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Synaptic protein expression in human postmortem brain tissue of autism spectrum disorder

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By

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ABSTRACT

It is estimated that one in 59 children in the US are affected by autism spectrum disorder (ASD). ASD is distinguished by social and communication deficits that can be displayed throughout a wide range of severity. This resulting spectrum of behaviors observed in ASD suggests that a complex etiology is involved. Previous studies have shown a genetic susceptibility to autism including paternal age, twin and sibling concordance. Genetic sequencing of those affected as well as first order relatives have identified alterations in genes associated with neuronal synaptic communication. However, very little information is available regarding the pathophysiology of synapses in ASD. Neuronal communication between anterior cingulate cortical neurons via synapses with other brain regions is vital in the execution of social behaviors in individuals. The aim of this study was to evaluate the protein expression of the synaptic marker spinophilin and post-synaptic density protein-95 (PSD-95) in postmortem ASD gray matter brain tissue from the anterior cingulate and frontal cortex to compare to typically developing (TD) control brain tissue. Postmortem brain tissue of ASD and TD subjects was acquired from nationally funded brain repositories previously matched by brain area, age and gender. Immunoblotting for spinophilin and PSD-95 was performed using anterior cingulate and frontal cortical gray matter brain tissue from matched ASD and TD brain tissue. Spinophilin and PSD-95 protein amounts for all donors were normalized using GAPDH. Frontal cortical tissue demonstrated no significant differences in spinophilin protein expression between TD and ASD groups (N=6). Anterior cingulate tissue demonstrated no significant differences in spinophilin protein expression between TD and ASD groups (N=5). PSD-95 protein expression levels did not result in any significant differences between ASD donors and their control pairs for either brain tissue region. Although no changes were detected in the frontal cortex or anterior cingulate

cortex, more brain areas and subjects must be evaluated to determine if spinophilin or PSD-95 can be reliable markers for synaptic alterations in ASD. These data are critical in determining synaptic pathology in ASD which may lead to future treatments.

ABBREVIATIONS: Autism Spectrum Disorder (ASD), Gastrointestinal (GI), Typically Developing (TD)**,** Brodmann Area (BA), Anterior Cingulate Cortex (ACC), Magnetic resonance imaging (MRI), Functional MRI (fMRI), Prefrontal cortex (PFC), Gamma- aminobutyric acid (GABA), Postsynaptic density protein 95 (PSD 95), Immunohistochemistry (IHC), Diagnostic and Statistical Manual (DSM), Gray Matter (GM), White Matter (WM), Insulin-like growth factor (IGF1).

BACKGROUND

Autism Spectrum Disorder (Facts and Behaviors)

Autism Spectrum Disorder (ASD) poses a major concern to society as it is a disorder that affects many individuals, and has shown a growing trend in numbers. ASD is known as a spectrum disorder due to the wide range of behaviors that can be displayed in individuals. It can be categorized by repetitive and specific patterns of behavior that interfere with communication and social interactions. Assessment of children for ASD include measures of intellectual functioning and verbal language skills that display varying degrees of impairment. The resulting 'spectrum' of behaviors is attributed to a broad range of phenotypes that are classified by levels of severity suggesting that the complexity of this disorder is not the result of one causal or determining factor. Intelligence for example is the result of many contributing factors and has demonstrated varying patterns with ASD symptom severity. One study for example, analyzed the IQ's of 75 children diagnosed with ASD and found that 55% of the children fell within the "intellectual disability" category, 16% were categorized as "moderate to severe intellectual disability", 28% demonstrated "average intelligence", and 3% exhibited "above average intelligence" (Charman *et al.*, 2010). An older study in 2000 conducted on 27 children diagnosed with ASD demonstrated that early intervention in 31 to 65-month-olds is imperative and led to higher quality, educational placement post- treatment (Harris *et al*). Mayes and Calhoun's cross-longitudinal study examined 164 3 to 15-year-olds and established that any early speech delays demonstrated in ASD children did not determine their verbal IQ ability at a later age (2003). The study of IQ, speech, and behavioral patterns is just one example of the complexity of the disorder. It is likely an interplay of both genetic abnormalities and environmental factors at

critical stages of development interact to cause the varying degree of symptoms demonstrated by ASD.

The prevalence of ASD is astounding. According to the National Institute of Mental Health, a study done by the CDC's Autism and Developmental Disabilities Monitoring Network (ADDM) found that one in every 59 children were diagnosed with ASD in 2014. ASD affects individuals across all racial and ethnic groups, regardless of socioeconomic status. ASD is found to occur more commonly in boys than girls, with boys four times as likely to be identified with ASD (Baio *et al.*, 2014). Additionally, children are more likely to develop ASD if their parents already have a child with ASD (Ozonoff *et al.*, 2011; Sumi *et al.*, 2006). ASD has a complex etiology as 5% of the ASD population present with an "underlying chromosomal anomaly" (Folstein *et al.*, 2001). Multiple genes have been identified through genetic sequencing of affected individuals and family members (Jamain *et al.*, 2003). Genetic changes can directly affect phenotype; therefore, the genetic profile of individuals with ASD has contributed to our current knowledge but does not account entirely for development of the disorder. Identical or monozygotic twins, fraternal or dizygotic twin pairs and regular siblings have been investigated to determine the genetic similarity in ASD. ASD concordance levels in identical twin studies (where both twins are diagnosed with ASD) range between 36 to 95%, strongly supporting that additional factors contribute to the disorder (NINDS, 2015). One particular monozygotic twin study showed a concordance rate of 60% with the dizygotic pairs demonstrating 0% concordance (Bailey *et al.*, 1995). While the rates may vary slightly from study to study, it has been consistently shown that monozygotic twin pairs display a higher risk of ASD concordance than dizygotic twin pairs (Rosenberg *et al.*, 2009). But, concordance rates of less than 100% in monozygotic twins

suggests that genes do not entirely account for the ASD risk, but do play some role in the disorder.

Many possible risk factors have been associated with ASD including parental age (Durkin *et al.*, 2008). Paternal age played a factor in the diagnosis of ASD, for one study found children born to fathers 40 years or older were at a greater risk, by a factor of 5.75 (Reichenberg *et al.*, 2006). While ASD reports have shown a high prevalence in offspring of the non-Hispanic white race, the diagnosis is not limited to this population. One study examined the factor of maternal nativity and found that compared to US-born whites, children of foreign-born mothers of African-American, Central/ South American, Filipino, Vietnamese and US-born Hispanic and African American ethnicities had a higher risk of ASD diagnosis (Becerra *et al.*, 2014). This study is significant in that demonstrates the need for future research on how maternal race and nativity are related to the occurrence of ASD in children. Because ASD crosses all racial and socioeconomic borders, more studies on parental factors could lead to new findings in regards to the etiology and pathophysiology of ASD.

In terms of co-occurrence with other diagnoses, individuals with ASD are 83% more likely to have one or more non-ASD developmental disorders (Levy *et al.*, 2010). While the Diagnostic and Statistical Manual of Mental Disorders (5th Edition) prohibits ASD and ADHD from being co-diagnosed, the co-occurrence has recently been reported as common, especially between children who have ADHD and motor coordination issues (Reiersen & Todd, 2014). Children with ASD have also been found to suffer from more gastrointestinal symptoms as compared to control groups (McElhanon *et al.*, 2014). The idea of a "gut-brain axis" has been hypothesized and examined in numerous studies stemming from this relationship between GI disturbances and ASD (Rosenfeld, C. S., 2015). The connection between the gut-microbiome and brain is further

linked by the gut's closely associated role in the limbic system, controlling "emotionality" (Jones *et al.,* 2006). Further, it is noteworthy to state that studies have found that the severity of these GI disturbances is linked with the severity of ASD diagnosis (Adams *et al.,* 2011). Collectively, these studies strengthen the argument for more research on genetic risks and comorbid disorders associated with ASD.

The economic burden of ASD is rising as the number of reported cases increases. For example, in the UK one estimate by the Foundation for People with Learning Disabilities found the annual cost of supporting children with ASD to total 2.7 billion pounds and roughly 3.5 billion dollars (Knapp *et al.*, 2007). In the US, one study found that compared to children without ASD, the mean medical expenditure for Medicaid-enrolled children diagnosed with ASD was six times greater (Peacock *et al.*, 2012). Little research is available for other countries regarding the economic burden of ASD. However, one study in urban China found that 58% of families with a child with ASD affected employment outcome (Ou *et al.*, 2015). Additionally, a correlation was found between the occurrence of ASD and cost of emergency department (ED) visits, for adults with ASD exhibited mean total ED costs of 2.3 times higher than typically developing (TD) adults (Vohra *et al.*, 2015). The financial burden that has been associated with autism spectrum disorder is staggering and it is important to recognize that there are many costly concerns that families face with ASD.

The behavioral aspects of this disorder vary extensively with most diagnoses occurring shortly after two years of age. According to the latest version of the Diagnostic and Statistical Manual of Mental Disorders, the severity of autism is dependent upon the level of "social communication impairments, and restricted, repetitive patterns of behavior" (American Psychiatric Association, 2013). Individuals on the spectrum tend to find social interactions

challenging, and they prefer to spend time alone rather than playing with others (NINDS, 2015). Parents and educators report that people with ASD may repeat certain phrases, use a monotone voice, perform high-pitched singing, or simply refrain from talking (NINDS, 2015). It has been reported that children with ASD will fixate and discuss a particular topic, ignoring the second participant in the conversation (NINDS, 2015). Furthermore, children with ASD may struggle to understand and interpret behavioral, nonverbal cues such as waving goodbye, or body language and its implications. Another common characteristic behavior seen in ASD is an obsession with a particular activity or item, motion, or word (NINDS, 2015). For example, the action may involve one specific toy truck and driving it around in circles, or leaning from side to side regardless of the environment. Individuals with ASD present with varying phenotypes, therefore, an extensive evaluation consisting of the input of various medical professionals is necessary for an accurate diagnosis. While there is a spectrum of behaviors, the DSM-5 classifies ASD as being comprised of the following disorders: early infantile autism, childhood autism, Kanner's autism, high-functioning autism, atypical autism, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder, and Asperger's syndrome (American Psychiatric Association, 2013). The number of autism phenotypes indicates a very complex etiology consisting of multiple factors. It is imperative that more information become available for the pathophysiology of the disorder. Research that utilizes postmortem tissue can lead to the identification of the etiology or treatments for ASD.

Neuroanatomical and Neuroimaging Findings in ASD

The identification of neuroanatomical alterations has been a key factor in the current knowledge regarding the interplay of genetic and environmental contributions. These abnormalities are thought to begin during the early stages of brain development, thus leading to

alterations that can be identified through the use of postmortem tissue in deceased individuals that were diagnosed with the disorder. The pathological changes indicate that communication between cell types, specifically excitatory and inhibitory neurons in the brain, is not the same as in a TD age-matched control. Research is lacking for support of this claim. It is known that ASD has shown neuroanatomical differences as compared to TD age-matched controls. ASD studies support overgrowth of cortical cells in the frontal cortex of the brain during the early postnatal period (Donovan *et al.,* 2016). Schumann et al. (2010) provided one of the first major studies to demonstrate these significant differences where they found that both the gray and white matter of the cerebral cortex displayed substantial abnormalities in growth. This study also provided evidence that by the age of 2.5, the most notable discrepancies in volume and other age-associated differences were found in the anterior brain regions, such as the frontal, temporal and cingulate regions (Schumann *et al.,* 2010).

Volumetric differences in certain areas of the brain have also been examined in ASD research. One study found that 90% of boys diagnosed with ASD had a larger brain volume by years two to four, as compared to the normal average (Courchesne *et al.,* 2001). Researchers are still trying to determine why there are volumetric differences in postmortem ASD brains; for example, are there more neurons in a particular area of the brain? One particularly interesting study using mouse models found that by manipulating the second and third layers of neocortex by increasing the amount of excitatory neurons led to social-behavioral deficits similar to those in human ASD (Fang *et al.,* 2014). These findings are significant in showing support for the excitatory-inhibitory neuronal imbalance. The imbalance is theorized to stem from either changes to the composition of different neuronal cell types or an over-production of neurons, leading to an increase in synaptic activity in certain areas of the brain (Fang *et al.,* 2014).

Regardless of the specific pathophysiology, it is clear that abnormalities observed in ASD stem from some neuropathological alterations. Collectively, the previous studies mentioned all highlight the need to identify the pathophysiological differences at a cellular level.

In terms of diagnosis, ASD may be a classified as a behavioral diagnosis, but the underlying cause of the disrupted social interaction lies in the pathophysiology of the behavior. For this reason, it is important to examine the pathology of brain regions that contribute to social behaviors. The brain is one of two main components of the central nervous system, working with the spinal cord to process information and communicate with the rest of the body via the cranial and peripheral nervous system. The brain processes and interprets sensory information that is delivered from the spinal cord and cranial nerves. The frontal cortex plays a key role in higher-order cognitive function meaning that it mediates social behavior, learning, communicating, and decision making (Donovan *et al.*, 2016). Part of this research study examined Brodmann Area 10, or BA 10, which is solely located in the frontal cortex. The anterior cingulate cortex (ACC) is a particularly important region in mediating sensory information that is involved in social behaviors. The ACC receives neuronal signals from the amygdala and then routes those signals onto the prefrontal cortex where executive function planning takes place. Essentially, the cingulate coordinates between the sensory (amygdala) and decision making (frontal cortex). The cingulate cortex can be divided into the ACC and the posterior cingulate cortex (PCC). The ACC can be further sectioned into the ventral ACC, and the dorsal ACC. This study focuses on Brodmann Area 24, or BA 24, the ACC area that resembles a backwards "C" shape that surrounds the corpus callosum. While the ACC functions in a wide range of autonomic functions such as blood pressure and heart rate regulation, it is significant in ASD studies for its role in rational cognitive functions (Stevens *et al.,* 2011).

Magnetic resonance imaging studies have demonstrated differences in the social brain activation patterns involving the amygdala, anterior cingulate and prefrontal cortical regions during social tasks. Furthermore, studying these key brain areas through MRI studies can highlight deviations from typical brain development, providing researchers with more information on atypical developmental patterns in ASD. The amygdala is a component of the limbic system that is involved in emotional behaviors and motivations. Using magnetic resonance imaging, researchers have measured larger amygdala volumes in toddlers diagnosed with ASD, as compared to their typically developing counterparts (Schumann *et al.*, 2009). Another key social brain area for MRI studies is the ACC for its role in social impairment, one of the main deficits displayed in ASD. One functional MRI (fMRI) study found disrupted functional connectivity and decreased activity in the ACC (Mueller *et al.*, 2013). The prefrontal cortex is involved in goal-oriented behaviors such as decision making, problem solving, and social reasoning. For this reason, it has been examined in ASD studies to see if any structural or functional abnormalities are present. In a comprehensive study of connectivity in ASD, Rane *et al* (2015) evaluated numerous prior studies to state that the prefrontal cortex was "most often reported" to exhibit decreased connectivity with other brain regions. These such connectivity studies used MRI technology to demonstrate reduced resting state connectivity which could ultimately play a role in the social and behavioral impairments in ASD, as this reduced connectivity negatively affects the typical neural pathways (Rane *et al.*, 2015).

Excitatory and Inhibitory Neuronal Imbalances in ASD

Neurons specifically have their own specialized way of communicating within the cell, enabling the extensive connections previously described to quickly and effectively transmit neuronal messages. Communication within the nervous system is conducted through electrical

currents known as action potentials. The action potential is propagated along the axon from the cell body to the axon terminal (Barak, 2019). One factor that affects the speed of the action potential's transmission is the myelin (Barak, 2019). The myelin is an outer covering that protects the axon while simultaneously providing insulation and increasing the speed of conductivity (Barak, 2019). Action potentials are facilitated by neurotransmitters. Neurotransmitters are classified as excitatory or inhibitory due to their ability to promote or diminish the development of an action potential. Neurotransmitters alter the membrane potential of cells thus propagating or inhibiting an action potential. The propagation of an action potential can result in the release of neurotransmitter into the synaptic space and bind to the postsynaptic neuron to produce an excitatory or inhibitory effect that leads to the facilitation or diminishment of another action potential.

Glutamate is an excitatory neurotransmitter present in the central nervous system that is involved in interneural communication. Essentially, glutamate is the primary neurotransmitter responsible for nerve signaling- where a chemical message is passed from one presynaptic (sending) nerve cell to a post-synaptic (receiving) nerve cell. Glutamate is responsible for this communication because when it binds to a specific receptor on nerve cells, it causes positive ions to flood the cell and effects the action-potential threshold. Ultimately, this increase in membrane potential will produce a downstream affect where the nerve cell releases neurotransmitters into the synapse of the receiving cell. If concentrations of glutamate reach abnormally high levels, an over-excitation of nerve cells can occur. This over-excitation can be harmful as it can ultimately lead to cell damage or apoptosis, cell death. The glutamate pathways have also been questioned in ASD research, as this neurotransmitter is equally important in neuronal communication. One

particular study was able to establish a connection between the reduced density of glutamate and severity of ASD social impairments (Horder *et al*., 2018).

Gamma aminobutyric acid (GABA), is the inhibitory neurotransmitter that results in the diminishment of action potentials in the postsynaptic cells upon binding. In the mature human brain, GABA is the main inhibitory neurotransmitter, with about 20-30% of cortical interneurons exhibiting an inhibitory nature (Wu & Sun, 2015). GABA binding to the ionic GABA receptor will result in a conformational change to allow negative ions outside the cell to enter the neuron. GABA may also bind to G-protein-coupled receptors, which do not allow ions to follow straight through, but rather, initiate a signal to other proteins in the cell. This signal will eventually result in a downstream effect, causing other proteins to change ion channels. The negative ions reduce the membrane potential and squelch future action potential. Other chemicals can bind to the GABA receptor at different sites than the neurotransmitter GABA. These chemicals (such as benzodiazepine in certain medications used to treat anxiety) will work to reduce the neuron's activity level. Additionally, GABA-agonists have been used to treat seizure disorders. The high comorbidity of seizure disorders such as epilepsy with ASD supports an association between ASD and GABAergic functioning (Kang & Barnes, 2013).

There has been significant evidence that patients with ASD exhibit a substantial amount of dysregulation within the inhibitory GABA system, leading to issues in neuronal connectivity and communication (Blatt *et al.*, 2011). By examining postmortem brain tissue, researchers over the last three decades have found a connection between ASD and lower amounts of GABAergic cerebellar Purkinje cells in the cerebellar region (Blatt *et al.*, 2011). While the GABAergic discrepancies seen in ASD can be found in several brain regions, the ACC is of particular interest with its role in rational and cognitive function. Oblak et al (2009) found a reduction of

48% in the density of a specific type of GABAergic receptors located in the superficial layer of the cerebral cortex, and a 20.2% decrease in the deep layers of the ACC. Another study examined the association between GABA levels and children with ASD, finding decreased sensorimotor GABA levels in contrast with the TD controls (Puts *et al.*, 2016).

Clearly, GABA and glutamate alterations have been supported in the pathophysiology of ASD. The findings may be the result of increased glutamate activity or reduced GABAergic function at the synapse. It is simply unknown whether there is too much gas represented by glutamate or not enough brakes represented by GABA in ASD. Future studies are needed to determine neurotransmitter-specific changes.

Synaptic studies using postmortem tissue comparing donors with ASD and TD would greatly enhance the understanding of cellular communication in ASD. Spinophilin is a protein that is found in the spines of dendritic processes (Feng *et al*., 2000). Further, spinophilin is characterized as a marker for glutamatergic synapses, where excitation signaling occurs. The dendritic processes are very specific binding areas that serve as hosts in the central nervous system for a large amount of excitatory input (Feng *et al.*, 2000). Feng et al., found that spinophilin is essential for regulating the development of characteristics of dendritic spines as shown in spinophilin-deficient mice (2000). This study also demonstrated that the mice had adverse effects in their neuronal connections as a result of the "dysregulation of glutamate receptor currents" (Feng *et al.*, 2000). Further, spinophilin deficient mice showed a overall reduced brain size, particularly evident in the hippocampus (Feng *et al.*, 2000). Currently, spinophilin expression has not been examined in brain donors with ASD. It is important to show glutamatergic synaptic marker alterations in ASD as compared to TD brain donor tissue. ASD is

currently only described as a behavioral disorder; thus, highlighting the need for identification of molecular markers that could provide therapeutic or etiological targets at a molecular level.

Postsynaptic density protein 95, or PSD 95 is another synaptic marker protein that, as its name suggests, tags postsynaptic areas. Specifically, PSD 95 is localized to the postsynaptic areas which represent asymmetric synapses. In the cerebral cortex, synapses can be categorized as symmetrical or asymmetrical, depending upon the width of the synaptic junctions. Asymmetric synapses are characterized by a larger, more prominent postsynaptic density, as compared to the presynaptic density. These types of synapses represent about 80% of the total population, meaning that a majority of the synapses are excitatory in function, as compared to symmetric synapses which are inhibitory. In shape, asymmetric synapses are disproportionate as they are formed by axon terminals encapsulating spherical synaptic vesicles. There is a large postsynaptic density found at asymmetrical synapses, and this accounted for by dendritic shafts, spines and the cell bodies of the inhibitory neurons.

Both of these markers can be used to measure the synaptic density in a specific area of the brain. It is important to measure and examine synaptic density and its correlation to dendritic spines as this is important in relation to the quantification of proteins and subsequent pathophysiology of ASD. Immunohistochemistry (IHC) involves tagging specific proteins in a tissue sample with a particular primary antibody, and using detection reagents to create a visual of the target protein in the sample. The detection reagents used can create fluorescent staining images, such as those generated in this study on spinophilin. From the staining image, the protein's level and distribution in the tissue sample are revealed. For this reason, IHC was utilized in an analysis of spinophilin expression in post-mortem brain tissue of ASD.

HYPOTHESIS

We hypothesize that synaptic protein expression of spinophilin and PSD-95 will be increased in the frontal cortex and anterior cingulate cortex of donors that were diagnosed with ASD when compared to typically developing control brain tissue.

METHODS AND MATERIALS

Brain Tissue

The ASD and TD control donor tissue were obtained from AutismBrainNet (formerly the Autism Tissue Program and the Harvard Brain Tissue Resource Center in Belmont, MA) and NIH NeuroBioBank (formerly the NICHD Brain and Tissue Bank for Developmental Disorders in Baltimore, MD). Tissue brain banks provide medical records, clinical information, and postmortem tissue information including brain area, pH and RNA integrity number when available. ASD and TD control donor tissue were matched as closely as possible by brain region, age, gender, pH and RNA quality as shown in Table 1. Tissue was matched a-priori by the brain banks. Control donors died as a result of heart attack, asphyxia, drowning and trauma. Cause of death for the ASD donors included asphyxia, heart failure, respiratory distress, trauma, ketoacidosis, and bowel obstruction. To uphold a level of respect and protect the identity of the donors, cause of death for individuals is not included in *Table 1* with the donor. ASD donors were confirmed in their diagnosis through the postmortem Autism Diagnostic Interview- Revised (ADI-R) or through medical record analysis. Additionally, ASD donors met the standards established for diagnosis in the DSM V criteria.

Table 1. *Subject Demographic Information*

Pair	ID	Age	Gender	RIN	Assay	Tissue
	Controls					
1	AN14757	24	M	7.8	WB	BA10, BA24
2	4848	16	M	7.6	WB	BA10, BA24
3	5342	22	M	8.1	WB	BA10, BA24
4	5079	33	M	7.3	WB	BA10, BA24
5	M3231M	37	M	7.4	WB	BA10, BA24
6	AN12137	31	M	7.3	WB	BA10
MEAN		27.2		7.6		
SD		7.8		0.32		
	ASD					
1	AN04166	24	M	8.1	WB	BA10, BA24
2	5302	16	M	6.6	WB	BA10, BA24
3	5176	22	M	7.1	WB	BA10, BA24
4	5297	33	M	7.1	WB	BA10, BA24
5	5027	37	M	7.7	WB	BA10, BA24

6	AN11989	30	M	7.7	WB	BA10
MEAN		27		7.4		
SD		7.7		0.55		

RIN, RNA integrity number
WB, Western Blotting

Tissue Sample Preparation and Sectioning

Postmortem human brain tissue was sectioned at a varying thickness depending on the experiment at -20 degrees Celsius using a cryostat microtome (model Leica CM3050S). Tissue used for immunohistochemistry was sectioned at 20 μM thickness and placed on positively charged glass slides followed by storage at -80^{o}C. IHC also used control donor brain tissue that was paraffin embedded brain tissue after fixation with formaldehyde. Tissue was sectioned to 4μM thickness using a microtome. Tissue used for immunoblotting was sectioned at 50 μM thickness and punch-dissected at 3.5 μM using a trephine punch tool in gray or white matter for each brain regions. Tissue was placed in 1.5 ml eppendorf tubes and stored at -80^{o}C. All ASD and TD pairs were sectioned at the same time and subjected to the same storage time in the ultra-low storage freezers.

Immunohistochemistry

IHC using antibodies specific for spinophilin was used to visualize interneural synaptic areas, specifically at the dendritic spines. In brief, frozen tissue mounted onto glass slides was removed from the ultra-cold -80°C storage freezer and incubated at room temperature for 20 minutes in a

ventilated hood. Human tissue was fixed in acetone (10 minutes) and washed one time in phosphate buffered saline solution or PBS (3 times for 5 minutes). The tissue was enclosed with a silicone-based pen to create a waterproof barrier in which 1-3 drops of peroxidase blocking reagent were placed (5 minutes). A gentle rinse and wash using PBS were completed (5 minutes). Goat serum blocking reagent was placed on the tissue, 1-2 drops per sample on the slide (15 minutes). A quick rinse was performed with PBS. The attempted protocols for antibody specificity completed during the optimization process can be found in sequential order in Table 2. In brief, formalin-fixed paraffin embedded human brain tissue was sectioned at 4uM thickness onto supercharged slides. Slides were deparaffinized using a xylene and a series of ethanol hydration steps proceeding from 100%, 95%, 70%, and 50% followed with a cold-water rinse. Antigen retrieval was performed using sodium citrate at pH 6.0 at a 95oC incubation for 20 minutes, followed with three washes of buffer without detergent. The protocols using the peroxidase and serum blocking reagents were performed as stated above. Optimization steps are summarized in Table 2.

Table 2. *Attempted protocols for the Spinophilin immunohistochemical stain on fixed and frozen human brain tissue*

	Block	Primary Antibody Manufacturer and Dilution	Components for Primary Antibody Incubation	Secondary Antibody Manufacturer and Dilution	Components for Secondary Antibody Incubation	Developer
1 x PBS*****	Blocking Reagent Kit-Peroxidase Blocking Reagent and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:500	1mL PBS 2 uL rabbit spinophilin 20uL goat serum	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1000	1 mL PBS 1 uL goat anti rabbit 4412S	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:500	1mL PBS 2uL rabbit spinophilin 20uL goat serum	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1000	1mL PBS 1uL goat anti rabbit 4412S	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:250	1mL PBST 4uL rabbit spinophilin 15 uL goat serum (NGS)	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1000	1mL PBS 1uL goat anti rabbit 4412S	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:250	1mL PBST 4uL rabbit spinophilin 15 uL goat serum (NGS)	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1000	1mL PBS 1uL goat anti rabbit 4412S	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:250	Positive Tissue sample: 1mL PBST 4uL rabbit spinophilin 15 uL goat serum (NGS) Negative tissue sample: 1 mL PBS-T 15 uL goat serum (NGS)	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1000	Both Positive and Negative Tissue Samples Receive: 1mL PBS 1uL goat anti rabbit 4412S	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase and Serum Blocking G	Cell Signaling Technology cat. 14136S, 1:250	1mL PBST 4uL rabbit spinophilin 15 uL goat serum (NGS)	NA	Negative: Neither tissue received secondary AB	DAPI
1 x PBS	Blocking Reagent Kit-Peroxidase	NA	Negative: No Primary AB	Thermo Fisher Scientific cat. no. (HRP) 31460,	Take 2mL of 0.5% BSA in PBS-T, place 1uL in aliquot	DAPI

	and Serum Blocking G			1:1	and add 1uL goat anti rabbit	
1 x PBS	Blocking Reagent Kit-Peroxidase (5 minutes) and Serum Blocking G (15 minutes) and 3% BSA in PBS-T Block (1 hr)	NA	Negative: 1mL PBST 15 uL goat serum (NGS)	Thermo Fisher Scientific cat. no. (HRP) 31460, 1:1	1uL goat anti rabbit 1uL 0.5% BSA in PBS-T	DAPI
1 x PBS*	Blocking Reagent Kit-Peroxidase (5 minutes) and Serum Blocking G (15 minutes) and 3% BSA in PBS-T Block (1 hr)	NA	Negative: 1 mL PBST	1:500	0.0050 g BSA in 1 mL PBS-T 2 uL donkey anti-rabbit	DAPI

*indicates FROZEN human brain tissue, all other attempted protocols were fixed human tissue.
Blocking Reagent Kit-
BSA-Bovine Serum Albumin (BSA) (Fraction V) sourced from Fisher BioReagents cat. 9048-46-8
NGS- Normal Goat Serum in Secondary Antibody sourced from Jackson ImmunoResearch Laboratories
DAPI- ProLong Gold antifade reagent with DAPI sourced from Life Technologies Corporation

Immunoblotting

Brain tissue homogenates from either the anterior cingulate or the frontal cortex were obtained from the -80^{o}Celsius ultra cold freezer. Triton-EDTA (TE) homogenation buffer (pH 7.4) was added to tissue homogenates while in the freezer. Optimization steps included using radio immunoprecipitation assay (RIPA) buffer (50mM Tris·HCl, pH 8.0, 150 mM NaCl, .1% Triton X-100, .5% sodium doxycholate, .1% SDS) for homogenization, but did not provide the quality of homogenizing using TE buffer. Protease and phosphate inhibitors (PPI) were added at 100-fold concentrations to buffer prior to addition to tissue homogenates. Two hundred microliters of TE buffer was added to the frozen tissue homogenates. The mixture was placed on ice after vortexing for ten seconds. The tissue mixture was sonicated twice for ten seconds and

kept on ice prior protein assay. A five-time loading buffer consisting of betamercaptoethanol (BME) and Lameli buffer was prepared. Fifty microliters of the BME-Lameli mixture was added to 150 μL of tissue homogenate and heated to 95^{o}C for 5 minutes. Denatured proteins were cooled on ice, aliquoted and frozen at -80^{o}C until the next day. Protein concentrations were determined using a Lowery-based commercial assay with a standard curve of BSA in PBS solution (Pierce). Twelve micrograms of protein was determined to be the optimal protein amount for spinophilin in the frontal cortex. Six micrograms of protein was used in the anterior cingulate cortex. Denatured proteins were separated by an SDS-polyacrylamide gel electrophoresis (BioRad, Mini PROTEAN TGX Gels). Ten microliters of Precision Protein standard ladder (Bio-Rad) was used for each TGX gel. Gel electrophoresis was performed for 90 minutes at 100 volts. Proteins were transferred onto a nitrocellulose membrane (BioRad cat. #1620115 using 100 volts for 60 minutes being careful to not surpass 0.3 Amps). Protein transfer from the SDS-polyacrylamide gel onto the nitrocellulose membrane was confirmed through a Poncheau S Stain (SIGMA-ALDRICH- 0.1% Ponceau S, 5% acetic acid, cat #SLCB3855) for 10 minutes. Afterwards, the membrane was rinsed and washed with tris-buffered saline solution with tween 20 (.2%) or TBS-T (5 minutes) until the red stain color disappears. Membranes were then blocked with five percent nonfat milk solution (1.25 grams of evaporated milk in 25 mL of PBS-T) for one hour at room temperature. The membrane was incubated with primary antibody (6 mL of 5% milk in PBS-T with 6uL of spinophilin) in a Falcon tube on an oscillator overnight at 4^{o}C. The membrane was washed with PBS-T (3 times for 5 minutes each) the next morning, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Secondary antibody components for spinophilin were 20mL of PBS-Tween with 2 μL of anti-Rabbit HRP. The membrane was incubated for two hours on a rocking platform and thenwashed

with PBS-T (3 times for 5 minutes). Chemiluminescent signals were identified using Lumagen reagents and identified using the BioRad ChemiDoc. In preparation for a new primary antibody, the membrane was washed with TBS-T (3 times for 5 minutes) and protein was removed or stripped using a mild stripping buffer for 10 minutes. A wash with TBS-T (2 times for 5 minutes) was performed before blocking the membrane in 5% nonfat dry milk for one hour. A quick rinse with TBS-T was completed (3 times for 5 minutes) and the blot was ready for incubation with the second primary antibody of interest, PSD-95. Paired ASD and TD control tissue samples were run on the same gel and performed in duplicate. Table 3 highlights antibodies and their appropriate secondary antibody and Table 4 displays recipes for common buffers used throughout the immunoblotting process.

Data Analysis

Data were normalized to GAPDH measured on the same blot, after stripping Spinophilin and PSD-95 antibodies. The blots were then analyzed and quantified using the ImageLab software. The observed band intensities were measured densitometrically, and numerically expressed as volume changes relative to the normal, typically developing tissue pair, age and gender matched via NIH. Band intensities were converted into a numerical value or "adjusted volume" by creating identical boxes around the band measurements for each pair. Once an adjusted volume was found for each pair for each respective protein (spinophilin, PSD-95, and GAPDH), the values for each individual were averaged from the two blots. The protein expression levels were normalized by dividing their average expression level by GAPDH's average. This normalization provided a ratio for each control and ASD donor pair, representing a numerical value that can be used in comparison.

Micro BCA Protein Assay Kit used was sourced from ThermoScientific Ref. 23235 Lot. SI252807

Table 3: Antibodies

Protein	**Source**
Spinophilin*	Cell Signaling Technology cat. 14136S
PSD-95*	Cell Signaling Technology cat. 3450S
GAPDH*	EMD Millipore cat. 2955484
Goat anti Rabbit	GE Healthcare UK Limited cat. NA934-1
Donkey anti mouse IgG HRP	GE Healthcare UK Limited cat. NA931V

*indicates a primary antibody

Table 4: Buffers

Buffers	**Components or Source**
RIPA	Cell Signaling Technology cat. 4370
TE-lysis	(TrisHCl- EDTA) Fisher Scientific
Loading (Lameli)*	4x Lameli Sample Buffer, BioRad Laboratories cat. 161-0747
10x Transfer Stock**	800mL distilled water, 30.3g Tris (Tris base), 144.1 g Glycine Dilute to 1L
10x Running Stock***	800mL distilled water, 30.4g Tris (Tris base), 144.2 g Glycine,

	and 10.0 g of SDS. Dilute to 1L
Mild Stripping Buffer	15g Glycine, 1g SDS, 10mL Tween20, pH 2.2, bring to 1L

Note:
**A 10% BME (beta kappa ethanol) is added to the loading buffer, as it breaks the protein up and elongates it in the gel. An amount of 20 μL to 180 μL of loading buffer.*
***For the transfer buffer in transfer step, take 100mL from the 10X stock, add 200mL of methanol and then dilute to 700 mL with water. Place in the fridge for 20 minutes as it needs to be cold before use. Additionally, the methanol is only necessary if using nitrocellulose blotting paper, which this protocol uses.*
****The running buffer used in the gel electrophoresis is also a dilution of the 10x stock. To prep the running buffer, obtain 100mL of running buffer and add 900mL of distilled water.*

RESULTS

Immunohistochemistry Analysis of Gray Matter Spinophilin

ASD and TD control donor brain tissue were carefully paired by brain banks prior to the start of the study. Tissues were matched to reduce difference between the pairs for age, gender, RIN (RNA- integrity number), and post-mortem interval period. Representative images of the immunohistochemistry identification procedure is shown in Figure 1 using spinophilin primary antibodies in formalin fixed human postmortem BA10 Gray Matter (GM) brain tissue. Panel A is an image taken from brain tissue that was incubated with spinophilin antibody followed by an incubation with the green fluorescent-conjugated secondary antibody (Figure 1, Panel A). Panel B show 4'6-diamidino-2-phynylindole (DAPI) conjugated along with the green-conjugated secondary antibody (Figure 1, Panel B). Panels C and D are images that were taken from tissue that was serially sectioned but did not utilize the spinophilin antibody Figure 1, (Panel C, D). The illuminated blue dots are 405 fluorescent-conjugated molecule that produces a wavelength at 405 nanometers under a microscope as it binds to adenine-rich regions in DNA. DAPI serves as a useful indicator of the nucleus region of each individual cell in a tissue stain to visualize all

cells that are present in the tissue sample. It was found that there was non-specific secondary binding to the tissue in the negative stain as evident in Figure 1-*Panel C and D*. No green fluorescence should be visible in the negative gray matter tissue stain. Table 2 includes the specific details of every optimization procedure for the spinophilin antibody. Figure 2 exhibits representative images of the same spinophilin-positive (Panel A & B) vs Negative (Panel C &D) immunohistochemistry of fixed human postmortem BA10 brain tissue using white matter (WM) tissue of this tissue sample. Interestingly, there was no binding of the green-fluorescent conjugated antibody in the white matter. White matter only has ten percent of the neurons found in gray matter. Therefore, it was likely nonspecific antibody binding to only neurons.

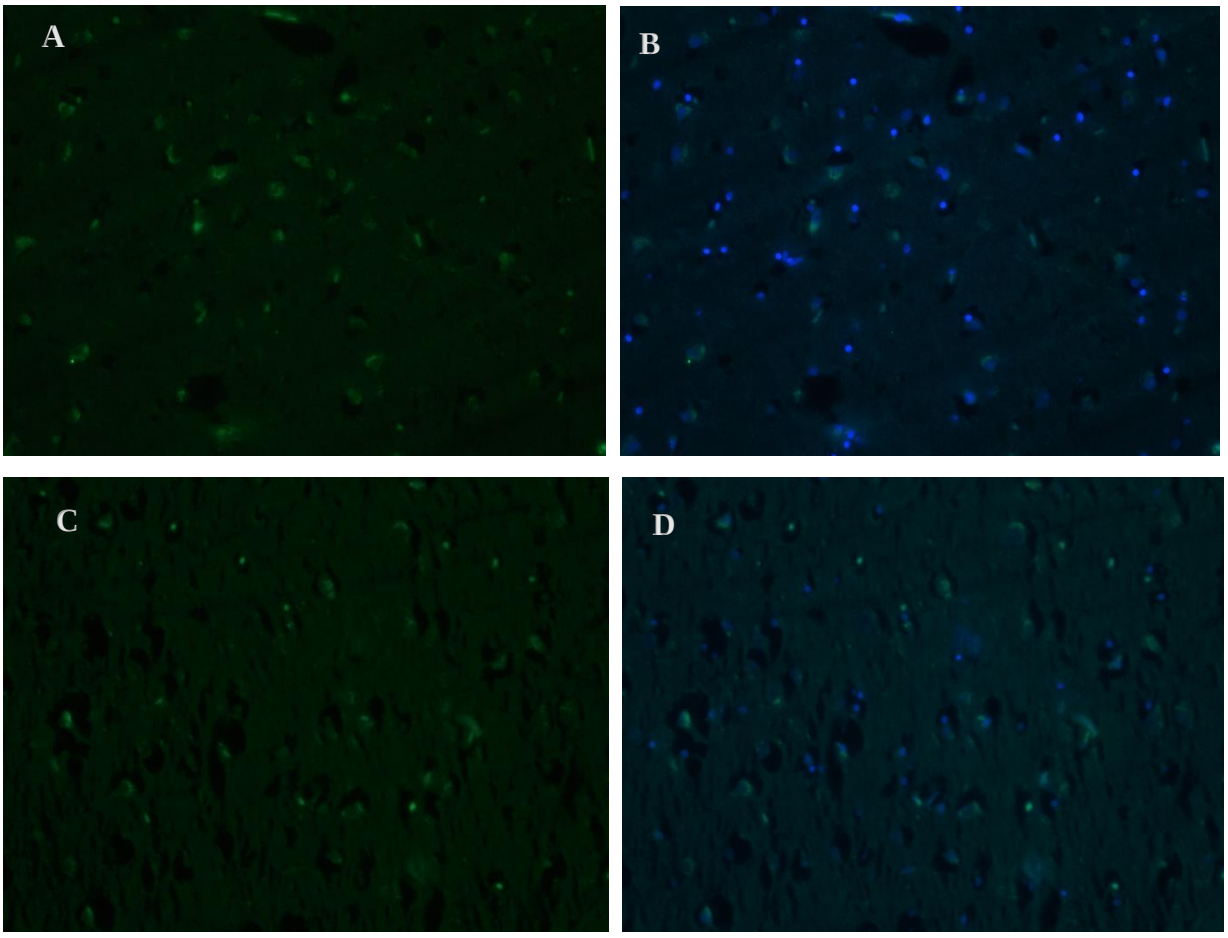

Figure 1: GRAY MATTER- Human gray matter (GM) brain tissue using spinipholin antibodies (positive) followed with GFP-conjugated secondary antibodies for immunohistochemical identification taken at 20X magnification (*Panel A*); Human GM 20X DAPI and GFP Positive

Stain (*Panel B*); GM 20X GFP immunohistochemistry without the use of spinophilin primary antibody (*Panel C*); Human GM 20X DAPI and GFP immunohistochemistry without the use of spinophilin primary antibody (*Panel D*).

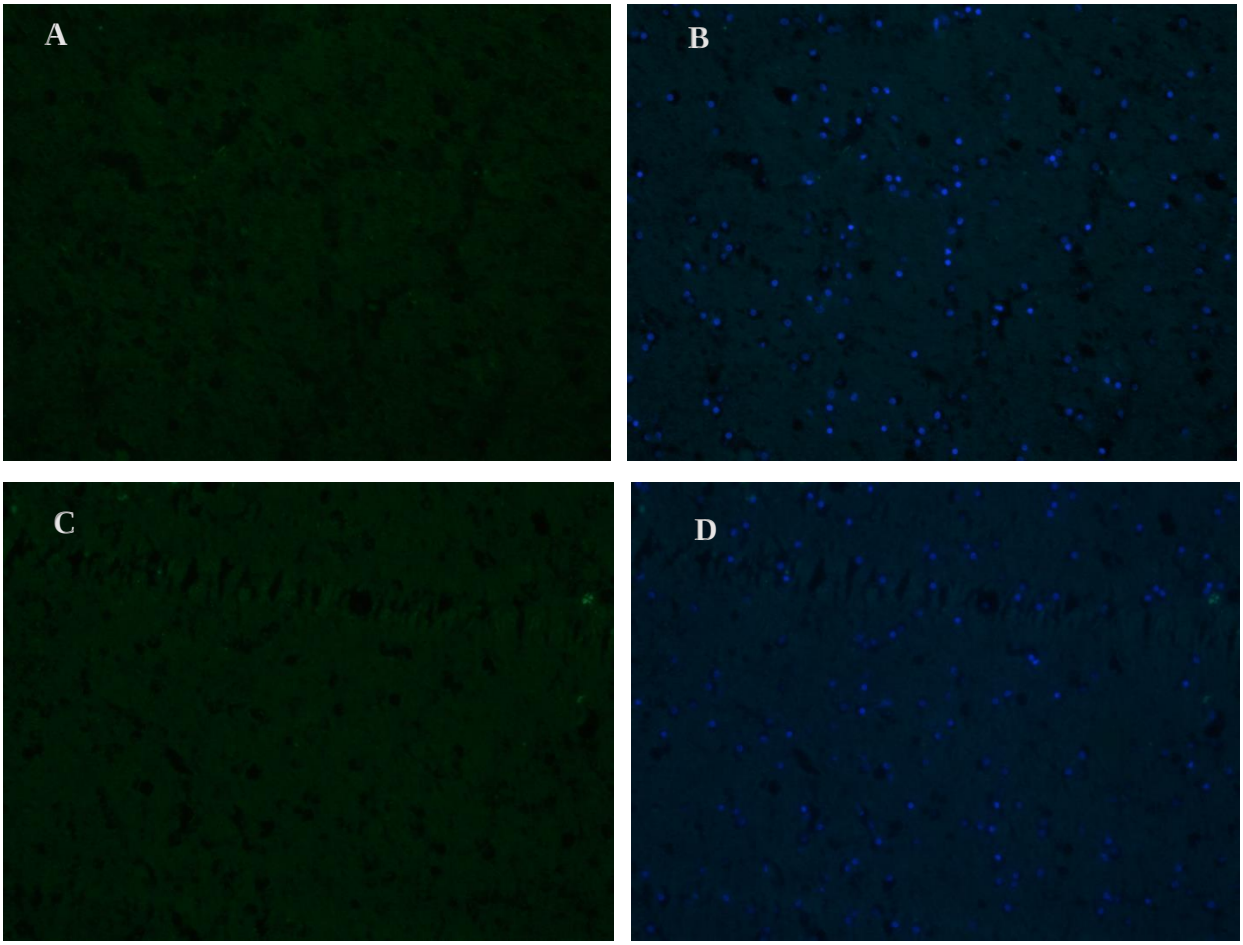

Figure 2: WHITE MATTER- Human white matter (WM) brain tissue using spinipholin antibodies (positive) followed with GFP-conjugated secondary antibodies for immunohistochemical identification taken at 20X magnification (*Panel A*); Human WM 20X DAPI and GFP Positive Stain (*Panel B*); WM 20X GFP immunohistochemistry without the use of spinophilin primary antibody (*Panel C*); Human WM 20X DAPI and GFP immunohistochemistry without the use of spinophilin primary antibody (*Panel D*).

Western Blot Analysis of Gray Matter Spinophilin and PSD95

Spinophilin and PSD-95 protein amounts were measured in two different human brain regions, including frontal and anterior cingulate cortical gray matter areas, using immunoblotting

or Western Blotting comparing ASD to TD control donor tissue. There was no statistical difference between typically developing tissue and ASD for either brain region, in both spinophilin and PSD-95.

Spinophilin protein expression is showin in Figure 3 for two different brain regions, BA10 or frontal cortex (N=6) and BA24 or anterior cingulate cortex (N=5). An unpaired Student's t- test was used to determine that no statistical significance was found in the frontal cortex (BA10) when comparing spinophilin protein amounts in ASD donor tissue to TD donor tissue. (Figure 3). Likewise, no statistical difference was found in spinophilin expression in the cingulate cortex (BA24) between TD and ASD donor tissue. A small sample was used, and more research must be completed to validate this finding.

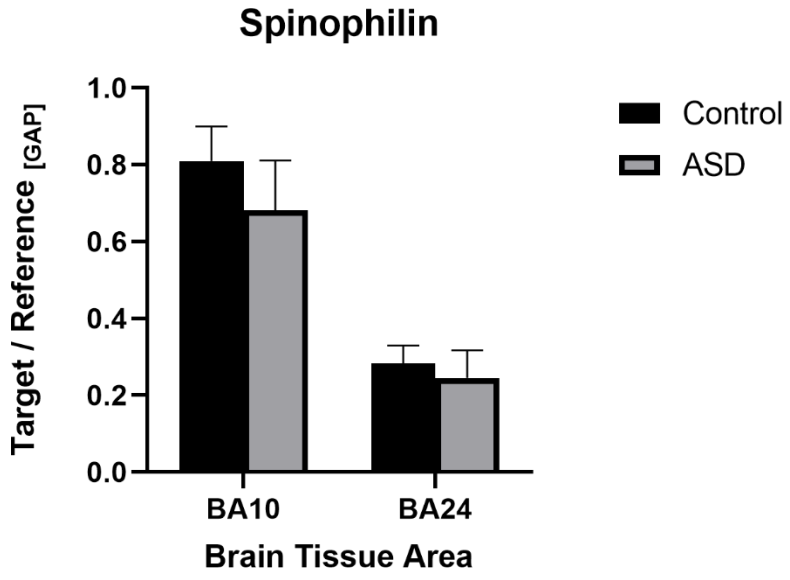

Figure 3. Spinophilin Protein Expression determined by immunoblotting in human postmortem tissue from frontal cortex or BA10 (N=6) and anterior cingulate or BA24 (N=5) punch dissected brain tissue from TD control tissue (black bars) and ASD tissue (gray bars). Spinophilin protein expression was normalized the protein expression of a stable reference protein (GAPDH) on the same blot. No significant difference was observed using an unpaired Student's t-test.

Protein expression levels for PSD95 for the two different brain areas, BA10 (N=6) and BA24 (N=5) are shown in Figure 4. Expression levels for PSD-95 expression were not significantly different in both frontal cortex (BA10) and cingulate (BA24) tissue samples, analyzed using an independent unpaired Student's t-test.

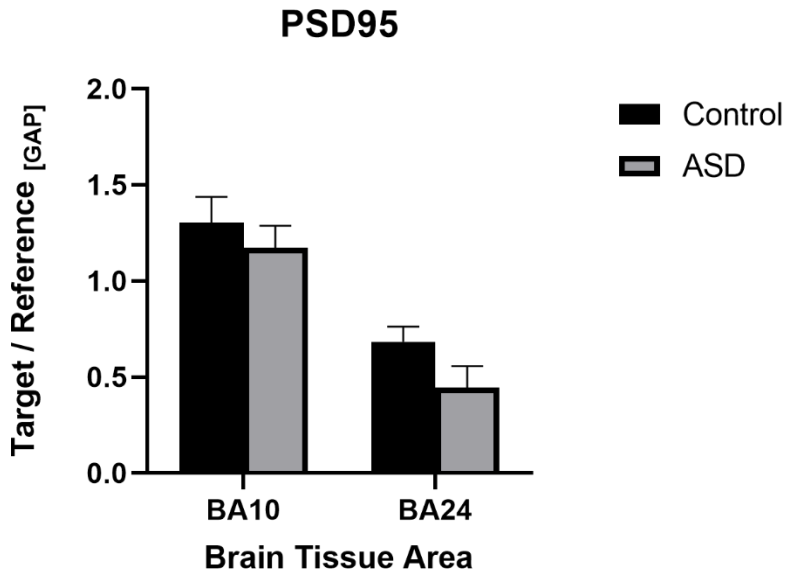

Figure 4. PSD-95 Protein Expression determined by immunoblotting in human postmortem tissue from frontal cortex or BA10 (N=6) and anterior cingulate or BA24 (N=5) punch dissected brain tissue from TD control tissue (black bars) and ASD tissue (gray bars). Spinophilin protein expression was normalized the protein expression of a stable reference protein (GAPDH) on the same blot. No significant difference was observed using an unpaired Student's t-test.

To determine if differences could be identified in isolated paired tissue samples, a paired Student's t-test analysis was performed. All subjects were gender, age, PMI, and RIN matched as closely as possible; therefore, the possibility exists that paired analysis may better reflect protein expression differences based on the different developmental stages of the tissue. It is important

to recognize if perhaps increases may occur in an ASD compared to the paired control donor, rather than looking at the group as a whole. Since subjects (as pairs) varied in age, it was determined that a paired analysis could account for the age difference. The data was evaluated for outliers using a Grubb's test. The results of a paired T-test for spinophilin protein expression is shown for each brain area in Figure 5. In each brain area, only one pair showed an increase in spinophilin protein expression, when comparing control to ASD donors.; however, this pair was not the same in both areas. Table 5 shows the P values that were gathered to assess the significance of the differences between control donors and ASD donors in terms of Spinophilin protein expression for the two different brain areas analyzed in this study.

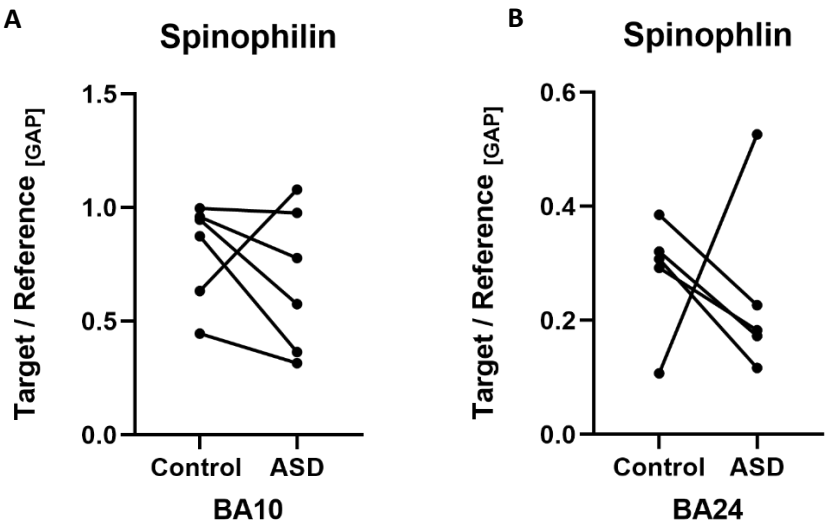

Figure 5. Levels of spinophilin protein expression in punch-dissected gray matter from frontal cortex or BA10 (N=6; panel A) and anterior cingulate cortex or BA24 (N=5; panel B) using a paired Student's t-test. Target protein or spinophilin in this figure was normalized to the reference protein, GAPDH. No significant differences were found.

T-Test	P-Value BA10	P-Value BA24
Unpaired	0.4368	0.674
Paired	0.3893	0.7598

Table 5. Statistical values of an unpaired and paired Student's t-test for human postmortem frontal (BA10) and cingulate (BA24) brain tissue of spinophilin protein expression between ASD and TD control tissue.

RESULTS (cont'd)

Figure 6 displays the results of a paired Student's t-test for PSD-95 expression levels in both BA10 and BA24 brain regions upon comparing ASD and TD donor tissue. No trending data emerged from the graphed information. Two pairs had increased PSD-95 expression in TD when compared to ASD tissue in BA10. In the paired analysis for BA24, one pair exhibited an increase in PSD-95 protein expression levels. It is important to compare what changes occurred in PSD-95 expression in individual pairs considering the ages of subjects varied. The p-values for differences between control donors and ASD donors in terms of PSD-95 protein expression for the two different brain areas analyzed in this study are shown in Table 6.

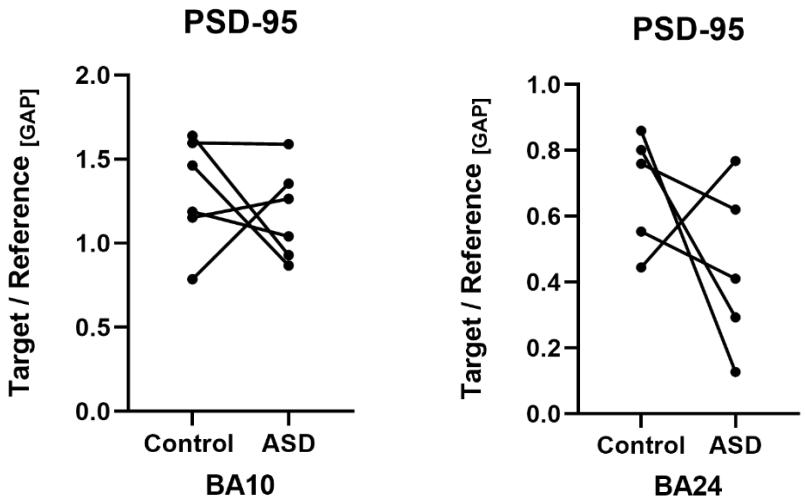

Figure 6. Levels of PSD-95 protein expression in punch-dissected gray matter from frontal cortex or BA10 (N=6; panel A) and anterior cingulate cortex or BA24 (N=5; panel B) using a paired Student's t-test. Target protein or PSD-95 in this figure was normalized to the reference protein, GAPDH. No significant differences were found.

T-Test	P-Value BA10	P-Value BA24
Unpaired	0.4736	0.1221
Paired	0.5301	0.2547

Table 6. Statistical values of an unpaired and paired Student's t-test for human postmortem frontal (BA10) and cingulate (BA24) brain tissue of PSD-95 protein expression between ASD and TD control tissue.

DISCUSSION

Previous studies have suggested that there is some sort of connectivity disfunction that causes the social and behavioral deficits observed in autism. It is useful to acknowledge that while MRI studies provide insight to what is happening structurally in the brain, these types of

studies do not discuss what is occurring at the synaptic level. One emerging study urged future studies to examine what is occurring pathophysiologically at the synapse, particularly the GABA inhibitory signaling system, for these neurodevelopmental disorders often manifest from "synapse to symptoms" (Coghlan *et al*, 2012). Other behaviorally diagnosed disorders such as schizophrenia have been identified as important avenues for future research on synaptic protein expression levels. Synaptophysin is another synaptic marker that is useful in providing insight to any abnormalities that could occur at the synapse. While the specific function of synaptophysin is unclear, some studies of another behavioral disorder, schizophrenia, have shown reduced levels of synaptophysin in prefrontal cortex (Glantz & Lewis, 1997). This study and additional papers on schizophrenia work to support the hypothesis that this disorder has alterations in synaptic connectivity (Lewis & Liebermann, 2000). It has been established that there is a disturbance in the circuitry and synaptic connectivity in patients diagnosed with schizophrenia. Thus, proving the need for further research on synaptic activity and particular protein levels through quantification as they may play a role in the pathophysiology of certain behavioral diseases like ASD.

Although there was no statistical significance between control and ASD donor groups, there are some takeaways to acknowledge from this study. Researchers are trying to distinguish between schizophrenia and ASD as they are both behaviorally diagnosed. Up to this point, this study was novel in its ASD research on spinophilin protein expression in human postmortem brain tissue in frontal and anterior cingulate tissue. One study found decreased spinophilin mRNA levels in four different brain areas of schizophrenic patients, compared to their normal comparison subjects, suggesting a possible underlying dendritic pathology in schizophrenia (Law *et al*, 2004). As autism is another behaviorally diagnosed mood disorder, it could be evident that

excitatory or glutamatergic synapses display similar abnormalities. While our study did not find any significant difference in spinophilin protein expression in frontal or cingulate brain tissue, this is a significant finding in that it could be one distinguishable difference in the pathophysiology of ASD and schizophrenia. More research is needed to solidify these findings, but it could be a starting point.

The results for the IHC were disappointing. IHC is plagued by what is known as background or nonspecific labeling of the secondary antibodies. It can interfere with the interpretation of the specific protein being analyzed. Useable immunofluorescence requires that the fluorescence of spinophilin be more distinguishable from the background; thus providing a more accurate quantification in analysis. Tissue sections were imaged using the EVOS microscope to provide a high-resolution picture. The images captured were taken in the gray matter, in consistent, specific locations determined by landmarks. Gray and white matter human post-mortem BA10 brain tissue was examined and the positive and negative stain ultimately revealed no difference in spinophilin expression above background or nonspecific staining. IHC was the initial experiment of choice for distinguishable, identifiable morphological changes in neurons.

The spinophilin antibody could not be utilized for protein quantification using the brain tissue; therefore, Western Blots, or immunoblotting was proposed. The alternative method of using immunoblotting was executed because they serve as another technique to identify and tag specific proteins in tissue samples. Similar to immunohistochemistry, an antibody is used in immunoblotting to specifically detect its antigen in the sample. By creating a highly specified interaction between a region of interest on the protein and a synthesized antibody, one can detect the protein in a complex tissue sample such as brain tissue. This technique of immunoblotting

provides an excellent visual comparison of how target protein expression can differ in response to a condition or disease.

Limitations

A possible limitation of the immunoblotting experimentation is half the quantity was used of the anterior cingulate (BA24) tissue as was used in the BA10 frontal cortical tissue for antibody probing. Thus, less tissue will lead to lower protein that may not be sensitive enough for antibody detection. We expected that the ratio of the target protein to the reference protein to be the same in both brain areas. However, it was found that values in the cingulate or BA24 were half the amounts in the frontal cortex. It could be that spinophilin and PSD-95 gene expression is greater in the frontal cortex. Additionally, this study utilized GAPDH as a reference gene. The reference protein should not change regardless of the condition, such as in in ASD or a typically developing brain. However, it has been found that GAPDH may not be as reliable as previously thought. Target proteins could be normalized to protein values obtained from a Poncheau S Stain to ensure the integrity of GAPDH.

Another limitation of the immunoblotting could be attributed to reprobing the same blot for several proteins. Multiple proteins were investigated for other research purposes on the same nitrocellulose blot while spinophilin and PSD-95 were examined. Repetitive stripping and reprobing with new primary antibodies could have caused the tissue samples to detatch as the membrane becomes fragile. In this study, a pair was lost in cingulate tissue because GAPDH (the last protein to be probed) expression was not quantifiable.

The western blot is a helpful tool, for when the results do not equal the expected data, it will provide hints as to what actually went wrong. For example, if the band appears smaller than

expected, that reveals that protein degradation has occurred and damaged the target protein. It is possible that some of this protein degradation did occur, considering not all bands looked identical when comparing the duplicate blots. With a small 'n' of 6 (BA10) and 5 (BA24), more subjects must be added to this study in order to solidify these overall findings. The addition of several more brain areas could prove advantageous to gaining more insight on any synaptic abnormalities in ASD. For example, the amygdala which provides information as to the sensory, or limbic system, could be a key area for future studies as it is involved in executive decision making in the social brain pathway. Disturbances in gaze-following in humans has already been related to impairments following damage to the normal role of the amygdala (Kennedy & Adolphs, 2010). It is possible that these same impairments may be present in ASD pathophysiology. It has also been theorized that difficulties in understanding and interpreting facial expressions could be related to dysfunction of the amygdala in ASD (Kleinhans *et al*, 2010). Clearly, the amygdala should be considered in future research for its role in any social deficits displayed in ASD. The cerebellum is another brain area that should be examined for protein expression levels. With its role in language and cognition, there are implications to study and distinguish any abnormalities in the structure of the cerebellum in ASD patients.

Future Studies

Alternative studies may also look to analyze other proteins involved in synaptic function and regulation in the frontal cortex and anterior cingulate cortex. Insulin-like growth factor (IGF1) is a molecule that can serve as a marker for inflammation, and it has been found to have increased gene expression levels in the anterior cingulate cortex of ASD donors (Sciara *et al*, 2018). This particular study proposed that in ASD, the signaling pathway of IGF1 may possibly be increased, and thus play some sort of role in the complex etiology of this disorder (Sciara *et al*, 2018).

While this study examined both gray and white matter of the ACC, evidence of an abnormality may also be present in the frontal cortex of ASD post-mortem brain tissue.

Perhaps the most important variable to examine in ASD is age. Age is a factor that is extremely revealing in ASD studies, as it is classified as a neurodevelopmental disorder. Thus, there is a large demand for research on age differences in ASD. This study mainly analyzed older donors (24-37 y/old) with only two pairs including younger individuals (16 and 22 y/old). Perhaps the findings of protein expression levels could have been profoundly different if the tissue donors were children, toddlers, or infants. A co-variant analysis in which age is examined could be quite informative in future studies. Those will be performed when more subjects have been added to this study. We completed a paired analysis of the data to account for age difference, and to see if we overlooked any differences between the individual pairs. However, the paired analysis did not reveal any evidence of overlooking any variables such as age.

Conclusion

This is the first study that examined Spinophilin protein expression in human postmortem brain tissue in ASD. PSD-95 expression levels were also analyzed for comparison to typically-developing and carefully matched controls since it has previously been explored in other brain areas. Although no statistically significant differences were found in either brain region for both spinophilin and PSD-95, these findings may be useful as guiding tool and precursor to other research avenues. Very little is known regarding the precise etiology of this complex disorder and these data serve to strengthen the present understanding. Absence of evidence does not have the same meaning as evidence of absence. This study featured a small study sample and only focused on two brain regions involved in the social behavior pathway. Future studies should aim to include more subjects, if possible, and examine any potential synaptic deficits in other key

brain areas such as the amygdala. Ultimately, any knowledge gained from these studies will aid the field of autism research in precisely identifying the pathophysiology of this disorder that could lead to developments in specific treatments.

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