# AN AUTISM-CAUSING VARIANT MISREGULATES SELECTIVE AUTOPHAGY TO ALTER AXON TARGETING AND BEHAVIOR

by

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ABSTRACT

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Neurodevelopmental disorders cause debilitating disruptions to the cellular mechanisms that underlie development of the brain. Unfortunately, the complexities of neurodevelopmental disorders make them difficult to study, and the molecular mechanisms perturbed by these disorders remain elusive. Better understanding of neurodevelopmental mechanisms, and the related genes involved, will likely yield new insight into neurodevelopmental disorders. A gene that has been associated with a number of neurodevelopmental disorders is the *calcium voltage-gated channel subunit alpha1 C (CACNA1C)* gene. Common and rare variants of the *CACNA1C* gene have been associated with autism and other neurodevelopmental disorders including schizophrenia, bipolar disorder and ADHD. However, little is known about how *CACNA1C* variants affect cellular processes to alter neurodevelopment. The Timothy syndrome mutation is a rare, gain-of-function variant in *CACNA1C* that causes autism with high penetrance, providing a powerful avenue into investigating the role of *CACNA1C* variants in neurodevelopmental disorders. A gain-of-function (gof) mutation in the *C. elegans CACNA1C* homolog known as *egl-19* causes an equivalent amino acid change to the Timothy syndrome mutation in humans. This work shows that this *egl-19(gof)* mutation can alter axon targeting and affect behavior in *C. elegans*. Wildtype *egl-19* functions independently of *Regulator of Presynaptic Morphology-1* (*rpm-1*) to negatively regulate axon termination. The *egl-19(gof)* mutation represses axon

termination to cause axon targeting defects that lead to the misplacement of electrical synapses and alterations in habituation to light touch. Moreover, genetic analysis indicates that selective autophagy acts downstream of the *egl-19(gof)* mutation to mediate its effects on both axon termination and behavior. These results reveal a novel mechanism whereby an autism-causing variant of *CACNA1C* misregulates selective autophagy to alter circuit formation and affect behavior.

Dedicated to:

This dissertation is dedicated to my wife Huong, whose endless support has allowed me to pursue my dreams and finish my dissertation.

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| | |
|---|---|
| ADHD | attention deficit hyperactive disorder |
| ALFY | autophagy-linked FYVE protein |
| ALM | anterior lateral microtubules |
| ALS | amyotrophic lateral sclerosis |
| AMAN | alpha-mannosidase |
| aPKC | atypical protein kinase C |
| Arp2/3 | actin-related proteins 2 and 3, protein complex |
| ARX | aristaless related homeobox gene |
| ASD | autism spectrum disorder |
| ATG | autophagy related genes |
| AUTS2 | activator of transcription and developmental regulator |
| BCL2L13 | BCL2-like 13 |
| BDNF | brain-derived neurotrophic factor |
| BECLIN-1 | coiled-coil, moesin-like BCL2-interacting protein |
| BOC | brother of CDO |
| *C. elegans* | *Caenorhabditis elegans* |
| cac | cacophony gene |

| | |
|---|---|
| CACNA1 | calcium channel alpha-1 |
| CACNA2D | calcium channel alpha-2-delta |
| CASPR2 | contactin-associated protein-like 2 (protein) |
| CHD8 | chromodomain helicase DNA binding protein 8 |
| CMA | chaperone-mediated autophagy |
| CNTNAP2 | contactin-associated protein-like 2 (gene) |
| COMT | catechol-o-methyltransferase protein |
| CRP | cargo receptor proteins |
| CUP-5 | coelomocyte-uptake defective protein 5 |
| Cvt | cytoplasm-to-vacuole targeting pathway |
| DLK-1 | delta like non-canonical notch ligand 1 |
| *Drosophila* | *Drosophila melanogaster* |
| DVL3 | dishevelled segment polarity protein 3 |
| EGL-19 | egg laying defective protein 19 |
| ENDO-A | endophilin-a |
| EPG | ectopic P granules protein |
| EPH | erythropoietin-producing human hepatocellular receptor proteins |
| EPHA | EPH Receptor A1 |

| | |
|---|---|
| EPHRIN | eph receptor-interacting proteins |
| EPHRINA | ephrinA ligand for EPHA receptor |
| Esrom | zebrafish myc-binding protein 2 |
| FGF | fibroblast growth factor |
| FGFR1 | fibroblast growth factor receptor 1 |
| fMRI | functional magnetic resonance imaging |
| FSN-1 | F-box/SPRY domain-containing protein 1 |
| Gem GTPase | GTP-binding protein of the ras superfamily |
| Gof | gain-of-function |
| GFP | green Fluorescent Protein |
| GLO | gut granule loss protein |
| GWAS | genome wide analysis studies |
| HSC | heat shock protein |
| HSP | cytosolic chaperone heat shock protein |
| L1CAM | L1 cell adhesion molecule protein |
| LAMP2A | lysosome-associated membrane glycoprotein protein 2A |
| LC3 | microtubule-associated proteins 1A/1B light chain 3B |
| LD | linkage disequilibrium |

| | |
|---|---|
| LGD | likely gene disrupting |
| LMP | latent membrane protein |
| lof | loss-of-function |
| MEC-7 | mechanosensory protein 7 |
| mito | mitochondria |
| µl | microliter |
| µm | micron |
| MRI | magnetic resonance imaging |
| NCOA4 | nuclear receptor coactivator protein 4 |
| NLGN-4 | neurologin protein 4 |
| NSF | N-ethylmaleimide sensitive fusion protein |
| PAM | protein associated with Myc, human PHR protein |
| PAS | preautophagosomal structure |
| PE | Phosphatidylethanolamine |
| PHR | PAM/Highwire/RPM-1 protein |
| PIK3C3 | phosphatidylinositol 3-kinase catalytic subunit type 3 |
| PLM | posterior lateral microtubules |
| PRS | polygenic risk score |

| | |
|---|---|
| PTEN | phosphatase and tensin homolog |
| Rac | ras-related C3 botulinum toxin substrate |
| RELN | reelin |
| RFP | red fluorescence protein |
| RIC-7 | resistant to inhibitors of cholinesterase 7 |
| ROBO | roundabout protein |
| RGM | repulsive guidance molecule |
| RPM-1 | regulator of posterior microtubules protein 1 |
| SCG10 | superior cervical ganglia protein 10 |
| SRCaTs | spontaneous regenerative calcium transients |
| SHANK | SH3 and multiple ankyrin repeat domains protein |
| SHH | sonic hedgehog |
| SNARE | soluble N-ethylma-leimide-sensitive factor attachment protein receptor protein |
| SQSTM1 | sequestosome protein 1 |
| SYNJ1-1 | synaptojanin protein 1 |
| TRAM-1 | translocating chain-associated membrane protein 1 |
| TrkB | tropomyosin receptor kinase B |
| ULK1/2 | unc-51 like autophagy activating kinase |

| | |
|---|---|
| UNC | uncoordinated protein |
| VAMP | vesicle-associated membrane protein |
| VGCC | voltage gated calcium channels |
| VMP | variable major protein |
| WASP | wiskott-aldrich syndrome protein |
| WDFY-3 | WD repeat and FYVE domain containing protein 3 |

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# Chapter 1 – Human Autism and Autism Genetics

## 1.1 Autism Overview

Autism spectrum disorder (ASD) is defined as a pervasive disorder that affects the development of the brain. In the United States, it is estimated that around 1 in 59 children are diagnosed with some form of ASD (Baio et al., 2018). People with ASD can exhibit severe neurological symptoms such as difficulties learning to speak, as well as problems with repetitive behavior, impaired social interaction, and general cognition deficiencies ("Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR)," 2000; Fombonne, 2009). While some forms of ASD can be mild, the more severe forms of ASD can lead to heavy financial and emotional costs. It is estimated to cost about 60 million USD annually for the lifelong care of people with ASD (Lavelle et al., 2014). Human and financial costs make understanding the causes of ASD paramount.

While ASD is a neurodevelopmental disorder, the exact mechanisms affected in the development of the disorder are not clear. The first portion of this chapter will focus on neurodevelopment, specifically the mechanisms governing the development of the long neurite projection known as the axon. The relationship between axon development and ASD will then be discussed. The remainder of the chapter will then discuss the genetics of ASD, and how we can use those genetics to better understand the disorder.

## 1.2 Neurodevelopment Background

### *1.2.1 Basics of axon outgrowth*

During the formation of neuronal networks, neurons undergo many developmental changes that culminate in the elongation of the axon in order to reach and selectively form synapses with its proper targets. New neuronal cells differentiate from their progenitors based on the concentration of specific proteins, which direct proper polarization of the neuron (Prehoda, 2009; Gallaud et al., 2017). For instance, the localization of aPKC directs the development of the basal domain of the neuron (Atwood & Prehoda, 2009; Prehoda, 2009). The further differentiation of the neuron gives rise to the polarized outgrowth of axons and dendrites (Polleux & Snider, 2010). Axon outgrowth has a number of key factors that govern how the axon develops, the directions it moves, and the point at which the axon stops growing and forms synapses with its intended targets.

In neurons, the enlarged tip of the elongating axon is known as the growth cone. The physical outgrowth of the axon is governed by many of the same mechanisms that govern cell migration in other cell types. Cell migration starts with the polarized protrusion of cytoplasm in the form of lamellipodia, filopodia, or membrane ruffles from the cell body (Small et al., 2002; Lämmermann & Sixt, 2009). There are high levels of actin in the peripheral region of the growth cone (Letourneau, 1983; Challacombe et al., 1996). The actin in the growth cone is a dynamic structure that is constantly reforming and expanding in precise directions, allowing for the elongating axon to move and change as needed during outgrowth. Growth cones promote neuron elongation through the expansion of cytoplasmic space (Letourneau, 1979; Challacombe et al., 1996). This expansion is generated by tensile force created by the polymerization of acting filaments (Symons & Mitchison, 1991; Carlier & Pantaloni, 2007; Kerstein et al., 2015). The

regulation of how the growth cone expands is controlled by actin mediator proteins. Actin nucleation is mediated by the Arp2/3 complex (Figure 1). Arp2/3 is made up of highly conserved proteins that, when active, bind to actin and induce the formation of short actin filaments (Svitkina & Borisy, 1999). The Arp2/3 complex is recruited to the leading edge of the growth cone by the Rac activated SCAR/WAVE complex, which is made of up five different proteins (Xu & Quinn, 2012; Vehlow et al., 2013). Furthermore, the Arp2/3 complex is activated by members of the WASP family (Higgs & Pollard, 2001). In addition to growing actin filaments at the leading edge of the growth cone, the force at the membrane is regulated by contracting myosin (Craig et al., 2012) Myosin is also integral in the turnover of actin for later use (Wilson et al., 2010; Craig et al., 2012). All of these proteins serve as precise regulatory machinery to control the actin dynamics at the growth cone, thus insuring the proper outgrowth of the axon to its intended targets.

Continued axon outgrowth, and the overall function of the neuron, is maintained by axon transport. Actin at the distal tip of the axon is eventually replaced by microtubules as the axon continues to grow (Letourneau, 1979; Challacombe et al., 1996). These microtubules are a more permanent structure for the neuron, and also allow for the shuttling of proteins, cargo, and organelles up and down the axons. Proteins and organelles are transported by motor proteins like dyneins and kinesins (Hirokawa et al., 2010; Maday et al., 2014). The importance of functional axon transportation mechanisms is highlighted by the fact that disruption of axon transport has been linked to neurodegenerative diseases such as ALS (Chevalier-Larsen & Holzbaur, 2006). Some examples of the cargo and organelles transported up and down the axon includes vesicles (Okada et al., 1995; Kamal et al., 2000), endosomes (Delcroix et al., 2003), autophagosomes (Maday et al., 2012) and mitochondria (Pilling et al., 2006; Misgeld et al., 2007). Cargo and

organelles having proper spatial and temporal localization is vital for the continued outgrowth and survival of the neuron. Understanding how two of these cargo/organelles play their roles in axon outgrowth and neuronal homeostasis will be of particular importance: autophagosomes and mitochondria. We will discuss the movement of autophagosomes up and down the axon in a later chapter, and instead focus on mitochondria's role in the developing neuron.

Mitochondria move bidirectionally in the axon, and their movements are closely tied to the outgrowth of the axon (Morris & Hollenbeck, 1993; Kiryu-Seo & Kiyama, 2019). Additionally, mitochondrial function in neurons is closely connected to the development and regeneration of axons (Han et al., 2016; Smith & Gallo, 2018). As an illustration of the importance of mitochondria in axon development, mitochondrial depletion at or before the initiation of axon outgrowth totally prevents the formation of the axon (Mattson & Partin, 1999). Indeed, proper mitochondrial numbers at the synaptic terminal is vital for the proper transportation of other vesicles and cargo along the axon. A lack of mitochondrial transport can lead to dysfunctional synapses (Verstreken et al., 2005; Lee & Peng, 2008). Therefore, mitochondrial function and localization are important for the proper development and function of neurons. These examples of cellular migration mediators are only some of the axon outgrowth regulators. On top of general outgrowth, there are also a multitude of regulators of axon pathfinding and the eventual termination of outgrowth, both of which are discussed below.

### *1.2.2 Axon guidance and termination*

Now that we have outlined the proteins and mechanisms involved in the physical outgrowth of axons, we will shift focus to the regulators of axon guidance. Axon guidance is the process by which axons navigate to their intended targets. This is primarily done by utilizing

axon guidance cues. Within axons, guidance receptors function at the leading edge of the growth cone. These receptors are used by the axon to detect either attractive or repulsive cues that act either over long distances or locally (Figure 2; Berzat & Hall, 2010; Stoeckli, 2018). These guidance receptors can be regulated by the neuron so that the makeup of the types of guidance receptors at the surface of the growth cone are in sync with the direction the axon needs to grow. As one example, mRNA transported from the cell body to the growth cone leads to the translation of the necessary guidance receptors locally in the growth cone. This results in alterations in receptor composition at the growth cone (Campbell & Holt, 2001; Brittis et al., 2002). Axon guidance receptors, when acting correctly, are what allow the axon to navigate to their final target.

Axon guidance cues can act as both attractants and repellents (Figure 2B,C). Some examples of proteins that have evidence showing they act as attractive cues are FGF and SFRP, which bind to the FGFR1 and frizzled guidance receptors, respectively (Shirasaki et al., 2006; Marcos et al., 2015). Some well-known examples of proteins that act as repulsive cues are SLIT, which binds to ROBO (Ypsilanti et al., 2010), and RGM which binds to neogenin (Monnier et al., 2002). There are also examples of proteins that can act as both attractive and repulsive cues, the function of which is dependent on a number of factors. Another well studied example are netrins, which are attractive cues when binding to the neurogenin receptor, but can also act as repulsive cues when they bind to the UNC5A netrin receptor (Sun et al., 2011). Then there is the axon guidance roles of the pervasive developmental proteins WNT and SHH. WNT will bind only to frizzled, while SHH binds only to BOC, and their chemotrophic effect is dependent on the subsequent signaling cascade that is triggered by the binding of the cue to the receptor (Charron & Tessier-Lavigne; Salinas, 2012). Finally, EPHA binds to the ephrinA receptor, but

depending on which co-receptor is also present during EPHA binding, will either act as an attractive or repulsive cue (Knoll, 2004; Suetterlin & Drescher, 2014). Guidance cue behavior can depend on the receptor it binds to and/or additional modifications, and can provide an incredible amount of complexity to the regulatory mechanisms governing axon path finding.

Axon guidance is further complicated by additional factors outside of the aforementioned guidance cues. There are many extracellular matrix components that can affect the outgrowth of an axon, as well as neuronal process self-avoidance, which is the neuron's tendency to grow axons or other branch-like structures away from one another (Kolodkin & Pasterkamp, 2013). In addition, the proteins that mediate axon cytoskeletal restructuring are also thought to play a role in regulating axon outgrowth. For example, in the growth cone of neurons, the Arp2/3 complex has been shown to negatively regulate axon outgrowth, despite being a positive regulator of axon guidance (Strasser et al., 2004). There are also axon outgrowth links to the cellular degradation and recycling during autophagy. Autophagy is a regulator of various forms of axon outgrowth, with some evidence of negative regulation of axon outgrowth by autophagy (Ban et al., 2013). Other findings show that autophagy is vital for proper microtubule stabilization and it is therefore key for axon regeneration (He et al., 2016). There will be more discussion of autophagy and neurodevelopment in a later chapter.

Axon termination is simply the growing axon's ability to stop its growth at a specified place and time (Figure 2D). Defects in axon termination are marked by overextended axons and are often coupled with weakened synaptic function (Wan et al., 2000; Wu et al., 2005; Grill et al., 2007; Borgen, M. et al., 2017). While the regulators of axon outgrowth are relatively well characterized, there is far less known about the ability of neurons to stop their axon outgrowth at the appropriate location. Axon termination has its own mechanisms and regulators to consider,

but many of these mechanisms and regulators remain to be identified. PAM/Highwire/RPM-1 (PHR) is one of the most studied axon termination regulators and is highly conserved in multiple organisms (Zhen et al., 2000; Borgen, M. et al., 2017). Most other known regulators of axon termination function in conjunction with or on the same targets of PHR (Tulgren et al., 2011; Tulgren et al., 2014). In *C. elegans,* RPM-1 has established a signaling pathway made up of well conserved proteins. RPM-1 acts upstream of two parallel pathways: the GLO-4 pathway (Grill et al., 2007) and the FSN-1 pathway (Liao et al., 2004). The RPM-1+FSN-1 ubiquitin ligase activity targets DLK-1, which activates a MAP kinase pathway that leads to the activation of P38 (Liao et al., 2004; Nakata et al., 2005). The parallel GLO-4 pathway is downstream of RPM-1 and works to activate the Rab GTPase GLO-1, which then promotes vesicular trafficking via late endosomes (Grill et al., 2007). These pathways govern the axon's ability to terminate its growth, and subsequently undergo synaptogenesis with its appropriate targets (Figure 3; Grill et al., 2007). These studies provide substantial evidence that axon termination is indeed governed by unique regulators that are separate from those controlling axon guidance or synaptogenesis. However, cross talk between the signaling pathways show how these axon termination mechanisms can influence and be influenced by other mechanisms.

Axon termination and its regulators have influence on and are influenced by neuron function and circuit formation. In zebrafish, the PHR homolog Esrom has roles in the proper organization of the visual system (D'Souza et al., 2005), and in mice, PHR-1 functions to regulate synapse formation for the neuromuscular junction and sensory neurons (Burgess et al., 2004). Axon termination also has close ties to axon guidance and repulsive cues, as well as the regulation of cargo transport up and down the axon, as is evidenced by RPM-1 being involved in growth cone integrity and microtubule stability (Borgen, M.A. et al., 2017). Further research will

need to be performed in order to identify additional mechanistic intricacies and specific targets of the axon termination regulators. Since there are limited findings regarding the regulators of axon termination, we also do not fully understand what role dysfunctional axon termination may play in neurodevelopmental disorders. As we learn more about the mechanisms governing axon development and termination, we will gain a better understanding of how disruptions of these mechanisms can lead to neurodevelopmental disorders. We will now shift our focus to one particular component of functional neurons; voltage gated calcium channels.

## 1.3 Voltage gated calcium channels and neurodevelopment

### *1.3.1 General function of calcium channels*

Voltage gated calcium channels (VGCC) function to control the amount of calcium that is allowed to enter the neuron. Like other neuronal ion channels that exist on neurons, VGCCs respond to action potentials, as well as contribute to the membrane potential that triggers action potentials of the neuron (Catterall, 2000; Bender et al., 2010). Calcium ions are not only important for the regulation of membrane potentials, but also play roles as important signaling molecules (Clapham, 2007). The opening of VGCCs results in an influx of calcium, which ultimately leads to elevated intracellular calcium (Wadel et al., 2007). The tight regulation of the activation of VGCCs thus results in specific spatial-temporal intracellular calcium transients that trigger a wide range of calcium-dependent processes. These processes include neurotransmitter release, neurite outgrowth, gene transcription, and the activation of signaling enzymes such as calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Wheeler et al., 1994; Wheeler et al., 2008; Wheeler et al., 2012). When a VGCC protein is mutated in a way that increases its permeability to calcium transients to lower the threshold for activation, or

increase the rate of inactivation of the channel, it is considered a gain of function mutation (Splawski et al., 2005; Laine et al., 2014). When the opposite occurs, and a mutation results in a constitutively inactive or difficult to activate channel, then it is considered a loss-of-function mutation (Templin et al., 2011).

There are three general families of VGCCs that share conserved functions across many organisms, each differentiated by their pharmacological and biophysical properties. They are L-type, P/Q type, and T-type channels (Catterall, 2011). In humans, the P/Q type channel can be further divided into R-type and N-type calcium channels (Catterall, 2011). The L-type calcium channels are characterized by their long-lasting activation (Catterall, 2000). The L-type calcium channels are important for muscle contraction, endocrine secretion, gene transcription, synaptic activity, neuronal function, and neuronal development (Catterall, 2000; Felizola et al., 2014; Kamijo et al., 2018). T-type calcium channels are known for their low voltage threshold for activation, as well as their fast voltage-dependent inactivation allowing for repetitive firing of action potentials (Catterall, 2011). The rhythmic action potentials generated by T-type calcium channels make them essential in the proper function of cardiac muscles, as well as dopaminergic neurons (Catterall, 2011). The P/Q-type calcium channel is a high-voltage-gated calcium channel important for neuronal excitation and the release of neurotransmitters (Bourinet et al., 1999; Ishikawa et al., 2005). The P/Q-type calcium channels are primarily expressed in the neurons of the central nervous system (Bourinet et al., 1999). The similar R-type and N-type calcium channels are also high-voltage-activated channels (Williams et al., 1992; Soong et al., 1993). The R-type calcium channels have roles in the cortex, hippocampus, striatum, and amygdala (Parajuli et al., 2012). The N-type calcium channels are also important in the nervous system, but also have known roles in the kidney and the heart (Adams & Berecki, 2013).

VGCCs differ in physical organization and number of subunits depending on their type and function, but each subunit of the VGCC are organized in a similar manner. The subunits consist of the alpha-1 subunit, the alpha-2-delta subunit, the beta subunit, and sometimes the gamma subunit (Figure 4; Catterall, 2011). The alpha-1 subunit is the pore forming subunit that spans across the membrane of the neuron and physically allows for the influx of calcium into the cell (Catterall, 2000). The alpha-1 subunit is composed of four domains that are made up of six transmembrane segments linked to each other by cytoplasmic loops (Catterall, 2011). L-type and P/Q-type VGCCs are also made up of extracellular alpha-2-delta subunit and cytosolic beta subunit, which are considered auxiliary subunits (Buraei & Yang, 2013; Dolphin, 2013). Alpha-2-delta subunits are vital for the proper localization of VGCC, in addition to affecting the biophysical properties of the channels, such as influencing the voltage sensing capabilities and inactivation rates of the channels (Felix et al., 1997; Qin et al., 1998; Sipos et al., 2000; Davies et al., 2006; Davies et al., 2010; Dolphin, 2013). Interestingly, there are likely additional roles for the alpha-2-delta subunits independent from the calcium channel complex, such as influencing synaptic morphogenesis (Eroglu et al., 2009; Kurshan et al., 2009; Dolphin, 2012). These listed functions of the alpha-2-delta likely vary depending on the type of VGCC and location in the organism (Dolphin, 2013). The beta subunit is important for delaying VGCCs from being flagged for degradation by the proteasome (Altier et al., 2011; Waithe et al., 2011). Beta subunits also function to promote proper trafficking of the VGCC to the plasma membrane (Pragnell et al., 1994). Finally, there are gamma subunits which are inconsistently present in the VGCC complex (Dolphin, 2013). The gamma subunit plays a role in the activation and inactivation properties of the VGCC, as well as influencing cellular trafficking (Wei et al., 1991; Chen et al., 2000; Burgess et al., 2001).

*1.3.2 Calcium channels in neurodevelopment*

VGCCs play a vitally important role in the development of neurons. As previously mentioned, VGCCs have roles beyond regulating membrane potential, including the activation of a variety of signaling cascades (Clapham, 2007). Specifically, calcium signaling has been shown to modulate the length of neurites in a variety of neuron types (Gomez & Spitzer, 1999; Wayman et al., 2008; Takemoto-Kimura et al., 2010). Likely related, calcium signaling also has roles in growth cone motility (Zheng, 2000; Henley & Poo, 2004). Additionally, VGCCs give rise to spontaneous fluctuation in intracellular calcium concentration during development (Tang et al., 2003), although the specific molecular details of this phenomenon remain elusive. One way to study the specifics in spontaneous regenerative calcium transients (SRCaTs) during neuronal development is to test VGCC mutations. Indeed, Mutations in VGCC genes have been associated with various neurodevelopmental disorders such as autism and schizophrenia (Ripke et al., 2011; Lee, S.H. et al., 2013; De Rubeis et al., 2014; Purcell et al., 2014). Such mutations have been recently shown to have observable effects on the SRCaTs as well as the overall development of individual neurons. In the developing neurons of mice, knocking out L-type VGCCs result in shorter axons and dendrites (Kamijo et al., 2018). Moreover, inducing gain-of-function mutations in L-type calcium channels during perinatal development in mice impaired cortical radial migration, and reversing the gain-of-function postnatally rescued this phenotype (Kamijo et al., 2018). The role of the spontaneous opening of VGCCs in neural development remains an important area of study. Mutations in VGCC genes is a logical place to continue investigating the neurodevelopmental roles of VGCCs. In the next section, we will shift focus back to ASD and how the many regulators of neuronal development have been tied to ASD.

### 1.4 Axon development as it relates to ASD, relevant genes and mechanisms

ASD is primarily a neurodevelopmental disorder with symptoms deriving from changes in brain function. It is pertinent to look at how the development of neurons in the brains of patients with ASD are different, and what mechanisms might be involved. Excess neurogenesis has been observed in patients with ASD, with 67% more neurons detected in the prefrontal cortex (Courchesne et al., 2011). Recent studies utilizing functional MRIs (fMRIs) to view the structures of the brains of those with ASD and those without have shed light on the morphological changes in the brain (Just et al., 2004; Just et al., 2007; Schipul et al., 2011). In addition, regions of abnormal neuronal positioning of cortical projection neurons have been observed in patients with ASD (Wegiel et al., 2014). Abnormal lamination of neurons has been detected in the cortex of brains in patients with ASD (Stoner et al., 2014). These are examples of concrete ties to neurodevelopmental defects that are observable in the brains of individuals with ASD. Besides ties to the physical changes in the brains and neurons of ASD patients, there are plenty of genetic ties to neurodevelopmental regulators.

There are a few examples of genes identified in higher frequencies in patients with ASD that also have ties to proper neuronal migration. RELN, a large secreted glycoprotein, is a mediator of axon termination and is vital for proper neuronal layering and the morphology of neurons (Falconer, 1951; D'Arcangelo et al., 1995; Hong et al., 2000). Studies have found abnormal RELN in brain samples of individuals with ASD (Persico et al., 2001; Bonora et al., 2003; Fatemi et al., 2005). There are also studies that show mutations within the *AUTS2* gene occurring in patients with ASD (Liu et al., 2015). The *AUTS2* gene encodes a protein that is part of a protein complex that maintains the repressed state of certain genes during brain development (Bedogni et al., 2010; Gao et al., 2014), and has been shown to regulate cortical neuronal

migration during axon outgrowth (Hori et al., 2014). *CNTNAP2,* which encodes a cell adhesion glycoprotein, is another such gene identified in a population of patients with ASD that also has potential roles in the proper migration of neurons (Strauss et al., 2006). There are also connections between ASD and genes that are associated with specific symptoms or neurological changes. This includes genes such as *PTEN*, encoding an enzyme vital for cell migration and apoptosis, and has been associated with macrocephaly in ASD (Orloff et al., 2013; Weston et al., 2014). There is also *CHD8*, encoding a calcium dependent cell adhesion protein, and has been associated with macrocephaly (Bernier et al., 2014). Finally, patients with mutations in the transcription factor encoding *ARX* gene display ASD-like symptoms along with epilepsy (Stromme et al., 2002; Turner et al., 2002). While these genetic connections exist, the affected mechanisms that lead to the development of ASD are unknown. Based on the statistical evidence alone, the mechanisms that are likely affected by mutations that lead to the development of ASD are numerous. It is also likely, due to the polygenic nature of ASD, that the development of ASD is the result of a combination of mutations and thereby multiple neurodevelopmental mechanism could be affected. Unfortunately, because of these genetic complexities, hard evidence linking specific mechanisms to ASD is difficult to establish.

As was the case in identifying many of the above genes, one way that we can determine the specific mechanisms affected by ASD is through the examination of the genetic data regarding ASD. An example of this is the recent connection of various missense mutations of the *WDFY-3* gene being associated with ASD (Iossifov et al., 2012; Iossifov et al., 2014). *WDFY-3* encodes WD repeat and FYVE domain containing protein 3 (WDFY-3), which is also known as autophagy linked FYVE protein (ALFY). WDFY-3 is a selective autophagy related protein, and thus gives us an additional clue as to which specific mechanisms are being affected in ASD.

WDFY-3 also has known roles in regulating axon outgrowth (Iossifov et al., 2012; Orosco et al., 2014; Dragich et al., 2016; Napoli et al., 2018). This selective autophagy connection with ASD and neurodevelopment will be further explored in a later section. The next section covers the many genetic connections to ASD that have recently been uncovered. The understanding of these genetic associations with ASD will be incredibly useful in uncovering the many mysteries still surrounding the development of ASD.

### 1.4 Autism Genetics Background

The identification of genetic markers has become a heavy focus in ASD research, and the importance of genetics in the development of ASD cannot be overstated. Since the first characterization of ASD, diagnosis has relied on the identification of specific symptoms. These symptoms may be detectable as early as 18 months of age, but the most common age of an ASD diagnosis is at 2 years or older (Lord et al., 2006). With the development of large-scale genome-wide analysis, as well as more advanced tools for individual gene analysis, the rate of discovery and characterization of key genetic markers for ASD has accelerated (De Rubeis et al., 2014; Iossifov et al., 2014; RK et al., 2017; Grove et al., 2019). There is still much to uncover regarding the genetic contributions and polygenic nature of ASD (Chaste et al., 2017). Moreover, the exact nature of the mechanisms involved in the development of ASD remain unknown. However, the thorough identification and characterization of the genetic causes of ASD would not only improve the efficiency of diagnosis and subsequent treatment outcomes, but would also shed light on the cellular mechanisms that underlie ASD. This would greatly increase our understanding and ability to treat the disorder.

Depending on the metrics utilized, ASD is estimated to be caused by genetic factors in 56% - 95% of the cases (Colvert et al., 2015). Twin studies place the heritability of ASD to be on the higher side, with older studies indicating heritability around 90% (Steffenburg et al., 1989; Bailey et al., 1995), and newer studies place heritability at 83% (Sandin et al., 2017). Regardless of the exact percentage, the genetic association to ASD is very high, suggesting that disruptions in a neurodevelopmental mechanism due to genetic changes is a certain contributor. Therefore, elucidating the genetic interactions and variations that lead to ASD is of great importance if we are to have any hope in understanding ASD. In this literature review, the different types of genetic data available on ASD will be discussed, while outlining the existing gaps in the literature. The three major categories of autism heritability are 1) common variants identified by genome wide association studies (GWAS), 2) rare *de novo* likely gene disrupting (LGD) mutations, and 3) missense mutations that are found in higher frequency in ASD patients. It is important to note that a causative role of missense mutations in ASD is difficult to establish, and therefore the role of these mutations remain poorly understood. This will establish a pretext for a discussion of the polygenic component of ASD inheritance, and the difficulties these genetic interactions create.

### 1.5 Common Variants

Common variants account for most of the heritability of ASD (Gaugler et al., 2014). These common variants are identified through the utilization of GWAS studies. GWAS studies provide extremely useful analyses of the entire genome across a large population of people. When using GWAS to study ASD related loci, the general approach is to take a population of individuals with ASD and scan their genome to find the genetic variations that they share. These

genetic variations are compared to those in a random control population of the same general traits without ASD. Researchers can compare variants between different types and severities of ASD, such as severe ASD compared to high functioning ASD. Additionally, GWAS can be used to compare the common variants identified in ASD to those identified in other neurodevelopmental disorders. Indeed, many of the genetic variants identified as indicators of ASD are shared with other neurodevelopmental disorders. For example, schizophrenia and major depression have shared loci identified through GWAS (Li et al., 2015; Grove et al., 2019). These shared loci might be comprised of conserved regions of genes important for the proper development of neurons or are indirectly related to a mechanism governing the development of the brain. However, it is important to note that without a large enough population or the use of proper statistical methodology, narrowing down the significant loci can be difficult, and make it almost impossible to identify individual genes of significance.

One difficulty in using GWAS to study polygenic disorders is that the original models used to analyze the genomic data did not take into consideration the effect of many heterozygous genes or other small genetic variants that create polygenicity. The use of polygenic scoring systems and analyses, such as linkage disequilibrium (LD) score regression analysis (Bulik-Sullivan et al., 2015) and polygenic risk score (PRS) analysis (Dudbridge, 2016), allow for comparison of gene variants in a population while distinguishing true polygenic signals from inflated distribution of test statistics. Utilizing these systems, the genetic variant candidates can be further narrowed down, which has led to a number of new discoveries that were otherwise buried beneath background noise (Grove et al., 2019). Once these significant "risk variants" for ASD have been established, they can be used for clinical screening, proper genetic counseling,

and treatments. These common risk variants must then be further studied to better understand ASD and the mechanisms involved in the rise of the disorder.

When common genetic variants are identified, further research is needed in order to elucidate the true mechanism behind the gene's role in the development of ASD. The type of mutation that is typically identified by GWAS are intergenic, which are non-coding regions of DNA that do not encode for proteins. Most of the intergenic regions of DNA have no known function, but these regions can contain sequences of DNA that have a regulatory function for other genes (Dunham et al., 2012). Some specific risk variants in genes that have been identified through GWAS studies and then examined for their association to ASD include variants in *calcium voltage-gated channel subunit alpha1 C* (*CACNA1C),* which encodes the alpha-1 subunit of the L-type calcium channels in mammals (Lu et al., 2012; Li et al., 2015). Many of these variants of *CACNA1C* incur risk of schizophrenia, (Li et al., 2015), as well as bipolar disorder (Ferreira et al., 2008). Intergenic regions of DNA that regulate *CACNA1C* have been identified as risk factors for other neurodevelopmental disorders such as schizophrenia (Roussos et al., 2014), so it is likely that the intergenic variants identified via GWAS may regulate genes with well-established connections to ASD. Another example of a gene identified via GWAS that is a well-known common variant for ASD is the *CNTNAP2* gene, which encodes the cell adhesion glycoprotein CASPR2 (Canali et al., 2018). Further studies into CASPR2 show that it plays a role in axon development and is thought to contribute to autism through selective mechanisms (Canali et al., 2018). These studies show the value of GWAS studies in identifying genes that are key contributors to the development of ASD.

### 1.6 *De novo* likely gene disrupting mutations

While there is a plethora of evidence regarding the contribution of common variants as a major cause of ASD, severe *de novo* mutations are a large factor in the development of ASD. Rare, likely gene disrupting (LGD) mutations that contribute to ASD are those expected to completely remove gene function, and can be frameshift, nonsense, or deletion mutations (Iossifov et al., 2012). Often these LGD variants exist in one allele in individuals, supported by the evidence of high levels of heterogeneity in individuals with such mutations (Iossifov et al., 2014). The percentage of cases of ASD that are attributed to *de novo* LGD mutations are estimated to be around 15-20% (Iossifov et al., 2012). While these rare variants can be passed down from a parent (Gaugler et al., 2014), they most often appear *de novo* in the individual without the mutation being in the genomes of the parents. Unlike the common variants discussed above, many of which actually follow a pattern of positive selection (Polimanti & Gelernter, 2017), the more severe phenotypes caused by LGD mutations are less likely to be passed down and are therefore more likely to occur *de novo* within the germline. Indeed, the categorization of rare *de novo* LGD mutations as heritable has been a point of inconsistency within the scientific community. While the nomenclature of *de novo* mutations makes it clear that these mutations in individuals are not truly inherited from the parent, but instead arise in the germline as a new mutation, these mutations can still be passed down from an individual to their progeny. Regardless, the *de novo* mutations that are discussed below are, at their most basic level, altered genes in an individual that arose independent from the parents, and have been shown to contribute to the development of ASD. Therefore, these rare *de novo* LGD mutations are vital to study and understand. In fact, rare genetic variation leads to a higher individual risk to develop ASD than common variants (Gaugler et al., 2014; Alonso-Gonzalez et al., 2018), despite only

making up around 15-20% of the cases of ASD (Iossifov et al., 2012). The significant contribution of *de novo* LGD mutations to the pathogenesis of ASD exemplifies the complex nature of its development. One of the examples of *de novo* LGD mutations that has been associated with ASD is *CACNA1C* (Lu et al., 2012), where a severe form of ASD known as Timothy syndrome is known to be caused by a rare mutation in the *CACNA1C* gene (Boczek et al., 2015). Some *de novo* mutations in the *WDFY-3* gene are also associated with the development of various forms of ASD (Iossifov et al., 2012). These *de novo* LGD mutations are powerful contributors to ASD, but are just a piece of a complex disorder associated with multiple genetic variants, including the more subtle missense mutations.

### 1.7 Missense mutations

In contrast to LGD mutations, individual missense mutations tend to have smaller effects. However, when missense mutations exist in multiple ASD related genes, or when they occur simultaneously with LGD mutations, then the effects can cause dramatic phenotypes (Stein et al., 2013; Guo et al., 2018). Not all missense mutations are the same, as some (including the *CACNA1C* mutation resulting in Timothy syndrome) can lead to gain-of-function effects that can cause severe ASD all on their own (Splawski et al., 2004; Napolitano & Antzelevitch, 2011; Diep & Seaver, 2015). For the sake of differentiating the missense mutations from other previously discussed genetic variants, the focus will be on less potent mutations that are still associated with ASD. It is important to note that missense mutations associated with ASD were identified because they were more prevalent in affected individuals than in controls. Therefore, it is difficult to know if these ASD associated missense mutations actually contribute to ASD. The

Timothy syndrome mutation is one of the few missense mutations where there is strong evidence that it causes ASD (Boczek et al., 2015).

There are a few missense mutations that have strong connections to ASD. One family of proteins that has been strongly associated with ASD is the SHANK proteins. The SHANK family of proteins are scaffolding proteins that regulate the development and organization of synapses (Naisbitt et al., 1999; Grabrucker et al., 2011). Various missense mutations in *SHANK1* have been found in individuals with ASD (Sato et al., 2012; Gong & Wang, 2015). *SHANK3* has more recently been implicated in the development of ASD, with missense mutations being the focus of a number of studies (Wang et al., 2019). As another example, a *NLGN-4* missense mutation has been associated with ASD (Zhang et al., 2009). NLGN-4 is a postsynaptic adhesion protein that controls the development and function of synapses (Varoqueaux et al., 2006; Hoon et al., 2011). While missense mutations of these families of proteins have been shown to be associated with the development of ASD, it is the collective effect of all the genetic variants that contribute to the symptoms of ASD.

### 1.8 Polygenic and gene interactions in autism

As established in the previous sections, research has shown that the clinical presentation of ASD is the result of polygenic factors, meaning it is the result of the combined action of multiple genetic variations (Niemi et al., 2018). There are many examples of polygenic inheritance influencing the risk of developing ASD. For example, *de novo* single nucleotide variants combined with nonsense mutation can confer five times the individual risk relative to carrying a single copy number variant (Stein et al., 2013). Epigenetic factors contributing to ASD have also been identified and add an additional layer of complexity to studying the disease

(Siu & Weksberg, 2017; Hannon et al., 2018). Indeed, differential methylation of genes increases the polygenic burden of ASD (Hannon et al., 2018). Additionally, GWAS studies are difficult in part due to the original models used to analyze the genomic data not taking into consideration the effect of many heterozygous or other small genetic effects that create polygenicity (Grove et al., 2019). As mentioned above, novel statistical models have been helpful in elucidating the less significant genetic variants (Grove et al., 2019). This will prove vital, as understanding the additive effects of small genetic changes are equally important in understanding the various individual variants that cause more severe symptoms on their own. Even though a few gene variants have stood out as primary contributors to certain forms of ASD (Splawski et al., 2004; Bader et al., 2011), it is clear that factors outside of these gene variants influence the severity of the symptoms. All of these examples demonstrate how difficult it is to pinpoint specific contributors to the development of ASD.

One of the most difficult parts of studying ASD is the fact that it is a polygenic disorder. The genetic component of ASD is complex and diverse. The disorder could be caused by a combination of mutations inherited by a parent or that arise *de novo*, and the number of genetic variations is staggering (De Rubeis & Buxbaum, 2015; Sanders et al., 2015). There are many genes that have been associated with ASD, with the number increasing depending on the criteria used to implicate a gene, but we still do not know how they interact with each other. This makes ASD difficult to study, as direct links between the genetic mutations and specific effects that lead to ASD are difficult to discern. Ultimately, the complexities of the disorder make the specific molecular mechanisms that underlie ASD elusive.

Another factor causing yet more complications in understanding ASD is the wide spectrum of symptom severity due to polygenic factors and the large number of gene variants

that likely contribute to ASD. Where one person can have mild symptoms that may go undetected for a long period of time, others are severe enough to warrant lifelong care and cause non-neural complications (Splawski et al., 2005; Jacquemont et al., 2014). This can make reaching broad conclusions about ASD difficult. This differing symptom severity is likely a result of the polygenicity of ASD, the large multitude of genetic variants, and complexity of the genetic interactions that contribute to the disorder. Monogenic causes of ASD are few and far between, making monogenic models for study hard to develop. It would be incredibly useful to have a model that utilizes the more severe genetic variants, preferably a monogenic variant, in order to understand the mechanisms underlying ASD.

**Chapter 1 Figures:**

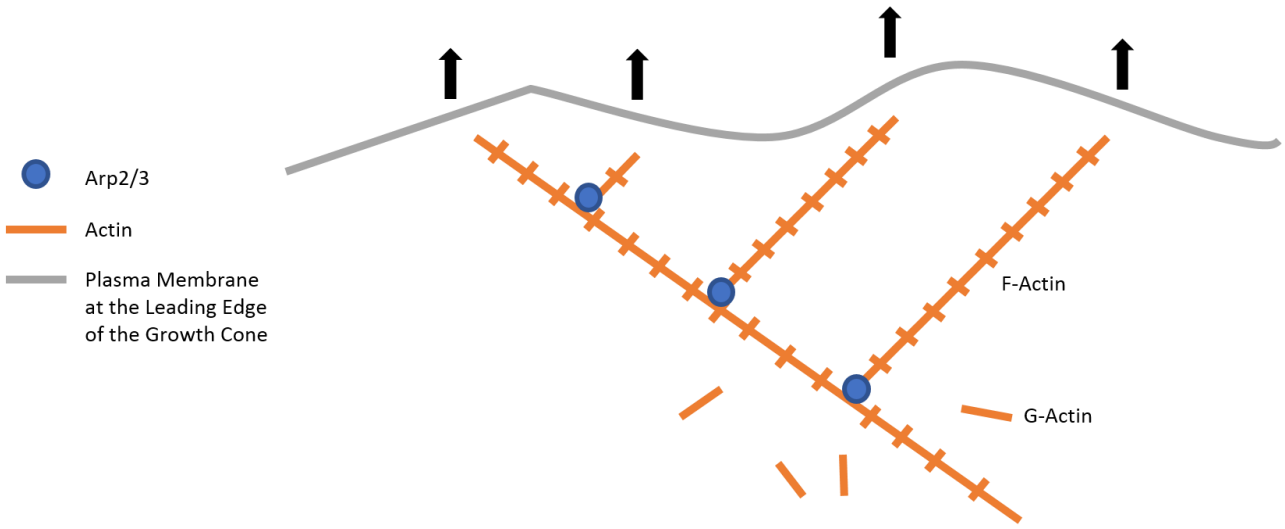


**Figure 1: Diagram of how Arp2/3 complex nucleates a branched network of actin filaments that produce a tension force at the growth cone membrane.**

Actin nucleation is mediated by the Arp2/3 complex. ATP globular (G)-actin monomers are then recruited to polymerize into long filamentous (F)-actin structures. Further nucleation of F-actin can occur along growing F-actin strands that polymerize perpendicular to the original strand, resulting in a branched like structure. These polymerizing F-actin strands push against the membrane produce the force that drives cell elongation and migration.

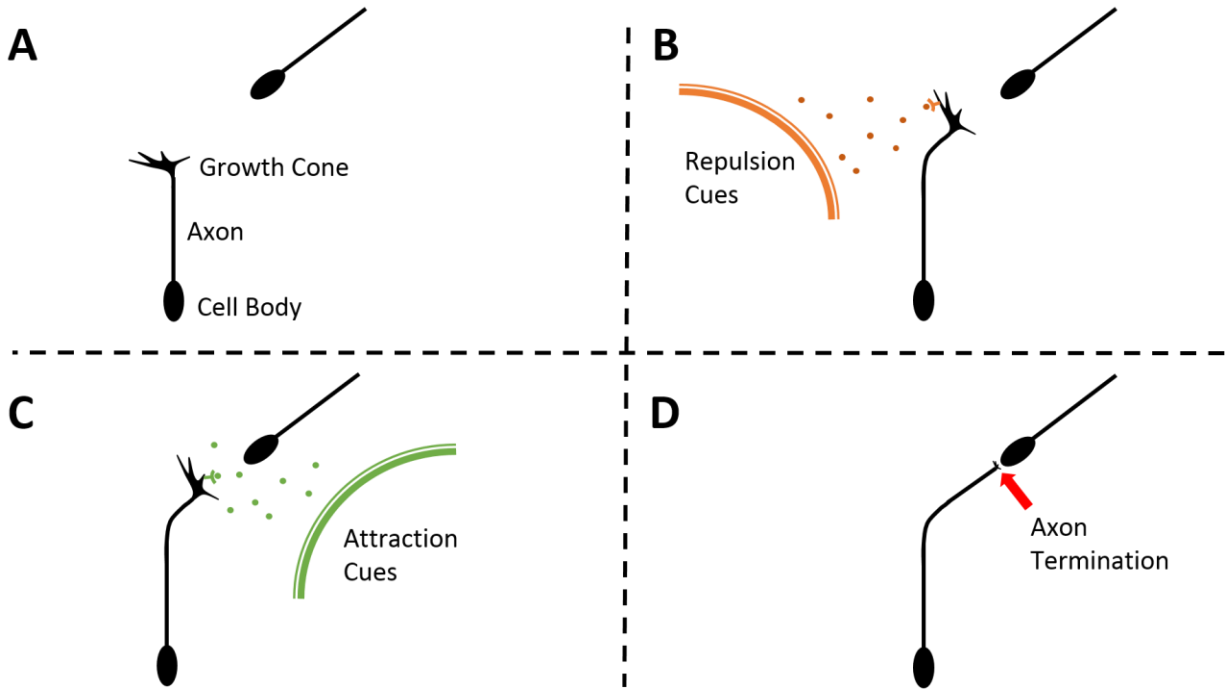


**Figure 2: Diagram of axon outgrowth and guidance.**

(A) Axon outgrowth is the process by which the axon extends away from the cell body, with the dynamic capabilities lying within the growth cone. (B) Repulsive cues will bind to receptors at the growth cone in order to direct the growth of the axon away from the repulsive cue. (C) Attractive cues can also bind to receptors at the growth cone, but instead instruct the axon to grow towards the attractive cues. (D) The final step in the axon outgrowth process is axon termination, where the axon extension stops, and connections are formed with the neuron's intended targets.

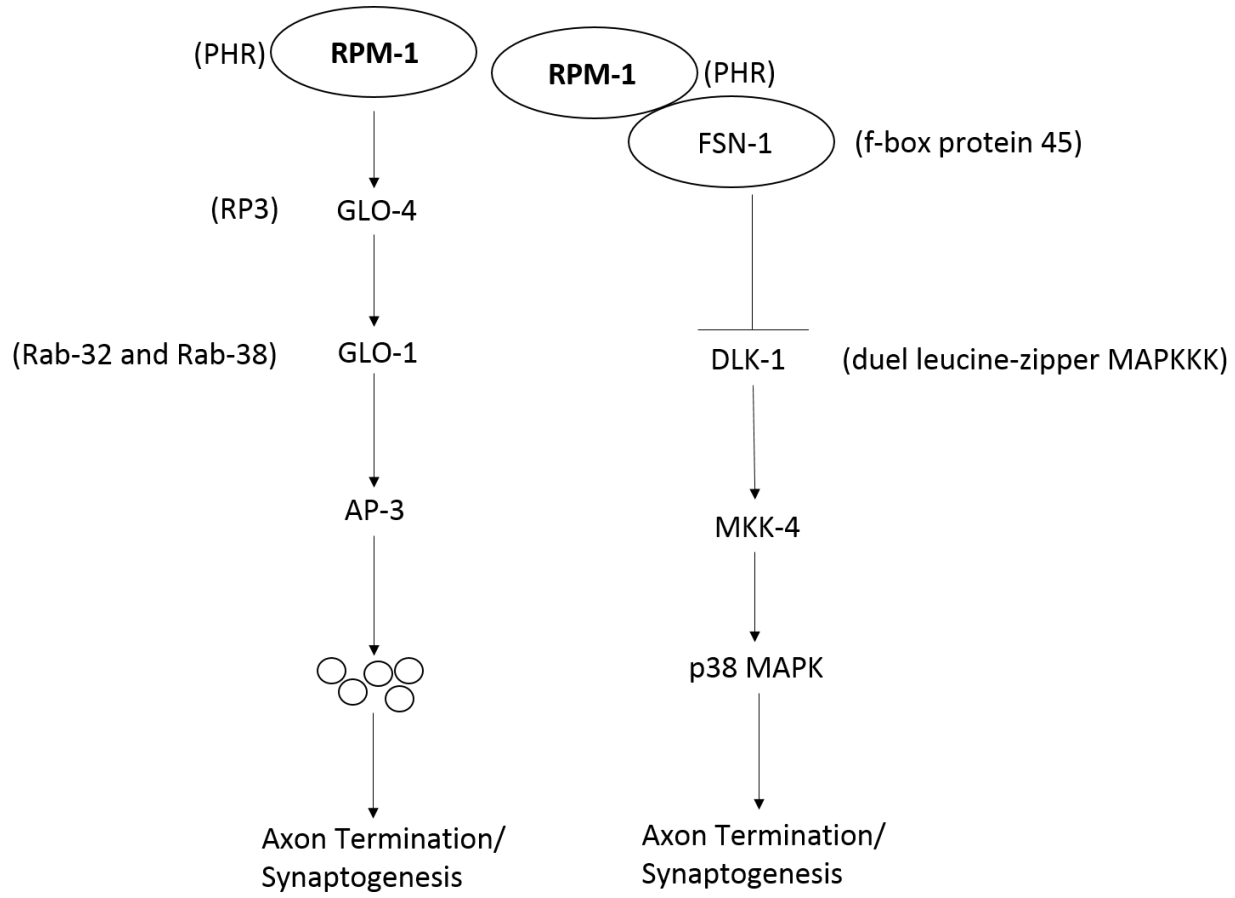


**Figure 3: Model of the RPM-1 pathway.**

RPM-1 is an E3 ubiquitin ligase containing an N-terminal RCC1-like GEF. It forms a complex with the FSN-1 f-box protein in order to promote the ubiquitination of the MAPKKK known as DLK-1. The DLK-1 pathway ultimately regulates the p38 MAPK which is involved in cell differentiation. Overall, the FSN-1 pathway leads to the regulation of endosomal and activity necessary for synaptogenesis. RPM-1 also interacts with GLO-4 to regulate the GLO-4 pathway, which runs in parallel to the FSN-1 pathway. GLO-4 is a guanine nucleotide exchange factor (GEF) which activates its downstream target GLO-1, a rab GTPase responsible for proper vesicle formation. The GLO-4 pathway ultimately regulates AP-3, which is predicted to be involved in the formation of vesicles and the biogenesis of lysosomes (Grill et al., 2007). Overall, the GLO-4 pathway leads to the regulation of vesicular transport and late endosomal/lysosomal activity

necessary for axon termination. Mammalian homologs to the *C. elegans* RPM-1 pathway are reported adjacent to the RPM-1 pathway members in parentheses.

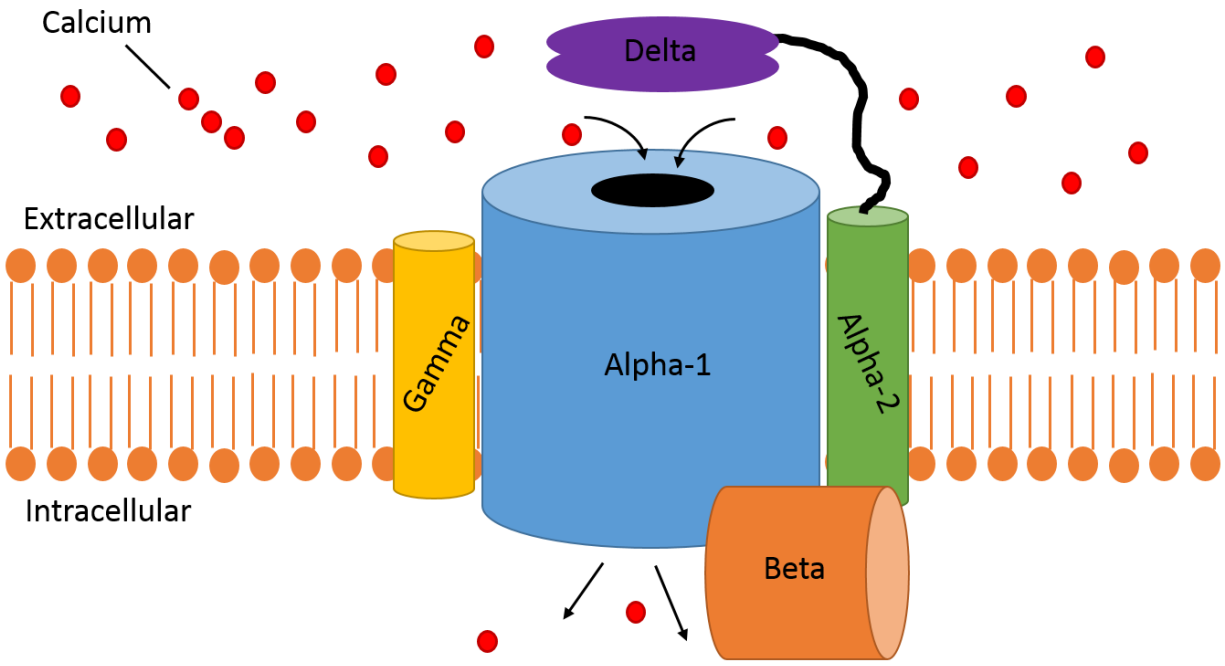


**Figure 4: Diagram of a voltage gated calcium channel with alpha-1, alpha-2-delta, beta, and gamma subunits.**

Example of a voltage gated calcium channel with all common subunits. The apha-1 subunit is the pore forming subunit that physically allows for calcium to enter the intracellular space when the channel is active. The alpha-2-delta subunit is responsible for the proper localization of the alpha-1 subunit, as well as a regulator of various biophysical properties of the channel such as the voltage sensing capabilities. The beta subunit is important for preventing VGCCs from being flagged for degradation by the proteasome. Beta subunits also function to promote proper trafficking of the VGCC to the plasma membrane. Finally, there are gamma subunits which are inconsistently present in the VGCC complex. The gamma subunit plays a role in the activation

and inactivation properties of the VGCC, as well as influencing cellular trafficking (Dolphin, 2013).

## Chapter 2 – Autophagy and Neurodevelopmental Disorders

### 2.1 Autophagy overview

Autophagy deals with the destruction of aged, damaged, misfolded, or otherwise unwanted cellular components within the cell. Autophagy maintains the homeostasis and normal functioning of a cell through the degradation and subsequent turnover of these old, aggregated, or simply non-functional proteins and cell organelles. The digested proteins and organelles are then recycled to create new intracellular components (Levine & Kroemer, 2008; Mizushima & Komatsu, 2011; Levine & Kroemer, 2019). The basic molecular mechanisms of autophagy have been well characterized. However, current research continues to identify proteins with previously unknown functions related to the different forms of autophagy. Moreover, while the existence of autophagy has been known for decades, its importance in the functioning cell and the overarching mechanisms affected by autophagy remain poorly understood. Studies of various diseases, developmental disorders, and degenerative disorders have implicated autophagy as a key part of their pathogeneses, and the results help uncover cellular processes and mechanisms where autophagy plays a role (Cushman et al., 2010; Iossifov et al., 2012; Lee, K.-M. et al., 2013). Indeed, the number of recent findings on malfunctioning autophagy leading to such detrimental disorders underscores the incredible importance of autophagy in organismal development, health, and physiology.

## 2.2 The molecular mechanisms of general autophagy

### *2.2.1 Autophagy Basics*

There are two major forms of autophagy that will be discussed: general autophagy and selective autophagy. Among the forms of general autophagy are macroautophagy and microautophagy. Macroautophagy is a bulk degradative pathway that generally occurs in the cytosol (Levine & Kroemer, 2008). Most research on autophagy focuses on this form of autophagy.

The process of general macroautophagy starts with the formation and elongation of a membrane known as the phagophore. The phagophore will continue to elongate around cytoplasmic material until the ends meet and fuse. This results in a sealed, large double membrane bound vesicle, known as the autophagosome, which at this stage has fully formed around the material to be degraded. The pathway ends with the autophagosome fusing with a lysosome, which creates the autolysosome, where the contents are finally degraded via lysosomal enzymes (Figure 5; Levine & Kroemer, 2008).

Microautophagy, although less understood, involves direct uptake of cargo into lysosomes for degradation. Microautophagy typically occurs in order to degrade soluble components. This uptake of the soluble components occurs through direct invagination of cargo into the lysosomal lumen (Figure 6; Kunz et al., 2004; Uttenweiler & Mayer, 2008).

### *2.2.2 Mediators of General Autophagy*

Autophagy is mediated by a group of highly conserved proteins, many of which are encoded by *ATG* (autophagy related) genes (Klionsky et al., 2003). In yeast, a structure known as the pre-autophagosomal structure (PAS) is where nearly all the ATG proteins co-localize (Suzuki

et al., 2001). There are likely similar structures in mammals (Itakura & Mizushima, 2010), although none have been concretely identified. The core proteins responsible for autophagosome formation are as follows, using the human versions of these proteins. The initial creation of the membranes that subsequently form the autophagosome requires activation of the ULK1/2-ATG-13 complex and the PIK3C3-BECLIN1 complex (Mizushima et al., 1998). ATG9A and VMP1 localize to the phagophore membrane and are thought to add membrane to the growing autophagosome (He et al., 2008; Yamamoto & Yue). The bulk of the elongation of the membranes is then attained by two conjugation steps, which are the formation of an ATG-12–ATG-5 conjugate and the formation of the LC3- PE conjugate (Mizushima et al., 1998; Ichimura et al., 2000; Kirisako et al., 2000). Finally, the accumulation of LC3-PE conjugate on the membrane is vital for the proper closure of the membrane (Figure 7; Nair et al., 2010).

The fusion of autophagosomes to the lysosome is also tightly regulated. One well characterized component of the autophagosome-lysosome fusion is the SNARE complex. Among the SNARE proteins are STX17 and SNAP-29, both of which are localized to the autophagosome and form a complex with VAMP8, which is localized to the lysosome (Itakura et al., 2012). The trans-SNARE protein complex forms, which drives the membrane fusion and subsequent creation of the autolysosome. Another layer of regulation at this step in the autophagy pathway has been recently discovered. *cac*, a *Drosophila* homolog of the *CACNA1A* P/Q alpha subunit gene, is required for the normal progression of autophagy in neurons (Tian et al., 2015; Wang, Y. et al., 2016). Moreover, it was found in mouse neurons that CACNA1A localizes to lysosomes and promotes fusion of lysosomes and autophagosomes (Tian et al., 2015). It is thought that this calcium channel-dependent mediation of autolysosome formation is tied to the calcium activation of the SNARE complex. Studies have indicated that calcium

activation of the SNARE complex can promote fusion of autophagosomes and lysosomes (Figure 7; Wang, Y. et al., 2016). This pathway of autophagic regulation has yet to be fully understood, but the evidence demonstrates the existence of an intricate system of regulators to control where and when autophagy takes place, showcasing that macro-autophagy may be more selective than previously thought.

## 2.3 The molecular mechanisms of selective autophagy

### *2.3.1 Selective autophagy overview*

The molecular mechanisms governing macroautophagy described above focus on the creation of autophagosomes around cytoplasmic cargo to be degraded by a subsequent fusion of the autophagosome with a lysosome. For a long time, it was thought that this relatively indiscriminate form of autophagy (often referred to as bulk autophagy) was the primary form of autophagy, with selective forms of substrate degradation being left to the likes of proteasomes. However, evidence of autophagy in maintaining cellular homeostasis begged the question of how autophagic pathways would selectively degrade unwanted substrates, while disregarding new and functional substrates, in order to maintain a homeostatic balance within the cell. The second category of autophagy that will be discussed is selective autophagy. Selective autophagy introduces a component to the autophagy pathway that allows the cell to choose specific cellular components to be degraded. In more recent years, research focused on selective autophagy has uncovered multiple pathways for autophagy to precisely target and degrade specific cellular materials.

*2.3.2 Chaperone mediated autophagy*

One of the most well-known forms of selective autophagy is chaperone-mediated autophagy (CMA). CMA can be described as the direct targeting and degradation of soluble cytoplasmic proteins that have a specific target sequence that is recognized by a chaperone protein (Cuervo, 2011; Kaushik & Cuervo, 2012). Chaperone proteins recognize specific pentapeptide motifs, known as the KFERQ-motif, on target proteins that allow for binding between the chaperone protein and the target protein (Fred Dice, 1990). These pentapeptide motifs are recognized based on their physical properties rather than their individual amino acid sequence (Fred Dice, 1990). The ability of the CMA pathway to select for old or misfolded proteins lies in the fact that, when properly folded, the KFERQ-motif is located inside the protein core, and it only becomes exposed to chaperone proteins if the protein undergoes some sort of unfolding or modification. Moreover, if the KFERQ-motif is located at the linker region between subunits, excess unassembled proteins can be eliminated via CMA (Wang et al., 2015). It has also been shown that the overall number of KFERQ-motifs on a protein does not influence the rate at which CMA selects and degrades such proteins (Massey et al., 2006; Dice, 2007). In summary, it is the specific condition of the substrate that allows for the selection of the substrate by CMA, not simply the presence or number of KFERQ-motifs located on the substrate.

It has been stated that HSC70 is the only chaperone protein known to function in substrate targeting for CMA (Chiang et al., 1989). However, co-chaperones such as BAG1, HSC40, HSC90, and HSC70-HSP90 organizing protein have been shown to interact with the substrate-chaperone complex, and could recognize the KFERQ-motif, or at least assist HSC70 in the CMA process (Agarraberes & Dice, 2001; Dice, 2007; Wang et al., 2015). The most basic breakdown of the CMA system is that the KFERQ-motif of the substrate protein is recognized by

the HSC70 which delivers the substrate protein to LAMP2A for degradation (Cuervo & Dice, 1996). LAMP2A is a transmembrane protein localized to the lysosome that delivers the substrate into the lysosome lumen for degradation (Bejarano & Cuervo, 2010). The substrate is then unfolded by HSC70-HSP90 and translocated across the lysosomal membrane with the assistance of lysosomal lumen HSC70 (Bejarano & Cuervo, 2010; Kaushik & Cuervo, 2012). The substrate is then degraded in the lysosome (Figure 8). Interestingly, LAMP2A is limiting for CMA, meaning cells can regulate the levels of LAMP2A to upregulate or downregulate CMA, thus assisting in the autophagy mediated homeostasis of the cell (Cuervo & Dice, 2000).

### *2.3.3 Selective microautophagy*

It is important to note that there exist forms of microautophagy that are considered selective autophagy. Microautophagy can lead to the selective uptake and subsequent degradation of specific proteins and organelles (Kunz et al., 2004). If the substrate in question contains the KFERQ motif, they can be selectively targeted to this autophagy pathway by HSC70 (Sahu et al., 2011). Unlike CMA, selective microautophagy is not reliant on LAMP2A. HSC70 instead binds to endosomal membrane phosphatidylserines to carry out the selective microautophagy (Sahu et al., 2011). Moreover, the starvation-stress response has been shown to increase selective endosomal microautophagy in *Drosophila melanogaster* (Mukherjee et al., 2016). While these methods of selective autophagy do select for specific substrates for degradation while excluding other substrates from the autophagosome, they do not take advantage of the macroautophagic machinery available in the cell.

*2.3.4 Selective macroautophagy*

Recent studies and interpretation of macroautophagy has suggested what was previously thought to be "bulk-autophagy" is instead far more selective for its cargo. In yeast, the selective form of macroautophagy is known as the cytoplasm-to-vacuole targeting (Cvt) pathway. Cargo receptor proteins (CRPs) are one of the facilitators of selective macroautophagy. CRPs function by binding specific cargo to core autophagic machinery (Figure 9; Zaffagnini & Martens, 2016). One core autophagic component in this selective process is LC3. LC3 and its family members are localized to the early autophagosome components, as discussed above. In the yeast Cvt, ATG-19 acts as a cargo receptor, binding to various cargo and to LC3 (ATG-8 in yeast), thereby facilitating the recruitment of core autophagic machinery to the bound cargo (Shintani et al., 2002; Sawa-Makarska et al., 2014). In mammals, some cargo receptors bind to their cargos directly, while others recognize poly-ubiquitin chains attached to the surface of cargos (Khaminets et al., 2016). An example of a cargo receptor is SQSTM1, which mediates the autophagic degradation of ubiquitinated aggregated protein and cytosolic bacteria (Figure 10; Pankiv et al., 2007; Zheng et al., 2009; Seto et al., 2013). However, SQSTM1 only functions to tag aggregated proteins at a high affinity when they are oligomerized and when there is a high amount of ubiquitination present on the cargo, thereby increasing the selectivity of the cargo receptor (Long et al., 2010; Wurzer et al., 2015; Zaffagnini & Martens, 2016). Another such cargo receptor protein is NCOA4, which works to degrade intracellular iron sequestered by ferritin (Dowdle et al., 2014; Mancias et al., 2015). Once again, only high amounts of ferritin will be successfully bound to NCOA4 (Dowdle et al., 2014; Mancias et al., 2015), insuring that NCOA4 is only acting on excess iron. While cargo receptor proteins are responsible for directly

interacting with core autophagic components and the cargo, a number of other proteins are integral for the selection process to be successful for selective macroautophagy.

Cargo receptors are aided by adaptor proteins in order to facilitate the proper selection of cargo. One adaptor protein of current research interest is WDFY-3. WDFY-3 is a membrane associated selective autophagy adaptor that functions by acting as a scaffold between a CRP, core autophagic machinery (namely SQSTM1 and ATG-5), and insoluble protein aggregates to be degraded by autophagy (Figure 11; Simonsen et al., 2004; Filimonenko et al., 2010; Lystad et al., 2014). WDFY-3 has been specifically associated with mitophagy, which is the selective degradation via autophagy of old and unwanted mitochondria in the cell (Napoli et al., 2018). To underscore the importance of WDFY-3 in cell homeostasis, *WDFY-3* has been recently identified as a genetic risk factor for multiple neurodevelopmental disorders (Iossifov et al., 2012; Orosco et al., 2014; Kadir et al., 2016; Napoli et al., 2018). One way WDFY-3 acts in neurons to regulate outgrowth is through the selective degradation of aggregated DVL3, a protein involved in the WNT signaling pathway (Kadir et al., 2016). ATG-11 is another such adaptor protein that works as a scaffold to recruit ATG proteins to cargo destined to be degraded (Delorme-Axford & Klionsky, 2015). ATG-11 is primarily known for its role in selective degradation of mitochondria during starvation through BCL2L13 (Kanki et al., 2009; Mao et al., 2013). The general trend in the literature is that macroautophagy is far more selective than previously believed, suggesting that the regulatory functions mediated by autophagy are more numerous and more important than previously thought.

## 2.4 Autophagy and cellular function

Autophagy is conserved in all eukaryotic organisms and is vital for overall cell and tissue structure and function, as well as for maintaining organismal physiology. Indeed, there are many diseases and disorders attributed to the malfunction of general autophagy. These include cardiovascular disorders, neurodegeneration, and cancer (Mizushima & Komatsu, 2011). For example, the pathology of Parkinson's disease has several links to malfunctioning autophagy (Levy et al., 2009; Lynch-Day et al., 2012). Autophagy also plays a crucial role in defending against intracellular pathogens (Seto et al., 2013; Wileman, 2013). Beyond these links to tissue health and physiology, there is evidence that autophagy has highly conserved roles in cellular adaptation to various stressors. Autophagy is important for the mitigation of damage caused by metabolic stress, and therefore has a role in counteracting aging (Madeo et al., 2010). Moreover, autophagy plays a crucial role in the starvation response in cells. When starvation occurs, autophagy in cells is induced by calcium activated ULK1/2 in order to digest unneeded cellular substrates and provide the cells with much needed nutrients (Scott et al., 2004; Shang & Wang, 2011). While these examples of autophagic processes are focused on how the cell utilizes autophagy during the life of the cell, there are many important roles for autophagy during the development and differentiation of cells.

Autophagy has conserved roles in the proper development of an organism. The developmental roles of autophagy are just beginning to be understood, but the importance and ubiquitous nature of autophagy in development cannot be understated (Mizushima & Komatsu, 2011). There is much evidence of autophagy playing key roles in the differentiation of cells from stem cells to differentiated cells (Boya et al., 2018). The roles of autophagy in a variety of cell

types could be discussed, however the primary focus of the next section will be on the role that autophagy plays in the proper development of highly specialized neurons.

### 2.5 Autophagy and neuronal function

Autophagy has many roles in proper neuronal function. The most obvious function of autophagy is maintaining homeostasis and cell survival (Yamamoto & Yue, 2014; Kulkarni et al., 2018). Autophagy in neurons is highly compartmentalized, which is important for the large length and specialized nature of neurons (Kulkarni & Maday, 2018). The vast majority of autophagosomes originate in the distal end of the axon and move towards the cell body throughout the life of the cell (Maday et al., 2012). The core machinery for autophagosome formation is similar in neurons to that in other cells, with the exception of a few neuron-specific proteins that regulate the rate of autophagosome biogenesis (Murdoch et al., 2016; Soukup et al., 2016; Okerlund et al., 2017; Vanhauwaert et al., 2017). One feature of autophagy that is unique in neurons is that autophagosomes fuse to late endosomes in order to initiate the migration of the autophagosomes toward the cell body (Maday et al., 2012; Cheng et al., 2015). Once the autophagosomes are moving along the axon, they can fuse with lysosomes to become autolysosomes, starting the degradative process of the cargo (Maday et al., 2012). Once the autophagosomes are in the cell body, they are sequestered to the somato-dendritic region, where further fusion with lysosomes can occur in order to finish degrading any cargo remaining in the autophagosomes (Maday & Holzbaur, 2016). The majority of autophagosomes originate at the distal end of neurons, but they can also be generated within the cell body (Maday & Holzbaur, 2016), and along the axon to perform mitophagy and other specific, local needs (Ashrafi et al., 2014). Maintaining homeostasis of the brain also involves coordination between neurons and

other types of cells nearby, such as glia. There is evidence that neurons will shuttle cellular cargo to neighboring glia, consisting of cellular garbage to be degraded by the glia's autophagic systems (Kulkarni et al., 2018). While this intercellular coordination between neurons and glia are outside the scope of this review, it does outline further complexities regarding autophagy regulated cellular homeostasis within the brain. Autophagy is indisputably important for the survival of neurons, however its importance in the proper development and function of neurons have also been well documented.

### 2.6 Autophagy and neurodevelopment

Autophagy has key roles in the proper development of neurons and neuronal networks (Shen & Ganetzky, 2009; Shen et al., 2015). One way this is demonstrated is by the axonal outgrowth defects caused by the loss of autophagic proteins (Ban et al., 2013; Stavoe et al., 2016; Kannan et al., 2017). Furthermore, autophagy is important in neuroregeneration (Huang et al., 2016). One way autophagy works in neurons to facilitate proper growth and maintenance is to stabilize microtubules through the degradation of a microtubule destabilizing protein SCG10, which helps promote axon growth and regeneration (He et al., 2016).

Autophagy is also vital for proper neuronal signaling and synaptic function. Autophagosomes can act as signaling conduits from the axon tip to the cell body in order to promote specific neuronal functions, as well as prevent neurodegeneration (Kononenko et al., 2017). This signaling is facilitated by transport of BDNF-activated TrkB receptors (Kononenko et al., 2017). One of the ways synaptic function is reliant on autophagy is through the turnover of synaptic cargoes, including synaptic scaffold proteins and synaptic vesicles (Wang et al., 2017; Liang & Sigrist, 2018). These synaptic proteins include WASP and NSF (Uytterhoeven et al.,

2015). There are additional synaptic proteins that act in the opposite manner, utilizing autophagy to regulate synaptic function. A protein known as bassoon has a role in controlling the activity levels of presynaptic autophagy, adding a new layer to the molecular mechanisms for synaptic regulation by autophagy (Okerlund et al., 2017). ATG-9, endophilin-A, and SYNJ1-1 also have roles in the regulation of synaptic function through autophagy (Soukup et al., 2016; Stavoe et al., 2016; Vanhauwaert et al., 2017).

There are additional proteins that act through autophagy in order to facilitate other forms of proper neuronal development and function. As mentioned previously, voltage gated calcium channels (VGCCs) have been shown to regulate autophagosomes in promoting homeostasis in the neurons of drosophila (Tian et al., 2015; Wang, Y. et al., 2016). These same studies placed the localization of the VGCC proteins on the membrane of lysosomes, implicating VGCCs as a mediator of autophagosome-lysosome fusion (Tian et al., 2015; Wang, Y. et al., 2016). The downstream targets of autophagy are still being elucidated, and the pathways that are uncovered will help us understand the complex mechanisms regulating the development and function of neurons and neuronal networks. Full understanding of these autophagic mechanisms will also help us find the elusive causes of neurodevelopmental disorders.

### 2.7 Autophagy and neurodevelopmental disorders

Defects in general autophagic machinery have been associated with various neurodevelopmental disorders (Lee, 2012; Lee, K.-M. et al., 2013). Moreover, impaired lysosomal function, which indirectly causes autophagic malfunction, have also been implicated in a variety of neurological disorders (Kang et al., 2010; Nixon, 2013; Ebrahimi-Fakhari et al., 2016). The primary role of autophagy is to clear away misfolded or unneeded proteins or

organelles. Accumulation of misfolded proteins is a hallmark of various neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS), Parkinson's, Huntington's and Alzheimer's disease (Cushman et al., 2010). The accumulation of misfolded and malfunctioning proteins plays one and perhaps more roles in autophagy during normal and abnormal neural development. Autophagy's role in polygenic neurodevelopmental disorders such as ASD and schizophrenia have been investigated (Sragovich et al., 2017; Napoli et al., 2018). Indeed, the mechanisms and downstream targets are unclear in any of the few identified autophagy related links to ASD. However, these studies focused on general macroautophagy and its role in neurodevelopmental disorders, and we have much to learn about the roles of selective autophagy in the development of neurodevelopmental disorders.

### 2.8 Selective Autophagy in neurodevelopmental disorders

Studies have identified selective autophagy genes as potential candidates for ASD (Iossifov et al., 2012). However, biological evidence for a role for selective autophagy in ASD has not been established. One selective autophagy related gene that has been associated numerous times to neurodevelopmental disorders is the cargo adaptor encoding *WDFY-3* discussed earlier (Iossifov et al., 2012; Orosco et al., 2014; Dragich et al., 2016; Kadir et al., 2016; Napoli et al., 2018). Selective autophagy has been implicated in having an axon guidance role through the degradation of ubiquitin proteins (Dragich et al., 2016). Widespread axonal wiring defects were observed in mouse brains, where the mice were carrying different alleles of *WDFY-3* mutants (Dragich et al., 2016). The homozygous null *WDFY-3* mutants caused major forebrain commissure defects, further demonstrating the importance of WDFY-3 in the proper development of the brain (Dragich et al., 2016). Additional screens of mice that had brains with

severe neuronal defects found a hypomorphic *WDFY-3* allele as a contributor (Orosco et al., 2014). Some of the possible mechanisms for WDFY-3 and selective autophagy in neurons is through the degradation of outgrowth signaling molecules like DVL3 (Kadir et al., 2016) or via mitophagy (Napoli et al., 2018). Defects in mitochondrial quality control in neurons affects the function of axon guidance proteins semaphorin, ROBO, L1CAM and EPH-EPHRIN signaling (Napoli et al., 2018). Overall, research supports a key role for WDFY-3 in neurodevelopment, possibly through mitochondrial homeostasis. These are the overall trends regarding the link between selective autophagy and neurodevelopmental disorders. However, a direct link between the key regulators of axon outgrowth machinery and selective autophagy have not been established. There is a great deal more we need to understand about the roles of selective autophagy and the various neurodevelopmental disorders to which they have been genetically associated.

**Chapter 2 Figures:**

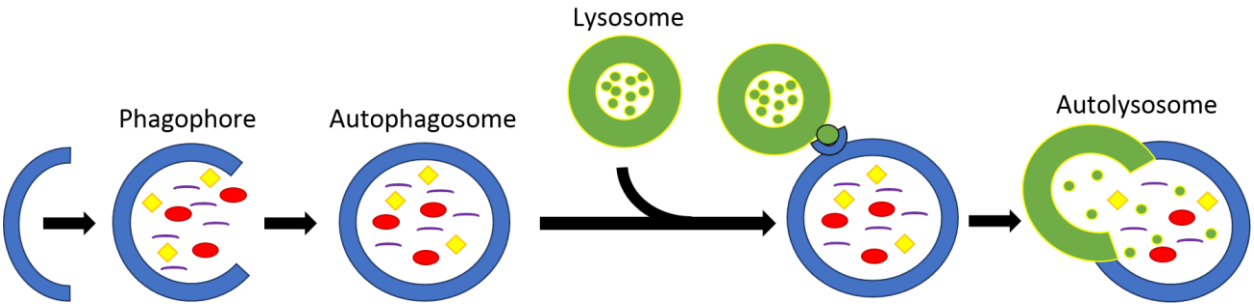


**Figure 5: General autophagy diagram.**

The process of general autophagy starts with the formation and elongation of a membrane known as the phagophore. The phagophore will continue to elongate around cytoplasmic material until the ends meet and fuse. This results in a sealed, large double membrane bound vesicle, known as the autophagosome, which at this stage has fully formed around the material to be degraded. The pathway ends with the autophagosome fusing with a lysosome, which creates the autolysosome, where the contents are finally degraded via lysosomal enzymes.

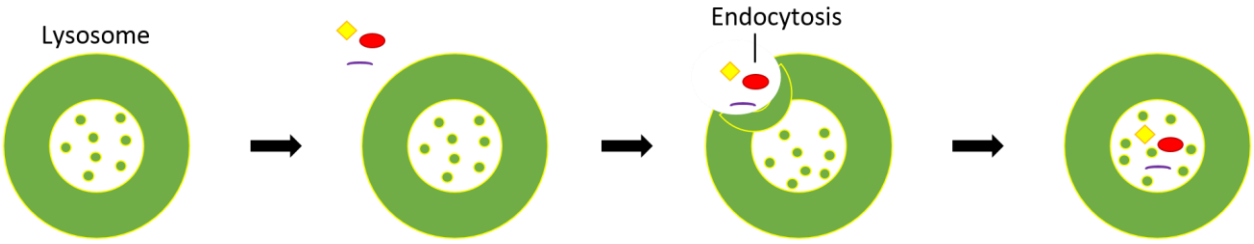


**Figure 6: Microautophagy diagram.**

Microautophagy involves direct uptake of cardo directly into lysosomes for degradation. This uptake of the soluble components occurs through endocytosis into the lysosomal lumen.

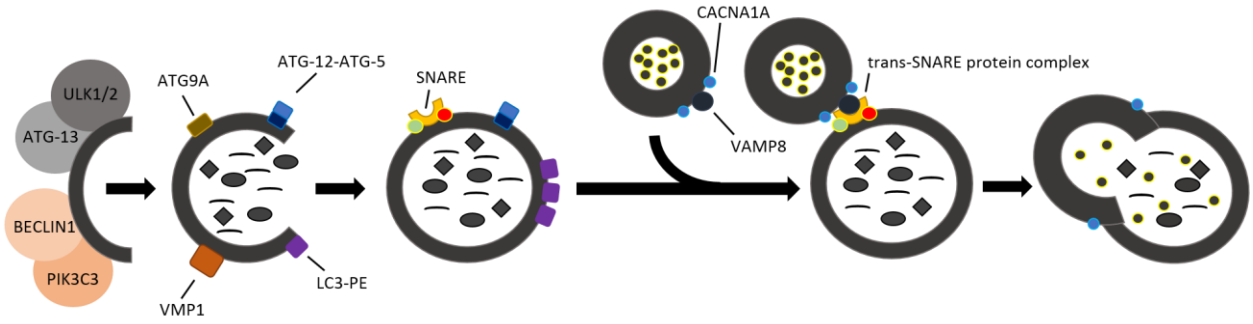


**Figure 7: Core autophagic machinery diagram.**

The initial creation of the membranes that subsequently form the autophagosome requires activation of the ULK1/2-ATG-13 complex and the PIK3C3-BECLIN1 complex (Mizushima et al., 1998). ATG9A and VMP1 localize to the phagophore membrane and are thought to add membrane to the growing autophagosome. The bulk of the elongation of the membranes is then attained by two conjugation steps, which are the formation of an ATG-12–ATG-5 conjugate and the formation of the LC3- PE conjugate. Finally, the accumulation of LC3-PE conjugate on the membrane is vital for the proper closure of the membrane. SNARE proteins are responsible for the fusion of the autophagosome with the lysosome. SNARE proteins localized to the autophagosome form a complex with vesicle-associated membrane protein 8 VAMP8, which is localized to the lysosome. CACNA1A localizes to lysosomes and promotes fusion of lysosomes and autophagosomes, likely through calcium activation of the SNARE complex.

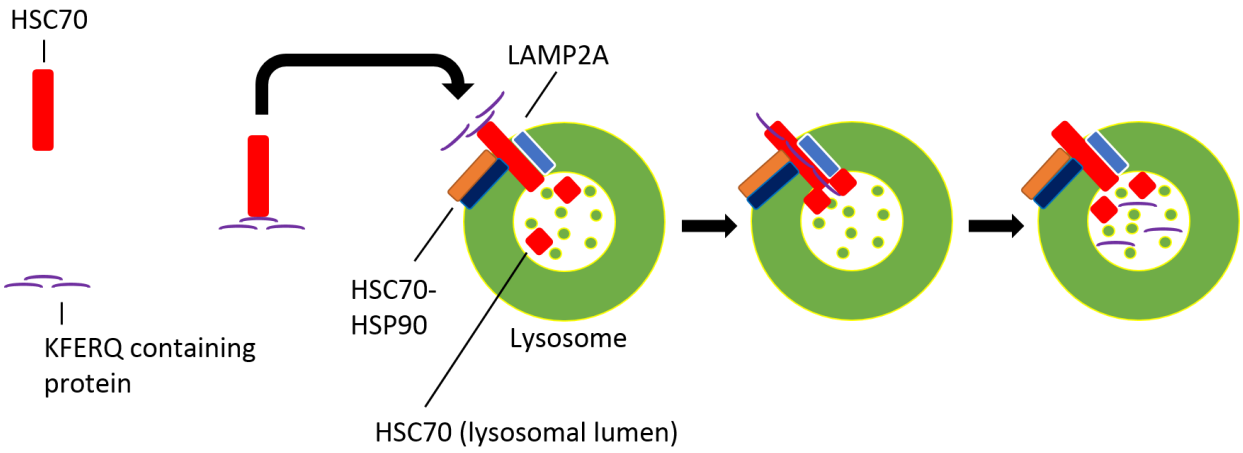


**Figure 8: Chaperone mediated autophagy diagram.**

The KFERQ-motif of the substrate protein is recognized by the HSC70 which delivers the substrate protein to the LAMP2A for degradation. The LAMP2A is a transmembrane protein localized to the lysosome that delivers the substrate into the lysosome for degradation. The substrate is then unfolded by HSC70-HSP90 and translocated across the lysosomal membrane with the assistance of lysosomal lumen HSC70. The protein is then degraded in the lysosome.

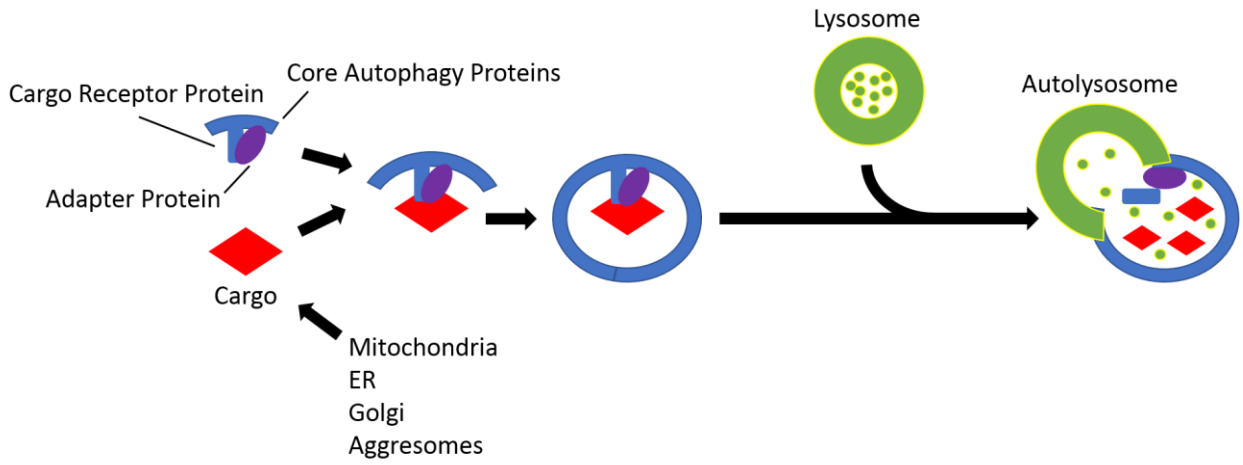


**Figure 9: Selective autophagy diagram.**

Cargo receptor proteins (CRPs) are one of the facilitators of the selective macroautophagy. These CRPs can be further assisted by adaptor proteins acting as a scaffold between the cargo, the CRP, and the core autophagic machinery. The remainder of the autophagy pathway proceeds the same way as general autophagy. The autophagosome forms around the cargo, then fuses to the lysosome to create the autolysosome and degrade the cargo.

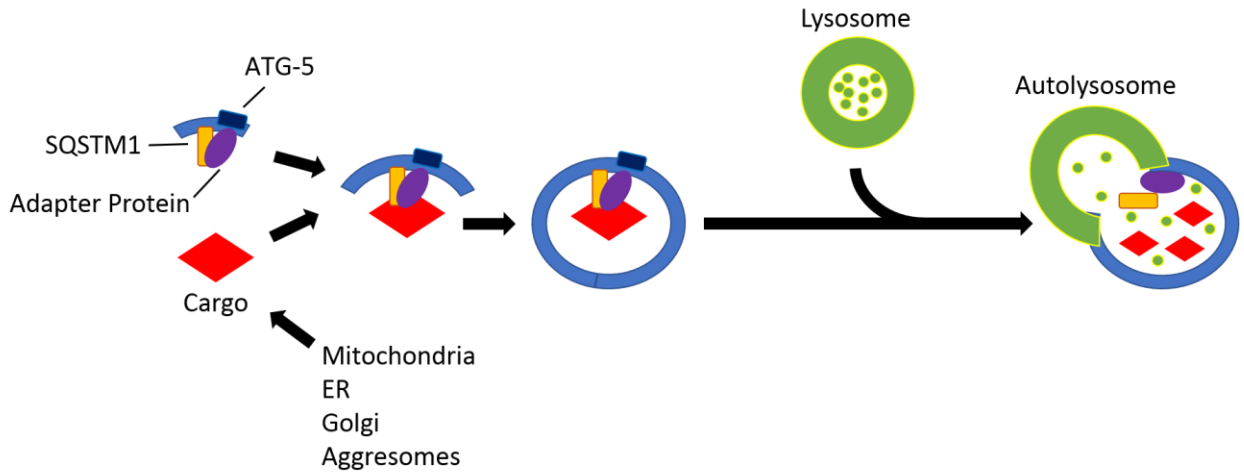


**Figure 10: Selective autophagy with cargo binding protein diagram.**

An example of a cargo receptor is SQSTM1, which mediates the autophagic degradation of ubiquitinated aggregated protein.

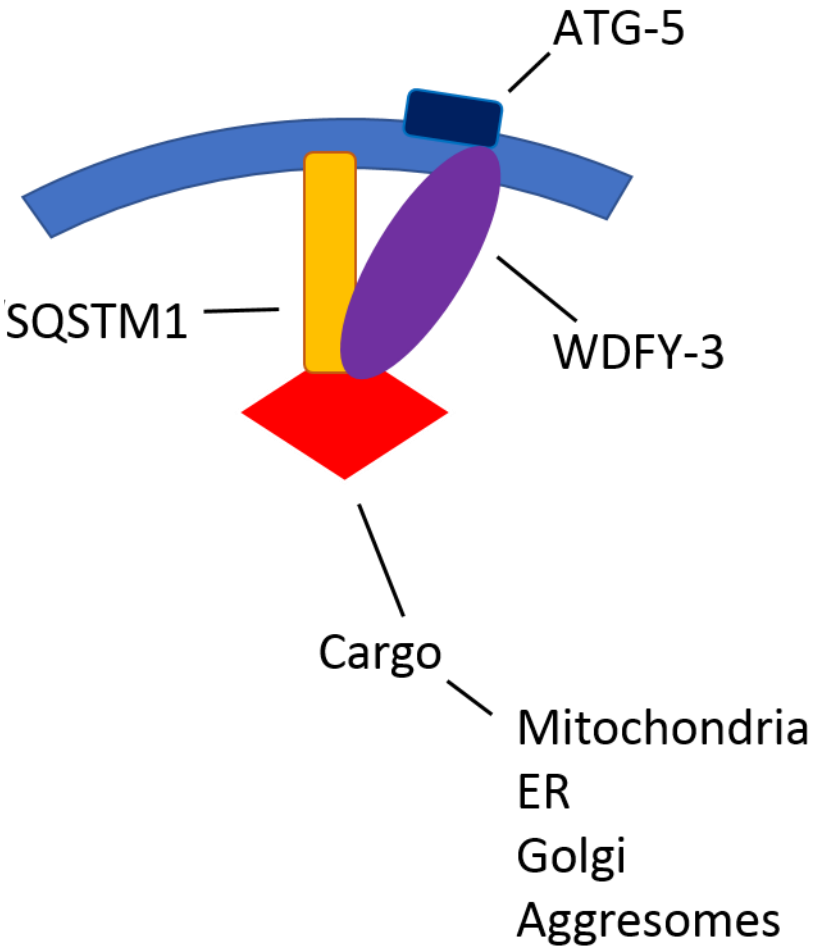


**Figure 11: WDFY-3 in selective autophagy diagram.**

WDFY-3 is an adaptor protein that acts as a scaffold between the cargo, the CRP, and the core autophagic machinery. As one specific example, WDFY-3 has been shown to act as a scaffolding for SQSTM-1, helping to maintain a connection between the cargo and the CRP protein SQSTM-1. Both WDFY-3 and SQSTM-1 are bound to ATG-5, a member of the core autophagic machinery. Once a cargo is successfully bound, the regular autophagy pathway is induced, and the cargo is eventually degraded.

## 2.9 Thesis statement

What are the mechanisms that function downstream of autism-causing gene variants to disrupt neuronal development? This fundamental question is the basis for this dissertation work. By answering this question, we can contribute to a biological explanation for the development of autism spectrum disorder (ASD). Additionally, we would be likely to uncover important pathways involved in critical steps of neurodevelopment.

Autism is a disorder that affects neuronal development, leading to alterations in cognition and behavior. Imaging studies have revealed alterations in axonal connectivity as a key feature of autism. However, the underlying perturbations in cell biology that drive these alterations remain largely unknown. To address this issue, we have taken advantage of the Timothy syndrome mutation, a variant in a voltage gated calcium channel that has the unusual property of causing ASD with high penetrance. We identify a role for wild-type voltage gated calcium channels in regulating axon termination in *C. elegans*. The *C. elegans* PLM axons are an ideal candidate for studying axon termination because they terminate outgrowth in a consistent and easily identifiable manner. The *C. elegans* PLM neurons start development in the posterior end of the worm and extend their axons anteriorly until reaching the midpoint of the worm, just posterior to the ALM cell body where the axons terminate their growth. *C. elegans* is also useful for genetic experimentation due to its rapid life cycle, compact genome, and two sexes: self-fertilizing hermaphrodite and male. For these reasons, *C. elegans* is an excellent model to study the complex basis of ASD.

We found that the Timothy syndrome mutation disrupts axon termination and alters behavior. We report that the effects of the Timothy syndrome mutation on both axon development and behavior are mediated by selective autophagy, the process through which

cellular components are selected for degradation. These results reveal a mechanism through which variants in voltage gated calcium channels can cause the disruptions in axonal connectivity that underlie autism.

The monogenic model we established is an important step towards understanding the mechanisms that are disrupted in the development of ASD. Continued study of autism-causing variants will lead to better understanding of ASD genetics. Our findings can be applied to ASD diagnostic procedures in order to improve treatment outlook. Additionally, molecular mechanistic discoveries derived from our findings can be used to develop new treatments that can be applied to ASD and other neurodevelopmental disorders.

## Chapter 3 – An autism-causing variant misregulates selective autophagy to alter axon targeting and behavior.

This chapter is a modified version of the paper published in the Journal *Plos Genetics* (Buddell et al., 2019).

### 3.1 Abstract

Common and rare variants of the *CACNA1C* gene have been associated with autism and other neurodevelopmental disorders including schizophrenia, bipolar disorder and ADHD. However, little is known about how *CACNA1C* variants affect cellular processes to alter neurodevelopment. The Timothy syndrome mutation is a rare *de novo* gain-of-function variant in *CACNA1C* that causes autism with high penetrance, providing a powerful avenue into investigating the role of *CACNA1C* variants in neurodevelopmental disorders. Here, we show that an *egl-19(gof)* mutation that is equivalent to the Timothy syndrome mutation can alter axon targeting and affect behavior in *C. elegans*. We find that wildtype *egl-19* negatively regulates axon termination. The *egl-19(gof)* mutation represses axon termination to cause axon targeting defects that lead to the misplacement of electrical synapses and alterations in habituation to light touch. Moreover, genetic interactions indicate that the *egl-19(gof)* mutation functions with genes that promote selective autophagy to cause defects in axon termination and behavior. These results reveal a novel genetic mechanism whereby a *de novo* mutation in *CACNA1C* can drive alterations in circuit formation and behavior.

## 3.2 Introduction

Variants in the *CACNA1C* voltage gated calcium channel (VGCC) gene are common risk factors for autism and other neurodevelopmental disorders including schizophrenia, bipolar disorder and attention deficit hyperactivity disorder (ADHD). For example, genome wide association studies (GWAS) have associated common variants in *CACNA1C* to autism (Lu et al., 2012; Li et al., 2015). Moreover, statistical analysis of whole genome sequencing data indicates that rare variants in *CACNA1C* are also associated with autism (Splawski et al., 2004; Schaaf et al., 2011; Jiang et al., 2013; Brett et al., 2014; Iossifov et al., 2014; D'Gama et al., 2015; Alvarez-Mora et al., 2016; RK et al., 2017). Whereas the evidence is strongest for *CACNA1C*, variants in other VGCC subunit genes are also associated with autism (Strom et al., 2010; Lu et al., 2012; Breitenkamp et al., 2014; RK et al., 2017). Despite these insights from statistical analysis, little is currently known about how variants in VGCC genes affect cellular processes to disrupt neurodevelopment.

A major impediment to understanding how autism-associated variants affect cellular processes is that most variants have a small effect size. Because each variant only has a small effect, it is thought that multiple variants engage in genetic interactions that give rise to the neurodevelopmental defects underlying autism (Robinson et al., 2016; Turner et al., 2017). Therefore, a key goal in understanding the biological basis for autism is to understand genetic interactions between autism-associated variants. However, currently little is known about how autism-associated variants interact with each other. Moreover, in most cases the cellular mechanisms perturbed by each variant are also unknown.

Morphological abnormalities in axon development are associated with autism and other neurodevelopmental disorders (Travers et al., 2012; Wolff et al., 2012; Koldewyn et al., 2014;

Lazar et al., 2014; Farias et al., 2017). Most mechanistic studies of autism-associated genes have focused on dendrite and synapse structure. However, imaging studies have suggested that alterations in axon targeting are a key feature of autism. For example, diffusion tensor imaging has revealed alterations in the Inferior Longitudinal Fasciculus in autistic individuals relative to healthy controls (Travers et al., 2012; Wolff et al., 2012; Koldewyn et al., 2014; Lazar et al., 2014; Farias et al., 2017). Moreover, functional MRI has revealed alterations in long range connectivity that can predict autism in individuals before the onset of symptoms (Just et al., 2004; Just et al., 2007; Schipul et al., 2011). These observations suggest that alterations in axon targeting are likely to underlie autism. However, little is currently known about how autism-associated variants can alter axon targeting.

In this work, we use the Timothy syndrome mutation as a platform to discover how autism-associated variants interact with each other to alter cellular processes and disrupt axon development. The Timothy syndrome mutation is a rare *de novo* mutation in *CACNA1C* that causes autism with high penetrance (Splawski et al., 2004; Bader et al., 2011). Although this is a rare mutation with a large effect, common variants with a small effect in *CACNA1C* are also associated with autism (Lu et al., 2012; Li et al., 2015). As the mechanisms by which CACNA1C affects axon development are unknown, studies of the Timothy Syndrome variant may uncover genetic mechanisms that also apply to the more common *CACNA1C* risk variants.

Here, we identify an *egl-19(gof)* mutation in *C. elegans* that is equivalent to the Timothy syndrome mutation. Using the mechanosensory nervous system (Figure 12), we find that this *egl-19(gof)* mutation causes overextension of the PLM axon, leading to the misplacement of electrical synapses. Moreover, we find that this *egl-19(gof)* mutation interacts genetically with a

homolog of the autism-associated *WDFY3* selective autophagy gene to disrupt axon development and alter behavior.

## 3.3 Results

### *3.3.1 A VGCC mutation that causes autism in humans and disrupts axon termination in C. elegans*

The *egl-19(n2368)* mutation is equivalent to the autism-causing Timothy syndrome mutation in *CACNA1C*. Both common and rare variants in the human *CACNA1C* gene have been associated with autism and other neurodevelopmental disorders (Strom et al., 2010; Schaaf et al., 2011; Lu et al., 2012; Li et al., 2015; Yuen et al., 2015; Al-Mubarak et al., 2017; RK et al., 2017; Stessman et al., 2017). The Timothy syndrome mutations, G402R, G402S and G406R in human CACNA1C, are of particular interest because each causes autism with high penetrance (Splawski et al., 2004; Napolitano & Antzelevitch, 2011; Diep & Seaver, 2015). To establish a model for investigating how the Timothy syndrome mutation affects neurodevelopment, we searched for an equivalent mutation in *egl-19*, the *C. elegans* orthologue of *CACNA1C.* We found that the *egl-19(n2368)* mutation (hereafter referred to as *egl-19(gof))* encodes a G365R variant of EGL-19 that is equivalent to the G402R Timothy syndrome variant in human *CACNA1C* (Figure 13A). In both human CACNA1C and *C. elegans* EGL-19, this Timothy syndrome mutation is a gain-of-function variant: it disrupts slow inactivation of the voltage gated channel, thereby increasing calcium permeation (Splawski et al., 2004). Specifically in the *egl-19(n2368)* mutation, the EGL-19 VGCC opens at a lower threshold compared to wild-type, and their activation times were about twice as long as wild-type worms (Laine et al., 2014).

To determine how the Timothy syndrome mutation affects neurodevelopment, we observed the PLM touch receptor neuron in *egl-19(gof)* mutants. The PLM cell body is located in the tail, with an axon extending along the lateral body wall (Figure 12). For this experiment we used two independently isolated *egl-19(gof)* alleles, *egl-19(n2368)* and *egl-19(tr134)*. Both of these alleles produce a G365R mutation in EGL-19, which is equivalent to the G402R Timothy syndrome mutation in humans (Figure 13A; Lee et al., 1997; Kwok et al., 2008). In wild-type animals, nearly all of the PLM axons terminate posterior to the ALM cell body (Figure 12A; Figure 13B,D). However, in the *egl-19(n2368)* and *egl-19(tr134)* G365R gain-of-function mutants, around 52% of the PLM axons terminate anterior to the ALM cell body (Figure 12B; Figure 13C,D). By contrast, *egl-19(lof)* mutants exhibit normal PLM axon termination (Figure 13D), with nearly all PLM axons terminating posterior to the ALM cell body. We also tested the *egl-19(ad695)* gain-of function mutation, which has previously been characterized as a weaker gain-of-function relative to *egl-19(n2368)* (Lee et al., 1997). Consistent with these prior observations, we found that the *egl-19(ad695)* gain-of-function mutation causes axon termination defects with a lower penetrance relative to *egl-19(n2368)* and *egl-19(tr134)* (Figure 13D).

In humans, Timothy syndrome is also caused by a G406R mutation in *CACNA1C* (Splawski et al., 2004). Therefore, we tested *egl-19(syb1243)*, a mutation that produces a G369R mutation in EGL-19, which is equivalent to G406R in human CACNA1C (Figure 13A). We found that the *egl-19(syb1243)* mutation is homozygous lethal, with no maternal rescue. However, we observed the PLM axon in *egl-19(syb1243)* heterozygotes and found that around 18% of the PLM axons had axon termination defects (Figure 13D). Together, these observations

indicate that mutations equivalent to the Timothy syndrome mutations can cause defects in axon targeting.

To determine if the Timothy syndrome mutation functions cell autonomously to disrupt axon termination, we used *Pmec-7::egl-19(gof)* transgenes to express the EGL-19 G365R mutant protein specifically within touch receptor neurons (PLM, ALM, AVM and PVM) (Figure 13E). We tested one transgene that was created by injecting *Pmec-7::egl-19(gof)* at a concentration of 5 ng/ul and another at 25 ng/ul. We found that both of these transgenes caused axon termination defects. These results suggest that EGL-19(GOF) functions cell autonomously in the PLM neuron to disrupt axon termination.

Finally, we repeated these experiments in the ALM in order to determine if the disrupted axon termination machinery was observed in additional mechanosensory neurons in *egl-19(gof)* mutants (Figure 14). In wild type animals, the ALM axon terminates its growth past the nerve ring and posterior to the mouth of the worm (Figure 14A,C). In *egl-19(gof)* mutants, we observed overextended ALM axons, which were scored when the axon terminated at the mouth of the worm (Figure 14 B,C). This result suggests that the axon termination mechanism regulated by EGL-19 functions in multiple axons.

It is important to note that an additional defect was observed in the ALM cell body of *egl-19(gof)* mutants. In wild type animals, the ALM cell body extends a singular axon in the anterior direction of the worm, and no large neurites are visible growing in a different direction (Figure 15A). However, in *egl-19(gof)* mutants, there is visible neurite growth extending from the posterior side of the ALM cell body (Figure 15B). We observed a 24% penetrance of posterior process defects in *egl-19(gof)* mutants (Figure 15C). These types of defects are often seen in animals with mutated genes important for regulating axon development (Kirszenblat et

al., 2013), or genes that govern cell orientation and polarity (Hilliard & Bargmann, 2006). This result further suggests that axon development mechanisms are perturbed in *egl-19(gof)* Timothy syndrome mutants.

### *3.3.2 Wild-type EGL-19 and other VGCC subunits negatively regulate axon termination*

To determine if and how wild-type EGL-19 regulates axon termination, we conducted genetic analysis with mutations in the genes that encode the RPM-1 (PAM, Highwire) signaling pathway. RPM-1 is an E3 ubiquitin ligase that promotes axon termination by ubiquitinating DLK-1 (MAP3K12), thereby marking it for proteasomal degradation (Schaefer et al., 2000; Zhen et al., 2000; Nakata et al., 2005). This function of RPM-1 is mediated through an interaction between RPM-1 and a SCF (Skp/Cullin/F-box) complex that includes the FSN-1 (FBXO45) F-box protein. In addition, RPM-1 also promotes axon termination by functioning with GLO-4 (RCBTB1), a guanine nucleotide exchange factor (GEF) for the GLO-1 (RAB38) Rab GTPase (Grill et al., 2007). GLO-4 functions with RPM-1, but in parallel to FSN-1, to promote axon termination (Figure 3).

Since the *egl-19(gof)* mutation disrupts axon termination, it is possible that wild-type EGL-19 also negatively regulates axon termination. Alternatively, it is possible that *egl-19(gof)* acts in a neomorphic role not normally controlled by wild-type EGL-19. To determine the function of wild-type EGL-19, we conducted genetic analysis with a loss-of-function allele of *egl-19*. For this experiment, we used a null allele of *fsn-1* that causes axon termination defects (Liao et al., 2004; Grill et al., 2007). In *fsn-1(null)* mutants, 53% of the PLM axons are overextended (Figure 16A). However, in *fsn-1(null)*; *egl-19(lof)* double mutants, the phenotype

is suppressed to only 33% of PLM axons overextended. These observations suggest that wild-type EGL-19 acts to negatively regulate axon termination.

EGL-19 is the pore forming subunit of the L-type VGCC. To further explore the role of voltage gated calcium channels, we tested loss-of-function mutations in other genes that encode VGCC components. The *unc-2* gene encodes the pore-forming subunit of the P/Q-type VGCC (Schafer & Kenyon, 1995). The human homolog of *unc-2*, CACNA1A, has also been associated with autism (Damaj et al., 2015; Li et al., 2015; Lelieveld et al., 2016). We found that a null allele of *unc-2* could also suppress axon termination defects caused by a *fsn-1(null)* mutation (Figure 16A). We also tested a null mutation in *unc-36*, which encodes the alpha2-delta3 subunit that works with both the EGL-19 and UNC-2 pore-forming subunits to modulate voltage dependence, activation kinetics, and calcium conductance (Frokjaer-Jensen et al., 2006; Saheki & Bargmann, 2009; Laine et al., 2011). The human homolog of UNC-36, CACNA2D3 has also been associated with autism (De Rubeis et al., 2014; Iossifov et al., 2014; RK et al., 2017). We found that a null allele of *unc-36* could also suppress axon termination defects caused by a *fsn-1(null)* mutation (Figure162A). Together, these observations suggest that both L-type and P/Q-type voltage gated calcium channels can negatively regulate axon termination. We note that the different levels of suppression observed between *unc-36(null)*, *unc-2(null)* and *egl-19(lof)* mutations are likely the result of differing roles played by each VGCC subunit or could reflect different strengths of the alleles that we used.

To determine if VGCCs function cell-autonomously to regulate axon termination, we constructed a *Pmec-7::unc-36::rfp* transgene, which uses the *mec-7* promoter to drive expression of UNC-36::RFP within the touch receptor neurons. If UNC-36 functions cell autonomously, we expect that the *Pmec-7::unc-36::rfp* transgene will reverse the suppression of axon termination

defects observed in *fsn-1(null)*;*unc-36(null)* double mutants relative to *fsn-1(null)* single mutants. Indeed, we found that *fsn-1(null)*;*unc-36(null)* double mutants with the *Pmec-7::unc-36::rfp* transgene had a higher penetrance of axon termination defects relative to *fsn-1(null)*;*unc-36(null)* double mutants without the *Pmec-7::unc-36::rfp* transgene (Figure 16A). These observations suggest that UNC-36 functions cell-autonomously to negatively regulate axon termination.

### *3.3.3 VGCC regulation of axon termination is specific to the FSN-1 pathway*

FSN-1 functions in parallel to GLO-4 to promote PLM axon termination (Hermann et al., 2005; Grill et al., 2007). Although both pathways promote axon termination, they do so through distinct molecular mechanisms. Whereas FSN-1 is an F-box protein that regulates a MAP Kinase cascade (Liao et al., 2004), GLO-4 is a guanine nucleotide exchange factor for the GLO-1 Rab GTPase (Hermann et al., 2005). To determine if VGCCs regulate the GLO-4 pathway, we constructed *glo-4(null)*;*unc-2(null)* and *glo-4(null)*;*unc-36(null)* double mutants. We found that neither loss of *unc-2* nor loss of *unc-36* function suppresses the axon termination defects caused by loss of *glo-4* function, suggesting that VGCCs do not regulate the GLO-4 pathway (Figure 16B). To further explore the role of VGCCs within the context of the parallel FSN-1 and GLO-4 pathways, we constructed an *fsn-1(null)*;*glo-4(null)*;*unc-36(null)* triple mutant and a *fsn-1(null)*;*glo-4(null)* double mutant (Figure 16C). Consistent with prior studies, we found that *fsn-1(null)*;*glo-4(null)* double mutants had termination defects in 86% of PLM axons (Grill et al., 2007). In the triple mutant, loss of *unc-36* function reduced this penetrance to 46%, which is similar to the *glo-4* single mutants. Together, these observations suggest that VGCCs can negatively regulate axon termination in response to the FSN-1 pathway, but not GLO-4 pathway.

The RPM-1 ubiquitin ligase functions with the FSN-1 F-box protein to negatively regulate downstream proteins (Liao et al., 2004; Grill et al., 2007). Loss-of-function mutations in these downstream proteins suppress the phenotype of loss-of-function mutations in FSN-1 and RPM-1. For example, FSN-1 and RPM-1 function together to negatively regulate the DLK-1 MAP Kinase (Nakata et al., 2005). Loss of DLK-1 function suppresses the phenotype that is caused by either loss of RPM-1 function or loss of FSN-1 function. Since loss of VGCC function can suppress the axon termination phenotype caused by loss of FSN-1 function, we considered the possibility that FSN-1 might function with RPM-1 to negatively regulate VGCCs. If this is true, loss of VGCC function should suppress the axon termination defect caused by loss of RPM-1 function. However, we found that axon termination defects caused by loss of RPM-1 function could not be suppressed by loss of function mutations in *unc-36, unc-2* or *egl-19* (Figure 16B). These observations suggest that VGCCs are not downstream targets of RPM-1 and FSN-1.

### *3.3.4 The egl-19(gof) mutation alters PLM axon connectivity*

To determine if the *egl-19(gof)* mutation affects connectivity of the PLM axon, we examined its chemical and electrical synapses. In wild-type animals, the PLM axon extends a synaptic branch that forms a cluster of chemical synapses on axons in the ventral nerve cord (Schaefer et al., 2000). We used a *mec-7::rfp* transgene (Bounoutas et al., 2009) to visualize the PLM synaptic branch and found that it appears normal in *egl-19(gof)* mutants (Figure 17A,D). We also used a *mec-7::gfp::rab-3* transgene (Marcette et al., 2014) to visualize synaptic vesicles and found that these also appear normal in *egl-19(gof)* mutants (Figure 17B,E). We measured the length of the synaptic vesicle clusters in wildtype and *egl-19(gof)* mutants and found no significant difference (Figure 17C,F,G). Moreover, consistent with prior findings (Grill et al.,

2007), we found that about 15% of *fsn-1* null mutants were missing the PLM branch (Figure 17H). However, this missing branch phenotype was not suppressed in *fsn-1(null)*; *egl-19(lof)* double mutants (Figure 17H). These observations suggest that the EGL-19(GOF) mutation does not affect the PLM synaptic branch or its chemical synapses. Moreover, wildtype EGL-19 does not affect the PLM branch. However, we cannot rule out the possibility that the synaptic branch and chemical synapses are affected in more subtle ways.

We next asked if the *egl-19(gof)* mutation affects electrical synapses. In wild-type PLM axons, electrical synapses are clustered in two distinct zones (Meng et al., 2016). Zone 1 electrical synapses are located close to the PLM cell body, whereas zone 2 electrical synapses are located at the PLM axon tip, which is posterior to the ALM cell body (Figure 18A). Since the *egl-19(gof)* mutation causes overextension of the PLM axon, it is possible that it could also cause misplacement of zone 2 electrical synapses to a location anterior to the ALM cell body. Alternatively, it is possible that *egl-19(gof)* causes axon overextension, but leaves the zone 2 synapses in their normal location posterior to the ALM cell body. To differentiate between these two possibilities, we used a *mec-7::unc-9::gfp* transgene (Meng et al., 2016) to visualize the UNC-9 Innexin, a marker for electrical synapses (Meng et al., 2016). We found that the *egl-19(gof)* mutation caused misplacement of the zone 2 electrical synapses to a point anterior to the ALM cell body (Figure 18B). Misplacement of the electrical synapse occurred in approximately 53% of *egl-19(gof)* PLM axons, but only in 7% of wildtype PLM axons (Figure 18C) Therefore, the PLM axon overextension caused by *egl-19(gof)* is also associated with misplacement of zone 2 electrical synapses.

The zone 2 electrical synapses are formed between the PLM mechanosensory neuron and the BDU interneuron. The BDU interneuron begins its axon extension in the nerve ring of *C.*

*elegans*, which grows in a posterior direction until it approaches the PLM. The BDU axon then maneuvers towards the PLM and continues to grow until it reaches the PLM axon tip where the connection is formed (White et al., 1986). Despite not being a true mechanosensory neuron, the BDU interneuron is vitally important for the touch response (Li et al., 2011). Therefore, we wanted to know whether or not the BDU interneuron was disturbed in the *egl-19(gof)* mutants. In order to examine the integrity of the PLM-BDU connection, we used a *Pmec-7::gfp* to observe the PLM axon (Figure 19A,C), and a *Punc-86::myr gfp* to visualize the BDU axon (Figure 19B,D). In wild-type animals, the BDU axon maneuvers towards the PLM and forms a connection at the PLM axon tip (Figure 19B). Despite the overextension of the PLM, in the *egl-19(gof)* mutants the PLM-BDU connection appears undisturbed, and the BDU maneuvers towards the PLM axon tip before making a connection in the same manner as the wild-type animal (Figure 19D). This result suggests that the zone 2 electrical synapses are mislocalized in the *egl-19(gof)* mutant worm, but the overall PLM-BDU connection remains intact. It is important to note that, although the overall structure of the BDU neuron appears normal, we cannot say for certain that the BDU neuron is unaffected by the *egl-19(gof)* mutation.

### *3.3.5 The egl-19(gof) mutation interacts with selective autophagy genes to disrupt axon termination*

As part of an ongoing effort to test autism-associated genes for roles in axon development, we identified a genetic interaction between *egl-19(gof)* and *wdfy-3*, a homolog of the autism-associated *WDFY3* selective autophagy gene. For this experiment, we used the *wdfy-3(ok912)* deletion allele, hereafter called *wdfy-3(lof)*. This allele is likely to be a null or strong loss of function because it creates a frameshift that disrupts the FYVE domain, WD repeat

domain and nearly all of the beach domain. We found that the PLM axon was normal in *wdfy-3(lof)* single mutants. In *egl-19(gof)*; *wdfy-3(lof)* double mutants, the axon termination defects observed in *egl-19(gof)* single mutants were almost completely suppressed (Figure 20A). Moreover, PLM axon termination defects caused by transgenic expression of EGL-19(GOF) in touch receptor neurons were also suppressed by *wdfy-3(lof)*. The PLM neurons are likely to express WDFY-3 because RNAseq on purified touch receptor neurons identified *wdfy-3* mRNA transcripts (Kaletsky et al., 2016). The *wdfy-3* gene is an orthologue of the human autism-associated *WDFY3* gene that encodes a protein required for cargo selection during selective autophagy (Clausen et al., 2010; Filimonenko et al., 2010). Therefore, these observations establish a genetic pathway between two autism-associated genes that regulates axon termination.

To further explore a potential interaction between selective autophagy and EGL-19(GOF), we constructed double mutants between *egl-19(gof)* and mutations in two other genes that are expected to disrupt selective autophagy: *epg-7* (RB1CC1, FIP200) and *cup-5 (Mucolipin-3)*. The *epg-7* gene encodes an additional component required for selection of cargo for autophagy (Lin et al., 2013), and the *cup-5* gene encodes a scaffold protein that promotes lysosome biogenesis (Hersh et al., 2002; Treusch et al., 2004). We found that axon termination defects caused by the *egl-19(gof)* mutation could be suppressed by either a likely null mutation in *epg-7* or a hypomorphic mutation in *cup-5* (Figure 20A). These observations suggest that the *egl-19(gof)* mutation causes axon termination through a mechanism that requires selective autophagy.

*3.3.6 WDFY-3 negatively regulates PLM axon termination*

Having found that *wdfy-3* can negatively regulate axon termination in the *egl-19(gof)* mutant, we wanted to ask if *wdfy-3* could also regulate axon termination independently of the gain-of-function *egl-19* mutation. For this experiment, we used a *fsn-1(null)* mutation to induce axon termination defects. We found that loss of *wdfy-3* function completely suppresses the axon termination defects caused by the *fsn-1(null)* mutation (Figure 20B). Like the VGCC loss-of-function mutants, we also found that the *wdfy-3(lof)* mutant does not suppress axon termination defects caused by *rpm-1(lof)*. Together, these observations suggest that WDFY-3, like VGCCs, can regulate axon termination signaling downstream of FSN-1, but is not a downstream target of FSN-1 and RPM-1.

We next wanted to narrow down the possible selective autophagic mechanism involved in axon termination. One likely candidate is the cargo receptor protein SQSTM-1, as it has been shown to function with WDFY-3 in selective autophagy (Figure 11; Clausen et al., 2010)). The homolog of mammalian SQSTM-1 in *C. elegans* is encoded by the *sqst-1* gene. In order to determine if SQST-1 mediated selective autophagy functions as a regulator of axon termination, we used a *fsn-1(null)* mutation to induce axon termination defects. We found that the *sqst-1(lof)* mutation does not suppress axon termination defects caused by the *fsn-1(null)* mutation (Figure 20C). This is in contrast to both *wdfy-3* and *VGCC* loss-of-function mutations, which result in the suppression of *fsn-1(null)* induced axon termination defects (Figure 16A; Figure 20A). These results taken together suggest that WDFY-3 mediated selective autophagy functions with EGL-19 to regulate axon termination. Although we did not see an effect from the loss-of-function of *sqst-1*, its important to note that there are many *sqst* genes in *C. elegans*. Therefore, the lack of observed effect is likely due to redundant function of other *sqst* genes.

As an additional experiment, we wanted to see if other proteins that have been associated with ASD would act as regulators of axon termination. One family of proteins that have statistical association with the development of ASD are the SHANK proteins (Gong & Wang, 2015; Wang et al.). In order to test this relationship, we utilized the *C. elegans* homolog to human SHANK2, encoded by the gene *shn-1.* These experiments used the *fsn-1(null)* mutation to induce axon termination defects. We found that two different *shn-1(lof)* alleles caused suppression of axon termination defects caused by the *fsn-1(null)* mutation (Figure 21). However, the axon termination defect suppression induced by the *shn-1(lof)* mutations was weaker than that of the *wdfy-3* and *VGCC* loss-of-function mutations (Figure 16A; Figure 20A; Figure 21). This weaker affect by the *shn-1(lof)* as compared to *wdfy-3(lof)* does imply specificity in selective autophagy as a regulator of axon termination. Additionally, due to the preliminary nature of these results, we cannot rule out pleiotropic effects as a possible cause for the *shn-1(lof)* induced changes in axon termination defects. Additional experiments using *shn-1* mutations will be required before any inferences can be made.

### *3.3.7 The egl-19(gof) mutation interacts with the wdfy-3 selective autophagy gene to regulate habituation to light touch*

The PLM neuron is a mechanosensory neuron that is responsible for sensing light touch in the posterior of *C. elegans* (Chalfie et al., 1985). When light touch is applied to the tail, the animal responds by moving forward (Rankin et al., 1990; Hobert et al., 1999; Zhang & Chalfie, 2002). However, after repeated touches, the animal habituates and becomes less likely to respond to each touch. Since we observed that the *egl-19(gof)* mutation alters the morphology of the PLM neuron, we wanted to determine if this mutation also alters the response to light touch.

We conducted a touch assay to determine if the *egl-19(gof)* mutation affects the response to light touch. Each animal was subjected to ten eyelash touches alternating between the head and tail . For the initial touch, there was no significant difference in the response rate between *egl-19(gof)* mutants and wild-type animals. However, for each subsequent touch, the *egl-19(gof)* mutants had a significantly lower response rate relative to wild-type animals (Figure 22). These observations suggest that the *egl-19(gof)* mutation enhances habituation to light touch.

Because we found that the *egl-19(gof)* mutation interacts with *wdfy-3* to disrupt axon termination, we next asked if this genetic interaction can also affect habituation to light touch. If the *egl-19(gof)* mutation functions with *wdfy-3* to alter habituation, we expect that loss of *wdfy-3* function will reduce the effect of the *egl-19(gof)* mutation on habituation. Indeed, we found that in *egl-19(gof);wdfy-3(lof)* mutants, the habituation to light touch was not significantly different relative to wild-type animals (Figure 22). Together, these observations suggest that the *egl-19(gof)* mutation acts through *wdfy-3* to affect both axon termination and habituation to light touch.

### *3.3.8 WDFY-3 as it fits in the RPM-1 pathway*

A question we wanted to answer regarding WDFY-3 and its role in axon termination was where the protein existed in the context of the RPM-1 pathway. We previously determined that *wdfy-3(lof)* mutations suppress *fsn-1(null)* induced axon termination defects, and that *wdfy-3(lof)* mutations do not suppress *rpm-1(lof)* induced axon termination defects (Figure 20B). However, we did not yet examine how WDFY-3 relates to GLO-4. In order to answer this question, we examined the effect *wdfy-3(lof)* mutations have on axon termination defects induced by *glo-4(null)* mutations (Figure 23). In contrast to the *VGCC(lof)* mutants (Figure 16B), *wdfy-3(lof)* did

appear to suppress the axon termination defects caused by the *glo-4(null)* mutation (Figure 23). However, there were additional defects observed in the *glo-4(null);wdfy-3(lof)* double mutants, including some shortening of the PLM axon, instances of more severe than *glo-4(null)* single mutants, and the occasional missing PLM axon. These defects are not observable in any other mutation discussed in this research and are therefore indicative of pleiotropic affects. Additional experiments will be necessary to rule out this affect as the reason for a lower penetrance in the *glo-4(null);wdfy-3(lof)* double mutants.

### *3.3.9 Determining how UNC-36 acts to severely suppress the* fsn-1(null) *induced axon termination defects*

We further tested the enhanced suppression of *fsn-1(null)* caused axon termination defects by *unc-36(null)* mutations. UNC-36 is important for the proper localization of the UNC-2 alpha-1 subunit and the proper function of the EGL-19 calcium channel in *C. elegans* (Laine et al., 2011; Caylor et al., 2013). We therefore wanted to test if the enhanced *unc-36(null)* suppression of axon termination defects was due to the additive effects caused by the disrupted UNC-2 and EGL-19 calcium channels. In order to test this, we used axon termination defects caused by the *fsn-1(null)* mutation. We found that loss-of-function of both *egl-19* and *unc-2* did not suppress axon termination defects, and instead increased the penetrance of the axon termination defects (Figure 24). This is in contrast to the suppression effect of *unc-36(null)* mutations (Figure 16A), and therefore suggests that UNC-36 does not regulate axon termination via UNC-2 and EGL-19. The results are also opposite of what was observed in the single mutant *unc-2(null)* and *egl-19(lof),* both of which suppressed axon termination defects caused by the *fsn-1(null)* mutation (Figure 24). This suggests that the loss of both *unc-2* and *egl-19* function caused

additional developmental mechanisms to fail, possibly due to pleiotropic effects. More experiments will be needed to further understand these mechanisms.

### *3.3.10 VGCCs co-localize with mitochondria in the PLM cell body*

In order to determine the target of the mechanism by which VGCCs regulate axon termination, we utilized transgenic expression of *unc-36::RFP* in the PLM cell body. We expressed the *unc-36::RFP* in conjunction with markers for organelles associated with selective autophagy and neuronal outgrowth. These include lysosomes, autophagosomes, golgi, and mitochondria. We found that UNC-36::RFP co-localized with mitochondria (Figure 25F), but not with any of the other organelles (Figure 25A,D,E). This result is interesting in regards to neuronal development, given that mitochondria have important roles in axon outgrowth and neuronal health (Han et al., 2016; Smith & Gallo, 2018). However, we cannot say with certainty if and how UNC-36 and mitochondria interact, and which mechanisms are being regulated. In addition, the co-localization of mitochondria and UNC-36 is seen in the cell body but not in the axon. These locations in the cell body may be sites of mitophagy, but could also be aggregated proteins trapped in the cell body. The latter option is less likely considering that co-localization seems to be specific to the mitochondria marker and UNC-36, and not the other markers tested. Eventually we will also want to know if selective autophagy is involved, as mitophagy is a vital part of maintaining functional and homeostatic levels of mitochondria in neurons (Kanki et al., 2009; Mao et al., 2013). It will also be important to follow up with additional experiments to identify the exact nature of the connection between UNC-36 and mitochondria.

We also tested UNC-36::RFP co-localization with GLO-1::GFP, which is a downstream target of RPM-1 in the GLO-4 pathway (Figure 3; Grill et al., 2007). We found that UNC-

36::RFP and GLO-1::GFP did not co-localize in the PLM cell body (Figure 25B). This result suggests that UNC-36 and GLO-1 do not directly interact in the cell body of the PLM. However, they both could act on an intermediate protein or organelle. Finally, due to the role of the GLO-4 pathway as regulators of late endosomes/lysosomes (Grill et al., 2007), we tested the localization of GLO-1::RFP relative to lysosomes, and found that GLO-1::RFP did not co-localize with lysosomes (Figure 25C). Therefore, GLO-1 does not appear to directly interact with lysosomes in the PLM cell body.

## 3.4 Discussion

The Timothy syndrome mutation in *CACNA1C* has the unusual property of being causative for autism with high penetrance, providing an opportunity to discover the downstream cellular processes that are perturbed to cause autism. However, the cellular processes that interact with this mutation to give rise to autism have remained unknown. To address this question, we created a disease model in *C. elegans* that utilizes a mutation equivalent to the Timothy syndrome mutation in humans. Our results reveal that selective autophagy genes interact with the Timothy syndrome mutation to disrupt axon termination and alter behavior. Because common variants of *CACNA1C* are associated with autism, it is likely that this mechanism will be broadly applicable to autism in humans.

### *3.4.1 An understanding of genetic interactions between variants is key to understanding autism*

Only a small fraction of autism cases are thought to be caused by a single variant. Rather, most cases of autism are thought to be caused by genetic interactions between variants (Schaaf et al., 2011; Grice et al., 2015; Turner et al., 2017). For example, likely gene-disrupting (LGD)

mutations have been associated with 15-20% of autism cases. In addition, autistic individuals carry more missense mutations in autism-associated genes relative to healthy controls (Schaaf et al., 2011; Geisheker et al., 2017).

However, both LGD and missense mutations are rare and therefore almost always heterozygous. Therefore, in most cases, it is thought that each of these mutations have little or no effect on their own. Thus, it is likely that the disorder arises from genetic interactions between autism-associated variants. Indeed, statistical analysis of sequencing data suggests that autism arises from the combined action of multiple variants (Schaaf et al., 2011; Turner et al., 2017; Chen et al., 2018).

Although the heritability of autism has been estimated at 83% (Sandin et al., 2017), the complexity of the genetic interactions that give rise to autism make it difficult to predict and diagnose autism from whole genome sequencing data. In fact, with current knowledge, no genetic cause can be found from whole genome sequencing data for most cases of autism. The solution to this challenge could come from genetic analysis. For example, in most cases, single heterozygous null mutations have no phenotype. However, many cases exist to show that an animal carrying two heterozygous null mutations can exhibit a phenotype when each of the mutated genes function in the same genetic pathway (Yook et al., 2001; Xu & Quinn, 2012). Thus, if an individual is heterozygous for two LGD variants in each of two autism-associated genes that function in a pathway, this individual would carry a higher risk for autism. Therefore, knowledge of the pathways that link autism-associated genes will help promote our understanding of the genetic basis of autism.

*3.4.2 Selective autophagy functions with EGL-19(gof) to alter axon development and behavior*

A key finding of our study is the identification of a genetic interaction between the homologs of two autism-associated genes, *egl-19* and *wdfy-3*. Our genetic analysis indicates that *wdfy-3* and other selective autophagy genes are required for the *egl-19(gof)* mutation to disrupt axon termination. Moreover, we also find that *wdfy-3* can negatively regulate axon termination independently of the *egl-19(gof)* mutation. These observations suggest that *wdfy-3* functions with *egl-19* to negatively regulate axon termination. Based on these genetic interactions, we propose a model where the Timothy syndrome mutation induces excessive selective autophagy that causes a disruption of axon termination. As an alternative, it is also possible that selective autophagy could function upstream of the EGL-19(GOF) mutant protein. In this scenario, selective autophagy could promote the function of the EGL-19(GOF) protein by affecting its turnover, stability or localization.

The genetic interaction between *egl-19* and *wdfy-3* also regulates habituation to light touch. It is possible that this genetic interaction affects behavior by functioning in the developing nervous system to regulate connectivity. Alternatively, it is possible that the genetic interaction between *egl-19* and *wdfy-3* functions in the mature nervous system to regulate neural function. Although our data cannot distinguish between these two possibilities, recent work on mice favor the former possibility (Dedic et al., 2018). Conditional knockout of *CACNA1C* in forebrain neurons during development results in anxiety in adult mice, whereas knockout of *CACNA1C* during adulthood does not. These observations lend support to the possibility that CACNA1C acts during development to alter circuit formation, which in turn affects behavior in the adult.

The interaction between the Timothy syndrome mutation and *wdfy-3* provides biological evidence for a role of selective autophagy in autism. A major challenge in autism genetics is to

confirm and characterize the roles of autism candidate genes. For example, *WDFY3* is a candidate gene for autism because whole genome sequencing has found that 3 out of 6707 sequenced autism genomes contain a heterozygous *de novo* likely-gene-disrupting mutation in *WDFY3* (De Rubeis et al., 2014; Iossifov et al., 2014; Wang, T. et al., 2016; RK et al., 2017). However, despite this association, it is not possible to determine if *WDFY3* variants contribute to the cause of autism. Our results place genes that promote selective autophagy in a pathway with a mutation that is causative for autism in humans, thereby providing the first biological evidence for a role of selective autophagy in autism.

Selective autophagy is also required for normal axon development. Aside from its function with EGL-19(GOF) in inducing axon defects, our results suggest that WDFY-3 also functions independently of EGL-19(GOF) to regulate axon development. Consistent with this idea, loss of *WDFY3* causes the disorganization and loss of many commissural axon tracts in mice (Dragich et al., 2016). Loss of *WDFY3* in mice also attenuates the response to guidance cues *in vitro*, suggesting that selective autophagy could regulate the response to guidance cues. Despite these insights, the mechanism through which selective autophagy regulates axon targeting is currently unknown. Based on our genetic analysis, we propose a mechanism whereby selective autophagy functions with voltage gated calcium channels to regulate the response to axon targeting cues.

Selective autophagy and bulk autophagy may have distinct functions in neurodevelopment. Whereas our results suggest a mechanism for selective autophagy in the negative regulation of axon termination, prior studies have reported a role for bulk autophagy in promoting synapse development and inhibiting axon growth. In cultured mouse neurons, knockdown of an autophagy gene promotes axon growth, whereas induction of autophagy

inhibits axon growth (Ban et al., 2013). In *Drosophila*, autophagy promotes development of the neuromuscular junction (Shen & Ganetzky, 2009). In *C. elegans*, autophagosomes form at synaptic sites and are required for presynaptic assembly (Stavoe et al., 2016; Hill et al., 2019). This role for autophagy in synaptogenesis is specific to bulk autophagy, since mutations in selective autophagy genes do not affect synaptogenesis (Stavoe et al., 2016). Interestingly, our results suggest that selective autophagy may regulate axon termination without affecting synaptogenesis.

Selective autophagy could function with VGCCs to regulate other aspects of autism-related pathology. Whereas our study focuses on the role of the Timothy syndrome mutation in misregulating axon termination and behavior, previous work has found that the Timothy syndrome mutation can promote activity-dependent dendrite retraction in cultured mouse neurons and can inhibit the elaboration of mouse dendrites *in vivo* (Krey et al., 2013). The downstream cellular mechanisms for this effect on dendrites are not yet known, but it is possible that selective autophagy could also be involved in this process. Alternatively, it is possible that the Timothy syndrome mutation functions through distinct mechanisms to affect dendrite development and axon development.

### *3.4.3 Potential role for VGCCs in regulating the RPM-1 pathway.*

Our results identify specific genetic interactions between VGCC genes and RPM-1 pathway genes. These genetic interactions indicate that loss of VGCC function suppresses axon termination events that are caused by loss of *fsn-1* function, but not loss of *glo-4* function. Moreover, we find that loss of VGCC function does not suppress defects caused by loss of *rpm-1* function, but can partially suppress defects caused by the double loss of *fsn-1* and *glo-4*. Taken

together, these observations suggest that VGCCs specifically regulate axon termination signaling downstream of FSN-1, but are not themselves downstream targets of RPM-1 and FSN-1.

The genetic interactions between the VGCC genes and RPM-1 pathway genes could be explained by a model where VGCCs negatively regulate an unknown protein that functions with RPM-1 to enhance signaling events that promote axon termination downstream of FSN-1, but not GLO-4 (Figure 26). In fact, prior work has found that FSN-1 binds to RPM-1 and promotes axon termination by negatively regulating the DLK-1 MAP kinase signaling pathway (Liao et al., 2004; Nakata et al., 2005). Moreover, the PPM-1 phosphatase also binds to RPM-1 and promotes axon termination by negatively regulating DLK-1 MAPK signaling (Tulgren et al., 2011). Thus, it is possible that EGL-19 might repress axon termination by negatively regulating PPM-1, or another protein that plays a similar role (Figure 27).

### *3.4.4 Role for VGCC-mediated calcium transients in axon growth.*

Our study focuses on the genetic mechanisms that mediate the role of VGCC genes in axon termination, but does not address how alterations in calcium permeation might be involved in this process. Interestingly, a recent study of cultured prenatal mouse neurons has revealed that VGCCs function during axon outgrowth to produce calcium transients that have very different properties compared to those produced during synaptic transmission (Kamijo et al., 2018). These transients have been named Spontaneous Regenerative Calcium Transients (SRCaTs) and are mediated by $Ca_V1.2$, which includes the homolog of EGl-19, CACNA1C. Unlike its function in adult neurons, $Ca_V1.2$ appears to open near resting potential, suggesting that the $Ca_V1.2$ channel may open spontaneously in developing axons. Knockout of *CACNA1C* in these cultured neurons causes a decrease in axon growth. Thus, $Ca_V1.2$ functions in axons to regulate axon growth,

using a mechanism that is very different than how it functions in synaptic transmission. The role of SRCaTs in regulating axon growth are unknown. However, our results suggest the possibility that these SRCaTs may regulate signaling downstream of FSN-1 (FBXO45), utilizing a mechanism that involves selective autophagy.

### *3.4.5 Potential role for common CACNA1C variants in affecting selective autophagy and axon development in autism*

We propose that the effect of *CACNA1C* variation in altering axon development is not limited to the Timothy syndrome mutation, but rather extends to the other autism-associated *CACNA1C* variants. This idea is supported by our genetic analysis suggesting that the effect of the *egl-19(gof)* Timothy syndrome mutation on axon termination is not neomorphic, but rather reflects an increase in the normal function of *egl-19*. Therefore, other gain-of-function and loss-of-function variants in CACNA1C could contribute to autism by altering axon development. Consistent with this idea, statistical analysis has identified some candidate variants in VGCC genes that are likely to be gain-of-function and others that are likely to be loss-of-function (Splawski et al., 2004; Breitenkamp et al., 2014; De Rubeis et al., 2014; Iossifov et al., 2014; Limpitikul et al., 2016). Therefore, we speculate that both under-activation and over-activation of the signaling pathways that promote axon termination could contribute to autism.

The Timothy syndrome mutation is a very rare *de novo* mutation, and is therefore only responsible for a very tiny fraction of autism cases. However, several common variants in *CACNA1C* have also been associated with autism (Lu et al., 2012; Cross-Disorder Group of the Psychiatric Genomics, 2013; Li et al., 2015). For example, the A genotype at the rs1006737 locus in *CACNA1C* confers risk for autism and is present in about 33% of the human population.

This A genotype at rs1006737 is located within a large intron and is thought to cause *CACNA1C* gain-of-function because neurons with the risk genotype have higher levels of *CACNA1C* mRNA and increased L-type calcium currents relative to neurons with the non-risk genotype (Yoshimizu et al., 2015). Therefore, this risk variant may be associated with a gain-of-function of *CACNA1C* that could disrupt axon development in a way analogous to the Timothy syndrome mutation. However, the small effect size of the rs1006737 locus suggests that this is a relatively weak *CACNA1C* gain-of-function.

Although common alleles have a small effect size relative to the Timothy syndrome mutation, they could interact with risk variants in other genes that function in a genetic pathway with *CACNA1*C. For example, the rs1006737 risk variant could provide a weak gain-of-function in *CACNA1C* that does not cause autism on its own. However, the rs1006737 risk variant could synergize with a gain-of-function risk variant in *WDFY3* to contribute to autism. Alternatively, a weak loss of function in *CACNA1C* could synergize with a weak loss-of-function in *WDFY3* to give rise to autism.

### 3.5 Methods

***C. elegans* genetics** *C. elegans* strains were cultured and maintained on nematode growth medium (NGM)-agar plates using standard methods at 20°C (Brenner, 1974). The following alleles were used in this study: wild-type N2, *rpm-1(ok364), glo-4(ok362), fsn-1(gk429), unc-2(e55), unc-36(e251), egl-19(n2368), egl-19(n582), egl-19(syb1243), cup-5(ar465), epg-7(tm2508), wdfy-3(ok912), ric-7(nu447); egl-30(n686), cca-1(gk30); msi-1(os1)*. Unless otherwise noted, double and triple mutants were constructed following standard procedures, and were confirmed by the associated phenotypes and by PCR/sequence genotyping.

**Transgenic fluorescent markers** The *muIs32* transgene was obtained from the CGC and encodes *Pmec-7::gfp + lin-15(+)* (Ch'ng et al., 2003) and was used to observe the PLM axon. The *jsls973* and *jsls821* transgenes were obtained from Michael Nonet. The *jsls973* transgene encodes *Pmec-7::rfp* (Marcette et al., 2014) and was used to observe the PLM axon. The *jsls821* transgene encodes *Pmec-7::gfp::rab-3* (Bounoutas et al., 2009) and was used to observe the localization of chemical synapses in the PLM axon. The *yadIs12* transgene was obtained from Dong Yan and encodes *Pmec-4::GFP::unc-9* (Meng et al., 2016) and was used to observe electrical synapses in the PLM axon. The *kyIs262* transgene was obtained from the CGC and encodes *Punc-86::myr GFP + odr-1::rfp* (Zhang et al., 2013) and was used to observe the BDU neuron. The *bzIs62* transgene was obtained from Monica Driscoll and encodes *Pmec-7::lmp-1::gfp* (Melentijevic et al., 2017) and was used to observe lysosomes in the mechanosensory neurons. The *uIs145* transgene was obtained from Martin Chalfie and encodes *Pmec-4::aman-2::yfp* (Chen et al., 2016) and was used to observe golgi in the mechanosensory neurons. The *sqIs24* transgene was obtained from the CGC and encodes *Prgef-1::lgg-1::gfp* (Palmisano et al., 2016) and was used to observe autophagosomes in the mechanosensory neurons. The *jsIs609* transgene was obtained from Ralf Baumeister and encodes *Pmec-7::mito::gfp* (Fatouros et al., 2012) and was used to observe the mitochondria in the mechanosensory neurons. The *egl-19(syb1243)* mutation was obtained from SunyBiotech. The *cueEx17* and *cueEx18* transgenes were created by injecting *Pmec-7::unc-36::rfp at 5 ng/ul + Pstr-1::gfp at 50 ng/ul.* The *cueEx19* and *cueEx20* transgenes were created by injecting *Pmec-7::egl-19(gof)* at 5 ng/ul + *Podr-1::rfp at 50 ng/ul.* The *cueEx21* transgene was created by injecting *Pmec-7::egl-19(gof)* at 25 ng/ul + *Podr-1::rfp at 50 ng/ul.* The *cueEx22* transgene was created by injecting *Pmec-7::glo-1::gfp* at 5

ng/ul + *Podr-1::rfp at 50 ng/ul.* The *cueEx23* transgene was created by injecting *Pmec-7::glo-1::gfp* at 5 ng/ul + *Pstr-1::gfp at 50 ng/ul.*

**Microscopy** Unless otherwise specified, the microscope used for imaging and phenotype analysis was the Zeiss Axio Imager M2. Images were acquired using the AxioCam MRm camera. Fluorescence was illuminated using the X-cite series 120Q. Images were taken under a 40x objective unless otherwise specified. All images acquired from the microscope were analyzed using Axiovision 4 software. Images were edited into figures using Adobe Photoshop.

**Analysis of phenotypes** For analysis of axon termination phenotypes, animals were mounted on a 5% agarose pad and observed with a 40x objective. For PLM axon termination, an axon was scored as defective if it grew anterior to the ALM cell body. PLM neurons were visualized with the *muIs32* transgene which encodes *Pmec-7::gfp* and is expressed in all mechanosensory neurons.

For analysis of the PLM chemical synapses, a *Pmec-7::gfp::rab-3* transgene that expresses the RAB-3 synaptic vesicle marker in the touch receptor neurons was used to visualize synaptic vesicle clusters (Bounoutas et al., 2009). The size of each synaptic cluster was measured as previously described (Xu & Quinn, 2016). For analysis of PLM electrical synapses, a *Pmec-4::gfp::unc-9* transgene was used to express the UNC-9 innexin fused to GFP in the touch receptor neurons (Meng et al., 2016).

For analysis of mechanosensation, we adopted an eyelash touch assay (Chalfie et al., 2014). We assayed gentle touch responses by touching the lateral side of animals with an eyebrow hair. Each animal was subjected to five touches alternating between the anterior and posterior ends and scored by the number of responses elicited. Assays were performed blind to genotype. Three independent samples of 20 animals each were collected by three independent observers and reported as mean percentage scores.

***C. elegans* male creation using RNAi** In order to create male *C. elegans* for performing genetic crosses, we adopted a protocol originally established by Killian & Hubbard, described in Killian and Hubbard (2001). 4 to 6 worms at the L3-L4 stage were placed on a plate freshly coated with HT115(DE3) bacteria which produce *him-14* dsRNA. The plates were incubated at 22 degrees Celsius for 3-4 days. The F1 generation from these plates contained males. RNAi created males were then used to mate with hermaphrodites of the same genotype in order to produce a second generation of males. The F2 generation contained more fertile males that were one generation separated from the RNAi treatment. The males were then used in genetic crosses.

### 3.6 Acknowledgements

We thank Shohei Mitani, Michael Nonet, Dong Yan, Monica Driscoll, Martin Chalfie, Ralf Baumeister, Peter Roy and the Caenorhabditis Genetics Center for strains and reagents. We also thank Claire de la Cova for discussion of the manuscript.

**Chapter 3 Figures:**

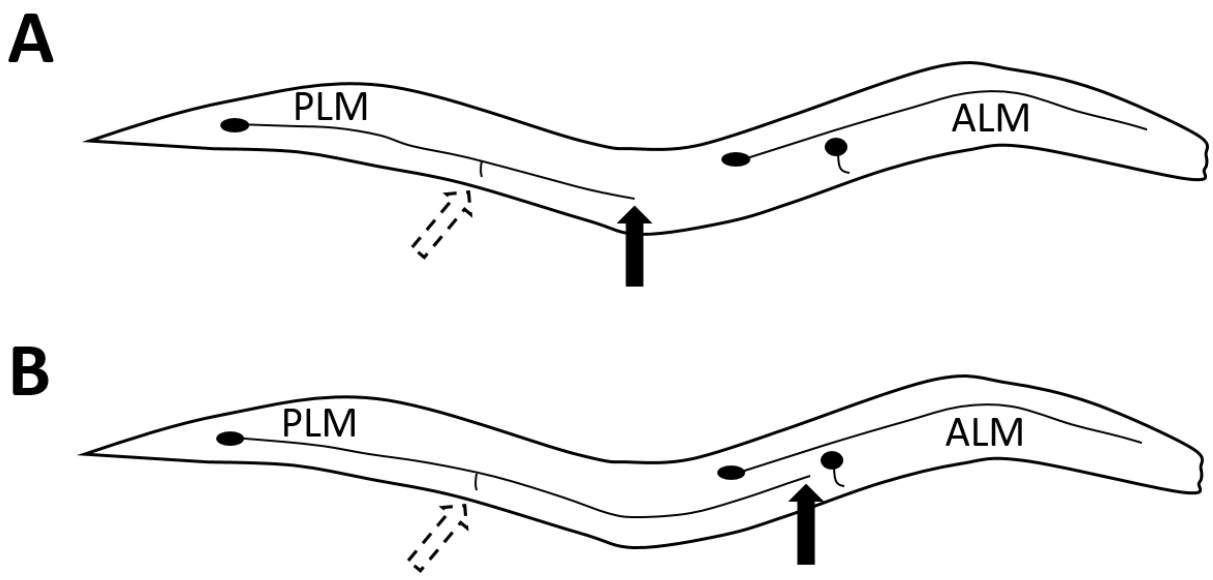


**Figure 12: *C. elegans* mechanosensory neuronal network.**

**(A)** A diagram of a wildtype mechanosensory neuronal network. The PLM axon terminates growth near the midline, posterior to the ALM cell body. **(B)** A diagram of a mechanosensory neuronal network with an overextended PLM. The PLM axon terminates growth anterior to the ALM cell body. The black arrow indicates the PLM axon tip. The dotted arrow indicates the ectopic branch formed between the PLM and the ventral nerve chord. The posterior end of the worm is on the left, the anterior end of the worm is on the right.

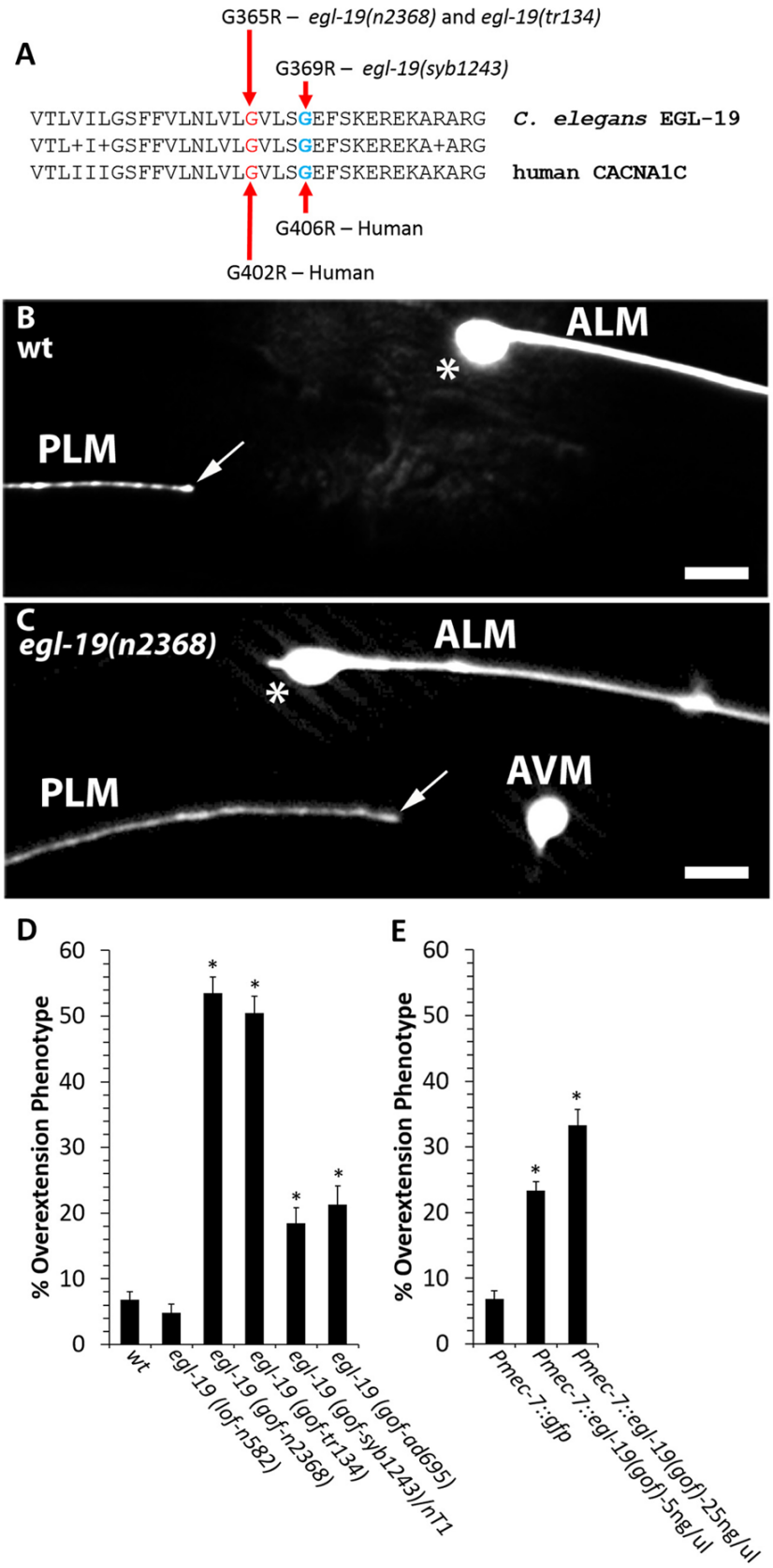
A
G365R – egl-19(n2368) and egl-19(tr134)
G369R – egl-19(syb1243)
VTLVILGSFFVLNLVLGVLSGEFSKEREKARARG C. elegans EGL-19
VTL+I+GSFFVLNLVLGVLSGEFSKEREKA+ARG
VTLIIIGSFFVLNLVLGVLSGEFSKEREKAKARG human CACNA1C
G406R – Human
G402R – Human
B
wt
ALM
PLM
C
egl-19(n2368)
ALM
AVM
PLM
D
% Overextension Phenotype
0
10
20
30
40
50
60
wt
egl-19 (lof-n582)
egl-19 (gof-n2368)
egl-19 (gof-tr134)
egl-19 (gof-syb1243)/nT1
egl-19 (gof-ad695)
E
% Overextension Phenotype
0
10
20
30
40
50
60
Pmec-7::gfp
Pmec-7::egl-19(gof)-5ng/ul
Pmec-7::egl-19(gof)-25ng/ul

**Figure 13: Mutations equivalent to the G402R and G406R Timothy syndrome mutations cause PLM axon termination defects.**

**(A)** The *egl-19(n2368)* and *egl-19(tr134)* mutations are equivalent to the G402R Timothy Syndrome mutation in *CACNA1C* that causes autism in humans. The *egl-19(syb1243)* mutation is equivalent to the CACNA1C G406R mutation that causes Timothy Syndrome in humans. **(B)** Example of normal axon termination in a L4 stage wild-type PLM neuron, where the axon terminates posterior to the ALM cell body. **(C)** Example of axon termination defect in a L4 stage *egl-19(gof)* mutant, where the axon terminates anterior to the ALM cell body. Axons are visualized with the *muIs32* transgene that encodes *Pmec-7::gfp*. Arrows point to the tip of the PLM axon. Asterisk marks the ALM cell body. Scales bars are 10um. **(D)** Gain-of-function mutations in *egl-19* cause axon termination defects. The *egl-19(lof)* mutation does not affect axon termination. **(E)** Transgenic expression of EGL-19(GOF) specifically within touch receptor neurons causes axon termination defects. The *Pmec-7::egl-19(gof)* transgenes use the *mec-7* promoter to drive expression of an *egl-19* cDNA that includes a mutation identical to the *egl-19(n2368)* mutation. For *Pmec-7::egl-19(gof)*-5 ng/ul, 2 independent transgenic strains were analyzed and the results were averaged. For *Pmec-7::egl-19(gof)*-25 ng/ul, 1 transgenic strain was analyzed.

Between 200 and 400 axons were observed in L4 stage hermaphrodites per genotype. Asterisks indicate statistically significant difference, Z-test for proportions (*$p<0.0001$). Error bars represent the standard error of the proportion.

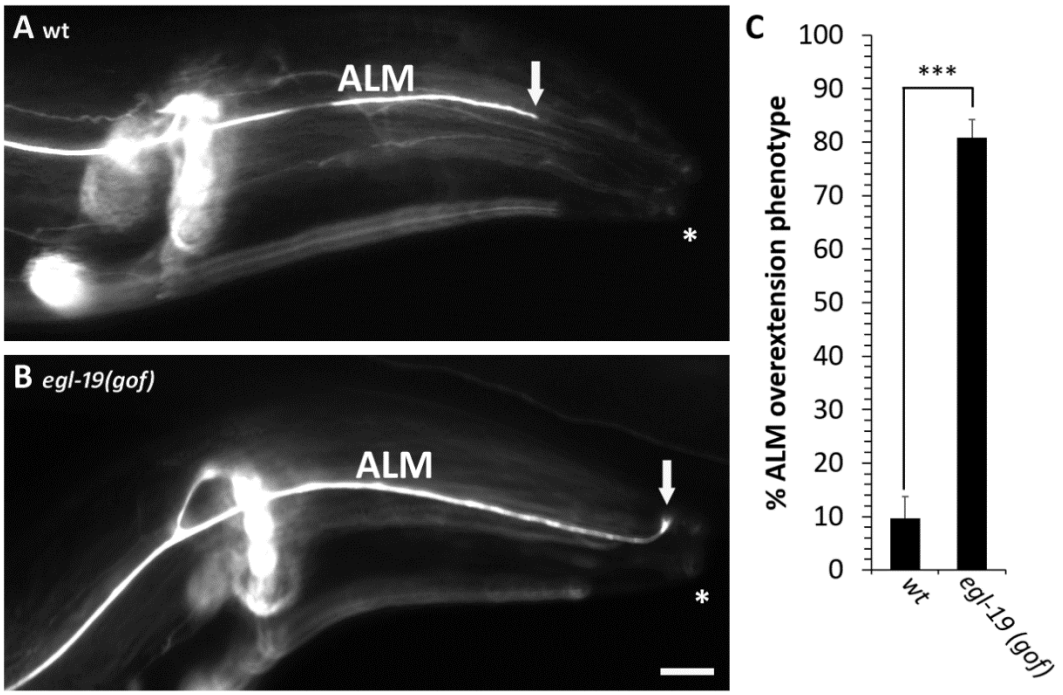


**Figure 14: The *egl-19(gof)* Timothy syndrome mutation causes ALM axon termination defects.**

**(A)** Example of normal axon termination in a L4 stage wild-type ALM neuron, where the axon terminates posterior to the mouth of the worm. **(B)** Example of axon termination defect in a L4 stage *egl-19(gof)* mutant, where the axon extends to the anterior most point of the worm. Axons are visualized with the *muIs32* transgene that encodes *Pmec-7::gfp.* Arrows point to the tip of the ALM axon. Asterisk marks the anterior-most part of the worm. Scales bar is 10um. **(C)** Gain-of-function mutations in *egl-19* cause axon termination defects.

Between 100 and 150 axons were observed in L4 stage hermaphrodites per genotype. Asterisks indicate statistically significant difference, Z-test for proportions (***$p<0.0001$). Error bars represent the standard error of the proportion.

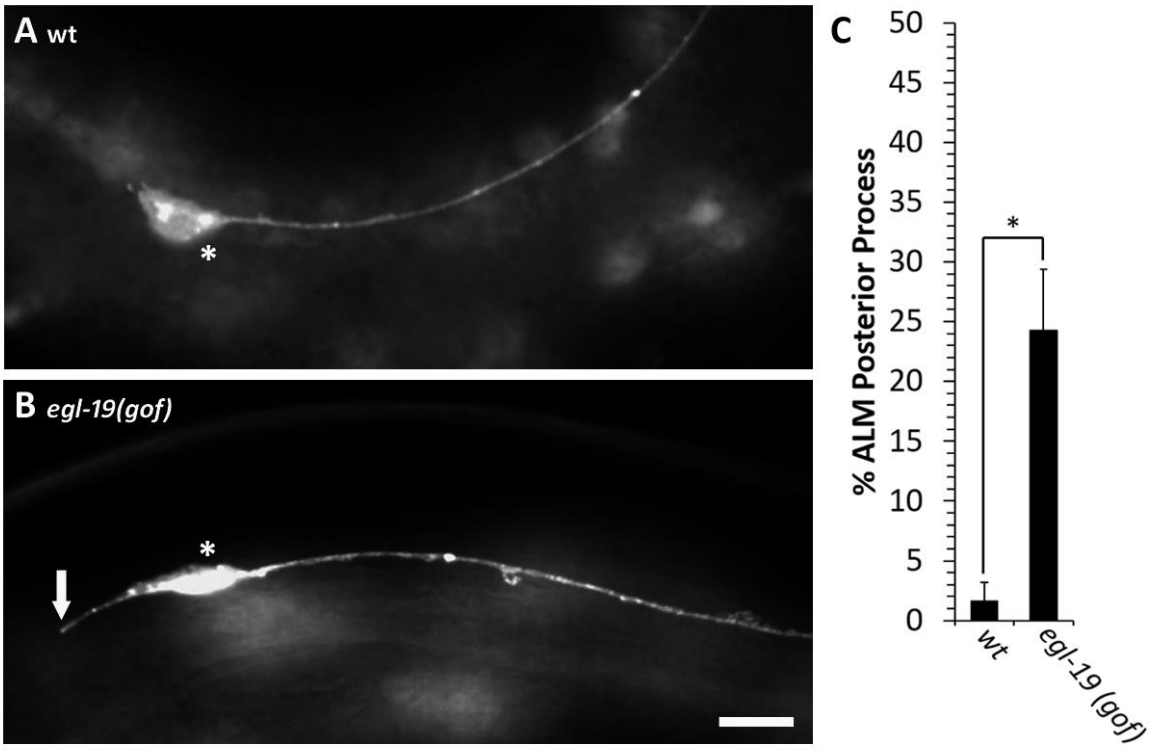


**Figure 15: The *egl-19(gof)* Timothy syndrome mutation causes ALM posterior process defects.**

**(A)** Example of a normal cell body in a L4 stage wild-type ALM neuron, where there are no visible processes extending from the posterior side of the ALM cell body. **(B)** Example of a posterior process phenotype in a L4 stage *egl-19(gof)* mutant, where a visible process extends from the posterior side of the ALM cell body. Axons are visualized with the *muIs32* transgene that encodes *Pmec-7::gfp.* Arrows point to the tip of the posterior process formed from the ALM cell body. Asterisk marks the anterior most position on the worm. Scale bar is 10um. **(C)** Gain-of-function mutations in *egl-19* cause posterior process defects in the ALM.

Between 100 and 150 axons were observed in L4 stage hermaphrodites per genotype. Asterisks indicate statistically significant difference, Z-test for proportions (*$p<0.0005$). Error bars represent the standard error of the proportion.

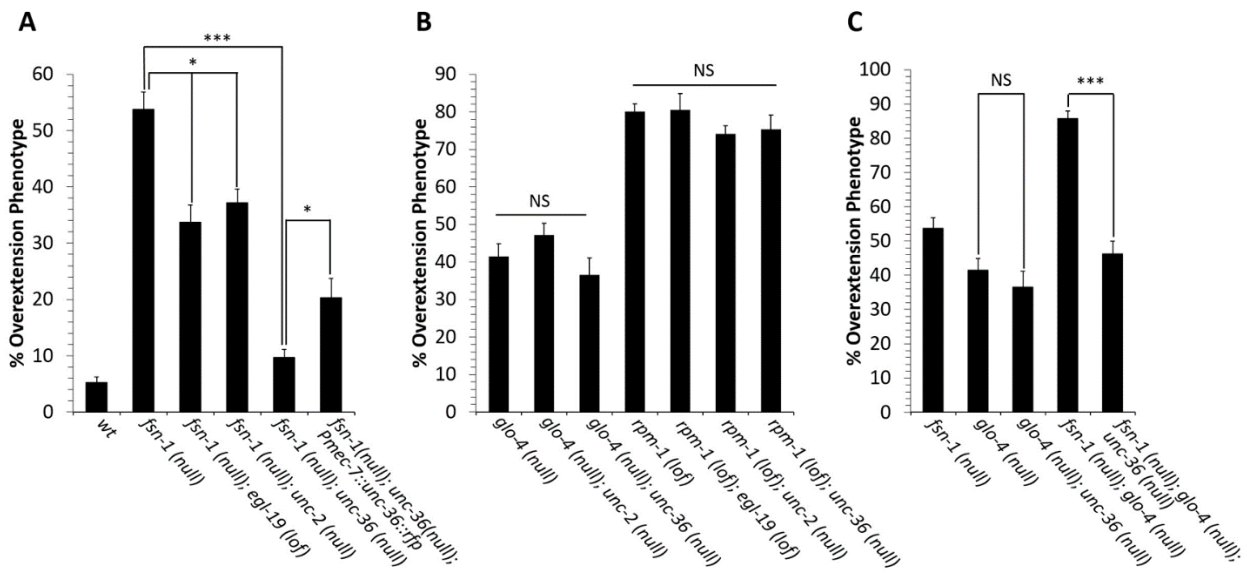


**Figure 16: *VGCC* genes negatively regulate axon termination.**

**(A)** Loss-of-function mutations in VGCC genes suppress axon termination defects caused by the *fsn-1(null)* mutation. The *egl-19* gene encodes the pore-forming subunit of the L-type VGCC. The *unc-2* gene encodes the pore-forming subunit of the P/Q-type VGCC and the *unc-36* gene encodes the alpha2-delta3 subunit that works with both the L-type and P/Q-type VGCCs. **(B)** Mutations in VGCC genes do not suppress axon termination defects caused by loss-of-function mutations in *glo-4* or *rpm-1*. **(C)** Loss of VGCC function can partially suppress axon termination defects caused by the *fsn-1(null)*; *glo-4(null)* double mutant background. Asterisks indicate statistically significant difference, Z-test for proportions (*p<0.005, ***p<0.0001). Between 200 and 400 axons were observed in L4 stage hermaphrodites per genotype using the *muIs32* transgene. Error bars represent the standard error of the proportion. For *Pmec-7::unc-36::rfp*, 2 independent transgenic strains were analyzed and the results were averaged. Alleles: *fsn-1(null)* is *fsn-1(gk429)*, *egl-19(lof)* is *egl-19(n582)*, *unc-2(null)* is *unc-2(e55)*, *unc-36(null)* is *unc-36(e251)*, *glo-4(null)* is *glo-4(ok362)*, *rpm-1(lof)* is *rpm-1(ok364)*

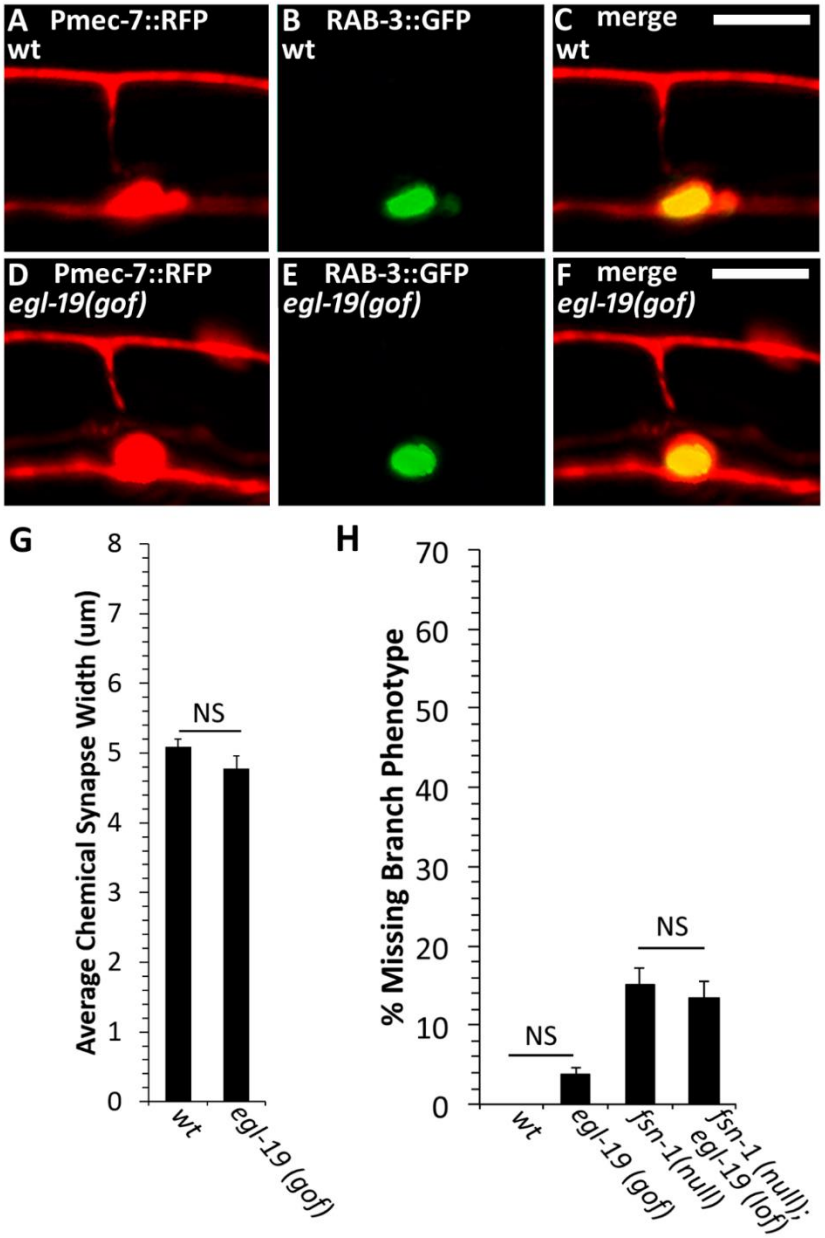


**Figure 17: The *egl-19(gof)* Timothy syndrome mutation causes no visible change to PLM chemical synapse and branch formation.**

**(A)** Example of normal PLM chemical synapse in a wild-type animal. The PLM axon and branch of a wild-type animal are shown here in the L4 stage. **(B)** The PLM chemical synapses of a wild-type animal are shown here in the L4 stage. **(C)** The merged image showing the PLM chemical

synapse in a wild-type animal. **(D)** Example of chemical synapses in an *egl-19(gof)* mutant. The PLM axon and branch appear morphologically unchanged by the *egl-19(gof)* mutation, shown here in the L4 stage. **(E)** The PLM chemical synapses appear morphologically unchanged by the *egl-19(gof)* mutation, shown here in the L4 stage.. **(F)** The merged image showing the PLM chemical synapse in an *egl-19(gof)* animal. The axon and its synaptic branch are shown in red as visualized with the *jsls973* transgene that encodes *Pmec-7::rfp.* The chemical synapse is shown in green as visualized by the *jsls821* transgene that encodes *Pmec-7::gfp::rab-3*. The synaptic vesicles appear yellow in merged images due to overlap with the red signal. **(G)** The PLM chemical synapses are unaffected by changes in *egl-19* function. The average width of the chemical synapse cluster is not changed by the *egl-19(gof)* mutation. Between 50 and 100 chemical synapse clusters were observed in L4 stage hermaphrodites per genotype using the *jsls821* transgene. Error bars represent the standard error of the proportion. **(H)** The PLM synaptic branch is unaffected by changes in *egl-19* function. The *egl-19(gof)* mutation does not cause defects in the PLM synaptic branch. The *egl-19(lof)* mutation does not suppress the PLM synaptic branch defect caused by the *fsn-1(null)* mutation. Z-test for proportions. Between 200 and 400 synaptic branches were observed in L4 stage hermaphrodites per genotype using the *muIs32* transgene. Error bars represent the standard error of the proportion. Alleles: *egl-19(gof)* is *egl-19(n2368), fsn-1(null)* is *fsn-1(gk429), egl-19(lof)* is *egl-19(n582).*

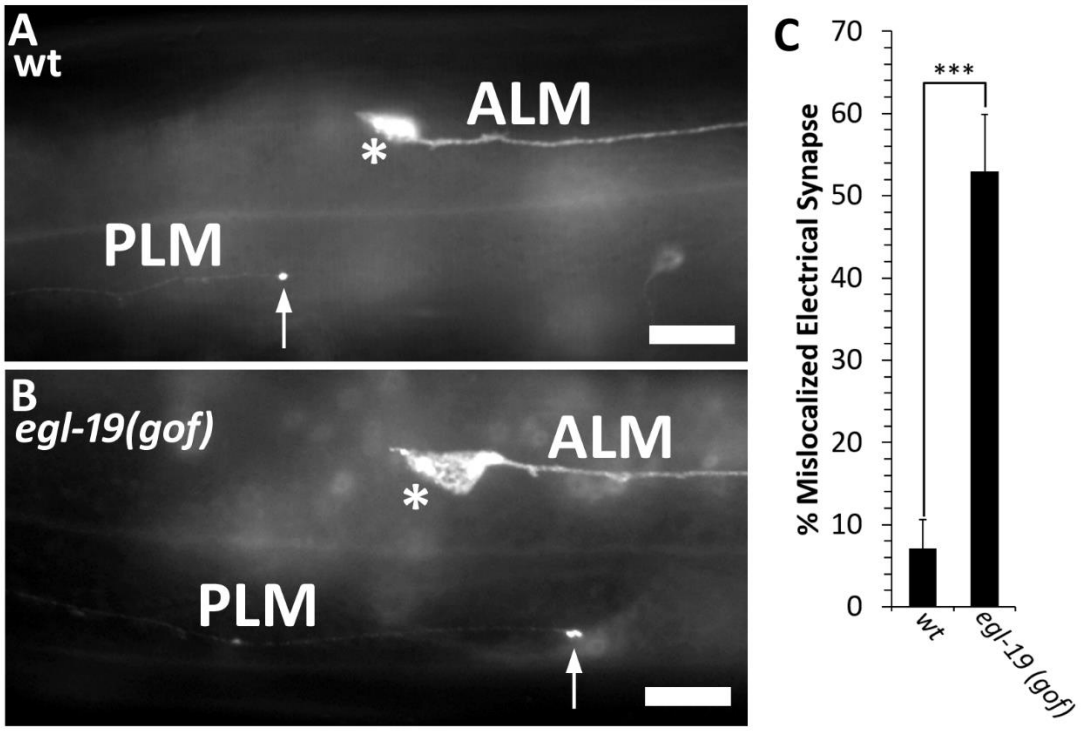


**Figure 18: The *egl-19(gof)* Timothy syndrome mutation causes mislocalization of PLM electrical synapses.**

**(A)** Example of zone 2 electrical synapses at the tip of the PLM axon in wildtype animals. **(B)** Example of zone 2 electrical synapses at the tip of the PLM axon in *egl-19(gof)* mutants. The zone 2 electrical synapses should be localized posterior to the ALM cell body. However, in *egl-19(gof)* mutants, the zone 2 electrical synapses are aberrantly localized anterior to the ALM cell body. Electrical synapses were visualized with the *yadIs12* transgene that encodes *Pmec-4::gfp::unc-9 (Meng et al., 2016).* Axons were observed under the Zeiss Axio Imager M2 microscope, and images were acquired using the Axiocam 702mono camera. Fluorescence illumination was performed with an X-cite 120LED boost by Lumen Dynamics. Arrow marks electrical synapses at the tip of the PLM axon. Asterisk marks the ALM cell body. Scalebars are

10um. **(C)** The *egl-19(gof)* mutation causes mislocalization of PLM zone 2 electrical synapses. Z-test for proportions (***p<0.0001). Between 200 and 400 zone 2 electrical synapses were observed in L4 stage hermaphrodites per genotype using the *yadIs12* transgene.. Alleles: *egl-19(gof)* is *egl-19(n2368)*.

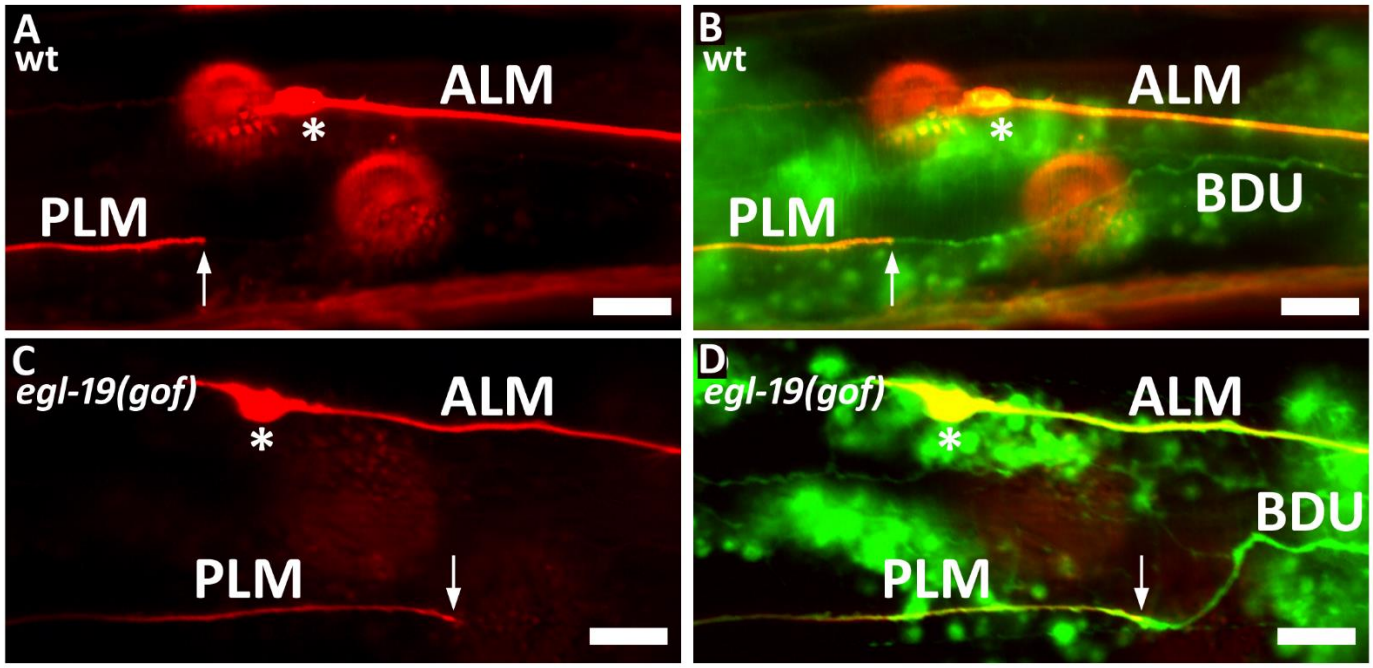


**Figure 19: PLM-BDU connection is not disrupted by the *egl-19(gof)* Timothy syndrome mutation.**

**(A)** An example of normal axon termination in a L4 stage wild-type PLM neuron, where the axon terminations posterior to the ALM cell body. **(B)** Example of a normal PLM-BDU connection. The BDU migrates towards PLM to form a connection with the axon tip of the PLM. **(C)** Example of axon termination defect in a L4 stage *egl-19(gof)* mutant, where the axon terminates anterior to the ALM cell body.. **(D)**Example of PLM-BDU connection in *egl-19(gof)* mutants. Despite the overextension of the PLM, the BDU migrates towards the PLM and forms a connection with the axon tip of the PLM. ALM and PLM axons are visualized with the *muIs32* transgene that encodes *Pmec-7::gfp*. BDU axons are visualized with the *kyIs262* transgene that encodes *Punc-86::myr gfp + odr-1::rfp.* Axons were observed under the Zeiss Axio Imager M2 microscope, and images were acquired using the Axiocam 702mono camera. Fluorescence

illumination was performed with an X-cite 120LED boost by Lumen Dynamics. Arrows point to the tip of the PLM axon. Asterisk marks the ALM cell body. Scales bar are 10um. Alleles: *egl-19(gof)* is *egl-19(n2368).*

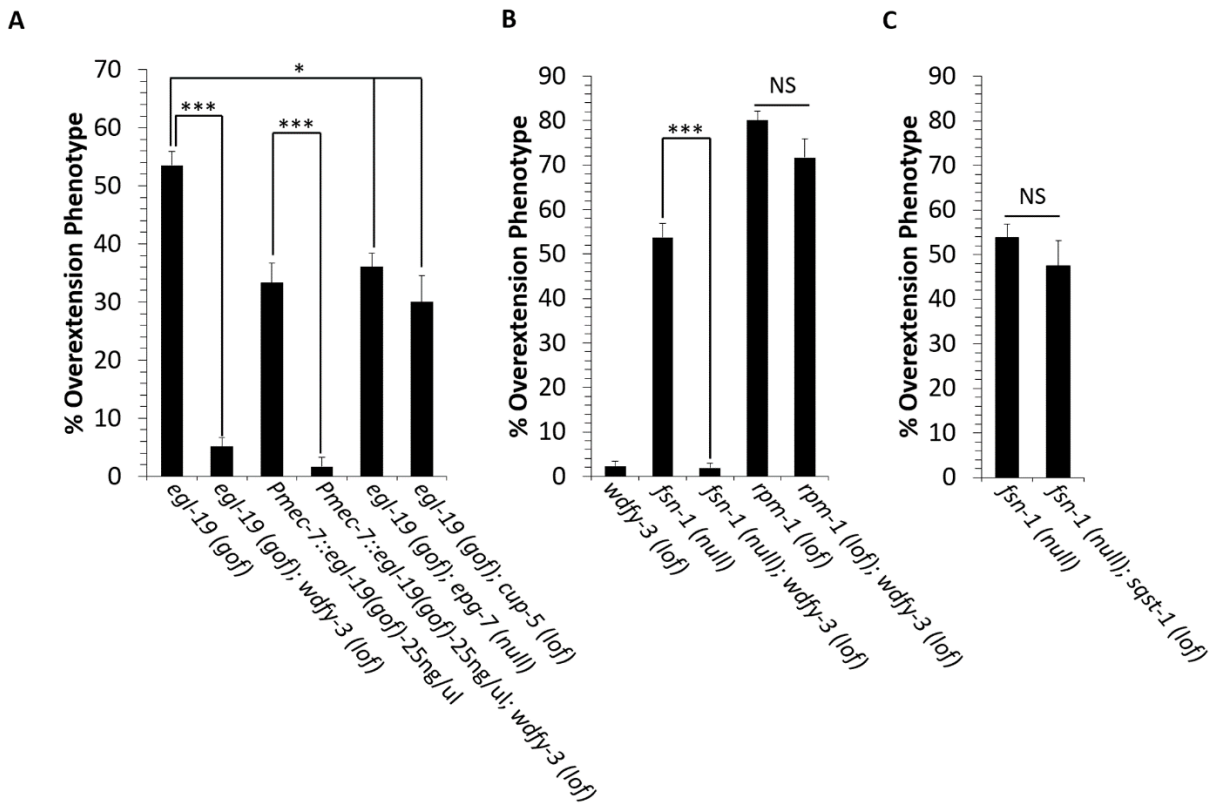


**Figure 20: EGL-19(GOF) functions with selective autophagy to cause axon termination defects.**

**(A)** Axon termination defects caused by the *egl-19(gof)* mutation are suppressed by loss-of-function mutations in *wdfy-3(lof)*, *epg-7(null)* and *cup-5(lof)*. **(B)** Axon termination defects caused by the *fsn-1(null)* mutation are suppressed by the *wdfy-3(lof)* mutation. However, axon termination defects caused by *rpm-1(lof)* mutation are not suppressed by the *wdfy-3(lof)* mutation. **(C)** Axon termination defects caused by the *fsn-1(null)* mutation are not suppressed by the *sqst-1(lof)* mutation. The *sqst-1* gene encodes a cargo receptor protein that functions with WDFY-3 in selective autophagy. Axons are visualized with the *muIs32* transgene that encodes *Pmec-7::gfp.* Asterisks indicate statistically significant difference, Z-test for proportions (*p<0.005, ***p<0.0001). Error bars represent the standard error of the proportion. n=200-400

axons per genotype. Alleles: *egl-19(gof)* is *egl-19(n2368), wdfy-3(lof)* is *wdfy-3(ok912), epg-7(null)* is *epg-7(tm2508), cup-5(lof)* is *cup-5(ar465), fsn-1(null)* is *fsn-1(gk429), rpm-1(lof)* is *rpm-1(ok364).*

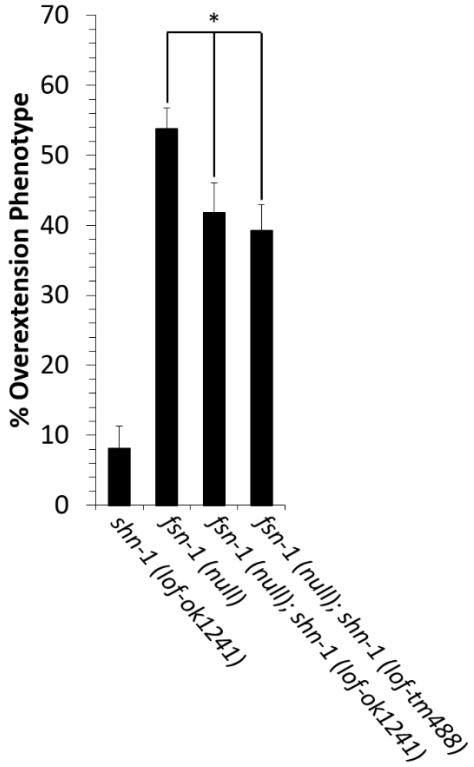


**Figure 21: Loss-of-function mutations in the autism associated gene *shn-1* suppress the axon termination defects caused by *fsn-1(null)* mutation.**

Axon termination defects caused by the *fsn-1(null)* mutation are partially suppressed by loss-of-function mutations in *shn-1*. Z-test for proportions (*$p<0.05$). Between 200 and 400 synaptic branches were observed in L4 stage hermaphrodites per genotype using the *muIs32* transgene. Error bars represent the standard error of the proportion. Alleles: *fsn-1(null)* is *fsn-1(gk429)*.

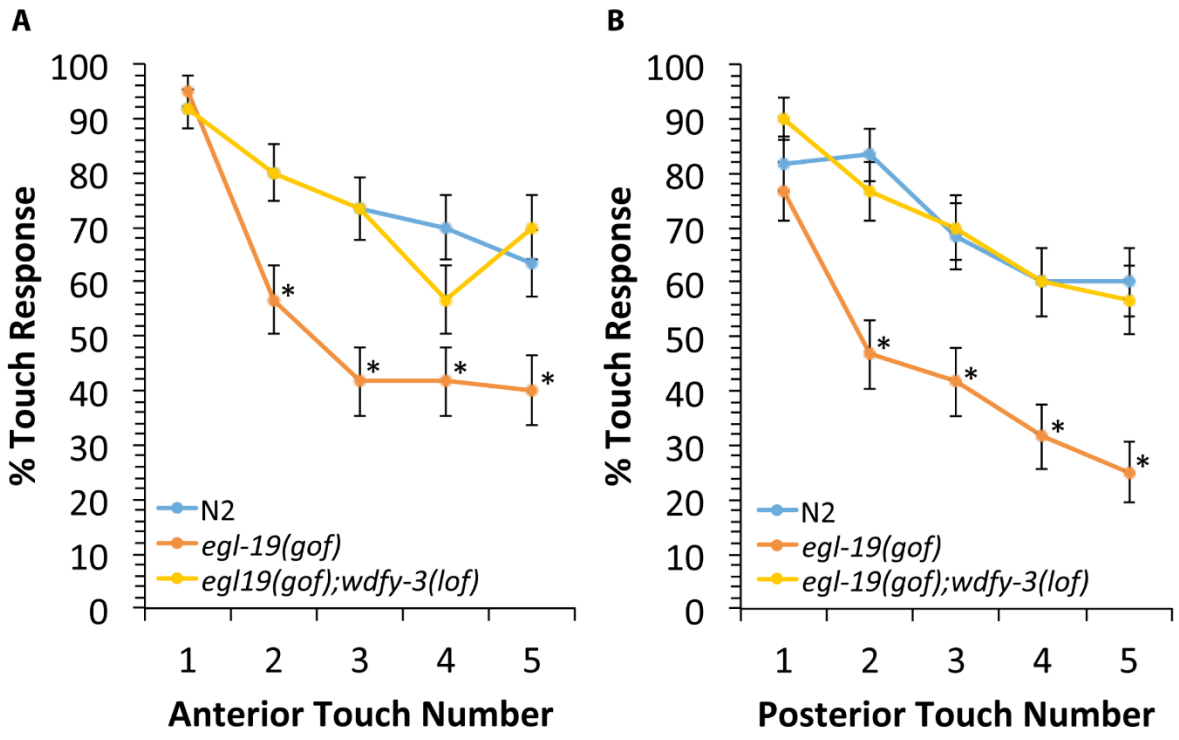


**Figure 22: The *wdfy-3* gene functions with the *egl-19(gof)* mutation to alter habituation to light touch.**

Animals were subjected to eyelash touches alternating between the head and tail and the response rate was recorded after each touch. **(A)** Response rate for anterior touches. **(B)** Response rate for posterior touches. For both anterior and posterior touches habituation was significantly increased in *egl-19(gof)* mutants relative to wild-type. This change in habituation in *egl-19(gof)* mutants was suppressed by *wdfy-3(lof).* Asterisks indicate statistically significant difference compared to wild type worms, Z-test for proportions (*p<0.0001). Error bars represent the standard error of the proportion. For each genotype, the assay was repeated 3 times by 3 different observers who were blind to the genotype. Each experiment included 20 worms for a total n of 60 for each genotype. Alleles: *egl-19(gof)* is *egl-19(n2368), wdfy-3(lof)* is *wdfy-3(ok912).*

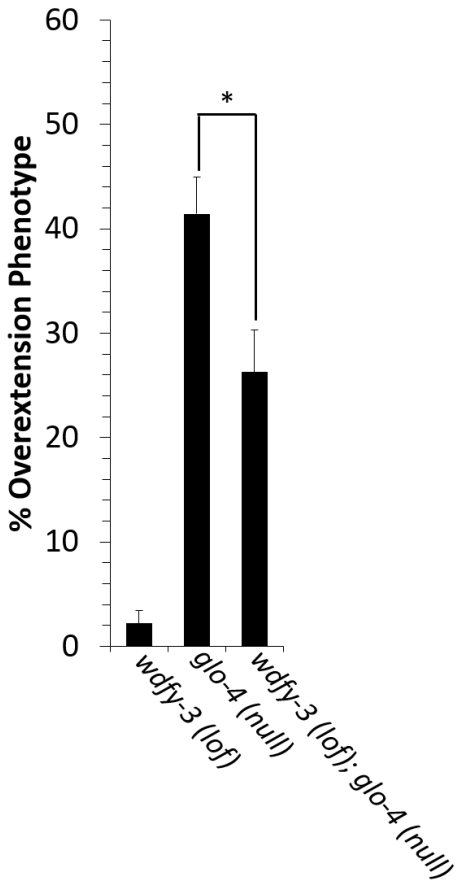


**Figure 23: loss-of-function of *wdfy-3* partially suppresses overextension phenotype caused by *glo-4(null)* mutation.**

Axon termination defects caused by the *glo-4(null)* mutation are partially suppressed by the *wdfy-3(lof)* mutation. The suppression appears to be a pleiotropic affect due to the many additional and unique defects in the PLM axon in the *wdfy-3(lof);glo-4(null)* mutant. Asterisks indicate statistically significant difference compared to wild type worms, Z-test for proportions (*$p<0.01$). Error bars represent the standard error of the proportion. Between 200 and 400 synaptic branches were observed in L4 stage hermaphrodites per genotype using the *muIs32*

transgene. Error bars represent the standard error of the proportion. Alleles: *wdfy-3(lof)* is *wdfy-3(ok912)*, *glo-4(null)* is *glo-4(ok362)*.

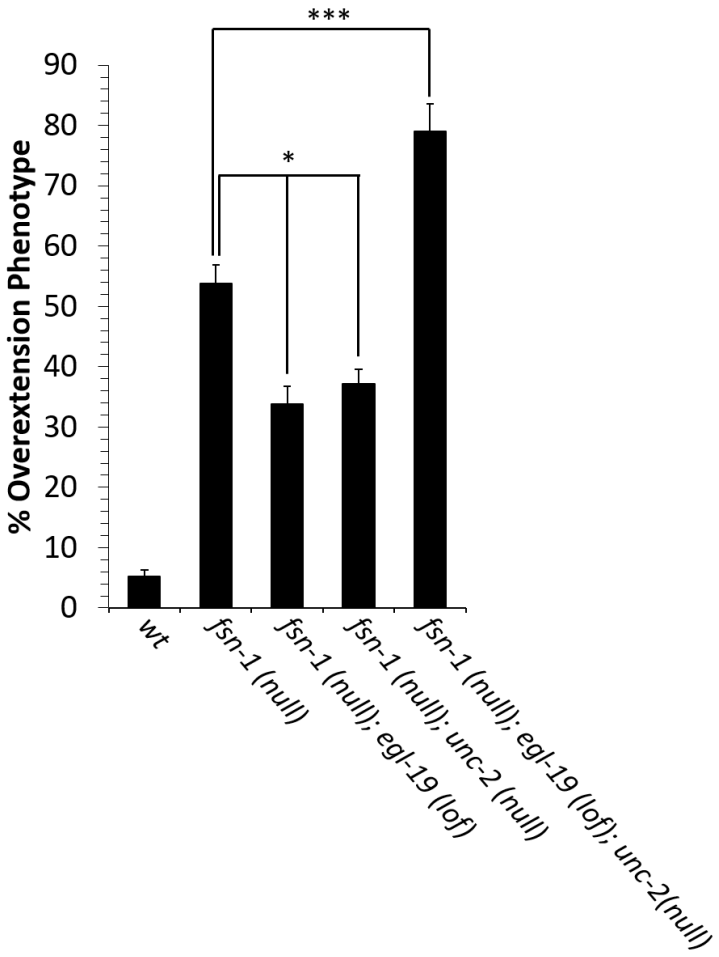


**Figure 24: Loss-of-function of both *egl-19* and *unc-2* genes enhances the axon termination defects caused by the *fsn-1(null)* mutation.**

Axon termination defects caused by the *fsn-1(null)* mutation are suppressed by the *egl-19(lof)* mutation. Axon termination defects caused by the *fsn-1(null)* mutation are suppressed by the *unc-2(null)* mutation. Loss of *egl-19* and *unc-2* function enhance axon termination defects caused by the *fsn-1(null)* mutation. Asterisks indicate statistically significant difference compared to wild type worms, Z-test for proportions (*$p<0.005$, ***$p<0.0001$). Error bars represent the standard error of the proportion. Between 200 and 400 synaptic branches were

observed in L4 stage hermaphrodites per genotype using the *muIs32* transgene. Error bars represent the standard error of the proportion. Alleles: *fsn-1(null)* is *fsn-1(gk429), egl-19(lof)* is *egl-19(n582), unc-2(null)* is *unc-2(e55).*

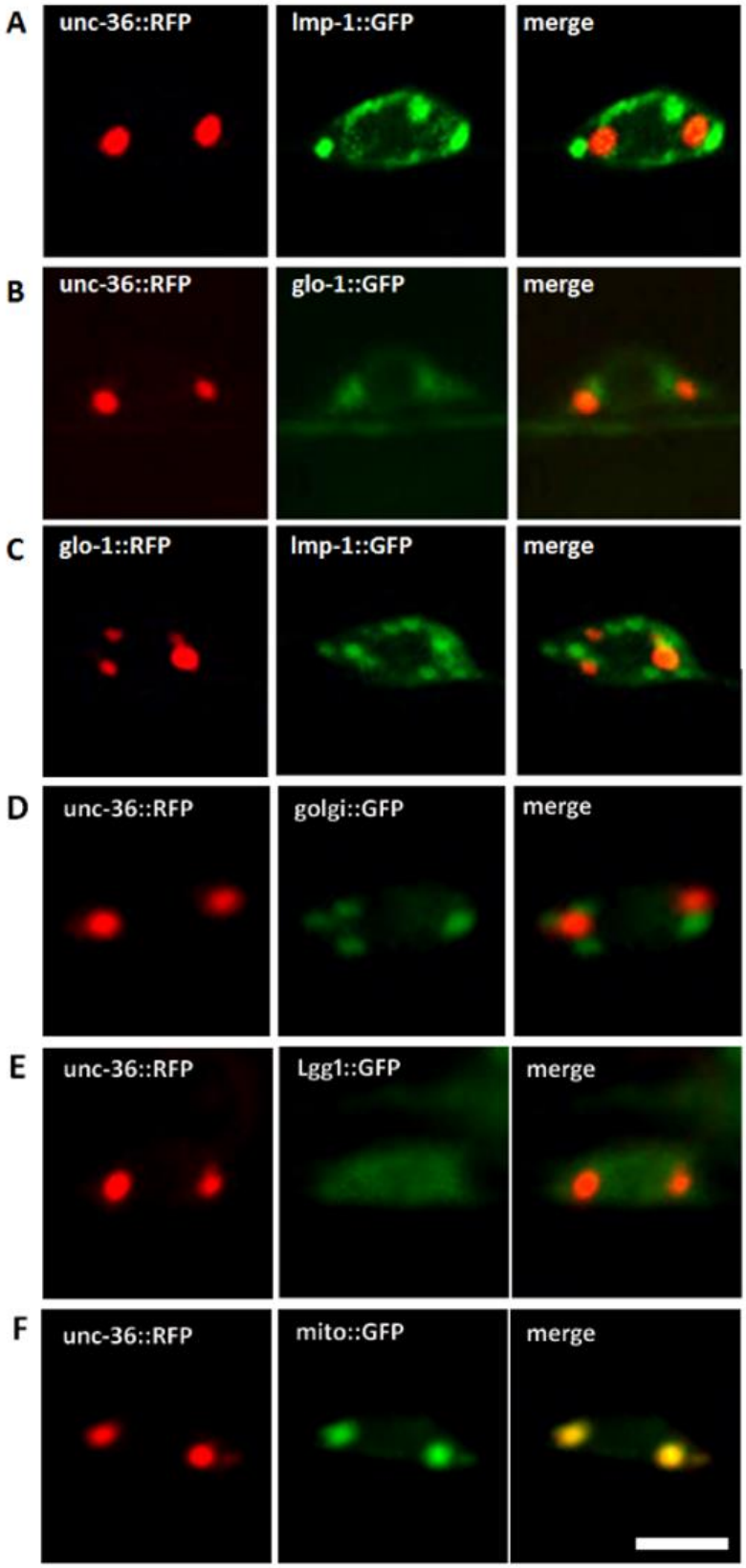
A
unc-36::RFP
lmp-1::GFP
merge
B
unc-36::RFP
glo-1::GFP
merge
C
glo-1::RFP
lmp-1::GFP
merge
D
unc-36::RFP
golgi::GFP
merge
E
unc-36::RFP
Lgg1::GFP
merge
F
unc-36::RFP
mito::GFP
merge

**Figure 25: UNC-36 co-localizes with mitochondria in the PLM cell body.**

**(A)** Expression of *mec-7p::unc-36::RFP* was used to visualize the voltage gated calcium channel UNC-36 localization in the PLM cell body. Expression of the transgene *bzIs62* which encodes P*mec-7::lmp-1::GFP* was used to visualize lysosomes. No co-localization was detected. **(B)** Expression of *mec-7p::unc-36::RFP* was used to visualize the voltage gated calcium channel UNC-36 localization in the PLM cell body. Expression of *mec-7p::glo-1::GFP* was used to visualize the endosomal related protein GLO-1. No co-localization was detected. **(C)** Expression of *mec-7p::glo-1::RFP* was used to the endosomal related protein GLO-1 localization in the PLM cell body. Expression of the transgene *bzIs62* which encodes *mec-7p::lmp-1::GFP* was used to visualize lysosomes. No co-localization was detected. **(D)** Expression of *mec-7p::unc-36::RFP* was used to visualize UNC-36 localization in the PLM cell body. Expression of the transgene *uIs145* which encodes *mec-4p::aman-2::GFP* was used to visualize golgi bodies. No co-localization was detected. **(E)** Expression of *mec-7p::unc-36::RFP* was used to visualize UNC-36 localization in the PLM cell body. Expression of the transgene *sqIs24* which encodes *rgef-1p::lgg-1::GFP* was used to visualize autophagosomes. No co-localization was detected. **(F)** Expression of *mec-7p::unc-36::RFP* was used to visualize UNC-36 localization in the PLM cell body. Expression of the transgene *jsIs609*, which encodes *mec-7p::mito::GFP* was used to visualize mitochondria. Co-localization was detected. Zeiss imaging software was used to capture images and to test for co-localization. Scalebar is 5um.

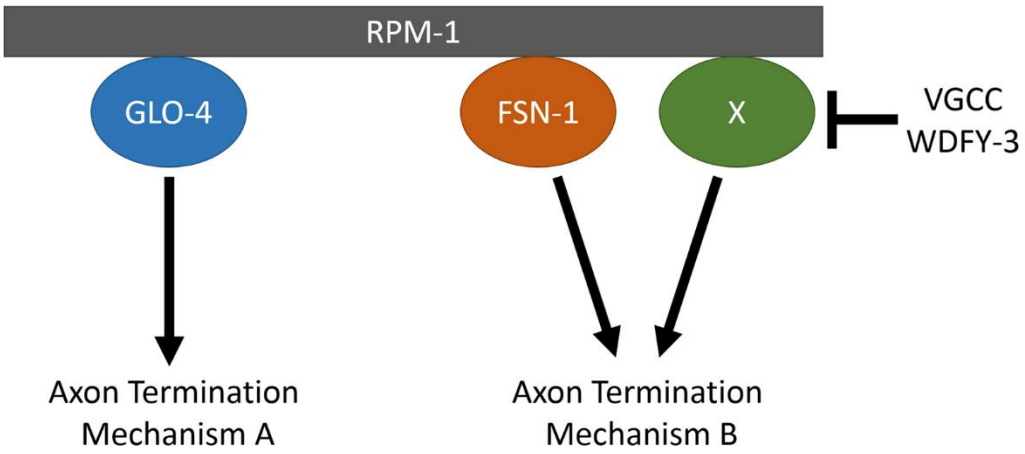


**Figure 26: Model for function of VGCCs in relation to the RPM-1 pathway.**

This hypothetical model could explain the genetic interactions observed between VGCC genes and genes that encode members of the RPM-1 pathway. In this model, VGCCs function with WDFY-3 to negatively regulate an unknown protein (labeled as X). Protein X functions with RPM-1 to promote signaling events downstream of FSN-1 that promote axon termination. Loss of VGCC function causes an increase in protein X function. The additional protein X function works with RPM-1 to promote axon termination mechanism B, thereby compensating for loss of FSN-1 function. Loss of VGCCs does not suppress loss of RPM-1 function because the function of protein X requires RPM-1.

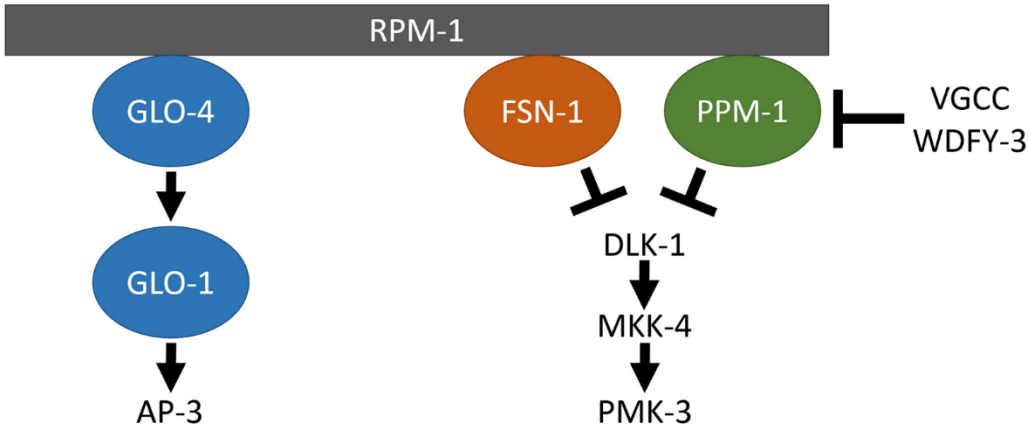


**Figure 27: Model for function of VGCCs relative to specific members of the RPM-1 pathway.**

PPM-1 is a protein that functions with RPM-1 to promote signaling events downstream of FSN-1 that promote axon termination. It is possible that PPM-1, or another protein with a similar role, could be protein X (see Figure 26). Both FSN-1 and PPM-1 promote axon termination by functioning with RPM-1 to negatively regulate the DLK-1 MAP kinase pathway. Thus, it is possible that VGCCs and WDFY-3 could negatively regulate PPM-1, or a protein with a similar role. The extra PPM-1 function could enhance negative regulation of the DLK-1 pathway, thereby compensating for loss of FSN-1 function.

**Table 1: List of mutations.**

| *Gene* | Description |
|---|---|
| *egl-19(n2368)* | Encodes L-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1C, contains gain-of-function point mutation equivalent to G402R mutation that causes Timothy syndrome in humans |
| *egl-19(tr134)* | Encodes L-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1C, contains gain-of-function point mutation equivalent to G402R mutation that causes Timothy syndrome in humans |
| *egl-19(syb1243)* | Encodes L-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1C, contains gain-of-function point mutation equivalent to G406R mutation that causes Timothy syndrome in humans |
| *egl-19(n582)* | Encodes L-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1C, contains loss-of-function mutation |
| *fsn-1(gk429)* | Encodes an F-box protein that regulates a map kinase cascade, important for axon termination, homolog to human FBXO45, contains null mutation |
| *unc-2(e55)* | Encodes P/Q-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1B, contains null mutation |
| *unc-36(e251)* | Encodes P/Q-type voltage gated calcium channel alpha-2-delta subunit, homolog to human CACNA2D3, contains null mutation |
| *glo-4(ok362)* | Encodes a guanine nucleotide exchange factor, important for axon termination, homolog to human RCBTB1, contains null mutation |
| *rpm-1(ok364)* | Encodes an E3 ubiquitin ligase, important for axon termination, homolog to human/drosophila PAM/Highwire proteins, contains loss-of-function mutation |
| *wdfy-3(ok912)* | Encodes a selective autophagy adaptor protein, variants statistically associated with autism, homolog to human WDFY-3, contains loss-of-function mutation |
| *egl-19(syb4935)* | Encodes L-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1C, contains gain-of-function point mutation equivalent to G402R mutation that causes Timothy syndrome in humans |
| *egp-7(tm2508)* | Encodes a selective autophagy adaptor protein, homolog to yeast ATG-11, contains null mutation |
| *cup-5(ar465)* | Encodes a scaffold protein essential to lysosome formation, homolog to MCOLN3, contains hypomorphic loss-of-function mutation |
| *glo-1(zu391)* | Encodes a Rab GTPase downstream of GLO-4, important for axon termination, homolog to human RAB38, contains a loss-of-function mutation |

| | |
|---|---|
| *ric-7(nu447)* | Encodes a protein important for proper mitochondrial localization in the axon, contains loss-of-function mutation |
| *egl-30(n686)* | Encodes a G protein subunit, important for learning and memory, homolog to human GNAQ, contains a loss-of-function mutation |
| *cca-1(gk30)* | Encodes a T-type voltage gated calcium channel alpha-1 subunit, homolog to human CACNA1G and CACNA1I, contains loss-of-function mutation |
| *msi-1(os1)* | Encodes an RNA binding protein, involved in neurodevelopment, homolog to human MSI1, contains loss-of-function mutation |

**Table 2: List of transgenes.**

| *Transgene* | Description |
|---|---|
| *cueEx19 + cueEx20* | *Pmec-7::egl-19(gof)* at 5 ng/ul + *Podr-1::rfp at 50 ng/ul* used to observe Timothy syndrome mutation in mechanosensory neurons |
| *cueEx21* | *Pmec-7::egl-19(gof)* at 25 ng/ul + *Podr-1::rfp at 50 ng/ul* used to observe Timothy syndrome mutation in mechanosensory neurons |
| *cueEx17 + cueEx18* | *Pmec-7::unc-36::rfp at 5 ng/ul + Pstr-1::gfp at 50 ng/ul* used to observe Timothy syndrome mutation in mechanosensory neurons |
| *muIs32* | *Pmec-7::gfp* is expressed in all mechanosensory neurons, used to observe defects in the mechanosensory neurons |
| *jsls973* | *Pmec-7::rfp* is expressed in all mechanosensory neurons, used to observe defects in the mechanosensory neurons |
| *jsls821* | *Pmec-7::gfp::rab-3* used to observe the localization of chemical synapses in the PLM axon |
| *yadIs12* | *Pmec-4::gfp::unc-9* used to observe electrical synapses in the PLM axon |
| *kyIs262* | *Punc-86::myr GFP + odr-1::RFP* is expressed in touch neurons and interneurons, used to observe the BDU neuron |
| *cueEx22* | *Pmec-7::glo-1::gfp* used to view glo-1 expression patterns in the mechanosensory neurons |
| *cueEx23* | *Pmec-7::glo-1::rfp* used to view glo-1 expression patterns in the mechanosensory neurons |
| *bzIs62* | *Pmec-7::lmp-1::gfp* used to view lysosomes in the mechanosensory neurons |
| *uIs145* | *Pmec-4::aman-2::yfp* used to view golgi in the mechanosensory neurons |
| *sqIs24* | *Prgef-1::lgg-1::gfp* used to view autophagosomes in the mechanosensory neurons |
| *jsIs609* | *Pmec-7::mito::gfp* used to view mitochondria in the mechanosensory neurons |

## Chapter 4 – General Discussion

### 4.1 Summary of key findings

The results of this dissertation research yield a novel model from which to continue autism research in neurons. The model is a neuron morphology defect caused by a genetic variant that is causative for autism. Moreover, this genetic variant also causes a related behavioral defect. This autism model is incredibly useful for studying neuronal development. By using the model system, we uncovered a number of primary findings.

The first finding was genetic evidence for a role for voltage gated calcium channels (VGCC) as regulators of axon termination. We showed that loss of function of VGCC genes suppress the axon overextension phenotype that is caused by loss of *fsn-1* function. Moreover, the autism-linked Timothy syndrome mutation known as *egl-19(gof)* caused an axon overextension phenotype on its own. We also have evidence that synapse integrity remains unaffected by the *egl-19(gof)* mutation, suggesting a synaptogenesis-independent mechanism. As mentioned in the first chapter, the human homolog for *egl-19* known as *CACNA1C* has been associated with a number of different neurodevelopmental disorders (Ferreira et al., 2008; Li et al., 2015), including ASD (Lu et al., 2012; Boczek et al., 2015; Li et al., 2015). The *egl-19(gof)* mutation results in the exact same amino acid change that can occur in *CACNA1C* to cause Timothy syndrome in humans. Therefore, we have uncovered a cellular/genetic mechanism by which the calcium channel CACNA1C could act to influence neuronal development, and when mutated leads to neurodevelopmental disorders. Indeed, the direct connection of the gain-of-function Timothy syndrome mutation in the *CACNA1C* homolog in *C. elegans* is a new piece of evidence associating the ASD-causing mutation to a neurodevelopmental change in the form of axon termination defects. The genetic interactions between the Timothy syndrome mutation and

other known mechanisms governing axon termination only strengthens this link between the ASD-causing mutation and neurodevelopmental changes. Moreover, the resulting axon overextension in the mechanosensory network is a potential contributor to the behavioral defects observed in the Timothy syndrome mutant worms, as the overextension displaces the electrical synapses located at the tip of the PLM to the anterior portion of the worm. The aforementioned electrical synapses located at the PLM axon tip and their role in the mechanosensation of *C. elegans* is the subject of a future study in our lab, as the exact contribution of that synaptic connection with the BDU neuron is not well understood. Regardless, the morphological and behavioral defects induced by the monogenic Timothy syndrome mutation make our ASD model one of great interest for future studies of ASD. However, this model has additional depth which we also uncovered that could link *CACNA1C* and ASD to another mechanism; autophagy.

In addition to the link between an ASD-causing *CACNA1C* variant and axon overextension, the second major finding was a connection between selective autophagy and *CACNA1C* in the development of ASD. Autophagy has been statistically associated with ASD (Krey et al., 2013; Tang et al., 2014; Sragovich et al., 2017; Napoli et al., 2018) and other neurodevelopmental disorders (Lee, 2012; Lee, K.-M. et al., 2013). Selective autophagy is less understood as a cellular mechanism compared to other forms of autophagy, and the evidence linking selective autophagy to neurodevelopmental disorders is limited (Iossifov et al., 2012; Iossifov et al., 2014). We have provided biological evidence for the suppression of *egl-19 (gof)* Timothy syndrome mutation-induced overextension phenotypes by *wdfy-3* selective autophagy gene loss-of-function mutation. While the selective autophagy protein WDFY-3 has been shown to be essential for the development of neurons (Iossifov et al., 2012; Orosco et al., 2014; Dragich et al., 2016; Napoli et al., 2018), our evidence is the first that puts it in a pathway with a genetic

variant that causes autism in humans. Considering that WDFY-3 is an adaptor protein and acts as a scaffold for the targeting and degradation of a variety of different cargo (Simonsen et al., 2004; Pohl & Jentsch, 2009; Filimonenko et al., 2010; Lystad et al., 2014; Napoli et al., 2018), identification of the targets for selective autophagy in this process are likely to yield mechanistic insights. Therefore, identification of the selective autophagy targets in the ASD model we have developed is the subject of a current study.

The evidence we gathered from our research suggests a strong role for selective autophagy as a regulator of axon termination during both normal and pathological development. Indeed, selective autophagy appears to be both an important target for the Timothy syndrome mutation, but also regulates normal axon development in the absence of the Timothy syndrome mutation. As mentioned, we have demonstrated that loss of *wdfy-3* function suppresses the overextension caused by the *egl-19(gof)* Timothy syndrome mutation. Our evidence also shows selective autophagy mutant genes *wdfy-3* and *epg-7* suppress the *fsn-1(null)* phenotype, similar to the *VGCC* loss-of-function mutant suppression on the *fsn-1* lof phenotype. This evidence further strengthens the connection between selective autophagy and the regulation of axon termination. In addition, similar suppression using lysosome hypomorphic lof mutations agree that autophagy is a key regulator of axon termination, as lysosomal degradation of autophagic cargo is the final step of the autophagy pathway (Levine & Kroemer, 2008). The connection extends to the behavioral defects shown in the *egl-19(gof)* Timothy syndrome mutants, as the defects can be suppressed by *wdfy-3(lof)*, further strengthening our model. Overall, our findings strengthen the connection between the *CACNA1C* and *WDFY-3* genes and their role in neurodevelopment.

Finally, our model ties VGCCs and selective autophagy back to ASD. There is a strong ASD connection to the EGL-19 and selective autophagy pathway that will help us understand the development of ASD. Both *CACNA1C* and *WDFY-3* have statistical connections to ASD (Lu et al., 2012; Lee, S.H. et al., 2013; De Rubeis et al., 2014; Iossifov et al., 2014; Li et al., 2015; Wang, T. et al., 2016; RK et al., 2017). Our genetic, mechanistic, and behavioral data further connect these ASD risk variants. The morphological and behavioral changes make for a good model to use for better understanding of ASD. Having a model such as this is vitally important since most ASD linked variants have little effect on their own. Therefore, ASD is likely to arise from interactions between variant genes, and this model can be a useful system to test such interactions. Future research can continue to use this model to further elucidate the mechanistic causes of ASD.

## 4.2 EGL-19 and WDFY-3

### *4.2.1 Selective autophagy and neurodevelopment*

There is much evidence of autophagy influencing axon outgrowth, but not as much concerning the importance of selective autophagy. In cultured mouse neurons, loss of autophagy leads to axon growth, while inducing autophagy inhibits axon growth (Hara et al., 2006). In *Drosophila*, autophagy has been shown to promote development of the neuro-muscular junction (Shen & Ganetzky, 2009). In *C. elegans,* autophagosomes form at synaptic sites and are required for presynaptic assembly (Stavoe et al., 2016; Hill et al., 2019). These studies each provide insight into the importance of autophagy in neurodevelopment. However, the studies were focused on autophagy in a general sense. What we have produced is evidence of selective autophagy regulating axon outgrowth in a manner independent of general autophagy pathways.

Our evidence shows selective autophagy mediates the specific axon outgrowth step of axon termination. Unlike the work done to show that loss of autophagy leads to axon growth in mouse neurons (Hara et al., 2006), we show loss of selective autophagy does not increase outgrowth in *C. elegans.* The pathway we have established shows that loss of function of the selective autophagy gene *wdfy-3* can suppress axon termination defects created by both the *egl-19(gof)* mutation and the *fsn-1(null)* mutation. This suppression of axon termination defects is done without affecting synaptogenesis. This is consistent with the literature, as mutations in selective autophagy genes do not affect the regulation of synaptogenesis (Stavoe et al., 2016). This also suggests that selective autophagy regulation of axon termination is a separate pathway from general autophagic activity at the synapses in *C. elegans*. Overall, this isolation of selective autophagy as a mediator of axon termination is a specialized pathway and could help us further elucidate how selective autophagy affects neurodevelopment.

### *4.2.2 Our findings and their connection with Selective Autophagy and Neurodevelopment*

As outlined in the background, the selective autophagy gene *WDFY-3* has been statistically associated with ASD in multiple studies (Iossifov et al., 2012; Iossifov et al., 2014). In addition, studies done in mice have shown that the loss of WDFY-3 function results in altered neurogenesis and a reduction in neuronal connectivity (Orosco et al., 2014; Dragich et al., 2016). These studies showcase the importance of WDFY-3 during neurodevelopment, and provide examples of what can go wrong when WDFY-3 is not functioning properly. Our findings provide the first biological evidence that associates WDFY-3 with ASD and uncover a neurodevelopmental mechanism that WDFY-3 regulates. In addition, given the known links between autophagy and neurodevelopmental disorders (Krey et al., 2013; Tang et al., 2014;

Sragovich et al., 2017; Napoli et al., 2018), our findings are consistent and support a major role for autophagy in neuronal development. However, the connection between selective autophagy and neurodevelopmental disorders is lacking in terms of clear biological data. We provide novel biological evidence that is consistent with the role of WDFY-3 in regulating neurodevelopment. Moreover, our evidence linking selective autophagy and the ASD-causing Timothy syndrome mutation adds one of the first pieces of biological evidence for selective autophagy playing a role in ASD development

### *4.2.3 The role of CACNA1C in regulating selective autophagy*

There are previously established roles for calcium channels in the regulation of autophagy (Tian et al., 2015; Wang, Y. et al., 2016). Studies show that VGCCs can promote autophagy in *Drosophila* and mice (Tian et al., 2015) and that CACNA1A has a direct connection with autophagosome fusion with lysosomes (Wang, Y. et al., 2016). We have provided biological evidence of a role for CACNA1C mediating selective autophagy for neurodevelopment. Our evidence puts selective autophagy downstream of the EGL-19 calcium channel, seen in the suppression of the overextension induced by the *egl-19(gof)* Timothy syndrome mutation by the *wdfy-3(lof)* mutation. The interaction between CACNA1C and selective autophagy is a novel connection. However, while we have provided a connection between CACNA1C and selective autophagy, the exact way the calcium channel proteins are interacting with the autophagy pathway are something we are still investigating. Because many organelles are important in the autophagy pathway, it could be that VGCCs interact with the lysosome related GLO-1, golgi bodies, late endosomes, or directly with lysosomes. Late

endosomes are a strong candidate for a potential interactor with VGCCs, as there is already evidence of interaction between the two (Tian et al., 2015).

### 4.3 Mechanisms for VGCCs as regulators of neurodevelopment

The novelty of our findings is that they place selective autophagy in a pathway with voltage gated calcium channels in the regulation of axon outgrowth. Variants in *CACNA1C* have been statistically shown to be an ASD risk factor and a risk factor for other neurodevelopmental disorders (Ferreira et al., 2008; Lu et al., 2012; Li et al., 2015). However, the actual mechanisms involved in *CACNA1C* mutation induced neurodevelopmental disorders are still unknown. Our research further strengthens the connection between CACNA1C and ASD by tying CACNA1C to the function of the selective autophagy gene *WDFY-3*, which has also been statistically associated with ASD (Iossifov et al., 2012; Iossifov et al., 2014). Our evidence also adds a possible mechanism for the regulation of axon termination by selective autophagy. Looking beyond CACNA1C to the other VGCCs may allow us to find more context for interpreting our evidence.

Axon termination was not previously recognized as a VGCC mediated step of axon outgrowth. To underscore the importance of VGCCs in axon termination, our data show enhanced suppression of *fsn-1(null)* overextension phenotype when coupled with *unc-36(lof)*, compared to *fsn-1(null)* double mutants with other *VGCC* loss-of-function mutations. This is significant as UNC-36 regulates the function of EGL-19 and the localization of UNC-2 calcium channels (Caylor et al., 2013), thus providing a possible explanation for the enhanced phenotype. However our *fsn-1(null);egl-19(lof);unc-2(null)* showed an enhancement of axon termination defects, suggesting *unc-36* functions to regulate axon termination independent of EGL-19 and

UNC-2. While our data is the first to suggest VGCCs are regulators of axon termination, we are not the first to find that VGCCs affect the general outgrowth of axons. Many studies have linked calcium signaling to the regulation of neurite outgrowth (Gomez & Spitzer, 1999; Zheng, 2000; Henley & Poo, 2004; Wayman et al., 2008; Takemoto-Kimura et al., 2010). A recent study showed that knocking down *CACNA1C* results in shorter axons in mice (Kamijo et al., 2018). Although this study did not focus on axon termination, but instead found that overall neurite length decreases in *CACNA1C* mutants, our findings are in line with these findings, and add more evidence to the regulatory abilities of VGCCs during axon development.

### 4.4 RPM-1 pathway revelations

Our data suggest EGL-19 and WDFY-3 are acting outside of the RPM-1 pathway. While the suppression of the *fsn-1(null)* axon overextension phenotype by both *egl-19(lof)* and *wdfy-3(lof)* mutants means they could be downstream targets of FSN-1, our remaining data suggest they act outside of the RPM-1 pathway. The triple mutant analysis of *fsn-1(null);glo-4(null);unc36(null)* also suggest that EGL-19 and WDFY-3 suppression is specific to FSN-1 only. We also found that *wdfy-3(lof)* caused pleiotropic affects to the PLM axon, which was not observed in any of the VGCC and RPM-1 pathway double mutants. This could be indicative of the variety of roles WDFY-3 plays in mechanosensory neurons, or an interaction between WDFY-3 and the RPM-1 pathway that requires further experimentation. Regardless, there is strong evidence suggesting EGL-19 and WDFY-3 function outside of the RPM-1 pathway.

The primary evidence for the EGL-19 and WDFY-3 pathway being outside the RPM-1 pathway is that there is no *VGCC(lof)* or *wdfy-3(lof))* suppression of the *rpm-1(lof)* severe overextension phenotype. Moreover, in examining the localization of PLM-BDU electrical

synapses in the overextended axon of *egl-19(gof)* mutants, we found that the synapses appear anterior to the ALM cell body at the very tip of the overextended PLM axon. However, work on the RPM-1 mutants examining the same synapses has demonstrated that the *rpm-1(lof)* mutants have overextension of the PLM, but the PLM-BDU synapses are in their normal location and not at the tip of the PLM axon (Borgen, M.A. et al., 2017). We also determined that the PLM-BDU connection in the *egl-19(gof)* worms appeared to be intact. This difference between the *egl-19(gof)* and *rpm-1(lof)* phenotypes, along with our genetic data, suggest that the mechanism of axon termination affected by the *egl-19(gof)* mutation is separate from the RPM-1 pathway.

There are a few explanations for the data we have gathered regarding the RPM-1 pathway. For instance, FSN-1 could also be acting outside of its known function of working with RPM-1 to ubiquitinate DLK-1 (Liao et al., 2004). Thus, FSN-1 could degrade a different target, such as EGL-19, by partnering with a different E3 ubiquitin ligase domain containing protein. Or it could be that EGL-19 and WDFY-3 act on a pathway adjacent to the FSN-1 pathway. There is evidence of additional parallel pathways that regulate the downstream DLK-1 independently of FSN-1 (Tulgren et al., 2011). While PHR proteins are known as axon termination regulators, it could be that WDFY-3 and EGL-19 establish their own pathway, possibly incorporating separate proteins to regulate axon termination.

Additionally, research on RPM-1 has opened up a possible mechanism by which the WDFY-3 mediated selective autophagy functions to regulate axon termination. RPM-1 was found to regulate the trafficking of the SAX-3 (homolog to human ROBO) and UNC-5 receptors to the cell membrane (Li et al., 2008). This is thought to be a potential mechanism for axon termination regulation via RPM-1; via the tight control of the localization of axon outgrowth mediators SAX-3 and UNC-5. We would need to test if the selective autophagy mediated by

WDFY-3 is somehow involved in the regulation of axon outgrowth receptors to determine if the EGL-19 and WDFY-3 pathway is using a similar mechanism to regulate axon termination. That is why determining the target of the WDFY-3 selective autophagy is paramount going forward.

## 4.5 Future Directions

### *4.5.1 AIM 1: Autophagy and Axon Development*

The primary goal for future research is to determine how selective autophagy is regulating axon outgrowth and axon termination. The GFP::LGG1 marker, a well-established and stable marker for autophagosomes, had been used successfully to quantify autophagosome formation (Stavoe et al., 2016; Hill & Colón-Ramos, 2018). We will utilize this marker to examine changes in autophagosome numbers and trafficking in multiple VGCC mutant backgrounds. Specifically, we will image GFP::LGG1 in *egl-19(gof), egl-19(lof)* and *unc-36(lof)* mutant worms. If the Timothy Syndrome mutation promotes autophagy, we would expect to see increased autophagosome clusters in the *egl-19(gof)* mutant background.

We will examine further evidence of a direct relationship between VGCCs and targets related to selective autophagy. One way to address this is to continue examining the different possible targets of selective autophagy in VGCC mutant backgrounds to see what is relevant. These targets could be aggresomes, golgi, ER, lysosomes, lysosome related organelles, and mitochondria. We will focus on the PLM neuron for these experiments, and will utilize the following fluorescent markers: a-synuclein::YFP for aggresomes, AMAN-2::YFP for Golgi, YFP::TRAM-1 for ER, LMP::1::GFP for lysosomes, GFP::GLO-1 for lysosome related organelles, and mitochondria-targeted::GFP (mito::GFP) for mitochondria. These transgenes are readily available and have been used in a number of studies (Hermann et al., 2005; van Ham et

al., 2008; Chen et al., 2016; Melentijevic et al., 2017). We can also test each marker in the following genetic backgrounds*: egl-19(gof), egl-19(lof),* and *unc-36(lof).* We can also utilize *wdfy-3(lof)* and *epg-7(lof)* to test if any changes are dependent on selective autophagy. We can use mitochondria, as we have already observed a possible connection between mitochondria and VGCCs, and given the connection between mitochondria, axon outgrowth, and mitophagy (Morris & Hollenbeck, 1993; Han et al., 2016; Napoli et al., 2018; Smith & Gallo, 2018). If VGCCs are negatively regulating mitophagy, then we would expect loss-of-function mutations of *VGCC* genes to result in increased mitophagy. A previous study suggests upregulated mitophagy increases mitochondria numbers in the axon (Napoli et al., 2018). Therefore, we would expect that the number of mitochondria clusters would increase in *VGCC* loss-of-function backgrounds. Following these morphological experiments, we will do genetic experiments utilizing mutant genes of the various targets of autophagy to support any connections we find. For example, we can use *ric-7(lof)* and *unc-116(lof)* mutants, both of which are vital for the proper localization of mitochondria in the axon (Rawson et al., 2014). If VGCCs promote selective autophagy of mitochondria, we would expect the *ric-7(lof)* and *unc-116(lof)* mutants to exhibit suppression of the overextension caused by the background *egl-19(gof)* Timothy syndrome mutation in a similar way to *wdfy-3(lof).*

Finally, additional selective autophagy genes tested against the *egl-19(gof)* Timothy syndrome mutation may narrow down which type of selective autophagy is downstream of EGL-19. These include *epg-2* and various *sqst* genes, both of which encode for adaptor proteins for selective autophagy (Lamark et al., 2009; Tian et al., 2010; Lin et al., 2013). If any of these selective autophagy adaptor proteins function downstream of EGL-19, then we expect to see a suppression of the overextension caused by the *egl-19(gof)* Timothy syndrome mutation. These

same sets of experiments would also be carried out in regard to the behavioral defects observed in the *egl-19(gof)* Timothy syndrome mutants. We would expect the loss-of-function of selective autophagy proteins functioning downstream of EGL-19 to suppress the increased mechanosensory adaptation. Additional experiments can be done in reverse to test whether genes responsible for axon termination are upstream of selective autophagy. We can use the *wdfy-3(lof)* and *epg-7(lof)* mutations as backgrounds to see if they suppress the effects of the loss-of-function mutant genes that cause overextension of the PLM, such as genes encoding members of the RPM-1 pathway. Further experiments can be done to elucidate the role of the electrical synapses formed between the PLM and the BDU, and whether or not the localization of these electrical synapses play a role in the behavioral changes observed in the *egl-19(gof)* Timothy syndrome mutants. Understanding the PLM-BDU connection would help us determine if the interaction between EGL-19 and WDFY-3 acts during development to alter PLM-BDU connection, or alternatively, if EGL-19 and WDFY-3 function elsewhere in the mature nervous system to affect behavior. This particular line of experiments is not as imperative as those concerning selective autophagy mechanisms, but still may run ancillary to primary studies to test additional cellular mechanisms. Ultimately, all of these future experiments would aid in understanding the specificity of the selective autophagy required for proper axon termination, and how these mechanisms can go awry to lead to neurodevelopmental disorders such as ASD.

### *4.5.2 AIM 2: Human Variants*

Studies have found that missense mutations in *CACNA1C* occur at a higher frequency in individuals with ASD compared to control populations (Schaaf et al., 2011). In this study, a total of 13 out of 376 control individuals carried missense mutations in *CACNA1C,* whereas 26 out of

339 autistic individuals carried missense mutations in *CACNA1C*. Moreover, studies in both humans and mice have associated both gain-of-function and loss-of-function *VGCC* variants to ASD (Splawski et al., 2004; Bader et al., 2011; Bettt et al., 2012; Lee et al., 2012; RK et al., 2017; Dedic et al., 2018). It is therefore important to test human variants of *VGCC* genes of unknown significance in relation to neurodevelopmental disorders. This is especially true given that the frequency of specific autism causing *VGCC* variants is low (Splawski et al., 2004; RK et al., 2017), suggesting many other *VGCC* variants can also contribute to ASD. Since we found that gain-of-function *VGCC* mutations cause axon termination defects, we can use that as an indicator of gain-of-function mutations. The same can be done with loss-of-function variants, as we found that loss-of-function *VGCC* mutations suppress axon termination defects in the *fsn-1(null)* background. We can start by testing additional human variants associated with Timothy syndrome by utilizing CRISPR mutations. An example of a Timothy syndrome variant that we can induce with CRISPR is *CACNA1C* R240C (*egl-19* R193C). We will also test *unc-36/CACNA2D3* variants and *ccb-1/CACNB2* variants, as both genes have variants that have also been associated with ASD (Breitenkamp et al., 2014; Iossifov et al., 2014; Yuen et al., 2015). The findings can be strengthened through experiments showcasing enhanced phenotypes when coupling multiple ASD risk factors. As human variant experiments in *C. elegans* are becoming more efficient with improving genome editing technologies, these kinds of experiments will provide a convincing means of linking human disorders with the evidence gathered through the use of model organisms.

*4.5.3 AIM 3: RPM-1 pathway investigation*

In regard to continued experimentation with the RPM-1 pathway, there are a number of avenues we can consider. Genetic experiments utilizing additional RPM-1 pathway mutants, such as DLK-1, can be useful in further specifying where EGL-19 and WDFY-3 are acting. Rounding out the VGCC genetic testing with RPM-1 and GLO-4 would also be useful in that regard, as we only tested *unc-36(lof)* and *unc-2(lof)* against the loss-of-function background of the majority of the RPM-1 pathway. Another set of experiments we can do is to test the involvement of WDFY-3 in other parts of the RPM-1 pathway. One experiment is to see if *wdfy-3(lof)* suppresses the habituation defects also observed in *fsn-1(null)*, *glo-4(null),* and *rpm-1(lof)* mutant animals (Giles et al., 2015). However, these habituation defects are the opposite of what we observed in the *egl-19(lof)* worms, whereby the *rpm-1(lof)* mutants show a lack of habituation after repeated mechanosensory stimulations (Giles et al., 2015). Regardless, the effect *wdfy-3(lof)* has in these in *fsn-1(null)*, *glo-4(null)*, and *rpm-1(lof)* mutant backgrounds will give us another piece of evidence as to the role of the EGL-19 and WDFY-3 interaction in regard to the RPM-1 pathway. Another is to see if *wdfy-3(lof)* suppresses the axon termination defects of other RPM-1 pathway loss-of-function genes. If the results of these experiments show that *fsn-1(null)* is the only mutant background to be suppressed by *wdfy-3(lof)* and *egl-19(lof)* mutants, then we have further reason to suspect that the EGL-19 and WDFY-3 pathway is outside of the RPM-1 pathway. However, if other members of the RPM-1 pathway are influenced by *wdfy-3(lof)*, then we will need to assess the function of selective autophagy as a broad regulator of axon termination both within and apart from the RPM-1 pathway.

*4.6 Concluding remarks*

In the case of ASD and similar polygenic disorders, uncovering the genetic variants that contribute to the disorder will allow for early detection and preventative treatments long before the development of severe symptoms. Moreover, understanding the genetics of neurodevelopmental disorders will define the eventual therapeutic targets. The prevalent roles of selective forms of autophagy in neurodevelopmental disorders is backed by genetic findings. Additional cellular functions will likely be implicated in various human diseases by our increased understanding of genetics. Determining the mechanisms that regulate the proper development of the neuron is equally important in understanding the underlying causes of neurodevelopmental disorders. Considering the many complexities of studying individual axons in the developing brain, our monogenic ASD model, as well as the relatively simple nervous system of *C. elegans*, will aid in overcoming some of the barriers of understanding neurodevelopment. By progressing in our understanding of the molecular mechanisms that govern neurodevelopment, future findings can extend to other human diseases involving the brain and the rest of the nervous system.

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**CURRICULUM VITAE**

# Tyler Buddell

## EDUCATION

**University of Wisconsin-Milwaukee**, Milwaukee, WI
Doctorate of Philosophy (Ph.D.), Biology, December 2019
Areas of Concentration: Molecular Neuroscience and Genetics

**Knox College**, Galesburg, IL
Bachelor of Arts (B.A.) in Biology**,** June 2014
Areas of Concentration: Molecular Biology, Cell and Developmental Biology
Minors: Chemistry and History

## RESEARCH EXPERIENCE

Dissertation: An Autism-Causing Variant Misregulates Selective Autophagy to Alter Axon Targeting and Behavior **August 2014 – August 2019**
University of Wisconsin-Milwaukee, Milwaukee, WI
PI: Dr. Christopher C. Quinn

Senior Research: Localization of Intersectin1 in *Xenopus laevis* **September 2013 – June 2014**
Knox College, Galesburg, IL
Mentor: Dr. Judith Thorn

Research Assistant: C2C12 myogenic cells as a model for studying cell pathways of Duchenne muscular dystrophy in mammals **June 2013 – August 2013**
Rosalind Franklin University, North Chicago, IL
Mentor: Dr. Joseph X. DiMario

Independent Research: Characterization and localization of a novel gene in *Drosophila melanogaster* **September 2012 – May 2013**
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Research Assistant: Isolating quail myoblast cell line **June 2012 – August 2012**
Rosalind Franklin University, North Chicago, IL
Mentor: Dr. Joseph X. DiMario

Research Intern **June 2010 – August 2010**
Rosalind Franklin University, North Chicago, IL
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## TEACHING EXPERIENCE

Teaching Assistant – Laboratory Cell Biology and Genetics, University of Wisconsin-Milwaukee **August 2014 – May 2019**
**Average Evaluation Score: 4.7 out of 5**

Teaching Assistant – Laboratory Anatomy & Physiology, University of Wisconsin-Milwaukee **August 2019 – December 2019**

## PUBLICATIONS

*PLOS Genetics,* doi: 10.1371/journal.pgen.1008488
**Buddell, T.**, Friedman, V., Drozd, C., Quinn, C.C. "An autism-causing calcium channel variant functions with selective autophagy to alter axon targeting and behavior"

## NOTABLE PRESENTATIONS

"An autism-causing variant misregulates selective autophagy to alter axon targeting and behavior" UWM, Dissertation Defense, **August 21st 2019**

"An autism-causing variant misregulates selective autophagy to alter axon targeting and behavior"
University of Illinois-Chicago, 2nd Chicago Are Worm Meeting, **May 23rdth 2019**

"An autism-causing mutation disrupts axon termination by misregulating selective autophagy"
UWM, Colloquium, **March 8th 2019**

"An autism-causing mutation disrupts axon termination by misregulating selective autophagy"
UW-Madison, Ce NEURO International Conference, Oral Presentation, **June 26th 2018**

"Autism Spectrum Disorder: How Calcium Channel Mutations Can Change the Brain"
UWM, Inaugural 3MT Competition Finalist, **April 4th 2018**

"The role of lamellipodin in regulating axon outgrowth"
UWM, Qualification Exam Defense, **January 10th 2017**

"The role of voltage gated calcium channels in mediating axon termination"
UWM, Dissertation Proposal Defense, **September 23rd 2016**

"The role of voltage gated calcium channels in mediating axon termination"
UWM, Biological Sciences Research Symposium, Poster **April 22nd 2016** + CMB Talk **April 21st 2017 + April 14th 2018**

"The role of voltage gated calcium channels in mediating axon termination"
UWM, Neurosymposium Micro-talk & Poster Presentation, **March 11th 2016 + March 3rd 2017 + March 16th 2018**

"Investigation of how the *unc-2* and *unc-36* calcium channel genes regulate axon outgrowth"
UWM, Cell and Molecular Biology Seminar Presentation, **April 1st 2015 + February 17th 2016**

## ADDITIONAL CONFERENCES ATTENDED

1st Chicago Area Worm Meeting (published abstract), University of Illinois-Chicago, May 24th **2018**

Milwaukee Worm Meeting, Marquette University, Milwaukee, WI, October 23rd **2015**

*C. Elegans* 20th International Meeting, University of California, Los Angeles, CA, June 24th-28th **2015**

## AWARDS AND HONORS

Chancellor's Graduate School Award **2014-2019**
Clifford H. Mortimer Award **2017**
UWM Inaugural 3MT Finalist **2018**
Ruth I Walker – Graduate Grant-in-Aid Award **2018**
James J Magnino, MD Scholarship **2019**