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Endosomal System Genetics and Autism Spectrum Disorders: A Literature Review

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Abstract

Autism spectrum disorders (ASDs) are a group of debilitating neurodevelopmental disorders thought to have genetic etiology, due to their high heritability. The endosomal system has become increasingly implicated in ASD pathophysiology. In an attempt to summarize the association between endosomal system genes and ASDs we performed a systematic review of the literature. We searched PubMed for relevant articles. Simons Foundation Autism Research Initiative (SFARI) gene database was used to exclude articles regarding genes with less than minimal evidence for association with ASDs. Our search retained 55 articles reviewed in two categories: genes that regulate and genes that are regulated by the endosomal system. Our review shows that the endosomal system is a novel pathway implicated in ASDs as well as other neuropsychiatric disorders. It plays a central role in aspects of cellular physiology on which neurons and glial cells are particularly reliant, due to their unique metabolic and functional demands. The system shows potential for biomarkers and pharmacological intervention and thus more research into this pathway is warranted.

Keywords

Autism spectrum disorder (ASD); genetics; endosomal system; endosome dysregulation; recycling; trafficking; mTOR

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1: INTRODUCTION

1.1: Overview of Autism Spectrum Disorder

1.1.1: Clinical Pathology—Autism spectrum disorders (ASDs) are a group of impairing, pervasive neurodevelopmental disorders that affect 1 in 68 children (Prevention, 2015). The core symptoms used for the diagnosis of ASDs are persistent deficits in social interactions and communication across multiple contexts with restricted repetitive behavioral patterns (DSM-V). Despite advances in our understanding of the etiology of these disorders, no current medication is markedly efficacious for the core symptoms of ASDs (Prevention, 2015), although some medications effectively control associated features such as comorbid aggression (McCracken et al., 2002) or attention-deficit/hyperactivity disorder (ADHD) (Antshel et al., 2013). Co-occurring psychiatric disorders are common in patients with ASDs and represent a challenge for healthcare providers. A study of 60 Arabic autistic children showed that 63% were diagnosed with at least one additional comorbid disorder (Amr, 2012). The most commonly reported comorbidities were anxiety (58.3%), ADHD (31.6%), and conduct disorder (23.3%) (Amr, 2012). This study did not include other well-known comorbidities such as epilepsy, which is reported to be as high as 30% (El Achkar and Spence, 2015; Jeste and Tuchman, 2015; Speaks, 2015; Viscidi et al., 2013) and gastrointestinal symptoms, such as constipation and abdominal pain, reported between 9%-90% according to a literature review (Arlene Mannion, 2014).

1.1.2: ASD Genetics—Autism is one of the most heritable of psychiatric disorders with a monozygotic concordance rate of 60-91% and a family recurrence risk of 20% (Muhle et al., 2004). Syndromic autism, with a single gene etiology and co-occurring with other physical features in addition to autism, accounts for approximately 10% of cases (Betancur, 2011; De Rubeis and Buxbaum, 2015; Devlin and Scherer, 2012; Iossifov et al., 2014; Krumm et al., 2014; Miles, 2011). Examples of ASDs with single gene disruptions are fragile X, tuberous sclerosis, and Rett syndrome, among others (Devlin and Scherer, 2012; Miles, 2011). Non-syndromic ASDs represent the larger percentage of cases. The proposed genetic alterations are copy number variants (CNV's), which cause gene dosage effects, and common genetic variation, such as single nucleotide polymorphisms (SNP's). There are a number of known CNV's associated with ASD, such as 16p11.2 and 15q13.3 (Ben-Shachar et al., 2009), but common variation has been implicated in upwards of 50% of cases of ASD (Gaugler et al., 2014).

Although no SNP has achieved genomewide significance for ASDs, gene enrichment studies have given insight into a number of pathways associated with ASDs (Anney et al., 2010; Pinto et al., 2014). Some examples are: “neuronal signaling and development”, “synaptic function” and “chromatin regulation” (Pinto et al., 2014). An exome-sequencing study of sporadic autism families (simplex families) showed a four times paternal bias toward *de novo* mutation and mutations were enriched in the highly integrated beta-catenin protein network (O’Roak et al., 2012). In a familial whole-exome sequencing study of a subset of the Simons Simplex Collection *de novo* single nucleotide variants (SNV's) and more specifically, non-synonymous *de novo* SNV's including highly disruptive nonsense and splice-site mutations, were found to be associated with ASDs (Sanders et al., 2012).

Additionally the authors report three significantly associated genes *SCN2A* (a sodium channel alpha subunit associated with ASD and epilepsy), *KATNAL2* (katanin p60 subunit), and *CHD8* (a helicase DNA-binding protein) (Sanders et al., 2012). Additional loss of function mutations were found in *CHD8* and *KATNAL2* in a separate exome study of ASD probands, further solidifying their association with the disorder (Neale et al., 2012). A smaller exome-sequencing study of 20 sporadic probands and their families revealed four possibly causal genes *SNC1A* (a sodium channel), *FOXP1* (a foxhead box transcription factor), *GRIN2B* (an inotropic glutamate receptor), and *LAMC3* (laminin, an extracellular matrix glycoprotein) (O'Roak et al., 2011).

Despite these promising leads, the pathogenesis of ASDs remains elusive. Although the pathway enrichment studies suggest there could be differences in neural connectivity between autistic and control brains, the evidence for connectivity is conflicting (Courchesne et al., 2007). Whereas some studies that have found hyperconnectivity to be associated with ASDs, others have found hypoconnectivity (Di Martino et al., 2014; Supekar et al., 2013; Washington et al., 2014; Watts, 2008). To address this debate, the “autism brain imaging data exchange” consortium pooled imaging studies of ASDs (Di Martino et al., 2014) and reported both hyper- and hypoconnectivity, with hypoconnectivity being more prevalent (Di Martino et al., 2014). Areas of hyperconnectivity were mainly in subcortical regions, such as the thalamus, globus pallidus and primary parietal sensorimotor regions (Di Martino et al., 2014). The consortium also found a decrease in autism related functional connectivity in the corpus callosum (Di Martino et al., 2014). Because the corpus callosum is implicated in high-load neural processing, it is thought that perturbations in its structure contribute to deficits in higher order reasoning (Di Martino et al., 2014; Gazzaniga, 2000).

There is evidence to suggest that a subset of ASDs are caused by defects in neuronal migration, such as cerebral cortical malformations, decreased neuronal density, decreased cortical thickness, and displaced grey matter (Watts, 2008). This evidence is bolstered by studies of reelin (RELN), an ASD risk gene that coordinates cellular migration and positioning, and has been found to be decreased among ASD patients in post-mortem brain studies (Magdaleno et al., 2002; Watts, 2008).

1.1.3: Imaging and Structural Findings—Neuroanatomical imaging has associated ASDs with abnormalities in several brain regions. The main regions implicated being the hippocampal formation, amygdala, cortex, and the cerebellum. The hippocampus is enlarged across all stages of development in ASDs (Schumann et al., 2004). With one study indicating perturbations in shape (Dager et al., 2007). The amygdala was reported to have an altered developmental course characterized by rapid enlargement, which is associated with disturbances in facial emotional processing (Amaral et al., 2008; Kliemann et al., 2012; Tottenham et al., 2014). There are mixed reports concerning alterations of the amygdala, depending on age (Amaral et al., 2008). For example, neuroimaging studies of autistic males 8-12 showed an increase in amygdala volume compared to controls, but no volume differences when 13-18 year olds ASD patients were compared with controls (Schumann et al., 2004). There are multiple pathological conditions affecting the hippocampus and amygdalar regions that are associated with epilepsy (Amaral et al., 2008), which is frequently comorbid with ASDs.

Structural and functional perturbations in the cerebral cortex have been associated with ASDs. Loss of verbal expression, one of the most prominent diagnostic signs in ASDs, is associated with Broca's area and the inferior frontal gyrus (Amaral et al., 2008). Lesions of the motor cortex have also been implicated in the loss of expressive language in ASDs (Amaral et al., 2008). Disturbances in the development of cortical minicolumn, with respect to neuronal number and dendritic arborization have been linked to ASDs (Casanova et al., 2006). Post-mortem comparison of cortical structure showed systematic differences between autistic and control brains (Casanova et al., 2002). Minicolumns were found to be narrower and in increased number with a greater amount of cellular dispersion, which caused a decrease in the alignment of pyramidal cells and a disorganization of the circuitry in the minicolumn core (Casanova et al., 2002). Neuronal density was increased by 23% in ASD patients compared with normal controls (Casanova et al., 2006).

One study summarized MRI studies comparing people with ASDs and controls. It showed a period of rapid cortical expansion throughout development of both white and grey matter (Amaral et al., 2008). These findings are consistent with the minicolumn findings of increased neuronal number. Additionally, the genetics of the rapid brain expansion have been linked to a down regulation of DNA-repair, apoptosis and cell cycle genes, including *BRCA1*. Furthermore there was an increase in cell survival and proliferation genes, such as *WNT* as well as *PTEN* and *TSC1* of the mTOR pathway (Chow et al., 2012).

ASDs are serious neurological disorders associated with devastating comorbidities. Current treatments do not markedly address the core symptoms of the disorder and, despite many advances in our understanding of the genetics and neurobiology of ASDs, we are far from fully understanding the causes and pathophysiology of these disorders. In this article we describe a relatively new, but burgeoning area of research, that has associated ASDs with the cellular endosomal system. Because this new pathway can, potentially, be targeted by drugs, it could lead to new approaches to treating a subset of ASDs.

1.2: Overview of the Endosomal Pathway

1.2.1: Vesicular Formation—A description of the clathrin-dependent endosomal pathway begins at the cell membrane, where the internalization of lipids and surface receptors forms primary vesicles (see Figure 1) (Schmidt and Haucke, 2007). The signal for a transmembrane protein, such as a receptor, to be internalized via clathrin-mediated endocytosis is a specialized sequence motif found on the cytoplasmic domain that interacts with clathrin, a scaffolding protein (Maxfield and McGraw, 2004). The vesicles are formed when clathrin binds to the c-terminal of the receptor and forms a network of triskelions (three interlocked clathrin molecules) and other adaptor proteins, such as adaptor-protein-2 (AP-2) and clathrin assembly lymphoid myeloid leukemia (CALM) (Matsuoka et al., 2001; Schmidt and Haucke, 2007). Accessory proteins are critical for the formation of clathrin-coated pits, which mediate the binding of clathrin to the cargo present in vesicles. The vesicles are 'pinched-off', by a protein called dynamin, a GTPase, noose-like protein, to complete their formation. It was once thought that these vesicles were released from their clathrin-protein triskelion-coat immediately; but research suggests that the clathrin coat

actually helps to guide the vesicle on its course and is even involved in the tethering process between the vesicle and its destination fusion endosome (Trahey and Hay, 2010).

Eventually, the vesicles are uncoated and fused with pre-existing, intracellular endosomes, or early endosomes, now more accurately termed sorting endosomes (SE's) (see Figure 2) (Maxfield and McGraw, 2004; Trahey and Hay, 2010). The fusion between vesicles and endosomes requires Rab5, early endosomal antigen 1 (EEA1), and SNARE proteins. It is clear that Rab5 is not just a signal for motor proteins, but also a recruiter. Since Rab proteins localize to specific endosomes, it has been proposed that they could recruit other proteins to establish a membrane environment that is permissive to the maturation of those endosomes into later-stage endosomes, such as sorting endosomes or lysosomes. (Maxfield and McGraw, 2004; Schmidt and Haucke, 2007). These, slightly larger, endosomes are acidic in nature and serve as major sorting stations for vesicle contents. SE's eventually undergo maturation and take on the role of late stage endosomes (Maxfield and McGraw, 2004), labeled by an entirely different set of Rab proteins and effectors, such as Rab7 and ESCRT (Jovic et al., 2010). The mechanism of maturation and regulation is not completely understood; however, it is likely that pH plays a role in the maturation process because when V-ATPases are inhibited the rate of maturation decreases (see Figure 3) (Maxfield and McGraw, 2004).

Sorting endosomes are the major site of geometric sorting, a specialized method of sorting membrane receptors and unbound soluble ligands based on spatial coordination (Maxfield and McGraw, 2004). The tubular-vesicular organization of the sorting endosome is iterative and sorts based on luminal volume (Maxfield and McGraw, 2004). The fusion point between the incoming vesicles and sorting endosome contains more volume as well as acidic pH and thus concentrates soluble ligand. Conversely, the tubular outgrowths of the sorting endosome have low luminal volume and concentrate in the membrane receptor fraction (Schmidt and Haucke, 2007). From here the cargo can then be transferred to the recycling endosomes (RE's) (Mayle et al., 2012; Trahey and Hay, 2010), the late-endosome-to-lysosome pathway, or they can pass directly back to the membrane without passing through the recycling endosomes (Schmidt and Haucke, 2007). The regulation of these pathways provides a method of controlling the density of synaptic receptors, thereby regulating neural transmission. These pathways will be discussed in detail in the order just outlined.

1.2.2: The Endosomal Recycling Compartment—The endosomal recycling compartment (ERC), is a distinct vesicular body located in the perinuclear space, close to the mitotic organizing center (see Figure 2) (Lin et al., 2002). The ERC is a collection of tubular endosomes, which is functionally distinct from other endosome compartments. It temporarily accumulates and stores receptors that are queued for recycling rather than being sent to the lysosomes for degradation (Lin et al., 2002). The morphology of the ERC can be dramatically changed in a cell-specific and temperature dependent manner, but this does not significantly affect the rate of internalization of receptors (Lin et al., 2002). The depolymerization of microtubules causes dispersal of the ERC. However, this does not affect the rate of internalization or recycling of the transferrin receptor (TfR), thus movement on microtubules is not the rate determining step for internalization or recycling (Trahey and Hay, 2010). It is conceivable that the ERC is important for either kinetics or the

internalization of the TfR and other receptors. They can stall and accumulate here before returning back to the membrane, although the exact determination of why and with what consequences have not been elucidated to date (Mayle et al., 2012). It may be that this compartment itself is simply a central hub from which the cell samples its receptor-mediated milieu and can regulate from here accordingly through the concerted efforts of the trans-golgi network, the recycling endosome, and the lysosome.

1.2.3: Late endosome-lysosome pathway—In the context of signaling, the late endosome-to-lysosome pathway is important because it controls the number of receptors present within the cell. Degraded receptors lose their signaling capabilities (Maxfield and McGraw, 2004). Receptors that are destined for the late endosome have been found to be ubiquitinated and it also known that hepatocyte growth factor regulated tyrosine kinase substrate (HRS) protein links ubiquitin and clathrin, which is thought to cause these receptors to be retained for a longer period in maturing endosomes (Maxfield and McGraw, 2004). The “endosomal-sorting-complex-required-for-transport” (ESCRT) has also been shown to be involved in the sorting of ubiquitinated proteins, however it is only been demonstrated in yeast (Maxfield and McGraw, 2004). The receptors are internalized in a specialized fashion called invagination, in which they are engulfed into the endosomal lumen forming a multi-vesicular body (MVB) (Traer et al., 2007). MVB's fuse with hydrolytic lysosomes and this causes degradation (Traer et al., 2007).

1.2.4: Direct membrane recycling—The cell has the capability to sidestep the endocytic pathway and directly recycle receptors back to the membrane surface during high activity and increased demand. The direct recycling pathway occurs in a Rab4-dependent manner and bypasses the recycling compartments (Jovic et al., 2010). It has been estimated, using fluorescent lipid analogs, that approximately half of the lipid components internalized return to the surface through the direct pathway. It is conceivable that the cell employs this path to regulate the amount of lipid at the cell surface to quickly control volume and surface area (van Meer et al., 2008).

1.2.5: The Transferrin Receptor (TfR)—The TfR is a common receptor with high basal-level expression that has been very well documented. Thus, it is frequently used as a tool for investigating endocytosis. The canonical view of the cellular physiology of the TfR starts on the cell membrane, where it binds ferrotransferrin (transferrin that has bound to two Fe³⁺-atoms). It is internalized via clathrin-mediated endocytosis and as the vesicle migrates through the cytoplasm, the clathrin is shed. The vesicle fuses to the sorting endosome and, as this compartment matures, its pH decreases (≈ 5.9 -6.0), causing the iron atoms to dissociate from transferrin. The receptor-apotransferrin complex stays intact as it is shuttled through the recycling endosomes until it reaches the membrane at the cell surface where the now neutral pH causes dissociation, releasing transferrin from the receptor (Lodish, 2013). The TfR can also be stalled in the perinuclear space, as previously stated, and a large percentage of TfRs are often found there (3).

Latif et al (2002) were the first to describe a high rate of iron deficiency in autistic patients (Latif et al., 2002). Since then, another study found that 24% of autistic patients were iron deficient (Herguner et al., 2012). However, a more recent study contradicted these findings

(Reynolds et al., 2012). It is of value to note that dysregulated iron metabolism has been associated not only with ASDs, but also with ADHD (Ayhan Bilgic, 2010; Dosman et al., 2006; Herguner et al., 2012). However, a systematic review concerning iron metabolism and ADHD, found that of the 20 studies cited, there were both significant and non-significant results (Cortese et al., 2012). These findings are interesting in an etiological sense and for the fact that the receptor and its pathway may constitute an available drug target or delivery system for the treatment of the underlying pathological cellular physiology in ASDs.

1.2.6: A Word on Neural endosomal systems—As many aspects of the endosomal system have been outlined above, it is important to note that there are differences between cell types and their consequent endosomal pools. For example, neuronal and epithelial endosomal systems are polarized, creating a more diverse and specialized pathway in order to accomplish their cellular functions (Yap and Winckler, 2012). The recycling endosomal system within neurons is highly specialized and differs in its ability to sort receptors as well as recruit binding partners (Folsch et al., 2009). Due to the spatial architecture of neurons, their recycling endosomal system has been found throughout the soma as well as in the dendrites and axons. In contrast, the recycling system is usually concentrated in a perinuclear region in non-neuronal cells (Ascano et al., 2009). The recycling endosome is required for the development and maintenance of dendrites (Park et al., 2006). Additionally, there are even differences within the unique endosomal subsets within neurons, such as, the somatodendritic vs. the axonal systems (Yap and Winckler, 2012). One noteworthy example is the early endosomal regulator EEA1, which is only found within the somatodendritic early endosomes and not in the axonal endosomes (Wilson et al., 2000; Yap and Winckler, 2012).

2: REVIEW

2.1: Review of Studies Implicating the Endocytic Pathway in ASDs

2.1.1: Literature Search and Screening—Because the literature on ASD gene associations is huge, we relied on the Human Gene Module of the Simons Foundation Autism Research Initiative (SFARI). We used the SFARI database as our reference for scoring genes, requiring them to have at least “minimal evidence” within the literature (see Table 1) (Basu et al., 2009). Although the SFARI database may not include some newer findings, it is a well-curated, -well-accepted and highly-utilized tool for summarizing genetic associations and, thus, provides a comprehensive view of the literature.

The endosomal system plays a key role in neuronal functioning, but is it relevant for the neuropathology of autism? To find the answer to this question, we downloaded and compared the entire gene list from the SFARI database to an endosomal pathway gene list generated in Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). The IPA list was assembled from combining genes from the “canonical” and “Disease and Function” pathways for the endosomal system in IPA. GeneVenn was used to compare the gene lists for overlap (Pirooznia et al., 2007). There were 24 endosomal genes in the SFARI database (figure 4). This represents a significant enrichment of autism risk genes within this pathway ($p = 0.018$ by Fisher's Exact Test). Next, we searched PubMed for

articles pertaining to ASDs and the endosomal pathway using the following search algorithm: (“autism spectrum disorders”[MeSH] OR ASD OR autism OR autistic disorder) AND (endocytosis OR endosomal OR endosome OR recycling endosome OR receptor recycling OR receptor trafficking OR membrane trafficking). The search produced 98 articles. After excluding articles, as described in Figure 1, 58 articles remained (see Figure 5). 85% of the studies retrieved from PubMed had been published in the last 5 years indicating that the endosomal system is a novel area of ASD research. This is in part due to genetic studies as well as bioinformatics analysis of large publicly available data sets, such as “Brainspan”.

Some broad molecular mechanisms that have been described as underpinning the pathophysiology of ASDs are “regulation of gene expression”, “protein localization”, “synaptic transmission”, “cell signaling”, “cytoskeletal remodeling” and “cell adhesion” to name a few (Pescosolido et al., 2012). These categories are broad, but as the previous authors propose, there is some convergence on late synapse development perturbations (Pescosolido et al., 2012). Of noted importance is the fact that all of these biological mechanisms can be affected by endocytic dysregulation, solidifying the role of this pathway as a fundamental biological process and a possible source for drug targeting. One example is the Delta/Notch-like EGF related receptor. Delta/Notch-like EGF receptor is specifically found in dendrites and is important for the development and functionality of the nervous system and it has been shown to be controlled by the process of clathrin-mediated endocytosis (Kurusu et al., 2010).

There are a multitude of endosomal pathway genes that have been associated with ASDs (Pescosolido et al., 2012; Yap and Winckler, 2012). Some of the better-known examples are *SLC9A6*, *NEUROBEACHIN*, and multiple Rab proteins (Pescosolido et al., 2012). Additionally, in a large re-sequencing study of the X-chromosome searching for rare variants associated with intellectual disability two of the top six genes, *SLC9A6* and *APIS2*, were well-established endosomal genes (Tarpey et al., 2009), indicating the importance of the endosomal system for neurological perturbations. It has been shown that 31% of ASD individuals exhibit comorbid intellectual disability (Prevention, 2014). To describe the pathophysiological role of the endosomal system in ASDs we divided the studies from our search into genes that are regulated by the endosomal system and genes that regulate the endosomal system. We found 19 genes that met the criteria of ‘minimal evidence per the SFARI gene database summary. In Table 1, four genes fell under ‘syndromic’ classification, one gene was classified as ‘high confidence, syndromic’, three genes were classified as ‘strong confidence, syndromic’, two were classified as ‘strong confidence’, two were classified as suggestive evidence and six genes that were classified as ‘minimal evidence’. Table 1 summarizes our findings.

2.2: Genes that are regulated by the endosomal system

2.2.1: The Dopamine Transporter—The dopamine transporter (DAT) that is localized presynaptically, removes dopamine from the synaptic cleft and therefore, modulates the strength and efficacy of dopamine signaling (Giros and Caron, 1993). DAT has been implicated in ADHD by the efficacy of stimulant medications, which treat the disorder and

block the DAT (Yanli Zhang-James, 2015) along with suggestive findings from genetic association studies (Faraone et al., 1999; Faraone et al., 2014). Some reports of DAT associated with ASD and ADHD have focused on mutations, such as the V559 and the T356M variants, that cause the transporter to anomalously efflux dopamine into the synaptic cleft (Hamilton et al., 2015; Mazei-Robison et al., 2008). In addition to anomalous dopamine efflux during basal level neurotransmission, the V559 coding variant was found in two autism probands and was found to have trafficking disruptions (Bowton et al., 2014). Finding that the V559 variant was not responsive to amphetamine-associated redistribution away from the plasma membrane, the authors investigated dopamine receptor activation, which is responsible for controlling surface expression of DAT through PKC activity (Bowton et al., 2014). The authors postulated that there is increased PKC signaling due to D2R activation in the mutant causing the redistribution and trafficking perturbation (Bowton et al., 2014). Additionally, using a novel experimental approach that employed quantum dot labeling of DAT, Kovtun *et al.* showed that neuropsychiatric-associated variants in DAT caused an increase in membrane diffusion and constitutive endocytosis and recycling (Kovtun et al., 2015). These studies linking DAT to ASDs show how disturbances in its function and its membrane trafficking have the ability to directly affect neurological phenotypes, but they also intimately connect its surface trafficking to its function.

2.2.2: The Serotonin Transporter—Disruption in serotonin transporter (SERT) function has been associated with a subset of ASDs in a number of studies (Prasad et al., 2009). Reduced dietary tryptophan, the biochemical precursor of serotonin, exacerbated the symptoms of repetitive and anxious behaviors in ASD patients (McDougale et al., 1996). In a study involving heterologous cell lines, primary culture of mouse raphe nucleus, and lymphocytes from autistic patients *N*-ethylmaleimide-sensitive factor (NSF) was found to be a novel binding partner of SERT and had reduced expression in autistic brains (Iwata et al., 2014). Knock down of NSF decreased SERT cell membrane expression and serotonin uptake. The mechanism was postulated to be decreased trafficking to a functional position (Iwata et al., 2014). 4-phenylbutylate increased the trafficking of SERT in COS-7 cells, thus modulating its function and again lending credence to the endosomal system as a source for etiological and pharmacological intervention for ASDs (Fujiwara et al., 2013). Trafficking and cell surface expression dysfunction were also associated with SERT in ASDs in another study that used HeLa cells (Prasad et al., 2009).

2.2.3: Oxytocin receptor—The oxytocin receptor was first implicated in ASDs in a family based association study of Han Chinese, which identified two SNPs significantly associated with ASD (Wu et al., 2005). This gene has also been associated to a number of disorders and traits that co-occur with ASDs, such as epilepsy, aggression (Sala et al., 2011), social recognition skills (Skuse et al., 2014) and increased amygdalar volume (Inoue et al., 2010). In a genomic sequencing and functional analysis study of 132 ASD patients, six patients were found to have the rs35062132; c.1126C>G SNP associated with ASDs (Ma et al., 2013). Functional analysis in HEK293 cells using confocal microscopy showed that the mutant form of the oxytocin protein had faster rates of oxytocin-induced receptor internalization and recycling when compared to cells expressing the wild-type version (Ma et al., 2013).

2.2.4: CADM1—CADM1, a member of the immunoglobulin superfamily, is a cell adhesion molecule that is localized to both sides of the synaptic cleft (Zhiling et al., 2008). The extracellular domain is calcium-independent and homophilic with cell-adhesion activity. The intracellular domain has interaction sites for calmodulin-associated serine/threonine kinases (Biederer et al., 2002). A study of 195 autistic patients found an intronic copy number variant within CADM1 in one patient (Nava et al., 2014). In a study of 195 autistic patients two missense mutations were found in two male patients and their family members (Zhiling et al., 2008). The mutations caused defects in glycosylation and ultimately trafficking that resulted in the retention of CADM1 in the endoplasmic reticulum (ER) (Zhiling et al., 2008). These mutations were confirmed to cause trafficking defects and ER stress in C2C5 cells (Fujita et al., 2010).

2.2.5: CNTNAP2—Contactin-associated protein-like 2 (*CNTNAP2*) is a neurexin-related cell adhesion molecule associated with ASDs, epilepsy, intellectual disability, schizophrenia, and bipolar disorder (Varea et al., 2015). Genetic variants of *CNTNAP2* associated with ASDs caused endosomal trafficking disruptions that increased protein retention in the endoplasmic reticulum (Falivelli et al., 2012). The proper localization and function of Caspr2 depends on endosomal trafficking (Bel et al., 2009). Although CNTNAP2 mutations did not induce synapse formation in culture or affect axonal length, they caused dysfunctional trafficking of glutamate receptors (Varea et al., 2015). This work suggests that dysregulated dendritic trafficking may be a pathophysiologic feature of ASDs. Additionally, loss of CNTNAP2 function caused neuronal migration issues in a knockout mouse model, in particular ectopic neurons in the corpus callosum, increased neuronal number in the deep cortex, and a decreased number of interneurons (Penagarikano et al., 2011). Correlating dysregulation in the endosomal system to perturbations in cellular migration is an interesting perspective to consider in the context of neurodevelopment because neurons must migrate to form functional clusters within the brain.

2.3: Genes that regulate the endosomal system

2.3.1: AHI1—Abelson helper integration site 1 (AHI1) is a cytoplasmic protein that forms a stable complex with Huntington-associated protein 1 (HAP1) and is responsible for associating with microtubules involved in the internalization and trafficking of transporters and membrane receptors (Sheng et al., 2008). Mutations in AHI1 cause Joubert syndrome, in which autism is 40% comorbid (Alvarez Retuerto et al., 2008). Joubert syndrome is an autosomal recessive neurological disorder characterized by agenesis of the cerebellar vermis, hypotonia, ataxia, intellectual disability, and characteristic breathing patterns (Barreirinho et al., 2001). A large three-part sequencing study of subjects from the Autism Genetic Resource Exchange (AGRE) determined AHI1 haplotypes were associated with ASDs, with two common variants reaching significance (Alvarez Retuerto et al., 2008). Using heterogeneous cell populations and cultured neurons it was shown that AHI1 deficiency causes TrekB internalization and degradation and thus a lesser BDNF signaling cascade (Xu et al., 2010). It was shown that the AHI1-HAP1 complex stabilized the Trek B receptor, inhibiting degradation through the lysosomal pathway (Xu et al., 2010). How exactly this complex coordinates vesicular trafficking has yet to be resolved, however it does show promise as an endosomal stabilizer that may be able to divert lysosomal degradation.

2.3.2: SHANK3—*SHANK3* is a postsynaptic scaffolding protein that localizes neurotransmitter receptors to the postsynaptic density. It is associated with ASDs and intellectual disability (Bonaglia et al., 2006; Durand et al., 2007; Sheng and Hoogenraad, 2007) (Uchino and Waga, 2013). The localization of receptors in the postsynaptic membrane is intimately related to endo- and exocytosis. In a synaptic modeling experiment it was demonstrated that haploinsufficiency of *SHANK3* caused a dispersal of glutamate receptors across the post-synaptic density, reducing the effective neurotransmission by upwards of 40% (Freche et al., 2012). In hippocampal neurons, *SHANK3* binds to a Rho-GAP protein, thus regulating the recycling of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor via the endosome-recycling compartment (Raynaud et al., 2013). In concordance with these findings, it was also reported that siRNA knockdown of *SHANK3* decreased transferrin recycling (Raynaud et al., 2013). It is postulated that *SHANK3* affects dendritic spine density and morphology through its regulation of the endocytic system (Raynaud et al., 2013; Uchino and Waga, 2013).

2.3.3: Neurexin—Neurexin is a pre-synaptic cell adhesion molecule (Chanda et al., 2015). It is localized to the presynaptic membrane and binds neuroligin as well as other receptors and adhesion molecules. Aoto et al. (2013) used a novel experimental method, which employed homologous recombination to change a splice site for neurexin-3 constitutively. They showed that presynaptic neurexin controlled AMPA receptor endocytosis and trafficking at the *post*-synaptic membrane (Aoto et al., 2013). They concluded that long-term potentiation, a postsynaptic event, relies on presynaptic signals and that all of the molecular events therein are orchestrated by endocytosis and proper localization through the endocytic system (Aoto et al., 2013). Neurexin gene expression has also been strongly associated with the expression of two ASDs candidate genes that localize to the endosomal system and regulate its function and kinetics, the sodium hydrogen exchanger's isoform 6 and 9 (Schwede et al., 2014). The sodium hydrogen exchangers are discussed in greater detail later in this review.

2.3.4: Neuroligin—A large sequencing study consisting of 36 affected sibling pairs and 122 trios implicated neuroligin 3 in the pathophysiology of ASDs (Jamain et al., 2003). Additional exome and sequencing studies have reinforced this original association. Molecular analysis has determined that neuroligin is a post-synaptic cell adhesion molecule (Chanda et al., 2015). Mutations in this gene impact the protein's cell surface localization and function (Comoletti et al., 2004; De Jaco et al., 2008; De Jaco et al., 2010). Several studies show that neuroligin regulates the intracellular trafficking of post-synaptic AMPA receptors, NMDA receptors and post-synaptic density proteins, such as PSD-95 (Barrow et al., 2009; Chanda et al., 2015). The R704C mutation in neuroligin-3 enhances its protein-protein interaction with the AMPA receptor and internalizes and consequently decreases postsynaptic AMPA receptor density (Chanda et al., 2015).

2.3.5: DYRK1A—Also known as *minibrain kinase (mnb)*, is a synaptically located endocytic protein that recently emerged as a putative risk gene for ASDs. *De novo* loss of function mutations were identified in the Simons Simplex Collection and the Autism Sequencing Consortium found it to have high statistical significance (Basu et al., 2009). *mnb*

is a kinase that enhances synaptojanin phosphoinositol phosphatase activity and consequently accelerates endocytosis. A large exome sequencing study determined it as one of 59 genes with function disrupting mutations in their ASD cohort (Iossifov et al., 2012). Its overexpression has been associated with Down syndrome (Guimera et al., 1999). Chen et al. (2014) used the *Drosophila* neuromuscular junction to show that in an activity-dependent manner, *mnb* enhances endocytosis by activating synaptojanin, its putative substrate (Chen et al., 2014). Furthermore, *mnb* is required for synaptic growth and rapid synaptic vesicle recycling. This endocytic regulatory protein is essential for the normal development of synaptic morphology and function by its ability to strengthen the synapse through efficient endosomal recycling in an activity dependent manner. The authors showed that hypomorphic expression of *mnb* causes a significant decrease in the number of synapses, but an increase in the size of synapses that were present in a *Drosophila* model (Chen et al., 2014).

2.3.6: Neurobeachin—Neurobeachin is an intracellular scaffolding protein that anchors protein kinase A (PKA) to various intracellular vesicles (Wang et al., 2000). Its role in binding protein kinase A has been confirmed in platelet studies of haploinsufficient mice showing platelet dense granules, a phenomenon also seen in haploinsufficient autistic patients (Nuytens et al., 2013). Additionally, it has neuron specific expression, is necessary for dendritic morphology and synaptogenesis (Miller et al., 2015) and is associated with trans-*golgi* trafficking of intracellular, tubulovesicular endomembranes (Wang et al., 2000). There is a cytosolic pool of neurobeachin that is recruited to endosomes and vesicles in a protein-coat fashion (Wang et al., 2000). In a single case report, Castermans et al. (2003) described a three-year-old male, followed into adolescence, with regression, echolalia and stereotypical speech, hand flapping, borderline I.Q., and diagnosed with autism. Molecular analysis showed that the boy had a balanced reciprocal translocation between chromosomes 13q13.2, defined as fragile site FRA13A (Savelyeva et al., 2006), and 5q12.1 that disrupted the neurobeachin gene (Castermans et al., 2003).

The developmental role of neurobeachin was shown using a knock out mouse model. Using biochemical assays, electrophysiological studies and microscopy, Medrihan et al. (2009) showed that neurobeachin knockout mice had altered composition of synaptic proteins, decreased spontaneous neurotransmission, and reduced synaptic numbers. The frequency of excitatory neurotransmission and the frequency and amplitude of inhibitory neurotransmission was decreased by the knock out (Medrihan et al., 2009). The authors concluded that neurobeachin regulates the formation of synapses and their subsequent role in neurotransmission (Medrihan et al., 2009). The authors did not report any global changes in cellular excitability or major brain architectural deficits (Medrihan et al., 2009), suggesting that perturbations in the endosomal system within neurons can have drastic behavioral consequences with only minor effects on cellular physiology.

Drosophila knockout models of neurobeachin showed decreased odor learning and gross disturbances in brain architecture, which were rescued by transgene expression (Volders et al., 2012). Additionally, social deficits including locomotor deficiency, increased rate of habituation, hyperactivity, decreased avoidance to stress odors, and increased social space for neurobeachin loss of function mutants were also reported in *drosophila* models (Wise et al., 2015).

Neurobeachin affects the endosomal trafficking of glycine receptors, implicating its role in recovery and post-*golgi* trafficking of neurotransmitter receptors (del Pino et al., 2011). Neurobeachin also regulates the surface density and trafficking of glutamate and GABA receptors, thereby controlling basal levels of neurotransmission (Nair et al., 2013). The regulation of surface glutamate receptors was associated with neurobeachin binding to synapse-associated protein 102, whereas localization of surface GABA receptors was associated with the pleckstrin homology (PH) domain of neurobeachin (Farzana et al., 2015). Taken together these studies implicate neurobeachin in the binding, recovery, and trafficking of neurotransmitter receptors. It is an integral scaffolding protein in the endosomal system, which coordinates the binding and function of regulatory kinases.

2.3.7: REEP3—Receptor expression enhancing protein 3 (REEP3) is a probable regulator of vesicular trafficking from the endoplasmic reticulum to the Golgi apparatus, as it interacts with Rab proteins. Its overexpression blocks the transport of proteins to the Golgi apparatus from the ER (Calero et al., 2001). It was shown in another study, using elegant imaging, that REEP3 is a morphogenic protein that stabilizes the curvature of the endoplasmic reticulum (Voeltz et al., 2006). It was first associated with ASD in a case report of an autistic male patient with a paracentric inversion in chromosome 10 (Castermans et al., 2007).

2.3.8: GRIP1—Glutamate receptor interacting protein 1 (GRIP1) is a neuronal scaffolding protein that is responsible for binding and localizing glutamate receptors at the synaptic surface (Dong et al., 1997; Dong et al., 1999). It has been mapped to autism risk loci 12q14.3 (Ma et al., 2007). The rate of GRIP1-mediated recycling of glutamate receptors determines the strength of the excitatory synapse through proper receptor localization (Trotman et al., 2014). A study that employed GRIP1 knock out mice and primary hippocampal neurons determined that ASD associated mutations involved a gain of function in GRIP1 (Mejias et al., 2011).

GRIP1 gain of function mutants increased interactions with glutamate receptor A2, which, in turn, increased recycling and surface distribution. A knockout of GRIP1 caused the opposite effect (Mejias et al., 2011). GRIP1 was elevated in cerebellar and hippocampal cell lysates from the stargazer mouse model (Trotman et al., 2014). Since the stargazer mouse lacks TARP proteins responsible for the trafficking of glutamate receptors, the overexpression of GRIP1 is thought to be a compensatory mechanism of the cell to allow proper localization of receptors (Trotman et al., 2014). This upregulation and compensatory cellular response suggests that a novel pharmacological intervention might accomplish the same biological end.

2.3.9: Sodium/Hydrogen Exchangers (NHEs)—NHEs were studied in 22.5% of the articles returned by our targeted literature search and thus represent a concentrated effort within the field. There are nine sodium/hydrogen exchanger genes: *SLC9A1* through *SLC9A9* (Donowitz et al., 2013; Kondapalli et al., 2014; Zhang-James et al., 2011). These code the nine NHE proteins: NHE1 to NHE9. NHE genes show differential expression throughout the cell, such that *SLC9A1* through *5* reside in the plasma membrane, *SLC9A6* in various endosomes, *SLC9A7* in the trans-*golgi* network, *SLC9A8* in the *Golgi* apparatus and *SLC9A9* in endosomes (Kondapalli et al., 2014). All of the NHEs control

some aspect of cellular physiology through their action as electroneutral ion exchangers. Some regulate the cytosolic pH. NHE6 through 9 regulate endosomal pH and, hence, endosomal trafficking (Kondapalli et al., 2014). A growing body of evidence supports the idea that NHE6 through 9 regulate the acidification of endosomal compartments by acting as a leak pathway in which the stoichiometry is that of 1:1 for hydrogen and sodium (see Figure 6) (Hill et al., 2006; Nakamura et al., 2005; Ohgaki et al., 2010).

The secretory carrier membrane proteins or ‘SCAMP’ family directly binds and localizes NHEs to their respective positions intracellularly. SCAMP5, a gene returned in our literature search, is a protein that coordinates endocytosis and is recruited under excessive excitatory load (Castermans et al., 2010). Although not present in the SFARI database, we chose to discuss it here due to the connection it has to NHEs. Additionally, a 40% reduction of SCAMP5 gene expression has been linked to idiopathic autism (Castermans et al., 2010). Using primary hippocampal neuron culture from Sprague Dawley rats and consequent exocytic/endocytic analysis assays, Zhao et al. (2014) showed that SCAMP5 knockdown results in endocytic defects when neurons were strongly electrically stimulated, while there were no effects reported for the exocytic pathway.

2.3.9a: *SLC9A6* (NHE6)—*SLC9A6* has been associated with epilepsy, Angleman syndrome and X-linked intellectual disability (Gilfillan et al., 2008). NHE6 localizes to the early recycling and sorting endosomes (Guterman, 2013; Ohgaki et al., 2010). It acts as a proton leak pathway in the earlier endosomal system and shares some functional redundancy with NHE9 (Kondapalli et al., 2013; Nakamura et al., 2005). NHE6 was recently implicated in long-term-potential, showing enhanced localization within dendritic spines and mediating glutamatergic endosomal translocation (Deane et al., 2013).

Stromme et al. (2011) were the first to report the consequences of *SLC9A6* knockout in a mouse model. The knockout caused an accumulation of GM2 ganglioside and cholesterol in the neuronal endo-lysosomal system which, in turn, led to the formation of axonal spheroids (Stromme et al., 2011). They also observed increased aggregations of misfolded proteins (aggresomes) in these neurons (Stromme et al., 2011). Whether these accumulations are a cause or effect of the overabundance of misfolded proteins is unknown. Consistent with the effects of the *SLC9A6* knockout, an in frame deletion (370)Trp-Ser-Thr(372) of *SLC9A6* eliminates the effects of NHE6 on arborization, increased uptake of transferrin, and clathrin-mediated endocytosis (Ilie et al., 2014).

Christianson syndrome was first reported in a South African family with X-linked intellectual disability in which there were 16 affected males and ten carrier females (Christianson et al., 1999). Linkage analysis implicated *SLC9A6* (Christianson et al., 1999). This syndrome exhibits components of autism, intellectual disability, regression, ataxia, microcephaly and epilepsy (Pescosolido et al., 2014). There are also reports of tau deposition and lysosomal storage disease-like phenotypes (Garbern et al., 2010; Stromme et al., 2011). Schwede et al. (2014) found that NHE6 was consistently downregulated in postmortem autistic brain. Consistent with this finding, the NHE6 knockout mouse shows impoverished dendritic spines with decreased circuit functionality along with decreased axonal and dendritic arborization with reduced synaptic field potentials through

hippocampal sections (Ouyang et al., 2013). The biological mechanism responsible was an overacidification of endosomes, which caused decreased BDNF-TrkB signaling (Ouyang et al., 2013).

Neuronal cultures were used to show that the decreased arborization and dendritic spine density could be rescued with exogenous BDNF treatment, which suggests an avenue of research for treatment development (Ouyang et al., 2013; Pescosolido et al., 2014). The endosomal and vesicular trafficking systems are intimately connected to BDNF-TrkB signaling, one of the most well characterized neurotrophic factors responsible for dendritic spine development and survival (Chapleau et al., 2009).

2.3.9b SLC9A9 (NHE9)—Like other NHE's, *SLC9A9* is predicted to have 12 transmembrane spanning regions and a long, C-terminal tail that is projected into the cytoplasm (Zhang-James et al., 2011). The C-terminal tail, which is predicted to be the site of regulation, contains the binding sites for cellular signal transduction (Ohgaki et al., 2010; Wakabayashi et al., 1992; Zhang-James et al., 2011). Binding partners include receptor for activated kinase 1 (RACK1), calcineurin homologous protein CHP, and phosphatidylinositol 4,5 biphosphate (PIP2) (Malo and Fliegel, 2006; Zhang-James et al., 2011). *SLC9A9* expression is mostly postnatal and found prominently in the olfactory bulb, hippocampus, amygdala, cortex, and cerebellum ([Internet]), #151], #151], #151], 2015 #151], four brain regions implicated in ASDs. Its expression is significantly higher in astrocytes, when compared with neurons, and localizes with Rab7 and Rab11 to the late and recycling endosomes (Guterman, 2013; Kondapalli et al., 2013; Nakamura et al., 2005).

A study of a single family cosegregating ADHD and intellectual disability found a rare pericentric inversion affecting *SLC9A9* and *DOCK3* (de Silva et al., 2003). A genome wide association study (GWAS) of ADHD found *SLC9A9* to have the strongest association among 51 candidates (Lasky-Su et al., 2008). Additionally, several studies have associated *SLC9A9* with addiction (Vink et al., 2009; Wang et al., 2013), a known comorbidity of ADHD (Groenman et al., 2013; van de Glind et al., 2014; van Emmerik-van Oortmerssen et al., 2014).

Morrow et al. (2008) were one of the first to describe an association between *SLC9A9* and autism in a groundbreaking study of shared ancestry employing homozygosity mapping. They reported ten separate mutations of *SLC9A9* in ASD individuals with and without epilepsy. One generated a stop codon (R423X) in the last transmembrane spanning region. The latter mutation generates a truncated protein without a C-terminal tail (Morrow et al., 2008). Morrow et al. found R423X in a patient with severe epilepsy and was present in the same region as another stop codon mutation in *SLC9A1*, which produces the slow-wave epileptic (*swe*) mouse (Morrow et al., 2008). A deletion spanning exon two of *SLC9A9* has also been reported in a male autistic patient with focal cortical dysplasia (Wagle, 2014).

We previously reported five novel single nucleotide polymorphisms (SNP's) within the coding region of *SLC9A9* in a rat model of ADHD (Zhang-James et al., 2011). Two of these were non-synonymous and were inherited together and demonstrated an increased affinity between NHE9 and calcineurin homologous protein (CHP) protein interaction (Zhang-James

et al., 2011). We confirmed the interaction between NHE9 and RACK1 and found an association between *SLC9A9* expression and synaptophysin expression, which suggests that *SLC9A9* regulates synaptic proliferation (Zhang-James et al., 2011). Additionally, RNA interference knockdown of *NPAS4*, a neuron activity dependent transcription factor, significantly altered gene expression levels of *SLC9A9*, demonstrating that it exhibits activity related expression patterns (Morrow et al., 2008).

We collected gene expression data from hippocampus, prefrontal cortex, substantia nigra, and ventral tegmental area tissue from Spontaneous Hypertensive, Wistar-Kyoto and Sprague Dawley rats. The gene expression data showed *SLC9A9* to be coregulated with *CHP*, *RACK1*, *calmodulin (CaM)* and other genes coding for signaling proteins (Zhang-James et al., 2012). We also reported a 2.5 percent genomic difference between two WKY rat strains: one from Harlan Laboratories and the other from Charles River Laboratories (Zhang-James et al., 2014). The differential regions included *SLC9A9* that were enriched for ASD candidate genes as defined by the SFARI Human Gene Module. There were differences between the two WKY strains on behavioral parameters such as lower social interest and lower ultrasonic vocalization calls, suggested that the Charles River strain has traits consistent with ASDs (Zhang-James et al., 2014). We also reported that the *SLC9A9* knockout mouse showed ASD-like traits as evidenced by significant decreases in ultrasonic vocalization and increases in repetitive behaviors (Lina Yang, 2016). However the *SLC9A9* knockout did not produce other relevant ASD features, such as decreased social behavior (Lina Yang, 2016).

A very recent functional analysis by Kondapalli et al examined three ASD-associated mutations, which led to a loss of function phenotype in astrocytes (Kondapalli et al., 2013). Overexpression of NHE9 increased the pH of the endosomal system, which consequently increased the amount of transferrin and glutamate uptake. The authors formulated a model of ASDs caused by decreased functional expression of *SLC9A9*. Mutant astrocytes showed a down regulation in surface expression of glutamate transporters compared to wild type. Improperly localized transporters leave excess levels of glutamate within the tripartite synapse (consistent with ASD and epilepsy), consequently leading to increased seizure activity and neurotoxicity (Kondapalli et al., 2013). Additional mechanisms possibly linking *SLC9A9* to ASDs include actin remodeling, neurotransmitter loading, and decreased neurotropic factor signaling (Kondapalli et al., 2014).

In contrast to the hypothesis put forward by the Kondapalli group, Schwede et al. (2014) found that *SLC9A6* was significantly downregulated and *SLC9A9* was significantly upregulated in postmortem cerebral cortex of autistic patients. They also reported a general downregulation of synaptic genes with consequent upregulation of *SLC9A9* (Schwede et al., 2014). This implies that, in a subset of ASDs, there is increased *SLC9A9* and decreased synaptic gene expression, however we do not know the functional effects of these changes (Schwede et al., 2014). The authors did not report if the transcripts were functional and thus we cannot conclude if this was a compensatory upregulation.

Kondapalli et al reported that *SLC9A9* was overexpressed in glioblastoma multiforme (GBM) and that this had direct implications for the treatment effectiveness and survival rates

of patients (Kondapalli et al., 2015). The study reported an increase in the migration of glial cells *in vivo* and *in vitro* (Kondapalli et al., 2015). Additionally, they showed that *SLC9A9* directly increased the expression and recycling of epidermal growth factor receptor (Kondapalli et al., 2015). An interesting hypothesis that the authors put forward, and with which we agree, is that *SLC9A9* controls the pan-receptor milieu through endosomal sampling (Kondapalli et al., 2015). Increasing *SLC9A9* expression will increase the membrane expression of receptors and transporters. By regulating the posttranslational receptor levels through the endolysosomal system, *SLC9A9* directly impacts gene expression. We postulate that via dysregulation of not just receptors and receptor signals, but also the compartments that orchestrate trafficking throughout the cell, it is possible that gene expression could be impacted.

2.3.10 Mammalian Target of Rapamycin (mTOR)—mTOR is a serine/threonine kinase that forms regulatory complexes mTORC1/2 (Laplane and Sabatini, 2009). mTOR regulates protein synthesis, lipid synthesis, autophagy, cell survival and proliferation to name a few (see Figure 7) (Laplane and Sabatini, 2009). mTOR is intimately connected to the process of autophagy, as its signaling regulates the process of “self-eating” or the autodigestion of proteins and organelles (Laplane and Sabatini, 2012; Mizushima et al., 2008). This pathway is critical for neuronal survival because mTOR regulates growth in the developing brain and the turnover of misfolded proteins in developed neurons (Graber et al., 2013). There is accumulating evidence suggesting that malfunctions in autophagy can cause neurodegenerative diseases (Laplane and Sabatini, 2012; Lee et al., 2013), but the role of autophagy in neurodevelopment is fairly recent (Lee et al., 2013). Autophagy is constitutively active in healthy post-mitotic neurons (Lee et al., 2013; Nixon et al., 2005). Whole exome-sequencing studies have found network enrichment for autophagy related genes in autistic patients (Poultny et al., 2013) and there are functional studies implicating mTOR dysregulation and autophagy in ASDs. Autophagy is dependent on the endolysosomal system, because autophagic vesicles must fuse with multiple endocytic compartments, such as the early endosomes, late endosomes, MVBs, and lysosomes (Otomo et al., 2012; Razi et al., 2009).

The tuberous sclerosis complex, consisting of TSC1 and TSC2, regulates the mTORC1-signaling pathway by inhibiting Rheb. When dysfunctional, either TSC1 or 2 can produce benign tumors throughout the body. Both cause syndromic autism. There are conflicting studies regarding the effects of TSC2 expression levels on fluid-phase endocytosis. One study found that TSC2 physically associated Rab5, had Rab5-GTPase (GAP) activity, had localized to the perinuclear region where the recycling endosome is located, and was shown to regulate the fluid-phase endocytic rate (Xiao et al., 1997). It was postulated that TSC2 regulated the fusion, docking and maturation of early endosomes and thus acts as a negative regulator of endocytosis (Xiao et al., 1997). In another study in mouse embryonic fibroblasts and primary mouse astrocytes there was no detection of gene dosage effect on endocytosis and there was no change in endocytic rate when the null-mutant clones re-expressed recombinant TSC2 (Uhlmann et al., 2002). It was observed that astrocytes, heterozygous for TSC2, had conferred growth advantage over wild-type astrocytes, due to decreased expression of cell-cycle regulatory proteins (Uhlmann et al., 2002). Since Rab5 is a known

positive coordinator of the endosomal pathway (Bucci et al., 1992) and that it is associated with the regulation of autophagy (Ao et al., 2014) it would make sense that TSC2, which regulates Rab5, would affect the endosomal system. In a landmark study Tang et al found a molecular basis for increased cell survival, increased synaptic density and ASD-traits in *Tsc2*^{+/-} mice (Tang et al., 2014). These mice have overactive mTOR signaling, due to decreased inhibition by tuberous sclerosis associated proteins (Tang et al., 2014). Hyperactivity of the mTOR complex caused a loss of mTOR regulated neuronal autophagy required for synaptic pruning (Tang et al., 2014). Additionally, pharmacological correction of neuronal autophagy rescued the social behavior deficits and synaptic pathologies in ASD models (Tang et al., 2014).

2.3.11 MAGE-L2—As a member of the melanoma antigen protein family, MAGE-L2 is an ubiquitin ligase enhancer protein (Doyle et al., 2010). It is a paternally imprinted gene that maps to the Prader-Willi deletion locus (Hao et al., 2013). MAGE-L2 enhances ubiquitination of the WASH complex and thus facilitates endosomal F-actin polymerization as well as retromer-dependent transport (Hao et al., 2013). In this way, it is critical for endosomal recycling (Hao et al., 2013). Case reports of MAGE-L2 truncations cause ASDs, intellectual disability and Prader-Willi phenotypes (Schaaf et al., 2013). In addition, USP7 interacts with MAGE-L2, acting as a deubiquinating enzyme that functions to molecularly fine-tune WASH-dependent endosomal recycling. USP7 variants are associated with neurodevelopmental disorders with clinical features of ASDs and intellectual disability (Hao et al., 2015).

3: Discussion

In effort to summarize the current literature pertaining to genes within the endosomal system implicated in ASDs, we performed a systematic review of the literature. Available data suggest this is a valuable avenue for neuropsychiatric research and, potentially, intervention. Neurons are highly dependent on the endosomal system process. They must properly traffic and localize receptors, remove receptors to decrease signaling potential, maintain their membrane system, and regulate nutrient metabolism and resource recycling. The endosomal system also regulates the formation and maintenance of synapses, which has implications for learning and memory.

Our search found several studies of autism that implicated endosomal system genes. Mutations in these genes perturb the endosomal system's kinetics in a manner that might contribute to pathophysiology in some cases. Thus, it is possible that manipulating the function and kinetics of the endosomal system could be a pathway to treatment and, perhaps, prevention (Murphy et al., 2009). Interventions would not be limited to dysfunctional proteins that control the endosomal system. Mutations that decrease the functional localization of receptors and transporters could also be targeted. For example, if a receptor gene contains a mutation that decreases its surface localization, it might be beneficial to target the endo-lysosomal system in effort to decrease the degradation of the functional receptors present at the surface. Multiple genes highlighted in this review were reported as regulators of the endosomal system, such as Neurobeachin and the endosomal ion-

exchangers *SLC9A9* and *SLC9A6*. Learning about the molecular machinery of central regulatory proteins could lead to valuable insights into novel pharmacological inventions.

By modulating the intracellular and vesicular pH, sodium/hydrogen exchangers maintain a central role in regulating the endosomal system. An avenue less studied is the protein-protein interaction network of these central proteins, although some progress has been made in cancer research. One study showed that *SLC9A9* expression impacts esophageal cancer cell response to chemotherapy (Chen et al., 2015). It was found that *SLC9A9* overexpression was associated with an increase in signaling through RACK1 (Chen et al., 2015). This signaling cascade involves direct interaction and activation of Akt, specifically through the phosphorylation of Ser473 and consequently signaling beta-catenin and Bcl-2 (Chen et al., 2015). Functionally, this directly facilitated cell survival and decreased treatment response (Chen et al., 2015). This study emphasized the rich signaling cascades that are downstream of *SLC9A9* and the potential affect of loss or gain of function.

Evidence from structural and functional brain imaging has revealed mixed findings of connectivity in ASDs. There is evidence of both hypo- and hyperconnectivity in different patients. Additionally, within individual patients, there is evidence of local hyperconnectivity and long-range hypoconnectivity (Kana et al., 2014; Maximo et al., 2013; Moseley et al., 2015). Synaptic remodeling depends on mTOR-regulated autophagy (Shehata and Inokuchi, 2014; Shehata et al., 2012; Shen et al., 2015; Shen and Ganetzky, 2009; Tang et al., 2014). Thus, insufficient autophagy results in over connection and excessive results in over-pruning. From the work we reviewed, we postulate that *SLC9A9* could directly influence the mTOR pathway and that this could be a pathophysiologic pathway for ASDs (Prasad and Rao, 2015). Through RACK1, *SLC9A9* has the potential to hyperactivate mTOR causing a decrease in autophagy and an increase in the number of synapses (Prasad and Rao, 2015). Combined with the overall increase in cell survival signaling there would be an increase in cell number as well, consistent with the overgrowth and overabundance of neurons and synapses present in autistic brain during early development. This is also consistent with Schwede *et al's* findings of upregulated *SLC9A9* in autistic postmortem brain (Schwede et al., 2014; Tang et al., 2014). It is reasonable that if *SLC9A9's* ion-transport mechanism is dysfunctional then there could be a compensatory upregulation of gene expression. Upregulating the protein, that is dysfunctional, yet still retains protein-protein interaction and signaling capabilities could produce the situation described above.

However, there are an equal number of reports demonstrating that gene mutations in *SLC9A9* associated with ASDs cause null expression through nonsense mutations or exonic deletions that cause haploinsufficiency (Morrow et al., 2008; Wagle, 2014). Decreased functional expression of *SLC9A9* would decrease mTOR signaling through a decrease in network signaling and cause a consequent increase in autophagy. In addition this would decrease cell survival signals and could make neurons more susceptible to cellular stressors, such as reactive oxygen species, excitotoxicity and neuro-inflammation. Increased levels of autophagy could have the consequence of over-pruning and produce a regressive autistic phenotype, in which a child develops properly and then loses cognitive capabilities, such as verbal expression (Thomas et al., 2015). Conceivably, synaptic loss and neuronal death

would cause microcephaly and intellectual disability with severe autism. The precise mechanism has not yet been determined.

We postulate that mTOR is not only physically connected through sodium-hydrogen exchanger signaling and protein-protein interaction, but that the process of endosomal control is inseparable from autophagy. Autophagic processes rely on a functional endo-lysosomal system and require fusion with early endosomes and lysosomes. Both the endosomal system and autophagic systems require strict pH regulation. Dysregulation of either system causes ASD-like traits through faulty localization of cellular components and an inability to regulate cellular metabolism and proper cycling (Kondapalli et al., 2013; Ouyang et al., 2013; Tang et al., 2014). Regulated through a triad of kinases, including the AMP-dependent protein kinase ULK-1 and the mTORC complexes, there may be a number of available drug targets within this system (Di Nardo et al., 2014) (Dunlop and Tee, 2013).

mTOR-mediated autophagy maintains a cellular energy balance. Starvation is a powerful inducer of autophagy while abundant nutrient levels inhibit autophagy (Singh and Cuervo, 2011). The hypothalamic mTOR pathway integrates nutritional status and hormonal signaling to limit food intake and thus body weight (Cota, 2009). Activation of mTOR in the arcuate nucleus is required for transmission of satiety signals, such as leptin (Cota, 2009). Leptin, a molecule, secreted by adipose tissue, inhibits hunger signals (Valleau and Sullivan, 2014); it is an upstream effector of the mTOR pathway (Peter Ishola, 2015). Elevated leptin levels have been associated with autistic phenotypes (Valleau and Sullivan, 2014). This provides a molecular mechanism linking maternal nutritional status and fetal brain development.

Over or under weight mothers are at increased risk for birthing a child with ASDs (Dudova et al., 2014a; Dudova et al., 2014b; Krakowiak et al., 2012; Rivera et al., 2015). This could be due to a gene-environment interaction in which leptin, or the mTOR-pathway, are dysregulated and producing increased risk for developing autism. This idea implicates three non-mutually exclusive mechanisms: 1) the maternal environment dysregulates the fetal or placental environment, 2) the maternal environment interacts with faulty fetal genetics, or 3) faulty fetal genetics impacts the nutritional response system. For example, maternal nutritional status could dysregulate fetal pathways, such as mTOR or endocytosis, leading to ASDs. If the maternal environment is over or under nourished and the fetus has an inherited defect in a compensatory pathway this could represent a source of increased risk of autism. Or finally, the fetus could have an inherited defect in the genes that regulate these pathways. Maternal obesity results in increased leptin levels. Even if maternal nutrient intake is correctly monitored during pregnancy, obesity will result in the increase of leptin through excess adipose tissue. Although it remains to be determined whether or not leptin crosses the placental barrier, excess maternal leptin is associated with higher fetal leptin levels (Luo et al., 2013) and is associated with negative pregnancy outcomes (Tessier et al., 2013). We wonder if excess leptin could contribute to fetal dysregulation of mTOR. Additionally, the increased leptin could produce a degree of leptin resistance in the fetus, later translating to an increased risk of obesity in offspring (Tessier et al., 2013).

Endocytosis and autophagy are distinct yet intertwining systems that coordinate neuronal function and development. The endosomal system regulates basal level neuronal function by synchronizing signals and cellular reactions involving receptors and lysosomal trajectories of proteins. Autophagy remodels neurons and tightly regulates cellular energy based on available nutrients. The interplay between these two systems fine-tunes neuronal development through dendrite formation and elimination and maintains neuronal functions, such as long-term potentiation and long-term depression (Shen et al., 2015). A direction for pharmacological intervention is the development of separate inhibitors for the two systems, which is difficult because the two systems have similar upstream signals (Shen et al., 2015).

We are unsure of the proportion of cases of ASD that involve the endosomal and autophagic pathways. Though, both are implicated in ASDs by rare variants, we are unsure if they play a role in more common forms. That being said, Schwede et al's finding that common variant ASDs show a misregulated *SLC9A9* gene expression implies that the endosomal system could be involved in a larger set of ASD cases, possibly in a multifactorial manner. If this hypothesis is accurate, then the system should be further implicated in disease etiology when larger genome wide association studies are published.

There are limitations to our review. We cited articles written in English only, thus we could have missed articles in other languages. Additionally, our search was only conducted through the PubMed database, thus further limiting our results. Finally, the articles were screened by only one investigator, which could potentially add error to the results.

In summary, the biochemical mechanisms of endocytosis are important for many aspects of neuronal processes regulating brain function. Many lines of evidence suggest that this pathway might be valuable for diagnostics and treatment modalities. *SLC9A9* is a central regulator of the endosomal system with recent research demonstrating signaling networks to the mTOR pathway as well. However, the literature is conflicted as to whether this transporter is upregulated or haploinsufficient in ASDs, with theoretical and experimental evidence valid in both directions. Determination of the underlying mechanism could prove valuable for determining not just which genes are mutated in a given disease, but how those mutations affect protein function.

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Highlights

- Autism spectrum disorders (ASDs) are serious neurological disorders that are highly heritable.
- The literature on endosomal system genes and ASDs was summarized.
- Many genes within the endosomal pathway are implicated in ASDs.
- The endosomal system is crucial for development and function of the nervous system.
- It shows potential for biomarkers and intervention.

Figure 1. Vesicular formation and initiation of clathrin-mediated endocytosis

Receptors clump together on the surface as their transmembranous regions bind clathrin. Clathrin forms triskelions and a spherical structure as adapter proteins bind and cause the pinching mechanism that completes the vesicle.

Figure 2. The endosomal pathway

Primary endosomes are formed according to figure 1. They are trafficked throughout the cell on various trajectories. The sorting endosome is the site of geometric sorting and the first place where the vesicle will fuse. Based on biochemical signals receptors and internalized material will be sorted to their respective destinations. Proteins can be sorted back to the membrane in two ways: 1) fast recycling in which they go directly to the membrane and 2) through the ERC, where they stall and are recycled slowly. Alternatively, they can be sent to the lysosome through multivesicular body formation and progressive acidification.

Figure 3. Endosome compartments and acidification status

Acidification is one of the main regulatory processes of endosomal maturation. Progressive acidification will cause the endosomes to eventually mature into a lysosomal compartment. Alternatively, if the compartment is alkalinized then a recycling endosome causes the receptors to be sent back to the membrane. Recycling endosomes are important for their ability to retain molecules, which can preserve energy and specific lipids.

Figure 4. Venn diagram demonstrating overlap between IPA endosomal genes and SFARI database

Ingenuity pathway analysis generated endosomal gene list (IPA-endo) (red) compared to the SFARI gene database list (yellow). 24 genes were found in common (orange). Autism genes are significantly enriched with endosomal genes.

Figure 5. PRISMA diagram

This workflow for systematic screening articles describes the number and justification for removal. The total number of articles remaining after screening was 55.

Figure 6. Representative image of a sodium-hydrogen exchanger protein

The NHE family of proteins resides in different cellular compartments, but their general structure consists of 12 transmembrane domains that form the ion-exchange pore. There is a large C-terminal tail that is hypothesized to regulate the proteins action and is also the site of the interactome. The protein functions as an electroneutral proton-leak, exchanging hydrogen for sodium or potassium. The 1:1 stoichiometry alkalinizes the compartment in which the protein is localized.

Figure 7. mTOR pathway

The mTOR pathway is a conserved signaling cascade that results in various fundamental cellular processes, such as autophagy and protein translation. It is a regulator of cellular metabolism that integrates cellular energy and nutrient status signals.

Table 1

SFARI gene database summary.

Gene Symbol	Alternate Name	Association Level in SFARI	Number of Autism Reports	Highly Cited Associated Disorders	Number of Negative Reports	Recent Recommendations
SHANK3	SH3 + multiple ankyrin	Category 1S (high confidence; syndromic)	23	1	5	20
dyrk1A	Minibrain kinase (mnb)	Category 1S (high confidence; syndromic)	9	None	None	6
NRXN	Neurexin	Category 2 (strong confidence)	36	2	None	20
NLGN3	Neuroigin	Category 2 (strong confidence)	17	0	6	13
GRIP1	Glutamate receptor interacting protein 1	Category 2 (strong confidence)	3	3	None	4
CNTNAP2	Contactin-associated protein-like 2	Category 2S (strong confidence; syndromic)	29	1	2	13
MAGE-L2	Melanoma Antigen Family L2	Category 2S (strong confidence; syndromic)	1	None	None	1
USP7	Ubiquitin specific peptidase	Category 2S (strong confidence; syndromic)	1	None	None	None
SLC6A3	DAT	Category 3 (suggestive evidence)	11	1; ADHD	None	2
OXTR	Oxytocin receptor	Category 3 (suggestive evidence)	16	3	2	12
SLC6A4	SERT	Category 4 (minimal evidence)	15	1	4	4
NBEA	Neurobeachin	Category 4 (minimal evidence)	1	1	None	2
SLC9A9	NHE9	Category 4 (minimal evidence)	2	1	None	5
CADM1	Cell adhesion molecule 1	Category 4 (minimal evidence)	2	1	None	4
REEP3	Receptor accessory protein 3	Category 4 (minimal evidence)	1	2	None	None
SLC9A6	NHE6	Category S (syndromic)	4	1	None	4
TSC1	Tuberous sclerosis 1	Category S (syndromic)	6	2	1	7
TSC2	Tuberous sclerosis 2	Category S (syndromic)	8	2	1	7
AHI1	Ableson helper integration site 1	Category S (syndromic)	4	2	None	10