The Dissertation Committee for Scott Joseph Davis certifies that this is the approved version of the following dissertation:

Elucidating residues on the BK channel required for activation by alcohol and intoxication in *C. elegans*

Committee:

Jonathan Pierce-Shimomura, Supervisor

R. Adron Harris

S. John Mihic

Lawrence K. Cormack

Richard W. Aldrich

Nigel S. Atkinson

Elucidating residues on the BK channel required for activation by alcohol and intoxication in *C. elegans*

by

Scott Joseph Davis, B.A.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2014

Acknowledgements

I could not have completed this journey without the help of many wonderful people. To start, I would like to thank my advisor Dr. Jon Pierce-Shimomura for his support and guidance throughout graduate school. His love of *C. elegans* is infectious, as is his love of research. I could not have asked for a more encouraging, positive and thoughtful advisor. I would also like to thank my committee for their ideas, guidance and support. The Waggoner Center for Alcohol and Addiction Research has many current and past graduate students, postdocs and professors that also helped guide my research.

All of the wonderful members of the Pierce-Shimomura lab were invaluable, both academically and otherwise. Luisa Scott not only worked on projects presented in this dissertation, but also provided me with support and ideas during the most difficult times on this journey. Andrés Vidal-Gadea's creativity knows no bounds, and he was always willing to listen, give thoughtful comments and provide thought provoking questions related to my project, and science in general. Grabbing coffee and late night dinner with Andrés will be remembered by me with particular fondness. I am very thankful to Josh, my benchmate throughout, and in the trenches weekend after weekend. Layla, our lab manager was invaluable, working tirelessly to make sure all lab members had everything we needed. Many others in the lab, especially Sangeetha, Allison, Ashley, Jesse and Sarah have helped me during this journey. Very special thanks to Krysal Phu, the best graduate coordinator this world has ever known. She always went above and beyond with a smile that brightened my day every time I saw her.

I am eternally grateful to my family. My mother Elaine, aka Supermom, was instrumental in pushing me to pursue this dream. My stepfather Jeff has done so much to help me during this journey. My father Tom provided me with many lessons I will carry

throughout my life. I cherish all of my siblings who have provided me with support and advice. Also, I must acknowledge all of my wonderful nieces and nephews that have brought me so much joy.

I will forever be indebted to my wonderful wife, Laura, my best friend that provides me with never-ending support, love, patience and encouragement. Finally, I would like to acknowledge all of my furry companions, Bahama, Baxter, Millie and Reggie, who are constant reminders that life is about more than work and school.

Elucidating residues on the BK channel required for activation by alcohol and intoxication in *C. elegans*

Scott Joseph Davis, Ph.D.

The University of Texas at Austin, 2014

Supervisor: Jon Pierce-Shimomura

Alcohol produces changes in behavior through molecular effects on ion channels, enzymes and transporters. Many proteins have been elucidated that at least in part mediate behavioral changes induced by alcohol. However, it has been difficult thus far to uncover key amino acid residues within a protein that are necessary for the effects of alcohol. This information is critical, potentially leading to effective pharmacological treatments for alcohol use disorders (AUD) and identification of allelic variations that predispose an individual for AUD. The big conductance voltage- and calcium-activated potassium (BK) channel has recently emerged as a critical protein for the effects of alcohol across species. In this dissertation, we study the molecular action of alcohol on the BK channel, and how this action contributes to behavioral intoxication. To accomplish this, we first provide credence for using the nematode *C. elegans* for studying the behavioral effects of ethanol. We demonstrate how behavioral intoxication and internal ethanol concentration in *C. elegans* is altered by the osmolarity of the ethanol-solution, reconciling results from previous conflicting reports in the literature. We then identify the amino acid residue T381 on the BK channel in *C. elegans* is critical for

behavioral intoxication, but not other BK channel-dependent behaviors. These results suggest a functional BK channel resistant to ethanol. By knocking-in the human BK channel, we then demonstrate that the equivalent residue, T352 is also critical for behavioral intoxication in *C. elegans,* but not other BK channel-dependent behaviors. Using single-channel recordings, we find that the T352 residue is critical for the potentiating effects of ethanol on the human BK channel, without being critical for basal-function. Finally, we investigate the role of calcium-sensing residues on the worm BK channel for behavioral intoxication in *C. elegans.* We find that these residues are non-essential for intoxication, in contrast to *in vitro* reports in the mammalian channel suggesting the calcium-sensing residues are critical for ethanol-activation of the BK channel.

Table of Contents

List of Figures

1.0 - INTRODUCTION

For centuries alcohol has been imbibed for its intoxicating effects, enjoyed by the majority of drinkers without serious negative consequences. However, in a subset of individuals an alcohol use disorder (AUD) develops, which is a chronic and often progressive disease that includes problems controlling drinking, preoccupation with alcohol, continued use of alcohol despite negative consequences, increased tolerance to alcohol and withdrawal symptoms after cessation from alcohol (DSM-V, 2013). According to the NIAAA, about 8.5% of the US population meets the Diagnostic and Statistical Manual of Mental Disorders (DSM) criteria for AUD (Grant et al., 2004). Also, it is reported that in the US alcohol causes 88,000 deaths per year and costs the US economy over 223 billion dollars per year (Bouchery et al., 2006). Although alcohol is one of the most widely used and abused drugs, the molecular mechanisms through which ethanol produces its intoxicating and reinforcing effects are not fully understood.

Effective treatments for alcoholism are particularly lacking. With current treatment options, about two-thirds of individuals relapse with heavy bouts of drinking after treatment (Miller et al., 2001). In addition, every current pharmacological treatment results in at most modest and often controversial results (Franck and Jayaram-Lindström, 2013). Uncovering how the molecular actions of alcohol engender behavioral intoxication, tolerance and addiction may lead to insights into the development of new pharmacological treatments for AUDs and in identifying genetic factors for predisposition to addiction.

1.1 - The Action of Alcohol on the Nervous System

Alcohol is an organic compound that contains a hydroxyl functional group (-OH) bound to a carbon atom. The 2-carbon-alcohol, ethanol, is the drinking alcohol used by billions of people to produce intoxication.

Alcohols were first thought to produce their effects through nonspecific action on the lipid membrane of neurons. The first experimental evidence for this theory was found by Meyer and Overton, who reported that the anesthetic potency of alcohols correlated with their solubility in cell membranes (Peoples et al., 1996). Short chain alcohols, such as ethanol can disrupt the packing in the lipid bilayer, leading to changes in membrane fluidity (Chin and Goldstein et al., 1977), membrane permeability (Komatsu and Okada, 1995) and lipid lateral mobility (Chen et al., 1996). These disruptions to biological membranes could have substantial nonspecific effects on intrinsic membrane proteins, which in turn could engender intoxication (Frischknecht and Frink, 2006). Indeed, changes in the lipid bilayer can exert effects on signaling proteins. For example, mechanosensitive ion channel activity is mediated by changes in the lipid bilayer of cell membranes (Gullingsrud and Schulten, 2004). Also, evidence has emerged that the effect of alcohols on the potassium channel KcsA is mediated by alterations in lateral mobility of the membrane caused by alcohols (Van den Brink-van der Laan et al., 2004). In addition, Garcia-Cabrera and Berge (1988) demonstrated a reversal of the depressive effects of ethanol in rats that were exposed to a hyperbaric environment, providing evidence that altering the lipid membrane with pressure could reverse intoxication. According to the nonspecific theory of alcohol action, tolerance to ethanol occurs due to

changes in the lipid membrane as a result of chronic consumption. In support of this theory, Lyon and Goldstein (1983) found that mice treated chronically with ethanol have increased membrane rigidity compared to naïve mice.

The Meyer and Overton theory of nonspecific alcohol action on lipid membranes fell out of favor due to multiple inconsistencies with the observed effects of alcohols. First, the potency of some anesthetic compounds does not correlate with lipid solubility (Fang et al., 1997). Second, clinically relevant doses of ethanol only increase membrane disorder to the same levels as an increase in temperature by a few tenths of a degree Celsius (Pang et al., 1980). Third, stereoisomers that exert identical physiochemical effects on a lipid bilayer do not engender identical *in vivo* effects. An example of this can be seen with the R^+ isomer of the anesthetic compound etomidate, which is 10 times more potent than the S- isomer at potentiating the $GABA_A$ receptor (Belelli et al., 2003). Fourth, according to the Meyer and Overton theory, addition of methylene groups to alcohols should increase lipid solubility and therefore, anesthetic potency. However, alcohols exhibit a cutoff effect after a certain chain length, beyond which they fail to exhibit any anesthetic potency (Franks and Lieb, 1986). These inconsistencies suggest that intoxication by ethanol is irrespective of the effects on the lipid membrane.

Evidence for the direct action of alcohols on proteins was first demonstrated in 1986 by Franks and Lieb. This work tested the anesthetic potency of alcohols on the protein firefly luciferase. Luciferase activity was strongly inhibited by clinically relevant concentrations of halothane, hexanol and decanol. Because firefly luciferase is a soluble

protein, nonspecific effects on the membrane could not explain the demonstrated effect of these alcohols. In the 1990s, crystallography experiments provided definitive proof ethanol had a specific binding site on alcohol dehydrogenase (Ramaswamy et al.,. 1994). Shortly thereafter, an ethanol binding pocket was elucidated in the *Drosophila melanogaster* odorant-binding protein LUSH (Kruse et al., 2003). Further work identified ethanol directly interacts with LUSH through hydrogen bonding within a threonine-serine-threonine structural motif (Thode et al., 2009).

Explorations of neuronal targets that respond to clinically relevant concentrations (15-150 mM) of ethanol have uncovered many receptors and ion channels that may contribute to the behavioral effects of ethanol. This includes the glycine, GABA (Mihic et al., 1997), NMDA (Lovinger et al.,,. 1989), nicotinic (Godden et al., 2001), GIRK (Lewohl et al., 1999), TRPVI (Benedikt et al., 2007) and serotonin receptors, (Lovinger, 1999) as well as L-type calcium (Wang et al., 1994) and the large conductance voltage- and calcium activated potassium (BK) channels (Dopico et al., 1996). However, a question often remains: Does ethanol interact with the protein directly or through secondary mechanisms that influence protein function to elicit behavioral effects?

Before a protein can be considered a direct target for ethanol., Harris et al. (2009) has proposed a set of criteria that must be met: 1) Protein function must be altered by clinically relevant concentrations of ethanol (15-150 mM), 2) Mutation of specific amino acids should provide evidence for an ethanol putative binding site, 3) Manipulation of the protein *in vivo* should produce results consistent with a role in the behavioral response to ethanol and, 4) Physical structural studies should demonstrate the presence of ethanol

molecules within the protein (Harris et al., 2009). Generally, the fourth criterion is the most difficult to provide evidence for, and thus far has only been collected for a small number of ethanol's neuronal targets, such as the glycine, GABA (Mihic et al., 1997; Wick et al., 1998; Lobo et al., 2004) and GIRK (Aryal et al., 2009) proteins. An understanding of how ethanol acts at a molecular level is still in its infancy, but has already provided useful clues into what targets ethanol may act through. Further elucidating how ethanol acts on a molecular level will allow researchers to fully understand how ethanol produces its behavioral effects, including anxiolysis, reduced inhibition, intoxication and reinforcing effects. A major part of this work will be testing if there is a direct interaction between ethanol and proteins already known to be modulated by ethanol.

Uncovering the molecular actions of ethanol on specific proteins may also provide insights into the development of effective pharmacological interventions for the treatment of alcohol abuse. For example, pocket-based drug design plays a key role in *in silico* drug design studies (Zheng et al., 2012). However, for *in silico* drug design to take place characterization of the molecular actions of ethanol on a target must be uncovered.

The BK channel is now emerging as a potential direct target for ethanol that contributes to ethanol-mediated behaviors. However, currently the binding pocket and the molecular effects of ethanol binding have yet to be uncovered. The following sections of this dissertation will address the current state of knowledge regarding how the BK channel mediates the effects of ethanol, and how I used the nematode

Caenorhabditis elegans to start elucidating the molecular interaction between ethanol and the BK channel.

1.2 - BK Channel

The gene that encodes the BK channel was first cloned by Atkinson et al. (1991), who identified a mutant strain of *Drosophila melanogaster* that exhibited jerky movements. Atkinson (1991) then mapped and cloned the BK channel gene, terming it *slowpoke* after the mutant phenotype in fly. Shortly thereafter, homologous genes were identified in other species, including mammals (*mslo*) and *C. elegans* (*slo-1*) (Butler et al., 1993, Wei et al., 1996). All BK channels comprise a constitutive, pore-forming tetramer of α subunits trafficked to cellular membranes (Shen et al., 1994). Each α subunit consists of seven transmembrane spanning segments (S0-S6), a pore-forming loop (P-loop) between the S5 and S6 domains, and four hydrophobic segments (S7-S10) contained in the large intracellular C-terminus (Figure 1.1). Unlike other potassium channels, the BK channel contains an extra transmembrane domain (S0), which leads to the extracellular N-terminus (Wallner et al., 1996). The S6 domain is thought to contain the selectivity-pore of the channel, which determines the high flux rate of potassium (often >200 pS) compared to other ions (Jiang et al., 2002).

The BK channel is also unique in that it is sensitive to both an increase in intracellular Ca^{2+} and membrane depolarization. Early single-channel recordings established distinct closed and open states that indicated enhanced BK channel opening was dependent on both voltage and Ca^{2+} (Barrett et al., 1982). Later work determined

depolarization can activate BK channels in the absence of Ca^{2+}, suggesting that calcium-binding and voltage sensor activation interact in an allosteric fashion to influence channel opening (Cox et al., 1997; Horrigan et al., 1999; Horrigan and Aldrich, 1999; Horrigan and Aldrich 2002). These experiments led to the proposal of the Horrigan and Aldrich (HA) model of BK channel activation. This model proposes that calcium-binding and voltage-depolarization have independent gating paths that act upon a final concerted gating step to facilitate channel opening, and that the BK channel can undergo closed to open transitions in the absence of either Ca^{2+} or membrane depolarization.

1.21 - BK Channel Voltage Activation

The voltage sensing domain (VSD) structure is contained in the S0-S4 segments (Figure 1.1). Upon membrane depolarization, S0 acts as a pivot element against which S4 moves against and away from to facilitate channel opening (Pantazis et al., 2010). During this process, fluorescent labeling of tryptophan-substituted residues has demonstrated that S4 moves away from S1 and S2, while S2 moves closer to S0 during voltage activation of the channel (Pantazis and Olcese, 2012). Like other voltage-gated channels, the α subunit contains positively charged residues in the S4 domain. However, unlike other voltage-gated channels, only one amino acid in the S4 domain, R213, has been directly implicated in voltage sensing (Diaz et al., 1998; Ma et al., 2006). Mutations of positively charged residues have also implicated D153 and R167 in S2 and D186 in S3 as critical for voltage-induced conformation changes (Figure 1.1; Ma et al., 2006). Overall, these

studies provide the framework for how the BK channel detects depolarization to facilitate BK channel opening.

1.22 - BK Channel Ca^{2+}-sensing Regions

The large cytoplasmic domain that comprises 70% of the BK channel houses two domains, regulator of calcium conductance (RCK) one and two, which are positioned in tandem within the C-terminus of the BK channel (Jiang et al., 2002; Yuan et al., 2010). RCK1 is located within amino acid positions 340-610 and RCK2 is located within amino acid positions 640-1055 (Figure 1.1). The two RCK domains from the four α subunits form an octameric gating ring in the cytoplasm directly below the pore. This gating ring undergoes conformational changes upon Ca^{2+} binding to promote BK channel opening, as demonstrated by both crystallography and FRET signaling (Yuan et al., 2010; Miranda et al., 2013).

Two high and one low affinity Ca^{2+}- binding sites have been identified that are critical for the response of the BK channel to Ca^{2+} levels ranging from 100 nM to 10 µM (Cox et al., 1997). The Ca^{2+} bowl, contained within RCK2, was the first site to be identified as a high affinity Ca^{2+} sensing site. This was demonstrated by mutating a string of five aspartate residues (D897-901) on the mouse BK channel to asparagine (5D5N), reducing sensitivity to Ca^{2+} by roughly half (Figure 1.1; Schreiber and Salkoff, 1997). Further work with the *Drosophila* BK channel showed that the binding of radionucleotide $^{45}Ca^{2+}$ to a 280 amino acid C-terminal fragment was markedly reduced after introduction of the 5D5N mutation (Bian et al., 2001). These results suggest a conservation of the Ca^{2+}

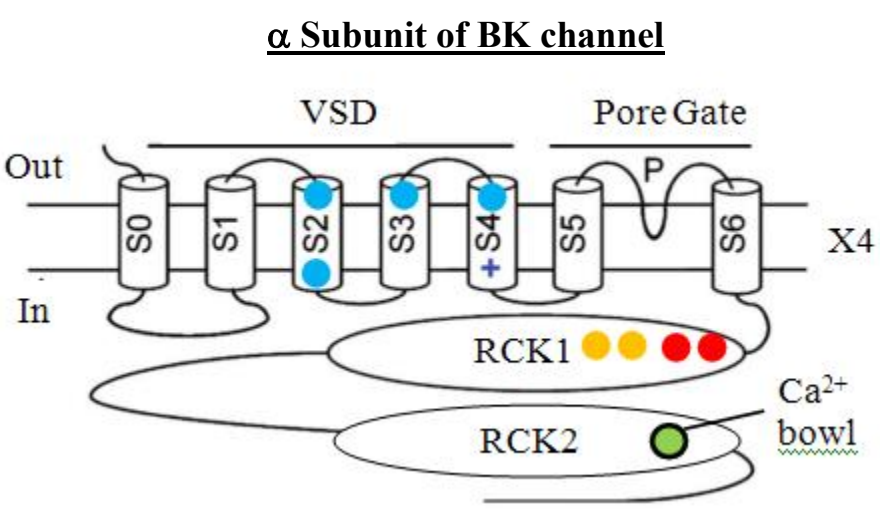
bowl across species as a high affinity calcium sensor. However, because knocking out the calcium bowl did not completely eliminate calcium sensitivity of the BK channel, these data suggested there must be at least one more calcium-binding site critical for channel gating (Schreiber and Salkoff, 1997; Bian et al., 2001). Shortly thereafter, an additional high affinity calcium-binding site was identified within RCK1, requiring aspartate residues (D362/367) (Figure 1.1). When these residues were mutated to alanine, a significant reduction in BK channel calcium sensitivity occurred (Xia et al., 2002). Also, a lower affinity calcium site responsive to millimolar concentrations of Ca^{2+} was located on glutamate residues (E374/399) (Figure1.1; Xia et al., 2002; Zeng et al., 2005). The low affinity site does not appear to be critical for channel gating because a combination of the D362/367A and 5D5N mutations completely abolishes the calcium sensitivity of the BK channel within the physiological range of Ca^{2+} (< 1 mM) typically reached. (Figure 1.1; Zeng et al., 2005).

Opposing the hypothesis of three distinct calcium-binding sites on the BK channel was an experiment performed by Piskorowski and Aldrich (2002), who reported that Ca^{2+} sensitivity of the BK channel remained after removal of the entire of C-terminus. These experiments suggest the purported calcium-sensing residues may not be binding sites, but residues that are necessary for allosteric calcium gating of the BK channel. Although seemingly incompatible with other work, the majority of previous studies that implicated the C-terminal sites were solely functional studies, which make it difficult to definitively conclude if a residue is binding calcium. The studies that directly measured calcium-binding were carried out in isolated c-terminals, which prevented the concurrent study of

BK channel function (Bian et al., 2001). In addition, some previous work had suggested there are calcium-binding sites outside of the C-terminus (Braun and Sy, 2001). However, the experiments by Piskorowski and Aldrich (2002) could also be explained by an unknown accessory subunit mediating Ca^{2+}-sensing of the truncated BK channel. More research will be needed in order to resolve this seemingly conflicting report from previous studies.

Figure 1.1 – Schematic of the BK channel

Diagram of the BK channel showing sites critical for calcium- and voltage-activation. Two aspartate residues (D362/367) are located in the RCK1 domain (red). The five aspartate residues are contained in the "calcium bowl" (green). These are high affinity sites that coordinate Ca^{2+} sensing at micromolar concentrations. Two glutamate residues (E374/399) contain a low affinity site, which coordinates Ca^{2+} sensing at millimolar concentrations (orange). R213 of S4, D153 and R167 of S2, and D186 of S3 coordinate voltage-sensing of the BK channel (blue). Extracellular (out), intracellular (in), the voltage-sensing domain (VSD) and the K+ selective pore gate are all depicted. Figure image is adapted from Cui et al. (2009). For simplicity, the numbering scheme of residues relate to the mouse BK channel.



1.23 - Gating of the BK Channel

The ubiquitous expression, functional diversity and ability of the BK channel to sense calcium and voltage results in a unique role for the BK channel as a coincidence detector between increases in intracellular calcium and cellular depolarization. This allows the BK channel to fill a myriad of physiological roles across species, including controlling vascular smooth muscle (Brenner et al., 2000) and urinary bladder tone (Petkov et al., 2001), as well as mediating hormone release (Maruyama and Peterson, 1982), tuning auditory hair cells (Oberholtzer, 1999), maintaining resting membrane potential (Snetkov and Ward, 1999), modulating action potential frequency (Gu et al., 2007), repolarizing cellular membranes (Shao et al., 1999) and mediating synaptic transmission (Hu et al., 2001).

The functional diversity of the BK channel is tailored through a variety of mechanisms, including alternative splicing, coassembly with auxiliary subunits and posttranslational modifications. Alternative splicing can alter functional properties of the BK channel, including gating kinetics and calcium- and voltage-sensing (Contreras et al., 2013). In vertebrates and flies, the BK channel gene encodes 10 and 13 alternative exons respectively, theoretically allowing for more than 1,000 transcripts (Fodor and Aldrich, 2009). In *C. elegans*, three splice sites exist with 12 possible variants (Glauser et al., 2011). Individual isoforms are differentially expressed in specific cell types (Chen et al., 2005), developmental stages (MacDonald et al., 2006) and in response to behavioral changes in an animal, such as stress (McCartney et al., 2005).

Another method for enhancing functional diversity of the BK channel is coassembly of auxiliary subunits with the α subunit of the BK channel. To date, four β subunits (β1- 4) and four γ gamma subunits (γ1-4) have been identified, all of which display distinct expression profiles (Contreras et al., 2013). For example, the β1 subunit is mainly expressed in vascular smooth muscle (Brenner et al., 2000), but is also found in the brain at low levels (Martin et al., 2004), while the β4 subunit is generally found in neurons and endocrine cells (Behrens et al., 2000; Brenner et al., 2005). Both β and γ subunits can alter BK channel sensitivity to calcium and voltage, gating kinetics and efficacy of pharmacological agents (Hoshi et al., 2013; Yan and Aldrich, 2010; Almassy and Begenisich, 2012).

Posttranslational modifications including phosphorylation, oxidation and palmitoylation further increase the BK channel's functional range (Hoshi et al., 2013). For example, there are 30 Ser/thr phosphorylation sites, 23 of which are located in the C-terminus and four of which are found in exon splice insertions (Yan et al., 2008). Posttranslational modifications can have either inhibitory or excitatory effects on the channel, as well as alter the sensitivity of the BK channel to modulators (Contreras et al., 2013). These methods to increase functional diversity of the BK channel also provide a platform for modulating the response of the BK channel to ethanol. In the subsequent section of this dissertation I will focus on what the known molecular effects of ethanol are on the BK channel to engender intoxication, tolerance and addiction to alcohol.

1.3 - Ethanol Action on the BK Channel

The first of the four criteria proposed by Harris et al. (2009) to establish a protein as a direct target of ethanol states that protein function must be altered by clinically relevant concentrations of ethanol. Many studies have shown that BK channel function is altered by clinically relevant concentrations (20-100mM) of ethanol *in vitro* across species from worm to human, and tissue types ranging from smooth muscle, pituitary and brain (Jakab et al., 1997; Widmer et al., 1998; Dopico et al., 1998; Walters et al., 2000; Dopico, 2003; Liu et al., 2004; Jakob et al., 2006; Malysz et al., 2014). In addition, the *C. elegans* BK channel has been shown to be activated by clinically relevant concentrations *in vivo* (Davies et al., 2003). The first single-channel recordings indicated ethanol reversibly increased BK channel opening frequency in neurohypophysial terminals. This study was performed in excised inside-out patches, suggesting BK channel modulation by ethanol does not require diffusible second messengers (Dopico et al., 1996). Further work identified that BK channels reconstituted in lipid bilayers were activated by alcohol, suggesting in this minimal preparation the actions of ethanol are conserved (Chu et al., 1998).

Differential expression of β subunits confer varied BK channel responses to ethanol. Feinberg-Zadek and Treistman (2007) first discovered that ethanol sensitivity of the BK channel was reduced when coexpressed with β1 or β4 subunits in HEK293 cells. In myocytes, BK channels consisting solely of α subunits are potentiated at low, and inhibited at high intracellular Ca^{2+} concentrations (crossover ~15 μM). However, the β1

subunit shifts the crossover of ethanol activation to inhibition to ~3 μM. This result explains why ethanol inhibits myocyte activity leading to cerebrovascular constriction (Bukiya et al., 2009). In the nucleus accumbens, low alcohol concentrations selectively potentiate BK channel activity in the soma, but not in the dendrites (Martin et al., 2004). This difference is mediated by dendritic expression of β1 subunits conferring ethanol insensitivity on BK channels. On the other hand, BK channels containing β4 subunits in the soma are ethanol sensitive. Similarly, in rat neurohypophysial neurons expression of the β1subunit in the soma and dendrites prevents ethanol potentiation of the BK channel, while in the terminals BK channel expression with β4 subunits is potentiated by ethanol (Wynne et al., 2009).

The tuning of BK channel responses to ethanol is further enhanced by an array of splice variants. If a splice variant contains the alternative exon (STREX), it is insensitive to ethanol (Pietrzykowski et al., 2008). However, if a BK channel does not contain any alternative exons (INSERTLESS), or the alcohol-regulated exon (ALCOREX), it can be potentiated by ethanol. Because these isoforms have the same phosphorylation sites, the alternative exon presence itself seems to be modifying the interaction between ethanol and the BK channel.

Posttranslational modifications, such as phosphorylation has also been shown to alter how ethanol mediates BK channel function. Specifically, phosphorylation of the T107 residue unique to the bovine BK channel switches ethanol activation to inhibition (Liu et al., 2006). More work must be done to test if any of the other 29 identified phosphorylation sites alter the response of the BK channel to ethanol (Yan et al., 2008).

Composition of the surrounding lipid environment is another factor that influences the effect of ethanol on the BK channel. Increased incorporation of cholesterol into the membrane reduces ethanol potentiation of the BK channel (Crowley et al., 2003). Composition of the phospholipids also appears to modulate the effects of ethanol. Conical phospholipids blunt, while cylindrical phospholipids favor BK channel potentiation by ethanol (Crowley et al., 2005). In addition, the response of the BK channel to ethanol switches from a strong potentiation in thin membranes, to inhibition in thick membranes (Yuan et al., 2007; Yuan et al., 2008). In agreement, behavioral experiments have demonstrated that putting rats on high-cholesterol diets results in cholesterol buildup in the arterial tissue. This buildup diminishes the effects of ethanol on the BK channel and results in reduced artery constriction by ethanol (Bukiya et al., 2014). Although the surrounding lipid membrane is clearly important, cholesterol tuning of the BK channel response to ethanol is enantioselective. This was demonstrated by experiments that expressed the α subunit of the BK channel in a minimal lipid bilayer, and tested if the addition of a synthetic enantiomer of cholesterol or the addition of natural cholesterol to the bilayer had a differential effect on the response of the BK channel to ethanol. While the synthetic enantiomer had no effect on the response of the BK channel to ethanol, an equal concentration of natural cholesterol strongly modulated the BK channel's response to ethanol (Yuan et al., 2011). These results strongly suggest that the action of ethanol is directly between the α subunit of the BK channel and ethanol, and mediated by the surrounding environment.

1.31- Ethanol Action on the BK Channel Underlies Alcohol-mediated Behaviors

The BK channel contributes to a vast array of an animal's behavioral responses to ethanol. A set of experiments has correlated alcohol exposure regulating BK channel isoform expression with acute behavioral tolerance to alcohol. Pietrzykowski et al. (2008) measured microRNA-9 (mir-9) expression in cultured neurons from mice in two brain regions that are implicated in alcohol abuse, the supra optic nucleus (SON) and the striatum. These researchers found mRNA levels of the alcohol insensitive isoform containing the stress regulated (STREX) exon doubles compared to other isoforms, along with an overall decrease of BK channel expression (Pietrzykowski et al., 2008). Further work expanded on this study by exposing mice to ethanol, followed by withdrawal. mRNA levels of the alcohol-insensitive STREX splice variant increased after withdrawal from 6 hours of ethanol exposure, mimicking the results from cultured neurons (Velázquez-Marrero et al., 2011). These changes in molecular composition of the BK channel provide potential mechanisms for acute tolerance to alcohol. In addition, this work provides an attractive mechanism for earlier studies that demonstrated acute tolerance of the BK channel to ethanol in neurohypophysial terminals, which results in a short-term ethanol-mediated reduction of peptide hormone released in rats (Knott et al., 2000; Knott et al., 2002; Pietrzykowski et al., 2004).

The third criterion proposed by Harris et al. (2009) to establish the BK channel as a direct target of ethanol is that the manipulation of the BK channel *in vivo* should produce results consistent with a role in the behavioral response to ethanol. Davies et al. (2003) demonstrated a central role for the BK channel in behavioral intoxication by

performing a genetic screen in *C. elegans*. In this unbiased genetic screen, every mutant that displayed a strong resistance to ethanol had a mutation in the gene that encodes the BK channel, *slo-1*, providing strong evidence that the BK channel plays a central role in intoxication. Subsequently, researchers demonstrated that in *Drosophila*, acute functional tolerance (AFT) to alcohol requires the BK channel (Cowmeadow et al., 2005; Cowmeadow et al., 2006). In mice, two recent studies have demonstrated that acute tolerance of the BK channel to ethanol correlates with behavioral acute tolerance and drinking behaviors in mice (Martin et al., 2008; Kreifeldt et al., 2013). Knocking out the β4 subunit of the BK channel causes rapid acute behavioral tolerance to a 2g/kg ethanol challenge compared to wild-type mice. These mutant mice also imbibed more compared to wild-type mice measured by a restricted access ethanol self-administration assay (Martin et al., 2008). Correlative effects were seen at the single-channel level in the striatum. The BK channel in β4 knock-out mutant mice develop a strong tolerance to the potentiating effects of ethanol, which is not seen in the wild-type animals. Tolerance was also recorded by measuring the firing rate of medium spiny neurons in the striatum. In wild-type mice, ethanol depresses spike pattering that persists for the duration of ethanol exposure. However, in β4 knock-out mice an initial reduction of firing frequency occurs followed quickly by a return to control levels.

This research in particular has laid the foundation for the interaction between ethanol and the BK channel contributing to behaviors in mammals that overlap with the DSM-V criteria for an AUD, namely drinking behaviors and acute tolerance. In addition, humans that carry gain-of-function mutations in the BK channel are hypersensitive to

intoxication (Du et al., 2005). Although research has established the importance of the molecular effects of ethanol on the BK channel in alcohol-mediated behaviors, the molecular interaction between ethanol and the BK channel has not yet been elucidated. Thus, the criteria for establishing the BK channel as a direct alcohol target proposed by Harris et al. (2009) has yet to be filled. Specifically, the second criterion, mutation of specific amino acids should provide evidence for an ethanol putative binding site, and the fourth criterion, physical structure studies demonstrating the presence of ethanol molecules within the protein have yet to be satisfied. Elucidating the ethanol binding site(s) and key residues involved in the BK channel response to ethanol will begin to forge a path to satisfy these final criteria. In addition, uncovering the ethanol-binding site/transduction pathway may lead to methods to pharmacologically alter the action of ethanol on the BK channel and may also help to uncover the general mechanisms through which ethanol acts.

1.4 - Conventional Methods to Uncover the Ethanol-BK Channel Molecular Interaction

A useful approach to identify critical residues on a protein involved in the response to ethanol is to study the protein in isolation within a heterologous system, substituting amino acid residues/regions until the protein no longer responds normally to ethanol, while retaining some level of basal function. This *in vitro* approach is critical to control for the functional diversity often observed with *in situ* and *in vivo* preps. Mihic et

al. (1997) used this approach by constructing a chimeric channel composed of portions of an α1 glycine receptor, which is normally potentiated by ethanol, fused with a GABA ρ1 receptor, which is normally inhibited by ethanol. By combining segments of these receptors the authors narrowed down the region to 45 amino-acid residues between TM2 and TM3 critical for the effects of ethanol. Next, by comparing sequences between the GABA ρ1 and glycine α1 receptors, 12 non-conserved amino acids were mutated to elucidate the critical residues for the action of ethanol. Two residues, S267 and A288 were found to be critical for the potentiating effects of ethanol. Substituting the S267 residue to amino acids of varying sizes demonstrated an inverse correlation between size and potentiation, revealing a putative binding pocket for ethanol (Ye et al., 1998).

This *in vitro* approach has also been used to begin elucidating the molecular interaction between the BK channel and ethanol. Liu et al. (2003) set out to determine the amino acids that are critical for the consistent potentiation by ethanol in human and mouse BK channels versus the inconsistent potentiation by ethanol in bovine BK channels (Crowley et al., 2003; Dopico et al., 1998; Dopico, 2003). Liu et al. (2003) constructed chimeras by interchanging the core and tail domains of the mouse and bovine BK channels, demonstrating that the core-linker is critical for the response to ethanol.

The presence of Ca^{2+} intracellularly is required for ethanol to modulate BK channel activity. Ethanol potentiates the BK channel at low levels, but inhibits activity at high levels of Ca^{2+} (Liu et al., 2008). Moreover, when the Ca^{2+}-sensing regions of the BK channel were neutralized (5D5N $^{+}$ D362/367A mutations) using site-directed mutagenesis, the BK channel no longer was affected by ethanol. However, these

alterations drastically shift the opening probability of the BK channel under basal conditions (Xia et al., 2002; Schreiber and Salkoff, 1997). Therefore, these results confound if the abolishment of the effect of ethanol is a result of altering the ability of ethanol to modulate the BK channel or nonspecifically altering the gating kinetics of the BK channel that in turn prevents ethanol modulation of the BK channel.

In a recent study, Liu et al. (2013) made multiple chimeric channels of the ethanol sensitive BK channel and related potassium channels that are insensitive to ethanol. When the intracellular tail of the BK channel was swapped with an ethanol insensitive channel, resistance to ethanol was conferred. In addition, a chimera of ethanol insensitive channels with the BK channel intracellular tail was sensitive to ethanol. Primary sequence alignment of the channels used to generate the chimeras identified 37 residues in the intracellular region that are likely to be critical for the actions of ethanol on the BK channel (Liu et al., 2013).

While these conventional *in vitro* approaches are powerful and have proved successful, much work remains to elucidate the molecular interaction between the BK channel and ethanol, including the identification of a binding pocket and residues that are critical for the action of ethanol without being critical for channel function. In addition, how the *in vitro* mutations to the BK channel relate to an animal's behavioral response to ethanol remains unknown.

The major drawback of the conventional approaches outlined above is that they cannot easily determine whether the mutations identified as important for ethanol's effects *in vitro* are also relevant to ethanol-mediated behaviors *in vivo*. In addition,

sometimes it is clear that the mutations identified from these *in vitro* approaches would be detrimental or fatal if the mutant replaced the wild-type protein *in vivo*. For instance, although single-residue mutations that render the glycine receptor insensitive to ethanol were described by Mihic et al. (1997), the knock-in replacement of the wild type with the mutant glycine receptor has not been successfully accomplished. Unfortunately, this mutation results in post embryonic lethality shortly after birth. It was determined that the abnormally low activity of the receptor likely leads to fatal seizure activity (Findlay et al., 2003). These problems were finally overcome for the α2 GABAA receptor after a decade of searching for a distinct combination of S270H/L277A mutations that produce a viable mutant knock-in mouse with altered ethanol but not basal behaviors (Blednov et al., 2011; Harris et al., 2011).

Many of the site-directed and chimeric constructions of the BK channel in previous *in vitro* studies may also result in gross basal behavioral changes or lethality when studied in knock-in mutant animals, which would make studying behavioral changes to ethanol impossible. For example, Liu et al. (2008) found that mutating calcium-binding residues in the Ca^{2+} bowl and RCK1 domain abolished the ability of ethanol to activate the BK channel *in vitro*. However, mutations of the calcium-binding residues also dramatically alter calcium-dependence which greatly reduces BK channel activity (Xia et al., 2002; Schreiber & Salkoff, 1997). Such radical differences in function would be expected to produce changes in basal behaviors that would obscure any changes in ethanol-mediated behaviors. Evidence supporting this is demonstrated in BK channel knock-out mice that contain severe deficits in breeding activity, bladder control issues,

circadian rhythms, forelimb grip force, inter-limb coordination, shorter stride lengths, sensorimotor gating and cognitive function (Mededith et al., 2004; Meredith et al., 2006; Mirkowski, 2013). Because these alterations have complicated breeding and other basal behaviors in BK channel knock-out mice, research to test the effects of ethanol on these mutant mice has yet to be published.

The conventional approaches above also suffer from two other complications that make it difficult to translate an *in vitro* finding to an animal's behavioral response to ethanol. First, the potential mutation site must be known *a priori* before making single point mutations to identify residues critical for the response to ethanol. This usually is contingent on identifying similar channels that have differing responses to ethanol, constructing a set of chimeric channels and successfully expressing, trafficking and recording from these chimeras in a heterologous system. In addition, this approach requires residue positions that show divergence within an ion channel family being the critical residue(s) that contribute to differential drug sensitivities. As a result, conserved mechanisms important for drug action may be missed. It is completely reasonable that a conserved site may confer sensitivity to ethanol in one channel and not another, as a result of differences in the secondary or tertiary protein structure.

1.41- A Genetic Approach to Uncover the Ethanol-BK Channel Molecular Interaction

Complementary strategies to the conventional site-directed mutagenesis method to uncover the molecular interaction between ethanol and the BK channel are through

unbiased *in vivo* genetic approaches. Using chemical mutagenesis of the genome, two approaches can specifically target the BK channel in this manner: 1) non-complementation screen targeting the BK channel gene and, 2) next generation sequencing after mutagenesis to find animals with mutations in the BK channel gene. In both cases, characterization of the mutant's behavior on and off ethanol will uncover changes in basal function and ethanol sensitivity. The mutant's behavioral response off ethanol will determine if the BK channel is functional. If the channel is not functional, no useful information into the molecular interaction between ethanol and the BK channel will be uncovered. If the channel is functional, then the mutant's behavioral responses to ethanol can be tested. If differences are found between the mutant and a wild-type animal, the mutation may reveal residue(s) critical for the molecular interaction between the BK channel and ethanol.

The advantages of this approach are multiple. First, an unbiased screen does not need to identify specific residues *in vitro* before *in vivo* analysis. This eliminates the need to identify candidate mutations *in vitro*, and then knock-in the equivalent mutation in an animal's genome prior to behavioral experiments. Also, this approach is not confined to non-conserved residues between channels that display differential responses to ethanol. As stated earlier, it is entirely possible conserved or non-conserved residues between insensitive and sensitive channels are critical for the response to ethanol. This includes an ethanol binding pocket or residues necessary for conformational changes induced by ethanol. Third, because this approach begins by identifying phenotypic changes *in vivo*, this strategy uncovers changes important to the living animal's behavior on and off

ethanol rather than changes to a protein *in vitro* expressed in a heterologous system. As such, this *in vivo* approach avoids the study of mutations that would cause lethality, and thus represent promising candidate sites to target for pharmacological treatment of AUD. Candidate sites for ethanol interaction derived from an *in vivo* study with proven importance for behavior can then be studied in more detail at the level of single channel molecule with the power of *in vitro* physiological tools.

1.5 - *C. elegans*

C. elegans is a free-living roundworm that grows to about a millimeter in length as an adult. The use of *C. elegans* as a genetic research tool was first described by Brenner (1974), who provided methods for isolation of the nematode and characterization of mutant worms. The primary form of reproduction in the worm is self-fertilization; however, males can also be used for mating to allow for genetic crosses. The adult hermaphrodite consists of 959 somatic cells, 657 of which make up primarily muscle, hypodermis, gut and gonad (Sulston and Horvitz, 1977). The simple nervous system is composed of 302 neurons, of which every synapse within the organism has been traced with serial electron microscopy to create a complete nervous system connectome (White et al., 1986).

The major advantages of the worm exploited in this dissertation include the short-life cycle, fecundity, small size, facile genetics and amenability to *in vivo* electrophysiological recordings. The fecundity, short life cycle and small size allow for

large scale behavioral tests in the worm, while the facile genetics allow for a myriad of methods to study genes, including chemical genetic screens and knock-in transgenic worms.

1.6 - *C. elegans* and Alcohol

As with mammals, the effects of ethanol on *C. elegans* is manifested as stimulation to incoordination to immobility and unresponsiveness with increasing concentrations (Morgan and Sedensky, 1995; Davies et al., 2003; Kwon et al., 2004; Hong et al., 2008; Speca et al., 2010; Bhandari et al., 2012; Alaimo et al., 2013; Dillon et al., 2013). Many genes in *C. elegans* are altered by ethanol, either indirectly or directly. Kwon et al. (2004) took a genomics approach to uncover the actions of ethanol on gene expression. In these experiments, RNA was collected at various times during treatment of wild-type worms with a high dose of ethanol. The RNA collected was used to probe microarrays that represented nearly all *C. elegans* open reading frames to determine what changes occurred in gene expression. It was found nearly 1% of the genes (230) change in response to ethanol.

Behavioral changes during ethanol exposure include a reduction in body bend depth during locomotion and a decrease in egg laying and pumping of its feeding organ (Davies et al., 2003; Mitchell et al., 2007; Dillon et al., 2013). Importantly, these effects are readily reversible after removal from ethanol, and worms demonstrate a similar cutoff effect seen in other organisms with alcohols and anesthetics (Morgan and Sedensky,

1995). These results suggest that ethanol acts upon the nervous system to elicit reversible behavioral responses analogous to those in higher animals. *C. elegans* also metabolizes alcohol similarly to mammals via homologous alcohol dehydrogenases (Williamson et al., 1991; Glasner et al., 1995; Alaimo et al., 2013).

Neuronal genes in *C. elegans* with clear mammalian homologues have been identified as critical for the response of *C. elegans* to ethanol. The mitochondrial gene, *gas-1*, increases ethanol sensitivity in complex I of the mitochondria (Kayser et al., 2003). Importantly, the mammalian complex I is strongly affected by ethanol as well (Chazotte and Vanderkooi, 1981). Loss-of-function mutations in the synaptic vesicle-associated *rab-3* gene and its guanosine triphosphate exchange factor *aex-3* gene confer resistance to the acute locomotor effects of ethanol (Kapfhamer et al.,2008). Similarly, mice lacking one or both copies of *rab-3* are resistant to the ataxic and sedative effects of ethanol, while increasing voluntary ethanol consumption. Similar to the mouse gene, *munc-18*, the worm homologue *unc-18* modulates changes in worm locomotion induced by ethanol (Graham et al., 2009). Preference to ethanol is mediated by similar neurotransmitter systems between *C. elegans* and mammals. *C. elegans* lacking enzymes necessary for biosynthesis of dopamine or serotonin fail to develop an ethanol preference demonstrated in wild-type animals (Lee et al., 2009). Recently, our own lab has uncovered that *dop-4*, a D1-like dopamine receptor, impairs ethanol-induced disinhibition of crawling behaviors when *C. elegans* is submerged in liquid (Topper et al., 2014).

Like mammals, the lipid environment plays a role in the development of tolerance to alcohol in the worm. By decreasing cholesterol levels in *C. elegans* through

environmental manipulation, Bettinger et al. (2012) demonstrated that development of AFT to ethanol was inhibited. Further, genetically increasing or decreasing expression of *lips-7*, a lipase known to regulate levels of triacylglycerols, results in opposing effects on AFT. In addition, in both mice and worms, the respective CRF receptors have been shown to mediate the development of AFT (Jee et al., 2013).

Withdrawal from ethanol also occurs in *C. elegans*. In wild-type worms this is exemplified as spontaneous deep body bends, which are relieved by a low dose of ethanol (Mitchell et al., 2010). A mutant deficient in a proprotein convertase that participates in peptide secretion, *egl-3*, is resistant to withdrawal. In addition, a CRF-like receptor has also been shown to be critical for withdrawal of *C. elegans* from ethanol (Jee et al., 2013).

Moderate alcohol consumption is thought to have beneficial effects on heart disease, osteoporosis, dementia and depression (Meister et al., 2000). Worms too display beneficial effects on low alcohol concentrations, including extended lifespan and a slower decline of mobility during aging (Zhao et al., 2011). However, as with their mammalian counterparts, high doses are harmful, significantly impairing mobility, shortening lifespan and reducing fertility (Zhao et al., 2011).

As with humans, a natural variation in the acute response to ethanol has been found in *C. elegans*. The Hawaiian and Bristol wild-type strains have allelic variation in the neuropeptide Y (NPY) receptor. The more active variation of NPY receptor in the wild-type Bristol strain results in a smaller degree of acute tolerance as measured by locomotion over time on ethanol, and prevents a withdrawal behavior identified as

induction of worm clumping (Davies et al., 2004). Overexpressing NPY in mice recapitulates the effect of NPY-mediated acute tolerance in *C. elegans*, slowing recovery from ethanol-induced sleep (Thiele et al., 1998). Intriguingly, a significant decrease in expression of NPY has been found in alcoholic brain tissue (Mayfield et al., 2002). In rats, reduced levels of NPY in multiple brain regions occurs during chronic ethanol exposure has been found (Roy and Pandey, 2002; Bison and Crews, 2003). Also, allelic variation in neuropeptide Y (NPY) is associated with higher alcohol consumption in Finnish male populations and with alcoholism in European Americans (Kauhanen et al., 2000; Lappalainen et al., 2002). These results suggest a conservation of NPY for the behavioral responses to ethanol across species from humans to *C. elegans*. Overall, the strong conservation of the behavioral and molecular effects of ethanol from humans to *C. elegans* gives credence to the prediction that identification of the molecular actions of alcohol on proteins important for the response to alcohol in *C. elegans* will translate to humans.

1.61- Controversial Studies Regarding *C. elegans* and Alcohol

When studying the behavioral response of *C. elegans* to ethanol, high exogenous concentrations (400-600 mM) are required to engender intoxication (Davies et al., 2003; Alaimo et al., 2012), and even higher (1 M) to produce anesthetic effects (Morgan and Sedensky, 1995). When internal ethanol has been measured, usually the concentration is found to be 10-20 times lower than ethanol in the external environment (Davies et al.,

2003; Davies et al., 2004; Kapfhamer et al., 2008). As a result, a concentration of 400 mM exogenous ethanol equates to 20-40 mM in the worm, which is near the legal limit to drive in humans of a ~21.7 mM blood alcohol concentration. The reason for such low permeability of ethanol is largely due to the cuticle exoskeleton forming a barrier that surrounds the worm, often resulting in researchers applying 10-20 times the internal target dose of a compound to elicit behavioral effects (Rand et al, 1995; Burns et al., 2010; Cox et al., 1981). An additional route of ethanol entry may be through the pumping of the pharynx, effectively ingesting ethanol. The barrier to ethanol may be greater when *C. elegans* is swimming in liquid, because ingestion is inhibited in *C. elegans* when submerged in liquid (Vidal-Gadea et al., 2012). Whether submerged or not, ethanol acutely inhibits ingestion, so it is likely that this is only a secondary route of entry (Mitchell et al., 2007).

A recent report by Mitchell et al. (2007) suggested that more ethanol enters the worm than previously thought, and that the cuticle does not form a barrier for ethanol entry into the worm. Specifically, these researchers proposed the internal concentration of ethanol may actually be equivalent to the external concentration. If true, this work argues that all previous ethanol studies using *C. elegans* may not have been using concentrations that humans reach when ingesting alcohol. Mitchell et al. (2007) demonstrated this with a series of experiments. First, it was found that *C. elegans* reach steady-state locomotion inhibition by ethanol rapidly and recovered from ethanol exposure within minutes. Second, inhibition of pharynx pumping occurred in intact animals, and animals with their pharynx exposed to an ethanol solution within minutes of exposure. From these data,

these authors surmised that ethanol passes through the worm cuticle freely and rapidly, reaching an internal concentration in *C. elegans* that is similar to the concentration of ethanol in the external solution.

These authors also concluded that measurements of internal ethanol concentrations by previous worm researchers were incorrect. Specifically, in previous studies *C. elegans* was washed in cold buffer prior to measuring ethanol concentration in the worm pellet (Davies et al., 2003). According to Mitchell et al. (2007), this wash step washed away ethanol inside *C. elegans* during ethanol exposure. Abolishing this wash step resulted in a measurement of ~300 mM internal ethanol concentration in *C. elegans* when exposed to 500 mM exogenous ethanol for 20 minutes. The overall conclusion of this work was that the cuticle does not prevent ethanol from entering or exiting the worm. Chapter 2 of my dissertation will demonstrate a simple solution for the apparent discrepancies of these results from previous studies, while strongly suggesting that during a high exogenous exposure to ethanol (500 mM) the internal concentration of ethanol in *C. elegans* that produces a behavioral response is well under 100 mM.

1.7 - *C. elegans* BK Channel

The gene *slo-1* was first identified by Wei et al. (1996), who found there is high homology from worm to human, with their respective genes encoding proteins that have a 58% percent sequence similarity using a percent identity matrix. Importantly, all major functional regions including the pore domain, voltage-sensor, Ca^{2+} bowl and RCK1 are

nearly identical from worm to human. In addition, Wang et al. (2001) expressed the worm BK channel in oocytes and found that BK channel activation by depolarization and Ca^{2+}, and the large conductance in symmetrical K^{+} (~250 pS) demonstrated in the mammalian BK channel were conserved. It should be noted that the worm BK channel conductance is significantly smaller (~50 pS) when measured by *in vivo* recordings (Davies et al., 2003). Functional conservation of the BK channel from mammals to worm is present in that the BK channel controls neurotransmitter release in neurons (Wang et al., 2001; Liu et al., 2007). In addition, the worm BK channel is expressed in muscle, critical for its proper function (Jospin et al., 2002; Carre-Pierrat et al., 2006; Kim et al., 2009; Sancar et al., 2011).

Modification to the *C. elegans* BK channel also occurs through expression of auxiliary subunits and alternative splicing. The *bkip-1*gene encodes an auxiliary subunit that decreases the activation rate and shifts the conductance-voltage relationship in a Ca^{2+} dependent manner, while increasing BK channel surface expression (Chen et al., 2010). In addition, the twelve predicted isoforms of the worm BK channel have been identified as being expressed in the worm, and critical for proper synaptic transmission (Johnson et al., 2011). Specific alternative exons have been identified that regulate both activation kinetics and Ca^{2+} sensitivity of the BK channel (Glauser et al., 2011).

Unlike their mammalian counterparts, when the worm gene equivalent is knocked-out only minor changes to basal behavior occur in the laboratory setting. These include displaying slightly slower and jerky locomotion (Wang et al., 2001; Guest et a., 2007; Davies et al., 2003), bending deeper at the head (Kim et al., 2009), laying fewer

eggs (Davies et al., 2003) and increasing reversal frequency (Crisford et al., 2011) compared to wild-type worms. These phenotypes of the *slo-1* null mutant are relatively minor compared to the BK channel knockout mouse mutant (Mirkowski, 2013), perhaps due to either a unique ability of the worm to compensate for lack of BK channel function, or a general phenomenon that simpler organisms can compensate for gene disruptions more effectively under laboratory conditions.

1.71- Using *C. elegans* to Elucidate BK Channel Modulation by Alcohol

The genetic tractability of *C. elegans* allowed Davies et al. (2003) to conduct an unbiased genetic screen that tested tens of thousands of mutants for behavioral resistance to ethanol. The screen employed two separate behaviors that are strongly suppressed by ethanol in wild-type worms: locomotion and egg-laying. Surprisingly, all mutants that displayed the strongest level of resistance measured had a null mutation in *slo-1*, which encodes the *C. elegans* BK channel.

By performing *in vivo* patch-clamp and single-channel recordings on the native BK channel in *C. elegans*, my advisor, Dr. Jon Pierce-Shimomura, demonstrated ethanol activated the BK channel *in vivo* at both 20 and 100 mM (Davies et al., 2003). This effect would inhibit neuronal activity throughout the nervous system and thus explain the decrease of locomotor and egg-laying behaviors in *C. elegans* exposed to ethanol. In addition, a recent study has demonstrated that in a dissected preparation, *slo-1* is responsible for inhibition of pharyngeal pumping induced by ethanol (Dillon et al., 2013).

Since it has been established that the highly conserved BK channel has a prominent role in the behavioral response to ethanol in species ranging from worms to mammals, the overarching goal of this dissertation is to harness the genetic power of the worm to uncover the conserved molecular interaction between ethanol and the BK channel. Specifically, I would like to identify conserved residues on the BK channel that are critical for the behavioral responses to ethanol, but not essential for BK channel basal function.

My first attempt to uncover the BK channel-ethanol molecular interaction included a non-complementation genetic screen to specifically identify mutations that would render the BK channel functional, but resistant to ethanol. I uncovered 23 mutants, all of which were putative nulls. Unfortunately, null worms are resistant because of a mutation that renders the BK channel non-functional, providing no new information on specific residues or regions of the channel that interact with ethanol. Next, I attempted to mutate the cDNA of the *slo-1* open reading frame (ORF) to generate functional, resistant BK channels. However, this approach proved difficult due to the number of mutations generated by error prone PCR on each unique cDNA. Next, I took advantage of the million mutation project, headed by the Moerman and Waterston labs. The goal of this project was to generate a collection of 2,000 mutagenized strains each with their entire genome sequenced (Thompson et al., 2013). To this end, I obtained 32 strains that had a unique missense mutation in *slo-1*, and tested each strain for basal behaviors mediated by *slo-1* and their response to ethanol. Last, I generated transgenic worms that expressed either the human or worm BK channel, and employed a site-directed approach to probe

for residues that are critical for ethanol modulating the BK channel to engender intoxication.

1.8 - Summary

The BK channel has been identified as being modulated by ethanol *in vitro*, and playing a role in intoxication, tolerance and drinking behaviors in mammalian model systems. The BK channel is the central protein in *C. elegans* to confer behavioral responses to clinically relevant concentrations of ethanol (Davies et al., 2003). Although it may be difficult (if not impossible) to measure addiction in *C. elegans*, uncovering how the molecular actions of ethanol produce intoxication will undoubtedly elucidate important information regarding alcohol-mediated behaviors dependent on the BK channel.

The remaining chapters of this dissertation expand on the establishment of *C. elegans* as a model system for studying the effects of ethanol and the work of Davies et al. (2003) by beginning to elucidate the molecular action of ethanol on the BK channel. Chapter 2 details experiments that proved solution osmolarity alters the intoxicating effects and the amount of ethanol entering *C. elegans*. In addition, this work used a novel method to measure internal ethanol concentration in *C. elegans* to demonstrate the cuticle represents a significant barrier to ethanol entry. These experiments are critical because it resolves previously irreconcilable results from Davies et al. (2003) and others with Mitchell et al. (2007), who called into question the use of high exogenous concentrations

of ethanol being appropriate for studying the intoxication effects of ethanol in *C. elegans*.

Chapter 3 details experiments that elucidate an amino acid residue within the BK channel critical for behavioral intoxication in *C. elegans.* I found that a strain with the SLO-1 substitution T381I near the RCK1 domain was highly resistant to intoxication. This mutation did not interfere with other BK-channel-dependent behaviors, suggesting that the mutant channel retained normal *in vivo* function. Knock-in of wild-type versions of the worm or human BK channel rescued intoxication and other BK-channel-dependent behaviors in a *slo-1* null-mutant background. By contrast, knock-in of the worm T381I or equivalent human T352I mutant BK channel selectively rescued BK-channel-dependent behaviors while conveying resistance to intoxication. Single-channel patch-clamp recordings confirmed that the human BK channel engineered with the T352I missense mutation was insensitive to activation by ethanol, but otherwise had normal conductance, potassium selectivity and only subtle differences in voltage dependence. This threonine amino acid within the RCK1 domain is the first residue found on the BK channel to be critical for the effects of ethanol without being critical for channel function.

Chapter 4 elucidates differences in how the worm and human BK channel respond to ethanol. Previous work by Liu et al. (2008) found that Ca^{2+}-sensing domains are critical for the mammalian channel to respond to ethanol. To determine if the conserved calcium-sensitive residues were also critical for the response of the worm BK channel to ethanol, I generated SLO-1 knock-in replacement mutants that render the RCK1, Ca^{2+} bowl, or both domains insensitive to calcium. As expected, mutating these domains appeared to perturb the *in vivo* function of the SLO-1 channel because resulting neck and

body curvature mimicked that of the BK null mutant. Unexpectedly, however, we found that mutating the calcium-sensitive domains singly or together had no effect on intoxication in *C. elegans*. Thus, for the worm SLO-1 channel, the calcium-sensitive domains appear critical for basal *in vivo* function, but not critical for *in vivo* ethanol action.

Chapter 5 is an overall discussion of the results of experiments presented in Chapters 2-4. General conclusions about the utility of using non-traditional genetic manipulations or tools, such as the million mutation project will also be discussed. In addition, the potential trajectories for future studies will be outlined.

2.0 - OSMOLARITY MODIFIES BEHAVIORAL RESPONSES TO ETHANOL IN *C. ELEGANS*

The majority of text, data and figures presented in this chapter have been published in a 2012 manuscript in the journal *Alcohol: Clinical and Experimental Research*:

Alaimo JT, Davis SJ, Song SS, Burnette CR, Grotewiel M, Shelton KL, Pierce-Shimomura JT, Davies AG, Bettinger JC (2012) Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*. *Alcohol Clin Exp Res* 36: 1840-50.

Co-author contributions: Alaimo JT, Burnette CR, Grotewiel M, Shelton KL, Davies AG and Bettinger JC focused on the ethanol metabolism portion of this manuscript. Specifically, these authors discovered that inactivating an alcohol dehydrogenase gene (*sodh-1*) or aldehyde dehydrogenase genes (*alh-6* or *alh-13*) resulted in an increase of internal ethanol concentrations and caused hypersensitivity to the acute effects of ethanol on locomotion. In addition, all internal alcohol concentration measurements and experiments testing ethanol inhibition of *C. elegans* crawling on an agar plate were run by Alaimo JT. I ran all experiments that studied the ethanol response of *C. elegans* when submerged in liquid. In all ethanol experiments, behavioral intoxication was measured in *C. elegans* in high and low osmolarity conditions. Song SS, an undergraduate research assistant that I trained helped me run behavioral experiments. Pierce-Shimomura JT is my P.I. who funded the study and provided guidance for the osmolarity experiments.

2.1 - Abstract

When studying the behavioral response of *C. elegans* to ethanol, high exogenous concentrations (400-600 mM) are required to engender intoxication (Davies et al., 2003). When the corresponding internal ethanol concentrations have been measured, it has been reported to be 10-20 times lower than ethanol in the external environment (Davies et al., 2003; Davies et al., 2004; Kapfhamer et al., 2008). The reason for such low permeability of ethanol is largely due to the cuticle forming a barrier that surrounds the worm. A contrasting report by Mitchell et al. (2007) suggested the internal concentration of ethanol is actual equivalent to the external concentration and the cuticle does not represent a significant barrier to ethanol. However, this report was unique in that it studied *C. elegans* submerged in a high osmolarity solution containing ethanol, and the method for measuring internal ethanol was altered compared to previous studies. To settle these conflicting reports, we used *C. elegans* to directly examine how changes in osmolarity alter behavioral responses to alcohol during an acute exposure. In addition, to investigate potential differences in permeation of ethanol in *C. elegans*, internal ethanol measurements were taken while varying ethanol solution osmolarity. We demonstrated that the sensitivity to intoxication is strongly influenced by the osmolarity of the exogenous ethanol solution. We also established that only a small amount of exogenously applied ethanol accumulated in the tissues of *C. elegans* and consequently tissue concentrations were similar to those that intoxicate humans. Moreover, the osmolarity of the surrounding solution significantly contributes to the amount of ethanol that permeates into *C. elegans*. These data suggest that either the behavioral responses to ethanol and/or ethanol permeability in *C. elegans* is dependent on solution osmolarity. These findings explain contrasting publications on the behavioral responses of *C. elegans* to ethanol.

Overall, these data reinforce the findings that using high exogenous concentrations of ethanol equates to internal concentrations in *C. elegans* below 100 mM. These concentrations are similar to clinically relevant concentrations of ethanol ingested routinely by people to achieve intoxication.

2.2 - Introduction

Alcoholism is a disorder with both genetic and environmental underpinnings. However, critical genes have yet to be identified that are strong predictors for alcoholism. The genetic tractability of the nematode *C. elegans* has been increasingly exploited to illuminate the genetic basis for alcohol-mediated behaviors (Morgan and Sedensky, 1995; Davies et al., 2003; Davies et al., 2004; Kwon et al., 2004; Kapfhamer et al.,2007; Graham et al., 2009; Bettinger et al., 2012; Jee et al., 2013 Topper et al., 2014).

Proteins and neurological pathways identified as important for alcohol-induced behaviors and withdrawal in mammals have also been reported to be critical in *C. elegans*, such as the BK channel and neuropeptide Y-like, dopamine and CRF signaling pathways (Davies et al., 2003; Davies et al., 2004; Topper et al., 2014; Jee et al., 2013). Because of the conservation of neurological pathways from worm to human, *C. elegans* is an extremely useful tool in identifying genetic clues relevant to alcohol-mediated behaviors in humans.

High exogenous concentrations (400mM-600mM) of ethanol are often used to study alcohol-mediated behaviors in worms (Davies et al., 2003). When internal ethanol has been measured, usually the concentration is reported to be 10-20 times lower than ethanol in the external environment. This concentration (20-40mM) is well within the clinically relevant dose of ethanol reached routinely by people (Davies et al., 2003; Davies et al., 2004; Kapfhamer et al., 2008). In the majority of experiments studying behavioral intoxication in *C. elegans*, ethanol is added to plates made with nematode growth medium (NGM) mixed with agar. NGM solution is used to make the agar plates

that *C. elegans* are cultured on. These studies typically measure ethanol-inhibition of egg-laying and locomotion of *C. elegans* (Davies et al., 2003; Davies et al., 2004). Also, Morgan and Sedensky (1995) tested the behavioral effects of submerging worms in nematode growth medium (NGM) solution mixed with 1 M ethanol. It is unclear if these concentrations represent an anesthetizing dose for the worms, or if the exoskeleton cuticle that encases the worm is harder for ethanol to breach while swimming in liquid versus crawling on a plate. In addition, ethanol may be ingested by a worm crawling on an agar plate through pumping of the pharynx. The barrier to ethanol may be greater when *C. elegans* is swimming in liquid because pharyngeal pumping is inhibited in *C. elegans* when submerged in liquid (Vidal-Gadea et al., 2012).

A contrasting report by Mitchell et al., (2007) reported that the internal concentration of ethanol is actually equivalent to the external concentration, suggesting the cuticle does not represent a significant barrier to ethanol. This was demonstrated with a series of experiments. First, animals were immersed in a solution commonly used for electrophysiological recordings, Dent's saline. The response of *C. elegans* to Dent's saline that contained ethanol was a steady-state inhibition by three minutes. Also, *C. elegans* recovered from ethanol two minutes post-removal. Second, electropharyngeogram (EPG) recordings were performed in intact whole worms and worms with their pharynx exposed to Dent's saline that contained ethanol. EPG recordings are extracellular recordings of the pharynx that describe the rate of pharyngeal pumping and the duration of each pump (Avery et al., 1995). It should be noted that serotonin was also used in the Dent's saline solution to stimulate pharyngeal pumping.

These authors found although the time course for the intact animal was slightly slower, inhibition of the pharynx occurred in both cases within 3 minutes. From the behavioral and EPG experiments, these authors surmised that ethanol passes through the worm cuticle freely and rapidly.

These authors also concluded that measurements of internal ethanol concentrations by Davies et al. (2003) were incorrect because *C. elegans* was washed in cold buffer prior to measuring ethanol concentration in the worm pellet. According to Mitchell et al. (2007), this wash step washed away ethanol that was inside *C. elegans* during ethanol exposure. To demonstrate their point, they set up an experiment with multiple conditions, in which they either did not wash the pellet, or washed the pellet with 50 or 500 µl of buffer. From these results they surmised that the internal ethanol measurements were ~300 mM in the no wash condition, ~100 mM in the 50 µl wash condition and ~20 mM in the 500 µl wash condition. Because Mitchell et al. (2007) assumed the wash step washed away ethanol that was present inside the worm during ethanol exposure, they concluded that the internal concentration of ethanol in worms was at least ~300 mM. The overall conclusion of this work was that the internal concentration in *C. elegans* is similar to the concentration of ethanol in the exogenous solution.

The differences in both the behavioral responses to ethanol and the vastly different internal ethanol measurements in these experiments compared to previous work caused us great concern. First, the behavioral effect of ethanol on *C. elegans* was not consistent with what we had observed on agar plates or when we submersed worms in NGM buffer containing ethanol. In addition, in our opinion there is an incorrect

assumption pertaining to their concentration measurements. In their no wash condition, we believed it was likely that ethanol in the external and internal environment was measured because no wash was performed to wash away the exogenous ethanol solution. Therefore, it would be expected that the measured concentration should be somewhere between the internal concentration in the worm and the external environment. However, these authors assumed the measured concentration of ~300 mM provided evidence supporting their hypothesis that ethanol rapidly and freely passes through the cuticle. Moreover, Mitchell et al. (2007) reported worms recover after two minutes post-ethanol exposure due to ethanol leaving the worm rapidly. However, because the wash step performed by Mitchell et al. (2007) was under one minute in both the 50 and 500µL wash conditions, there should still be a significant amount of ethanol inside *C. elegans*. In stark contrast, Mitchell et al. (2007) reported only a ~20 mM concentration in the 500 µL wash group. Nevertheless, we were concerned that these high internal measurements suggested using high exogenous concentrations of ethanol to induce ethanol-mediated behaviors in *C. elegans* may not generalize to relevant concentrations of ethanol reached by humans.

We decided to explore the differences in the ethanol-response of *C. elegans* between Mitchell et al. (2007) with our and other's previous work. To accomplish this, we tested the behavioral effects of ethanol on *C. elegans* in conditions similar to Davies et al. (2003) compared to conditions in experiments by Mitchell et al. (2007). This resulted in four unique conditions in which we tested *C. elegans* exposed to ethanol. Worms were either submersed in liquid or crawling on agar in both low (NGM) osmolarity solutions used by our and other groups, or high (Dent's saline) osmolarity

solutions used by Mitchell et al. (2007). In addition, we devised a new method to measure internal concentrations where worms were picked off of their ethanol plate directly and placed into a tube containing ddH2O, eliminating the need for the controversial wash step.

2.3 - Methods

Nematode culture and strains

Wild-type N2 C. elegans were maintained at room temperature using standard measures. (Brenner, 1974).

Analysis of Locomotion (crawling)

Age-matched 55 hour-old adult animals reared at 20°C were used. Locomotion on plates was assayed as described (Davies et al., 2003). Ten worms for each strain were tested. Two minute movies were recorded, and movies were analyzed using ImagePro Plus Version 6 (Media Cybernetics, Bethesda, MD, U.S.A.).

Time course analysis was performed as above except that single animals were placed on plates and video images were recorded every 0.5 seconds for 15 minutes. The speed for each interval was averaged for 6 worms. The speeds were binned for each 1-minute period and an average was calculated. A one-way ANOVA was performed with a significance value of $P < 0.01$ using Prism v5.0 (GraphPad Software), with Bonferroni post-hoc tests comparing all bins.

The effects of osmolarity on crawling were assessed as above, except that assay plates were either made of standard NGM or of Dent's Saline Solution (10 mM D-glucose, 10

mM HEPES, 140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) with 2% agar.

Analysis of Locomotion (swimming)

The effect of ethanol on swimming was assessed for both individuals and groups of animals. Individuals were video recorded (30 frames per second) in liquid medium (3 mL) over a 2% agarose surface in 6 cm diameter plates to promote continuous swimming. The midlines of the worms were determined with a custom-written image analysis macro using ImagePro as described (Pierce-Shimomura et al., 2008). Animals were gently transferred to identical conditions that contained 400 mM ethanol. Groups of animals were video recorded in identical conditions. The number of head bends made by each individual in a 30-second time window was quantified at 5, 10, 15 and 20 minutes of exposure to ethanol.

Internal ethanol Concentration calculations

Exposure to ethanol

55-hour old worms reared at 20°C were used. Several hundred worms were placed on unseeded plates containing the appropriate concentration of ethanol (prepared as for locomotion assays, see above) for 10 or 50 minutes. Worms were photographed for subsequent size analysis. Exactly 200 worms were picked from plates to a tube containing 20 µl ddH_2O. Tubes were placed at −80°C until analysis. Worms were thawed on ice and ground in the tube with a pestle (Kontes pellet pestle, Fisher Scientific, USA). Worm homogenate was stored at −20°C.

For 200 worms: Picking took an average of 1.5 minutes per condition. Picking itself caused a loss of between 0 μL and 0.25 μL of the water, evaporation in that time caused an undetectable loss of volume.

Size calculation

Photographs were analyzed using ImagePro Plus v6. Ten worms were used per strain per condition. A midline was drawn to determine the height, h. Three diameters were drawn, one at the vulva (the approximate center of the animal), one each at the approximate midpoint between the vulva and head or tail. These lengths were averaged and used as the diameter, ½ of this length was the radius, r. Volume was determined as for a cylinder: volume = $\pi r^2 h$.

2.4 - Results

Responses of *C. elegans* to ethanol is modulated by solution osmolarity

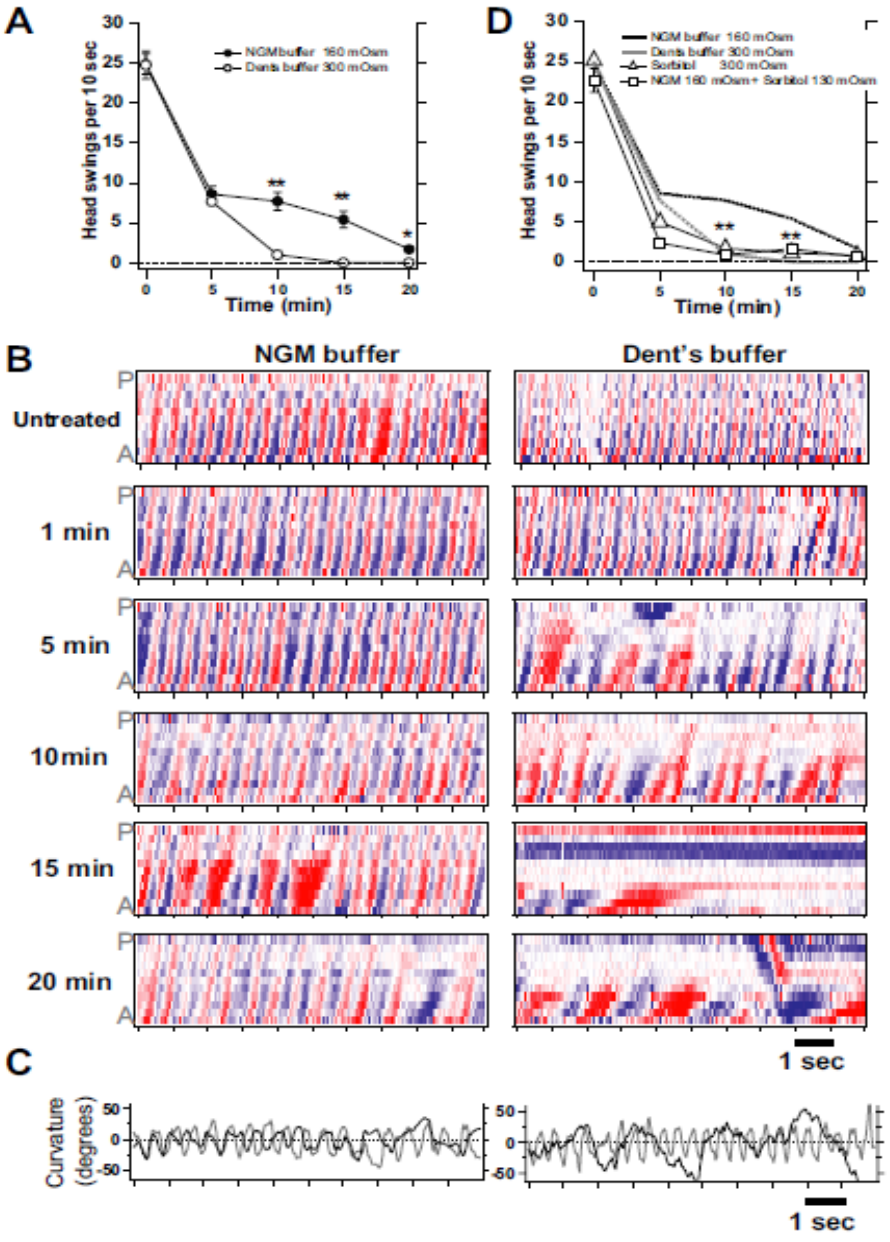
We demonstrated that ethanol inhibits swimming in *C. elegans*, although at a substantially higher dose than is required to inhibit crawling (Fig. 2.1). While our results are in agreement with the first study of ethanol's effects on swimming in *C. elegans* (Morgan and Sedensky, 1995), they differ significantly from those reported by Mitchell and colleagues (2007), who used slightly different conditions. We wanted to test if the difference of ethanol response found by our group compared to Mitchell et al. (2007) could be explained by the respective buffers. Our study used NGM solution which contains the same salts as the medium that animals are cultured on (Brenner, 1974), whereas the Mitchell study used Dent's buffer, a physiological saline normally used to

record the electrical activity of dissected pharynx muscle (Avery et al., 1995). While the basal swimming behavior of worms in the two buffers were indistinguishable (data not shown), we observed a striking difference in behavior in the two buffers when ethanol was added. Figure 2.1A shows the time course of intoxication in 500 mM ethanol in the two buffers. Swimming movement was quantified as the number of head swings during a 10-second time window. The motion of the animals exposed to ethanol decreased in both conditions by 5 minutes of exposure. However, animals assayed in Dent's buffer became essentially immotile by 10 minutes, whereas animals assayed in NGM buffer decreased motion but remained motile during the entire 20 minutes of treatment.

To examine the time course of intoxication during swimming more closely, we plotted matrices that represent body curvature along the anterior-posterior axis versus time (Fig. 2.1B). In this scheme, upward slanting grey and white "waves" in the matrix represent ventral and dorsal bends that pass from head to tail to generate forward motion. The representative plot for an animal swimming in NGM buffer (Fig. 2.1B, left column) shows little difference from its untreated condition even after 10 minutes in ethanol aside from a slight decrease in frequency and dampening of bends, which is evident in the muted grey and white color code. By contrast, the representative plot for an animal swimming in Dent's buffer (Fig. 2.1, right column) shows severe impairment of coordination, beginning at 5 minutes of exposure, reflected in the fact that many of the bends fail to propagate fully from head to tail. Portions of the animal also become immobile by 15 minutes, which is reflected in the fixed color pattern versus time. We also compared the swimming of untreated (gray line) versus 20-minute exposure to

ethanol (black line) by plotting the “neck” curvature versus time (Fig. 2.1C). The animal treated with ethanol in NGM buffer showed a slowing in frequency, while the animal in Dent’s buffer showed a much slower bend frequency and a rise in maximal bend amplitudes.

Figure 2.1 - Sensitivity to intoxication while swimming depends on exogenous osmolarity.
(A) Time course of intoxication while animals were swimming in 500 mM ethanol. Animals become significantly more immobilized by the same concentration of ethanol in Dent's buffer th an in NGM buffer (**p < 0.001). (B) Body curvature matrices during intoxication for 1 representative animal in NGM buffer and 1 representative animal in Dent's buffer. Grey scale along the anterior-posterior (A-P) axis versus time represents the amount of bending at given points along the body (grey = curved posture, white = straight posture). (C) Plots of neck curvature versus time. Untreated (gray) and 20-minute treatment in ethanol (black). (D) Time course of intoxication while animals were swimming in 500 mM ethanol. NGM and Dent's buffer data are replotted from panel (A). Animals treated with NGM+ sorbitol or sorbitol alone were as sensitive to ethanol as those animals treated in Dent's buffer.

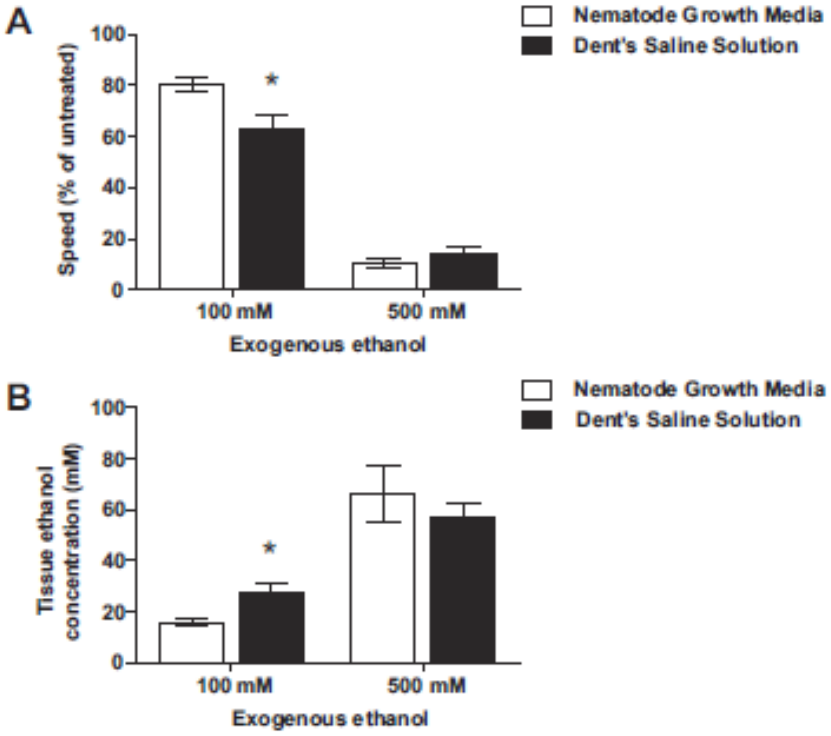


While searching for factors that could explain the vastly different results obtained in the different buffers, we noticed that the 2 buffers differed greatly in osmolarity: NGM is 160 mOsm, while Dent's buffer is 300 mOsm. We tested whether *C. elegans*' sensitivity to intoxication while swimming depends on osmolarity and demonstrated that worms became rapidly intoxicated when assayed in NGM buffer in which we had adjusted the osmolarity to match Dent's buffer by adding 130 mOsm sorbitol (Fig. 2.1D). Assaying worms in only 300 mOsm sorbitol without any salts reproduced a dose–response for intoxication that was characteristic of Dent's buffer (Fig. 2.1D). Moreover, we found that preincubation for 20 minutes in Dent's buffer or 300 mOsm sorbitol conferred enhanced sensitivity to intoxication when the animals were assayed in 150 mOsm NGM buffer (data not shown). Together, these results suggest that acute sensitivity to exogenous ethanol in *C. elegans* depends on osmolarity, that this sensitivity can be dynamically adjusted, and that this is an explanation that can resolve the conflicting reports of dose–response sensitivity to ethanol while swimming.

We next tested the effect of osmolarity on the response of *C. elegans* crawling on agar plates and found that the effect was significant, but more subtle than for swimming. At 10 minutes of exposure to 100 mM ethanol, the behavior of worms tested on NGM was less affected compared with worms tested on plates made with Dent's saline (Fig. 2.2A). We asked whether the effect of osmolarity on the dose–response for ethanol was attributed to altering the acute accumulation of ethanol. At 10 minutes of 100 mM ethanol exposure, worms on NGM plates accumulated significantly less ethanol than did animals on Dent's saline plates (Fig. 2.2B).

Figure 2.2 - Sensitivity to intoxication and tissue accumulation of ethanol while crawling depends on exogenous osmolarity.

(A) Animals were treated with exogenous ethanol for 10 minutes, 2-minute digital movies were recorded, and speed was determined by ImagePro image analysis software. A% relative speed was calculated by dividing treated speed by untreated speed to account for any baseline speed differences. Animals exposed to 100 mM of exogenous ethanol for 10 minutes on NGM) were less affected than animals exposed on Dent's saline plates. (B) Animals exposed to ethanol on NGM plates accumulated significantly less ethanol in tissue than animals exposed on Dent's saline plates.



2.5 - Discussion

Our investigation into the reported differences in ethanol sensitivity for swimming behavior led to the unexpected finding that the sensitivity of *C. elegans* to ethanol depends on the osmolarity of the external medium, and that the worms are able to change *their sensitivity to the effects of osmolarity based on experience. Therefore, the* constituents of the exogenous medium are critical to note in future alcohol studies using *C. elegans.* We speculate that the permeability of the worm to exogenous ethanol might change depending on the history of exposure. Dynamic permeability may be adaptive for an animal that must occasionally encounter dangerous chemicals, such as ethanol, while roaming through its natural soil environment. Future study of this phenomenon in *C. elegans* may give rise to novel strategies to alter excess permeation of ethanol into specific tissues to prevent toxicity. The effects of osmolarity may also be synergistic to the effects of ethanol. This would not be unique to *C. elegans*. One of the proposed methods of action for ethanol on proteins and lipid membranes involves the replacement of hydrogen-bonded water by ethanol (Erying et al., 1973). Osmotically active osmolytes can inhibit protein conformational changes in which there is a net uptake of water, instead favoring conformations in which a protein releases water into the bulk aqueous medium. This will increase the interaction between ethanol and target proteins. Experimental evidence for this has been demonstrated in human blood plasma, where 50 mM ethanol was 2.7 times more potent at 360 mOsm than at 0 mOsm (Mitchell and Litman, 2000).

We also developed a method to novel method without using a controversial wash step to measure the internal ethanol concentration in *C. elegans*, in which we used a known volume of worm tissue and measured ethanol concentration using gas chromatography. The concentrations of ethanol that we measured reflect the relative lack

of permeability of these nematodes to chemicals in their environment; for 500 mM of exogenous ethanol, the wild-type internal concentration is in the range of 70 to 90 mM (Fig 2.2B). This translates to blood alcohol concentration values of 0.32 to 0.41%, which would cause profound intoxication in a naıve human drinker. The degree of intoxication associated with these concentrations in *C. elegans* is also profound; on an agar medium, worms exposed to 500 mM ethanol move at approximately 20% of their untreated speeds (Davies et al., 2003, 2004), the amplitude of their body bends is significantly reduced (Davies et al., 2003), and their movement becomes severely uncoordinated.

Previously, we reported internal ethanol concentrations that were approximately half of the concentrations that we observe here (Davies et al., 2003, 2004; Kapfhamer et al., 2008). We found that we had overestimated the volume of worms in a pellet derived from spinning worms out of a solution because we assumed that the vast majority of the pellet was made of worm tissue, which we have found here to be incorrect. This observation may also explain why Mitchell and colleagues (2007) vastly overestimated the internal concentration in their animals; in their paradigm, they incubated worms in a high concentration of ethanol and then tested a pellet consisting of the resulting worm + ethanol solution. Our results suggest that the mixture probably contained relatively less worm tissue and more ethanol solution than they estimated, which would have contributed a significant amount of ethanol to the final concentration.

Whether differences in the behavioral response of *C. elegans* to ethanol was ultimately due to enhanced permeability and/or increased relative strength of ethanol in higher mOsm solutions, this paper provides a simple explanation for the differences in the behavioral effects of ethanol demonstrated by Mitchell et al., (2007) compared to other groups.

3.0 - CONSERVED SINGLE RESIDUE ON THE BK POTASSIUM CHANNEL REQUIRED FOR ACTIVATION BY ALCOHOL AND INTOXICATION IN *C. ELEGANS*

The majority of text, data and figures presented in this chapter have been submitted in a 2014 manuscript for publication in the journal *Journal of Neuroscience.*

Davis SJ, Scott L, Hu K, Pierce-Shimomura JT, (2014) Conserved Single Residue on the BK potassium channel required for activation by alcohol and intoxication in *C. elegans*.

Co-author contributions: I ran all the behavioral experiments that tested the inhibitory of effect of ethanol on egg-laying in *C. elegans* with the assistance of Hu K, an undergraduate research assistant that I trained. I ran all behavioral experiments that tested the inhibitory effect of ethanol on locomotion. Also, I generated all transgenic *C. elegans* used in this study. I also performed all recordings in *C. elegans* and HEK cells. Scott L trained me to transfect and record from HEK cells, and took the majority of the confocal images. Pierce-Shimomura JT is my P.I. who funded the study and provided guidance on experiments. The manuscript was written by me and Pierce-Shimomura JT with helpful comments from Scott L, Atkinson N., Aldrich R., and Harris A. Mayfield J. performed critical editing of the manuscript.

3.1 - Abstract

Alcohol directly modulates the BK potassium channel to alter behaviors in species ranging from invertebrates to humans. In the nematode *Caenorhabditis elegans*, mutations that eliminate the BK channel, SLO-1, convey dramatic resistance to intoxication by ethanol. We hypothesized that certain conserved amino acids are critical for ethanol modulation but not for basal channel function. To identify such residues, we screened *C. elegans* strains with different missense mutations in the SLO-1 channel. A strain with the SLO-1 missense mutation T381I near the RCK1 domain was highly resistant to intoxication. This mutation did not interfere with other BK-channel-dependent behaviors, suggesting that the mutant channel retained normal *in vivo* function. Knock-in of wild-type versions of the worm or human BK channel rescued intoxication and other BK-channel-dependent behaviors in a *slo-1* null-mutant background. By contrast, knock-in of the worm T381I or equivalent human T352I mutant BK channel selectively rescued BK-channel-dependent behaviors while conveying resistance to intoxication. Single-channel patch-clamp recordings confirmed that the human BK channel engineered with the T352I missense mutation was insensitive to activation by ethanol, but otherwise had normal conductance, potassium selectivity and only subtle differences in voltage dependence. Together, our behavioral and electrophysiological results demonstrate that the T352I mutation selectively disrupts ethanol modulation of the BK channel. The T352I mutation may alter a binding site for ethanol and/or interfere with ethanol-induced conformational changes that are critical for behavioral responses to ethanol.

3.2 - Introduction

The large-conductance voltage- and calcium-activated potassium (BK) channel mediates a wide variety of physiological processes, including neurotransmitter release, action potential bursts and afterhyperpolarization (Robitaille et al., 1993; Golding et al., 1999; Landcaster and Nicoll 1987). The BK channel also mediates ethanol intoxication and tolerance in worms, flies, mice and humans (Davies et al., 2003; Cowmeadow et al., 2005; Cowmeadow et al., 2006; Martin et al., 2008; Kreifeldt et al., 2013). Many studies have demonstrated BK channel activity is altered by pharmacologically relevant concentrations (20–100 mM) of ethanol (Chu and Triestman, 1997; Jakob et al., 1997; Dopico et al., 1998; Walters et al., 2000; Dopico, 2003; Brodie et al., 2007). Modulation of the BK channel by ethanol is also evident in reconstituted lipid bilayers demonstrating that ethanol directly acts on the protein (Chu et al., 1998).

A few studies have mechanistically explored how ethanol modulates the BK channel *in vitro*. For example, phosphorylation of two residues near the first two transmembrane domains switches the action of ethanol from activation to inhibition in the bovine BK channel (Liu et al., 2006). Ethanol modulation further depends on the presence of intracellular calcium and specific residues required for calcium activation, as well as the intracellular tail (Liu et al., 2008; Liu et al., 2013).

We previously showed that mutants with predicted null mutations in the worm BK channel SLO-1 are extremely resistant to ethanol intoxication as measured by egg laying and locomotion (Davies et al., 2003). In *C. elegans*, egg laying is a behavior governed by a defined neural circuit (Waggoner et al., 1998). Ethanol reduces the rate of

egg laying by 90% in wild-type worms compared to only 0–30% in *slo-1(null)* mutant worms (Davies et al., 2003). Ethanol reduces the rate of locomotion by 70% in wild-type worms. In addition, the native SLO-1 channel excised in patches of neuronal membrane from *C. elegans* was activated by ethanol (20–100 mM) (Davies et al., 2003).

Our previous work demonstrated that the BK channel is critical for intoxication in *C. elegans*, but did not divulge a molecular mechanism for ethanol action. In this study, we exploited the facile genetics of *C. elegans* and screened mutant worms with novel missense mutations in the BK channel. Our aim was to identify an amino acid residue that mediates ethanol effects but that is not critical for normal basal activity *in vivo*. We identified a mutation in a conserved residue that was essential for both ethanol modulation of the BK channel *in vitro* and intoxication *in vivo* without altering single channel properties or other BK-channel-dependent behaviors.

3.3 - Materials and Methods

Animals: *C. elegans* strains were grown at 20 °C and fed OP50 strain bacteria seeded on Nematode Growth Media (NGM) agar plates as described previously (Brenner, 1974). Worms cultured on plates contaminated with fungi or other bacteria were excluded from this study. The N2 Bristol strain of *C. elegans* was used as the wild-type reference. The *slo-1(null)* strain used in this study was NM1968 harboring allele *js379* (Wang et al., 2001). Additional strains from the million mutation project (Thompson et al., 2013) with predicted missense mutations in the *slo-1* gene were also used, including VC40372, as

well as:VC40853,VC40064, VC40938, VC30161, VC40899, VC40774, VC40641, VC40384, VC40804, VC40392, VC40468, VC40143, VC40062, VC20545, VC40416, VC20244, VC20444, VC40417, VC20417, VC20590,VC20642, VC40787, VC40642, VC30157, VC40692, VC41014, VC20468, VC40265, VC20240, VC40381, VC40221. The VC40372 strain was outcrossed with wild-type strain N2 six times, twice in parallel to generate strains JPS428 and JPS429, which are referred to as *slo-1(T381I)6x[#1]* and *slo-1(T381I)6x[#2]* in the results, respectively.

Transgenesis: The background for all transgenic worms generated in this study for behavior analyses included the characterized null allele, *js379*, of *slo-1* (Wang et al., 2001). Transgenic worms used for imaging carried the additional integrated reporter *Punc-17::gfp* for cholinergic neurons *vsIs48* (Chase et al., 2004) and worms used for electrophysiological recordings carried the additional integrated reporter *Punc-47::gfp* for GABA neurons (Hammarlund et al., 2007). Multi-site gateway technology (Invitrogen, Carlsbad, CA) was used to construct plasmids. 2501 kb of the native *slo-1* promoter (*Pslo-1*) and the traditional *unc-54* UTR were used in combination with either *slo-1(cDNA)::mCherry* or *hslo(cDNA)::mCherry* for wild-type transgenes. *hslo* cDNA was kindly provided by Dr. Richard Aldrich (University of Texas at Austin). Mutant versions were made as described below via site-directed mutagenesis. All plasmids were injected at a concentration of 20 ng/µl. The co-injection reporter PCFJ90 at a concentration of 1.25 ng/µl was used to ensure proper transformation of the following arrays. Two independent isolates were obtained for each of the four strains to help control for

variation in extrachromosomal arrays: strains JPS344 and JPS345 carried *vxEx344*[*Pslo-1::slo-1::mcherry::unc-54UTR,Pmyo-2::mcherry*], strains JPS327 and JPS 328 carried *vxEx327*[*Pslo-1::slo-1(T381I)::mcherry::unc-54UTR,Pmyo-2::mcherry*], strains JPS338 and JPS339 carried *vxEx338*[*Pslo-1::hslo::mcherry::unc-54UTR,Pmyo-2::mcherry*], strains JPS325 and JPS326 carried *vxEx325*[*Pslo-1::hslo(T352I)::mcherry::unc-54UTR,Pmyo-2::mcherry*]. For the control transgenic strain, the plasmid PCFJ150 (the backbone of which was used to construct the transgenic worms) was injected at a concentration of 20 ng/µl, along with 1.25 ng/µl of PCFJ90 plasmid to generate strain JPS383 that carried the extrachromosomal array *vxEx383*[*Pmyo-2::mcherry*].

Site-Directed mutagenesis: QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA). The primers

5'-ggacaccgaatcgtagatgatatggccacagac-3' and

5'-gtctgtggccatatcatctacgattcggtgtcc-3' were used to cause a C->T mutation in the *slo-1* gene that resulted in a T381I amino acid substitution. The primers 5'-gtggtctgcggacacatcattctggagagtgttt-3' and

5'-aaacactctccagaatgatgtgtccgcagaccac-3' were used to cause a C->T mutation in the *hslo* gene that resulted in a T352I amino acid substitution. All plasmids were confirmed by sequencing the full cDNA.

Locomotion posture assay: The movement of single worms was digitally recorded at 30 frames per second for 5-10 minutes and analyzed as described previously (Pierce-Shimomura et al., 2008). Briefly, custom-written software automatically recognizes the worm from each frame and assigns thirteen points spaced equally from the head to the tail along the midline of the body. The neck angle was defined as the angle formed by the most anterior three points for video frames selected when the head of the worm was maximally swung to the ventral side while crawling on an unseeded NGM-agar plate.

Egg laying response to ethanol: Plastic Petri plates (6-cm diameter) filled with NGM-agar (12 mL) were seeded with OP50 *E. coli* at least 20 hr before the assay and stored at 4 ^{0}C for no more than 2 weeks. Plates were brought to room temperature (20 ^{0}C) 1 hr before testing. Ethanol plates were prepared by adding 200-proof ethanol (Sigma Aldrich) beneath the agar 30 minutes prior to the assay to allow ethanol time to soak into the agar. For 600-mM plates, 420 μL was added. For 400-mM plates, 292 μL was added. It is important to note that these concentrations of exogenous alcohol are not equivalent to the concentration internally. Previous studies have demonstrated that internal concentrations are 40-60mM when exposed to 400-600 mM external concentrations (Davies et al., 2003; Kapfhamer et al., 2008; Alaimo et al., 2012). The 400-mM exogenous concentration of ethanol was required to prevent a floor effect in testing egg laying in the *hslo(+)* transgenic worms. At the start of the assay, ten young adult worms were placed on a control plate containing no ethanol. After 1 hr, worms were then transferred to an ethanol plate for another hour before being removed. The number of

eggs laid by the ten worms was counted on each plate. Average relative values of egg-laying frequencies per worm were determined for untreated and ethanol-treated conditions.

Locomotion response to ethanol: Plates were prepared as described in the egg-laying response to ethanol using 600-mM EtOH plates and seeded with 200 μL of OP50. At the start of the assay, five young adult worms were placed in the center of the OP50 on a control plate containing no EtOH and allowed to crawl for 5 minutes. Their position on the control plate was marked and worms were moved to an EtOH plate. After 20 minutes on the EtOH plate, worms were moved to another EtOH plate in the center of the OP50 and allowed to crawl for 5 minutes. Worms were removed and their position marked. For each worm, the mm distance from the center was recorded as a measure of worm locomotion on control and EtOH plates.

Aldicarb analysis: Assays were performed as described previously (Mahoney et al. 2006). Briefly, 20 young adult worms were picked to plates containing unseeded, NGM-agar with either 1.5 or 2 mM of aldicarb (Sigma). All worms were observed at the start of the assay to ensure that they were living and mobile. Every 30 minutes for the following 2 hours, the number of paralyzed worms was noted. A worm was defined as paralyzed when it showed no spontaneous movement or pharyngeal pumping.

Cell transfection: Human embryonic kidney 293 (HEK293) cells were cultured and transfected using standard procedures. Briefly, HEK293 cells were used over a passage range of 5–25. Cells were passaged every 3–5 days at approximately 80% confluency using trypsin-EDTA and maintained in 25 cm^2 Greiner flasks with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% Glutamax, 100 units/ml Penicillin, and 100 µg/ml streptomycin in a 37°C incubator with 95% (v/v) air and 5% CO_2.

For electrophysiological experiments, HEK293 cells were cultured on 13-mm diameter plastic thermanox coverslips (Thermo Scientific) in a sterile 12-well tissue culture plate until approximately 80% confluency. Cells were transiently transfected with 50 ng of the appropriate DNA and 10 µl of PolyFect Transfection Reagent (Qiagen). cDNA of *hslo* was contained in the mammalian expression vector pCDNA6 and kindly provided by Dr. Richard Aldrich (University of Texas at Austin). After transfection, HEK293 cells were used 24-72 hours later for electrophysiological assays.

Confocal Imaging: Worms were picked onto an unseeded plate and allowed to crawl for 5 minutes to remove any bacteria. Worms were then placed into a drop of NGM buffer on a glass slide containing 2% agarose and 3-mM azide. A coverslip was then pressed on top of the worms. All worms were used within 1 hour of placement onto slide. Confocal images were collected using a Zeiss LSM 710 microscope at 20x and 63x magnification, and processed using ImageJ software.

Electrophysiology: For *C. elegans* recordings, neurons were extruded from restrained worms (L4 and young-adult stage) for patch-clamp recording as described (Goodman et al., 1998). Inside-out patches were obtained from ventral cord motor neurons. During recordings, electrodes and the bath contained (in mM): 125 potassium gluconate, 18 KCl, 4 NaCl, 0.7 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 10 HEPES; pH 7.2 adjusted with KOH for a symmetrical potassium environment during single-channel recordings.

For HEK293 cells, single-channel recordings were obtained from inside-out membrane patches using standard procedures. The high-K^+ extracellular recording solution contained (in mM): 140 K^+ gluconate, 2.2 $CaCl_2$, 4 EGTA, 4 HEDTA, 1 $MgCl_2$, and 15 HEPES. The 1-µM calcium intracellular recording solution contained (in mM): 140 K^+ gluconate, 5 Na^+ gluconate, 0.43 $CaCl_2$, 2 HEDTA, 1 $MgCl_2$, and 15 HEPES. Solutions were adjusted to pH 7.35 with KOH as needed. These solutions were used previously by Chunbo et al., (2007) to study effects of alcohol on the BK channel.

Single-channel currents were low pass filtered at 2.9 KHz and digitized at 10 KHz using an EPC10 amplifier and patchmaster software (HEKA Elektronik). Data were stored on a PC and analyzed using QuB.

Statistical analysis: Data that passed Shapiro-Wilk normality test were analyzed using standard t- or ANOVA tests and the Holm-Sidak method for post hoc multiple comparisons test (Zar, 1999). Data that did not pass the Shapiro-Wilk normality test were analyzed using the Mann-Whitney Rank Sum Test or Kruskal-Wallis ANOVA on ranks and Dunn's test for post hoc multiple comparisons (Zar, 1999).

3.4 - Results

T381I missense mutation in the SLO-1 BK channel dramatically reduces ethanol intoxication without altering other BK channel-dependent behaviors

To elucidate residues of the BK channel that are selective for behavioral intoxication by ethanol but not basal function, we screened all mutant strains harboring missense mutations in the *slo-1* gene from the *C. elegans* Million Mutation Project (Thompson et al., 2013). This library contains 32 strains with unique amino acid missense mutations in the *slo-1* gene (shown in Figure 3.1). Intoxication was measured by the degree that ethanol inhibited egg laying behavior.

Figure 3.1 - Primary sequence of BK channel and ethanol sensitivity of missense mutants.
SLO-1 alpha subunit protein with residues color coded based on *C. elegans* strains with unique missense mutants available from Million Mutant Library (blue = ethanol (EtOH) sensitive, green = not tested (NT), and red = resistant to ethanol). Aspartate residues in the RCK1 domain and calcium bowl critical for calcium sensing are *underlined*. Mutant strains catalogued below with predicted missense mutation and behavioral sensitivity to ethanol. Strains defective in basal egg laying were not tested (N/A). Sensitivity to intoxication was determined by relative egg-laying behavior before and during ethanol treatment. Strain VC40372 contains a mutation in a conserved threonine residue 381 and was the only missense mutant tested that displayed strong resistance to intoxication.

SLO-1 BK Channel α subunit Protein

```
MGEIYSPSQSKGFNQPYGYPMNCNLSRVFMEMTEEDRKCLEERKYWCFLLSSITTFCASM 60
ILVVIWRVVTHLCCQRREKEFVEPIPAPEAVQINMNGSKHAPSETDPFLKQQEEKHLGWM 120
TEAKDWAGELISGQSLTGRFLVLLVFILSIGSLIIYFYDASFQNFQVETCIPWQDSPSQQ 180
IDLGFNIFFLVYFFIRFIAASDKVWFLLEMYSWIDFFTIPPSFVAIYLQRNWLGFRFLRA 240
LRLMTVPDILQYLNILKTSSSIRLTQLVTIFVAVCLTGAGLVHLLENSGDFFKGFINPHR 300
ITYADSVYFVLVTMSTVGYGDIYCTTLCGRLFMIFFILFGLAMFASYVPEIADLIGNRQK 360
YGGEYKGEHGKKHIVVCGHITYDSVSHFLQDFLHEDRDDVDVEVVFLHRVVPDLELEGLF 420
KRHFTKVEFFTGTVMDSLDLSRVKIGDADACLVLANKYSTNPDAEDAANIMRVISIKNYS 480
SDIRVIVQLMQYHNKAYLLNIPSWDWKRGDDVICLAELKLGFIAQSCLAPGFSTMMANLF 540
AMRSFKTSQTTPDWLNLYLCGAGMEMYTDTLSHSFVGMTFPEAVDLLFNRLGLLLLAIEL 600
KDEENKECNIAINPGPHIVIQPQTQGFFIAQSADEVKRAFFWCKQCHDDIKDVSLIKKCK 660
CKNLALFRRNTKHSTAARARATDVLQQFQPQAPAGPMGHLGQQVQLRMINQQSSTSDTHL 720
NTKSLRFAYEIKKLMPSSGGRRNSMSIPPDGRGVDFSKDFEQQFQDMKYDSTGMFHWCPS 780
RNLEDCVLERHQAAMTVLNGHVVVCLFADQDSPLIGLRNFIMPLRSSNFHYHELKHVVIV 840
GDLEYLRKEWKTLYNLPKISILNGSPLSRADLRAVNINLCDMCVIISARVPNTEDTTLAD 900
KEAILASLNIKAMQFDDTLGFFPMRHQTGDRSPLGSPISMQKKGAKFGTNVPMITELVND 960
SNVQFLDQDDDDDPDTELYLTQPFACGTAFAISVLDSLMSTTYFNDSALTLIRTLVTGGA 1020
TPELELILAEGAGLRGGYSTPETLSNRDRCRIAQISLQDNPYDGVVHNTTYGAMFTIALR 1080
RYGQLCIGLYRLHDQDNPDSMKRYVITNPPAELRIKNTDYVYVLEQFDPGLEYEPGKRHF 1140
```

Mutation	Strain	EtOH response	Mutation	Strain	EtOH response	Mutation	Strain	EtOH response
S6P	VC40853	Sensitive	D436E	VC40468	Sensitive	E789K	VC40787	Sensitive
P102S	VC40064	N/A	P462L	VC40143	Sensitive	R818Q	VC40642	Sensitive
S103N	VC40938	Sensitive	P462S	VC40062	Sensitive	S936A	VC30157	Sensitive
F140L	VC30161	Sensitive	G563E	VC20545	N/A	D975G	VC40692	Sensitive
P177L	VC40899	Sensitive	L595F	VC40416	Sensitive	T988I	VC41014	N/A
G234E	VC40774	Sensitive	R669H	VC20244	Sensitive	T1001I	VC20468	Sensitive
A304T	VC40641	Sensitive	L720F	VC20444	Sensitive	A1008V, I012S	VC40265	N/A
M333L	VC40384	N/A	Y729F	VC40417	Sensitive			
T381I	**VC40372**	**Strongly Resistant**	G753S	VC20417	Sensitive	G1036D	VC20240	Moderately Resistant
D401N	VC40804	Sensitive	G753D	VC20590	Sensitive	L1044F	VC40381	Sensitive
D436N	VC40392	Sensitive	G753S	VC20642	N/A	D1096N	VC40221	Sensitive

Most mutations in these strains were associated with wild-type-like sensitivity to intoxication, including VC20444 (L720F) and VC40804 (D401N) (blue coded residues in Fig. 3.1). We defined wild-type sensitivity to ethanol as =< 40%, of egg-laying for ethanol compared to control conditions, which was the reduction in egg laying on ethanol (19.3%) + 2 standard deviations (10.3%) demonstrated in wild-type *C. elegans*. Low level of resistance was defined as > 40% and =< 60.6%, moderate resistance > 60.6% and =< 81.2% and high resistance > 81.2%. One strain, VC20240, with missense mutation G1036D, displayed moderate resistance to intoxication (Fig 3.1; Fig. 3.2A,B). A few strains could not be assessed because they displayed basal defects in egg laying (Fig. 3.1). Defective egg laying may be due to gain-of-function mutations in the *slo-1* gene as observed previously (Davies et al., 2003), due to background mutations, or due to both.

Out of all of the missense mutants, however, only one strain, VC40372 (T381I), which harbors the *slo-1* allele *gk602291*, displayed strong resistance to intoxication. Similar to the *slo-1(null)* mutant, VC40372 mutant worms showed only a 6.3%% reduction in egg laying (red coded residue in Fig. 3.1; Fig. 3.2A,B). In contrast, ethanol reduced the rate of egg laying by 80.7% in wild-type worms (Fig. 3.2A,B). The *gk602291* allele results in a threonine to isoleucine mutation at position 381 of the worm SLO-1 channel. This is located only 10 and 15 residues away from the predicted calcium-binding aspartate residues in the RCK1 domain on the intracellular tail (Fig. 3.3). Importantly, the T381 residue is highly conserved across species (Fig. 3.3A).

Figure 3.2 - T381I mutation in the SLO-1 channel confers strong resistance to intoxication
A, Intoxication sensitivity as determined by egg laying on control versus ethanol plates for various strains: wild-type (WT) strain N2 (*N* = 41), *slo-1(null)* strain NM1968 (*N* = 30), VC40372 (*N* = 5), VC20444 (*N* = 3), VC20240 (*N* = 6) AND VC40804 (*N* = 3) where *N* represents an assay with 10 worms each. VC40372 was the only strain that displayed strong resistance to intoxication. ***B***, Intoxication as determined by relative egg-laying sensitivity to ethanol in the strains in panel *A*. The *slo-1(T381I)* and *slo-1(null)* mutants were significantly less intoxicated than WT. Other missense mutants displayed WT sensitivity to ethanol. ***C***, Intoxication as determined by relative egg-laying sensitivity to ethanol in strains derived from outcrossed *slo-1(T381I)* or *slo-1(null)* parents. *Horizontal lines* indicate behavior of *slo-1(null)* and WT worms. After independently outcrossing six times, the two resulting *slo-1(T381I)x6* mutants (*N* = 14, *N* = 9 respectively) displayed strong resistance to intoxication comparable to *slo-1(null)*. Strain *slo-1(T381I)x6[#2]* failed to genetically complement *slo-1(null)* (*N* = 9) indicating that T381I is associated with ethanol resistance. One WT copy of *slo-1(+)* was sufficient to rescue intoxication when crossed with *slo-1(null)* (*N* = 9). Intermediate ethanol sensitivity was displayed by a strain heterozygous for the T381I mutation and a WT copy of *slo-1* (*N* = 6). ***D***, Locomotion on control and ethanol plates for wild type, *slo-1(null)* and *slo-1(T381I)x6* mutants (*n* = 30 each). On ethanol, *slo-1(null)* and *slo-1(T381I)x6* mutants were significantly more displaced from the start location than WT (#, $p < 0.05$) In all panels, bars represent s.e.m.

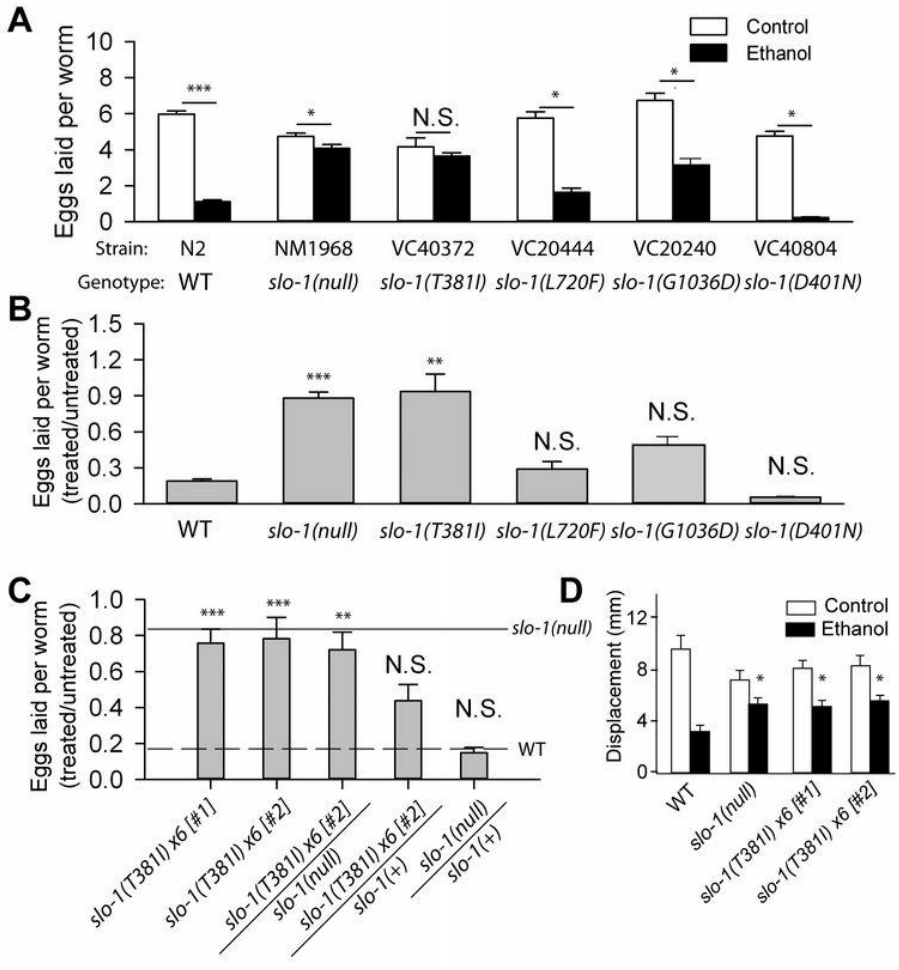
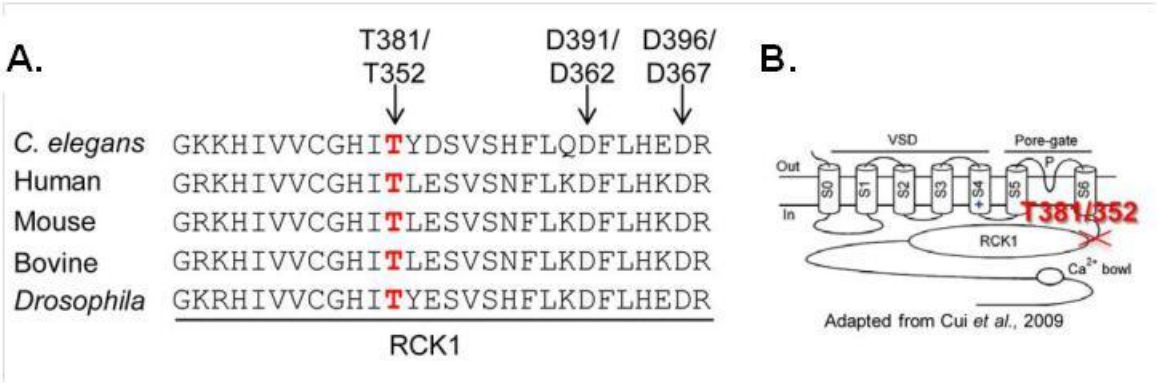


Figure 3.3 - The T381 residue in SLO-1 is conserved from worm to human and is equivalent to the mouse T352 position.

A, Primary sequence alignment of residues near the T381 on the worm BK channel compared to other species. The T381 residue is conserved as residue T352 in the mouse BK channel. This residue will be referred to T352 when referring to the human BK channel in this paper. This residue is located 10, and 15 residues away from the underlined calcium-sensing aspartate residues in the RCK1 domain. ***B,*** Diagram of SLO-1 alpha subunit with location of T381 residue in relation to transmembrane and calcium-binding domains (arrows).



The strong ethanol resistance of VC40372 may be due to additional background mutations or solely due to the *gk602291* mutation in the *slo-1* gene. To test the first possibility, we outcrossed the VC40372 strain six times, twice independently, with wild-type by tracking the *gk602291* genotype to generate strains *slo-1(T381I)x6[#1]* and *slo-1(T381I)x6[#2]*. Both of the outcrossed strains retained a strong level of resistance to ethanol that was statistically indistinguishable from the *slo-1(null)* strain (Fig. 3.2*C*). In addition, because background mutations have been known to persist after outcrossing, we asked whether one of the x6 outcrossed strains retained resistance after crossing with the *slo-1(null)* strain. We found that the F1 progeny from this cross, *slo-1(T381I)x6[#2]/slo-1(null),* displayed strong resistance to ethanol equivalent to the parent strains, a phenomenon called 'failing to complement' (Fig. 3.2*C*). This provides strong evidence that the ethanol resistance of the VC40372 strain resulted from the *gk602291* mutation in *slo-1* since recessive background mutations in the parental strains would be heterozygous in this F1 crossed strain and fail to account for ethanol resistance.

We performed additional complementation testing with wild-type worms to uncover the genetic relation between the null and T381I *slo-1* alleles. The F1 cross progeny of *slo-1(null)* and wild-type displayed intoxication indistinguishable from wild-type (Fig. 3.2*C*). This shows that only one wild-type copy of the *slo-1* gene is needed to rescue *slo-1*-dependent intoxication. Intriguingly, we found that the F1 cross progeny of *slo-1(T381I)x6[#2]* and wild-type strains displayed an ethanol sensitivity intermediate between wild-type and *slo-1(null)* strains (Fig. 3.2*C*). Given that BK channels are composed of four subunits (Shen et al., 1994), this result hints at the possibility that the

mutant SLO-1(T381I) channel alpha subunits may form heteromers with wild-type alpha subunits, resulting in a channel with intermediate ethanol sensitivity *in vivo*.

To test whether the T381I mutation conferred resistance to a distinct ethanol-mediated behavior, we tested the two 6x outcrossed *slo-1(T381I)* strains for resistance to the depressing effects of ethanol on locomotion. Both strains exhibited a robust resistance to intoxication on locomotion (61% and 62% of untreated levels), similar to *slo-1(null)* (70% of untreated level) (Fig. 3.2D). In contrast, ethanol significantly reduced the rate of locomotion by in wild-type worms (29% of untreated level) (Fig. 3.2D) in agreement with our previous findings. This suggests that the T381I mutation provides resistance to ethanol in distinct neuromuscular circuits in *C. elegans.* Taken together, our results above suggest that the T381I missense allele *gk602291* was the sole determinant explaining the robust resistance to intoxication in *slo-1* mutant strains carrying the T381I mutation.

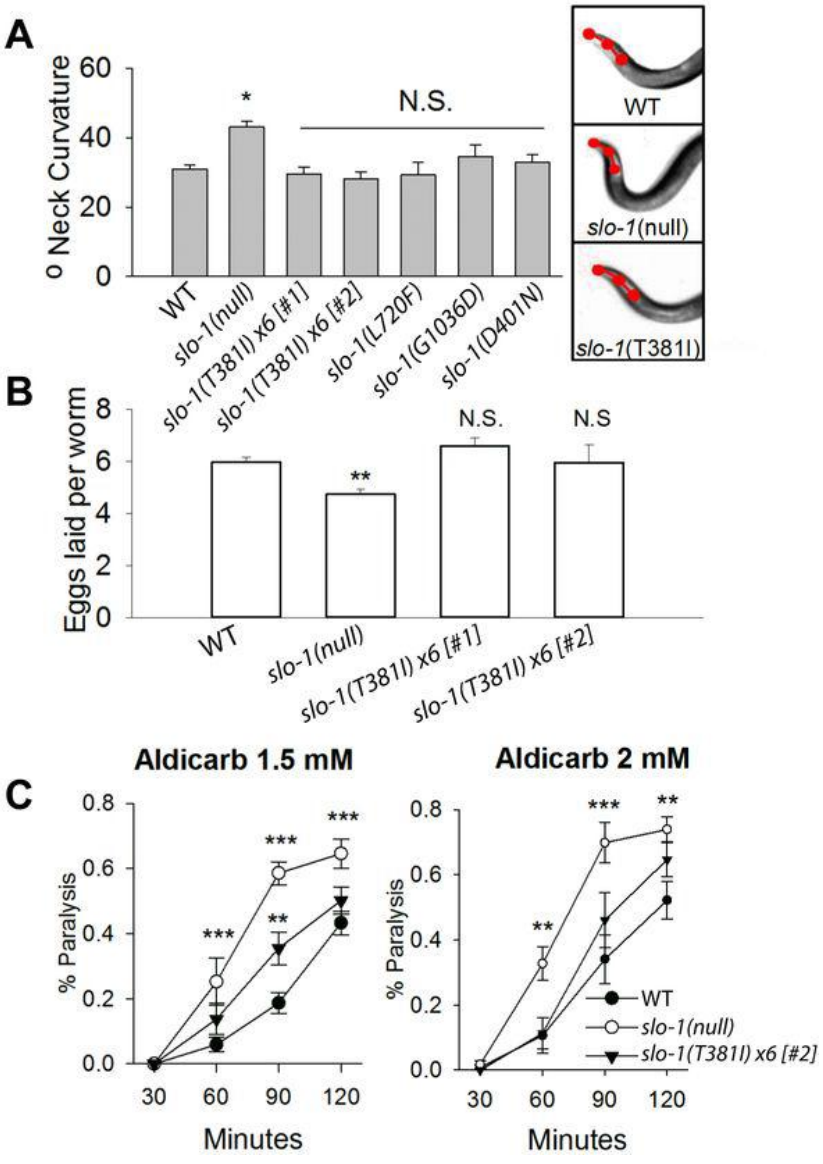
To determine if the mutation compromises basal function, leading to lack of ethanol sensitivity, we assessed BK channel function *in vivo* by analyzing three independent behaviors previously found to depend on normal BK-channel function: neck posture, egg laying and aldicarb paralysis.

Mutations in *slo-1* that are predicted to eliminate or strongly reduce BK channel function cause *C. elegans* to crawl with unusually sharp head bends — a phenotype described as a “crooked neck” posture (Kim et al., 2009). We performed quantitative image analysis on the *slo-1(T381I)x6* mutants to compare neck posture with wild-type and the *slo-1(null)* mutant. Similar to other mutants tested with wild-type sensitivity to intoxication, the *slo-1(T381I)* mutant displayed wild-type neck posture consistent with

functional BK channels *in vivo* (Fig. 3.4A). Unlike previously characterized *slo-1(null)* and loss-of-function alleles, *slo-1(T381I)* represents the first mutant that is both resistant to ethanol while displaying wild-type neck posture.

Figure 3.4 - T381I SLO-1 mutation has little or no effect on three ethanol-independent BK-channel-mediated behaviors.

A, Neck curvature is sharper in *slo-1(null)* mutants (n = 50) than in wild-type (WT) worms (n = 36). Both *slo-1(T381I)x6* strains (n = 17 each) display WT neck posture (p = 0.99 for each), as do other missense mutants Representative images of neck posture of WT and mutant worms are shown on the right. ***B***, In the absence of ethanol, egg laying is slightly, but significantly, lower in *slo-1(null) vs* WT worms (N > 30 each, p < 0.05). By contrast, both *slo-1(T381I)x6* strains display WT rates of egg laying (N > 9 each, p = 0.99). N represents an assay with 10 worms**.** ***C***, Aldicarb (1.5 mM) paralyzes *slo-1(null)* mutants (N = 8) significantly faster than *slo-1(T381I)x6#2* (N = 7, p < 0.001) and WT (N = 9) worms (N = 9, p < 0.001). Post hoc analysis shows that *slo-1(T381I)x6#2* worms were paralyzed slightly but significantly more than WT at the 90-minute time point only (p < 0.05). With 2-mM aldicarb, *slo-1(null)* worms (N = 6) were significantly more paralyzed than both WT (N = 7, p < 0.001) and *slo-1(null)x6[#2]* strains (N = 4, p < 0.001). N represents an assay with 20 worms. In all panels, bars represent s.e.m.



We next investigated basal egg-laying behavior which was previously shown to be slightly lower in *slo-1(null)* than in wild type worms (Davies et al., 2003). Here, we also found a slight but significant difference between *slo-1(null)* and wild-type egg-laying rates (Fig. 3.4*B*). However, no difference was observed between *slo-1(T381I)*x6 outcrossed strains and wild-type, providing further evidence for functional BK channels in the mutants (Fig. 3.4*B*).

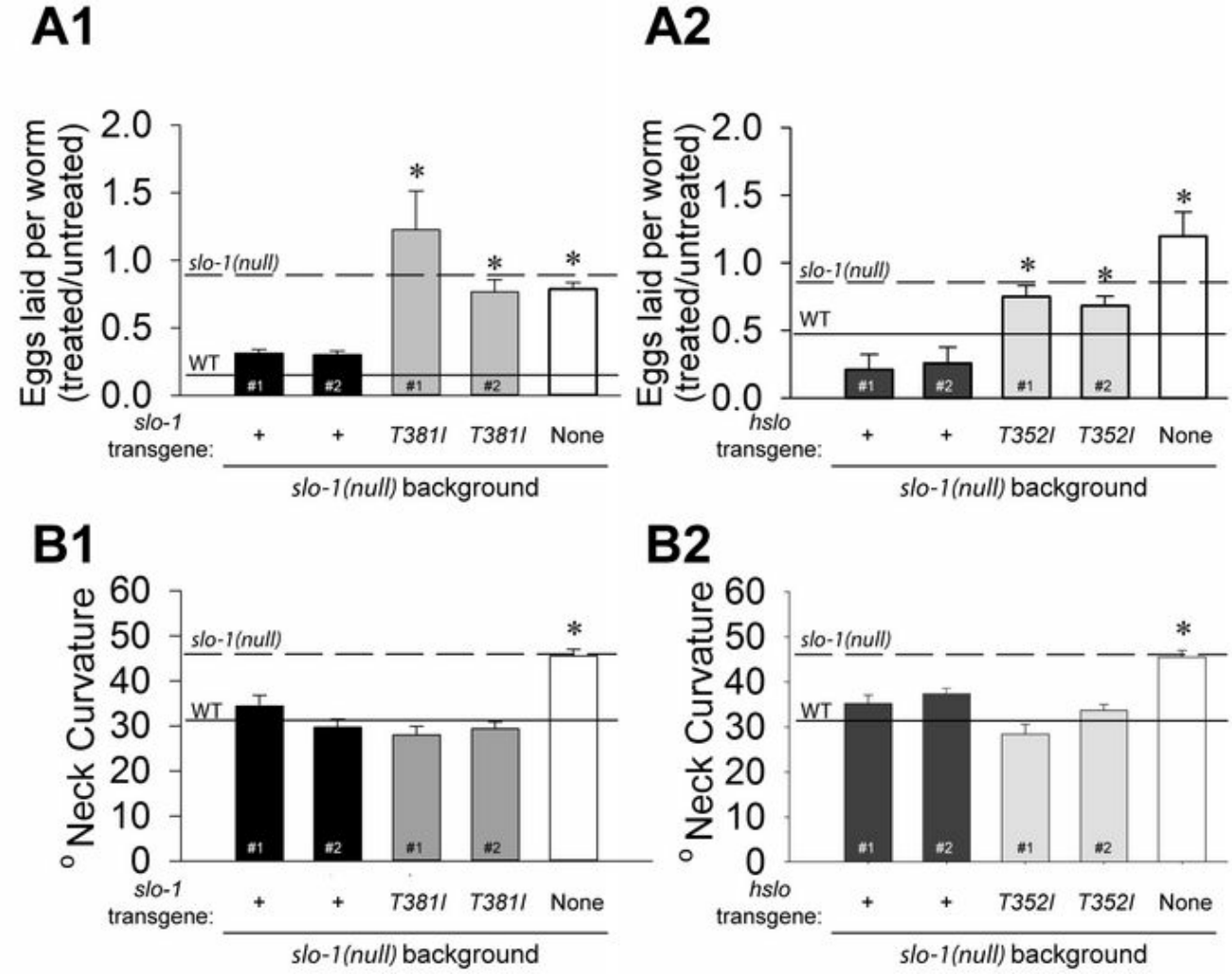
The BK channel is also an important negative regulator of neurotransmitter release at the neuromuscular junction in *C. elegans* (Wang et al., 2001). One manifestation of this is that loss of *slo-1* function causes hypersensitivity to paralysis by the acetylcholine-esterase inhibitor, aldicarb, due to abnormally rapid buildup of acetylcholine at the neuromuscular junction. Thus, chronic exposure to aldicarb causes a gradual paralyzing contraction of muscles that occurs faster in *slo-1(null)* relative to wild-type (Wang et al., 2001; Davies et al., 2003). In this study, we tested aldicarb sensitivity of the *slo-1(T381I)x6[#2]* mutant and found that it was more like wild type than *slo-1(null)* for both 1.5-mM and 2-mM aldicarb treatments (Fig. 3.4*C*). Overall, our results strongly suggest that the worm BK channel with the T381I missense mutation is both functional *in vivo* and resistant to ethanol.

Single conserved residue required for ethanol sensitivity of the human BK channel

We performed an additional test to determine if the T381I missense mutation results in an ethanol-resistant, but functional BK channel *in vivo*. We transformed the *slo-1(null)* strain with wild-type or T381I mutant cDNA for the *slo-1* isoform *slo-1a*. To visualize SLO-1 protein expression in the worm, we tagged the C-terminal (intracellular)

side of SLO-1 with the fluorophore mCherry. The *slo-1a::mCherry* transgene was driven by the native *slo-1* promoter *Pslo-1*. Transgenic worms harboring the wild-type or mutant *slo-1* transgene were identifiable as worms that also expressed a co-transformed mCherry reporter in the feeding organ (pharynx). The wild-type *slo-1(+)* transgene rescued both ethanol sensitivity (Fig. 3.5A1) and neck posture (Fig. 3.5B1). By contrast, transformation with the *slo-1(T381I)* transgene rescued neck posture, but failed to rescue ethanol sensitivity (Fig. 3.5A1,B1). Identical results were found for independently derived transformant strains (#1 and #2), suggesting that these results were not due to transgenesis efficiency. In addition, a control strain that was transformed with only the co-injection marker and empty plasmid failed to rescue ethanol sensitivity or neck posture (labeled None in Fig. 3.5A1,B1). These results provide strong independent evidence that the T381I mutant SLO-1 channel functions normally *in vivo* in addition to being insensitive to ethanol.

Figure 3.5 - Transgenic expression of T381I worm or T352I human BK channels selectively rescues neck curvature but not intoxication in the *slo-1(null)* mutant.
A, Expression of wild-type (WT) *slo-1(+)* rescues intoxication (**A1**) (N = 6 for each genotype) and neck curvature (**A2**) (n > 14 for each genotype) in the *slo-1(null)* mutant for two independent strains. Expression of *slo-1(T382I)* mutant channel fails to rescue intoxication (**A1,** $p < 0.05$ for both), but succeeds in rescuing neck curvature (**A2**) in the *slo-1(null)* mutant for two independent strains. ***B***, Likewise, expression of WT human channel *hslo(+)* in *slo-1(null)* mutant rescues both intoxication (**B1**) ($N = 6$ for each genotype) and neck curvature (**B2**) ($n > 26$ for each genotype) for two independent strains. Expression of the *hslo(T352I)* mutant BK channel fails to rescue intoxication for two strains (**B1,** $p < 0.01$, $p < 0.05$ respectively, $N = 6$ each) but succeeds in rescuing neck curvature (**B2**). In all graphs, the control transgenic strain with fluorescent reporter fails to rescue either behavior (n = 6-33). *Horizontal lines* indicate levels for *slo-1(null)* and WT strains. N represents an assay with 10 worms. In all panels, bars represent s.e.m.

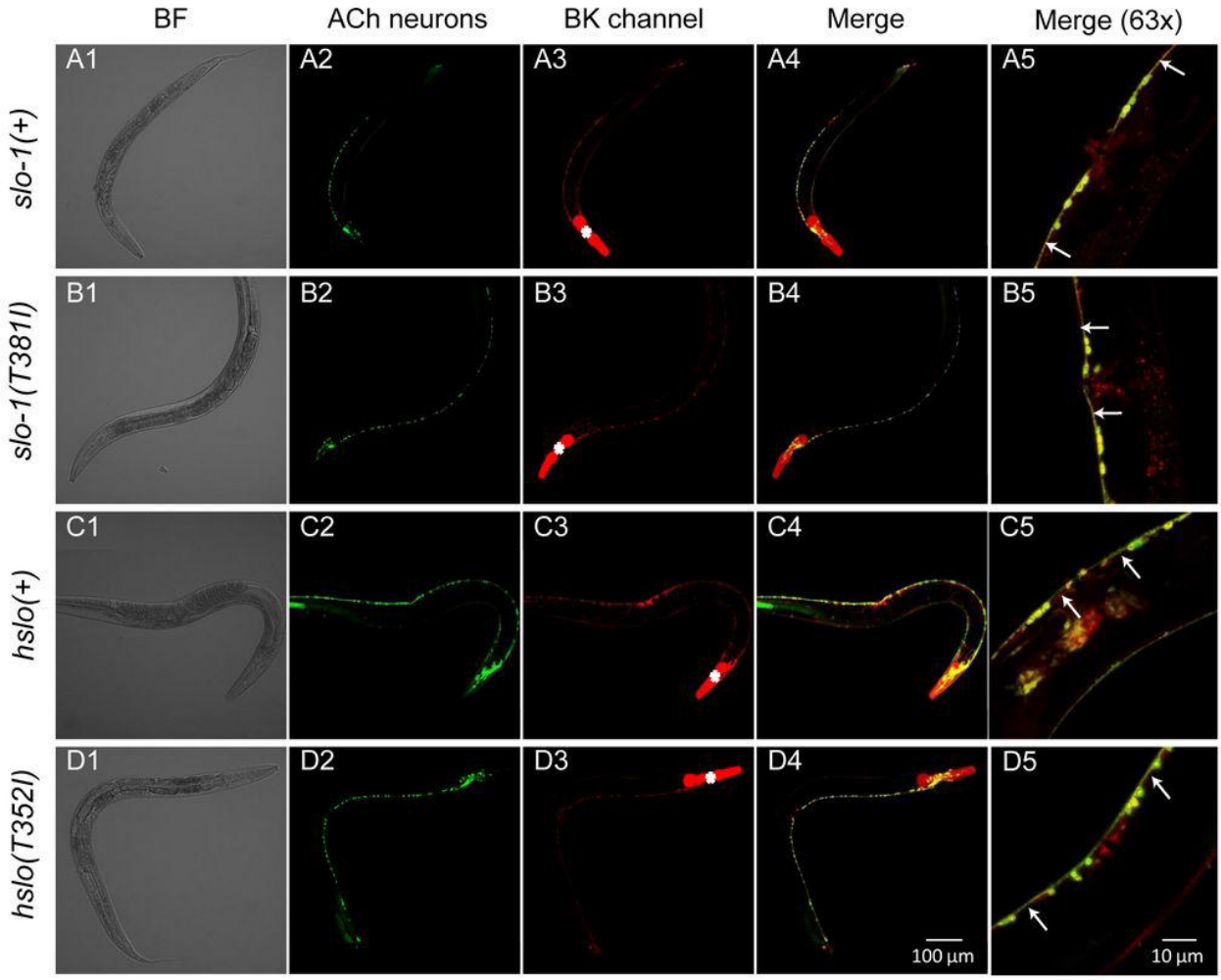


The T381 residue of SLO-1 is conserved across a wide range of species, including in the human BK channel (Fig. 3.3A). To determine whether this residue plays a conserved role in behavioral responses to ethanol, we transformed *slo-1(null)* mutants with wild-type or mutant cDNA for the human BK channel gene *hslo*. For the mutant, the equivalent T352 residue was mutated to encode isoleucine. As above, we used the *Pslo-1* promoter and tagged the human BK channel at the C-terminus with mCherry and co-transformed with the same fluorescent reporter. Consistent with our results with the worm *slo-1(+)* transgene, we found that the wild-type human *hslo(+)* transgene rescued ethanol sensitivity and crooked-neck posture for two transformant strains (Fig. 3.5A2,B2). Also consistent with our results in *slo-1*, we found that transformation with *hslo(T352I)* selectively rescued the neck posture phenotype but not ethanol sensitivity (Fig. 3.5A2,B2). In addition, a control strain that was transformed with only the co-injection marker and empty plasmid failed to rescue ethanol sensitivity or neck posture (Fig. 3.5A2,B2). This suggests a strong conservation from worm to human of the threonine 381/352 residue in contributing to behavioral intoxication by ethanol, without being critical for channel function.

The worm T381I and human T352I mutant BK channels might convey resistance to ethanol and yet rescue neck posture if they are expressed at abnormally low levels and/or are expressed in cells important for neck bending but not for intoxication. To address these possibilities, we examined the *in vivo* distribution of BK channels by visualizing their mCherry tag in the transgenic strains described above. Despite the widespread neuronal and muscle BK expression profile, expression of wild-type *slo-1* in

cholinergic neurons alone was previously found to be sufficient to rescue intoxication (Davies et al., 2003). Thus, we used a cholinergic GFP reporter to assess co-localization of mCherry-tagged transgenic BK channels in cholinergic neurons. By confocal microscopy, we found co-localization of mCherry signal in cholinergic motorneurons regardless of whether the transgenic BK channel was wild-type or mutant, worm or human (Fig. 3.6). Co-expression could be seen at high magnification (63x) in somata and neuronal processes throughout the ventral nerve cord across all strains (Fig. 3.6). These results are also consistent with the reported expression pattern of the *Pslo-1* promoter that was used to drive the transgenes (Chen et al. 2011). Importantly, punctate mCherry fluorescence was co-localized with GFP throughout the cholinergic motorneurons previously shown to be critical for the behavioral response to ethanol (Fig. 3.6). Thus, gross differences in expression pattern or in expression level do not appear to explain the dramatic ethanol resistance in the worm T381I or human T352I transgenic strains. Instead, our results strongly suggest that these mutations specifically affect the interaction between ethanol and the BK channel *in vivo*.

Figure 3.6 - Visualization of transgenic BK channels *in vivo*.
Each row shows photomicrographs of a representative worm expressing different BK channels tagged with mCherry on a *slo-1(null)* background. Bright-field images of individual worms displayed in column 1. Acetylcholine (ACh) neurons are labeled in all worms via a *Punc-17::gfp* fluorescent reporter in column 2. In all four transgenic strains, mCherry-tagged BK channels are observed in column 3 throughout the ventral nerve cord and tail neurons, matching previous expression profiles for the BK channel. Gross differences were not observed in expression level or pattern among the four transgenic strains. *White asterisks* indicate mCherry co-transformation marker expressed in the anterior feeding organ (pharynx). In addition, for all four strains mCherry co-localizes with green cholinergic neurons in column 4 as well as in representative magnified images at 63x in column 5. White arrows mark co-localization in neuronal processes. Scale for columns 1-4 -located in D4 right; scale for column 5 in D5.

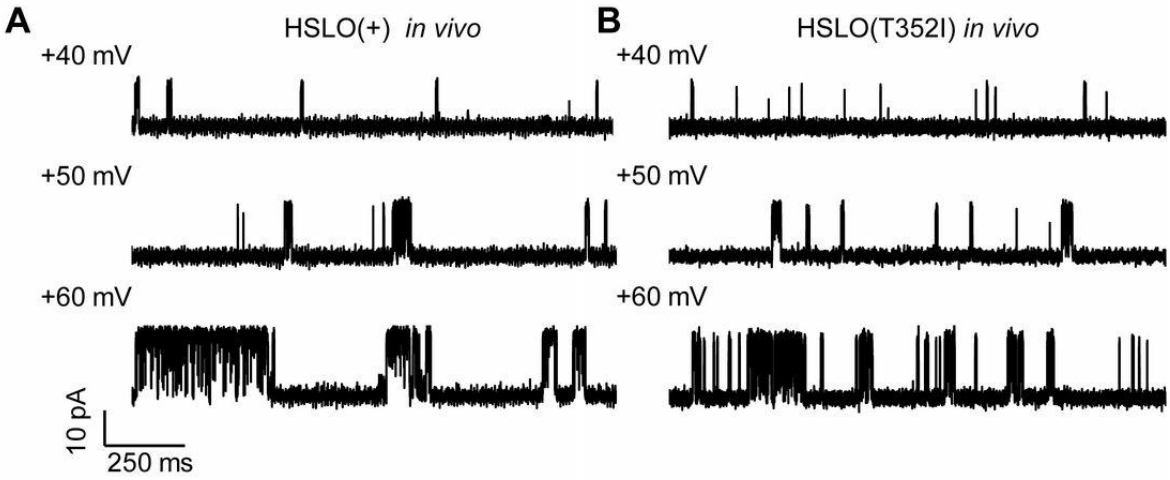


The T352I mutation selectively eliminates ethanol sensitivity of the BK channel

The behavioral and expression analyses described above suggest that the T352I mutant BK channel functions normally in *C. elegans*. For a direct measure of channel function, we performed *in vivo* single-channel patch-clamp recordings of identified neurons in *slo-1(null)* mutant worms that had been transformed with wild-type or T352I mutant human BK channels. Our previous work demonstrated that SLO-1 single-channel currents were completely absent in neurons of *slo-1(null)* mutants (Davies et al., 2003). By contrast, we found a high incidence of large conductance currents with inside-out recordings from ventral cord motor neurons in worms expressing the wild-type or T352I mutant HSLO channel (Fig. 3.7). These currents are likely mediated by the HSLO channel because they had a conductance of 292 ± 2 SEM pS (n = 4). This value is consistent with previous reports of the human channel, which notably higher than the 38 pS conductance of the worm SLO-1 channel (Dworetzky et al., 1994; Davies et al., 2003). In addition, these currents were blocked by the BK-channel-selective antagonist paxilline, and were voltage- and calcium-sensitive. These findings demonstrate functional human BK channels expressed in *C. elegans* neurons and indicate that the T352I mutation does not overtly affect single channel expression or function.

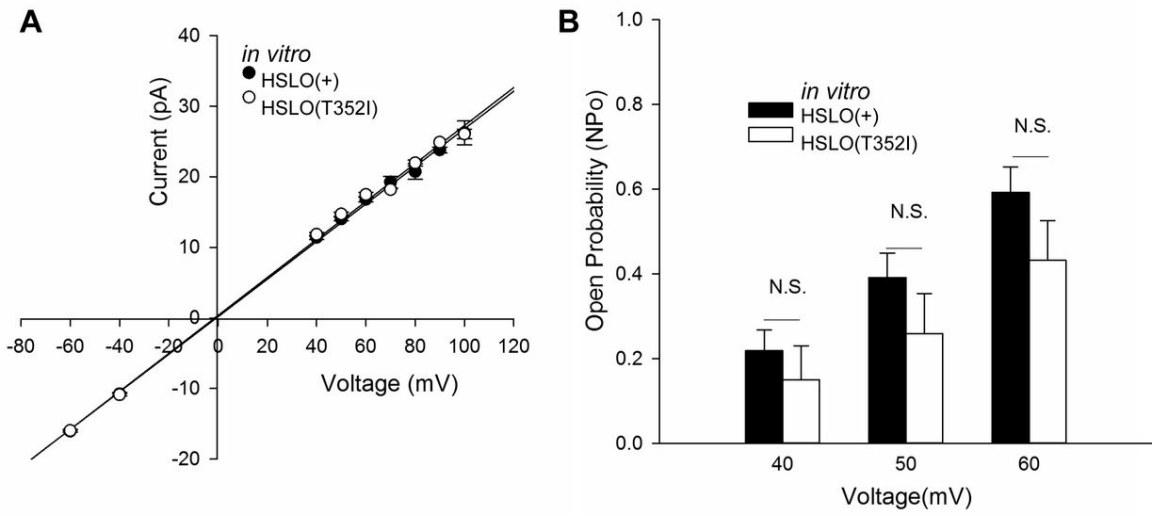
Figure 3.7 - HSLO(T352I) mutant currents qualitatively resemble HSLO wild-type currents *in vivo* in a *slo-1(null)* background.

A, Representative single-channel patch-clamp recordings from ventral motor cord neurons revealed large amplitude (292 pS), outward rectifying potassium currents characteristic of wild-type human BK channels. ***B***, Representative recordings of HSLO(T352I) mutant channel resembles those of the wild-type HSLO channel in worm neurons.



To quantitatively measure effects of the T352I mutation, we next studied HSLO channels transfected into HEK cells. Under basal conditions, no significant differences in conductance were observed between the wild-type and T352I mutant HSLO channel (Fig. 3.8A), and, only minor differences in open probability were seen across different voltages (Fig. 3.8B). ANOVA showed a small yet significant difference in the interaction between genotype and voltage (p = 0.045), but no significant difference was found in post hoc analyses comparing channel open-probability values at specific voltages (40 mV p = 0.5, 50 mV p = 0.19, 60 mV p = 0.12, n = 10). These results demonstrate that the T352I mutation has only subtle effects on basal function of the human BK channel at the level of single channel currents.

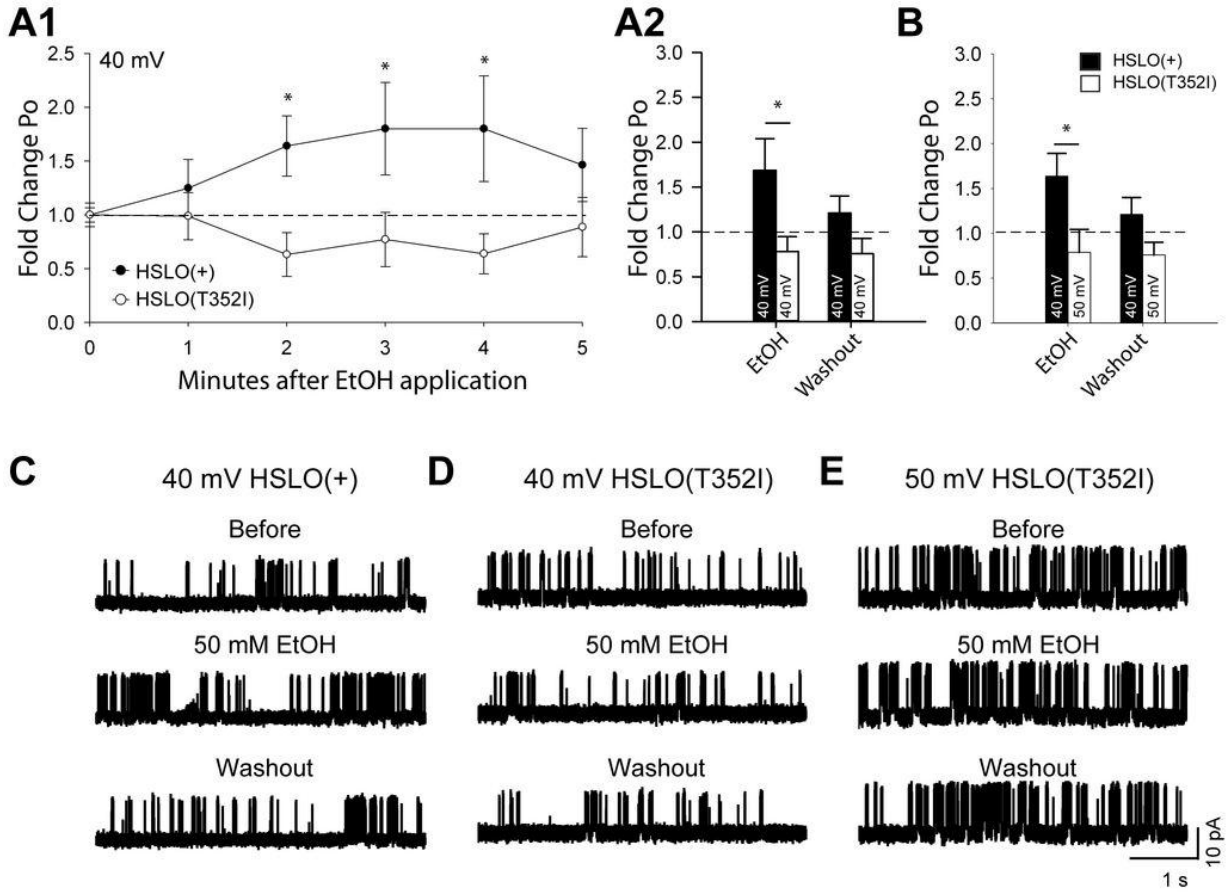
Figure 3.8 - T352I mutant human BK channel displays only minimal changes in basal function from wild-type *in vitro*.
Single-channel BK currents were studied in a symmetrical saline solution in patches from HEK cells injected with either WT or T352I mutant BK channel cDNA. ***A***, Reversal potential (-1.4 mV WT and -1.37 mV T352I) and conductance were the same for both channels (n = 7 for both, p = 0.17). ***B***, Analyzing opening probability at three holding potentials (n = 10 WT, n = 8 T352I) revealed a slight, yet significant effect of the T352I mutation compared to WT BK channel ($p < 0.05$). However, post hoc tests did not reveal differences at any voltages tested (40 mV p = 0.5, 50 mV p =0.19, 60 mV p = 0.12). In all panels, bars represent s.e.m.



We next tested effects of the T352I mutation on ethanol responses in HEK cells. Previous work has demonstrated that ethanol modulates single-channel currents from BK channels excised from neuronal membranes or minimal planar lipid bilayers, suggesting a direct interaction of the BK channel and ethanol (Chu et al., 1998). Consistent with these reports for the wild-type human BK channel, we found that ethanol application caused a significant increase in open probability of 50-80% above basal levels (40-mV holding potential; Fig. 3.9A,C). This increase had a rapid onset and was reversible upon removal of ethanol (Fig. 3.9A1,A2,C). In contrast, ethanol had no effect on the T352I mutant BK channel at this holding potential (Fig. 3.9A1,A2,D).

The mutant also showed a slight, but insignificant difference in open probability at 40 mV (P_o WT = 0.22 *vs* T352I = 0.15). Such a change might lower the activation state of the mutant channel and prevent potentiation by ethanol. To compensate for subtle differences in activation and observe channel function under similar activation conditions, we compared the ethanol response of the mutant at 50 mV to the wild-type channel at 40 mV (Basal P_o WT = 0.22 *vs* T352I = 0.26). After two minutes of ethanol application, when the WT channel reaches maximal potentiation, the T352I mutant demonstrated no change in channel opening (Fig. 3.9B,E). Our single-channel analysis showed that the T352I mutation eliminates potentiation by ethanol without significantly altering basal function of the human BK channel.

Figure 3.9 - T352I mutant human BK channel is resistant to ethanol. Patches containing a single BK channel were tested for potentiation by ethanol in HEK cells.
A1, Fold change in open probability (P_o) during ethanol exposure. Baseline (4 min) followed by ethanol (EtOH) application (5 min), and washout (4 min). At 40 mV, ethanol application significantly potentiated WT compared to T352I mutant BK channels ($p < 0.05$). Post hoc analyses determined ethanol significantly potentiated WT compared to T352 mutant after 2 ($p < 0.05$), 3 ($p < 0.05$) and 4 minutes ($p < 0.04$) of ethanol application. ***A2,*** During the 5-minute time course of ethanol application, the WT BK channel activity was significantly increased compared to the T352I mutant ($p < 0.05$). During washout, WT and mutant values for P_o were indistinguishable ($p = 0.23$). ***B***, WT BK channel held at 40 mV was compared to T352I mutant at 50 mV to control for subtle differences in basal opening (40 mV P_o WT = 0.22 *vs* 50 mV T352I = 0.26). WT BK channel showed increased P_o after ethanol exposure (2 min) ($p < 0.05$), while T352I mutant did not. During washout, no significant difference was found between WT and mutant ($p = 0.19$). ***C***, Representative WT BK channel currents at 40 mV before, during (2 min) and after 50 mM ethanol, showing reversible increase in open probability. ***D***, Representative T352I BK channel currents under the same conditions in panel *C* show no potentiation by ethanol. ***E***, Likewise, representative T352I BK channel currents held at 50 mV show no potentiation. (n= 6 recordings for each measurement in all panels).



3.5 - Discussion

We screened *C. elegans* carrying missense mutations in the BK channel SLO-1 to identify conserved residues that are specifically required for intoxication but not for other BK-channel-dependent behaviors. We identified one residue, T381, that when mutated to an isoleucine met these criteria and was conserved in the human BK channel as T352. Subsequent analyses with *in vivo* and *in vitro* single-channel patch-clamp recordings verified that the T352I mutation resulted in a BK channel that was insensitive to activation by ethanol but otherwise largely retained normal basal function. The conserved T352 residue identified in this study might be part of an ethanol-binding pocket, permit ethanol binding at another site, and/or occupy part of the channel that undergoes conformational changes during ethanol binding.

The human BK channel can functionally replace the *C. elegans* BK channel

The BK channel is highly conserved across species from invertebrates to humans, including the functional domains such as RCK1 and the Ca^{2+} bowl (Fodor and Aldrich, 2009). The worm and human BK channel both respond to Ca^{2+} and voltage depolarization, as well as the antagonists paxilline and iberiotoxin (Xia et al., 2002; Davies et al., 2003; Johnson et al., 2013). However, the conductance of the worm SLO-1 channel is vastly smaller (38 pS) than the conductance of the human BK channel (180 to 300 pS) (Davies et al., 2003; Hille, 2001). This difference in conductance may explain why lower ethanol concentrations were able to produce intoxication in worms expressing the HSLO channel compared to the SLO-1 channel. Lower doses of ethanol may have been effective in producing a large potassium conductance from the human channel to

suppress neuronal activity. Despite the large difference in conductance, the T381 residue appeared to be functionally equivalent to the human T352 residue since the mutation selectively abolished behavioral sensitivity to ethanol. In fact, this entire region is highly conserved – only two out of the 20 closest residues to T381/352 are highly dissimilar between worm and human. This region is also highly conserved in other species, including fly, cow, rat and mouse. Because the mutant channel functioned normally *in vitro* and *in vivo*, we suspect that this residue represents a conserved site across species required for mediating intoxication that operates independent of the calcium-binding RCK1 domain.

Behavioral genetic approach to identify critical residues for drugs

Our "*in vivo* first" approach contrasts with conventional methodologies where an ion channel is first altered via site-directed mutagenesis at specific residues and studied with single-channel patch recordings *in vitro* in a heterologous system. A related conventional *in vitro* approach uses chimeras made of ethanol-sensitive and -insensitive components of related ion channels (e.g. Mihic et al., 1997, Liu et al., 2003 and Liu et al., 2013). While these conventional approaches are powerful and have proved successful, they cannot easily determine whether the mutations identified as important for ethanol effects *in vitro* are also relevant to ethanol-modulated behaviors *in vivo*. In addition, sometimes it is clear that the mutations identified from these *in vitro* approaches would be detrimental or fatal if the mutant channel replaced the wild-type *in vivo*. For instance, although single-residue mutations that render the glycine receptor S267 insensitive to ethanol have been known for over a decade, the knock-in replacement of the wild-type

with the mutant channel resulted in post embryonic lethality shortly after birth. The abnormally low activity of the channel likely leads to fatal seizure activity (Findlay et al., 2003). These problems were finally overcome for the α2 $GABA_A$ receptor after a decade of searching for a distinct combination of S270H/L277A mutations that produce a viable ethanol-insensitive mutant knock-in mouse with altered ethanol but not basal behaviors (Blednov et al., 2011; Harris et al., 2011). Thus, *in vivo*-first approaches may accelerate the discovery of ethanol-selective mutations for other targets of ethanol.

Additionally, because "*in vitro* first" conventional approaches generally postulate that positions showing divergence within an ion channel family contribute to differential drug sensitivities, conserved mechanisms important for drug action may be missed. Here we show that a relatively conservative substitution at T352 disrupts ethanol sensitivity. This residue is also conserved not just between different isoforms and across species, but also in other large conductance potassium channels like SLICK and SLACK that are not sensitive to ethanol (Lui et a., 2013). In a chimeric-based *in vitro* approach, the T352 residue would not be targeted, and as a result never identified.

Liu et al., (2013) demonstrated the importance of the intracellular portion of the BK channel, and Liu et al., (2008) found that mutation of calcium-binding residues in the Ca^{2+} bowl and RCK1 domain abolished the ability of ethanol to activate the BK channel *in vitro*. However, mutations of the calcium-binding residues also dramatically alter calcium-dependence and greatly reduce voltage sensitivity (Xia et al., 2002; Schreiber & Salkoff, 1997). Such radical differences in function would be expected to produce changes in basal behaviors that would obscure any changes in ethanol-mediated

behaviors. Our *in vivo*-first approach overcomes these problems, by screening a simple genetic model for missense mutations that abolish ethanol-dependent behaviors while leaving ethanol-independent behaviors intact. We identify a residue important for ethanol sensitivity earlier in the signal transduction pathway—prior to interaction with channel gating mechanisms. Evidence for maintenance of basal function in worms was substantiated by *in vivo* rescue of ethanol-independent behaviors that are known to depend on BK channel function such as neck posture, egg laying and aldicarb paralysis. Future studies will investigate whether the T352I mutation abolishes ethanol sensitivity at the single-channel level like it does for HSLO. In principle, this approach could be applied to search for additional residues in the BK channel important for the actions of ethanol, or residues in other proteins involved in the actions of ethanol or other drugs. Importantly, this approach may be especially useful for drug targets that cannot be assessed easily at the molecular level.

Implications for the role of BK channels in alcohol abuse behaviors

It is remarkable that mutation of a single residue could have such a dramatic specific effect on ethanol modulation while minimally affecting basal BK channel function. Mutation of individual residues is also critical for ethanol-sensitive ligand-gated ion channels, highlighting the specificity of ethanol action on protein targets and eluding to binding sites for ethanol on channel proteins (Howard et al., 2014).

Identification of the T352I mutation elucidates a molecular basis of ethanol-dependent modulation of the BK channel. The T352 residue is positioned close to the conserved RCK1 domain, which is thought to be critical for activation by intracellular

calcium (Xia et al., 2002). This residue is not predicted to be a phosphorylation site (Blom et al., 1999; Dinkel et al., 2013; Huang et al., 2005). The T352I mutation results in a larger amino acid at position 352 and changes the residue from polar to nonpolar. Future studies will need to explore how alternative mutations at and near T352 influence ethanol responses of single channels. Such analyses might reveal whether this location provides a specific binding site for ethanol and/or represents a portion of the channel that undergoes conformational change in the presence of ethanol.

The BK channel has been implicated in behavioral responses to alcohol such as tolerance and consumption in mutant mice lacking BK channel auxiliary subunits (Martin et al., 2008; Kreifeldt et al., 2013). However, the basal behavioral impairments of the BK channel knockout mouse have made it difficult thus far to probe the role of the channel itself. These results suggest that knocking-in the T352I mutation in rodent models may alter ethanol-dependent behaviors without causing gross behavioral impairments, which would allow us to further advance our understanding of the role of the BK channel in alcohol-mediated behaviors.

4.0 - CA^{2+}-SENSING DOMAINS OF THE BK CHANNEL ARE DISPENSABLE FOR INTOXICATION IN CAENORHABDITIS ELEGANS

The majority of text, data and figures presented in this chapter have been submitted in a 2014 manuscript for publication in the journal *Genes, Brain and Behavior*

Davis SJ, Pierce-Shimomura JT, (2014) Ca^{2+}-sensing domains of the BK channel are dispensable for intoxication in *Caenorhabditis elegans*

Co-author contributions: I ran all of the experiments in this study. Also, I generated all transgenic *C. elegans* used in this study. Pierce-Shimomura JT is my P.I. who funded the study and provided guidance on experiments. The manuscript was written by me and Pierce-Shimomura JT with helpful comments from Scott L. Mayfield J. performed critical editing of the manuscript.

4.1 -ABSTRACT

Alcohol modulates the highly conserved, voltage- and Ca2+-activated potassium (BK) channel, which contributes to alcohol-mediated behaviors in species as diverse as worms and humans. Previous studies showed that the Ca2+-sensitive domains, RCK1 and the Ca2+ bowl, are required for ethanol activation of the mammalian BK channel in vitro. In the nematode C. elegans, ethanol activates the BK channel in vivo, and deletion of the worm BK channel, SLO-1, conveys strong resistance to intoxication. We recently identified a conserved residue (T381), located near the Ca2+-sensing residues of the RCK1 domain of the SLO-1 channel, required for intoxication. The equivalent residue (T352) of the human channel was also critical for ethanol activation and intoxication in C. elegans. To determine if the conserved Ca2+-sensitive domains were critical for intoxication and basal BK channel-dependent behaviors in C. elegans, we generated SLO-1 knock-in replacement mutants that render the RCK1, Ca2+ bowl, or both domains insensitive to Ca2+. As expected, mutating these regions inhibited the function of the SLO-1 channel in vivo since neck and body curvature of these mutants mimicked that of the BK null mutant. Unexpectedly, mutating the Ca2+-sensitive domains singly or together had no effect on intoxication in C. elegans. Thus, for the worm SLO-1 channel, the Ca2+-sensitive domains appear critical for basal in vivo function but not critical for in vivo ethanol action. One possibility, then, is that the Ca2+ gating pathway is less important for an effect of ethanol on channel opening in worm SLO-1 channels than human BK channels

4.2 - Introduction

Ethanol elicits intoxication in part by acting on membrane-bound ion channels (Harris et al., 2009; Morozova et al., 2012). The large-conductance, voltage- and Ca2+-activated potassium (BK) channel has emerged as a key mediator of behavioral intoxication and tolerance across species. In the nematode C. elegans, knocking out the slo-1 gene that encodes the BK channel produces strong resistance to intoxication (Davies et al., 2003). In Drosophila, acute functional tolerance to alcohol requires the BK channel (Cowmeadow et al., 2005; Cowmeadow et al., 2006). In mice, acute tolerance at the single BK channel level correlates with acute tolerance and ethanol ingestion at the behavioral level (Martin et al., 2008; Kreifeldt et al., 2013). Likewise, humans and worms that carry gain-of-function mutations in the BK channel are hypersensitive to intoxication (Du et al., 2005; Davies et al., 2003).

Clinically relevant concentrations of alcohol activate the BK channel in different species, suggesting a conserved mechanism of activation. Examples include homologues of the BK channel in C. elegans (Davies et al., 2003), mice (Dopico et al., 1998; Liu et al., 2008), and humans (Yuan et al., 2008). Although the molecular interactions between ethanol and the BK channel remain largely unknown, channel activation occurs in reconstituted lipid bilayers, suggesting a direct ethanol action on the channel that does not require diffusible second messengers or accessory subunits (Chu et al., 1998).

Ethanol modulates the mammalian BK channel in vitro independently of voltage and magnesium gating (Liu et al., 2008). Furthermore, Ca2+ must be present intracellularly for ethanol to activate the channel (Liu et al., 2008). Consistent with this finding,

abolishing Ca2+ sensitivity of the RCK1 and Ca2+ bowl prevents ethanol action on the mammalian BK channel in vitro (Liu et al., 2008). The importance of these domains in the behavioral responses to ethanol remains to be tested.

We recently determined that mutating residue T381 of the worm BK channel, SLO-1, conveyed dramatic resistance to intoxication (Davis et al., in press). This residue is conserved in mammals, and T352 is the equivalent residue in the human BK channel. This conserved residue is positioned 10 and 15 residues upstream of the two critical RCK1 Ca2+-sensitive residues, respectively, in worm, mouse and human BK channels. Thus, disruption of RCK1 might account for the resistance to intoxication as predicted by mammalian in vitro studies (Liu et al., 2008). However, the T352I mutation did not alter other behaviors that are dependent on BK channel function as would be expected if the RCK1 regions lost Ca2+sensitivity (Davis et al., in press). Therefore, it is unclear whether the RCK1 domain, the Ca2+ bowl domain, or both are important for intoxication in C. elegans.

Here, we investigate the function of the Ca2+-sensitive domains of the worm BK channel in mediating ethanol intoxication and basal BK channel-dependent behaviors. Both the RCK1 and Ca2+ bowl regions appear to be important for basal behaviors in C. elegans, but are surprisingly unnecessary for intoxication.

4.3 - Materials and Methods

Animals: *C. elegans* strains were grown at 20 °C and fed OP50 strain bacteria seeded on Nematode Growth Media (NGM) agar plates as described previously (Brenner, 1974). Worms cultured on plates contaminated with fungi or other bacteria were excluded. The reference wild-type strain was N2 Bristol. The reference *slo-1(null)* strain was NM1968, harboring the previously characterized null allele, *js379* (Wang et al., 2001).

Transgenesis: The background for all transgenic worms generated in this study used the *slo-1(null)* strain NM1968. Multi-site gateway technology (Invitrogen, Carlsbad, CA) was used to construct plasmids. 2501 kb of the native *slo-1* promoter (*Pslo-1*) and the traditional *unc-54* UTR were used in combination with *slo-1(cDNA)::mCherry* for wild-type transgenes. Mutant versions were constructed using site-directed mutagenesis as described below. All plasmids were injected at a concentration of 20 ng/µl. The co-injection reporter PCFJ90 (1.25 ng/µl) was used to ensure proper transformation of the arrays. Two independent isolates were obtained for each of the four strains to help control for variation in extrachromosomal arrays: strains JPS344 and JPS345 carried *vxEx344*[*Pslo-1::slo-1::mcherry::unc-54UTR,Pmyo-2::mcherry*], strains JPS360 and JPS361 carried *vxEx360*[*Pslo-1::slo-1(D391/396A)::mcherry::unc-54UTR,Pmyo-2::mcherry*], strains JPS350 and JPS351 carried *vxEx350*[*Pslo-1::slo-1(5D5N)::mcherry::unc-54UTR,Pmyo-2::mcherry*], and strains JPS358 and JPS359 carried *vxEx358*[*Pslo-1::slo-1(D391/396A+5D5N)::mcherry::unc-54UTR,Pmyo-2::mcherry*]. For the control transgenic strain, the plasmid PCFJ150 (the backbone of

which was used to construct the transgenic worms) was injected at a concentration of 20 ng/µl, along with 1.25 ng/µl of PCFJ90 plasmid to generate strain JPS383 that carried the extrachromosomal array *vxEx383*[*Pmyo-2::mcherry*].

Site-Directed mutagenesis: QuikChange II XL mutagenesis kit (Stratagene, La Jolla, CA) was used. The primers 5'-ccattttcttcaaaatttcctacacgagaaccgtgatgacgtgga-3' and 5'-ccattttcttcaagatttcctacacgaggaccgtgatgacgtgga-3' were used to generate two A->G mutations in the slo-1 gene that resulted in D391A and D396A amino acid substitutions. The primers 5'- atgtgcaattcctcgaccagaacaacaacaacaatccggacaccg-3' and 5'- atgtgcaattcctcgaccaggacgacgacgacgatccggacaccg-3' were used to induce five A>G mutations in the slo-1 gene that resulted in 5D5N substitutions in the Ca2+ bowl. All plasmids were confirmed by sequencing the full cDNA.

Neck posture assay: The movement of single worms was digitally recorded at 30 frames per second for 5-10 minutes and analyzed as described previously (Pierce-Shimomura et al., 2008). Briefly, custom-written software automatically recognizes the worm from each frame and assigns thirteen points spaced equally along the midline of the body from the head to the tail. The neck angle was defined as the angle formed by the most anterior three points when the head of the worm was maximally swung to the ventral side while crawling on an unseeded NGM-agar plate.

Overall body posture assay: Worm movement was recorded as described in the neck posture assay. The thirteen points spaced equally along the midline of the body from the

head to the tail were used to gather 11 body angles. The absolute sum of the angles was added to account for the overall body posture.

Egg laying response to ethanol: Plastic Petri plates (6-cm diameter) filled with NGM-agar (12 mL) were seeded with *E. coli* at least 20 hour before the assay and stored at 4 °C for no more than 2 weeks. Plates were brought to room temperature (20 °C) 1 hour before testing. Ethanol plates were prepared by adding 200-proof ethanol (Sigma Aldrich) beneath the agar 30 minutes before the assay to allow ethanol time to soak into the agar. For 600-mM plates, 420 mL was added. At the start of the assay, ten young adult worms were placed on a control plate containing no ethanol. After 1 hour, worms were then transferred to an ethanol plate for another hour. The number of eggs laid by the ten worms was counted on each plate. Average relative values of egg-laying frequencies per worm were determined for untreated and ethanol-treated conditions.

Locomotion response to ethanol: Plates were prepared as described in the egg-laying response to ethanol using 600-mM EtOH plates and seeded with 200 μL of OP50. At the start of the assay, five young adult worms were placed in the center of the OP50 on a control plate containing no EtOH and allowed to crawl for 5 minutes. Their position on the control plate was marked and worms were moved to an EtOH plate. After 20 minutes on the EtOH plate, worms were moved to another EtOH plate in the center of the OP50 and allowed to crawl for 5 minutes. Worms were removed and their position marked. For each worm, the mm distance from the center was recorded as a measure of worm locomotion on control and EtOH plates.

Statistical analysis: Sigmaplot 12.5 was used for all statistical analyses to determine significance ($p \leq 0.05$, two tailed) between two or more groups. If the groups being compared passed the Shapiro-Wilk normality test, they were analyzed using standard t- or ANOVA tests where appropriate. If needed, post hoc multiple comparisons were performed using the Holm-Sidak method. If the groups being compared did not pass the Shapiro-Wilk normality test, the groups were analyzed using the Mann-Whitney Rank Sum Test (unpaired) or Wilcoxon singed rank t test (paired) or Kruskal-Wallis ANOVA on ranks where appropriate. If needed, post hoc multiple comparisons were performed with the Bonferroni method.

4.4 - Results

Ca^{2+}-sensing domains in the worm BK channel are not required for intoxication

Following acute ethanol exposure, wild-type *C. elegans* displays intoxication by decreasing rates of egg laying and locomotion. Also, deletion of *slo-1* produces strong resistance to intoxication (Davies et al., 2003; Davis et al., in press). Transgenic rescue of the *slo-1(null)* strain with a wild-type *slo-1(+)* cDNA using the endogenous *Pslo-1* promoter restores the intoxicating effects of ethanol. Similar to previous reports, we found *slo-1(null)* mutant worms are markedly resistant to the inhibitory effects of ethanol on egg laying in wild-type worms (Figure 1; Kruskal-Wallis ANOVA, genotype, $H=111.19$, $df=10$ $p<0.001$; Dunn's multiple comparison, *slo-1(null),* $t(73)=7.44$, $p<.05$; n

= 32-43). In addition, *slo-1(null)* mutant worms are strongly resistant to the inhibitory effects of ethanol on locomotion in wild-type worms (Figure 2; Kruskal-Wallis ANOVA, genotype, H =36.65, df =10 $p<0.001$; Dunn's multiple comparison, *slo-1(null)*, t(32)=3.42, $p<.05$; n = 17).After expressing cDNA using the endogenous *Pslo-1* promoter, the ethanol response on egg laying was restored to wild-type levels for two independently derived *slo-1(+)* transgenic strains (#1 and #2) (Figure 1; Dunn's multiple comparison, *slo-1(null)* + *slo-1(+)#1*, t(53)=1.90, n.s.; *slo-1(null)* + *slo-1(+)#2*, t(63)=1.88, n.s.; n = 12-43). Additionally, the inhibitory effects of ethanol on locomotion were restored to wild-type levels. (Figure 2; Dunn's multiple comparison, *slo-1(null)* + *slo-1(+)#1*, t(33)=0.63, n.s.; *slo-1(null)* + *slo-1(+)#2*, t(35)=0.45, n.s.; n = 17-20).By contrast, a control fluorescent reporter transgene did not rescue ethanol sensitivity to egg laying(Figure1; Dunn's multiple comparison, *slo-1(null)* + *co. inj*, t(47)=3.74, $p<.05$; n = 6-43) or locomotion (Figure 2; Dunn's multiple comparison, *slo-1(null)* + *co. inj*, t(34)=3.13, $p<.05$; n = 17-19)

Figure 4.1 - Ca^{2+}-sensing domains of the *C. elegans* BK channel are not critical for ethanol-induced reduction in egg-laying.

A, Intoxication sensitivity as determined by egg laying in control *versus* ethanol plates for various strains: wild-type (WT) strain N2 (*N* = 41), *slo-1(null)* strain NM1968 (*N* = 30), *slo-1(+)* strains rescued with wild-type *slo-1* cDNA (#1 *N* = 12, #2 *N* =22), *slo-1(RCK1)* strains rescued with *slo*-1(D3912/396A) mutant transgene (#1 *N* = 6, #2 *N* =11), *slo-1(*Ca^{2+} *bowl)* strains rescued with *slo-1(5D5N)* transgene (#1 *N* = 6, #2 *N* =8), *slo-1(DM)* double-mutant strains rescued with *slo-1* transgene that contained both sets of Ca^{2+}-domain mutations (#1 *N* = 16, #2 *N* =6) and the co-injection control strain (*N* =6) (*N* represents an assay with 10 worms each). All groups displayed a significant reduction in egg-laying on ethanol plates compared to control plates. The co-injection control was the only transgenic strain that displayed resistance to intoxication as measured by egg laying (Paired Wilcoxon singed rank t test t=2.00, n.s; n = 6; all other groups (Paired t test/wilcoxon singed rank t test, $p < 0.05$; n = 6-43) Note that while the *slo-1(null)* strain displayed significant reduction in egg laying in the presence of ethanol, the effect was extremely small (Mean values for control = 4.8 and ethanol 4.1) compared to other strains. ***B***, Intoxication as determined by relative egg-laying sensitivity to ethanol in the strains in panel b. The *slo-1(null)* and co-injection control *strains* were significantly less intoxicated than WT ($p < 0.05$). All Ca^{2+}-domain mutant BK channels rescued ethanol sensitivity when transgenically expressed in *slo-1(null)* worms. In both panels, bars represent s.e.m. #1 and #2 refer to independently isolated strains expressing the same cDNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

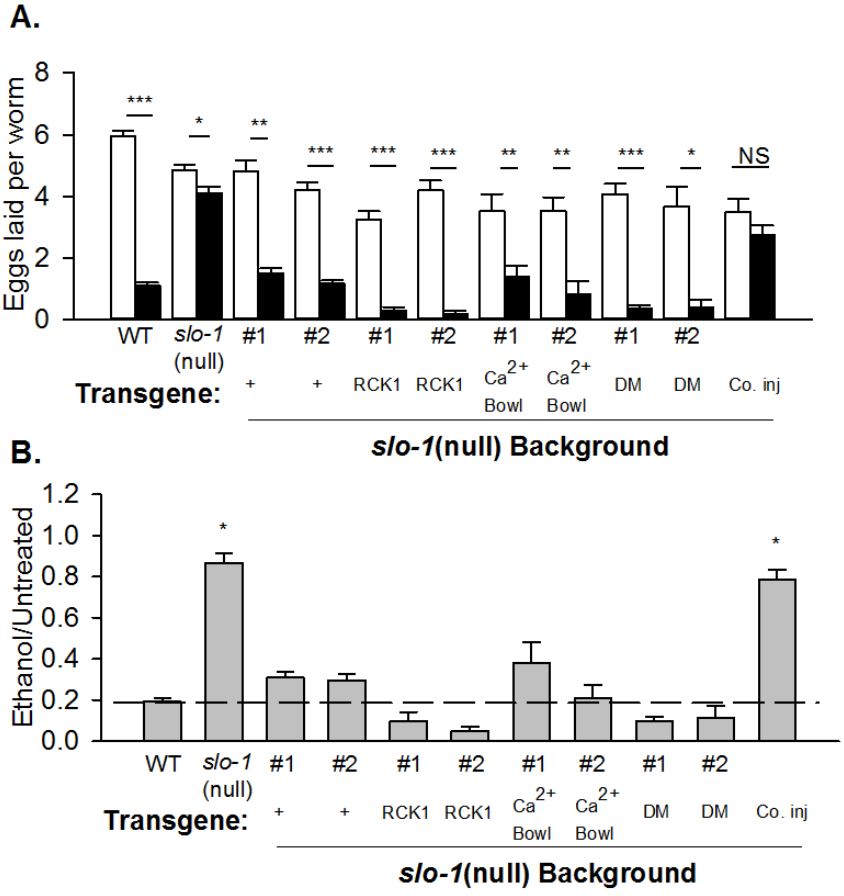
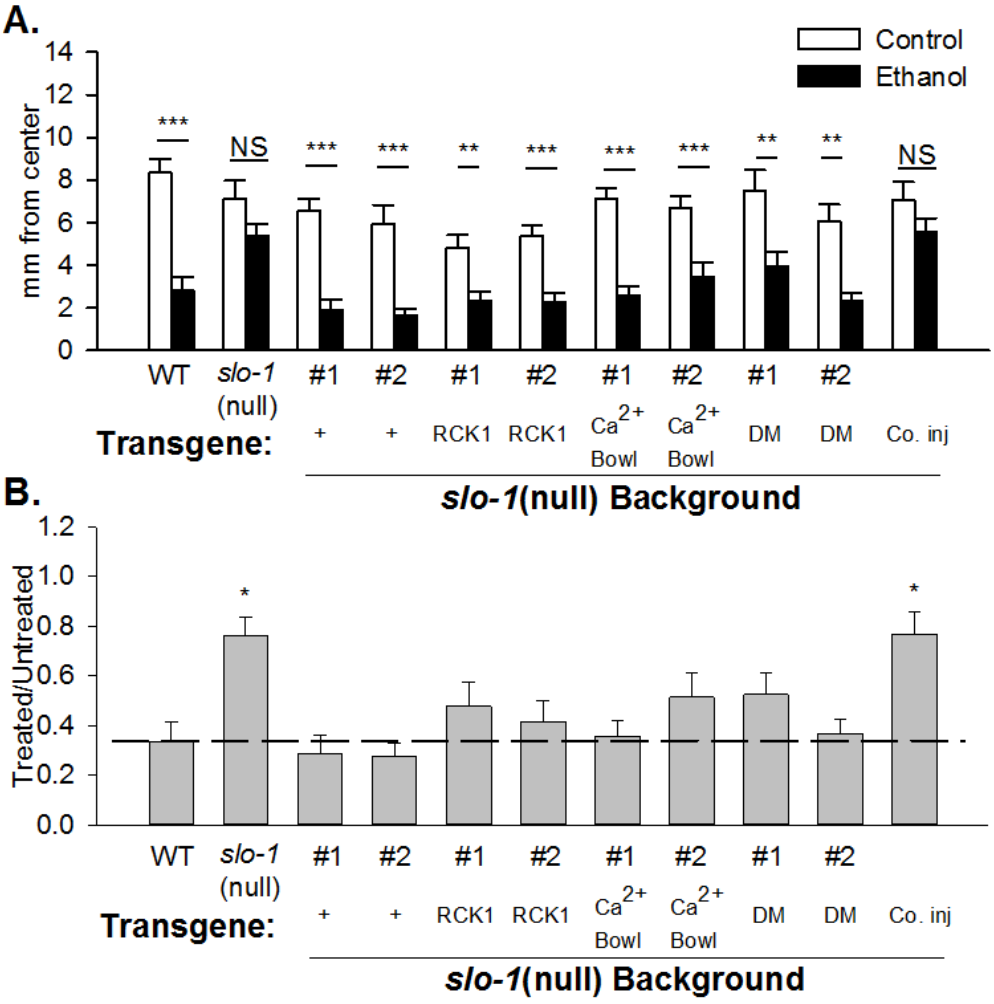


Figure 4.2 - Ca^{2+}-sensing domains of the *C. elegans* BK channel are not critical for ethanol-induced reduction in locomotion.

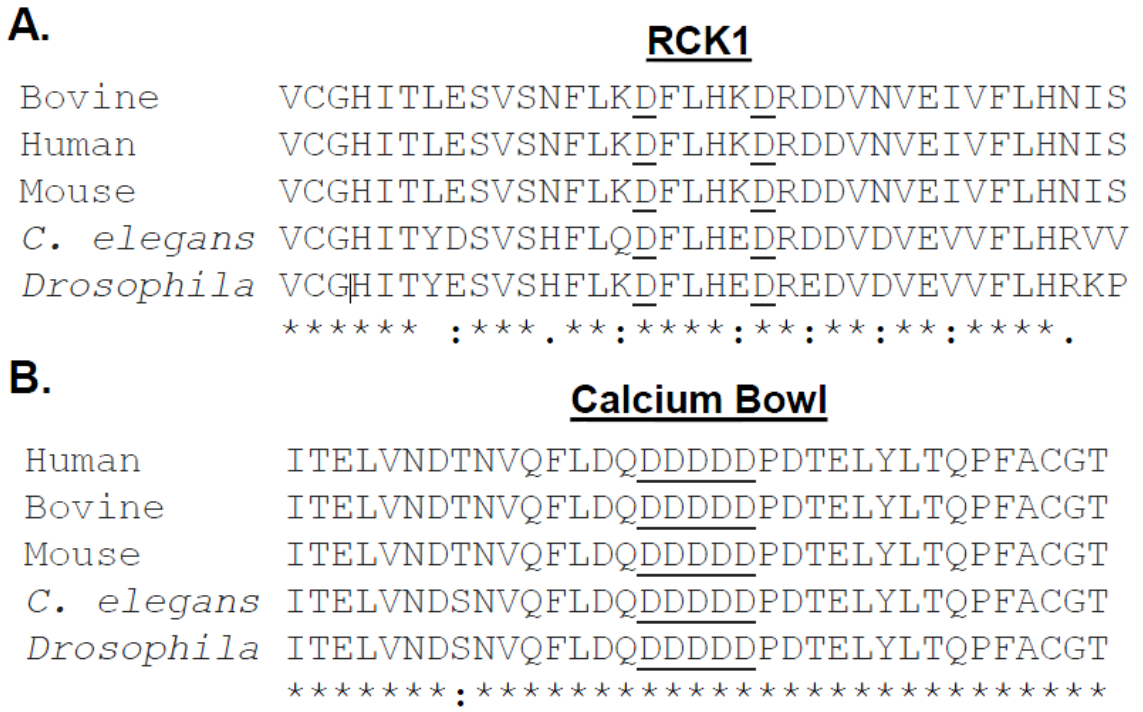
A, Locomotion on control and ethanol plates for various strains: wild-type (WT) strain N2 (*n* = 17), *slo-1(null)* strain NM1968 (*n* = 19), and *slo-1* rescue strains (#1 n = 18, #2 n =20), *slo-1* (RCK1) rescue strains (#1 *n* = 20, #2 *n* = 20), *slo-1(*Ca^{2+} *bowl)* rescue strains (#1 *n* = 19, #2 *n* = 20), *slo-1(DM)* rescue strains (#1 *n* = 19, #2 *n* = 18) (*n* represents number of worms). Abbreviated names for the strains are the same as in Figure 1. Intoxication as measured by locomotion was rescued in all versions of *slo-1* transgenes regardless of whether one or both Ca^{2+}-sensitive domains were mutated (Paired t test/wilcoxon singed rank t test, $p < 0.05$; n = 17-20). The only resistant strains were the *slo-1(null)* mutant (Paired t test t(34)=1.69, n.s., n = 17) and the co-injection control (Paired t test t(36)=1.38, n.s., n = 19) ***B***, Intoxication as determined by relative locomotion on control *vs* ethanol plates. The *slo-1(null)* and co-injection control strains exhibited resistance to intoxication demonstrated by significantly more relative movement compared to WT (* $p < 0.05$).



The Ca^{2+}-sensitive domains of the mammalian BK channel were previously shown to be important for ethanol action (Liu et al., 2008). To test the importance of these domains in the worm BK channel, we attempted to rescue the *slo-1(null)* mutant strain with versions of the *slo-1* gene containing missense mutations in conserved Ca^{2+} binding residues (Figure 3). As discussed above, we used the native *slo-1* promoter to express the transgenes and generated two independently derived strains for each transgene to control for variation in transgenesis.

Figure 4.3 - Primary sequence comparison of the RCK1 and Ca^{2+} bowl Ca^{2+}-sensitive domains of the worm BK channel.

A, The RCK1 domain is highly conserved from *C. elegans* to humans. Both negatively charged aspartate (D) residues critical for RCK1 Ca^{2+}-sensing (underlined) are completely conserved, while amino acids surrounding this region contain only one residue that is highly dissimilar between worm and human. ***B***, The region of the BK channel containing the Ca^{2+} bowl domain is highly conserved across species. All underlined aspartate (D) residues are conserved, while only one amino acid residue is not identical in the adjacent region. * = Residues are completely conserved; : = Residues are strongly similar in properties, (> 0.5 in the Gonnet PAM 250 matrix); and . = Residues are weakly similar (≤ 0.5 in the Gonnet PAM 250 matrix).



First, we tested the role of the RCK1 domain *in vivo* by generating a knock-in mutant with a *slo-1* transgene, *slo-1(RCK1)*, which contained two mutations (D391A and D396A, equivalent to mouse D362A and D367A, respectively). These mutations were previously shown to strongly reduce Ca^{2+} sensitivity of the mammalian BK channel and RCK-type domains in other proteins (Cui et al., 2009; Smith et al., 2013). We found that ethanol sensitivity of egg laying and locomotion in both transgenic *slo-1(RCK1)* mutant strains resembled wild-type levels for both egg-laying (Figure 1; Dunn's multiple comparison, *slo-1(null)* + *slo-1(RCK1)#1*, t(47)=1.34, n.s.; *slo-1(null)* + *slo-1(RCK1)#2*, t(52)=2.62, n.s.; n = 6-43) and locomotion (Figure 2; Dunn's multiple comparison, *slo-1(null)* + *slo-1(RCK1)#1*, t(35)=1.03, n.s.; *slo-1(null)* + *slo-1(RCK1)#2*, t(35)=0.55, n.s.; n = 17-20).

Second, we tested the role of the Ca^{2+} bowl domain *in vivo* by generating a knock-in mutant with a *slo-1* transgene, *slo-1(Ca^{2+} bowl)*, containing mutations that neutralize the negatively charged aspartate residues D969-973 (equivalent to mouse D897-901) to asparagine (5D5N), in order to abolish Ca^{2+}-sensing by the Ca^{2+} bowl (Schreiber and Salkoff, 1997; Bian et al., 2001). Similar to the results with the *slo-1(RCK1)* mutants, the two different *slo-1(Ca^{2+} bowl)* mutants displayed wild-type ethanol sensitivity of egg laying (Figure 1; Dunn's multiple comparison, *slo-1(null)* + *slo-1(5D5N)#1*, t(47)=1.73, n.s.; *slo-1(null)* + *slo-1(5D5N)#2*, t(49)=0.09, n.s.; n = 6-43) and locomotion (Figure 2; Dunn's multiple comparison, *slo-1(null)* + *slo-1(5D5N)#1*, t(35)=0.08, n.s.; *slo-1(null)* + *slo-1(5D5N)#2*, t(35)=1.05, n.s.; n = 17-20).

Our success in restoring wild-type ethanol sensitivity suggests that neither Ca^{2+}-sensitive domain acts alone to mediate intoxication in *C. elegan*s. To determine if the two domains work together and/or have overlapping actions, we generated a knock-in mutant with a *slo-1* double mutant transgene, *slo-1(DM),* which contained both the D391/396A and 5D5N mutations. These combined mutations eliminate all Ca^{2+} sensitivity (within the physiological Ca^{2+} concentration range) of the mammalian BK channel *in vitro* (Xia et al., 2002). Surprisingly, we found that ethanol sensitivity was rescued for two *slo-1(DM)* mutant strains, as measured by egg-laying (Figure 1; Dunn's multiple comparison, *slo-1(null) + slo-1(DM)#1*, t(47)=1.28, n.s.; *slo-1(null) + slo-1(DM)#2*, t(47)=1.31, n.s.; n = 6-43) and locomotion (Figure 2; Dunn's multiple comparison, *slo-1(null) + slo-1(DM)#1*, t(34)=1.32, n.s.; *slo-1(null) + slo-1(DM)#2*, t(34)=0.39, n.s.; n = 17-19). These results suggest that mutations in the Ca^{2+}-sensitive domains of the worm BK channel either do not compromise channel function as severely as demonstrated for mammalian channels *in vitro* or that ethanol sensitivity of the worm BK channel does not depend on known Ca^{2+}-sensing mechanisms.

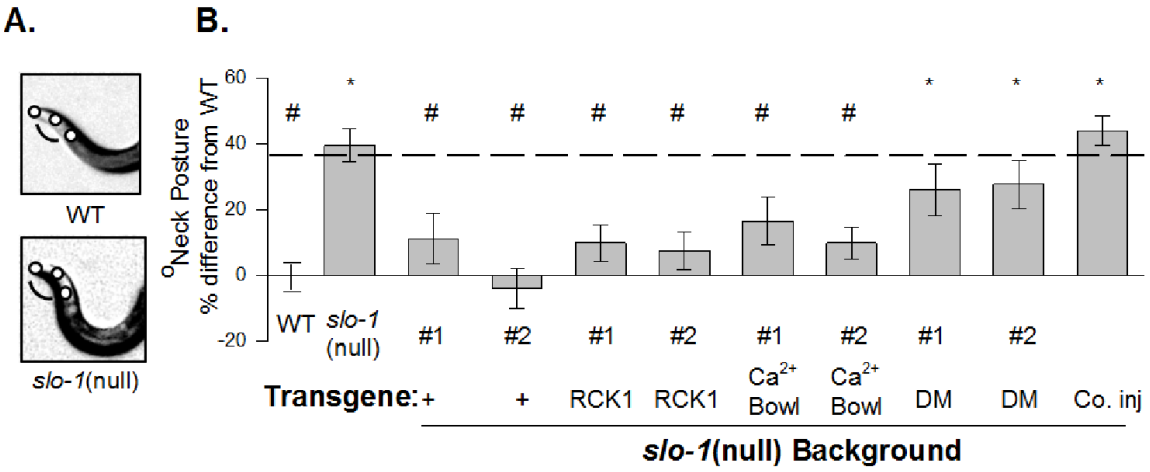
Ca^{2+}-sensing domains in the worm BK channel are functionally redundant for basal behaviors

The *slo-1(null)* mutant worms exhibit a characteristic head-bending phenotype, termed a "crooked-neck" (Kim et al., 2009). This is characterized by a significantly greater degree of bending at the anterior tip, or "neck" of the mutant compared to wild type (Figure 4; one-way ANOVA, genotype, $F(10,258)=9.33$, $p<.001$; Holm-Sidak multiple comparison, *slo-1(null),* $t(84)=6.26$, $P<.001$, n = 36-50). To determine whether

the Ca^{2+}-domain mutations compromise the basal function of the channel *in vivo*, we quantified the neck curvature of each transgenic strain studied above and compared with wild-type and *slo-1(null)* mutant strains.

Figure 4.4 - Neck curvature depends on Ca^{2+}-sensitive domains of the worm BK channel.

A, Representative photos of wild-type and *slo-1(null)* worms crawling on an agar surface. The three white circles near the head of the worm form the neck angle. Note the sharper "crooked" neck of the *slo-1(null)* mutant worm. ***B***, Quantitative analysis demonstrates neck curvature is sharper in *slo-1(null)* mutants (n = 50) than in wild-type (WT) worms (n = 36). Expressing either *slo-1(+)*, *slo-1(RCK1)* or *slo-1(*Ca^{2+} *bowl)* transgenes in *slo-1(null)* worms fully or partially rescued wild-type like neck curvature, suggesting that BK channel function was at least partially intact. In contrast, both *slo-1(DM)* mutant strains had significantly greater neck curvature than WT. Both of these strains are not significantly different than *slo-1(null).* The co-injection control also failed to rescue neck curvature. * $p < 0.05$ from WT, # $p < 0.05$ from *slo-1(null).*



The crooked-neck phenotype of the *slo-1(null)* mutant was rescued with a wild-type *slo-1(+)* transgene in two strains (Figure 4b; Holm-Sidak multiple comparison, *slo-1(null) + slo-1(+)#1*, t(51)=1.32, n.s.; *slo-1(null) + slo-1(+)#2*, t(49)=0.46, n.s.; n = 15-36). By contrast, a control fluorescent reporter transgene did not rescue this phenotype (Figure 4b; Holm-Sidak multiple comparison, *slo-1(null) + co. inj*, t(67)=6.80, P<.001; n = 33-36). The crooked-neck posture was also fully or partially rescued to wild-type levels with *slo-1* transgenes containing mutations in either the RCK1 domains (Figure 4b; Holm-Sidak multiple comparison, *slo-1(null) + slo-1(RCK1)*#1, t(63)=0.61, n.s.; *slo-1(null) + slo-1(RCK1)#2*, t(50)=0.63, n.s.; n = 16-36) or the Ca^{2+} bowl (Figure 4; Holm-Sidak multiple comparison, *slo-1(null) + slo-1(5D5N)#1*, t(51)=1.95, n.s.; *slo-1(null) + slo-1(5D5N)#2*, t(59)=1.31, n.s.; n = 17-36), These results suggest that compromising the function of individual Ca^{2+}-sensitive domains does not dramatically perturb *in vivo* function. To determine if the two domains are functionally redundant for neck curvature, we analyzed the *slo-1* double mutants. Both *slo-1(DM)* mutant strains displayed neck curvature that was significantly greater than wild type, (Figure 4; Holm-Sidak multiple comparison, *slo-1(null) + slo-1(DM)*, t(50)=3.02, p<.05.; *slo-1(null) + slo-1(DM)#2*, t(49)=3.12, p<.05; n = 15-36), but slightly less than the *slo-1(null)* mutant worms (Figure 4). Thus, the basal function of the worm BK channel is severely compromised *in vivo* when both Ca^{2+}-sensitive domains are mutated.

In addition to neck posture, the *slo-1(null)* mutant strain exhibits changes in body curvature (Kim et al., 2009). As another measure of BK channel function, we determined if the Ca^{2+}-sensitive domains are also required for body curvature. Quantitative

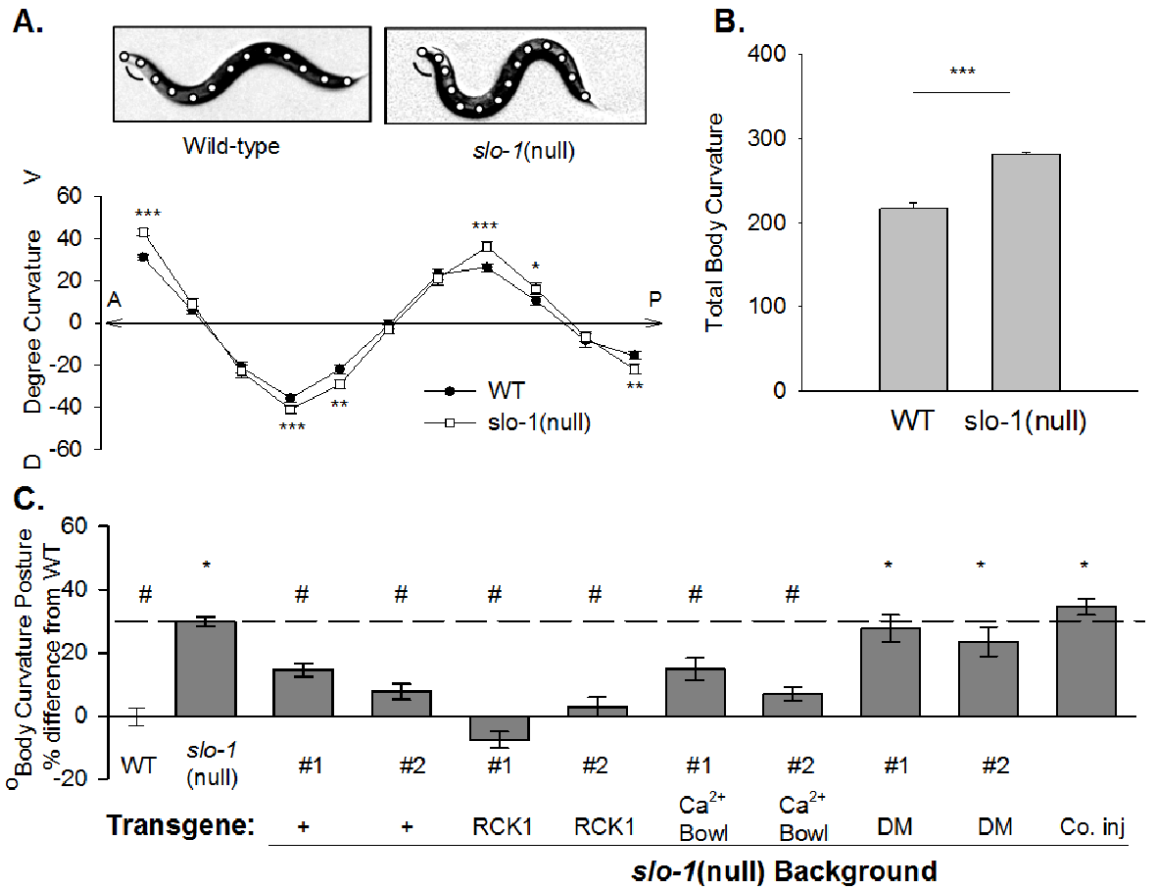
assessment of body curvature along the anterior-posterior axis showed that six different body portions of the *slo-1(null)* worms showed greater dorsal or ventral curvature than wild type (dorsal and ventral are assigned positive and negative values; Figure 5a; Paired t test, point 5, $t(84)=2.70$, $p<.01$; points 1,4,8,9 and 11, Mann-Whitney Rank Sum test, $U=323\text{-}664$, $p<.05\text{-}p<.001$; n = 36-50) To compare total body curvature values of individual worms, we computed a single *body curvature* metric as the sum of the absolute value of the 11 angles along the midline of the worm. The *slo-1(null)* mutant strain displayed significantly higher body curvature when compared to wild type (Figure 5b; Mann-Whitney Rank Sum Test, $U=119$, $p<.001$; n = 36-50). We used the body curvature metric as another measure quantitatively compare the degree of rescue for each transgenic strain versus wild-type and *slo-1(null)* mutant strains.

As observed for neck curvature, body curvature of the wild type rescue, and the RCK1 and Ca^{2+} bowl single-mutant strains was not significantly different than wild type (Figure 5c; Kruskal-Wallis ANOVA, genotype, $H=152.68$, $df=10$ $p<0.001$; Dunn's multiple comparison, *slo-1(null),* $t(84)=7.40$, $p<.05$; *slo-1(null)+ slo-1(+)*#1, $t(51)=2.60$, n.s.; *slo-1(null)+ slo-1(+)*#2, $t(49)=1.15$, n.s.; *slo-1(null)+ slo-1(RCK1)*#1, $t(63)=1.38$, n.s.; *slo-1(null)+ slo-1(RCK2)*#2, $t(50)=0.40$, n.s.; *slo-1(null)+ slo-1(5D5N)*#1, $t(51)=2.56$, n.s.; *slo-1(5D5N)*#2, $t(59)=1.23$, n.s.; n = 15-50). This suggests that each mutant with only a single perturbed Ca^{2+}-sensitive domain retained a significant level of BK channel function *in vivo*. For the double mutant strains and co-injection control strains, body curvature was not significantly different than *slo-1(null)* (Figure 5c; Dunn's multiple comparison, *slo-1(null)+ slo-1(DM)*#1, $t(64)=0.78$, n.s.; *slo-1(null)+ slo-*

1(DM)#2, t(63)=1.25, n.s.; *slo-1(null)+ co. inj*. t(67)7.49, $p<.05$; n = 15-50), indicating that BK channel function was severely compromised *in vivo* after mutation of both domains.

Figure 4.5 - Body curvature depends on Ca^{2+}-sensitive domains of the worm BK channel.

A, Representative photos of wild-type and *slo-1(null)* mutant worms crawling on an agar surface. The thirteen white points along the mid-body of each worm form 11 consecutive angles, which are quantified below the pictures. *C. elegans* crawls on its left or right side propagating bends to the dorsal (D) and ventral (V) sides from anterior (A) to posterior (P). ***B***, Quantitative analysis demonstrates body curvature is higher in *slo-1(null)* mutants (n = 50) than in wild-type (WT) worms (n = 36). ***C***, Expressing either *slo-1(+)*, *slo-1(RCK1)* or *slo-1(Ca^{2+} bowl)* transgenes fully or partially rescued body curvature in *slo-1(null)*, suggesting that channel function is at least partially intact for each type of BK channel. In contrast, expressing a *slo-1(DM)* transgene failed to rescue body curvature. The co-injection control transgene also failed to rescue body curvature. * $p < 0.05$ from WT, # $p < 0.05$ from *slo-1(null).*



4.5 - Discussion

In this study, we examined the functional role of the two conserved Ca^{2+}-*sensitive domains* (RCK1 and Ca^{2+} bowl) of the worm BK channel, SLO-1, in basal and ethanol-mediated behaviors. Our most salient finding was that the purported Ca^{2+}-sensing residues of the worm BK channel are critical for normal *in vivo* basal function, but are not essential for *in vivo* action of ethanol on the channel. Under *in* vivo conditions, ethanol can influence gating of the worm BK channel in the absence of the synergistic influence of calcium binding.

Intact calcium gating is necessary for proper SLO-1 channel function *in vivo.* We found that introducing mutations in both Ca^{2+}-sensitive domains of the worm BK channel perturbed basal behaviors (neck and body curvature) that depend on proper function of SLO-1. These findings suggest that, as for the mammalian channels recorded in heterologous systems, mutations of the negatively charged aspartate residues that knock out function of both RCK1 (D362/367A) and the Ca^{2+} bowl (5D5N) dramatically shift the voltage range of activation to non-physiological levels (e.g., voltage of half-activation > +120 mV; Xia et al., 2002). Although knocking out both Ca^{2+} binding domains was deleterious, leaving either Ca^{2+}-sensitive domain intact recapitulated WT behavior.

The RCK1 and Ca^{2+} bowl domains contribute to Ca^{2+} sensitivity via distinct mechanisms (Cui et al., 2009), so it is likely that the loss of either Ca^{2+} binding domain reduced SLO-1 Ca^{2+} sensitivity. Neutralizing the aspartate residues in either RCK1 or the Ca^{2+} bowl reduces Ca^{2+} sensitivity of mammalian BK channels recorded *in vitro*. For the Ca^{2+} bowl mutants, a reduction in Ca^{2+} sensitivity by half has been shown to be shared

between species, including *Drosophila*, mouse and human BK channels (Bian et al., 2001; Zeng et al., 2005; Savalli et al., 2012). Given this likely reduction in Ca^{2+} sensitivity for the single Ca^{2+} binding domain mutants, then, why did such a change in channel properties leave SLO1-dependent behaviors intact? One possibility is that wild type invertebrate BK channels are not as sensitive to Ca^{2+} (Bian et al., 2001; Johnson et al., 2011), and thus the loss of a single Ca^{2+} binding domain does not dramatically alter channel function under physiological conditions. Another possibility is that the transgenic strains can successfully compensate for the reduced function of the BK channels with neutralized aspartate residues in either RCK1 or Ca^{2+}. For example, increased expression of partially functional BK channels may be able to rescue posture in the transgenic rescue strains. In contrast, a loss of both Ca^{2+} binding domains produces a substantial enough change in channel function to disrupt behavior, supporting an important physiological role for these conserved Ca^{2+}-sensing domains.

Only under nonphysiological conditions has ethanol been shown to influence gating of the mammalian BK channel. *In vitro* recordings of mammalian channels have shown that perturbing RCK1 Ca^{2+} sensitivity (D362/367A) disrupts ethanol-induced changes in gating at high Ca^{2+} levels (> 0.1 mM), neither the RCK1 or Ca^{2+} bowl (5D5N) mutant at low Ca^{2+} levels (>10 uM) disrupted ethanol modulation (Liu et al., 2008). Our data suggest that individually, the RCK1 and Ca^{2+} bowl domains are not essential for ethanol modulation of the SLO-1 channel. We found that worms with a knock-in replacement of the SLO-1 channel containing individually mutated Ca^{2+}-sensitive domains exhibited normal intoxication. Because the physiological range of Ca^{2+}

concentrations is ordinarily lower than 10 μM *in vivo*, this change in ethanol sensitivity seen at high Ca^{2+} levels for RCK1 mutation in the mammalian channel is unlikely to play a role under *in vivo* conditions. Thus, from these data, across species there appears to be similar effects of ethanol on the mammalian BK channels *in vitro* and SLO-1 *in vivo*,

We also found that ethanol influences SLO-1 gating even when both calcium binding domains are disposed. Worms with a knock-in replacement of the SLO-1 channel containing mutations in both Ca^{2+}-sensitive domains exhibited normal intoxication. Conversely, ethanol fails to modulate the mammalian BK channel *in vitro* when both the RCK1 and Ca^{2+} bowl domains are neutralized (Liu et al., 2008). One potential source for this difference is that invertebrate BK channels may not require the synergistic influence of Ca^{2+}-gating for ethanol modulation because they are energetically closer to the open state in the absence of Ca^{2+} than mammalian channels. Invertebrate BK channels are less sensitive to Ca^{2+} levels than mammalian BK channels (Johnnson et al., 2011, Bian et al., 2001), suggesting a lower dependence on Ca^{2+} binding for SLO1 channel gating. Another possible source of ethanol modulation in the absence of Ca^{2+} gating in our experiments is that endogenous conditions including protein binding partners and phosphorylation states may enhance BK channel susceptibility to ethanol modulation *in vivo*. Such influences may be found if mammalian channels could be probed under physiological conditions *in vivo*. But, it will prove easier to compare how neutralizing the Ca^{2+}-binding domains influences worm BK channel gating in the absence and presence of ethanol using electrophysiological recordings of SLO-1 *in vitro*.

Despite potential differences in sensitivity to calcium gating, the high level of BK channel sequence conservation across species suggests that ethanol modulation occurs through a largely conserved mechanism. The BK channel is highly conserved with 58% protein sequence similarity between the worm and mammalian BK channels (Wang et al., 2001). Only two residues in the RCK1 region are highly dissimilar between *C. elegans* and the mammalian channel. Conservation in the Ca^{2+} bowl is even higher, with only one amino acid differing in this region. Our recent work establishes the importance of the RCK1 domain in the BK channel's response to ethanol across species. We found that mutating a conserved threonine (T381) to an isoleucine in SLO-1 produced mutant worms that were extremely resistant to intoxication, but otherwise normal for other behaviors that depend on a functional BK channel (Davis et al., in press). This mechanism is conserved in the human channel because knock-in replacement of the worm channel with a human BK channel containing the corresponding mutation (T352I) also produced a similar resistance to intoxication. Moreover, this T352I mutant human channel was not potentiated by ethanol *in vitro* (Davis et al., in press).

While high sequence conservation and a shared critical residue for ethanol sensitivity suggest a largely conserved mechanism for ethanol modulation, divergent residues may modify aspects of BK channel ethanol modulation across species. One interesting possibility is that a differential requirement for Ca^{2+} gating in ethanol binding or signal transduction underlies the potential ethanol modulation differences between the worm and mammalian BK channels discussed above. For example, in the absence of Ca^{2+} the binding pocket for ethanol may be occluded or in a state energetically unfavorable to

opening in the mammalian, but not worm BK channel. A recent report by Bukiya et al., (2014) established a putative ethanol-binding site involving residue K361 of the mouse BK channel. While this is at the opposite end of the same alpha helix as T352, a residue key for ethanol modulation in both worm and human BK channels, K361 is the one of the few residues in the RCK1 domain not conserved from the human to worm BK channel. Future studies will investigate if this residue contributes to the differential Ca^{2+}-dependence in the BK channel response to ethanol with *in vitro* patch-clamp and further *in vivo* behavioral analysis with *C. elegans*. In addition, exploring the differences in other amino acid residues between the worm and mammalian channel within RCK1 may lead to novel insights into channel kinetics and ethanol activation of the BK channel.

5.0 - DISCUSSION AND FUTURE DIRECTIONS

The overarching goal of this dissertation was to further *C. elegans* as a useful model to uncover the conserved molecular basis for behavioral responses to ethanol, and to use *C. elegans* to elucidate the molecular interaction between ethanol and the BK channel.

5.1 - *C. elegans* as A Model System for Intoxication

In my dissertation I first explored the discrepancies between behavioral responses to ethanol in previous work by my advisor and other groups with Mitchell et al. (2007). I found that osmolarity unexpectedly alters behavioral responses to ethanol in *C. elegans* by either influencing the amount of ethanol entering the worm and/or altering the apparent strength of ethanol that has entered the worm. Specifically, in high osmolarity solutions more ethanol enters *C. elegans*, and the worm's behavioral responses to ethanol are more severe than that seen in low osmolarity solutions with equal exogenous concentrations of ethanol. These experiments explain the differences in the behavioral responses to ethanol Mitchell et al., (2007) reported that conflicted with previous ethanol studies.

With the Bettinger and Davies laboratories, we explored a novel way to measure how much ethanol permeates the cuticle of *C. elegans* during exposure. This was done by modifying a protocol used previously by Davies et al. (2003) to measure the internal concentration of ethanol in *C. elegans* after exposure to high exogenous concentrations (500mM). For the measurements set forward in Chapter 2, we skipped a controversial

wash step previously used by Davies et al. (2003). The driving force behind this decision was that Mitchell et al. (2007) claimed this wash step was washing away ethanol that was inside *C. elegans* during exposure. Mitchell et al. (2007) also skipped this wash step and demonstrated that the internal concentration of ethanol in *C. elegans* was ~300 mM after exposure to 500 mM exogenous ethanol. However, Mitchell et al. (2007) also submerged the worms in the exogenous ethanol solution. Therefore, these authors were likely measuring a mix of external and internal ethanol. In our measurements, *C. elegans* were incubated on an agar plate that was mixed with 500 mM ethanol. After ethanol exposure, worms were picked directly into a container where internal ethanol concentration was measured. In this experiment, an external ethanol concentration of 500 mM produced an internal ethanol concentration in *C. elegans* of ~40 mM. These results provide further evidence that the cuticle represents a significant barrier for ethanol to enter into *C. elegans*. In addition, using high exogenous concentrations of ethanol will lead to internal concentrations in *C. elegans* near clinically relevant doses reached routinely by people (20-100 mM). Considering the natural ecology of *C. elegans*, which is found living in rotting vegetation and fruits where ethanol may be present up to 50 mM, it would make sense that this worm may have evolved mechanisms to resist ethanol penetration.

5.2 - The T381I Residue on the BK Channel is Critical for Worm Intoxication

The BK channel has previously been shown to be activated by ethanol in *C. elegans* (Davies et al., 2003). In addition, worms harboring a null mutation in the BK channel gene, *slo*-1, are extremely resistant to the intoxicating effects of ethanol (Davies et al., 2003). To explore the molecular interaction between ethanol and the BK channel, I obtained 32 strains from the million mutation project (MMP), each with a unique missense mutation in *slo-1*. After screening through all of these strains, I identified one strain that displayed a robust resistance to intoxication indistinguishable from that displayed by *slo-1(null)* worms. This strain, VC40372, had a threonine to isoleucine mutation at position 381 (T381I) of the BK channel. To verify that this mutation was the cause of ethanol resistance, we performed non-complementation testing with a *slo-1(null) C. elegans*. In addition, we outcrossed two sets of isolated VC40372 strains six times with wild-type worms to minimize background mutations. In both cases, the resulting strains were also resistant to ethanol, demonstrating the mutation that conferred resistance was *slo-1(T381I)*.

The T381I BK mutation could have resulted in a non-functional BK channel, which would provide us no additional information pertaining to the molecular interaction of the BK channel and ethanol. To test BK channel function *in vivo*, I performed experiments testing BK channel-dependent-behaviors. These included sensitivity to the paralytic agent aldicarb, and posture analysis. Unlike *slo-1(null)* worms, strains harboring

the *slo-1(T381I)* mutation displayed wild-type-like sensitivity to aldicarb and posture, suggesting the T381I mutation did not interfere with *in viv*o function.

I then obtained transgenic worms driving the expressing of *slo-1(T381I)* with the native *slo-1* promoter in a *slo-1(null)* background. I showed that posture dependent on BK channel function was rescued, while intoxication was not. These results provided further evidence that the T381I BK channel functioned *in viv*o, and was resistant to ethanol. To test if BK channel expression was altered, we took confocal images of *C. elegans* expressing the wild-type or T381I BK channel tagged with the fluorophore mcherry. A similar expression pattern was seen in strains expressing wild type and a mutant T381I BK channel. Visualization of mcherry in cholinergic neurons, the ventral nerve cord and muscle was seen in both cases. These results provide strong evidence that the T381I BK channel has a similar expression pattern and function compared to the wild-type BK channel. In contrast, while the wild-type channel is significantly modulated by ethanol, the T381I mutant channel appears to be resistant to ethanol. Overall, these are the first experiments that identified an amino acid substitution on the BK channel (T381I) that results in *C. elegans* that is extremely resistant to ethanol, while phenocopying wild type *C. elegans* in other BK channel-dependent-behavior. This suggests that T381 is critical for ethanol modulation of the BK channel, without being critical for channel function.

5.21 – The Conserved Residue on the Human BK Channel is Critical for Worm Intoxication

I next tested if the equivalent residue on the human BK channel, T352, resulted in an ethanol-resistant, functional BK channel. To accomplish this, I first obtained *slo-1(null)* strains that expressed the human BK channel driven by the *slo-1* promoter. I demonstrated that the human BK channel rescued ethanol sensitivity and posture to wild type levels in a *slo-1(null)* background. These results are significant, suggesting *hslo* functions in the worm properly, and rescues intoxication in *slo-1(null)* worms. Next, I transgenically expressed *hslo(T352I)* driven by the *slo-1* promoter in a *slo-1(null)* background. I found that although wild-type posture was rescued in these mutants, these worms remained resistant to the inhibitory effects of ethanol on locomotion and egg-laying. To test for differences in expression, confocal images were taken of *C. elegans* expressing wild-type or the T352I human BK channel tagged with mcherry. A similar expression pattern was seen, with mcherry visualized in Ach neurons, the ventral nerve cord and in muscle. These results demonstrate that the T352 residue on the human BK channel is critical for the behavioral response of *C. elegans* to ethanol, but not critical for *in vivo* channel function. In addition, these results demonstrate conservation from the worm to human BK channel regarding the T381/352 residue being critical behavioral intoxication.

5.22 - The Molecular Interaction Between the Human BK Channel and Ethanol

To explore the interaction between ethanol and the BK channel, I used single-channel *in vivo* and *in vitro* recordings. The *in vivo* recordings were performed in *C. elegans* neurons that expressed wild-type or T352I-mutant human BK channels. I found that both channels expressed, as evident from recordings and fluorescent tags, and appeared to have similar sensitivities to calcium and voltage. Due to the relative difficulty of *C. elegans* neuronal recordings, for further quantitative analyses I used human BK channels expressed in HEK cells.

I found a slight, yet significant difference in the basal properties of the wild-type and T352I BK channels. From these results we concluded that BK channel function remains largely intact in the T352I human BK channel. We also found that this mutation results in the complete abolishment of the potentiating effects of ethanol. From these results, I concluded that this mutation either represents an ethanol binding site and/or prevents the signal caused by ethanol binding from being transduced to drive BK channel opening.

5.3 - Potential Divergence for Ethanol Modulation Requiring Functional Calcium-Sensing Residues on the BK Channel

After identifying that the T381/352 conserved residue was selectively critical for worm intoxication and ethanol activation, I probed the importance of calcium-sensing residues of the channel for intoxication in *C. elegans*. This was based off of previous work by Liu et al. (2008), who reported calcium-sensing residues were critical for

modulation of the mouse BK channel by ethanol *in vitro*. The primary goal was to extend this work to an *in vivo* system, and test for conserved function of these residues in ethanol responses. Mutations in the mammalian BK channel gene that results in substitutions to calcium-sensing residues in the RCK1 (D362/367A) and Ca^{2+} bowl(5D5N) are known to abolish calcium-gating and the response of the BK channel to ethanol (Liu et al., 2008). I made a series of strains in a *slo-1(null)* background that transgenically expressed the worm BK channel with the equivalent calcium-sensing residue substitutions to RCK1(D391/396A) and the Ca^{2+} bowl (5D5N) singly and together.

I expected to see results that recapitulated Liu et al. (2008) in the worm BK channel, especially after identifying that the T381/352 residue was critical for the mammalian and the worm BK channel's response to ethanol. However, I found that the worm BK channel did not require calcium-sensing residues to engender behavioral intoxication, measured by egg-laying and locomotion. On the other hand, knocking out the calcium-sensing residues in the RCK1 and Ca^{2+} bowl resulted in a "crooked neck" posture reminiscent of *slo-1(null)* animals. Overall, these results suggest that although knocking out the calcium-sensing residues located in the RCK1 and Ca^{2+} bowl severely compromise BK channel function, it still strongly responds to ethanol. This is in sharp contrast to Liu et al. (2008), who found the mammalian channel no longer responds to ethanol after knocking out the calcium-sensing residues in the RCK1 and Ca^{2+} bowl. Overall, the calcium-sensing residues on the worm BK channel are not critical for behavioral intoxication in the worm. A few different explanations can be proposed for the contrast in the requirement for functional calcium-sensing residues on the worm and

mammalian BK channel in order to responds to ethanol: 1) the human BK channel enters a state where ethanol can drive potentiation only when calcium is bound, 2) there are differences in the ethanol/calcium activation pathways or, 3) the ethanol-binding pocket on the human BK channel is occluded when calcium is not bound to the channel.

5.4 - Genetic Screening with the Million Mutation Project

The relative simplicity, small size, hermaphroditic reproduction, fecundity, transparent body and short life cycle of *C. elegans* has been exploited with forward genetic screens to elucidate the genetic basis for a vast array of phenotypes. This includes uncovering the genetic basis for many compounds that affect the nervous system. The function of these screens is to identify mutants that are either hypersensitive or resistant to a compound to elucidate a pathway of action. For example, Ranganathan et al. (2001) discovered that mutations in the serotonin reuptake transporter (SERT) results in *C. elegans* that is resistant to fluoxetine. RNAi screens have also been a powerful tool to systematically screen for genes critical for a behavioral response of *C. elegans* to a compound. Traditional forward genetic screens and RNAi screens are limited to elucidating genes involved in a response to a compound. These loss-of-function screens are not designed to uncover the molecular interaction between a compound and a gene.

At the beginning of my doctoral research, I targeted *slo-1* using a non-complementation screen to uncover specific residues important for the response of the BK channel to ethanol that were not critical for channel function. In theory, there are 1000 or more mutations that will disrupt BK channel function rather than confer a

missense mutation that preserves function but selectively eliminates ethanol action; so many mutants were expected to be uncovered that would be *slo-1(null)* variants. This is especially true if only a few residues confer resistance to similar levels as *slo-1(null)* mutants. Although my screen uncovered many mutants, all ended up to be putative nulls. Thus, this screen failed in providing any novel information into the molecular interaction between the BK channel and ethanol. Next, I attempted an error prone genetic screen with the intention to generate mutations in *slo-1* cDNA, and then obtain transgenic strains that expressed *slo-1* cDNA with the native *slo-1* promoter. Unfortunately, the error-prone technique generated multiple errors in every cDNA fragment, which resulted in the probability that the vast majority *slo-1* cDNA fragments would result in a BK channel with compromised basal activity.

My attention then turned to the MMP to genetically probe the molecular interaction between the BK channel and ethanol. By doing this, we discovered that a sole conserved residue (T381/352I) is critical for ethanol to modulate BK channel activity and behavioral intoxication in *C. elegans* without being critical for basal BK channel function. Our results suggest that if a gene target is known strains obtained through the MMP project can be used to rapidly identify residues that are critical for a select phenotype (resistance to intoxication in our case), without being critical for overall basal function of the molecule (as inferred by normal posture, egg laying and aldicarb sensitivity in our case). This may prove especially useful to elucidate the residues critical for *in vivo* action of a drug on a conserved molecular target. As long as the drug elicits a

response in the worm, this approach could be used successfully irrespective of how difficult it is to probe the *in vitro* action of the molecule on the target.

In addition, problems that arise when a researcher starts a new non-complementation screen are solved by using the MMP strains. First, using the strains from the MMP will allow for an unlimited number of *C. elegans* with a particular mutation to screen, where a gradient of compound could be used to assess sensitivity of each mutant strain. Of course, in this example the compound screen would need two or more phenotypes to test. One phenotype is needed to test basal function and another to test drug response of the target molecule. We would encourage other researchers to use the MMP project to probe the molecular interaction of a gene and compound. In addition, MMP strains that are likely null could be very useful in place of or in addition to RNAi screens. This is especially true if the RNAi screen is dependent heavily on nervous system function, since RNAi expression in the nervous system is generally poor. The MMP is essentially a genetic screen that is partially completed. All mutations for each strain have already been identified and mapped, waiting for a phenotypic screening to elucidate the importance of particular mutations.

5.5 - General Conclusions and Future Directions

The nematode *C. elegans* is particularly attractive to researchers because of its genetic tractability. The genes and neurotransmitter systems are well conserved compared to humans. The low cost, small size and short life cycle are routinely exploited to effectively uncover the genetic basis for a variety of behavioral and other phenotypes.

My first project in this dissertation has further defended the use of *C. elegans* as a model system to study clinically relevant doses of intoxication by ethanol. This work provided a simple explanation for differences between Mitchell et al. (2007) and previous findings of other worm researchers. In addition, collaborating with the Betttinger and Davies labs resulted in a new method to accurately measure internal ethanol concentration in the worm. However, there still is contention among certain groups pertaining to ethanol permeating the cuticle. To this end, *C. elegans* researchers must continue to correlate findings of changes in the behavioral responses to ethanol with physiological responses when possible. For example, I demonstrated that expressing the wild-type BK channel in *slo-1(null)* worms rescued intoxication as measured by egg-laying and locomotion, while expressing the human T352I BK channel in *slo-1(null)* worms failed to rescue intoxication when *C. elegans* were exposed to a 400-600 mM exogenous ethanol concentration. Further work using single-channel recordings provided a strong correlation with the behavioral experiments using 50 mM ethanol. At this clinically relevant concentration, the wild-type, but not T352I mutant BK channel was potentiated by ethanol. Combining the behavioral experiments with physiological experiments using clinically relevant ethanol concentrations lends credence to the theory that only a small amount of ethanol permeates the cuticle of *C. elegans*. In addition, the controversy surrounding how much ethanol enters the worm becomes inconsequential. The end result is the same either way; I have provided information into a conserved residue (T381/352) that prevents clinically relevant concentrations of ethanol to potentiate the BK channel, while retaining basal channel function.

An important future direction of this work is to continue exploring the molecular interaction between the BK channel and ethanol. Although we have demonstrated a complete break of ethanol-signal in the T381/352I mutants, we cannot definitively conclude all aspects of basal channel function remains unchanged in all contexts. To continue to study this issue, a thorough analysis of T381/352I BK channel function across known calcium-concentrations and voltages, and in different cell types should be done to assess any minor changes to basal function.

It remains to be seen whether the T381/352 residue is part of an ethanol binding pocket or simply transduces the ethanol-signal after binding. To determine the possibility of T381/352 being a critical residue of the ethanol-binding pocket and/or ethanol-signal transduction, T381/352 could be mutated to the other 19 amino acids. If the molecular volume of T381/352 correlates with the ethanol-signal, evidence for a binding pocket would emerge. In addition, these experiments can be combined by testing n-alcohols of varying sizes to determine what the cutoff is in the wild-type channel, and test if this cutoff changes as a result of mutating T381/352 to amino acids of different sizes. Next, use of the substituted cysteine accessibility method (SCAM) in combination with the anesthetic-like reagent propyl methanethiosulfonate (PMTS) can test if T381/352C produces an irreversible alcohol-like potentiation, and if permanent occupancy of T381/352C by PMTS prevents certain n-alcohols from modulated the channel. These results could provide further evidence for T381/352 either being part of an ethanol-binding pocket or being necessary for ethanol-signal transduction.

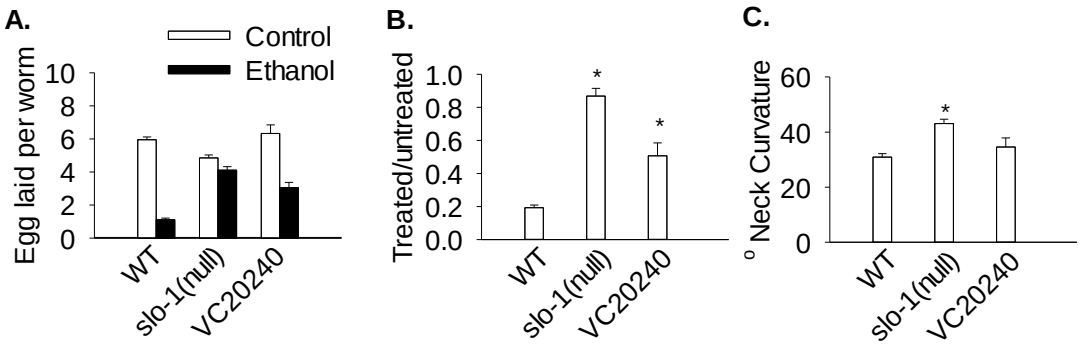
In most cases when mutant proteins are identified that lead to resistance at the single-channel level *in vitro,* the mutation cannot knocked into an animal model due to basal changes in channel/receptor function. These changes are often lethal, or at least cause severe changes to basal behaviors that complicate drawing any conclusions from potential differences in ethanol behaviors displayed by these knock-in replacement animals. In this case, however, the T352I mutation caused minimal changes to basal channel function *in vitro* and *in vivo,* and resulted in minimal changes in basal worm behavior off ethanol, while providing extreme resistance to intoxication. Recently, another residue, K361, has been identified that is critical for the effects of ethanol on the BK channel, without being critical for channel function *in vitro* (Bukiya et al., 2014). Although this residue does in fact seem critical, there is no *in vivo* data that demonstrate this channel will function properly *in vivo*. Perhaps the K361 mutation should be made in the human BK channel and knocked-in to *C. elegans* to assess function in a similar manner as we did with the T381/352 residue. However, because we have demonstrated that the T352 residue produces a functional BK channel *in vivo,* it would seem prudent to choose this mutation for a knock-in mammalian model study. A critical future direction of this work is to obtain a knock-in mouse with the T352I mutation in place of the native BK channel. From our analysis, it is likely that changes to basal behavior will not be severely altered. If this is the case, the relative importance of the BK channel for various alcohol-induced behaviors may finally be elucidated in the knock-in mouse.

Although the VC40372 strain, which expressed the T381I BK channel demonstrated the strongest level of resistance to ethanol of all MMP strains tested, the

strain VC20240, which harbors an allele that results in a G1036D substitution also displayed moderate resistance to the inhibitory effects of ethanol on egg laying (Figure 5.1). An important future direction would be to test if this mutant is also resistant to the inhibitory effects of ethanol on locomotion. Additional tests should also be run on VC20240 for neck posture, because preliminary results suggest a trend that it is significantly greater than wild-type worms, which indicates at least a partially broken BK channel (Figure 5.1). Currently, it remains to be determined whether the resistance in this strain is due to a BK channel that is not fully functional and/or the G1036D BK channel is not as sensitive to ethanol as the wild-type BK channel.

Figure 5.1 - VC20240 is moderately resistant to ethanol while retaining wild-type neck posture.

A) Egg-laying on and off ethanol in N2 wild-type (*N* = 43), NM1968 *slo-1(null)* (*N* = 32) and VC20240 (*N* = 6) **B)** There is a significantly smaller reduction in egg-laying on ethanol plates for VC20240 and *slo-1(null)* compared to wild-type worms. *N* represents a single assay with 10 worms **C)** VC20240 (n = 13) neck-posture is not significantly different than wild-type worms (n = 36), while *slo-1(null)* (n = 50) worms demonstrate a significantly greater neck curvature than wild-type worms. * represents $p < .05$.



In contrast to the mammalian channel, ethanol modulation of the worm BK channel without functional calcium-sensing residues was perhaps the most surprising result I obtained throughout this dissertation. Neutralizing the charged calcium-sensing residues compromised the worm BK channel function, measured by neck and body posture. However, the worm channel appeared to remain sensitive to ethanol, measured by egg-laying and locomotion in the presence of ethanol. Further research using single-channel recordings must be done to test the effects of mutating calcium-sensitive residues on the worm BK channel, both in the absence and presence of alcohol. These recordings may uncover if the difference of ethanol-response demonstrated by the human and worm BK channel is due to channel state in the absence of calcium, ethanol/calcium-gating, ethanol binding and/or transduction of the ethanol signal. In addition, differences in residues nearby the calcium-sensing residues in RCK1 between the worm and mammalian channel could be exploited with future site-directed mutation studies. For example, the two highly dissimilar residues in this immediate region could be mutated to their homologue counterpart. This may determine if these residues are critical for ethanol modulation of the channel being dependent on the presence of calcium.

The results of this dissertation have provided further credence for the use of *C. elegans* as a model system to study ethanol intoxication. I also elucidated a missense mutation on the worm (T381I) and human BK (T352I) channel that confers resistance to the behavioral effects of ethanol in *C. elegans.* The T352I BK channel also results in a functional channel completely resistant to ethanol. This demonstrates both conservation in how ethanol modulates the BK channel across species, and provides the first step in

delineating how ethanol interacts with specific residues on the BK channel. I also uncovered a species specific difference in the requirement of calcium for ethanol modulation of the BK channel. These results provide a framework to study how non-conserved residues may mediate the need for calcium to be present in order for ethanol to modulate the BK channel. This research would provide further information into the molecular interaction between ethanol and the BK channel. Overall, I hope that this work propels further research to fully describe the molecular interaction between the BK channel and ethanol. After this occurs, the potential for the development of pharmacotherapies and the identification of SNPs on the BK channel gene that confer susceptibility to alcohol use disorders increases dramatically. In addition, fully describing this molecular interaction may also provide insight into how ethanol interacts with other proteins that contribute to intoxication, tolerance and addiction.

References

Alaimo, J. T., Davis, S. J., Song, S. S., Burnette, C. R., Grotewiel, M., Shelton, K. L., Pierce-Shimomura, J. T., Davies, A. G., & Bettinger, J. C. (2012). Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*. *Alcohol Clin Exp Res*, **36**, 1840–50.

Almassy, J., & Begenisich, T. (2012). The LRRC26 protein selectively alters the efficacy of BK channel activators. *Mol Pharmacol*, **81**, 21–30.

Aryal, P., Dvir, H., Choe, S., & Slesinger, P. A. (2009). A discrete alcohol pocket involved in GIRK channel activation. *Nat Neurosci*, **12**, 988–95.

Atkinson, N. S., Robertson, G. a, & Ganetzky, B. (1991). A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science*, **253**, 551–5.

Avery, L., Raizen, D., & Lockery, S. (1995) Electrophysiological methods. *Methods Cell Biol*, **48**, 251–269.

Barrett, J. N., Magleby, K. L., & Pallotta, B. S. (1982). Properties of single channel-activated potassium channels in cultured rat muscle. *J Physiol*, **331**, 211–30.

Behrens, R., Nolting, A., Reimann, F. Y., Schwarz, M., Waldschu, R., & Pongs, O. (2000). hKCNMB3 and hkCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel L subunit family. *FEBS Lett*, **474**, 99–106.

Belelli, D. Muntoni, A. L., Merreywest, S. D., Gentet, L. J., Casula, A., Callachan, H., Madau, P., Hamilton, N. M., Lambert, J. J., Sillar, K. T., & Peters, J. A. (2003). The *in vitro* and *in vivo* enantioselectivity of etomidate implicates the $GABA_A$ receptor in general anaesthesia. *Neuropharmacology*, **45**, 57–71.

Blednov, Y. A., Borghese, C. M., McCracken, M. L., Benavidez, J. M., Geil, C. R., Osterndorff-Kahanek, E., Werner, D. F., Iyer, S., Swihart, A., Harrison, N. L., Homanics, G. E., & Harris, R. A. (2001). Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol insensitive α2-containing $GABA_A$ receptors. *J Pharmacol Exp Ther*, **336**,145-54.

Benedikt, J., Teisinger, J., Vyklicky, L., & Vlachova, V. (2007). Ethanol inhibits cold-menthol receptor TRPM8 by modulating its interaction with membrane phosphatidylinositol 4,5-bisphosphate. *Jf Neurochem*, **100**, 211–24.

Bettinger, J. C., Leung, K., Bolling, M. H., Goldsmith, A. D., & Davies, A. G. (2012). Lipid environment modulates the development of acute tolerance to ethanol in *Caenorhabditis elegans. PloS One*, **7**, e35192. doi:10.1371/journal.pone.0035192.

Bhandari, P., Hill, J. S., Farris, S. P., Costin, B., Martin, I., Chan, C. L., Alaimo, J. T., Bettinger, J. C., Davies, A. G., Miles, M. F., & Grotewiel, M. (2012). Chloride Intracellular Channels modulate acute ethanol behaviors in *Drosophila*, *C. elegans* and mice. *Genes Brain Behav*, **11**, 387-97.

Bian, S., Favre, I., & Moczydlowski, E. (2001). Ca^{2+}-binding activity of a COOH-terminal fragment of the *Drosophila* BK channel involved in Ca^{2+}-dependent activation. *Proc Natl Acad Sci U S A.*, **98**, 4776–81.

Bison, S., & Crews, F. (2003). Alcohol withdrawal increases neuropeptide Y immunoreactivity in rat brain. *Alcohol Clin Exp Res*, **27**, 1173–83.

Blednov, Y. A., Borghese, C. M., Mccracken, M. L., Benavidez, J. M., Geil, C. R., Werner, D. F., Iyer, S., Swihart, A., Harrison, N. L., Homanics, G. E., & Harris, R. A. (2008). Loss of ethanol conditioned taste aversion and motor stimulation in knockin mice with ethanol-insensitive α2-containing GABA(A) receptors. *J Pharmacol Exp Ther,* **336**, 145-54.

Bouchery, E. E., Harwood, H. J., Sacks, J. J., Simon, C. J., & Brewer R. D. (2006). Economic costs of excessive alcohol consumption in the U.S., 2006. *Am J Prev Med,* **41**, 516-24.

Braun, A. P., & Sy, L. (2001). Contribution of potential EF hand motifs to the calcium-dependent gating of a mouse brain large conductance, calcium-sensitive $K(^{+})$ channel. *J Physiol*, **533**, 681–95.

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L., & Aldrich, R. W. (2005). BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci*, **8**, 1752–9.

Brenner, R., Peréz, G. J., Bonev, a D., Eckman, D. M., Kosek, J. C., Wiler, S. W., Patterson, A J., Nelson, M. T., & Aldrich, R. W. (2000). Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature*, **407**, 870–6.

Brenner, S. (1974). The Genetics of *Caenorhabditis elegans*. *Genetics*, **77**, 71–94.
Brodie, M. S., Scholz, A., Weiger, T. M., & Dopico, A. M. (2007). Ethanol interactions with calcium-dependent potassium channels. *Alcohol Clin Exp Res*, **31**, 1625–32.

Bukiya, A., Kuntamallappanavar, G., Edwards, J., Singh, A. K., Shivakumar, B., & Dopico, A. M. (2014) An alcohol-sensing site in the calcium- and voltage- gated, large conductance potassium (BK) channel. *Proc Natl Acad Sci U S A.*, doi:10.1073/pnas.1317363111

Bukiya, A., Dopico, A. M., Leffler, C. W., & Fedinec, A. (2014). Dietary cholesterol protects against alcohol-induced cerebral artery constriction. *Alcohol Clin Exp Res*, **38**, 1216-20.

Bukiya, A., Liu, J., & Dopico, A. (2009). The BK channel accessory β 1 subunit determines alcohol-induced cerebrovascular constriction. *FEBS Lett*, **583**, 2779–84.

Burns, A. R., Wallace, I. M., Wildenhain, J., Tyers, M., Giaever, G., Bader, G. D., Nislow, C., Cutler, S. R., & Roy, P. J. (2010). A predictive model for drug bioaccumulation and bioactivity in *Caenorhabditis elegans*. *Nat Chem Biol*, **6**, 549–57.

Butler, A., Tsunoda, S., Mccobb, D. P., Wei, A., & Salkoff, L. (1993). *mSlo* , a Complex Mouse Gene Encoding " Maxi " Potassium Channels Calcium-Activated, *Science*, **261**, 221–24.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S., & Snyder, M. (2010). Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing. *PLoS Pathogens*, **6**, e1000834. doi:10.1371/journal.ppat.1000834.

Carre-Pierrat, M., Grisoni, K., Gieseler, K., Mariol, M. C., Martin, E., Jospin, M., Allard, B., & Ségalat, L. (2006). The SLO-1 BK channel of *Caenorhabditis elegans* is critical for muscle function and is involved in dystrophin-dependent muscle dystrophy. *J Mol Biol*, **358**, 387–95.

Chase, D. L., Pepper, J. S., Koelle, M. R. (2004) Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. *Nat Neurosci*, **7**,1096-1103.

Chazotte, B., & Vanderkooi, G. (1981). Multiple Sites of Inhibition of Mitochondrial electron transport by local anesthetics. *Biochim Biophys Acta*, **636**, 153–61.

Chen, B., Ge, Q., Xia, X., Liu, P., Wang, S. J., Zhan, H., Betty, A., & Wang, Z. (2010). A novel auxiliary subunit critical to BK channel function in *C. elegans*. *J Neurosci*, **30**, 16651–61.

Chen, B., Liu, P., Zhan, H., Wang, Z. W. (2011) Dystrobrevin controls neurotransmitter release and muscle Ca^{2+} transients by localizing BK channels in *Caenorhabditis elegans*. *J Neurosci*, **31**, 17338-47.

Chen, L., Tian, L., MacDonald, S. H., McClafferty, H., Hammond, M. S., Huibant, J. M., Ruth, P., Knaus, H. G., & Shipston, M. J. (2005). Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) alpha-subunits generated from a single site of splicing. *J Biol Chem*, **280**, 33599–609.

Chen, S. Y., Yang B., Jacobson, K., & Sulik, K. K. (1996). The membrane disordering effect of ethanol on neural crest cells in vitro and the protective role of GM1 ganglioside. *Alcohol,* **13**, 589-95.

Chin, J. H., & Goldstein, D. B. (1977). Effects of low concentration of ethanol on the fluidity of spin-labeled erythrocyte and brain membranes. *Mol Pharmacol*, **13**, 435–441.

Chu, B., Dopico, A. M., Lemos, J. R., & Treistman, S. N. (1998). Ethanol potentiation of calcium-activated potassium channels reconstituted into planar lipid bilayers. *Mol Pharmacol,* **54**, 397–406.

Chu, B., Triestman, S. N. (1997). Modulation of two cloned potassium channels by 1-alkanols demonstrates different cutoffs. *Alcohol Clin Exp Res,* **21**,1103-7.

Chunbo, Y., O'Connell, R. J., Wilson, A., Piestrzykowski, A. Z., Triestman, S. N. (2007) Acute alcohol tolerance is intrinsic to the BK_{Ca} protein, but is modulated by the lipid environment. *J Biol Chem* **283**, 5090-8.

Contreras, G. F., Castillo, K., Enrique, N., Carrasquel-ursulaez, W., Castillo, J. P., Milesi, V., Neely, A., Alvarez, O., Ferreira, G., Gonzalez, C., & Latorre, R. (2013). A BK (Slo1) channel journey from molecule to physiology, *Channels(Austin),* **7**, 442–58.

Cowmeadow, R. B., Krishnan, H. R., & Atkinson, N. S. (2005). The *slowpoke* gene is necessary for Rapid Ethanol Tolerance in *Drosophila*. *Alcohol Clin Exp Res*, **29**, 1777–86.

Cowmeadow, R. B., Krishnan, H. R., Ghezzi, A., Al'Hasan, Y. M., Wang, Y. Z., & Atkinson, N. S. (2006). Ethanol tolerance caused by *slowpoke* induction in *Drosophila*. *Alcohol Clin Exp Res*, **30**, 745–53.

Cox, D. H., Cui, J., & Aldrich, R. W. (1997). Allosteric gating of a large conductance Ca-activated K^+ channel. *J Gen Physiol*, **110**, 257–81.

Cox, G. N., Kusch, M., & Edgar, R. S. (1981). Cuticle of *Caenorhabditis elegans*: its isolation and partial characterization. *J Cell Biol*, **90**, 7–17.

Crisford, A., Murray, C., Connor, V. O., Edwards, R. J., Kruger, N., Welz, C., Samson-himmelstjerna, G. Von, Harder, A., Walker, R. J., & Holden-dye, L. (2011). Selective

Toxicity of the Anthelmintic Emodepside Revealed by Heterologous Expression of Human KCNMA1 in *Caenorhabditis elegans*, *Mol Pharmacol,* **79**, 1031–43.

Crowley, J. J., Treistman, S. N., & Dopico, A. M. (2003). Cholesterol antagonizes ethanol potentiation of human brain BKCa channels reconstituted into phospholipid bilayers. *Mol Pharmacol*, **64**, 365–72.

Crowley, J. J., Treistman, S. N., & Dopico, A. M. (2005). Distinct Structural Features of Phospholipids Differentially Determine Ethanol Sensitivity and Basal Function of BK Channels, *Mol Pharmacol*, **68**, 4–10.

Cui, J., Yang, H., & Lee, U. S. (2009). Molecular mechanisms of BK channel activation. *Cell Mol Life Sci*, **66**, 852–75.

Davies, A. G., Bettinger, J. C., Thiele, T. R., Judy, M. E., & McIntire, S. L. (2004). Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C. elegans*. *Neuron*, **42**, 731–43.

Davies, A. G., Pierce-Shimomura, J. T., Kim, H., VanHoven, M. K., Thiele, T. R., Bonci, A., Bargmann, C. I., & McIntire, S. L. (2003). A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell*, **115**, 655–66.

Diaz, L., Meera, P., Amigo, J., Stefani, E., Alvarez, O., Toro, L., & Latorre, R. (1998). Role of the S4 segment in a voltage-dependent calcium-sensitive potassium (h*Slo*) channel. *J Biol Chem*, **273**, 32430–36.

Dillon, J., Andrianakis, I., Mould, R., Ient, B., Liu, W., James, C., O'Connor, V., & Holden-Dye, L. (2013). Distinct molecular targets including SLO-1 and gap junctions are engaged across a continuum of ethanol concentrations in *Caenorhabditis elegans*. *FASEB J,* **27**, 4266-78.

Dinkel H, Van Roey K, Michael S, Davey NE, Weatheritt RJ, Born D, Speck T, Krüger D, Grebnev G, Kuban M, Strumillo M, Uyar B, Budd A, Altenberg B, Seiler M, Chemes LB, Glavina J, Sánchez IE, Diella F, Gibson TJ (2014) The eukaryotic linear motif resource ELM: 10 years and counting. *Nucleic Acids Res*, **42**, D259-D266.

Dopico, A. M., Anantharam, V., & Treistman, S. N. (1998). Ethanol increases the activity of Ca^{++}-dependent K^{+} (*mslo*) channels: functional interaction with cytosolic Ca^{++}. *J Pharmacol Exp Ther,* **284**, 258–68.

Dopico, A. M., Lemos, J. R., & Treistman, S. N. (1996). Ethanol increases the activity of large conductance, Ca^{2+}-activated K^{+} channels in isolated neurohypophysial terminals. *Mol Pharmacol*, **49**, 40–8.

Dopico, A. M. (2003). Ethanol sensitivity of BK_{Ca} channels from arterial smooth muscle does not require the presence of the β1-subunit. *Am J Physiol Cell Physiol*, **284**, 1468–80.
DSM-V. (2000). Diagnostic and Statistical Manual of Mental Disorders: Dsm-V (Washington, DC: American Psychiatric Association).

Eyring, H., Woodbury, J. W., and D'Arrigo, J. S. (1973). A molecular mechanism of general anesthesia. *Anesthesiology***, 38**, 415–424.

Fang, Z., Ionescu, P., Chortkoff, B. S., Kandel, L., Sonner, J., Laster, M. J., & Eger, E. I. (1997). Anesthetic potencies of n-alkanols: results of additivity and solubility studies suggest a mechanism of action similar to that for conventional inhaled anesthetics. *Anesth Analg*, **84**, 1042–8.

Feinberg-Zadek, P. L., Martin, G., & Treistman, S. N. (2008). BK channel subunit composition modulates molecular tolerance to ethanol. *Alcohol Clin Exp Res*, **32**, 1207–16.

Feinberg-Zadek, P. L., & Treistman, S. N. (2007). Beta-subunits are important modulators of the acute response to alcohol in human BK channels. *Alcohol Clin Exp Res*, **31**, 737–44.

Findlay, G. S., Phelan, R., Roberts, M. T., Homanics, G. E., Bergeson, S. E., Lopreato, G. F., Mihic, S. J., Blednov, Y. A., & Harris, R. A. (2003). Glycine receptor knock-in mice and hyperekplexia-like phenotypes: comparisons with the null mutant. *J Neurosci*, **23**, 8051–9.

Fodor, A. A., & Aldrich, R. W. (2009). Convergent evolution of alternative splices at domain boundaries of the BK channel. *Annu Rev Physiol*, **71**, 19–36.

Franck, J., & Jayaram-Lindström, N. (2013). Pharmacotherapy for alcohol dependence: status of current treatments. *Curr Opin Neurobiol*, **23**, 692-9.

Franks, N. P., & Lieb, W. R. (1986). Partitioning of long-chain alcohols into lipid bilayers: implications for mechanisms of general anesthesia. *PNAS*, **83**, 5116–20.

Frischknecht, A. L., & Frink, L. J. (2006). Alcohols reduce lateral membrane pressures: predictions from molecular theory. *Biophys J*, **91**, 4081–90.

Garcia-Cabrera, I., & Berge, O. G. (1988). Pressure reversal of the depressant effect of ethanol on spontaneous behavior in rats. *Pharmacol Biochem Behav*, **29**, 133–41.

Glasner, J., Kocher, T., & Collins, J. (1995). *Caenorhabditis elegans* contains genes encoding two new members of the Zn-containing alcohol dehydrogenase family. *J Mol Evol*, **41**, 46–53.

Glauser, D. A., Johnson, B. E., Aldrich, R. W., & Goodman, M. B. (2011). Intragenic alternative splicing coordination is essential for *Caenorhabditis elegans slo-1* gene function. *Proc Natl Acad Sci U S A.*, **108**, 20790–5.

Godden, E. L., Harris, R. A, & Dunwiddie, T. V. (2001). Correlation between molecular volume and effects of *n*-alcohols on human neuronal nicotinic acetylcholine receptors expressed in *Xenopus oocytes*. *J Pharmacol Exp Ther*, **296**, 716–22.

Golding, N. L., Jung, H. Y., Mickus, T., Spruston, N. (1999). Dendritic calcium spike initiation and repolarization are controlled by distinct potassium channel subtypes in CA1 pyramidal neurons. *J Neurosci*, **19**, 8789–98.

Graham, M. E., Edwards, M. R., Holden-dye, L., Morgan, A., Burgoyne, R. D., & Barclay, J. W. (2009). UNC-18 Modulates Ethanol Sensitivity in *Caenorhabditis elegans*. *Mol Biol Cell*, **20**, 43–55.

Grant, B. F., Stinson, F. S., Dawson, D. A., Chou, S. P., Dufour, M. C., Compton, W., Pickering, R. P., & Kaplan K. (2004). Prevalence and co-occurrence of substance use disorders and independent mood and anxiety disorders: Results from the National Epidemiologic Survey on Alcohol and Related Conditions. *Arch of Gen Psychiatry*, **61**, 807-16.

Gu, N., Vervaeke, K., & Storm, J. F. (2007). BK potassium channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. *J Physiol*, **580**, 859–82.

Gullingsrud, J., & Schulten, K. (2004). Lipid bilayer pressure profiles and mechanosensitive channel gating. *Biophys J*, **86**, 3496–509.

Guest, M., Bull, K., Walker, R. J., Amliwala, K., O'Connor, V., Harder, A., Holden-Dye, L., & Hopper, N. A. (2007). The calcium-activated potassium channel, SLO-1, is required for the action of the novel cyclo-octadepsipeptide anthelmintic, emodepside, in *Caenorhabditis elegans*. *Int J Parasitol*, **37**, 1577–88.

Hammarlund, M., Jorgensen, E. M., Bastiani, M. J. (2007). Axons break in animals lacking beta-spectrin. *J Cell Biol*, **176**, 269-275.

Harris, R. A., Osterndorff-Kahanek, E., Ponomarev, I., Homanics, G. E., & Blednov, Y. A. (2011). Testing the silence of mutations: Transcriptomic and behavioral studies of GABAA receptor α1 and α2 subunit knock-in mice. *Neurosci Lett.*, **488**, 31–35.

Harris, R. A., Trudell, J. R., & Mihic, S. J. (2009). Ethanol's molecular targets. *Sci Signaling*, **1**, re7. doi:10.1126/scisignal.128re7.
Hille, B. 2001. Ion Channels of Excitable Membranes, 3rd edition. Sinauer Associates, Inc., Sunderland, M.A., Hong, M., Choi, M. K., & Lee, J. (2008). The anesthetic action of ethanol analyzed by genetics in *Caenorhabditis elegans*. *Biochem Biophys Res Commun*, **367**, 219–25.

Horrigan, F. T., & Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels II. *mslo* channel gating charge movement in the absence of Ca^{2+}. *J Gen Physiol*, **114**, 305–36.

Horrigan, F. T., & Aldrich, R. W. (2002). Coupling between Voltage Sensor Activation, Ca^{2+} Binding and Channel Opening in Large Conductance (BK) Potassium Channels. *J Gen Physiol*, **120**, 267–305.

Horrigan, F. T., Cui, J., & Aldrich, R. W. (1999). Allosteric voltage gating of potassium channels I. *mslo* ionic currents in the absence of Ca^{2+}. *J Gen Physiol*, **114**, 277–304.

Hoshi, T., Pantazis, A, & Olcese, R. (2013). Transduction of voltage and Ca^{2+} signals by Slo1 BK channels. *Physiology (Bethesda.)*, **28**, 172–89.

Howard, R. J., Trudell, J. R., Harris, R. A. (2014). Seeking structural specificity: direct modulation of pentameric ligand-gated ion channels by alcohols and general anesthetics. *Pharmacol Rev* **66**, 396-412.

Hu, H., Shao, L. R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., Laake, P., Pongs, O., Knaus, H. G., Ottersen, O. P., & Storm, J. F. (2001). Presynaptic Ca^{2+}-activated K^+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release. *J Neurosci*, **21**, 9585–97.

Huang, H. D., Lee, T. Y., Tzeng, S. W., Horng, J. T. (2005). KinasePhos: a web tool for identifying protein kinase-specific phosphorylation sites. *Nucleic Acids Res*, **33**, W226-9.

Jakab, M., Schmidt, S., Grundbichler, M., Paulmichl, M., Hermann, A., Weiger, T., & Ritter, M. (2006). Hypotonicity and ethanol modulate BK channel activity and chloride currents in GH4/C1 pituitary tumour cells. *Acta Physiol (Oxf)*, **187**, 51–9.

Jakab, M., Weiger, T. M., & Hermann, A. (1997). Ethanol activates Maxi Ca^{2+}- activated K^+ Channels of Clonal Pituitary (GH3) Cells. *Nature*, **245**, 237–245.

Jee, C., Lee, J., Lim, J. P., Parry, D., Messing, R. O., & McIntire, S. L. (2013). SEB-3, a CRF receptor-like GPCR, regulates locomotor activity states, stress responses and ethanol tolerance in *Caenorhabditis elegans*. *Genes Brain Behav*, **12**, 250–62.

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T., & MacKinnon, R. (2002). Crystal structure and mechanism of a calcium-gated potassium channel. *Nature*, **417**, 515–22.

Johnson, B. E., Glauser, D. A., Dan-glauser, E. S., Halling, D. B., Aldrich, R. W., & Goodman, M. B. (2011). Alternatively spliced domains interact to regulate BK potassium channel gating. *Proc Natl Acad Sci U S A.*, **108**, 20784-9.

Jospin, M., Mariol, M. C., Segalat, L., & Allard, B. (2002). Characterization of K^+ currents using an in situ patch clamp technique in body wall muscle cells from *Caenorhabditis elegans*. *J Physiol*, **544**, 373–84.

Kapfhamer, D., Bettinger, J. C., Davies, A. G., Eastman, C. L., Smail, E. A., Heberlein, U., & McIntire, S. L. (2008). Loss of RAB-3/A in *Caenorhabditis elegans* and the mouse affects behavioral response to ethanol. *Genes Brain Behav*, **7**, 669–76.

Kauhanen, J., Karvonen, M. K., Pesonen, U., Koulu, M., Tuomainen, T. P., Uusitupa, M. I., Salonen, J. T. (2000). Neuropeptide Y polymorphism and alcohol consumption in middle-aged men. *Am J Med Genet*, **93**, 117-21.

Kayser, E. B., Hoppel, C. L., Morgan, P. G., & Sedensky, M. M. (2003). A mutation in mitochondrial complex I increases ethanol sensitivity in *Caenorhabditis elegans*. *Alcohol Clin Exp Res*, **27**, 584–92.

Kim, H., Pierce-Shimomura, J. T., Oh, H. J., Johnson, B. E., Goodman, M. B., & McIntire, S. L. (2009). The dystrophin complex controls BK channel localization and muscle activity in *Caenorhabditis elegans*. *PLoS Genetics*, **5**, e1000780. doi:10.1371/journal.pgen.1000780

Knott, T. K., Dayanithi, G., Coccia, V., Custer, E. E., Lemos, J. R., & Treistman, S. N. (2000). Tolerance to acute ethanol inhibition of peptide hormone release in the isolated neurohypophysis. *Alcohol Clin Exp Res*, **24**, 1077–83.

Knott, T. K., Dopico, A. M., Dayanithi, G., Lemos, J., & Treistman, S. N. (2002). Integrated channel plasticity contributes to alcohol tolerance in neurohypophysial terminals. *Mol Pharmacol*, **62**, 135–42.

Komatsu H., & Okada, S. (1995). Increased permeability of phase-separated liposomal membranes with mixtures of ethanol-induced interdigitated and non-interdigitated structures. *Biochim Biophys Acta,* **1237**, 169-75.

Kreifeldt, M., Le, D., Treistman, S. N., Koob, G. F., & Contet, C. (2013). BK channel β1 and β4 auxiliary subunits exert opposite influences on escalated ethanol drinking in dependent mice. *Front Integr Neurosci*, **7**, 105. doi:10.3389/fnint.2013.00105

Kruse, S. W., Zhao, R., Smith, D. P., & Jones, D. N. (2003). Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila* melanogaster. *Nat Struct Biol*, **10**, 694–700.

Kwon, J. Y., Hong, M., Choi, M. S., Kang, S., Duke, K., Kim, S., Lee, S., & Lee, J. (2004). Ethanol-response genes and their regulation analyzed by a microarray and comparative genomic approach in the nematode *Caenorhabditis elegans*. *Genomics*, **83**, 600–14.

Lancaster, B., Nicoll, R. A. (1987). Properties of two calcium-activated hyperpolarizations in rat hippocampal neurones. *J. Physiol*, **389**, 187–203.

Lappalainen, J., Kranzler, H. R., Malison, R., Price, L. H., Van Dyck, C., Rosenheck, R. A., Cramer, J., Southwick, S., Charney, D., Krysta, J., & Gelernter, J. (2002). A functional neuropeptide Y Leu7Pro Polymorphism associated with alcohol dependence in a large population sample from the United States. *Arch Gen Psychiatry*, **59**, 825–31.

Lee, J., Jee, C., & McIntire, S. L. (2009). Ethanol preference in *C. elegans*. *Genes Brain Behav*, **8**, 578–85.

Lewohl, J. M., Wilson, W. R., Mayfield, R. D., Brozowski, S. J., Morrisett, R. A., & Harris, R. A. (1999). G-protein-coupled inwardly rectifying potassium channels are targets of alcohol action. *Nat Neurosci*, **2**, 1084–90.

Liu, J., Asuncion-Chin, M., Liu, P., & Dopico, A. M. (2006). CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels. *Nat Neurosci*, **9**, 41–9.

Liu, J., Vaithianathan, T., Manivannan, K., Parrill, A., & Dopico, A. M. (2008). Ethanol Modulates BK_{Ca} Channels by Acting as an Adjuvant of Calcium. *Mol Pharmacol*, **74**, 628–640.

Liu, P., Chen, B., & Wang, Z. W. (2013). Postsynaptic current bursts instruct action potential firing at a graded synapse. *Nat Commun*, **4**, doi:10.1038/ncomms2925

Liu, P., Liu, J., Huang, W., Li, M. D., & Dopico, A. M. (2003). Distinct regions of the *slo* subunit determine differential BK_{Ca} channel responses to ethanol. *Alcohol Clin Exp Res*, **27**, 1640–4.

Liu, P., Xi, Q., Ahmed, A., Jaggar, J. H., & Dopico, A. M. (2004). Essential role for smooth muscle BK channels in alcohol-induced cerebrovascular constriction. *Proc Natl Acad Sci U S A.*, **101**, 18217–22.

Liu, Q., Chen, B., Ge, Q., & Wang, Z. W. (2007). Presynaptic Ca^{2+}/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at *Caenorhabditis elegans* neuromuscular junction. *J Neurosci*, **27**, 10404–13.

Lobo, I. A., Mascia, M. P., Trudell, J. R., & Harris, R. A. (2004). Channel gating of the glycine receptor changes accessibility to residues implicated in receptor potentiation by alcohols and anesthetics. *J Biol Chem*, **279**, 33919–27.

Lovinger, D. M. (1999). 5-HT_3 receptors and the neural actions of alcohols□: an increasingly exciting topic, *Neurochem Int,* **35**, 125-30.

Lovinger, D. M., White, G., & Weight, F. F. (1989). Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science*, **243**, 1721–4.

Lyon, R. C., & Goldstein, D. B. (1983). Changes in synaptic membrane order associated with chronic ethanol treatment in mice. *Mol Pharmacol*, **23**, 86–91.

Ma, Z., Lou, X. J., & Horrigan, F. T. (2006). Role of Charged Residues in the S1 – S4 Voltage Sensor of BK Channels. *J Gen Physiol*, **127**, 309–328.

MacDonald, S. H., Ruth, P., Knaus, H. G., & Shipston, M. J. (2006). Increased large conductance calcium-activated potassium (BK) channel expression accompanied by STREX variant downregulation in the developing mouse CNS. *BMC Dev Biol*, **6**, 37. doi:10.1186/1471-213X-6-37

Mahoney, T. R., Luo, S., & Nonet, M. L. (2006). Analysis of synaptic transmission in *Caenorhabditis elegans* using an aldicarb-sensitivity assay. *Nat Protoc*, **1**, 1772–7.

Malysz, J., Afeli, S. a Y., Provence, A., & Petkov, G. V. (2014). Ethanol-mediated relaxation of guinea pig urinary bladder smooth muscle: involvement of BK and L-type Ca^{2+} channels. *Am J Physiol Cell Physiol*, **306**, C45–58.

Martin, G. E., Hendrickson, L. M., Penta, K. L., Friesen, R. M., Pietrzykowski, A. Z., Tapper, A. R., & Treistman, S. N. (2008). Identification of a BK channel auxiliary

protein controlling molecular and behavioral tolerance to alcohol. *Proc Natl Acad Sci U S A.*, **105**, 17543–8.

Martin, G., Puig, S., Pietrzykowski, A., Zadek, P., Emery, P., & Treistman, S. (2004). Somatic localization of a specific large-conductance calcium-activated potassium channel subtype controls compartmentalized ethanol sensitivity in the nucleus accumbens. *J Neurosci*□, **24**, 6563–72.

Martire, M., Barrese, V., D'Amico, M., Iannotti, F. A., Pizzarelli, R., Samengo, I., Viggiano, D., Ruth, P., Cherubini, E., & Taglialatela, M. (2010). Pre-synaptic BK channels selectively control glutamate versus GABA release from cortical and hippocampal nerve terminals. *J Neurochem*, **115**, 411–22.

Maruyama, Y., & Peterson, O. H. (1982). Single-channel currents in isolated patches of plasma membrane from basal surface of pancreatic acini. *Nature*, **299**, 159–161.

Mayfield, R. D., Lewohl, J. M., Dodd, P. R., Herlihy, A., Liu, J., & Harris, R. A. (2002). Patterns of gene expression are altered in the frontal and motor cortices of human alcoholics. *J Neurochem*, **81**, 802–13.

McCartney, C. E., McClafferty, H., Huibant, J. M., Rowan, E. G., Shipston, M. J., & Rowe, I. C. M. (2005). A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits. *Proc Natl Acad Sci U S A.*, **102**, 17870–6.

Meister K. A., Whelan, E. M., & Kava, R. (2000). The health effects of moderate alcohol intake in human: an epidemiologic review. *Crit Rev Clin Lab Sci*, **37**, 261-96.

Meredith A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T. and Aldrich, R. W. (2004). Overactive bladder and incontinence in the absence of the BK Ca^{2+} - activated K^+ channel. *J of Bio Chem*, **279**, 36746-52.

Meredith, A. L., Wiler, S. W., Miller**,** B. H., Takahashi**,** J. S**.**, Fodor, A. A. Ruby N. F., & Aldrich, R. W. (2006). BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nature Neuroscience*, **9**, 1042-49.

Mihic, S. J., Ye, Q., Wick, M. J., Koltchine, V. V, Krasowski, M. D., Finn, S. E., Mascia, M. P., Valenzuela, C. F., Hanson, K. K., Greenblatt, E. P., Harris, R. A, & Harrison, N. L. (1997). Sites of alcohol and volatile anaesthetic action on $GABA_A$ and glycine receptors. *Nature*, **389**, 385–9.

Miller, W. R., Walters, S. T., & Bennett, M. E. (2001). How effective is alcoholism treatment in the United States? *J Stud Alcohol*, **62**, 211-220.

Miranda, P., Contreras, J. E., Plested, A. J. R., Sigworth, F. J., Holmgren, M., & Giraldez, T. (2013). State-dependent FRET reports calcium- and voltage-dependent gating-ring motions in BK channels. *Proc Natl Acad Sci U S A.*, **110**, 5217–22.

Mirkowski, M. N. (2013). *Cognitive function in BK channel knock-out mice*. Western University. Retrieved from http://ir.lib.uwo.ca/cgi/viewcontent.cgi?article=2703&context=etd

Mitchell, D. C., & Litman, B. J. (2000). Effect of Ethanol and Osmotic Stress on Receptor Conformation. *J Biol Chem*, **275**, 5355–60.

Mitchell, P. H., Bull, K., Glautier, S., Hopper, N. A, Holden-Dye, L., & O'Connor, V. (2007). The concentration-dependent effects of ethanol on *Caenorhabditis elegans* behaviour. *Pharmacogenomics J*, **7**, 411–7.

Mitchell, P., Mould, R., Dillon, J., Glautier, S., Andrianakis, I., James, C., Pugh, A., Holden-Dye, L., & O'Connor, V. (2010). A differential role for neuropeptides in acute and chronic adaptive responses to alcohol: behavioural and genetic analysis in *Caenorhabditis elegans*. *PloS One*, **5**, e10422. doi:10.1371/journal.pone.0010422

Morgan, P. G., & Sedensky, M. M. (1995). Mutations Affecting Sensitivity to Ethanol in the Nematode, *Caenorhabditis elegans*. *Alcohol Clin Exp Res*, **19**, 1423–29.

Oberholtzer, J. C. (1999). Frequency tuning of cochlear hair cells by differential splicing of BK channel transcripts. *Journal of Physiology*, **518**, 629.

Pang, K,Y., Braswell, L. M., Chang, L., Sommer, T. J., & Miller, K. W. (1980). The Perturbation of Lipid Bilayers by General Anesthetic: A Quantitative Test of the Disordered Lipid Hypothesis. *Mol Pharmacol*, **18**, 84–90.

Pantazis, A., Kohanteb, A. P., & Olcese, R. (2010). Relative motion of transmembrane segments S0 and S4 during voltage sensor activation in the human BK_{Ca} channel. *J Gen Physiol*, **136**, 645–57.

Pantazis, A., & Olcese, R. (2012). Relative transmembrane segment rearrangements during BK channel activation resolved by structurally assigned fluorophore-quencher pairing. *J Gen Physiol*, **140**, 207–18.

Peoples, R. W., Li, C., & Weight F. F. (1996). Lipid vs protein theories of alcohol action in the nervous system. *Annu Rev Pharmacol Toxicol*, **36**, 185-201.

Petkov, G. V, Bonev, A. D., Heppner, T. J., Brenner, R., Aldrich, R. W., & Nelson, M. T. (2001). β1-subunit of the Ca^{2+}-activated K^+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J Physiol*, **537**, 443–52.

Pietrzykowski, A. Z., Friesen, R. M., Martin, G. E., Puig, S. I., Nowak, C. L., Wynne, P. M., Siegelmann, H. T., & Treistman, S. N. (2008). Posttranscriptional regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. *Neuron*, **59**, 274–87.

Pierce-Shimomura, J. T., Chen, B. L., Mun, J. J., Ho, R., Sarkis, R., McIntire, S. L. (2008) Genetic analysis of crawling and swimming locomotory patterns in *C. elegans*. *PNAS*, **105**, 20982–87.

Piskorowski, R., & Aldrich, R. W. (2002). Calcium activation of BK(Ca) potassium channels lacking the calcium bowl and RCK domains. *Nature*, **420**, 499–502.

Rand, J. B., Johnson, C. D., (1995). Genetic Pharmacology: Interactions between drugs and gene products in *Caenorhabditis elegans*. *Methods Cell Biol*, **48**, 187-204.

Ramaswamy, S., Eklund, H., & Plapp, B.V. (1994). Structures of horse liver alcohol dehydrogenase complexed with NAD^+ and substituted benzyl alcohols. *Biochemistry*, **33**, 5230-7.

Ranganathan, R., Sawin, E. R., Trent, C., & Horvitz, H. R. (2001). Mutations in the *Caenorhabditis elegans* serotonin reuptake transporter MOD-5 reveal serotonin-dependent and -independent activities of fluoxetine. *J Neurosci*, **21**, 5871–84.

Robitaille, R., Garcia, M. L., Kaczorowski, G. J., Charlton, M. P. (1993). Functional co-localization of calcium and calcium-gated potassium channels in control of transmitter release. *Neuron*, **11**, 645–655.

Roy, A., & Pandey, S. C. (2002). The decreased cellular expression of neuropeptide Y protein in rat brain structures during ethanol withdrawal after chronic ethanol exposure. *Alcohol Clin Exp Res*, **26**, 796–803.

Sancar, F., Touroutine, D., Gao, S., Oh, H. J., Gendrel, M., Bessereau, J.-L., Kim, H., Zhen, M., & Richmond, J. E. (2011). The dystrophin-associated protein complex maintains muscle excitability by regulating Ca^{2+}-dependent K^+ (BK) channel localization. *J Biol Chem*, **286**, 33501–10.

Schreiber, M., & Salkoff, L. (1997). A novel calcium-sensing domain in the BK channel. *Biophys J*, **73**, 1355–63.

Shao, L. R., Halvorsrud, R., Borg-Graham, L., & Storm, J. F. (1999). The role of BK-type Ca^{2+}-dependent K^+ channels in spike broadening during repetitive firing in rat hippocampal pyramidal cells. *J Physiol*, **521**, 135–46.

Shen, K., Lagrutta, A., Davies, N. W., Standen, N. B., Adelman, J. P., & North, R. A. (1994). of *Slowpoke* calcium-activated potassium channels expressed in *Xenopus oocytes*□: evidence for tetrameric channel formation. *Pflugers Arch*, **426**, 440-5.

Snetkov, V. A., & Ward, J. P. (1999). Ion currents in smooth muscle cells from human small bronchioles: presence of an inward rectifier K^+ current and three types of large conductance K^+ channel. *Exp Physiol*, **84**, 835–846.

Speca, D. J., Chihara, D., Ashique, A. M., Bowers, M. S., Pierce-Shimomura, J. T., Lee, J., Rabbee, N., Speed, T. P., Gularte, R. J., Chitwood, J., Medrano, J. F., Liao, M., Sonner, J. M., Eger, E. I., Peterson, A. S., & McIntire, S. L. (2010). Conserved role of *unc-79* in ethanol responses in lightweight mutant mice. *PLoS Genet*, **6**. doi:10.1371/journal.pgen.1001057.

Sulston, J. E., & Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Developmental Biology*, **56**, 110–56.

Thiele, T. R., Marsh, D. J., Marie, L. S., Bernstein, I. L., & Palmiter, R. D. (1998). Ethanol consumption and resistance are inversely related to neuropeptide Y levels. *Nature*, **396**, 366–9.

Thode, A. B., Kruse, S. W., Nix, J. C., & Jones, D. N. (2009). The role of multiple hydrogen bonding groups in specific alcohol binding sites in proteins: Insights from structural studies of LUSH. *J Mol Biol*, **376**, 1360–1376

Thompson, O., Edgley, M., Strasbourger, P., Flibotte, S., Ewing, B., Adair, R., Au, V., Chaudhry, I., Fernando, L., Hutter, H., Kieffer, A., Lau, J., Lee, N., Miller, A., Raymant, G., Shen, B., Shendure, J., Taylor, J., Turner, E. H., Hillier, L. W., & Waterston, R. H. (2013). The million mutation project: a new approach to genetics in *Caenorhabditis elegans*. *Genome Res*, **23**, 1749–62.

Topper, S. M., Aguilar, S. C., Topper, V. Y., Elbel, E., & Pierce-Shimomura, J. T. (2014). Alcohol disinhibition of behaviors in *C. elegans*. *PloS One*, **9**, e92965. doi:10.1371/journal.pone.0092965

Velázquez-Marrero, C., Wynne, P., Bernardo, A., Palacio, S., Martin, G., & Treistman, S. N. (2011). The relationship between duration of initial alcohol exposure and persistence of molecular tolerance is markedly nonlinear. *J Neurosci*, **31**, 2436–46.

Vidal-gadea, A. G., Davis, S., Becker, L., & Pierce-shimomura, J. T. (2012). Coordination of behavioral hierarchies during environmental transitions in *Caenorhabditis elegans*. *Worm*, **1**, 5–11.

Wallner, M., Meera, P., & Toro, L. (1996). Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca^{2+}-sensitive K^{+} channels: an additional transmembrane region at the N terminus. *Proc Natl Acad Sci U S A.*, **93**, 14922–7.

Walters, F. S., Covarrubias, M., & Ellingson, J. S. (2000). Potent inhibition of the aortic smooth muscle maxi-K channel by clinical doses of ethanol. *Am J Physiol Cell Physiol*, **279**, C1107–15.

Wang, X., Wang, G., Lemos, J. R., & Treistman, S. N. (1994). Ethanol directly modulates gating of a dihydropyridine-sensitive Ca^{2+} channel in neurohypophysial terminals. *J Neurosci*, **14**, 5453–60.

Wang, Z., Saifee, O., Nonet, M. L., & Salkoff, L. (2001). SLO-1 Potassium Channels Control Quantal Content of Neurotransmitter Release at the *C . elegans* Neuromuscular Junction. *Neuron*, **32**, 867–81.

Wei, a, Jegla, T., & Salkoff, L. (1996). Eight potassium channel families revealed by the *C. elegans* genome project. *Neuropharmacology*, **35**, 805–29.

White, J. G., Southgate, E., Thomson, J. N., & Brenner, S. (1986). The Structure of the Nervous System of the Nematode *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci*, **314**, 1–340.

Widmer, H., Lemos, J. R., & Treistman, S. N. (1998). Ethanol reduces the duration of single evoked spikes by a selective inhibition of voltage-gated calcium currents in acutely dissociated supraoptic neurons of the rat. *J Neuroendocrinology*, **10**, 399–406.

Williamson, V. M., Long, M., & Theodoris, G. (1991). Isolation of *Caenorhabditis elegans* mutants lacking alcohol dehydrogenase activity. *Biochem Genet*, **29**, 313–23.

Wynne, P. M., Puig, S. I., Martin, G. E., & Treistman, S. N. (2009). Compartmentalized subunit distribution determines characteristics and ethanol sensitivity of somatic , dendritic , and terminal large-conductance calcium-activated potassium channels in the rat central nervous system, *J Pharmacol Exp Ther*, **329**, 978–86.

Xia, X. M., Zeng, X., & Lingle, C. J. (2002). Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature*, **418**, 880–4.

Van den Brink-van der Laan, E., Chupin, V., Killian, J. A., & de Kruijff, B. (2004). Small alcohols destabilize the KcsA tetramer via their effect on the membrane lateral pressure. *Biochemistry*, **43**, 5937–42.

Waggoner, L. E., Zhou, G. T., Schafer, R. W., Schafer, W. R. (1998) Control of alternative behavioral states by serotonin in *Caenorhabditis elegans*. *Neuron,* **21**, 203-14.

Wick, M. J., Mihic, S. J., Ueno, S., Mascia, M. P., Trudell, J. R., Brozowski, S. J., Ye, Q., & Harris, R. A (1998). Mutations of gamma-aminobutyric acid and glycine receptors change alcohol cutoff: evidence for an alcohol receptor *PNAS,* **95**, 6504–9.

Yan, J., & Aldrich, R. W. (2010). LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium, *Nature,* **466**, 513-6.

Yan, J., Olsen, J. V, Park, K. S., Li, W., Bildl, W., Schulte, U., Aldrich, R. W., Fakler, B., & Trimmer, J. S. (2008). Profiling the phospho-status of the BK_{Ca} channel alpha subunit in rat brain reveals unexpected patterns and complexity. *Mol Cell Proteomics*, **7**, 2188–98.

Ye, Q. (1998). Enhancement of Glycine Receptor Function by Ethanol Is Inversely Correlated with Molecular Volume at Position alpha 267. *J Biol Chem*, **273**, 3314–19.

Yu, X., Zhao, W., Ma, J., Fu, X., & Zhao, Z. J. (2011). Beneficial and harmful effects of alcohol exposure on *Caenorhabditis elegans* worms. *Biochem Biophys Res Commun*, **412**, 757–62.

Yuan, C., Chen, M., Covey, D. F., Johnston, L. J., & Treistman, S. N. (2011). Cholesterol tuning of BK ethanol response is enantioselective, and is a function of accompanying lipids. *PloS One*, **6**, e27572. doi:10.1371/journal.pone.0027572.

Yuan, C., O'Connell, R. J., Jacob, R. F., Mason, R. P., & Treistman, S. N. (2007). Regulation of the gating of BK_{Ca} channel by lipid bilayer thickness. *J Biol Chem*, **282**, 7276–86.

Yuan, C., O'Connell, R. J., Wilson, A., Pietrzykowski, A. Z., & Treistman, S. N. (2008). Acute alcohol tolerance is intrinsic to the BK_{Ca} protein, but is modulated by the lipid environment. *J Biol Chem*, **283**, 5090–8.

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y., & MacKinnon, R. (2010). Structure of the human BK channel Ca^{2+}-activation apparatus at 3.0 A resolution. *Science*, **329**, 182–6.

Zar JH (1999) Biostatistical Analysis, fourth Addition. Prentice Hall, Upper Saddle River, NJ.

Zeng, X. H., Xia, X. M., & Lingle, C. J. (2005). Divalent cation sensitivity of BK channel activation supports the existence of three distinct binding sites. *J Gen Physiol*, **125**, 273–86.

Zheng, X., Gan, L., Wang, E., & Wang, J. (2013). Pocket-based drug design: exploring pocket space. *AAPS J*, **15**, 228–41.