Degradation of SLO-1 BK channels in C. elegans

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Degradation of SLO-1 BK channels in *C. elegans*

Abstract
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Degradation of SLO-1 BK channels in *C. elegans*

by

James J. Haney

April 24th 2017

The report of the investigation undertaken as a Senior Thesis, to carry two courses of credit in The Department of Biology

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Abstract

SLO-1 is a large conductance potassium channel which when inhibited in *C. elegans* results in complete ethanol resistance. The protein ERG-28 is responsible for transporting SLO-1 from the endoplasmic reticulum to the golgi apparatus in *C. elegans*. *erg-28* mutants show greatly reduced SLO-1 signaling. We therefore hypothesize that SLO-1 undergoes ER associated degradation via the ubiquitin-proteasome system. We screen for ubiquitin ligase responsible for the degradation of the SLO-1 channel by performing fluorescent microscopy and behavioral studies in *C. elegans*. The mutants for this ubiquitin ligase showed both increased fluorescence and sensitivity to ethanol. In order to better elucidate the *erg-28* and *slo-1* pathways we also performed mutagenesis tests on *C. elegans* with MCHERRY tagged ERG-28. We created multiple mutants expressing a change in fluorescence. These experiments identify different molecules involved SLO-1 signaling pathway.
Acknowledgments

I think it is important to understand that research is a collaborative effort, that the overwhelming majority of the research papers I have read have been written in the first person plural tense. That is to say, they used, “we” where they might have used, “I.” That’s because research is typically the result of a group effort. People support and challenge each other all in the pursuit of saying something that is true. Here are some people who deserve recognition for the support and challenges they have provided me.

Thank you Dr. Hongkyun Kim and Dr. Kelly Oh for allowing me to work in your lab. You both have been a constant source of support throughout this process, and I am truly grateful. The knowledge and resources you have provided me with are simply unquantifiable. Thank you Dr. Shubhik DebBurman for being the chair of my thesis committee. Your insight has drastically improved the quality of this work. Thank you Dr. Jason Cody and Dr. Ann Maine for also providing your expertise to improve this work. Also thank you to Tim Cheung for cataloging some of the many procedures done in the lab.

There are a host of other people who deserve recognition for my development as a scientist, however a comprehensive list of their names could be a paper in its own right. Instead, I’ll only name the people directly related to this work. However, to all the people I owe my talent and fascination with science, dating back to the very first, “I wonder how that works?” thank you.
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Introduction

Alcohol is a widely used drug. In the United States, more than 80% of adults report using alcohol at some point in their lifetime (Substance Abuse and Mental Health Services Administration). Of the twenty-seven amendments to the United States Constitution, two have been expressly related to the sale and consumption of alcohol. The history and cultural significance of this one drug is staggering. Alcohol appears to be a permanent fixture in our culture. The source of alcohol’s popularity is its effects on the body. To understand why, first we must examine some basic structures in human physiology.

The Nervous System

Alcohol affects many different systems, organs, and tissues in the body. This includes detrimental effects toward the heart and liver. These effects are by no means trivial, but the impact on the nervous system can be far more severe. The nervous system is divided into two parts, the peripheral nervous system and the central nervous system. The peripheral nervous system is the network of nerves outside of the brain and spinal cord. Included in this are the nerves responsible for action and sensation. The central nervous system is the network of nerves which make up the brain and spinal cord. These are responsible for interpreting and responding to information received by the peripheral nervous system (Reece, et al., p. 564).

Both the central and peripheral nervous systems are made up of neurons and glial cells. Neurons communicate with each other using chemical and electrical signals.
This communication is facilitated by glial cells, either by protecting neurons, or increasing signal speed (Reece, Taylor, Simon, Dickey, & Hogan, 2015, p. 564). An electrochemical signal will travel across the neuron causing the release of a chemical signal which will interact with an adjacent neuron. This chemical signal is called a neurotransmitter, and can either activate or deactivate the neuron it binds to. This activation and deactivation will change the likelihood that a neuron will fire a signal. The neurotransmitter must cross a small junction, called a synapse, in order to reach the adjacent neuron. (Reece, et al., p. 565). Alcohol can affect the nervous system by interfering with neurotransmitters and synapses, specifically those located in the brain.

**The effects of alcohol on the nervous system**

Alcohol is a broad term. It refers to a large group of molecules characterized by a specific chemical structure. These molecules can vary widely in their effects on the body or in other how they react with other molecules. The alcohol most commonly consumed is drinking alcohol, or ethanol. For this paper alcohol and ethanol will be used synonymously.

Alcohol interacts with reward pathways in the central nervous system, leading to addiction (Herz, 1997). Addiction is a primary chronic disease associated with dysfunction in reward, motivation, and memory functions, all of which occur in the central nervous system (American Society of Addiction Medicine, 2017). Approximately 7% of the adult population in the U.S. has a disorder related to alcohol use (NIAAA, 2016). Annually alcohol accounts for 31% of all driving fatalities and is the fourth largest
preventable cause of death (NIAAA, 2016). However, the most tragic effects of alcohol occur in infants.

Over 40,000 infants suffer from fetal alcohol syndrome disorders, which are caused by pregnant mothers consuming alcohol. Fetal alcohol syndrome disorders (FASD) are spectrum disorders which are over four times more prevalent than Down Syndrome in the U.S. (FASD, 2007). People with fetal alcohol syndrome suffer from learning disabilities, growth defects, and difficulty making judgements (FASD, 2007). These problems result from neurodegeneration throughout the brain (Ikonomidou, et al., 2000). The most tragic part of fetal alcohol syndrome is it is completely preventable. Pregnant mothers simply have to not drink alcohol. Yet, this still occurs partly because of the addictive effects of alcohol.

Alcohol changes mood and behavior (NIAAA; Oscar-Berman & Marinković, 2007). In the central nervous system, alcohol damages the cerebellum, resulting in loss of motor coordination and cognitive function (Oscar-Berman & Marinković, 2007). As a result, excessive consumption of alcohol affects memory, balance, and one’s emotional state. Alcohol can also damage the cerebral cortex, resulting in reduced learning and problem solving skills (NIAAA; Oscar-Berman & Marinković, 2007). Alcohol’s effects on these systems occur on the cellular level.

The cell

The cell is the most basic unit of life. By definition, all of life is composed of cells (Reece, et al., p. 4). A cell is surrounded by a cell membrane comprised of a
phospholipid bilayer with integrated membrane proteins. These proteins could have a variety of functions such as the transportation of molecules into or out of the cell. Another protein function is signal transduction in which a molecule binds to the protein, causing a reaction inside the cell (Reece, et al. p. 54).

At the center of the cell is the nucleus, which contains the genetic information (DNA) necessary to make proteins. To make a protein, DNA must first be transcribed into a messenger RNA, or mRNA which is then translated into a protein at ribosomes in either the endoplasmic reticulum (ER) or cytoplasm. Proteins translated at the ER are then transported to the Golgi Apparatus, where they will be sent to the cell membrane, an organelle, or excreted. (Reece, et al., p. 58-59).

This transportation to the Golgi Apparatus occurs by through vesicles. In the ER, a protein enters a vesicle, or can be imbedded into the vesicle, which then moves to the Golgi Apparatus in order to be sent to its final destination (Connerly, 2010). The formation of this vesicle is mediated by a complex of proteins called COP II (Jensen & Schekman, 2011). This vesicle is then moved to the Golgi apparatus, to be sent off to its final destination (Glick & Malhotra, 1998). Occasionally this process needs to be mediated by a cargo protein (Herzig, Sharpe, Elbaz, Munro, Schuldiner, 2012). Alcohol can these different cellular processes.

**The effects of alcohol on the cell**

In order for alcohol to affect higher functioning tissues, it must cause changes to the individual cells that make up those tissues. Fetal alcohol syndrome is characterized
by neurodegeneration of the cerebellum resulting from exposure to alcohol (Ikonomidou. et al., 2000). In order to understand why this neurodegeneration occurs, we must examine the cellular level. The neurodegeneration can be explained by alcohol getting metabolized into other reactive oxygen species inside the cell, resulting in oxidative stress (Wu & Cederbaum, 2003; Das & Vasudevan, 2007; Manzo-Avalos & Saavedra-Molina, 2010). We can examine addiction with a similar method. cAMP, a molecule found throughout the cell, is an important part of the cellular mechanism involving addiction (Nestler, 1992; Nestler & Aghajanian, 1997). cAMP concentration increases in the nucleus accumbens after alcohol administration (Nestler & Aghajanian, 1997).

Alcohol can also act specifically on the nervous system by affecting certain neurotransmitters in the brain such as GABA and glutamate (Manzo-Avalos & Saavedra-Molina, 2010). GABA must cross the synapse and activate the GABA receptor, while glutamate must activate the NMDA receptor. When GABA binds to the GABA receptor, the receptor opens, causing an influx of negatively charged chloride ions (Davies, 2003; Gottesmann, 2002). This inhibits the cell, making it less likely to send a signal. Glutamate does the opposite, binding to the NMDA receptor, causing an influx of positively charged calcium ions (Blanke & VanDongen). This excites the neuron, making it more likely to fire (Dandolt, n.d.).

Alcohol enhances the effects of GABA signaling, and decreases the effects of glutamate signaling (Manzo-Avalos & Saavedra-Molina, 2010; Edenberg, 2004). Alcohol
cannot directly activate GABA receptors in the same sense that GABA can. Instead alcohol potentiates the GABA receptor, almost as if it is priming the receptor for the GABA neurotransmitter. When GABA binds to the ethanol induced potentiated receptor, it results in the receptor staying open longer, inhibiting the cell further (Davies, 2003). The potentiation of the GABA receptor by alcohol is only one example of alcohol’s inhibitory effects. Alcohol also directly blocks the binding of glutamate to NMDA receptors (Nagy, 2008). This will also contribute to alcohol’s inhibitory effects, but also causes sensitivity to glutamate. This may result in withdrawal symptoms, which further explains alcohol’s addictive effects (Nagy, 2008). The result of both of these receptors is an inhibitory effect on the cell, and therefore the nervous system.

The effects of alcohol on the cell are summarized in Figure 1. These are the general effects of alcohol that on the cellular level can cause changes in higher functioning tissues. The experiments presented here will examine a specific pathway important for the cellular response to alcohol. This pathway centers around the SLO-1 potassium channel. SLO-1 responds to ethanol differently in different organisms, as do other structures involved in this pathway. Therefore, before examining the specific relationships between alcohol, SLO-1, and the other structures, it is helpful to have some context on the organism being examined and how it relates to other model organisms.
Figure 1. Effects of ethanol on the cell. Alcohol can interact with molecules inside the cell to create reactive oxygen species, which can damage the cell. Alcohol can also affect receptors in the plasma membrane such as GABA or glutamate receptors, causing an influx of negative ions and preventing an influx of positive ions. While ethanol reacts differently with GABA and glutamate receptors, the result in both cases is an inhibitory effect on the cell.
Model Organisms

In order to explore the lesser understood cellular effects of alcohol, appropriate organisms are necessary to test on. Multiple model organisms are available to use, and each has specific advantages and disadvantages.

Humans are not the ideal model species for these experiments. Human trials are expensive to perform and are strictly regulated, which will greatly limit the nature of experiments that can be performed. Statistics about the prevalence of alcohol presented in this paper already can be considered to be human trials. However, these are observational statistics. For the purpose of studying behavior and even cellular effects, it would be necessary to examine the behaviors and cells of intoxicated people. While these studies are possible, they present a large number of health risks and financial obstacles, ultimately making humans a poor choice of organism.

A better option is to use mice. Mice are less expensive to use than humans. The generation time for mice is approximately 10 weeks. A mother will give birth to around 5-10 mice at once (Silver). Behavior and cellular studies can be performed on mice. Genetically, mice are relatively similar to humans compared with other invertebrate organisms. This similarity allows mice to be used to study analogous structures in the human body, such as the liver (Yin, et al. 1999; Xu, et al. 2011). They can even be used to study things that would be too dangerous to study in humans, such as fetal alcohol syndrome (Chernoff, 1977). However, while cellular studies are definitely possible in mice, invertebrate species will be more effective and less expensive to use.
Invertebrate species such as yeast, *Drosophila*, and *C. elegans* have almost no legislative protection, which allows for almost any type of experiment to be performed. They are also much less expensive, have shorter generation times, and typically give birth to more organisms. Drosophila have a generation time of approximately 9 days, *C. elegans* need 3-4, and yeast need just over an hour. Invertebrates share many genes with humans, but are still very distantly related. As a result, studies done with invertebrates may not directly translate to humans.

Yeast cells are an enticing option because of their short generation time. However, behavioral studies cannot be performed on yeast. Studies of alcohol using yeast are largely biochemical studies, such as examinations of alcohol dehydrogenase (Hayes & Velick, 1954; Vallee & Hoch, 1955; Wratten & Cleland, 1963). *D. melanogaster* and *C. elegans* serve as an acceptable middle ground for the purposes these experiments, as behavioral studies are possible, while they are still ideal for cellular studies.

The experiments conducted here will only be done with *C. elegans* because of their cost effectiveness, short generation time, and versatility with experiments that can be performed. The entire genome of *C. elegans* has been mapped out, making genetic modification easy to perform (C. elegan sorting consortium, 1998). *C. elegans* lack many structures common in humans, such as a brain, but share many analogous gene sequences (C. elegan sorting consortium, 1998). They are typically hermaphrodites, but can produce males (Wormatlas). Most importantly, they have a nervous system.
(Wormatlas). This allows us to study the potassium channel SLO-1, which is present in both humans and *C. elegans*.

**SLO-1 and ERG-28**

Alcohol affects the cell in a variety of ways. Alcohol causes oxidative stress by reacting inside the cell to form reactive oxygen species. Alcohol can also interact with membrane bound proteins such as GABA and glutamate receptors, as well as many other channel proteins. SLO-1 is a large conductance potassium channel, also called a BK channel, activated by Ca\(^{2+}\) (Wang, Saifee, Nonet, Salkoff, 2001; Wei, Solaro, Lingle, Salkoff, 1994). Ca\(^{2+}\) activates the channel by binding to the cytoplasmic tail of the protein (Xia, Bing, Lingle, 1999). SLO-1 is well conserved in mice, Drosophila, and humans (Wang, et al., 2001; Wei, et al. 1994). A study done on mice found that a calcium activated BK channel KCNMA1 was important to the pacemaker function of the superchiasmatic nucleus (Meridith, *et al.*, 2006).

In *C. elegans*, loss of function slo-1 mutants exhibit increased tolerance to ethanol (Davies, *et al.*, 2003). Tolerance was tested by measuring the movement patterns, speed, and egg laying. Davies, *et al.* (2003) further tested this tolerance using patch clamping electrophysiology. They showed that slo-1 mutants’ tolerance to alcohol is the lack of alcohol-mediated SLO-1 potentiation. This can be seen in Figure 2. SLO-1 is therefore intimately involved with the cellular response to alcohol.

Different model organisms may have analogous proteins, however, they may not always be directly related to one-another. In *D. melanogaster*, the SLO-1 homolog
Figure 2. Slo-1 BK channel. Alcohol binds to the channel, resulting in a conformational change which opens the channel. A.) shows the channel closed and unbound to alcohol. B.) shows the open channel as a result of ethanol binding. The result is an efflux of potassium leading to a hyperpolarized cell. This is similar to alcohol binding to GABA receptors in that both resulted in the hyperpolarization of the cell. However, there is an important difference with SLO-1. Ethanol was able to bind to the GABA receptor, but could only keep the channel open for a longer period of time once GABA bound to it. SLO-1 channels can actually be opened by ethanol.
slowpoke is important for the development of ethanol tolerance (Crownmeadow, Krishnan, Atkinson, 2005). Ethanol tolerance is controlled by a different protein in C. elegans. Davies, Bettinger, Thiele, Judy, and McIntire (2004) found NPR-1 regulated ethanol tolerance in C. elegans. In rats, ethanol activates similar BK channels, leading to an inhibitory effect (Dopico, Anantharam, Treistman, 1997).

SLO-1 is translated in the endoplasmic reticulum before being transported to the Golgi Apparatus (Oh, et al., 2017). Transportation to the Golgi Apparatus is facilitated by a complex of proteins, called COPII, and ERG-28, a cargo protein. Mutants for erg-28 do not show a buildup of SLO-1 in the endoplasmic reticulum. Instead, erg-28 mutants show an overall decrease in SLO-1 signaling. Control of SLO-1 concentration is therefore likely regulated by degradation of the SLO-1 channel in the endoplasmic reticulum. This is called endoplasmic reticulum associated degradation and typically involves the ubiquitin proteasome system.

**Endoplasmic reticulum associated degradation**

Membrane and secreted proteins are translated from mRNA in the endoplasmic reticulum. However, proteins are not translated into their three-dimensional shape. Proteins, during translation, take the form of a chain of amino acids. This chain will spontaneously fold into a three-dimensional shape. This process occurs in the endoplasmic reticulum (Reece, et al., p. 60).

One problem that can occur in this process is a misfolded protein. Protein conformational disorders can be very serious, and include diseases such as Parkinson’s
If there is a misfolded protein, the cell will attempt to degrade the protein. The mechanisms by which misfolded proteins in the endoplasmic reticulum are degraded is called endoplasmic reticulum associated degradation, or ERAD (Christianson & Ye, 2014). The cell accomplishes this by tagging the problematic protein with ubiquitin. Ubiquitin bound to a substrate will result in the binding of a proteasome to the substrate, and will result in degradation.

**Ubiquitin**

Ubiquitin is tagged to misfolded proteins in order to transport them to a proteasome. The proteasome then destroys the misfolded proteins. This combination of ubiquitin and proteasome make up the ubiquitin proteasome system or UPS (Christianson & Ye, 2014; Hershko & Ciechanover. 1998). Other proteins are involved in this process to help ubiquitin bind and transport the substrate, yet the crucial part is ubiquitin. Once bound to a substrate, ubiquitin can transport it to the proteasome without the help of a transport protein (Baldrige & Rapoport, 2016). Ubiquitin can be attached in chains to increase its effectiveness, removed, and at times both may occur to a single substrate (Christianson & Ye, 2014). While this is not energetically effective, it does improve the selectivity of ubiquitin (Christianson & Ye, 2014).
Figure 3. **Ubiquitination of a substrate.** The substrate is typically thought of as a misfolded protein, but this is not necessarily the case. Once ubiquitin is attached to the substrate, it is transported to a proteasome to be degraded. Multiple ubiquitin molecules can be bound to a substrate, and ubiquitin can also be removed before the substrate is degraded. While this is energetically unfavorable, it does make degradation of a substrate more selective. Ubiquitination occurs in the cytosol of the cell.
In order to attach to a misfolded protein, ubiquitin uses three proteins. This process is explained in Figure 3. First it is activated by an ubiquitin activating enzyme or E1. Once this occurs, ubiquitin can work with a conjugating enzyme (E2) to bind to a substrate which has been bound to an ubiquitin ligase (E3) (Christianson & Ye, 2014). Whereas there are only a few E1s or E2s, there are many different types of ubiquitin ligases (E3) (Christianson & Ye, 2014). This makes sense given that the ubiquitin ligase binds to the specific misfolded protein, while E1s and E2s bind to each other and ubiquitin. However, the ubiquitin ligases do not work in a one to one ratio with substrates. Instead, one ubiquitin ligase can bind to multiple substrates (Bays, Gardner, Seelig, Josaziero, 2001; Jolliffe, Harvey, Haines, Parasivam, Kumar, 2000) and two ubiquitin ligases can even bind to the same substrate (Swanson, et al., 2001). Controlling the expression of glutamate receptors via ubiquitination has already been seen in C. elegans (Burbea, Dreier, Dittman, Grunwald, Kaplan, 2002). The number of different ubiquitin ligases is surprisingly large. In humans, estimates suggest there are over 500 different ligases (Li, et al. 2008). In C. elegans, it decreases to about 100 (Kipreos, 2005).

Currently we do not know how SLO-1 is degraded in the cell. Based on the information we do know about SLO-1, we hypothesize that SLO-1 degradation occurs in the endoplasmic reticulum via the ubiquitin proteasome system. If this hypothesis is true, then there must be an ubiquitin ligase responsible for the binding of ubiquitin to SLO-1.
Aims

An overview of the relevant background information can be seen in Figure 4. The purpose of these experiments is to learn more about the degradation of SLO-1. If SLO-1 is degraded through the UPS system, then it must first bind to an ubiquitin ligase. If the ubiquitin ligase cannot bind to SLO-1, then SLO-1 cannot be degraded through this pathway, resulting in an increase in SLO-1 signaling. Increased SLO-1 signaling will result in both cellular and behavioral changes. We also want to discover other parts of the SLO-1 pathway.

Identifying the SLO-1 ubiquitin ligase

To test SLO-1 degradation SLO-1 signaling will be decreased by mutating erg-28. SLO-1 signaling will then be rescued by inhibiting ERAD ubiquitin ligases through RNA inhibition (RNAi). Decreased mRNA will result in decreased protein synthesis If we inhibit the SLO-1 ubiquitin ligase in an erg-28 mutant, we should see increased SLO-1 signaling. We hypothesize that this ligase will be MARC-6.

Behavioral changes in erg-28 mutants

The increased ethanol resistance shown by erg-28 mutants is a result of the decreased SLO-1 signaling. If the ubiquitin ligase which binds to SLO-1 is mutated, erg-28 mutants will show a rescue of ethanol sensitivity. erg-28 mutants which also have a loss of function mutation for the SLO-1 ubiquitin ligase will show ethanol sensitivity.
**Figure 4. SLO-1 functional overview.** SLO-1 is transported from its synthesis in the ER to the Golgi Apparatus, to its final destination in the cell membrane. The purpose of these experiments is to identify the ubiquitin ligase (E3) responsible for ubiquitinating SLO-1 in *C. elegans*, as well as further elucidate the signaling pathway of the cargo protein. The focus of this research is therefore on the ubiquitin ligase (E3) and the cargo protein. It is important to note that a secondary mechanism not shown here exists that is also capable of moving SLO-1 to the plasma membrane, as inhibiting the cargo protein does not completely eliminate SLO-1 signaling. However, this secondary mechanism is not as effective as the cargo protein, as null mutants for the cargo protein show decreased SLO-1 signaling.
comparable to wild type *C. elegans*. We therefore hypothesize that *marc-6* null mutants will exhibit ethanol sensitivity characteristic of an increase in SLO-1 signaling.

*erg-28*

To examine other parts of the SLO-1 pathway, we will examine ERG-28. By further mutating *C. elegans* with ERG-28 tagged with red fluorescent protein, we hope to uncover different mutations affecting SLO-1 through ERG-28. These mutations may result in gain of function or loss of function.
Methods

Inhibition of targeted ER active genes using RNAi

RNAi

Reduction of the ubiquitin ligase was first attempted by using RNAi to inhibit different genes. RNAi reduces the amount of mRNA coding for a particular protein, making that protein more difficult to produce. This is accomplished by feeding worms with double stranded RNA (dsRNA) for a target gene. dsRNA works on the cellular level. The dsRNA is cleaved into small portions in the cell. These portions are used to identify other strands of RNA. As a result, if mRNA from its own nucleus has the same code as the dsRNA it has been fed. Since mRNA is a crucial part of the translation of DNA into proteins, reduction of mRNA will result in a reduction of the protein. The cell will still produce the selected protein, but not as much.

In order for dsRNA to enter the cell, it must be fed to the worms. The best way to accomplish this is to induce dsRNA expression of a target gene in bacteria, then feed that bacteria to the worms. Each target gene was amplified from N2 genomic DNA using PCR with Q5 polymerase. The PCR product was engineered to have a recognition site for the NotI restriction enzyme, as well as the sequence for the target gene. The product was digested with NotI and ligated with an empty vector. The vector, (L4440) was digested with NotI and alkaline phosphatase in order to ensure successful ligation. Once ligated, the plasmid was introduced into DH5α competent bacteria. Successful subcloning was confirmed using gel electrophoresis, then the plasmid was purified and
introduced into HTTP competent cells. The dsRNAi cannot be induced in DH5α cells, however it is very difficult to introduce a plasmid into HTTP cells. The HTTP cells were cultured onto agar plates made with carbinocilin and IPTG where they were fed to the worms. IPTG induces the expression of the dsRNA.

*C. elegans*

Worm strains that were tested were *slo-1::gfp erg-28 uls69; lin-15b* and *slo-1::gfp; uls69; lin15b*. SLO-1::GFP was obtained using CRISPR/Cas9 to attach GFP to the SLO-1 protein. This worm was then crossed with an *erg-28* mutant, then crossed with a *uls69* mutant, then finally crossed with a *lin-15b* mutant. A similar process was performed to obtain the *slo-1 gfp; uls69; lin15b* strain. A loss of function mutation in *erg-28* causes improper transport of SLO-1 to the cell membrane, allowing increased ERAD of SLO-1. *uls69* promotes the transportation of RNAi from the bacteria to the neurons of the *C. elegans*. *lin-15b* is a mutation that decreases the worm’s ability to eliminate RNAi, thus increasing the effectiveness of RNAi. A control strain of *slo-1 gfp; uls69; lin15b* was also tested. The worms were fed a control RNAi as well as RNAi for *gfp, sel-11, marc-6, ddi-1, ddb-1, crbn, and cul-4*.

RNAi for GFP and DDI-1 were both used as controls. GFP was a negative control. Inhibiting GFP should result in decreased fluorescence compared to control. DDI-1 was used as a positive control. Previous research shows that DDI-1 acts as a proteasome for SLO-1 (Oh, et al., 2017). Therefore, inhibiting DDI-1 should result in increased SLO-1 fluorescence. *Sel-11, marc-6, ddb-1, crbn, and cul-4* had all been previously identified as

Feeding protocol & microscopy

Worm strains were picked onto the RNAi induced plates. The worms were grown, but were not allowed to starve. Adults were then picked onto a second plate of the same RNAi, allowed to lay eggs, then the adults were removed, leaving only the eggs. This synchronized all the worms being tested ensuring they were all the same age. The fluorescence of the GFP tagged SLO-1 was then examined in the nerve ring and dorsal cord using fluorescence microscopy. RNAi that showed an increase of SLO-1 was mutated further to completely eliminate the ligase. These strains were further tested using fluorescent microscopy.

Characterization of marc-6 null mutation

Fluorescent Microscopy

marc-6 was chosen for further mutation based on the results of the fluorescent assay. A null mutation for marc-6 was obtained and then crossed with males from two worm strains, slo-1::gfp and slo-1::gfp erg-28. The result of this was four worm strains, slo-1::gfp, slo-1::gfp marc-6, slo-1::gfp erg-28, and slo-1::gfp erg-28 marc-6. Fluorescent microscopy was then performed on these worm strains, similar to the method of microscopy performed on the worms fed dsRNA. No special feeding protocol was
necessary since inhibition was determined by the presence or absence of a mutation. The dorsal cord and nerve ring were still examined.

**Behavioral Studies**

The fluorescence of *slo-1::gfp, slo-1::gfp marc-6, slo-1::gfp erg-28*, and *slo-1::gfp erg-28 marc-6* worm strains were tested for the rescue of alcohol sensitivity. Plates containing nematode growth media (NGM) were dried at room temperature for 2 hours, then 3 copper rings were melted into the media to allow three strains to be tested simultaneously. Ethanol was then pipetted onto the plate. 10 worms of each strain were first transferred to a plate with no food for 30 minutes before being transferred to the respective ring on the ethanol plate. The worms were allowed to adjust to the ethanol plate for 20 minutes before their speed was recorded. The speed of the worms was measured using a CCD camera Image-Pro Plus 6.3 software. The movements of the worms were recorded for 2 minutes, and the average speed of the worms was calculated. The ubiquitin ligase mutant was tested with an *erg-28* mutant and a wild type N2 worm.

**Mutagenesis of mcherry::erg-28**

**Integration**

In order to elucidate the interactions of ERG-28 and SLO-1, we integrated a transgene for ERG-28 tagged with MCHERRY into a wild type N2 worm. MCHERRY is a fluorescent molecule similar to GFP. Integration was accomplished by inserting the
transgene into the gonads of a wild type N2 worms. The progeny of those worms were then examined to confirm the successful insertion of the fluorescent transgene into the genome of the worm.

The worm strain that showed the strongest fluorescence then had the transgene integrated into their genome. To accomplish this, the worms were exposed to a 50µg/ml solution of trimethyl psoralen (TMP) in DMSO, then 350 µJ (x100) long wave UV radiation. The combination of TMP and UV radiation allows TMP to form cross linkages on the DNA strand. These cross-links, if left unrepaired, make DNA replication impossible and lead to cell death. In order to repair the DNA, the cross-linked section is broken off. When the cell repairs this break, the DNA insert can be integrated into the genome. However, this is uncommon and requires many worms to perform successfully.

In order to find the worms with the integrated insert, 350 worms were picked onto individual plates from the offspring of the worms exposed to TMP. Only worms which displayed fluorescence were picked. Those plates were allowed to grow and produce offspring. Plates that had non-sterile worms with heterozygous worms for fluorescence then had 3 worms picked onto individual plates in order to find a homozygous genotype for fluorescence.

Mutagenesis

In order to learn more about erg-28, a mutagenesis experiment was performed. The worm strain integrated with erg-28 tagged with MCHERRY was exposed to the
mutagen ethyl methanesulfonate (EMS). The second generation of offspring was then screened for an increase or decrease in fluorescence of MCHERRY.
Results

The purpose of these experiments is to identify an ubiquitin ligase responsible for the degradation of SLO-1. To accomplish this, we first targeted multiple genes known to code for proteins active in the ER using RNAi. Successful RNAi of the ubiquitin ligase should show increased fluorescence of SLO-1. Upon identifying a candidate from RNAi experiments, we then sought to examine this candidate further by mutating the target gene. Null mutants for the ubiquitin ligase should show increased SLO-1 signaling and therefore increased ethanol sensitivity. Finally, we sought to elucidate the SLO-1/ERG-28 signaling pathway by performing mutagenesis experiments of mcherry::erg-28 mutants.

Inhibition of targeted ER active genes using RNAi shows increased SLO-1 signaling in MARC-6 target nerve rings

The first goal of this study was to identify the ubiquitin ligase responsible for the ubiquitination and degradation of SLO-1. This was done by performing a fluorescent assay with RNAi for select ubiquitin ligases. If the ubiquitin ligase responsible for the degradation of SLO-1 is reduced, the result should be an increase in SLO-1 channels over a control. If SLO-1 is tagged with a fluorescent protein, the result should be an increase in fluorescence.
Figure 5. Fluorescent assay of worms fed dsRNA for ER active gene targets

A.) nerve rings used for qualitative assessment of the different levels of fluorescence. The control is an empty vector, meaning no inhibition of RNA. GFP was used as a negative control.

B.) Qualitative analysis of the dsRNA fed worms (n=39). No difference in fluorescence is seen compared to control in the slo-1::gfp erg-28 uls69; lin-15b worm strain. slo-1::gfp uls69; lin-15b was used as a control strain to confirm decreased fluorescence from erg-28 mutants.
RNAi was performed in two different worm strains, *slo-1::gfp erg-28 uls69 lin-15b* and *slo-1::gfp uls69 lin-15b*. No significant difference was seen in *slo-1::gfp erg-28 uls69 lin-15b* worms fed different dsRNA. With a lack of quantitative evidence, no behavioral studies were performed. Figure 5A shows qualitative analysis of the *slo-1::gfp erg-28 uls69 lin-15b* worms fed different dsRNA. With a lack of quantitative evidence, no behavioral studies were performed. Figure 5A shows qualitative analysis of the fluorescence in the nerve rings for each type of RNAi. Figure 5B shows quantitative analysis of the fluorescence in the dorsal cord. Qualitatively, *marc-6* RNAi appears to result in a slight increase in SLO-1 signaling in the nerve ring.

Characterization of *marc-6* null mutations show increased SLO-1 signaling and ethanol sensitivity

Analysis of SLO-1 levels using fluorescence microscopy

MARC-6 RNAi studies showed a qualitative increase in the fluorescence of SLO-1 channels. This was examined further by studying the effects of a *marc-6* null mutation on SLO-1 signaling. This was accomplished by first performing a fluorescent assay. The results of this assay can be seen in Figure 6. Similar to the RNAi experiments, Figure 6A shows the qualitative analysis of the nerve rings while Figure 6B shows the quantitative analysis of the dorsal cords. *slo-1::gfp erg-28 marc-6* is a double mutant for *marc-6* and *erg-28* with GFP tagged SLO-1. This was tested with *slo-1::gfp erg-28* as a control. *slo-1::gfp marc-6* was used to assess the effects of *marc-6* in the absence of the *erg-28* mutation. The fluorescent assay in mutant animals has the same goal as the RNAi assay and applies the same principles. The difference is that MARC-6 is reduced not using
Figure 6. Fluorescent assay of marc-6 mutants A.) sample stacked images of the neuronal ring in order to allow for qualitative analysis. The nerve rings seem to suggest an increase in SLO-1 signaling in slo-1::gfp erg-28 marc-6 over slo-1::gfp erg-28. B.) the quantitative analysis of the dorsal cord fluorescence of the four worm strains (n=28). slo-1::gfp marc-6 shows increased fluorescence over slo-1::gfp. slo-1::gfp erg-28 marc-6 does show increased fluorescence over slo-1::gfp erg-28. However, none of the worms showed a difference in fluorescence that was statistically significant.
RNAi, but instead removing the DNA responsible for making MARC-6. This will result in a knockout of marc-6 as opposed to a reduction seen in RNAi.

Qualitative analysis in the nerve ring suggests increased fluorescence of the marc-6 mutants. Quantitatively measuring the fluorescence in the dorsal cord shows increased fluorescence in the slo-1::gfp erg-28 marc-6 over slo-1::gfp erg-28. However, t-test analysis shows that this difference is not significant (p>0.5). slo-1::gfp marc-6 also showed increased fluorescence over slo-1::gfp. However, this too failed to show statistical significance (p<0.2).

Behavioral Studies

If MARC-6 is negatively affecting SLO-1 signaling, then marc-6 mutants should show an increase in ethanol sensitivity. This means that the average speed of the marc-6 mutant should decrease in ethanol compared with a control. To test this, slo-1::gfp, slo-1::gfp marc-6, slo-1::gfp erg-28 marc-6, and slo-1::gfp erg-28 were each tested for their speed in ethanol. Figure 7 shows the results of this behavioral assay. Figure 7A shows the speed of the different worms in the absence of ethanol, while Figure 7B shows the speed of the worms in ethanol. The absence of ethanol resulted in different movement speeds in each strain of worm. This difference cannot be attributed to any individual gene being examined. In ethanol marc-6 null mutants show decreased movement speed, suggesting an increase in ethanol sensitivity. slo-1::gfp erg-28 moved significantly faster than slo-1::gfp erg-28 marc-6 (p<0.0001). slo-1::gfp also moved significantly faster than slo-1::gfp marc-6 (p<0.0001).
Figure 7. Behavioral Study of marc-6 mutants A.) movement speed on normal agar plates (n=591). All four strains move at statistically different speeds. This difference does not correlate with any single gene being examined in the experiment. B.) movement speed of the worms in ethanol (n=367). The marc-6 mutants move significantly slower in ethanol both in the presence and absence of an erg-28 mutation.
Mutagenesis of *mcherry::erg-28* successfully created mutants expressing varying levels of fluorescence

In order to better understand the SLO-1/ERG-28 pathway, mutagenesis experiments were performed on *mcherry::erg-28* worms. *mcherry::erg-28* was first obtained by integrating mCherry tagged ERG-28 into the genome of wild type N2 worms. These *mcherry::erg-28* worms were then mutated with EMS. The resulting mutants were characterized based on their level of fluorescence compared to the original *mcherry::erg-28* strain. This classification was simply whether or not they showed increased or decreased levels of fluorescence. Figure 8 shows each of the different mutants. Figure 8A shows mutants found to be expressing a decreased level of fluorescence while Figure 8B shows mutants found to be expressing an increased level of fluorescence. Mutated worm strains showed high levels of sterility.
Figure 8. Expression of fluorescence in EMS exposed \textit{mcherry::erg-28} transgenic animals. A.) The mutant worm strains that show a significant decrease in \textit{mcherry::erg-28}. B.) The mutant worm shows a significant increase in \textit{mcherry::erg-28}. It's noteworthy that mutagenesis seemed to preferentially favor increased expression of \textit{mcherry::erg-28} over decreased expression. This information combined with the knowledge that each of these strains displayed an above average degree of sterility may suggest ERG-28 plays some role in development.
Discussion

SLO-1 is the large conductance potassium (BK) channel which can be activated by ethanol in *C. elegans*. This results in inhibitory behaviors in *C. elegans* including reduced movement speed. Loss-of-function slo-1 mutants exhibit resistant behavior in ethanol. SLO-1 BK channels need to be transported to the cell membrane in order to function as ion channels. This first involves transport from the Endoplasmic Reticulum (ER) to the Golgi apparatus by COP II proteins. This process is facilitated by the ERG-28 transport protein. Loss of function mutants for *erg-28* exhibit similar ethanol resistance to loss of function slo-1 mutants.

*erg-28* mutants also show reduced SLO-1 signaling, but not a full knockout of SLO-1 signaling. This suggests the existence of a secondary mechanism of transport from synthesis in the ER to the cell membrane. *erg-28* mutants also do not show a buildup of SLO-1 in the ER. In *erg-28* mutants, SLO-1 cannot be moved out of the ER as effectively. This results in SLO-1 trapped in the ER, increasing the chances for degradation, a process called Endoplasmic Reticulum Associated Degradation (ERAD). This likely includes the tagging of ubiquitin, which must be facilitated by a ubiquitin ligase.

Inhibition of the ubiquitin ligase responsible for the degradation of SLO-1 would therefore lead to increased SLO-1 signaling, as well as a reversal of ethanol resistance previously observed in *erg-28* mutants. The purpose of these experiments has been to identify this ubiquitin ligase, as well as further elucidate the SLO-1 signaling pathway. To accomplish this, proteins known to be active in the ER were targeted using RNAi, and the effect on SLO-1 signaling was then measured.
Characterization of *marc-6* null mutations show increased SLO-1 signaling and ethanol sensitivity

SLO-1 signaling was measured in *marc-6* mutants compared to a control in both the presence and absence of a loss of function mutation for *erg-28*. This resulted in four worm strains, *slo-1::gfp, slo-1::gfp marc-6, slo-1::gfp erg-28*, and *slo-1::gfp erg-28 marc-6*. Qualitative analysis of the neuronal rings seemed to show increased fluorescence in *slo-1::gfp erg-28 marc-6* over *slo-1::gfp erg-28*. This appeared to be confirmed by quantitative measurements of fluorescence in the dorsal cords. However, this difference was not statistically significant. No apparent difference in the fluorescence of the nerve rings was seen in *slo-1::gfp marc-6* compared to *slo-1::gfp*. Measurements in the dorsal cords showed an increased level of fluorescence in the *marc-6* mutants, but again not to a statistically significant level.

The lack of statistical significance must be attributed to either the means being the same, the variance being too large. The results seem best explained by an overly large level of variance. *slo-1::gfp erg-28 marc-6* has almost twice the average fluorescence of *slo-1::gfp erg-28*. Variance in the data can result from a lack of precision in the technique which creates outliers. Variance might also result from a small sample size than cannot average out those outliers.

The method for measuring fluorescence in the dorsal cord involves measuring the number of white pixels on a black and white image in the defined area. This is then compared to an average measurement of the dark background of the worm. The
background measurement accounts for factors such as the lighting of the room, but not the actual fluorescence of the worm. It seems reasonable to suspect that an increase in SLO-1 signaling in the dorsal cord may lead to increased signaling elsewhere in the worm. If the background measurement was inconsistent, the result may be darker worms appearing brighter and brighter worms appearing darker. Another issue may be the sample size. The sample size used was about 30 animals, which was consistent with other research of a similar nature.

If the lack of statistical significance is purely a result of variance in the data, then marc-6 mutants have increased SLO-1 signaling. If this is true, then marc-6 mutants should also show increased ethanol sensitivity consistent with increased SLO-1 signaling. This would mean marc-6 mutants move slower in ethanol when compared to a control.

Movement speed was measured in the four worm strains slo-1::gfp, slo-1::gfp marc-6, slo-1::gfp erg-28, and slo-1::gfp erg-28 marc-6. Measurements were done both on dried control plates as well as ethanol plates. Ethanol plates were used to measure behavioral changes caused by the different mutations in the presence of ethanol. The control plates were used to confirm no effects of the mutations on movement speed that are independent of the presence of ethanol.

Ethanol induced plates showed results consistent with predictions. marc-6 mutants showed decreased movement speed both in the presence and absence of an erg-28 mutation. slo-1::gfp erg-28 marc-6 moved significantly slower than slo-1::gfp erg-28, and slo-1::gfp marc-6 moved significantly slower than slo-1::gfp. This shows
increased ethanol sensitivity, which when combined with the data from the fluorescent assay, provides strong evidence of MARC-6 affecting SLO-1 signaling. These results are however undermined slightly by the control plates.

The control plates were done to test for the presence of movement changes caused by the mutations that were not specific to the presence of ethanol. Ideally, these would have all had the same speed. Instead, the speeds are wildly inconsistent. The different speeds do not display any noticeable pattern. *marc-6* mutants are not faster than their respective controls, worms with *erg-28* mutations do not move faster than worms without, and the absolute number of mutations also does not correlate with speed.

One explanation is simply random error resulting from an unknown source. Another explanation may be the process of drying the plates. Plates were dried slightly before adding ethanol, while control plates were simply dried as a control. It’s possible the plates were overly dried resulting in effects to movements. This would explain some anomalies in the data such as *slo-1::gfp* actually having a higher average speed in ethanol than it does in the control plate. However, this would affect the plates uniformly, and therefore should affect the worms uniformly, which is not quite what happens.

Using movement speed is a well-established method for measuring behaviors in *C. elegans*. Beyond being used to specifically study SLO-1 signaling in *C. elegans*, (Davie, *et al.*, 2003) it has been used to study oxygen reduction (Cheung, Cohen, Rogers,
Albayram, Bono, 2005) as well as food response (Bono & Bargmann, 1998).

Fluorescence assays are also a common practice in *C. elegans* (Mohan, Chen, Hsieh, Wu, Chang, 2010; O’Rourke, Soukas, Carr, Ruvkun, 2009). These are common methods used with *C. elegans*, being performed with comparable sample sizes. While anomalies exist in both the behavioral and fluorescent assays, they are unlikely to be the result of a flaw in the experimental design.

Both the fluorescent assay and the behavioral studies provide evidence that MARC-6 is the ubiquitin ligase for SLO-1. Other research has identified MARC-6 as an ubiquitin ligase important for development in *C. elegans* (Sasagawa, et al., 2007). As previously discussed, ubiquitin ligases do not work in a one to one ratio (Bays, et al. 2001; Jolliffe, et al. 2000). Therefore, it’s reasonable that MARC-6 could degrade multiple proteins.

**Inhibition of targeted ER active genes using RNAi suggests increased SLO-1 signaling in MARC-6 target nerve rings**

RNAi experiments failed to show differences between the different targets in the dorsal cord. The reason for this was initially attributed to having not yet tested the SLO-1 ubiquitin ligase. This was supported by the decrease in fluorescence seen in the worms fed dsRNA for GFP. This was later contradicted by RNAi experiments on DDI-1, a proteasome which degrades SLO-1. DDI-1 should show a change in the level of SLO-1 signaling (Oh, K., et al., 2017). When this was not seen, it was concluded that RNAi did not sufficiently reduce the ubiquitin ligase enough to noticeably affect the level of SLO-1 fluorescence. This led to fully mutating the target ubiquitin ligase.
MARC-6 was mutated based on qualitative analysis of the fluorescent microscopy. No significant differences were seen in the measurements of dorsal nerve cords in any of the targeted proteins. However, worms fed dsRNA for MARC-6 consistently appeared to have increased levels of fluorescence in the neuronal rings. If MARC-6 is the SLO-1 ubiquitin ligase, a full knockout of MARC-6 should result in increased SLO-1 signaling. This would mean both an increased level of fluorescence in *C. elegans* with fluorescently tagged SLO-1, as well as increased ethanol sensitivity over controls. This would be particularly true for *erg-28* mutants, but could also be expected in the absence of the *erg-28* mutation.

The reason no difference was seen in the dsRNA fed slo-1::gfp erg-28 uIs69 lin-15b worm strain is unclear. One explanation may be that simply not enough tests were done to see a difference caused by the different feeding protocols. Another explanation is that RNAi was not an effective method for our experiment. dsRNAi has been shown to be an effective method for altering gene expression in *C. elegans* (Fire, *et al.*, 1998). RNAi doesn’t fully knockout expression of the target, but instead reduces its expression. This can even be dependent on the location of where the protein was expressed (Tabara, Grishok, Mello, 1998). A major problem with this explanation is RNAi was able to reduce expression of GFP in our study, and other studies as well (Knight & Bass, 2001; Duchaine, *et al.*, 2006). Since RNAi experiments were ended before sufficient data was collected to quantitatively assess the effectiveness of RNAi, it is possible RNAi worked, but did not produce a change in fluorescence large enough to be detected by our instrumentation.
Mutagenesis of mCherry::erg-28 successfully created mutants expressing varying levels of fluorescence

In order to further elucidate the SLO-1 pathway, the erg-28p::mCherry::erg-28 transgene was integrated into wild type N2 worms. A resulting transgenic line was then mutagenized to create random mutations in its genome. F2 progeny of these worms were then screened for changes in the level of mCherry fluorescence, both increases and decreases. Worms screened for increased fluorescence seemed to show increased egg laying behavior, but lower rates of eclosion. This may ERG-28 plays a role in development. However this is based purely on observation. No formal measurements of these behaviors were performed.

Figure 9 shows an overview of the signaling pathway, now updated with the appropriate ubiquitin ligase. Future studies would further examine the mutants created from the mutagenesis experiments. Changes in fluorescence should correspond to changes in the expression of ERG-28. It is therefore likely some of the mutants are simply erg-28 mutants. However, some of the mutants likely have other mutations affecting the expression of ERG-28.

Conclusion

The experiments performed provide evidence that MARC-6 is the ubiquitin ligase responsible for the ER associated degradation of SLO-1 BK channels. These experiments include both fluorescent assays and behavioral studies. However, the fluorescent assays merely approached significance, while behavioral studies could not definitively exclude the possibility of behavioral changes independent of the presence of ethanol.
Figure 9. Conclusion An updated SLO-1 functional overview to fit the work performed in this thesis. Similar to the overview figure, this figure shows MARC-6 as the ubiquitin ligase responsible for the degradation of SLO-1. This fits with previous work identifying MARC-6 as an ubiquitin ligase in *C. elegans*. Mutants have also been developed to allow further examine the function of ERG-28.
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