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Dendritic Spine Morphology in Autism Spectrum Disorders

A thesis submitted in partial fulfillment of the
requirements for the degree of
Bachelor of Science in Neuroscience and the Honors Program

by

Alexandra N. Scurry

Dr. Jeffrey Hutsler, Thesis Advisor

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ABSTRACT

Autism Spectrum Disorders (ASD) has become an extremely prevalent neurological disorder in society that is characterized by a number of social and behavioral deficits. The particular cause of ASD is unknown however continuous research is being conducted to expand the current knowledge on the disorder. The present research focuses on dendritic spine morphology and how this affects the function of spines, the locations of excitatory synapses throughout a neurological system. Abnormal morphologies and lengths of spines were observed in ASD cases when compared to neurotypical cases implicating alterations in morphology as the basis for altered neurological function. Brain tissue from both neurotypical cases and ASD cases were examined in the present study and spine data was collected from 45 cells per cases. The results of this study did show significant differences between the two types of cases and implicated a high proportion of immature and underdeveloped spines in ASD neural networks. These results have a great impact for the understanding of altered development associated with ASD and are in accordance with previous ASD studies gaining even further significance.

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1. INTRODUCTION

Autism Spectrum Disorders (ASD) has become an extremely prevalent neurological disorder. According to a report by the CDC done in 2010, ASD affects 1 in 110 of American children and of this, 1 in 70 boys (Centers for Disease Control and Prevention [CDC], 2011). Previous studies by the CDC had shown that ASD affects 1 in 150 children, demonstrating the rapid growth of the disorder. Behavioral therapies have become highly adept at offsetting some of the abnormal behavioral effects caused by ASD, however therapies must be given at an early age in order to have any beneficial impact.

ASD incorporates a variety of abnormalities in social and mental development that incorporate a wide range depending on the severity for that individual. Some various manifestations of social and behavioral impairments are: delays in spoken language, a lack of social or emotional reciprocity, repetitive motor manners, persistent preoccupation with parts of objects, impaired ability to sustain a conversation despite adequate speech, inflexible adherence to specific and nonfunctional routines and rituals (DSM-IV).

The cause of ASD is still unknown, however an enormous amount of research has been conducted to understand the disorder more thoroughly. The most logical model that has been proposed for ASD is few strengthened long distance connections underneath significant amounts of underdeveloped local connections. Specifically, neurological information is transferred at locations called synapses, the small spaces between neurons where chemical information is transferred to be converted into neurological information.

Dendritic spines are the locations for excitatory synapses, the key component in a neurological system, and will be the focus for this project. In particular, the morphology of dendritic spines and the implication this has on spine function.

There are a few key molecules imperative for spine morphology and function. Interactions between pre and postsynaptic molecules, neurexins and neuroligins, form complexes that enable a synapse to properly form. These induct other important molecules such as Epac2 and PSD-95 that allow a stronger connection and higher functioning of excitatory synapses. The interactions between these molecules not only contribute to the function of synapses, but also to dynamics of spines.

The ability for spines to change their morphology is imperative to a key function of the neurological system, synaptic plasticity. Strengthening of various connections based on reinforcements of experiences with the external environment allow for learning and formation of new memories throughout a lifetime. The dynamics of dendritic spine morphology is a major aspect in this feature of the neurological system. As experiences are reinforced, the synapses specific for those particular learned behaviors, movements, or memories become strengthened due to spine morphology undergoing changes to become more stable.

Spine morphology is inherently related to dendritic spine function which leads to proper formation and function of synaptic connections. In order to further the understanding of the relationship between spine morphology and function and the effects this has on the development of a neurological system of an ASD case, a morphological spine study is necessary. In the present study, spine morphological data is collected and analyzed to assess differences in morphologies between neurotypical and ASD cases. It is

expected to find significant differences between the two cases implying a causal effect of spine morphology on spine function and neural connectivity.

2. THE TYPICAL DEVELOPMENT OF A NEUROLOGICAL SYSTEM

Neurological development begins as early as the fifth week of gestation time and continues years later into the young adult life. During this development, synapses form in abundance at a very early period and become differentiated later on. As novel experiences occur with external stimuli, these synapses are strengthened and stabilized, or if unused they become weakened and eventually eliminated. Synapses develop based on a variety of interrelated molecular mechanisms that revolve around the dynamic function and morphology of dendritic spines. These spines are the sites of excitatory synapses and are critical to the overall connectivity and function of the neurological system. Abnormalities in morphology, function, or density of dendritic spines have been implicated in a number of neurological disorders (Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011).

Neurological disorders typically have a genetic basis that causes inappropriate and altered neural development and connectivity. To understand how these changes affect neural circuitry and function, it is first important to understand typical neural development. Gestation time through the first two years of the postnatal period in a subject's life incorporate the major aspects of development and organize the framework of a neural network. The initial connections that form at this time are followed by "critical periods" of development throughout the individuals first few years of life when the brain's sensitivity to stimuli is extremely heightened (Belmonte et al., 2004 and Takahashi, T., Svoboda, & Malinow, 2003). During these periods, the individual creates many synapses and neural connections concerning emotional and social development, language acquisition, and motor skills. Disruptions of the neural system, such as a lack in

pruning or abnormal amounts of excitatory synapses, are detrimental for the development that occurs during the "critical periods." If development at these times is prevented, the functions that are typically acquired at this time will be completely lost (Tau & Peterson, 2010).

2.1 Initial Developmental Stages in Utero

The first synaptic connections are formed during gestation age (GA) week 5 in the cortical layer called the preplate. Immature neurons are generated and form temporary connections with developing neurons in the thalamus and brainstem that become reorganized later in development (Tau & Peterson, 2010 and Levitt, 2003). Between GA weeks 18 and 22, the subplate develops within the preplate and contains a high density of synapses (Bystron et al, 2008; Judas, Sedmak, Pletikos, & Jovanov-Milosevic, 2010; Kostovic, Judas, & Sedmak, 2010). The neurons found here exhibit the same molecular features and overall framework of major neurotransmitter systems (Tau & Peterson, 2010). Similarly, these initial synapses serve as temporary connections that eventually get refined during GA weeks 24 – 28. This reworking of synaptic connections continues into the perinatal period of development when the different cortical layers can be distinguished by GA weeks 32 and 34. At this point, pyramidal neurons are found in each layer and a connectional organization is established (Belmonte et al., 2004). A peak number of neurons is observed at GA week 28 while an increase is observed in dendritic arborization and synaptogenesis resulting in a peak of these processes by GA week 34 (Lossi & Merighi, 2003; Tau & Peterson, 2010). An excessive production of neurons and synaptic connections occurs during these initial developmental stages that are followed

by pruning and destabilization. A significant portion of this destabilization is also seen at the molecular level in the dendritic spines that are produced along dendrites of neurons.

2.2 Postnatal synaptic development

Newly formed dendrites do not have characteristic protrusions typical of an adult brain; therefore, synapses are not being formed. However during this period, filopodia, finger-like projections, begin to form from the dendrites and are characteristically extremely motile and are distinguishable from dendritic spines in length and higher density of actin the cytoskeleton (Fiala, Spacek, & Harris, 2002). The filopodia are able to create synapses with axons or with the filopodia of other axons. As these connections happen, filopodia are able to retract into the dendrite and change their morphology into more spine-like shapes. Dendritic spines are then produced either by forming from these retracted filopodia or by emerging from the dendrite (Fiala, Spacek, & Harris, 2002). As normal spines increase in density, filopodia decrease and are not often seen in mature adult brains. As spines mature, their motility decreases and they become much more stable, especially when compared to filopodia (Fiala, Spacek, & Harris, 2002; Irwin et al., 2002; Matus, 2005). Spine production and increases in spine density also occur during this increased period of synaptogenesis characteristic of early developmental stages. Periods of significant brain growth that occur during the first few postnatal years are correlated to the extremely high number of dendritic spines and synapses undergoing formation and reorganization.

During the first postnatal year, the brain grows to about 70% of its adult size and grows another 10% by the second year (Tau & Peterson, 2010). As previously described by Tau & Peterson, during gestation a great number of neurological developments take

place. These include synaptogenesis, pruning of neurons, myelination, and synaptic remodeling resulting in the basic organization and function of a neural framework (Tau & Peterson, 2010). This remodeling incorporates both the local connections within a cortical circuit, as well as more complex long-range connections between cortical circuits. The combination of genetic factors, novel experience inputs, and behavioral responses leads to further development and finer organization of the neural network (Bystron, Blakemore, & Rakic, 2008 and Tau & Peterson, 2010).

2.3 Pruning and reorganization of synaptic connections

Pruning of unused and insignificant spines and synapses is critical to the functionalization and organization of a neuronal system. The first signs of pruning are observed as early as GA week 7 when the cell-cycle regulation of apoptosis, a self-regulatory mechanism of cells leading to cell destruction, occurs. Later at approximately GA weeks 19 through 23, synaptic activity initiates the regulatory process that leads to pruning of the system (Tau & Peterson, 2010; Goda & Davis, 2003). Pruning targets those spines and synapses that have not been differentiated or strengthened. Synapses that are either not functioning properly or have not been sufficiently adapted and stabilized will be eliminated (Tau & Peterson, 2010; Goda & Davis, 2003).

2.4 Abnormalities in connectivity typical of Autism Spectrum Disorders (ASD)

A change in the neuronal functioning in Autism Spectrum Disorders (ASD) is due to a disruption in the development of these neuronal networks causing abnormalities in local and long-range connectivity, the underlying basis of ASD (Just, Cherkassky, Keller, & Minshew, 2004 and Belmonte et al., 2004). Local connections occur through excitatory synapses between neighboring neurons while long-range connectivity encompasses

connections between neurons of separate layers or regions (Belmonte et al., 2004). ASD abnormalities result in overproduction of local connections that are unused or weak. Conversely, amounts of long-range connections are scarce although highly strengthened. Belmonte et al. further distinguishes two other types of connectivity that relate to these previously described types, physical and computational. Physical connectivity is observed in the actual neuronal connections found in cortical tissue and has elevated densities in ASD subjects. This high density of physical connections is due to the lack of pruning of irrelevant and non-functioning or insignificant synaptic connections. High physical connectivity is conversely related to low computational connectivity, or inefficiency in interpreting neural signals, resulting in the failure to differentiate signal from noise (Belmonte et al., 2004). Overproduction of synaptic connections results in a high density of spines and synapses that are extremely underdeveloped. These overabundant and immature connections cause excessive traffic within the neural system and lead to difficulties in differentiating noise from actual neuronal signals (Belmonte et al., 2004; Hutsler & Zhang, 2010; Lewis & Elman, 2008; Penzes, Cahill, Jones, VanLeeuwen, & Woolfrey, 2011).

While the majority of brain growth is observed during the first two postnatal years of an infant, the progression of brain growth seen in those with ASD is abnormal and distinguishable from neurotypicals. The dramatic growth seen by a regularly developing infant is due to extensive myelination and synaptogenesis in gray matter, especially in sensorimotor and visual cortices (Tau & Peterson, 2010). If neurological systems are underdeveloped, the brain size will follow the trend of decreased growth, while overproduction of synapses and neurons leads to an increase and acceleration of brain

growth. Interestingly, the brains of children with ASD have been smaller than those of normal subjects at birth and then between the ages of 6 to 14 months, a dramatic acceleration of growth happens (Zoghbi, 2003). According to Hardan et al., MRI testing has found expanded gray and white matter compartments in individuals with autism or ASD (Hardan et al., 2006). The cause of this expansion is theorized to be the failure to eliminate faulty or incorrectly operating synapses as well as diminished function of the process apoptosis (Tau & Peterson, 2010; Zoghbi, 2003). This development time (6 – 14 months) is characterized by significant synaptogenesis, dendritic arborization, myelination, and pruning. Neurological networks are incapable of proper function and organization without remodeling and fine-tuning of immature, newly developed connections. The periods of accelerated brain growth for autism and the typical developmental progression of neuronal networks are correlated to the initial manifestations of ASD. Early in a neurotypical infant's life, the child will have behaviors and responses typical of normal social development such as smiling, responding to faces, and the development of appropriate motor control. However, around the age of 10 months children with autism begin to show deficits in their response to social cues, a lack of interest in normal social cues or extreme interest in abnormal behaviors or objects (Zoghbi, 2003). This age falls in the development period (6 – 14 months) that pruning and specified organization take place in typical neuronal systems, however in instances of ASD these organizational mechanisms prove faulty.

The importance and functions of dendritic spines will be discussed in the following chapters. In order to properly describe the significance and function of dendritic spines, a more complete understanding of the development of synaptic

connections is necessary. Specific proteins and molecules for the pre- and post-synaptic terminals regulate the dynamic nature of spines and the destabilization or stabilization of them. A lack of pruning results in a significantly higher density of spines than found in normal subjects which increases underdeveloped spines and an overproduction of synapses typical of neuronal networks of ASD subjects (Irwin et al., 2002 and Hutsler & Zhang, 2010). The molecular mechanisms of spines, their function, and their changing morphologies are extensive and provide the basis for the abnormalities seen in the development and the organization of neuronal networks of ASD subjects and will be examined thoroughly.

3. MOLECULAR MECHANISMS OF SYNAPSE FORMATION

The neural system is an equilibrium balance of excitatory and inhibitory synapses necessary for the proper regulation and function of neural circuitry. Many neurological disorders are a result of an imbalance of this synaptic ratio that impairs the normal brain function and results in either excessive inhibition or excessive excitation. Autism spectrum disorders (ASD) have characteristic deficits in neural development and function caused by this imbalance of the excitatory/inhibitory (E/I) ratio (Tabuchi, et al., 2007).

As development of the neurological system occurs, synapses form due to the induction and interactions of synaptic specific molecules. The binding of neuroligins with neurexins is an important step that induces synapse formation (Chih, Engelman & Scheiffele, 2005). The neuroligin-neurexin complex initiates binding of other synaptic molecules necessary for the production and stabilization of synapses. Several mutations specific to autism inactivate the effectiveness of neuroligins and alter the expression of specific molecules that have been identified in ASD, such as PSD-95 and Epac2. These mutations cause depressed production of inhibitory synapses, but do not affect the overall number of synapses. This leads to an increased number of excitatory synapses creating an imbalance of inhibitory and excitatory synapses that is associated with ASD (Chih et al., 2005). The excess excitatory synapses seen in this unfavorable E/I ratio create a less differentiated cortex that is very weakly inhibited and cannot self-regulate (Chih et al., 2005 and Matsuzaki, 2007). Random and overused firing of excitatory synapses occurs followed by improper expression of PSD-95 and Epac2. These molecules, along with the neuroligin-neurexin complex, are highly incorporated in the proper formation and stabilization of excitatory synapses.

3.1 Neuroligin-Neurexin Function

Before looking at the specific changes induced by the mentioned neuroligin mutations, it is first important to understand the molecular functions of both the neuroligin and the neurexin family in a typically developing neurological system. Neuroligins are cell adhesion molecules that are designated as postsynaptic molecules. They are members of the family called cholinesterase-like adhesion molecules. This family of molecules mediates enzyme-substrate interactions (Lise & El-Husseini, 2006). The characteristic that distinguishes neuroligins as a subset in this family is a lack of a residue within the cholinesterase-like domain (CLD) that allows neuroligins to mediate receptor/ligand interactions rather than enzyme/substrate interactions (Lise & El-Husseini, 2006). Five different genes have been identified to code for neuroligins leading to five known forms of neuroligins, 1 -5. Neuroligins 1 – 3 are more closely associated with autism. Specifically Neuroligins 1 and 2 (NLGN-1, NLGN-2) are exclusively found in brain tissue.

Upon formation of a synapse, these postsynaptic molecules bind with neurexins, presynaptic molecules, to create a more stable environment for chemical and electrical signaling. Neurexins are single transmembrane proteins that contain distinct extracellular N-terminal sequences with a receptor-like structure (Lise & El-Husseini, 2006). Two forms of neurexins, α-neurexin and β-neurexin, have been identified, however the main focus is β-neurexin due to its interactions with neuroligin-1 and neuroligin-3 differentiating a synapse as excitatory.

As synapses form, recruitment of both of these pre- and post-synaptic adhesion molecules occurs, and the neuroligin-neurexin complexes that form stabilize the synapse.

As neuroligins are recruited to the post-synaptic site and the neurexin to the presynaptic site, both molecules become anchored in the membrane and generate a complex that allows for synapse formation by alignment of postsynaptic sites to presynaptic terminals (Dean & Dresbach, 2006 and Shapiro, Love, & Coleman, 2007). Recruitment of neuroligins to the synapse is complimented with the recruitment of other molecules and proteins that all contribute to the differentiation and stabilization of synapses (Levinson et al., 2005 and Dean & Dresbach, 2006).

3.2 PSD-95 Function

PSD-95 (Post synaptic density) is a scaffolding protein that binds to *N*-methyl *D*-aspartate (NMDA) receptors and to neuroligins at the PDZ-domain motif. This motif is located at the intracellular region of the neuroligin and is the binding site for three different proteins, one of which is PSD-95. When PSD-95 is overexpressed, there is an observed reduction of NLGN-2 at inhibitory synapses and increased expression of NLGN-1 at excitatory synapses. When PSD-95 expression is decreased, the opposite effects occur (Craig & Kang, 2007). Levels of PSD-95 expression can alter the E/I ratio, thus PSD-95 levels significantly affect the regulation of E/I balance as well as the maturation and stabilization of excitatory synapses (Craig & Kang, 2007). While recruitment of NLGN-1 to the membrane is associated with recruitment of PSD-95, expression of PSD-95 is also colocalized with Epac2 (described in detail in the next section), another marker for excitatory synapses (Woolfrey et al., 2009 and Penzes, Woolfrey, & Srivastava, 2011). It has been proposed that the complex formed between Epac2 and PSD-95 is necessary for Epac2 to bind and interact with other proteins at the synapse (Penzes, Woolfrey, & Srivastava, 2011).

3.3 Epac2 Function

The activation of Epac2 promotes spine shrinkage and spine motility as well as the removal of GluR 2/3 (glutamate receptor type 2/3)-containing AMPA receptors (Woolfrey et al., 2009). Strengthened synapses are strongly correlate to high amounts of AMPA receptors that are responsible for excitatory transmission. While Epac2 was found to have a high selectivity for interaction with Neuroligins 1 and 3, there were no significant interactions between Epac2 and Neuroligin 2, attributable to Epac2's colocalization with PSD-95. Both are markers of excitatory synapses, similar to the specificity of Neuroligins 1 and 3 for excitatory synapses, whereas Neuroligin 2 is predominately localized in inhibitory synapses (Woolfrey et al., 2009) Epac2 inhibition results in spine enlargement and spine stability (Woolfrey et al., 2009). Woolfrey et al. also found that overexpression of Epac2 removed AMPA receptors from synapses further contributing to weaker synaptic connections. Long periods of Epac2 activation promotes spine shrinkage and motility resulting in the persistence of decreased spine stability and spine pruning; however, Epac2 does not promote spine and synapse degeneration (Woolfrey et al, 2009).

A critical neuroanatomical classification of ASD is overextensive synaptic connections with limited cognitive abilities. Better understanding of Epac2 function indicates that these symptoms from ASD are the result of heightened Epac2 levels. Normal functioning and levels of Epac2 are required for the continuous remodeling and plasticity of synapses in typical neural development. Increased levels of Epac2 could plausibly result in an increase of Epac2 function in turn leading to a much greater number of destabilized spine, although Epac2 would not eliminate spines and affect the total

number. Synapse connections would become much weaker while maintaining abundance within the system. This type of framework is representative of the proposed model of the local overconnectivity along with the long-range underconnectivity involving numerous destabilized spines rather than extensive elimination and reorganization of spines seen in a typically developing system.

The next step in understanding the abnormal development characteristic of ASD is looking at interactions between each of the mentioned molecules. The relationship between these molecules is imperative for the formation of proper synapse structure and formation leading to correctly firing synapses. Mutations or altered expression of these molecules leads to immature spine morphologies and functions causing abnormal connectivity in ASD.

3.4 Interactions of Synaptic Molecules

Alterations for a molecule have direct effects on the activities of other molecules due to the high degree of interaction between these at the subcellular level. For instance, expression of NLGN-3 results in Epac2 translocating to dendrite membranes in order for the neuroligin to bind with Epac2 that activates the protein (Chih, Engelman & Scheiffele, 2005 and Woolfrey et al., 2009). Heightened Epac2 levels increase interaction with neuroligins and neurexins, so long as there are enough molecules for the excess Epac2 to interact with. This heightened interaction with neuroligins and neurexins increases the number of activated Epac2 molecules, thus increasing Epac2 activity.

One key aspect of synapse differentiation incorporates the majority of NLGN-1 concentrated to excitatory synapses while NLGN-2 is concentrated at inhibitory

synapses. In turn, the colocalization of specific proteins and receptors to each of these neuroligins become good indicators of whether a synapse is excitatory or inhibitory.

To understand the effects of neuroligin-1 on excitatory synapses, Chih et al. performed a study where NLGN-1 was overexpressed (2005). This overexpression resulted in irregular shapes of spines with increased numbers of presynaptic terminals for the vesicular glutamate transporter 1 (vGlut1), a definitive marker of excitatory synapses, as well as increased amounts of PSD-95 (Chih et al., 2005).

To further understand the function and mechanism of NLGN-1, two mutants of NLGN-1 were engineered and inserted into mice. The results of the experiments displayed the necessity of proper functioniong of β-neurexin binding sites for the proper alignment with presynaptic markers for vGlut1 to establish an excitatory synaptic connection (Chih et al., 2005). This study also demonstrated that β-neurexin binding followed recruitment of NMDA receptors to the membrane; however, intracellular binding of PSD-95 was not necessary for the mediation of this process. Improper functioning of PSD-95 binding sites did, however, result in a lack of stabilization of excitatory synapses (Chih et al., 2005, Dean & Dresbach, 2006).

To investigate the effects of reduced neuroligin function, silencing experiments were used. Lise and El-Husseini observed that simultaneous knockdown of all three neuroligins, 1, 2 and 3 resulted in decreased numbers of both inhibitory and excitatory synapses (2006). The decrease in inhibitory synapses was significantly higher than that of the excitatory synapses. They also observed reductions in spine numbers and GluR1 puncta (Lise & El-Husseini, 2006).

Chih et al. performed similar silencing experiments but focused on independently silencing NLGN-1 and NLGN-3 (2005). While they did not observe any significant differences in protein densities for either of these two sets of experiments, they did observe a decrease in spine density and maturation. They also observed decreased formation of excitatory synapses, identifiable by a reduction in vGlut-1 markers at the membrane (Chih et al., 2005). Many mutations associated with autism result in reduced or altered functions of neuroligins (Chubykin et al., 2005 and Laumonnier et al., 2004) demonstrated by the silencing experiments mentioned above. The mutations demonstrate increases in excitatory synapses and decreases in inhibitory synapses while not changing the total number of synapses. Overall, mutations that alter or silence neuroligin function create exaggerated numbers of excitatory synapses, imbalanced E/I ratios, and weakened unstable spines.

The overexpression of excitatory synapses seen in ASD has significant implications for the molecules necessary for the proper formation, pruning, and stabilization of synaptic development. Dendritic spines are the sites for these excitatory synapses, and the various interactions that occur here are significant to the understanding of altered neural development in ASD. Neuroligins 1 and 3, PSD-95, and Epac2 all colocalize with each other and are indicators of excitatory synapses. When increase in activation of one of these molecules occurs, paralleled increases in expression of the others follow. Understanding the role these molecules play on synapses stabilization and spine morphology and dynamics gives a greater understanding of how mutations for genes coding for each one of these molecules affects synapse development, spine morphology, and overall neural function.

3.5 Overview of interrelation between molecular mechanisms

Epac2 has been identified as one of the most significant contributors to regulation of dendritic spines and therefore synapse formation. Epac2 levels also affect PSD-95 and neuroligin-neurexin interactions and, therefore, abnormal functioning and expression of each of these has led to overconnectivity typical for ASD subjects. Normal neural development involves an overgeneration of spines and synapses followed by pruning of excess, unused spines (Fiala & Harris, 2002). If the mechanisms required for spine pruning during development are disrupted, spine density will significantly exceed the normal amounts. These increased densities lead to an overcomplicated network of neurons with excessive connections. The underdeveloped and abnormally high amounts of synaptic connections could result in chaotic signaling and the inability to distinguish between useful information signals and other signals that are only "noise" within the neuronal network system (Hutsler & Zhang, 2010).

4. DENDRTITIC SPINE PROCESSES

Dendritic spines are the sites for excitatory synapses and play a major role in neurological systems. Their capability for a dynamic morphology enables processes for synaptic plasticity to occur. When this morphology is altered and the majority of spines throughout a neurological system displays signs of weakened function and underdeveloped morphology, major deficits can occur. In ASD, spines exhibit abnormally high numbers in density while also demonstrating these immature states. Due to a lack of synaptic pruning, E/I ratios are imbalanced and excess numbers of excitatory synapses are found in weakened, extremely unstable states.

4.1 Spine Function

Initially, it was thought that spines helped to increase the area of the dendrite and help connect neurons. This first theory led to more developed theories of spine function in neuronal connections that involve the ability of spines to create maximum amounts of connections while eliminating extensive axons and axonal connections that cause wiring traffic within the cortex (Yuste & Majewska, 2001). The dynamic properties of spines and the rapid changes they undergo in their morphology also act as the main mechanism for synaptic plasticity (Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010). Synaptic plasticity is characteristic of neuronal networks and is based in the dynamic behavior of spines and the effects this has on synapses.

4.2 Calcium Dynamics in Spines

The vast amount of connections and synapses that can be made by spines is due to their unique morphology, leading to other ideas about spine function, in particular chemical compartmentalization. Spine dynamics influence synapse plasticity and are

dependent on the structure of spines that is intimately linked with calcium concentrations. A large fraction of protein in the spine is Ca^{2+}/Calmodulin kinase II, an enzyme that acts as a calcium-activated molecular switch and induces synaptic plasticity processes (Holcman, Korkotian, & Segal, 2005). Further indications that spines act by calcium regulated mechanisms and therefore are compartments for calcium comes from the Ca^{2+} dependence of the B-neurexin-neuroligin complex.

Recent studies by Holcman, Korkotian, and Segal have implicated calcium dynamics as being the molecular mechanism underlying changes in spine morphology (2005). Synaptic plasticity is proposed as a function of the change in calcium concentration in a spine over the change in time. The cytoskeleton of dendritic spines is composed of dynamic actin filaments that are regulated by calcium mechanisms (Oertner & Matus, 2005). Changes in the Ca^{2+} concentration in the cytoplasm of the spine results in rearrangement of the spines cytoskeleton which affects the morphology and stability of the spine (Oertner & Matus). Studies by Oertner & Matus showed the regulatory functions of Ca^{2+} in spine motility by inducing an increase in motility when Ca^{2+} was removed from the spine cytoskeleton. Overall, calcium dynamics are the driving force behind spine motilitiy and subsequently for processes that cause synaptic plasticity.

Synaptic plasticity involves the processes of long-term potentiation (LTP) and long-term depression (LTD); both are directly affected by changes in spine morphology. LTP requires elevations in calcium concentration at sites of the activated synapse whereas LTD requires a decrease in calcium concentration (Yuste & Majewska, 2001 and Yuste, Majewska & Holthoff, 2000). Spine head size can greatly vary indicating the differences in Ca^{2+} volumes they can contain (Yuste & Majewska, 2001 and Yuste,

Majewska & Holthoff, 2000). Oertner & Matus performed a study that showed long-lasting increases in the volume of spine heads following LTP based on the activation of Ca^{2+}/calmodulin-dependent protein kinase II (2005). This activation was also indicative of LTP by its correlation with increased numbers of AMPA receptors thereby creating a more stable excitatory synapse (Oertner & Matus, 2005).

Spines that express high levels of CA-ATPases can maintain high concentrations of Ca^{2+} for short periods because calcium ions diffuse quickly from the spine cytoplasm. Stimuli that elicit large calcium influxes are less likely to cause potentiation at these synapses as compared with synapse with low Ca-ATPase expression because high influxes would not be able to maintain that amount of volume for an extended period of time since the Ca^{2+} would all rapidly diffuse.

Differences in spine neck length result in differing diffusion barriers. Spines with shorter necks are less likely to be indicative of potentiation of a synapse because high Ca^{2+} concentration will not be very stable and readily diffuse into the dendrite. On the other side, spines with long necks are more isolated from the dendrite and are, therefore, better able to maintain high Ca concentrations and can be easily potentiatied (Yuste, Majewska, & Holthoff, 2000). It is evident that spines are individual in their calcium extrusion rates, neck length, patterns and expression of calcium channels and pumps. This individualism leads to differences in calcium concentrations and regulations among spines along a dendrite and varying stabilizations of excitatory synapses (Yuste & Majewska, 2001). Variation among morphology of spines demonstrates the high degree of independence that dendritic spines posses accounting for the differences in spine morphologies and calcium dynamics along a dendrite.

4.3 Spine Morphology:

Studies conducted by Irwin et al. in 2002 used genetic engineering in mice to compare wildtype (WT) mice with Fragile X mice. Fragile X and ASD have a very high comorbidity rate and the alterations in the neural developments for both disorders display a high degree of relatedness. Further, the abnormalities in neuronal connections for both Fragile X and ASD share the same types of underdeveloped connections and overproduced spines. The results of this study showed abnormal morphologies and lengths of spines for Fragile X mice when compared to WT mice. The spines observed in this case were labeled as immature and consisted of abnormal length, very thin necks, and small or no heads. Spines labeled as mature signified proper stabilization and function and were classified by normal length; therefore, the heads of these spines were located closer to the dendrite than those of Fragile X mice. The mature spines also displayed either a wide, large head connected to the dendrite by a neck, or a single stubby outgrowth with no indentations distinguishing a neck from a head. The classification of spines used in this study are similar to the scheme used in the present study.

Normal development of neurological systems involves the overgeneration of spines and synapses followed by pruning, LTP, and LTD to strengthen and stabilize important, specific connections and eliminate non-functional and excessive connections not necessary in the system. In cases of ASD, there is an extreme lack of function in this process, and spine densities are greatly increased, thereby increasing numbers of excessive and underused or nonfunctional synaptic connections (Hutsler & Zhang, 2010 and Van Spronsen & Hoogenraad, 2010). The morphology of spines, as previously noted, is constantly changing aided by calcium dependent mechanisms. The specific

morphologies within a neurological system play key roles in identifying maturity and stability of synapses. The present study investigates this spine morphology, comparing ASD cases with neurotypical cases, and seeks to identify the distinct differences in spine morphology and spine length between the two. Spines will be examined across regions and throughout different cortical layers in order to examine how altered spine morphology in ASD can affect the neurological mechanisms in a system. The most important features being investigated are thinness or thickness of a spine, the absence or presence of a head, and spine length.

5. METHODOLOGY

This study used cortical tissue samples provided by the Autism Tissue Program (ATP, Los Angeles, CA). The Biomedical Institutional Review Board at the University of Nevada, Reno reviewed the procedures to collect and identify the tissue as well as the data storage protocol. Samples were from four male ASD individuals as well as four neurotypicals, matched for both age and sex. Classification of the ASD subjects was based on the medical and psychological records available and confirmed by the Revised Autism Diagnostic Inventory (ADI-R). The control cases were selected based on age, PMI, tissue block availability, and an absence of neurological disorders in order to have a final set of four age- and sex-matched pairs. The case information for this study is represented in Table 1.

Table 1
Case Information

Case ID	Diagnosis	R/L	Age	PMI (h)	Brain Weight (g)	BA7	BA9	BA22
1	ASD	L	29	24	1340	x	x	x
2	Neurotypical	L	29	24	1350	x		x
3	Neurotypical	L	17	7	NA	x	x	x
4	Neurotypical	R	11	20	1420	x		
5	Neurotypical	L	51	24	1600	x	x	x
6	ASD	L	25	26	1220	x	x	
7	ASD	R	44	31	1530	x	x	x
8	ASD	R	20	24	1140	x	x	

From these four ASD cases and four control cases, formalin-fixed cortical tissue samples for all subjects were collected from three cortical locations: the lateral surface of the middle temporal gyrus, Brodmann's area 22 (BA22); the dorsolateral frontal lobe, Brodmann's area 9 (BA9); and the dorsal lateral parietal lobe, Brodmann's area 7 (BA7). Different locations of the cortex are implicated in

playing more significant roles in ASD, such as the temporal lobe. Further, the previously mentioned areas, BA22, BA9, and BA7, all display anatomic similarity leading to an anatomical control. There are no major differences in the organization of each region that could lead to reasons for differences observed in spines between the three layers examined during this study.

Some variation in spine density and morphology, regardless of cortical area, was expected. A study by Hutsler & Zhang in 2010 found increased levels of spine densities in all ASD cases. Further, the difference in spine densities was highest in layer 2 of all three regions indicating a layer-specific alteration of neural organization in ASD. The present study was conducted in Dr. Jeffrey J. Hutsler's lab at the University of Nevada, Reno. Five cells from each cortical layer 2, 3, and 5 were examined per each of the three regions (BA7, BA9, BA22) for each case resulting in a total of 45 cells per case.

The present case used a modified Golgi-Kopsch technique, a staining process ideal for well-fixed samples and specific for the staining of pyramidal neurons. This class of neurons has thousands of dendritic spines located on their dendrites implicating them as key cells in the neurological system for excitatory synapses (Spruston, 2008). This staining technique was utilized for this study due to its high specificity for pyramidal neurons, decreasing any staining of other types of cells within the system.

Collection and analysis of spine data were performed on the apical dendrites of cortical pyramidal cells. The typical morphology of a pyramidal cell involves several short basal dendrites and one long apical dendrite that splits and connects

the soma with several dendrites (Spruston, 2008). This organization creates multiple locations for excitatory synapses located on spines and is an ideal schematic for the present data collection. Spine morphologies were observed and classified based on a system derived from the principles outlined by Irwin et. al in 2002. Irwin used an 8-type scheme to effectively differentiate mature versus immature spines. Therefore, a deconstructed classification system was devised specific for this morphological study that incorporated three main features. The three classifications included thin versus thick, presence or absence of a head, and spine length (Figure 1).

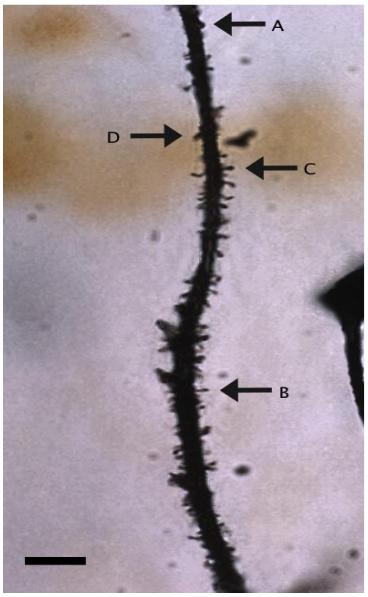

Figure 1. **Dendritic spines along an apical dendrite.** This is an example of multiple dendritic spines with varying morphologies along an apical dendrite. A: thick spine; B: thin spine; C: spine with a head; D: spine without a head; Scale Bar = 10 um.

The presence of a head is very important for spine function and the idea that the head serves as a chemical compartment. Spines lacking in a spine head would play a very diminished role in calcium mechanisms upon stimulation of the synapse.

Further, previous studies have shown that some very immature and unstable spines do not even present with a head, this has been an indicator of immature, weakly or improperly functioning spines (Penzes et al., 2011; Irwin et al., 2002; Fiala, Spacek, & Harris, 2002).

Cells were located at 100x magnification and then spines were examined and classified at 1000x magnification. The microscope and camera used for this study were the Olympus BX51 microsocope and an Hitachi DP20A camera, (Olympus, Inc.). This classification process utilized ImageJ software to perform accurate and consistent classification approaches. Due to the fine boundary between a thin spine and a thick spine, data collectors underwent extensive training prior to performing spine data collection for this study. This training ensured the same bias among collectors for differentiating between a thin and a thick spine, as well as a spine head or no head (refer to Figure 1), creating consistency throughout the entire spine classification process.

Once a cell was identified for further data collection, a grid was imposed on the screen composed of nine 25 x 25 μm bins. The camera was adjusted to make the apical dendrite parallel to these bins and classification processes ensued. Spines were classified by the cell number (dependent on the particular case), the bin number (1 – 9), thick or thin (designated by 0 or 1), and head or no head (designated by 0 or 1). A length measurement of the spine was also taken and following completion of classification along the entire dendrite, the cortical layer that the cell was in was determined.

The overall length of the spine is very informative for deducing spine function; the further away the spine head is from the dendrite the more effective the neck functions as a diffusion barrier (Yuste & Majewska, 2001 and Yuste, Majewska, & Holthoff, 2000). Additional measurements taken include cell body size and the diameter width of the dendrite along its length.

The extremely large amount of data, including spine length and spine classification, was analyzed across layers and regions. The results were averaged among layers and collapsed across regions to locate significant differences between the ASD cases and the neurotypical cases. Two different types of analyses were performed for the categorical and the non-categorical data. For the categorical data (morphology), an Odd's Ratio was calculated and a Fisher's Exact Test was performed to assess significant differences in morphology. For the non-categorical data (spine length), mean comparisons and an independent sample's t-test were applied where necessary.

6. RESULTS

ASD subjects showed a significantly lower proportion of spines with heads relative to neurotypical subjects (Odds Ratio = 1.945, $p < .001$). These results show that ASD subjects exhibit a lower number of synaptic spines that contain heads compared to neurotypical subjects. This proportion of spines without a spine head is an indication of differences in the calcium dynamics of these dendritic spines.

The length of spines showed notable differences between ASD subjects and the control cases. On average, ASD subjects had significantly longer spines. Furthermore, when comparing spine lengths across the three different layers from which cells were located and examined, there were no significant differences among ASD subjects (Figure 2). All of the spine lengths for the ASD cases were comparable across layers 2, 3 and 5; however, when observing the control cases, a trend presented toward reduction in spine length with increasing cortical depth. This trend was especially apparent in region 9 (Figure 3), and this effect was statistically significant in layer 5 averaged across regions ($t = -2.35$, $p < .05$, DF = 17).

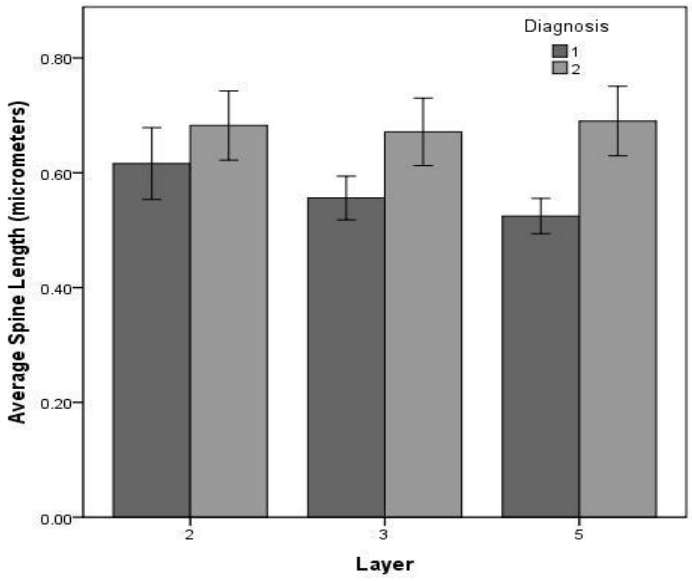

Figure 2. **Average spine lengths analyzed across layers**. This graph displays the average lengths of spines collapsed across regions and presented across cortical layers. Diagnosis 1 (dark gray bars) signifies neurotypical cases and Diagnosis 2 (light gray bars) signifies ASD cases.

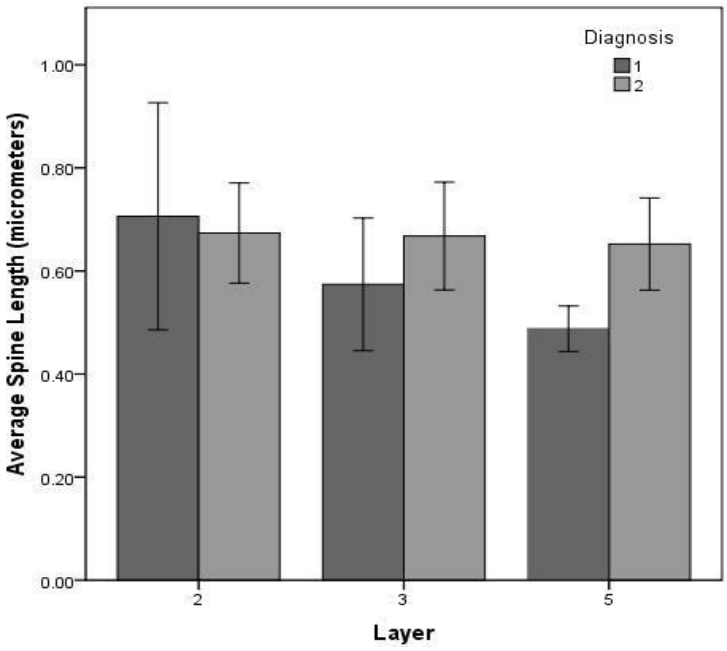

Figure 3. **Average spine lengths for region 9**. This graph depicts average spine lengths only from region 9. These lengths are are displayed across the layers from which cells were selected for collection. Diagnosis 1 (dark gray bars) signifies neurotypical cases and Diagnosis 2 (light gray bars) signifies ASD cases.

Overall, these results point to disruptions in the length and morphology of dendritic spines in the autistic brain. As previously discussed, features such as length and morphology have functional implications, and alterations to these characteristics in the autistic brain can be related to an abnormal neuronal network. These implications are discussed in more detail in the following section.

7. CONCLUSIONS

Significant differences in spine morphology and length were observed between the spines from ASD cases and the spines from neurotypical cases. Penzes et. al., 2011, produced findings on the relationship between spine morphology and function. Likewise, Yuste et al. in 2000 provided further evidence for a direct relationship between spine form and function. The amount of calcium that a spine head is able to hold is directly related to the size of the spine head. The length of a spine neck translates to the rate of diffusion of calcium and therefore rate of chemical information transfer. Yuste et al. also showed the independent functionalization of spines by showing the vast differences in calcium dynamics among spines located on the same dendrite. Overall, the ability of spines to alter morphology (this includes length) thereby adjusting their rates of diffusion has a significant impact on synapse plasticity. The size of a spine head is also directly related to the strength of the synapse (Yuste et al., 2000). The results of this morphological study agree with the previous studies and lead to important implications for how morphological changes affect spine function, thereby affecting synaptic development, function, and plasticity.

7.1 Implications for absence of a spine head

The results of this study showed significant differences in the amounts of spines with a head compared to those without a head in the ASD cases. The high reduction of spines without a head for ASD cases indicates an overexaggerated system with large numbers of spines lacking in a key functional component. The spine head plays a major role in spine dynamics and in the ability of spines to alter

their morphology thereby affecting plasticity of synapses. The significant number of spines lacking a spine head in ASD circuits implies major deficits in spines ability in regulating calcium dynamics. Without a head, there is a significant decrease in the proportions of spines that can carry volumes of Ca^{2+}, causing a weakened control of the Ca^{2+}-dependent actin cytoskeleton in order to alter their morphology. Further, spines that could not hold Ca^{2+}or only hold a limited amount would result in lessened function of the chemical information transfer that occurs at synapses. Overall, the absence of a spine head on such a large proportion of spines within a neural system leads to declined function of the calcium mechanisms necessary for spine motility.

7.2 Changes in calcium dynamics

Synaptic plasticity is an extremely important component of a neural system and is dependent on the motility of dendritic spines. A properly functioning neural system relies on significant amounts of spines with the ability to rapidly potentiate Ca^{2+} signals and self-regulate their motility through calcium-dependent mechanisms. As previously discussed, synapse stabilization and formation is highly dependent on neuroligin-neurexin complexes and the stabilizing proteins this complex induces. These complexes are highly dependent on Ca^{2+} and the calcium processes that occur in dendritic spines. If these spines do not have the proper morphology to mediate these processes, the neuroligin-neurexin complex would not be efficiently adept for synapse formation. The end result would be highly destabilized excitatory synapses with improper functioning. If these complexes were unable to form efficient synaptic sites on spines, the induction of other

important proteins to the synaptic system would be faulty. Altered expression of PSD-95 and Epac2 would result in irregular spine motility and weakened spines without elimination of spines. This absence of elimination of destabilized spines would produce an exaggerated number of excitatory synapses expressing abnormal and unreliable signaling. Without elimination of these faulty synapses, E/I ratio becomes imbalanced and excess traffic is created in the ASD neural system.

7.3 Spine length stasis across layers for ASD cases

Results of this study have indicated deficits in spine function due to significant proportions of immature spines, classified as long spines without a head. Shorter spines are more able to rapidly exchange chemical signals. The observance of spine length reduction across layers displays the importance in typical neural circuitry to have more rapid diffusion rates as cortical depth increases. For ASD cases, spine length remained static across cortical layers indicating slower diffusion rates. This stasis could result in delayed function of synapses and delays in transfer of chemical information.

The results of this study are in accordance with Irwin et al.'s findings from 2002 and 2001 based on Fragile X Syndrome. The underdeveloped connectivity that is due to Fragile X is the same organization as seen in ASD. Irwin et al. found significantly greater proportions of longer, thin, and immature type spines in Fragile X cases, similar to the results of the present study that indicated longer, immature spine morphologies in ASD cases. Strength of a synapse comes from the transition of a long, thin, headless spine to a shorter, thicker, stubby spine. Therefore, the

abundance of longer headless spines found in this study shows a significant dysfunction in strengthening a synapse.

7.4 Implications of differences in spine length across layers

Cortical layers 2 and 3 are designated for cortical-cortical transfer of information while cortical layer 5 is specific for transfer of information across subcortical areas. Normally, spine lengths decrease with increasing depth of the cortical layers. The results of this study showed a significant loss in this spine length reduction for ASD cases. This result was especially apparent in region 9 (BA9), which plays a significant role in integrating sensory information. Longer spines in this region indicate delayed diffusion rates that directly affect the proper relay of sensory information in the neuronal system.

One of the main classifications used in this study was the length of a spine. Thick, short, stubby spines exhibit very strengthened function and typically are sites for stable excitatory synapses. The classification used in the present study to designate stubby spies was thin, short spines without a head (A in Figure 1). Previous studies indicate the preference for these stubby spines by Thalamic (Th) inputs (Richardson, Blundon, Bayazitov, & Zakharenko, 2009). Highly significant increases in lengths for spines in layer 5 observed in ASD cases could account for an extreme absence of these short stubby spines indicating abnormally functioning and altered connections for the thalamus.

The thalamus is a key factor in distributing proper information to the appropriate areas of the brain. Its primary function is routing sensory information to the appropriate areas of the brain for further discernment (Dowell, 2007). The

"brain-behavior connection" implicates abnormalities in the relationship of the thalamus with sensory routing as a basis for ASD (Dowell, 2007). Dysfunctions in synaptic inhibition or selectivity could account for certain behavioral abnormalities typical of ASD such as, avoidance of touch or a need for deep pressure stimulation.

7.5 Future research

The results of this study provide evidence on alterations in spine morphology and length in ASD. The significant differences seen in spine length across layers between neurotypical cases and ASD cases implicate slower diffusion rates as cortical depth increases. Future research could be done to map the cortical connections in layers 2, 3, and 5 to try to identify any morphology preference for specific types of inputs. This type of study could also compare the cortical circuitry map for an ASD subject and a neurotypical subject to find slower rates of neural information transfer or reduced amounts of neuronal connections.

Further research is necessary to gain more knowledge on the calcium mechanisms in spines. Significant proportions of spines lacking a spine head in ASD could greatly contribute to alterations in function of spines that are regulated by calcium. This study was the first spine morphological study specific for ASD and contributed to knowledge on causes of deficits in ASD circuitry. Future research could be conducted to detail the form-function relationship of dendritic spines.

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