI-32ir cells.

Discussion

Previous research suggested that there is a decrease in the overall neuron density of neurons in BA 10 of WS subjects (Lew et al., 2017). When analyzed separately, layers II/III also displayed a trend toward lower neuronal density in WS (Lew et al., 2017). Here, we demonstrated that same pattern by examining one subset of neurons -- SMI-32ir neurons – located at the deep level III in BA 10 in two of the subjects used in Lew et al (2017) study. The same population of neurons displayed lower average cell body size, with even the largest neurons in most cases falling below the average for the control subject

(Fig 3.2b). It is thus possible that the differences seen between WS and controls in neuronal density could be driven -- at least in part – by selective pathologies in SMI-32ir neurons in lower layer III. If SMI-32ir neurons indeed represent a neuronal population selectively affected in WS, we would expect that the pathologies in their distribution are not limited to the lower parts of layer III, but that they extend throughout the depth of the layer III. Alternatively, additional sub-populations of pyramidal cells could be affected specifically in the lower layer III, and SMI-32ir neurons may represent only one population of affected neurons. In either case, the present findings warrant use of additional cell-specific markers to examine pathologies across layers in BA 10 of WS subjects.

Current findings on the differences in distribution of SMi-32ir neurons in deep layer III carry implications for the connectivity between BA 10 and other cortical area. SMI-32 typically stains neurons with long ipsilateral projections within the cortex (Hof et al., 1995a), and the decrease in SMI-32ir neurons in WS suggests lesser connectivity between BA 10 and other cortical areas in the same lobe. As BA 10 shares connections with the neighboring BA9, BA 46 in the lateral PFC, and BA 7 in the parietal lobe (reviewed by Mega and Cummings 1994), weakening the connectivity could explain compromised function of the 'executive circuit' within PFC. Behavioral manifestations seen in WS, including different responses to social situations compared to age-matched controls (Jones et al., 2000) may at least in part be explained by the effects lower density of long-projecting cortical neurons may have on corticocortical connectivity.

It is interesting that some social aspects of WS appear relatively early, and different responses to social situations are already present in late infancy/early childhood (Jones et al., 2000). In contrast, pathologies in other neurodevelopmental disorders are not present until much later – 3 years in autism and late childhood in Down syndrome. The appearance of symptoms in ASD and DS post-dates rapid development of cortical microciruitry and growth spurt in lower layer III pyramidal neurons. The neurons in lower layer III of PFC are specific for displaying two growth spurts – from birth until 2.5 months and from 18 months until 2.5 years (Petanjek et al., 2007; Sedmak et al., 2018). In neurodevelpmental disorders in which pathologies in dendritic branching and length occur, they typically emerge after the second period of rapid neuronal growth (Vuksic et al., 2002; 2011), and behavioral symptoms do not occur until much later. Early development of hypersocial behavior in WS, indicative of compromised executive circuit within the brain (Mega and Cummings 1994), but predating maturation of layer IIIC neurons challenges the hypothesis that the behavioral phenotype in WS in mostly driven by IIIc neurons, suggesting instead that other – earlier maturing -- parts of cortical microcircuitry are affected in WS.

Conclusions

By examining the distribution of SMI-32ir neurons in BA 10 of WS, we have demonstrated the importance of examining specific neuronal subclasses in neurodevelopmental disorders to better understand the link between a structure's organization and functional implications of the compromised neuronal organization. Previously, SMi-32 staining has been used to study the pathology of Alzheimer's disease (Hof and Morrison, 1990), since SMI-32ir neurons appear especially vulnerable to the decline in Alzheimer's disease. Earlier work on WS has suggested decreased neuronal

density in BA 10 in WS (Lew et al., 2017) and compromised organization of dendrites on pyramidal neurons in BA 10 (Hrvoj-Mihic et al., 2017). Here, we have expanded the analysis and presented pilot data that suggests that a specific class of neurons staining positive for SMI-32 neurofilament, located mostly in lower layer III of BA 10, may be particularly compromised in WS. Given the role of lower layer III in specifying cortico-cortical circuitry, it is possible to hypothesize that the decrease in long distance connections between PFC and other association areas in the cortex may underline the phenotype typical of the disorder.

Further analyses focused on neurodevelopmental disorders can yield important insights into the function of SMI-32ir neurons. Given that SMI-32ir neurons are sparse at birth and appear postnatally – making largest appearance 30-42 post-natal days in the visual cortex of macaques (Kogan et al., 2000), -- their development in humans can yield important insights into the role of environment in specifying the early establishment of cortical microcircuitry. It is worth nothing that lower layer III neurons in human PFC are characterized by slow development that spans the first 2.5 years (Petanjek et al., 2008; Sedmak et al., 2018), and the maturation of spines continues until the third decade of life (Petanjek et al., 2011). Given their long development and the exposure to various factors influencing neuronal development, examining lower layer III neurons in PFC can be informative for the patterns leading to the development of pathologies.

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Chapter III, in part, is currently being prepared for submission for publication of the material. Preliminary results presented in this chapter are currently in press in Hrvoj-Mihic, B., and Semendeferi, K. (in press). Neurodevelopmental disorders of the prefrontal cortex in an evolutionary context. *Progress in brain research*. The dissertation author was the primary investigator and author of this paper.

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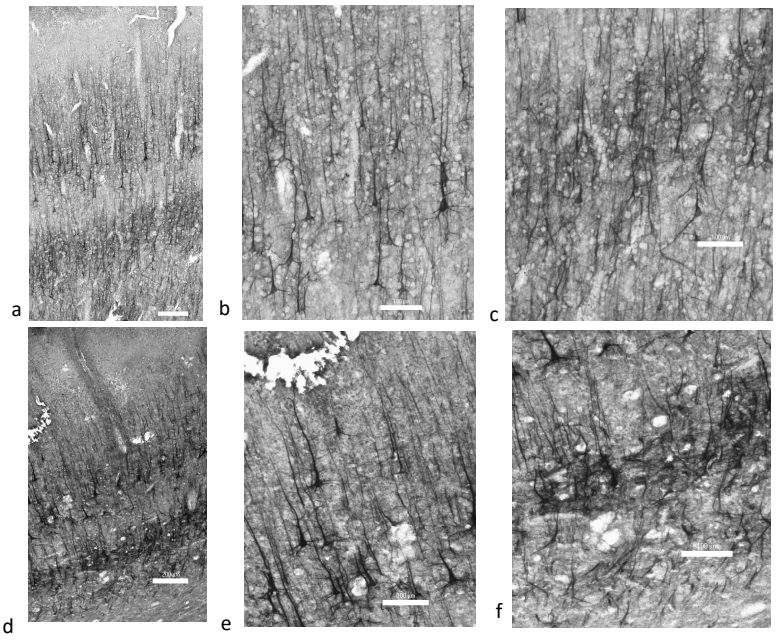

Figure 3.1 Photomicrographs of SMI-32 stained section of BA 10 in non-affected controls (subject 5552) and WS (subject WS 9). Image of both supra- and infra-granular layers taken at a low (4x) magnification in controls (a) and WS (e). Scale bar: 200 μm. A higher (10x) magnification image illustrating distribution of SMI-32ir neurons in supra-granular layers in controls (b) and WS (f), and in infragranular layers in controls (c) and WS (g). Scale bar: 100 μm.

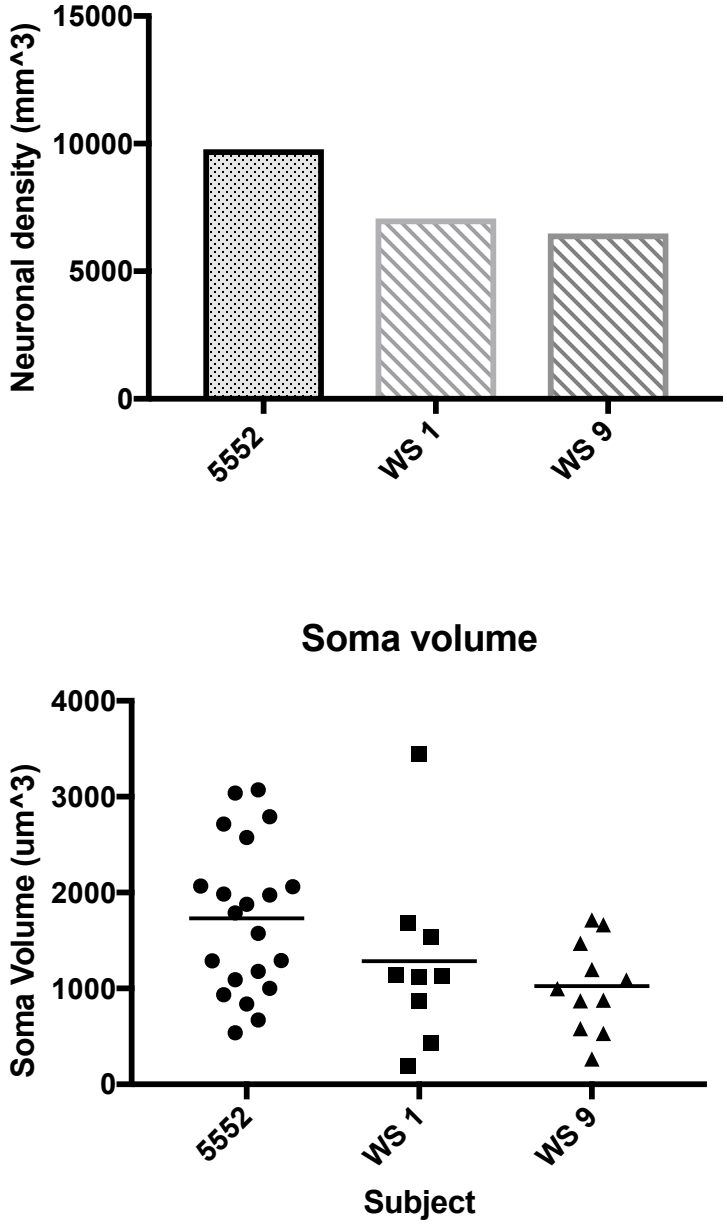

Figure 3.2 Graphs showing differences between the control subject in density of SMI-32ir neurons (top) and volume of cell bodies in the same subjects (bottom).

CONCLUSIONS

The research presented in this dissertation suggests that two aspects of cortical information flow – organization of dendrites on pyramidal neurons and the density of long projecting SMI-32ir neurons – display differences in PFC between WS and unaffected controls. Given that pyramidal neurons form the basic units of cortical microcircuitry and represent the only sources of output from a specific cortical area into other areas of the cortex and subcortical structures (Spruston, 2008), the observed changes carry implications for higher-level social and emotional processing in WS. In neurotypical subjects, dendrites of PFC neurons are longer, more branched, with a higher number of dendritic spines compared to the dendrites on neurons in unimodal areas (Jacobs et al., 2001), thus integrating inputs from a wider network than neurons in unimodal areas (Jacobs and Scheibel, 2002). In WS, dendritic length and branching do not display differences between the two PFC area and the primary motor, sensory, and visual cortices (Chapter II), suggesting that the behavioral profile typical of WS could be underlined -- at least in part -- by a decreased connectivity between PFC and the rest of the cortex.

The analysis of density of SMI-32ir neurons (Chapter III) carries additional implications for understanding the connectivity between PFC areas and the rest of the cortex in WS. Neurons expressing nonphosphorylated epitopes of neurofilament protein (SMI-32) are typically interpreted as supplying long cortico-cortical connections (Hof et al., 1990). Their decrease in WS can be interpreted as a decrease in the executive role BA 10 can exhibit onto the interconnecting cortical areas. More specifically, the association areas in temporal and parietal lobes (Burns, 2006). Further research, focused on the

association areas receiving inputs from PFC, can provide further insights into the organization of areas sharing connectivity with PFC. If the differences between WS and controls are found among the neurons receiving inputs from PFC, it may be possible to start examining WS as a disorder of cortical – i.e. higher-order -- social brain areas.

A similar approach has been suggested previously for schizophrenia (Burns, 2006), another disorder with involvement of PFC areas (Glantz and Lewis, 2000; Wible et al., 2001; Zhou et al., 2007). Whereas behavioral manifestations of WS differ from those in schizophrenia, both disorders are associated with atypical processing of socially relevant behavior – including face processing and interpretation of others' emotions -- and inadequate responses to social cues (Deruelle et al., 1999; Burns et al., 2006). Alternatively, given that WS displays differences with controls in the amygdala (Lew et al., 2018) and the striatum (Hanson et al., 2018), it is possible that the primary differences in WS social behavior can be attributed to processing in subcortical structures, and the deficiencies in PFC are secondary to the initial processing in the amygdala and striatum. Perceived that way, an explanation for the WS social phenotype can be sought at two separate levels: compromised emotional processing, as illustrated by an increased neuronal density in the lateral nucleus of the amygdala (Lew et al., 2018) and of inhibitory control, based on the findings in the striatum (Hanson et al., 2018).

Results of the dendritic branching analysis in WS (Chapter II) require additional attention. Previous research has suggested that, in comparison to controls, WS neurons are characterized by longer and more branched dendrites in primary processing areas compared to controls (Chailangkarn et al., 2016). Focus on only basal dendrites – in

comparison to Chailangkarn et al., (2016) study that took into consideration both apical and basal dendrites -- suggests that PFC areas do not differ in length and branching of dendrites from unimodal areas, opposite to the pattern observed in controls (Jacobs et al., 2001). Analysis of neuronal activity in iPSC-derived neurons expressing *CTIP2* -- indicative of their affinity as layer V/Vi neurons -- suggests differences in calcium signaling between iPSC-derived WS neurons and controls (Chailangkarn et al., 2016). These findings provide a link between the observed morphological changes in WS dendrites and their functional implications. However, given that layer II/III PFC neurons do not show increased dendritic complexity compared to primary processing areas (Chapter II), as it would be expected based on the controls (Jacobs et al., 2001), it can be suggested that the differences in function between WS and control neurons may differ across cortical areas, affecting unimodal and association cortices differently.

Plausible explanation for the observed patterns between PFC and primary processing areas could reflect the developmental uniqueness of PFC layer III pyramidal neurons. In other words, dendrites on layer III PFC neurons take longer to mature, with the period of intensive dendritic development spanning the first three years of life (Petanjek et al., 2008; Sedmak et al., 2018). In comparison, primary processing areas, such as the motor cortex, typically reach near-adult morphology within the first several postnatal months (Marin-Padilla, 1970). It is possible that the protracted maturation makes PFC neurons more vulnerable to pathological changes (Brüne, 2000), resulting in different dendritic phenotype in layers II/III of PFC compared to layers V/VI of primary motor areas. Given that iPSC model has already been applied to the analysis of WS neurons, further research

can expand onto development of layer II/III neurons in the dish and their inclusion into the analysis. In parallel, developmental studies – especially those focused on the period of rapid postnatal dendritc development – can yield insights into potential vulnerable periods at which the differences between WS and controls become visible. It is my hope that this dissertation provides enough baseline data to inform further research on this topic, and tht it has left enough questions open to expand analyses of post-mortem WS tissue in the future.

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