Behavioral and Functional Analysis of a Calcium Channelopathy in Caenorhabditis elegans

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BEHAVIORAL AND FUNCTIONAL ANALYSIS OF
A CALCIUM CHANNELOPATHY IN CAENORHABDITIS ELEGANS

A Dissertation Presented

By

YUNG-CHI HUANG

Submitted to the Faculty of the
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NEUROBIOLOGY
BEHAVIORAL AND FUNCTIONAL ANALYSIS OF
A CALCIUM CHANNELOPATHY IN CAENORHABDITIS ELEGANS

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April 4th, 2017
Dedicated to my family, for their unconditional love and support.
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ABSTRACT

The brain network is a multiscale hierarchical organization from neurons and local circuits to macroscopic brain areas. The precise synaptic transmission at each synapse is therefore crucial for neural communication and the generation of orchestrated behaviors. Activation of presynaptic voltage-gated calcium channels (CaV2) initiates synaptic vesicle release and plays a key role in neurotransmission. In this dissertation, I have aimed to uncover how CaV2 activity affects synaptic transmission, circuit function and behavioral outcomes using *Caenorhabditis elegans* as a model. The *C. elegans* genome encodes an ensemble of highly conserved neurotransmission machinery, providing an opportunity to study the molecular mechanisms of synaptic function in a powerful genetic system. I identified a novel gain of function CaV2α1 mutation that causes CaV2 channels to activate at a lower membrane potential and slow the inactivation. Cell-specific expression of these gain-of-function CaV2 channels is sufficient to hyper-activate neurons of interest, offering a way to study their roles in a given circuit. CaV2(gf) mutants display behavioral hyperactivity and an excitation-dominant synaptic transmission. Imbalanced excitation and inhibition of the nervous system have been associated with several neurological disorders, including Familial Hemiplegic Migraine type 1 (FHM1) which is caused by gain-of-function mutations in the human CaV2.1α1 gene. I showed that animals carrying *C. elegans* CaV2α1 transgenes with corresponding human FHM1
mutations recapitulate the hyperactive behavioral phenotype exhibited by $Ca_v2(gf)$ mutants, strongly suggesting the molecular function of $Ca_v2$ channels is highly conserved from $C. elegans$ to human. Through performing a genome-wide forward genetic screen looking for $Ca_v2\alpha(gf)$ suppressors, we isolated new alleles of genes that required for $Ca_v2$ trafficking, localization and function. These regulators include subunits of $Ca_v2$ channel complex, components of synaptic and dense core vesicle release machinery as well as predicted extracellular proteins. Taken together, this work advances the understanding of $Ca_v2$ malfunction at both cellular and circuit levels, and provides a genetically amenable model for neurological disorders associated with excitation-inhibition imbalance. Additionally, through identifying regulators of $Ca_v2$, this research provides new avenues for understanding the $Ca_v2$ channel mediated neurotransmission and potential pharmacological targets for the treatments of calcium channelopathies.
# TABLE OF CONTENTS

**Title Page** ................................................................................................................................. i

**Signature Page** ............................................................................................................................. ii

**Acknowledgements** ................................................................................................................... iv

**Abstract** ................................................................................................................................... vi

**Table of Contents** ................................................................................................................... viii

**List of Tables** ........................................................................................................................... x

**List of Figures** ........................................................................................................................... xi

**List of Symbols, Abbreviations, or Nomenclature** ................................................................. xiii

**Preface** ...................................................................................................................................... xv

**Chapter I: Introduction** ........................................................................................................... 1

**Chapter II: Behavioral and Functional Consequences of a Gain of Function Neuronal Voltage Gated Calcium Channel**

Introduction ................................................................................................................................. 18

Results ......................................................................................................................................... 20

Discussion ..................................................................................................................................... 36

Materials and Methods ............................................................................................................ 41

Figures ......................................................................................................................................... 50

**Chapter III: A Forward Genetic Screen for Regulators of Calcium Channel Function in Synaptic Transmission**

Introduction ................................................................................................................................. 76
List of Tables

Table I-1: Voltage-gated calcium channel subtypes ............................................ 16

Table III-1: Forward suppressor screen identifies new alleles of genes required for Cav2 trafficking, localization and function ................................................................. 95
List of Figures
Figure I-1: Subunits and structure of Cav2 channels.................................15

Figure II-1: zf35 animals are hyperactive in locomotion and the egg-laying behavior...............................................................50

Figure II-2: zf35 is a novel allele of the Cav2α1 subunit gene unc-2. ..............52

Figure II-3: Cav2α1/unc-2(zf35) mutation is a gain-of-function allele of unc-2. ...54

Figure II-4: Cell-specific expression of the unc-2(zf35gf) transgene is sufficient to hyperactivate targeted neurons..........................................................56

Figure II-5: The UNC-2(ZF35) corresponding G1518R mutation in human Cav2α1 subunit results in increased channel activity. ........................................59

Figure II-6: FHM1 mutations in unc-2 result in a hyperactive phenotype. ........61

Figure II-7: GLR-1 mediated signaling contributes to the hyperreversal phenotype of unc-2(zf35gf) mutants........................................................63

Figure II-8: unc-2(zf35gf) mutants have increased ACh neurotransmission. ......64

Figure II-9: unc-2(zf35gf) mutants have increased cholinergic and decreased GABAergic transmission.................................................................66

Figure II-10: unc-2(zf35gf) mutants have decreased GABA receptor expression at the NMJ. ..............................................................68

Figure II-11: unc-2(zf35gf) mutants are resistant to the GABA agonist muscimol.................................................................71

Figure II-12: The reduction of GABA receptor in unc-2(zf35gf) mutants is dependent on nicotinic acetylcholine receptor mediated signaling..................72

Figure II-13: Proposed model of the imbalance between excitatory and inhibitory neurotransmission in unc-2(zf35gf) mutants ........................................74

Figure III-1: Genes required for Cav2 channel trafficking, localization and function in synaptic transmission. .................................................96

Figure III-2: zf121 is a novel allele of the Cav2β subunit gene ccb-1. ...............97
Figure III-3: *ccb-1(zf121)* mutants are defective in locomotion and egg-laying behaviors. ........................................................................................................................................99

Figure III-4: *ccb-1(zf121)* mutants are hypersensitive to the cholinergic agonist, levamisole. ........................................................................................................................................100

Figure III-5: GK domain is essential for the function of CaVβ/CCB-1.................101
List of symbols, abbreviations, or nomenclature

ACh: acetylcholine
ASD: autism spectrum disorder
ChR2: channelrhodopsin-2
CoLBeRT: Control of Locomotion and Behavior in Real Time
DIC: differential interference contrast
EA2: Episodic ataxia type 2
ER: endoplasmic reticulum
E/I balance: excitation/inhibition balance
EMS: Ethyl methanesulfonate
EPSC: excitatory postsynaptic current
FHM1: Familial Hemiplegic Migraine type 1
GABA: gamma-aminobutyric acid
GFP: green fluorescent protein
GPCR: G protein-coupled receptor
IPSC: inhibitory postsynaptic current
L1- L4: C. elegans larval stages 1-4
NMJ: Neuromuscular Junction
NGM nematode grown media
mCherry red fluorescent protein
SCA6: Spinocerebellar ataxia type 6
SNP: single nucleotide polymorphism
VGCC: voltage gated calcium channel
VNC: ventral nerve cord
α: alpha
β: beta
δ: delta
nt: nucleotide
Preface

The work presented in Chapter II represents unpublished work that is in preparation for publication.

Yung-Chi Huang, Jennifer K. Pirri, Diego Rayes, Shangbang Gao, Ben Mulcahy, Yasunori Saheki, Cornelia I. Bargmann, Michael M. Francis, Mei Zhen and Mark J. Alkema

A Gain-of-function Mutation in the CaV2 Channel Leads to Behavioral Hyperactivity and Excitation-dominant Transmission in Caenorhabditis elegans.

The work presented in Chapter III represents unpublished work that is in preparation for publication.

Yung-Chi Huang, Jeff Grant, Wookyu Kang, Mark J. Alkema

The Role of CaVβ/CCB-1 in Regulating Presynaptic CaV2 Channel Function in Caenorhabditis elegans
CHAPTER I

Introduction
The brain network is a multiscale hierarchical organization from neurons, local circuits to macroscopic brain areas. Individual neurons are wired into neural circuits to perform specific functional outputs in response to environmental stimuli. The anatomical structure of the circuits and the precise neurotransmission at each synapse are therefore crucial for information processing in the brain network. Synapses are specialized structures where neurons communicate with each other through neurotransmitters. The key component that defines a synapse is the active zone, which is a small, electron dense region located in the plasma membrane of the presynaptic terminal. An active zone contains a large core protein complex: RIM (Rab3-interacting molecules), RIM-BP (RIM biding proteins) and Munc13; as well as the components for synaptic vesicle fusion and exocytosis: SNARE and Munc18. This core protein complex docks and primes synaptic vesicles at the active zone and recruits the presynaptic voltage-gated calcium channel (VGCC) to the vesicle release sites (Südhof, 2012). When an action potential arrives, depolarization of the nerve terminal activates VGCCs. Neurotransmitters, which are packed into synaptic vesicles, are then released by calcium-dependent synaptic vesicle exocytosis into the synaptic cleft, where they bind postsynaptic receptors and trigger signaling cascades in postsynaptic neurons. During this process, the presynaptic VGCCs are necessary and crucial for converting the electrical signal of the action potential into released chemical signal across the synaptic cleft.
**Voltage-gated calcium channels**

VGCCs activate upon membrane depolarization and mediate Ca$^{2+}$ influx, which initiate many physiological events in different cell types. In cardiac and muscle cells, opening of the VGCC initiates the excitation-contraction coupling process by activating the ryanodine receptors that release the intracellular Ca$^{2+}$ from the sarcoplasmic reticulum (Bers, 2002; Catterall et al., 1991; Mikami et al., 1989). In neurons and some endocrine cells, the activation of VGCCs leads to the release of synaptic vesicles and secretion of hormones respectively (Dunlap et al., 1995; Tsien et al., 1988; Yang and Berggren, 2006). In addition, in many different cell types VGCC-mediated Ca$^{2+}$ entry into cytoplasm is involved in the regulation of enzyme activity and gene expression (Flavell and Greenberg, 2008). Based on the voltage-dependent and pharmacological properties, VGCCs can be grouped into three major types: the high-voltage activated (HVA) channels: Ca$\alpha$1 family (Ca$\alpha$1.1- Ca$\alpha$1.4, mediate L-type currents) and Ca$\alpha$2 family (Ca$\alpha$2.1, mediates P/Q-type; Ca$\alpha$2.2, mediates N-type; and Ca$\alpha$2.3, mediates R-type currents), and the low-voltage-gated (LVA) channels: Ca$\alpha$3 family (Ca$\alpha$3.1- Ca$\alpha$3.3, mediate T-type currents) (Table I-1) (Catterall 2011). In this chapter, I will focus on the structure and regulation of Ca$\alpha$2 channels that are exclusively expressed in neurons.

Ca$\alpha$2 channels, which structurally similar to Ca$\alpha$1 channels, are heteromultimeric protein complexes composed of a pore-forming Ca$\alpha$1 subunit
(~ 190 kDa), plus two essential auxiliary subunits: CaVβ (~ 55 kDa) and CaVα2δ (~ 170 kDa) subunits (Liu et al., 1996; Witcher et al., 1993) (Figure I-1a). In some mammalian cell types, there is one extra auxiliary CaVγ subunit (Klugbauer et al., 2000; Takahashi et al., 1987). The 3D structure of the CaV1 channel complex was solved for the first time in 2015 by single-particle cryo–electron microscopy (Wu et al., 2015, 2016). As previously predicted, the CaVα1 subunit is a transmembrane protein consisting of one single polypeptide chain that is organized into four repeated domains (TM I to IV) of six transmembrane helices (S1 to S6) (Figure I-1b). In each domain, the S4 segment functions as the voltage-sensing helix, while the S5 and S6 helices constitute the pore helices. The CaVα1 subunit therefore is the key determinant of the CaV2 channel kinetics and is also the target for most second-messenger modulation and pharmacological agents. The cytosolic CaVβ subunit binds the CaVα1 subunit through the α1 interaction domain (AID), which is localized in the first intracellular loop between domain I and II of the CaVα1 subunit (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). CaVβ subunits have been shown to facilitate trafficking of the CaVα1 subunit from endoplasmic reticulum (ER) to the membrane surface (Bichet et al., 2000) as well as modulate the voltage-dependent activation/inactivation threshold of VGCCs (Buraei and Yang, 2010; He et al., 2007a). The CaVα2 subunit is an extracellular, extrinsic membrane protein attached to the membrane through disulfide linkage to the transmembrane CaVδ subunit (Gurnett et al., 1996). Because the CaVα2 and
CaVδ subunits are encoded by the same gene and are produced by post-translational proteolytic processing and then disulfide-bonded, they are usually referred as the CaVα2δ subunits (De Jongh et al., 1990). CaVα2δ subunits interact with the CaVα1 subunit through extracellular loops of domain I to III in the CaVα1 subunit (Wu et al. 2015). CaVα2δ subunits are able to modify channel biophysical properties, but their main role is to increase calcium currents by promoting trafficking of the CaVα1 subunit to the plasma membrane and by increasing its retention there (Bernstein and Jones, 2007; Cantí et al., 2005; Davies et al., 2007).

Regulation of the presynaptic CaV 2 channels

Opening of CaV2 channels results in Ca\(^{2+}\) influx from the extracellular space and gives rise to a localized elevation of intracellular Ca\(^{2+}\) concentration. This triggers a wide range of biophysical process from gene expression, synaptic transmission to the activation of various calcium-dependent enzymes, such as calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Wadel et al., 2007; Wheeler et al., 2012). To precisely regulate these physiological events and prevent excitotoxicity triggered by excess intracellular Ca\(^{2+}\) (Kass and Orrenius, 1999), the activity of CaV2 channels must be tightly regulated by both intrinsic gating and modulation by other interacting proteins.

Ca\(^{2+}\)/Calmodulin-dependent inactivation and facilitation
Calcium-dependent inactivation and facilitation of CaV2 channels has been observed in the rat neurohypophysis as well as the calyx of Held synapse (Branchaw et al., 1997; Cuttle et al., 1998; Forsythe et al., 1998). A novel calmodulin binding site, calmodulin-binding domain (CBD), in the C-terminus of the CaV2α1 subunit was identified in mammalian cells expressing CaV2 channels (Lee et al. 1999). This calcium-dependent binding has multiple functions: it increases the rate and extent of voltage-dependent inactivation, enhances recovery from inactivation and augments further calcium influx by facilitating the calcium current. Electrophysiological recordings have shown that accumulation of intracellular calcium during a train of impulses would increase inactivation, shorten calcium transients and contribute to terminating the calcium signal following the train. Meanwhile, the binding of calmodulin also increases recovery from inactivation, thereby preparing the channels to respond more fully to a subsequent train of stimuli, and enhances that response by facilitation of the calcium current (Lee et al. 1999; Lee, Scheuer, and Catterall 2000). This dual feedback regulation of CaV2 channels provides an important mechanism for the activity-dependent synaptic plasticity.

**G-protein regulation**

CaV2 channel-mediated currents are regulated through multiple G protein-coupled pathways. While the G protein α subunits are thought to confer specificity in receptor coupling and activate a wide range of signaling pathways,
the G protein βγ subunits can directly interact with intracellular loop between domain I-II and the N-terminal region of the Ca\textsubscript{v}2\textalpha subunits (Herlitze et al., 1996; Ikeda, 1996; De Waard et al., 1997; Zamponi et al., 1997). This interaction results in an inhibitory effect on Ca\textsubscript{v}2 channel activity, stabilizing the closed conformation of the Ca\textsubscript{v}2 channel, causing a positive shift in the voltage dependence and slowing activation (Bean, 1989). This Gβγ subunit mediated inhibition can be suppressed by application of large voltage pulses or by a series of short membrane depolarizations, and is therefore termed voltage-dependent inhibition. The strong depolarization causes a temporary dissociation of Gβγ subunits from the channel, thus briefly reverting the channel back to non-modulated state (Bean, 1989).

In addition to Gβγ subunit mediated inhibition, it is becoming increasingly clear that the Ca\textsubscript{v}2 channels form signaling complexes (through direct or indirect interactions) with a range of different types of G-protein coupled receptors (GPCR), for instance: mGluR1, opioid receptor family and dopamine receptors (Evans et al., 2010; Kisilevsky et al., 2008; Kitano et al., 2003). Such channel-GPCR signaling complexes have several potential functions: 1) optimize the efficiency of coupling between the receptor and the channels, 2) allow for agonist-dependent internalization of channel-GPCR complexes as a means of acutely controlling channel density in the plasma membrane via receptor activation (Altier et al., 2006; Kisilevsky et al., 2008). Together, the voltage-dependent Gβγ subunit mediated inhibition along with channel-GPCR complexes
are critical for controlling the calcium entry through Ca\textsubscript{V}2 channels (Huang and Zamponi, 2017).

\textbf{Ca\textsubscript{V}\textbeta subunit regulation}

Auxiliary Ca\textsubscript{V}\textbeta subunits interact with the Ca\textsubscript{V}\textalpha1 subunits in a 1:1 stoichiometry and reversible interaction (Tareilus et al., 1997) via the alpha interaction domain (AID) in the intracellular loop I-II of Ca\textsubscript{V}\textalpha1 subunits (Chen et al., 2004). It has been well established that this interaction is required for membrane expression of Ca\textsubscript{V}\textalpha1 subunits, both in heterologous expression systems and \textit{in vivo}. The increased surface expression of VGCCs can be detected by surface staining, gating charge measurement and calcium currents (Gregg et al., 1996; Josephson and Varadi, 1996; Kamp et al., 1996; Obermair et al., 2010). Two possible mechanisms explain how the Ca\textsubscript{V}\textbeta subunit enhances the VGCC surface expression: 1) Binding of the Ca\textsubscript{V}\textbeta subunit masks the endoplasmic reticulum (ER) retention signal in the Ca\textsubscript{V}\textalpha1 subunit, which facilitates Ca\textsubscript{V}\textalpha1 subunits trafficking from the ER to plasma membrane (Bichet et al., 2000). 2) This interaction prevents the ubiquitination and proteasomal degradation of Ca\textsubscript{V}\textalpha1 subunits (Altier et al., 2011).

After the VGCC complex reaches the membrane, Ca\textsubscript{V}\textbeta subunits, except for one isoform, Ca\textsubscript{V}\textbeta\textsubscript{2a}, modulate the channel gating enhancing both the voltage-dependent activation (VDA) and voltage-gated inactivation (VDI) (Cahill et al., 2000; He et al., 2007a). Structural, biochemical and functional studies have
revealed a model for this modulation. Through the interaction with the Caββ subunit, the entire interacting region of the α1 subunit (including the S6 of TM I and the intracellular loop between TM I and II) becoming a continuous α-helix. This conformational change likely affects the voltage-dependent movement of the pore gate (cytoplasmic region of S5 to S6 in each TM), thereby changing the voltage dependence and kinetics of the activation and inactivation (Buraei and Yang, 2010; Findeisen and Minor, 2009; Opatowsky et al., 2004).

**SNARE protein interaction**

CaV2 channels are crucial mediators of depolarization-evoked fast synaptic transmission. To ensure the efficient coupling of calcium influx and vesicle release, CaV2 channels have to be localized within active zones and synchronize with the synaptic exocytosis machinery. The CaV2α1 subunit contains a synaptic protein interaction site (synprint) in the intracellular loop between TM I-II. This synprint site directly interacts with the SNARE proteins in a calcium-dependent manner (Sheng et al. 1994; Sheng et al. 1996; Rettig et al. 1997). In addition to the functional role of interaction between CaV2 channels and SNARE proteins in facilitating the synaptic transmission, this interaction also regulates CaV2 channel function by promoting voltage-dependent inactivation and Gβγ modulation (Bezprozvanny et al., 1995; Jarvis and Zamponi, 2001). It is interesting to note that some invertebrates and mammalian isoforms of the CaV2α1 subunit have no synprint site (Kaneko et al., 2002; Spafford et al., 2003).
These CaV2 channels are recruited to the synaptic vesicle release site through other active zone proteins, such as Mint-1, CASK, RIM and RIM-binding proteins (Graf et al., 2012; Kaezer et al., 2011; Spafford et al., 2003). These findings may reveal other potential regulatory mechanisms for CaV2 channels at active zones independent of the SNARE proteins.

Altogether, the mechanisms mentioned in this section provide multiple layers of regulation for CaV2 channels to ensure the calcium entry, synaptic release and calcium-dependent signaling pathways are precisely and tightly controlled.

**CaV2 channel dysfunction: calcium channelopathies**

Dysregulation of CaV2 channel function is associated with severe neurological disorders including ataxia, epilepsy, migraine and autism-spectrum disorders (Adams and Snutch, 2007; Ophoff et al., 1996; Pietrobon, 2010; Zhuchenko et al., 1997). Mutations in the human \textit{CACNA1A} gene, encoding the P/Q $\alpha_1$ subunit (CaV2.1$\alpha_1$), have been associated with three neurological disorders: (1) Episodic ataxia type 2 (EA2), whose clinical features include the lack of voluntary coordination of muscle movements, headaches and epileptic seizures. (2) Spinocerebellar ataxia type 6 (SCA6), a late onset, cerebellar atrophy resulting in progressive gait ataxia, limb incoordination and dysarthria. (3) Familial hemiplegic migraine 1 (FHM1), a severe variant of migraine with aura that can co-occur with tonic-clonic seizures.
EA2 is associated with a wide range of mutations throughout the CACNA1A gene and include missense mutations, nonsense-, and splice site mutations (Jen et al., 2004). SCA6 patients have a polyglutamine expansion (CAG) at the 3’ end of CACNA1A gene (Zhuchenko et al., 1997). FHM1 mutations are primarily found near the voltage sensors and flanking domains of the CaV2α1 subunit (Pietrobon, 2007; Thomsen et al., 2007). Although these three autosomal dominant disorders are conventionally distinguished, they exhibit considerable overlap in clinical features. This is further complicated by the fact that even family members with identical mutations can suffer from different pathological features.

The functional consequences of several CACNA1A mutations have been investigated using electrophysiology in Xenopus and mammalian expression systems. EA2 missense mutations appear to cause a reduction in CaV2.1 mediated currents (Guida et al., 2001; Spacey et al., 2004). Heterozygous nonsense mutations also show reduction in current density, most likely due to a dominant negative effect that results in fewer channels being properly folded and reaching the membrane (Jeng et al., 2008; Mezghrani et al., 2008). Thus EA2 mutations seem to lead to reduced channel function, likely resulting in decreased neurotransmitter release (Jen et al., 2001). Studies from different heterologous expression systems and transfected neurons revealed that FHM1 mutations alter the CaV2.1 channel function in a much more complex way. Both gain- and reduction-of function phenotypes in channel function have been reported (Barrett
et al., 2005; Hans et al., 1999a; Tottene et al., 2002). In some cases, the different effects were dependent on the nature of the mutations; in other cases, the discrepancies may due to the specific isoform of the CaV2.1α1 subunit, as well as the CaVβ subunit it associates with.

**Study of CaV2 channels in Caenorhabditis elegans**

The nervous system of the *Caenorhabditis elegans* (*C. elegans*) adult hermaphrodite has only 302 neurons (Sulston and Horvitz 1977; White et al 1986) yet is able to perform a wide range of behaviors, including chemotaxis, thermotaxis, escape behaviors in response to touch, mating, social feeding, and both associative and non-associative learning. In addition, *C. elegans* is the only organism that has the complete neural wiring diagram mapped out (White et al 1986). Along with the recent developments in using optogenetics and neural imaging in free behaving animals (Leifer et al., 2011; Venkatachalam et al., 2016), *C. elegans* has become an increasingly important model to study neurobiological questions.

The *C. elegans* genome encodes synaptic transmission machinery that is highly conserved to vertebrates. Genes involved in neurotransmitter biosynthesis, synaptic release mechanisms, and neurotransmitter receptors, as well as the ion channels and second messenger pathways are highly conserved (Bargmann, 1998). One of the great advantages of using *C. elegans* to study synaptic biology is that the genome often has a single recognizable member of a
gene family where vertebrates have three or four very similar genes. This provides an opportunity to study the functional consequences of eliminating individual synaptic proteins in a living organism without compensatory effect from other gene family members. Recently, techniques have also been developed to perform electrophysiological recording in C. elegans in vivo (Francis and Maricq, 2006; Goodman et al., 2012). In addition, mutants that are defective in general synaptic transmission often exhibit pharmacological and behavioral phenotypes. This provides a valuable system to study the neural network, from genes and neurons to behaviors in vivo. These characteristics make C. elegans an attractive model organism to study synaptic function.

C. elegans genome encodes a single CaVα1 subunit for each VGCC type: egl-19 (Raymond et al., 1997), unc-2 (Schafer and Kenyon, 1995) and cca-1 (Steger et al., 2005) which appear to be orthologous to the CaV1(L-type), CaV2 (N-, P/Q- and R-type ) and CaV3 (T-type). Genes that encode the auxiliary protein CaVα2δ (unc-36) (Frøkjær-Jensen et al., 2006; Saheki and Bargmann, 2009) and CaVβ subunit (ccb-1) have been identified as well (Lainé et al., 2011). CaV2α1/UNC-2 is exclusively expressed in neurons and localizes to presynaptic active zones (Saheki and Bargmann, 2009; Schafer and Kenyon, 1995). unc-2 loss-of-function mutants are sluggish and uncoordinated and have defects in neurotransmitter release at the NMJ (Mathews et al., 2003; Richmond et al., 2001), which further underlines the conserved and important role of the CaV2 channels in synaptic transmission.
**Thesis outline**

In this dissertation, I have aimed to uncover how Ca\textsubscript{v}2 channel and its regulators affect synaptic transmission, circuit function and behavioral consequences using *C. elegans* as a model. In Chapter II, I will discuss a novel gain-of-function Ca\textsubscript{v}2\textalpha{}1 mutation that causes Ca\textsubscript{v}2 channel activation at a lower membrane potential with higher current density. The *Cav2a1(gf)* mutants display behavioral hyperactivity and excitation-dominant synaptic transmission. Elucidation of the underlying mechanisms of this imbalanced excitation and inhibition of the nervous system will potentially shed light on the pathology of human Familial Hemiplegic Migraine type 1 (FHM1), a calcium channelopathy resulted from increased Ca\textsubscript{v}2 channel function. Chapter III describes a genome-wide forward genetic screen for *Cav2a1(gf)* suppressors. New alleles of genes that functionally interact with Ca\textsubscript{v}2 channels and regulate its function in synaptic transmission were identified from this screen.

This thesis work provides a comprehensive understanding of Cav2 channel regulation *in vivo*, insights into the etiology of calcium channelopathies, and new avenues of discovering promising targets for the treatment of related neurological disorders.
Figure I-1. Subunits and structure of CaV2 channels (a) Calcium channel heteromeric complex: the pore-forming subunit, CaVα1, and two auxiliary subunits, CaVβ and CaVα2δ. (b) The CaV2α1 subunit is comprised of four major transmembrane domains (TM I-IV), which are connected by cytoplasmic linkers. Each domain contains six membrane-spanning α-helices with a re-entrant pore loop (shown in green), and the fourth transmembrane segment contains positively charged amino acids and forms the voltage sensor.
<table>
<thead>
<tr>
<th>Voltage Dependence</th>
<th>Ca\textsuperscript{2+} Current Type</th>
<th>Ca\textsubscript{v} Channel Type</th>
<th>Expression and Physiological functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>HVA</td>
<td>L-type</td>
<td>Ca\textsubscript{v}1.1</td>
<td><strong>Skeletal muscles</strong>&lt;br&gt;Excitation-contraction coupling; regulation of transcription</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca\textsubscript{v}1.2</td>
<td><strong>Cardiac muscles; Endocrine cells; Neurons</strong>&lt;br&gt;Excitation-contraction coupling in cardiac and smooth muscle; endocrine secretion; Ca\textsuperscript{2+} transients in cell bodies and dendrites; regulation of transcription</td>
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<td></td>
<td></td>
<td>Ca\textsubscript{v}1.3</td>
<td><strong>Endocrine cells; Neurons</strong>&lt;br&gt;Endocrine secretion; cardiac pacemaking, Ca\textsuperscript{2+} transients in cell bodies and dendrites; auditory transduction</td>
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<td></td>
<td></td>
<td>Ca\textsubscript{v}1.4</td>
<td><strong>Retina</strong>&lt;br&gt;Visual transduction</td>
</tr>
<tr>
<td>P/Q-type</td>
<td>Ca\textsubscript{v}2.1</td>
<td><strong>Neurons</strong>&lt;br&gt;Neurotransmitter release; Dendritic Ca\textsuperscript{2+} transients</td>
<td></td>
</tr>
<tr>
<td>N-type</td>
<td>Ca\textsubscript{v}2.2</td>
<td><strong>Neurons</strong>&lt;br&gt;Neurotransmitter release; Dendritic Ca\textsuperscript{2+} transients</td>
<td></td>
</tr>
<tr>
<td>R-type</td>
<td>Ca\textsubscript{v}2.3</td>
<td><strong>Neurons</strong>&lt;br&gt;Neurotransmitter release; Dendritic Ca\textsuperscript{2+} transients</td>
<td></td>
</tr>
<tr>
<td>LVA</td>
<td>T-type</td>
<td>Ca\textsubscript{v}3.1</td>
<td><strong>Skeletal and cardiac muscles; Neurons</strong>&lt;br&gt;Pacemaking and repetitive firing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca\textsubscript{v}3.2</td>
<td><strong>Cardiac muscles; Neurons</strong>&lt;br&gt;Pacemaking and repetitive firing</td>
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<td>Ca\textsubscript{v}3.3</td>
<td><strong>Neurons</strong>&lt;br&gt;Pacemaking and repetitive firing</td>
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Table I-1 Voltage-gated calcium channel subtypes
CHAPTER II

Behavioral and Functional Consequences of a Gain of Function

Neuronal Voltage Gated Calcium Channel
**Introduction**

Presynaptic Ca\(_{\text{V}2}\) channels are one of the most abundantly expressed calcium channel subtypes in the mammalian nervous system. They mediate depolarization-induced calcium influx, which is essential for triggering synaptic vesicle release, and thus act as transducers of electrical signals into chemical signals. The major Ca\(_{\text{V}2}\alpha_{1}\) subunit contains both the voltage-sensing mechanism and the calcium-selective pore, and largely determines the properties and kinetics of these channels. Mutations in the CACNA1A gene encoding the human Ca\(_{\text{V}2.1}\alpha_{1}\) subunit have been associated with three neurological disorders: Episodic ataxia type 2 (EA2), Spinocerebellar ataxia type 6 (SCA6) and Familial hemiplegic migraine 1 (FHM1) (Pietrobon, 2010). While missense and nonsense mutations attributed to EA2 appear to cause a reduction in Ca\(_{\text{V}2}\) channel mediated currents, both gain- and loss-of function phenotypes in channel function have been reported for FHM1 mutations in different expression systems. Reported effects of FHM1 mutations on channel function can vary considerably leaving a precise correlation of genotype to phenotype unresolved.

The unc-2 gene encodes the sole Ca\(_{\text{V}2}\alpha_{1}\) subunit in *C. elegans*. UNC-2 is exclusively expressed in neurons and localizes to presynaptic active zones (Schafer and Kenyon 1995; Saheki and Bargmann 2009). *unc-2* loss-of-function mutants have severe behavioral defects in locomotion, egg-laying and defecation, as well as deficits in neurotransmitter release at the NMJ (Mathews et al. 2003; Schafer and Kenyon 1995; Richmond, Weimer, and Jorgensen 2001).
These phenotypic defects are shared with animals harboring mutations in the Cav2 accessory subunit Ca\textsubscript{V}\textsubscript{2α2δ}/UNC-36, indicating a conserved role for Ca\textsubscript{V}2α1/UNC-2 and its auxiliary proteins as a presynaptic regulator of synaptic transmission.

In this study, we characterized a novel \textit{unc-2}(gf) mutant which, contrary to loss of function mutations, displays hyperactive behaviors in locomotion and egg-laying. We show that a G to R substitution in the highly conserved intracellular linker leads to a shift in the voltage dependence to more hyperpolarized potentials and reduces inactivation, suggesting a gain-of-function mutation. Cell specific expression of the UNC-2 gain-of-function channels is sufficient to increase synaptic transmission in neurons of interest. Transgenic animals carrying the \textit{unc-2} gene with human FHM1 mutations recapitulate the hyperactive behavioral phenotype. In addition, we showed that \textit{unc-2}(gf) mutants have an excitation-dominant synaptic transmission at the NMJ, and this enhanced excitatory neurotransmission is dependent on muscle acetylcholine receptors. Imbalanced cortical excitation and inhibition have also been associated with FHM1 patients (Vecchia and Pietrobon, 2012). Therefore, we believe \textit{unc-2}(gf) mutants may provide new insights into the pivotal role of Cav2 channels in the integration of circuit activity.
Results

zf35 mutants are hyperactive

*Caenorhabditis elegans* locomotion is biased towards forward movement, interrupted by periodic brief reversals. From a forward genetic screen for animals with locomotion defects, we isolated a mutant, zf35, which displayed a dramatic increase in the number of spontaneous reversals (43.1 ± 2.0/ 3 min, n=59) compared to wild-type animals (6.8 ± 0.4/ 3 min, n=59) (Figure II-1a and c). This seizure-like phenotype of zf35 mutants is accompanied with an increased locomotion rate during bouts of forward or backward locomotion. On average about zf35 mutants move 1.5 fold faster than wild-type animals in either direction (Figure II-1a and b). Animals that were heterozygous for zf35 mutation also displayed an increased reversal and locomotion rate albeit to a lesser extent compared to homozygous mutants, indicating the zf35 mutation is semi-dominant (Figure II-1a-c).

zf35 mutant animals had a reduced brood size (zf35: 150 ± 16, n=5, wild-type: 207 ± 11, n=5) and were slightly smaller than wild-type animals. Furthermore, zf35 mutant animals often had a protruding vulva, indicative of hyperactive egg-laying behavior (Figure II-1d). To study egg-laying behavior we compared the egg laying rate of zf35 mutant and wild-type animals. zf35 animals retained a reduced number of eggs in the uterus (3.6 ± 0.2, n =86) compared to the wild-type (14.1 ± 0.6, n=80). Furthermore, zf35 mutants laid their eggs at an earlier developmental stage than the wild-type (Figure II-1e), showing that the
time between fertilization and egg laying was reduced in zf35 mutants. This indicates zf35 mutants are hyperactive in both locomotion and egg-laying behavior.

The zf35 allele is a missense mutation in the Ca\textsubscript{\textalpha}2\textalpha 1/unc-2 gene

We mapped the zf35 mutation to the left end of chromosome X between the genetic markers lon-2 and dpy-3. This region contains the gene, unc-2, which encodes the \textalpha 1 subunit of the \textit{C. elegans} Ca\textsubscript{\textalpha}2 channel. Sequence analysis revealed that zf35 allele is a single-base transition (GGA -> AGA) in the 17th exon of unc-2 (Figure II-2a). The Ca\textsubscript{\textalpha}2\textalpha 1/UNC-2 consists of four homologous transmembrane domains (TMI-IV) each containing six hydrophobic membrane-spanning segments (S1–S6). The zf35 mutation results in a glycine to arginine substitution (G1132R) in the highly conserved intracellular linker between TM III-S6 and TM IV-S1 (Figure II-2b and c). To determine if the zf35 mutation is sufficient to confer the hyperactive phenotype we generated an unc-2(zf35) cDNA clone, which encodes the Ca\textsubscript{\textalpha}2\textalpha 1/UNC-2(G1132R) protein. Pan-neuronal expression of the unc-2(zf35) transgene induced hyperactive behavior similar to that of the zf35 mutant in both wild-type and loss-of-function unc-2(e55) background. On the other hand, transgenic expression of the wild type unc-2 transgene did not induce hyperactive behavior but did rescue the uncoordinated and lethargic phenotype of unc-2(e55) mutants (Figure II-3a), suggesting the zf35 mutation in the UNC-2 confers the behavioral hyperactivity.
unc-2(zf35) mutants did not display obvious defects in neural and axonal morphology. The wild type Ca_{1.2}/UNC-2 localizes to presynaptic zones, at sites of synaptic vesicle secretion (Saheki and Bargmann, 2009). To determine if the zf35gf mutation affected localization to presynaptic sites, we looked the subcellular localization of the UNC-2(zf35) in transgenic animals carrying GFP tagged unc-2(zf35) cDNA. UNC-2(wt)::GFP was present in the cell body and localizes to puncta in the nerve ring and the ventral nerve cord (Saheki and Bargmann, 2009). The fluorescence expression pattern of UNC-2(zf35)::GFP animals displayed no obvious difference with that of animals that express a UNC-2(wt)::GFP transgene (Figure II-3c), indicating that UNC-2(zf35) is properly processed and trafficked to presynaptic sites.

Identification of intragenic unc-2(zf35) loss-of-function suppressors

The sharp contrast between the unc-2(zf35) and unc-2(lf) mutant phenotypes suggests that the zf35 G1132R mutation is a rare gain-of-function mutation. Therefore, we sought to isolate intragenic mutations that suppress the hyperactivity phenotype of unc-2(zf35) mutants. From a screen of 10,000 haploid genomes we isolated 34 suppressors. SNP mapping and complementation tests identified 7 intragenic suppressor alleles of unc-2 (Figure II-2b). Four mutations, zf109, zf113, zf115 and zf124 caused severe locomotion defects similar to the canonical loss-of-function unc-2(e55) allele (Figure II-3b). Sequence analysis of the unc-2 gene of these mutants showed that two mutations, zf113(W551stop)
and zf124(M1371stop), result in a premature stop codons and therefore likely represent null alleles. zf115(C324Y) and zf109(L1357F) were found to be missense mutations that result in substitutions of highly conserved amino acids in the S5-S6 loop of domain I and IV, respectively. The S5-S6 loop forms re-entrant pore loop that is important in gating. Mutations in these loops are thus likely to impair Ca\textsubscript{v}2 channel function. While these nonsense and missense mutations result in severe uncoordinated locomotion phenotypes, the intragenic zf130 and zf134 suppressors caused moderate movement defects. The zf134(I970T) mutation results in a change an isoleucine to tyrosine domain in the conserved voltage sensor and the zf130(G1457D) mutation is predicted to result in a glycine to aspartate to the C-terminus in between a conserved EF-hand and the IQ like motif. The intragenic suppressor zf114(D892N) restored wild-type behavior in the \textit{unc-2(zf35)} mutant background. The zf114(D892N) mutation is predicted to change an aspartate to asparagine domain III S2. The change from a negatively charged group to a polar, uncharged group may have specific interaction with the \textit{zf35} mutation without generally weaken the Ca\textsubscript{v}2 channel function. These intragenic suppressors suppress \textit{unc-2(zf35)} phenotype and largely recapitulate \textit{unc-2} loss-of-function phenotype. This strongly supports the notion that the \textit{unc-2(zf35)} mutation is a gain-of-function allele of \textit{unc-2}.

\textbf{Cell-specific expression of the \textit{unc-2(zf35gf)} transgene}
Our data showed that gain-of-function Ca\textsubscript{\textit{v}}2\textalpha/UNC-2 channel leads behavioral hyperactivity in several aspects. Is cell-specific expression of the unc-2(zf35gf) transgene sufficient to hyperactivate individual neurons? To address this question we expressed the unc-2(zf35gf) transgene in specific subsets of neurons and analyzed their effect on behaviors. The hermaphrodite specific motor neurons (HSNs) play a critical role in the regulation of the \textit{C. elegans} egg-laying program (Trent et al., 1983). HSNs make extensive neuromuscular junctions with the vulval muscles, and also direct synaptic output to other egg-laying promoting motor neurons, VCs (Schafer 2005) (Figure II-4a and b). Transgenic animals that expresses the unc-2(zf35gf) transgene specifically in the HSNs have fewer eggs in their uterus and lay eggs at earlier developmental stage compared to the wild-type (Figure II-4c and d). These results suggest we are able to promote egg-laying by expressing unc-2(zf35gf) transgene in HSNs, and this effect is most likely due to the increased release of serotonin.

In \textit{C. elegans}, directional movements is controlled by a set of pre-motor interneurons: the AVB and PVC neurons control forward movement while the AVA, AVD and AVE neurons drive backing (Chalfie et al. 1985) (Figure II-4a and b). We therefore expressed the unc-2(zf35gf) transgene in subsets of pre-motor interneurons to determine whether they could recapitulate the hyper reversal phenotype of unc-2(zf35gf) mutants. Transgenic animals that express the unc-2(zf35gf) transgene in the PVC, AVA, AVD and AVE pre-motor interneurons exhibited a marked increase in reversal frequency. Expression of the unc-
2(zf35gf) transgene in the AVA backward premotor interneuron resulted in a milder but significant increase reversal frequency, while expression in the AVB neurons or cholinergic motor neurons had no appreciable effect on the reversal frequency (Figure II-4e). This indicates that the expression of the unc-2(zf35gf) transgene in pre- motor interneurons that drive backward locomotion increases the reversal rate. Transgenic animals that express the unc-2(zf35gf) transgene in pre-motor interneurons did not display apparent behavioral defects in the locomotion rate or egg laying. Furthermore, cell-specific expression of the unc-2(zf35gf) transgene in cholinergic motor neurons that have synaptic connection onto body wall muscles markedly increased the sensitivity to the aldicarb, while animals that carry unc-2(zf35gf) transgene GABAergic neurons were resistant the adicarb (Figure II-4f). Taken together, our data suggests that cell specific expression of unc-2(zf35gf) transgene is sufficient to increase excitability of specific neural subsets.

The characteristic sinusoidal movement of C. elegans is regulated by motor neurons in the ventral nerve cord, which alternate contraction and relaxation of the dorsal and ventral longitudinal muscle rows. When one side of the body wall muscles is activated, the contralateral side is inhibited. Animals that expresses the optogenetic protein, Channelrhodopsin-2 (ChR2), in either cholinergic or GABAergic motor neurons cease their locomotion when these subsets of neurons are activated by light. To dissect the role of cholinergic and GABAergic motor neurons in both generation and propagation of the sinusoidal
movement, we expressed the unc-2(zf35gf) transgene specifically in either class of the motor neurons. We observed that animals with increased cholinergic motor neuron activity have elevated body bend amplitude as well as head motion angle (Figure II-4g and h). By contrast, increased GABAergic motor neuron activity inhibits the head motion angle but does not alter the body bend amplitude (Figure II-4g and h). This decreased head motion is likely due to increased GABA released from the RMEs (Shen et al., 2016). Our data suggests that cell specific expression of unc-2(zf35gf) transgene not only allows us to hyperactivate specific neuron subsets but also to study theirs roles within a given neural circuit in its native context.

The biophysical consequences of the zf35 mutation

To investigate the functional consequences of the CaV2α1/UNC-2(ZF35) mutation, we introduced the human corresponding change, G1518R into the human P/Q type CaV2.1 channel α1 subunit CACNA1A. CaV2.1α1 expression constructs were transfected it in a stable HEK 293 cell line that expresses the auxiliary CaVβ1c and CaVα2δ subunits (Piedras-Renteria et al., 2001). We performed whole cell patch clamp experiments to determine the channel kinetics for the wild type and G1518R channel (Figure II-5a and b). The current-voltage relationships of the G1518R CaV2.1 channel shifted for about 10 mV to more negative potentials compared to the wild-type CaV2.1 channel (Figure II-5c). Furthermore, the maximal current density was 1.7-fold larger for the G1518R
Ca\textsubscript{v}2.1 channels (80.6 ± 5.7 pA/pF, n=11) compared to the wild type (47.5 ± 4.3 pA/pF, n=13). The slope of the activation curve was not significantly affected in the G1518R Ca\textsubscript{v}2.1 channels (Ka\textsubscript{wt} = 3.8 ± 0.2 mV; Ka G1518R = 4.1 ± 0.1 mV, Figure II-5c).

Currents of both wild-type and G1518R Ca\textsubscript{v}2.1 channels decayed with similar mono-exponential time courses (τ\textsubscript{inac wt} = 177 ± 45 ms and τ\textsubscript{inac G1518R} = 196 ± 32 ms at a 0 mV pulse). This suggested that the transition from the open to the inactive states was not affected by the G1518R mutation. To determine if inactivation following closed states were altered we compared steady-state inactivation properties of the wild-type and G1518R Ca\textsubscript{v}2.1 channel (Figure II-5d). The membrane potential at which half of the current was inactivated in the G1518R Ca\textsubscript{v}2.1 channels was 7.7 mV more positive compared to the wild type (V\textsubscript{0.5inact} = -55.0 ± 1.0 mV for wild-type channels and V\textsubscript{0.5inact} = -47.3 ± 1.0 mV for G1518R Ca\textsubscript{v}2.1 channels). The displacement to more depolarized membrane potentials indicates that the proportion of activatable channels at a given membrane potential is increased in G1518R Ca\textsubscript{v}2.1 channels.

Thus, the G1518R mutation in the intracellular linker between TM III and TM IV changes the voltage sensitivity, causing channels to activate at lower membrane potentials and inactivate at higher membrane potentials. Together with the increase in current density, these kinetic properties of the G1518R Ca\textsubscript{v}2.1 channels may lead to elevated synaptic release. The high level of conservation in the TM III-IV linker between \textit{C. elegans} and human strongly
suggest that the corresponding G1132R mutation in *C. elegans* Ca\(\nu\)2\(\alpha\)1/UNC-2 channel would lead to similar gain-of-function effects in activation and inactivation kinetics.

**Transgenic animals carrying FHM1 Ca\(\nu\)2\(\alpha\)1 mutations recapitulate the *unc-2(zf35gf)* mutant hyperactive phenotype**

Mutations in the human *CACANA1A* gene can result in Familial Hemiplegic Migraine type 1 (FHM1) (Pietrobon, 2010). Electrophysiological analysis of the effects of several FHM1 mutations on Ca\(\nu\)2.1 channel function have been shown to increase current density or shift the voltage dependence of activation to more hyperpolarized potentials (Hans et al., 1999a; Kraus et al., 1998; Van Den Maagdenberg et al., 2004). To determine if FHM1 mutations cause similar behavioral effects in *C. elegans* as the *unc-2(zf35gf)* mutation, we introduced two well-characterized human FHM1 mutations, R192Q and S218L, into the *unc-2* gene (Figure II-6a). Pan-neuronal expression of the *unc-2*(FHM1R192Q) or *unc-2*(FHM1S218L) transgene resulted in an increased reversal frequency (25.5 ± 0.9, n=34 and 16.5 ± 0.9, n=33, respectively) compared to the wild-type (4.2 ± 0.5, n=29) (Figure II-6b). Furthermore, the *unc-2*(FHM1R192Q) or *unc-2*(FHM1S218L) transgenic animals displayed hyperactive egg-laying behavior similar to *unc-2(zf35gf)* mutants (Figure II-6c). *unc-2*(FHM1R192Q) or *unc-2*(FHM1S218L) transgenic animals retained fewer eggs in the uterus (5.7 ± 0.4, n=37 and 8.4 ± 0.6, n=32, respectively) than the wild type
(16.5 ± 0.8, n=23), and laid eggs at an earlier developmental stage. Both FHM1 R192Q or S218L mutations in the unc-2 gene recapitulated unc-2(zf35gf) hyperactive behavioral phenotypes, providing clear genetic evidence that the FHM1 mutations are gain-of-function mutations.

**Elevated reversal frequency in unc-2(zf35gf) mutants is glutamate signaling dependent**

Why do CaV2α1/unc-2(zf35gf) mutants have an increased reversal frequency? Five pairs of interneurons (backward locomotion: AVA, AVD, AVE, and forward locomotion: AVB, PVC) are required for the control of directional movement in C.elegans (Chalfie et al. 1985). These pre-motor interneurons make reciprocal electrical and chemical synaptic contacts which mediate a bistable switch for forward and backward movement (Chalfie et al., 1985; Wicks and Rankin, 1995). glr-1, a gene encoding a non-NMDA glutamate receptor subtype, is expressed exclusively in this subset of neurons (Maricq et al., 1995). Interestingly, transgenic animals carrying a gain-of-function mutation in GLR-1 receptor display a rapid alternation between forward and backward movement similar to CaV2α1/unc-2(zf35gf) mutants, and the increased reversal rate is mediated by elevated depolarization frequency of the interneurons (Zheng et al., 1999). We therefore determined whether glutamate signaling is required for the hyper reversal phenotype of unc-2(zf35gf) mutants by analyzing locomotion behavior of unc-2(zf35gf) double mutants with glr-1 and eat-4 which encodes the
vesicular glutamate transporter. The hyperreversal phenotype of \textit{unc-2(zf35gf)} mutants (31.2 ± 1.4 reversals, n=10) was largely suppressed in \textit{glr-1;unc-2(zf35gf)} mutants (11.59 ± 0.8 reversals, n=27) and \textit{eat-4;unc-2(zf35gf)} double mutants (6.18 ± 0.9 reversals, n=22) (Figure II-7), underlining the importance of glutamate-mediated signaling in regulating directional movement. Our results suggest that the increased reversal frequency in \textit{Cav2a1/unc-2(zf35gf)} mutants may be due to an elevated glutamate release from glutamatergic sensory neurons onto pre-motor interneurons that control directional movement.

\textbf{\textit{Cav2a1/unc-2} gain-of-function mutations increase sensitivity to aldicarb}

To determine the functional consequences of \textit{unc-2(zf35gf)} mutation in synaptic transmission, we analyzed their sensitivity to the cholinesterase inhibitor, aldicarb. The \textit{C. elegans} body wall muscles receive excitatory cholinergic inputs. Aldicarb treatment causes the accumulation of acetylcholine (ACh) at the neuromuscular junction inducing hyper contraction of body wall muscles and acute paralysis. When placed on agar plates containing 1mM aldicarb approximately 50% of the wild type animals paralyzed within an hour. Consistent with previous results (Mathews et al., 2003; Miller et al., 1996), less than 10% \textit{unc-2(lf)} mutants paralyzed in this time frame. In sharp contrast, 100% \textit{unc-2(zf35gf)} mutants paralyzed within 30 minutes on aldicarb plates (Figure II-8a). All heterozygous \textit{unc-2(zf35gf)/+} mutants paralyzed in approximately one hour, whereas it took about two hours for all wild-type animals to paralyze,
confirming that \textit{unc-2(zf35gf)} mutation is semi-dominant. Pan-neuronal expression of the \textit{unc-2(zf35gf)} transgene, \textit{unc-2(FHM1R192Q)} or \textit{unc-2(FHM1S218L)} induced hypersensitivity to aldicarb. The hypersensitivity is not due to overexpression of the Ca\textsubscript{v}2 channels since the \textit{unc-2(wt)} transgene restored the locomotion defects in \textit{unc-2(lf)} mutants and wild-type sensitivity to aldicarb (Figure II-8b). Our data indicate gain-of function mutations in Ca\textsubscript{v}2\alpha1/UNC-2 result in increased ACh release.

\textit{unc-2(zf35gf)} mutants have increased cholinergic and decreased GABAergic transmission.

To directly assay the effect of the \textit{unc-2(zf35gf)} mutation on synaptic function we recorded miniature postsynaptic currents (mPSCs) at the body wall muscle. mPSCs are spontaneous release events caused the fusion of a single or a few synaptic vesicles from motor neurons. mPSC recordings were performed at a holding potential of -60mV under conditions which both cholinergic and GABAergic mPSCs appear as inward currents (see Material and Methods). \textit{unc-2(zf35gf)} mutants show a striking two-fold increase in the frequency of total mPSCs compared to wild-type animals with no significant changes in the amplitude (Figure II-9a to c). Since excitatory and inhibitory neurotransmitter systems appear differentially affected in FHM1 mouse models (Tottene et al., 2009), we analyzed the effect of \textit{unc-2(zf35gf)} mutation on cholinergic and GABAergic transmission separately. To isolate ACh currents, recordings were
made in an *unc-49* mutant background, which lacks inhibitory neurotransmission at the NMJ. The frequency of the endogenous excitatory postsynaptic currents (mEPSCs) is increased two-fold compared with the wild type (Figure II-9d to f). In contrast, the endogenous GABAergic postsynaptic currents (mIPSCs) showed a striking reduction in frequency (Figure II-9g to i). Thus, these experiments indicate *unc-2(zf35gf)* mutants shift the E/I balance towards excitatory transmission (McEwen et al., 2006; Vashlishan et al., 2008).

*unc-2(zf35gf)* mutations affect the development of cholinergic and GABAergic synapses

Changes in synaptic activity can modulate the distribution of synaptic proteins (Fauth and Tetzlaff, 2016; Turrigiano, 2012). To determine if the increased UNC-2 activity affects synaptic development, we examined the distribution and morphology of pre-synaptic and postsynaptic markers at the NMJ (Figure II-10a). Cholinergic synapses were labeled with the synaptic vesicle marker RAB-3::mCherry (*Pacr-2:: RAB-3*) and the nicotinic acetylcholine receptor subunits Punc-29::UNC-29::GFP as a post-synaptic markers (Figure II-10b). RAB-3::mCherry puncta were larger in *unc-2(zf35gf)* mutants compared to those in the wild-type background (Figure II-10b and c). This is consistent with the notion that increased calcium influx can recruit more synaptic vesicles to release sites. At the post synaptic sites we found a marked increase in the size of UNC-29::GFP clusters, indicating an increase in the post-synaptic ACh receptors.
To pharmacologically test if the enhanced level of ACh receptor at synapses leads to functional consequences, we examined the response of \textit{unc-2(zf35gf)} mutants to the ACh receptor agonist, levamisole. Levamisole directly binds to UNC-29 ACh receptors in body wall muscles and leads to hypercontracted paralysis (Barbagallo et al., 2010). \textit{unc-2(zf35gf)} mutants were hypersensitive to levamisole induced paralysis, supporting an increased ACh receptor expression at the NMJ (Figure II-11a). These pre- and post-synaptic morphological changes and pharmacological responses indicate that \textit{Ca}\textsubscript{V2a}/unc-2 gain of function mutations strengthen excitatory cholinergic synapses.

To gain insight into the mechanisms of reduced GABA signaling in \textit{unc-2(zf35gf)} mutants we visualized GABAergic synapses with the pre-synaptic vesicle marker RAB-3::mCherry (\textit{Punc-25:: RAB-3}) and the GABA receptor UNC-49::GFP (\textit{Punc-49::UNC-49::GFP}) as a post-synaptic marker. \textit{unc-2(zf35gf)} mutants displayed enhanced expression of the RAB-3::mCherry marker along the axons of GABAergic neurons (Figure II-10e and f), comparable to the increased expression of the presynaptic marker we observed in cholinergic motor neurons. However, in contrast to the increased expression of ACh receptors at the cholinergic synapse, UNC-49::GFP expression was strongly reduced and unevenly distributed in \textit{unc-2(zf35gf)} mutants (Fig.II-10e and g).
To determine if changes in UNC-49::GFP fluorescence reflect functional alterations in endogenous receptors expression, we analyzed the behavioral response of unc-2(zf35gf) mutants to the GABA agonist, muscimol. Muscimol treatment of body-wall muscle cells leads to hyperpolarization by an inward Cl⁻ flux that is dependent on the GABA receptor channel UNC-49 (Richmond and Jorgensen 1999). When touched on the head, wild-type animals treated with 1mM muscimol display a rubberband phenotype in which the body wall muscles contract and then relax without moving backwards (de la Cruz et al., 2003). To quantify muscimol responses, animals were scored according to the presence of a contraction–relaxation cycle and backward movement into five categories from unaffected locomotion (0) to complete flaccid paralysis (4) (see Materials and Methods). On muscimol plates, most wild-type animals displayed flaccid paralysis or rubberband phenotype where the animal failed to move backward in response to touch. In contrast, most unc-2(zf35gf) mutants were able to move away from the touch stimulus (Figure II-11b). The partial resistance of unc-2(zf35gf) mutants to muscimol-induced muscle relaxation is consistent with the reduction of UNC-49 expression at the NMJ and likely the cause of the decreased GABA neurotransmission.

*Increased cholinergic signaling in unc-2(zf35gf) mutants leads to the reduction of GABA receptor*
Why does a gain-function-mutation in the presynaptic Ca\textsubscript{v}2 channel lead to a reduction in postsynaptic GABA receptor clustering at the NMJ? To test if \textit{unc-2(zf35gf)} mediated increases neuronal excitability affect GABA receptor clustering, we analyzed UNC-49::GFP expression in \textit{acr-12; unc-2(zf35gf)} double mutants. Mutations in the gene that encodes the cholinergic ACR-12 receptor, reduce excitability of cholinergic and GABAergic motor neurons (Jospin et al., 2009; Petrash et al., 2013). We found that the loss of ACR-12 restored the GABA receptor clustering in \textit{unc-2(zf35gf)} mutants (Figure II-12c and d). This raises the possibility that increased presynaptic calcium transients and transmitter release from GABAergic motor neurons results in compensatory down regulation of GABA receptors at the post synapse. We therefore analyzed UNC-49::GFP clustering in transgenic animals that specifically express \textit{unc-2(zf35gf)} in GABAergic motor neurons. Animals carrying \textit{punc-47::unc-2(zf35gf)} transgenes are partially resistant to aldicarb indicative of increased in GABAergic signaling onto the body wall muscles (data not shown). Surprisingly, specific expression of \textit{unc-2(zf35gf)} in GABA motor neurons of resulted in increased UNC-49 fluorescence at the NMJ (Figure II-12c and d). This suggests that the increased GABA signaling results in strengthening of GABAergic synapses; and the reduction of GABA receptor clustering in \textit{unc-2(zf35gf)} mutants is not caused by elevated activity of GABAergic neurons.

Could the reduction in GABA receptor expression in \textit{unc-2(zf35gf)} be a secondary effect of the increased cholinergic signaling? Cholinergic activation of
GABAergic neurons has been shown to play a prominent role in the development of GABAergic outputs (Barbagello et al Development in press). Interestingly, hyperactivation of cholinergic motor neurons through a gain-of-function mutation in the acetylcholine receptor, ACR-2, leads to a strong reduction GABAergic signaling (Jospin et al., 2009). To test if cholinergic neurotransmission onto muscles affect the GABA receptor clustering, we genetically eliminated the postsynaptic acetylcholine receptor, UNC-29. Strikingly, the loss of UNC-29 restored the GABA receptor clustering in unc-29;unc-2(zf35gf) animals (Figure II-12c and d), This suggests the increased cholinergic depolarization affects the GABA receptor clustering on body wall muscles.

Discussion

Presynaptic voltage-gated calcium channels (Cav2) are crucial regulators of synaptic transmission. C. elegans has a single predicted neuronal Cav2α1 subunit, encoded by the unc-2 gene (Bargmann, 1998). unc-2 loss of function mutants are lethargic and sluggish, a phenotype similar to mutants with synaptic transmission defects (Schafer and Kenyon 1995; Miller et al. 1996). Here we report the isolation of a novel gain-of-function mutation in the unc-2 gene. unc-2(zf35gf) mutants are hyperactive in sharp contrast to unc-2(lf) mutants. zf35gf mutation results in a G to R substitution in a highly conserved region in the third intracellular linker between TM III and TM IV. Our electrophysiological analysis shows that this G to R substitution in the human Cav2.1 channel CACNA1A
causes a shift in the activation voltage to a more negative potentials and inactivation at higher potentials. These changes in channel properties are similar to those reported for several human FHM1 CaV2.1 channels (Hans et al., 1999a; Müllner et al., 2004; Tottene et al., 2005). While in heterologous expression system studies the effects on channel properties vary, most FHM1 mutations appear to lead to channel activation at lower voltages and increased channel open probability. The G1132R mutation increases calcium influx at lower membrane potentials, which would lead to an increase in neurotransmitter release probability. Interestingly, a similar G to R mutation in an intracellular linker of the human CaV1.2 channel results in defects in channel inactivation and is the underlying cause of Timothy syndrome, which is an autosomal dominant neurological disorder with heart QT-prolongation, heart arrhythmias, structural heart defects as well as autism spectrum disorders (Splawski et al., 2004). The G1132R mutation may change the CaV2 channel function by directly affecting the electrophysiological properties of CaV2α1/UNC-2, the central pore of the channel. Intracellular linkers of CaV2 channels have also been implicated in the modulation of channel kinetics through phosphorylation regulation as well as associating with the intracellular effector proteins, such as CaVβ subunit, G protein βγ subunits and calmodulin (Zamponi and Snutch, 2002). Thus we cannot exclude that the G1132R mutation affects the interaction with cytoplasmic proteins that modulate channel function.
Intragenic suppressors of the *unc-2(zf35gf)* mutant resemble those found in EA2 patients and include premature stop codons and missense mutations. Interestingly, similar to our C324Y *unc-2(zf35gf)* suppressor, a cysteine to tyrosine substitution (C287Y) in the domain I S5-S6 loop of CACNA1A has been associated with human EA2 patients (Wan et al., 2005). Furthermore, amino acid substitutions in a highly conserved leucine in the domain IV S5-S6 loop are found in both human EA2 patients (L1749P) and our *unc-2(zf35gf)* suppressor (L1357F). Thus *C. elegans* can be an efficient model to determine the *in vivo* consequences of CaV2α1 subunit mutations. While truncated mutations in EA2 show dominant-negative suppression (Jeng et al., 2008; Raike et al., 2007), *unc-2(zf35gf)* intragenic suppressors are recessive. This is likely due to the much longer intracellular loop between domain I and II in human CACNA1A. This region has been shown to be required for the dominant-negative suppression through interacting with cytoplasmic proteins including the CaVβ subunits (Raike et al., 2007).

Gain of function mutations in the *CACNA1A* gene, which encodes the human CaV2.1 α1 subunit, have been identified in patients with Familial Hemiplegic Migraine 1 (FHM1) (Hans et al., 1999a; Tottene et al., 2005). Data from the FHM1 knock-in mice suggest that the gain-of-function CaV2.1/CACNA1A channels exhibit an increase in calcium influx and are activated at lower membrane potentials (Tottene et al., 2005), which are similar functional consequences to the *zf35(G1132R)* mutation in CaV2.1/CACNA1A.
channels. Interestingly, transgenic animals that express corresponding FHM1 mutations in *unc-2* transgenes recapitulate the behavioral hyperactivity of *unc-2*(zf35gf) mutants. Together with the patch clamp recoding from the human G1518R CaV2.1 channels, our results provide an important proof-of-principle that the molecular function of CaV2α1 is highly conserved from *C. elegans* UNC-2 worms to human CACNA1A. Therefore the *unc-2*(zf35gf) mutant provides a powerful genetic handle to study the underlying molecular mechanisms of FHM1 pathology.

*unc-2*(zf35gf) mutants are extremely sensitive to the acetylcholinesterase inhibitor aldicarb, and show a two-fold increase in frequency of the endogenous postsynaptic currents. This is in sharp contrast with *unc-2* loss of function mutants, which are resistant to alidicarb (Miller et al., 1996) and have a significant reduction in endogenous postsynaptic currents (Richmond, Weimer, and Jorgensen 2001). This indicates that the shift to lower voltage of activation and a slow inactivation of the gain-of-function UNC-2 channel leads to a significant increase of synaptic transmission at the NMJ. Surprisingly, recordings from cholinergic and GABAergic synapses separately in *unc-2*(zf35gf) mutants showed the increased neurotransmission is specific to cholinergic synapses, and a significant reduction in GABA transmission was observed. We further show that this reduction is likely due to a decrease in GABA receptor expression at the postsynaptic domains and is dependent on the activation of muscle acetylcholine receptors. In cultured hippocampal neurons, enhanced excitatory synaptic
activity has been reported to decrease the cluster size of GABA$_A$Rs and GABAergic neurotransmission through NMDA receptor-dependent calcium influx and calcineurin dephosphorylation (Bannai et al., 2009, 2015). These studies are consistent with our findings in C. elegans and suggest a potential conserved mechanism of GABA receptor regulation. We speculate that constitutively activation of nicotinic receptors leads to sustained depolarization of body wall muscles, which in turn activates Cav1 channels. The elevated calcium influx then triggers calcium-dependent signaling and causes the dispersion or internalization of the GABA receptor (Figure II-13). Imbalanced excitation-inhibition neurotransmission has been associated with FHM1 (Vecchia and Pietrobon, 2012). Elucidation of underlying mechanisms of GABA receptor reduction in unc-2(zf35gf) mutants may provide insight into FHM1 pathogenesis.

In addition to analyzing behavioral and functional consequences of the unc-2(zf35gf) mutation, we showed that cell-specific expression of the unc-2(zf35gf) transgene is sufficient to recapitulate hyperactive behaviors. Transgenic animals that express the unc-2(zf35gf) transgene in the backward locomotion interneurons exhibit an increased reversal frequency, while expression in the hermaphrodite specific neurons (HSN) produces hyperactive egg-laying. This suggests that the unc-2(zf35gf) transgene can be used as a tool to genetically hyper-activate specific neurons by increasing neurotransmitter release in neurons of interest. Instead of acutely depolarizing the neurons, maximizing the output from particular neurons, the unc-2(zf35gf) transgene presents a way to
sensitize the neurons and chronically increases synaptic release from the neurons. This offers an opportunity to modulate neurons and circuits in the context of their natural circuit activity, and study the effect of chronic hyperactivity on circuit development and function.

**Materials and Methods**

**Strains**

All strains were cultured at room temperature (22-24 °C) on nematode growth media (NGM) agar plates with the *E. coli* strain OP50 as a food source. Experiments were performed on young adult animals (24 hr post-L4 larva) at room temperature (22-24 °C). The wild-type strain was Bristol N2. Transgenic strains were obtained by microinjection of plasmid DNA into the germline with coinjection marker *lin-15* rescuing plasmid pL15EK both at 80 ng/µl into *unc-2(e55); lin-15(n765ts)* or *lin15(n765ts)* animals. At least three independent transgenic lines were obtained. The data presented are from a single representative line. The following strains were utilized in this study:

CB55 *unc-2 (e55)*

QW37 *unc-2 (zf35gf)*

QW355 *unc-2 (zf109)*

QW359 *unc-2 (zf113)*

QW360 *unc-2 (zf114)*

QW441 *unc-2 (zf115)*
Transgenic strains were obtained by microinjection of plasmid DNA into the germline with coinjection marker lin-15 rescuing plasmid pL15EK both at 80 ng/µl into unc-2(e55); lin-15(n765ts) or lin15(n765ts) animals. At least three independent transgenic lines were obtained. The data presented are from a single representative line.

Molecular Biology and plasmids
Full length unc-2(wt) and unc-2(zf35gf) rescuing clones were obtained from Y. Saheki and C. Bargmann. Briefly, the unc-2 cDNA was cloned behind the pan neuronal promoter, Ptag-168, using restriction sites Ascl and XhoI to generate the Ptag-168::unc-2(wt) construct. The unc-2(zf35gf) mutation was introduced in the cDNA clone using site-directed mutagenesis. For cell-specific unc-2(zf35gf) transgene expression, cell specific promoters Pnmr-1, Pglr-1, Prig-3, Plgc-55s, Ptph-1 and Pacr-2 were amplified by PCR with FseI sequences at 5’ end and Ascl sequences at 3’ end. The Ptag-168::unc-2(zf35gf) construct was digested with FseI and Ascl to remove the Ptag-168 promoter and replace with cell specific promoters of interest.

Isolation of unc-2(zf35gf) mutants, Mapping and Cloning

unc-2(zf35gf) allele was isolated in a screen for animals that were resistant to the immobilizing effects exogenous tyramine as previously described. We mapped unc-2(zf35) to LG X based on its hyperactive locomotion phenotype using SNP mapping. Three-factor mapping placed unc-2(zf35) to the left of lon-2 and close to dpy-3.

Isolation and identification of intragenic unc-2(zf35gf) suppressors

unc-2(zf35gf) L4 animals (P0) were mutagenized with 0.5 mM N-ethyl-N-nitrosourea (ENU) for 4 hr. Approximately 10,000 F1 animals were bleached to obtain F2 eggs. F2 eggs were plated on NGM plates containing 0.25 mM alicarb
and examined for viable progeny after 7 and 14 days. Aldicarb resistant animals were individually transferred to fresh NGM plates, and their progeny were retested for aldicarb resistance. All suppressors isolated from the screen were first backcrossed with wild-type N2. Suppressors that showed linkage to the X-chromosome were tested for complementation with unc-2(lf) mutants. Molecular changes in unc-2 intragenic suppressors were identified by DNA sequencing of the unc-2 gene.

Behavioral and pharmacological assays

Spontaneous reversal frequency was scored on NGM plates with freshly seeded OP50. The animals were transferred from their culture plate to a new plate, and allowed to recover for 1 min. After the recovery period the number of reversals was counted for 3 min. To quantify the instantaneous velocity and average forward velocity, animals were transferred from their culture plate to a new NGM plate seeded with a thin bacterial lawn and allowed to recover for 1 min. After the recovery period the animals were tracked for 90 sec using a single worm tracker 50. Videos were recorded at 30 frames per second and each frame was analyzed with CoLBeRT associated software 51 to measure instantaneous velocity of single animals. Reversals, as well as 10 frames before and following each reversal, were discarded from the average forward velocity. To examine defects in movement, individual young adult worms were transferred into 96-well plates containing 50 µl M9 buffer in each well. After a 30 sec recovery period,
body bends were counted for 30 sec. A body bend was defined as a change in direction of bending at the mid-body. Egg-laying assays were performed as described by Koelle and Horvitz (1996). Rates of egg-laying behaviors were measured by two different assays: 1) the numbers of unlaid fertilized eggs accumulated inside of adult animals, and 2) the developmental stages of freshly laid eggs. Briefly, in both assays, L4 larvae were isolated and allowed to develop for 40 hr. In the first method, the adults were then incubated in 96-well plates containing 1% sodium hypochlorite until the bodies were dissolved. In the second method, the adults were transferred to a fresh plate. After 30 min, the developmental stage of each freshly laid egg was determined by viewing under a high magnification dissecting microscope.

All pharmacological assays were done in 3 cm petri dishes. For aldicarb and levamisole assay, young adult animals were transferred to NGM plates supplemented with final concentration 1 mM aldicarb and 0.5 mM levamisole respectively. The percentage of paralyzed animals was scored at 15 min intervals until every assayed animal was paralyzed. Animals were scored as paralyzed when they did not move when prodded with a platinum wire. For muscimol assays, young animals were placed onto NGM plates containing 1 mM muscimol and allowed to equilibrate for 1 hr. The rubberband response was scored by gently touching each worm across its body, just posterior to the pharynx. Each worm was scored five times. The responses to touch were scored according to the presence of a contraction–relaxation cycle and backward
movement in the following manner (de la Cruz et al., 2003): 0, worms did not contract and relax but moved away from the touch; 1, worms quickly contracted and relaxed and moved away from the touch; 2, worms contracted and relaxed while concurrently generating a small backward displacement (less than one-half of body length); 3, worms contracted and relaxed but failed to move backwards; 4, worms incompletely contracted and relaxed and produced no displacement.

**Electrophysiology**

A stable HEK293 cell line expressing the calcium channel auxiliary subunits β1c and α2δ 24 was used to transiently transflect 5 µg of the wild-type or G1518R CaV2.1 α1 subunit using the calcium phosphate method. A plasmid encoding the green fluorescent protein (pGreen lantern) was also transfected to allow identification of transfected cells. Cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum and 1000 U/ml penicillin–streptomycin.

Whole-cell inward currents were recorded 24–36 hr after transfection with a HEKA EPC-9 patch clamp amplifier. Recordings were filtered at 2 kHz and acquired using Patchmaster software (HEKA). The extracellular recording solution contained 5 mM BaCl2, 1 mM MgCl2, 10 mM HEPES, 40 mM TEACl, 10 mM glucose, and 87.5 mM CsCl, pH 7.4. Typically the pipettes exhibited resistances ranging from 2 to 4 MΩ and were filled with internal solution
containing: 105 mM CsCl, 25 mM TEACl, 1 mM CaCl2, 11 mM EGTA, and 10 mM HEPES, pH 7.2.

Cell capacitance (16.7 ± 6.7 pF; \(n = 24\)) and series resistance (9.7 ± 4.6 MΩ before compensation; \(n = 24\)) were measured from the current transient after a voltage pulse from -80 to -90 mV. Series resistance was typically compensated by 80–90%. Cells with large currents in which errors in voltage control might appear were discarded. I-V curves were generated by measuring the peak currents obtained after stepping the membrane potential from a holding potential of -120 mV to voltages between -55 and 40 mV in 5 mV increments for 200 msec. I-V curves were fitted with Equation 1.: 

\[
I = G( G - E_{rev}) (1+\exp(V0.5-V)/ka) -1
\]

where \(G\) is membrane conductance, \(E_{rev}\) is the reversal potential, \(V0.5\) is the midpoint, and \(ka\) the slope of the voltage dependence. Current densities were obtained by dividing the current peak amplitude to the cell capacitance for each experiment.

To measure steady-state inactivation profiles, conditioning prepulses (10 sec) from -90 to 20 mV in 10mV steps were applied, and the membrane was then stepped to the peak of the I–V curve. Currents were normalized to the maximal value obtained at the test pulse and plotted as a function of the prepulse potential. Data were fitted with Boltzmann equations: 

\[
\frac{I}{I_{max}} = \frac{1 + \exp((V-V0.5)/kin)}{-1}
\]

Data analysis was performed using the IgorPro software (WaveMetrics Inc., Lake Oswego, OR); figures, fitting and statistical analysis were done using
the SigmaPlot software (version 11.0; Systat Software Inc.). Data are presented as mean ± SD. Significant differences were determined using Student’s t test with the significance value set at \( p > 0.01 \).

Spontaneous postsynaptic currents were recorded from body wall muscles as previously described [Francis et al, 2005 Neuron]. All electrophysiology experiments were carried out at room temperature. The extracellular solution consisted of 150 mM NaCl, 5 mM KCl, 4 mM MgCl2, 1 mM CaCl2, 15 mM HEPES, and 10 mM glucose (pH 7.4, osmolarity adjusted with 20 mM sucrose). The intracellular fluid (ICF) consisted of 25 mM K-gluconate, 115 mM KCl, 0.1 mM CaCl2, 50 mM HEPES, 5 mM Mg-ATP, 0.5 mM Na-GTP, 0.5 mM cGMP, 0.5 mM cAMP, and 1 mM BAPTA (pH 7.4, osmolarity adjusted with 10 mM sucrose). The membrane potential was clamped at -60 mV. Data were digitized at 6.67 kHz and low pass filtered at 3.3 kHz. Membrane capacitance and the series resistance (at least 20%, up to 60%) were compensated, and only recordings in which the series resistance was stable throughout the course of the recording were included. Endogenous synaptic activity data were collected in continuous mode (saved as 30-s recording sweeps). Data analysis and graphing were performed using Excel (Microsoft), Igor Pro (WaveMetrics Inc.), and GraphPrism (GraphPad Software). Mini Analysis software (Synaptosoft Inc.) was used to detect and analyze the endogenous events off-line.

Microscopy
Animals were mounted on 2% agarose pads containing 60 mM sodium azide for 5 minutes before being examined for fluorescent protein expression and localization patterns. Images were captured under consistent detector settings with a Hamamatsu Photonics C2400 CCD camera on a Zeiss Axioplan2 Imaging System. Images were projected into a single plane using NIH ImageJ software.
Figure II-1. *zf35* animals are hyperactive in locomotion and the egg-laying behavior. (a) Representative traces from single worm tracking showing instant velocity of indicated genotypes on OP50 thin lawn plates. Positive and negative values indicate forward and backward locomotion, respectively. *zf35* mutants have increased velocity and reversal numbers compared with wild-type animals.
(b) Shown is the average instant forward velocity from single worm analysis for the wild type (0.118 ± 0.01 wormlength/sec, n=9), zf35 (0.156 ± 0.01 wormlength/sec, n=10), zf35/+ (0.155 ± 0.01 wormlength/sec, n=10) (c) Quantification of the reversal frequency in 3 minutes on regular OP50 plates: average reversal numbers made by wild type (6.8 ± 0.4 reversals, n=59), zf35 (43.1± 2.0 reversals, n=59) and zf35/+ (33.2 ± 1.6 reversals, n=23). Error bars represent SEM for at least three trials totaling 60 animals. Statistical difference from wild type *p<0.05, ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test (d) Representative Nomraski (DIC) images of unlaid eggs in adult wild type and zf35 animals. Arrows indicate eggs; asterisk denotes the position of the vulva. The average numbers of eggs in the uterus: wild type (14.1 ± 0.6 eggs, n=80), zf35 (3.6 ± 0.2 egg, n=86). (e) Distribution of stages of freshly laid eggs of wild type (1-16 cell stage: 5.2 ± 4.3%, 17-comma stage: 94.3 ± 4.5%, post comma stage: 0.8 ± 1.8%) and zf35 mutants (1-16 cell stage: 42.5 ± 16.7%, 17-comma stage: 57.5 ± 16.7%, post comma stage: 0%). Five independent trials totaling 75 animals for each genotype. Statistical difference from wild type ****p<0.0001, Chi-square.
Figure II-2. *zf35* is a novel allele of the CaV2α1 subunit gene *unc-2*. (a) The genetic map and gene structure of *unc-2*. Coding sequences are represented as black boxes. The *zf35* allele is a single nucleotide transition (GGA -> AGA) resulting in a glycine to arginine (G -> R) amino acid substitution at position 1132. (b) Diagram of the secondary structure of UNC-2. UNC-2 consists of four TM domains (TM I – TM IV) each containing six alpha-helix segments (S1 – S6). The UNC-2 (G1132R) mutation localizes in the intracellular loop between transmembrane (TM) domain III and IV, indicated by the blue circle. Purple
circles indicate positions of intragenic unc-2(zf35) suppressors, red circles indicate the location of human FHM1 mutations. (c) The G1132R mutation occurs in a highly conserved region of the CaV2 α1 subunit. Amino acid alignment of the transmembrane III alpha-helix segment 6 (TM III S6) and third intracellular loop of CaV2 α1 subunits from human (H.sapiens, CACNA1A), rainbow fish (P.reticulata, cacna1a), fly (D.melanogaster, Cacophony) and nematode (C. elegans. UNC-2). Identities are shaded in dark grey, similarities in light grey. Location of the G1132R mutation is indicated.
Figure II-3. CaV2α1/unc-2(zf35) mutation is a gain-of-function allele of unc-2

(a) Quantification of the reversal frequency: wild type (6.5 ± 0.4, n=73), unc-2(zf35gf) (41.0 ± 2.0, n=69), unc-2(lf) (2.2 ± 0.3, n=63), unc-2(lf) rescued with unc-2(wt) transgene (7.5 ± 0.6, n=10) and unc-2(lf) rescued with unc-2(zf35gf) transgene (29 ± 2.9, n=26). Statistical difference from wild type ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test (b) Intragenic unc-2(lf) suppressors suppress unc-2(zf35gf) hyperactive locomotion. Shown are numbers of thrashes in 30 seconds in M9 for the wild type (107.0 ± 14.0, n=60), unc-2(zf35gf) (128.1 ± 13.5, n=60), unc-2(lf) (4.8 ± 2.1, n=60), unc-2(zf35gf);unc-2(zf109) (6.9 ± 4.3, n=60); unc-2(zf35gf);unc-2(zf113) (5.6 ± 3.7 thrashes, n=60);
unc-2(zf35gf);unc-2(zf114) (80.2±9.9, n=60); unc-2(zf35gf);unc-2(zf115) (6.9±3.8, n=60); unc-2(zf35gf);unc-2(zf124) (5.3±3.1, n=60); unc-2(zf35gf);unc-2(zf130) (67.1±22.5, n=60); unc-2(zf35gf);unc-2(zf134) (31.2±17.9, n=60). Error bars represent SEM. Statistical difference from unc-2(zf35gf) mutants ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test. (c) Representative images of GFP tagged UNC-2(wt) and UNC-2(zf35gf) in the ventral nerve cord. Asterisks point the cell bodies of the motor neurons and arrows indicate the presynaptic sites. Both constructs are expressed under pan-neuroanal promoter tag-168. Scale bar, 10 µm.
### Transgene Neurons

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Neurons</th>
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</thead>
<tbody>
<tr>
<td>Ptph-1::UNC-2GF</td>
<td>all neurons</td>
</tr>
<tr>
<td>Ptgc-55s::UNC-2GF</td>
<td>AVB</td>
</tr>
<tr>
<td>Prig-3:: UNC-2GF</td>
<td>AEA, AVD, AVE, PVC</td>
</tr>
<tr>
<td>Pnmr-1::UNC-2GF</td>
<td>VB, DB, VA, DA</td>
</tr>
<tr>
<td>Pacr-2::UNC-2GF</td>
<td>RME, DD, VD</td>
</tr>
<tr>
<td>Punc-47::UNC-2GF</td>
<td>HSN</td>
</tr>
</tbody>
</table>

### Egg laying

- **Egg-laying**: HSN
- **Loeomotion**: PVC, AVB, AVA

#### Locomotion:
- **Forward locomotion**: AVB, PVC
- **Backward locomotion**: AVA, AVD

### Percentage of Laid Eggs

- **1 to 16-cell postcomma**: 100%
- **17-cell to comma postcomma**: 0%

### Reversals in 90 secs

- **Adicarb induced paralyzed (%):**
  - Wild type: 0%
  - unc-2(zf35gf): 100%
  - Ptph-1::UNC-2GF: 100%
  - Pacr-2::UNC-2GF: 100%
  - Ptph-1::UNC-2GF: 100%
Figure II-4. Cell-specific expression of the *unc-2(zf35gf)* transgene is sufficient to hyperactivate targeted neurons. (a) List of transgenes and expression patterns. *Ptag- 168, Plgc-55s(AVB), Prig-3, Pnmr-1, Pacr-2, Punc-47* and *Ptph-1*. (b) Schematic representation of neurons in egg-laying circuit and locomotion circuit. Synaptic connections as described by White *et al*. (c) Average numbers of eggs in the uterus of adult wild type (14.1 ± 0.6 eggs, n=80), *unc-2(zf35gf)* (3.6 ± 0.2, n=86) and *Ptph-1::unc-2(gf)* (7.9 ± 0.4, n=56). Animals carrying transgene which does not affect the egg laying circuit *Pacr-2::unc-2(gf)* are used as control (13.5 ± 0.7, n=20). Error bars represent SEM for at least three trials totaling 60 animals. (d) Distribution of stages of freshly laid eggs by indicated genotypes. *unc-2(zf35gf)* mutants and *Ptph-1::unc-2(gf)* animals lay eggs at an earlier stage (1-16 cell stage) compared with wild-type animals. ****p<0.0001, Chi-square. (e) Average numbers of reversals in 90 seconds of
wild type (4.1 ± 0.5, n=37), \textit{unc-2}(zf35gf) mutants (26.67 ± 2.2, n=9), \textit{Ptag-168}:\textit{unc-2}(gf) (24.80 ± 2.9, n=10), \textit{Plgc-55s}:\textit{unc-2}(gf) (3.1 ± 0.4, n=30), \textit{Prig-3}:\textit{unc-2}(gf) (7.1 ± 0.8, n=16), \textit{Pnmr-1}:\textit{unc-2}(gf) (14.0 ± 1.6, n=11) and \textit{Pacr-2}:\textit{unc-2}(gf) (2.2 ± 0.5, n=10), on OP50 thin lawn plate food. Error bars represent SEM for at least three trials totaling 60 animals. (f) Quantification of movement on 1 mM aldicarb. Each data point represents the mean ± SEM of the percentage of animals paralyzed every 15 minutes. \textit{Pacr-2}:\textit{unc-2}(gf) animals are more sensitive to 1 mM aldicarb compared with wild type, while \textit{Punc-47}:\textit{unc-2}(gf) animals are resistant to 1 mM aldicarb. Animals carrying transgene which does not affect the NMJ acetylcholine release \textit{Ptph-1}:\textit{unc-2}(gf) animals are used as control. (g) Maximum amplitude during movements on OP50 thin lawn plates of indicated genotypes: wild type (148.6 ± 4.1, n=22), \textit{unc-2}(zf35gf) mutants (200.5 ± 5.8, n=11), \textit{Plgc-55s}:\textit{unc-2}(gf) (153.1 ± 6.2, n=22), \textit{Prig-3}:\textit{unc-2}(gf) (158.5 ± 11.6, n=11), \textit{Pacr-2}:\textit{unc-2}(gf) (183.6 ± 6.3, n=13) and \textit{Punc-47}:\textit{unc-2}(gf) (146 ± 3.9, n=12). (h) Head tip motion during movements on OP50 thin lawn plates of indicated genotypes: wild type (0.72 ± 0.02, n=23), \textit{unc-2}(zf35gf) mutants (1.01 ± 0.03, n=12), \textit{Plgc-55s}:\textit{unc-2}(gf) (0.68 ± 0.02, n=23), \textit{Prig-3}:\textit{unc-2}(gf) (0.64 ± 0.02, n=12), \textit{Pacr-2}:\textit{unc-2}(gf) (0.79 ± 0.02, n=14) and \textit{Punc-47}:\textit{unc-2}(gf) (0.62 ± 0.02, n=13). Error bars represent SEM. Statistical difference from wild type * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, One way ANOVA with Dunnett’s multiple comparisons test.
Figure II-5. The UNC-2(ZF35) corresponding G1518R mutation in human Ca\textsubscript{v}2\textalpha{}1 subunit results in increased channel activity. (a) Representative macro-currents of wild type and G1518R Ca\textsubscript{v}2 channels. Currents were generated by stepping membrane potential to voltages between -55 and 40 mV in 5mV increments for 200 ms from a holding potential of -120 mV. (b) Voltage dependence of whole-cell current density for wild type and G1518R Ca\textsubscript{v}2 channels. Current density values were obtained by dividing current amplitudes and cell capacitance. (Wild type, n=9; G1518R, n=11). (c) Voltage dependence of Ba\textsuperscript{2+} current activation. The activation curve of G1518R exhibits a significant
shift of the $V_{0.5}$ value towards more negative membrane potentials. (d) Steady-State Inactivation curves. The G1518R mutation causes a slight positive shift in the midpoint voltage in the steady-state inactivation curves ($V_{0.5\text{inact}} = -55.0 \pm 1.0$ and $-47.3 \pm 1.0$ for wild type and G1518R, respectively). Currents were normalized to the maximal value obtained at the test pulse and plotted as a function of the prepulse potential. Data were fitted with the Boltzmann equation:

\[
\frac{I}{I_{\text{max}}} = \frac{1 + \exp\left(\frac{V - V_{0.5}}{k_{\text{in}}}\right)}{1 + \exp\left(\frac{V - V_{0.5}}{k_{\text{in}}^2}\right)}
\]
Figure II-6. FHM1 mutations in *unc-2* result in a hyperactive phenotype.

(a) The amino acid alignment of the conserved region of transmembrane domain I membrane-spanning segments 4 (TM I S4) and first intracellular loop from human (CACNA1A) and worm (UNC-2) CaV2.1α1 subunits. Identities are dark grey and similarities are light grey. Indicated are the known human FHM1 mutations: R192Q (Hans et al., 1999b) and S218L (Tottene et al., 2005). (b) Transgenic animals expressing pan-neuronal *unc-2* with either the R192Q or S218L mutation have an increased reversal rate compared to wild type. Shown is the average number of reversals in 90 seconds of wild type (4.2 ± 0.5, n=29), *unc-2(zf35gf)* (30.3 ± 1.2, n=20), *Ptag-168:: unc-2(R192Q)* (25.5 ± 0.9, n=34),
and *Ptag-168:: unc-2(R192Q)* (16.5 ± 0.9, n=33). (c) Transgenic animals expressing pan-neuronal *unc-2* with either the R192Q or S218L mutation display hyperactive egg laying behavior. Average numbers of eggs in the uterus of are: adult wild type (16.5 ± 0.8 eggs, n=23), *unc-2(zf35gf)* (2.7 ± 0.2, n=35), *Ptag-168:: unc-2(R192Q)* (5.7 ± 0.4, n=37), and *Ptag-168:: unc-2(S218L)* (8.4 ± 0.6, n=32). Each bar represents the mean ± SEM for at least three trials, totaling a minimum of 30 animals. Statistical difference from wild-type, ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons test.
Figure II-7. GLR-1 mediated signaling contributes to the hyperreversal phenotype of *unc-2(zf35gf)* mutants. Quantification of reversal numbers in 90 seconds of indicated genotypes. Average reversal numbers made by wild type (3.48 ± 0.7 reversals, n=15), *unc-2(zf35gf)* (31.2 ± 1.4 reversals, n=10), *eat-4* (0.78 ± 0.3 reversals, n=13), *glr-1* (0.5 ± 0.2 reversals, n=12), *eat-4; unc-2(zf35gf)* (6.18 ± 0.9 reversals,) and *glr-1; unc-2(zf35gf)* (11.59 ± 0.8 reversals, n=27). Error bars represent S.E.M. * represents statistical difference from the wild type, **** p<0.0001, one way ANOVA with Dunnett’s multiple comparisons test.
Figure II-8. *unc-2(zf35gf)* mutants have increased ACh neurotransmission.

(a) Quantification of movement on 1 mM aldicarb. Each data point represents the mean ± SEM of the percentage of animals paralyzed every 15 minutes. 50% of the wild type animals were paralyzed at 60 minutes. *unc-2(lf)* animals were resistant to the effects of aldicarb and reached 50% paralysis at 90 minute. Homozygous *unc-2(zf35gf)* mutants were sensitive to aldicarb; 50% of the *unc-2(zf35gf)* mutants were paralyzed at 20 minute. Heterozygous *unc-2(zf35gf)* mutants have 50% paralysis at 40 minutes, which further confirmed that the *unc-2(zf35gf)* allele is semi-dominant. (b) Quantification of paralysis percentage on 1 mM aldicarb at the 60-minute time point: 55.5% ± 4.5 of wild type, 56.7% ± 3.3 of *Ptag-168::unc-2(wt)*, 98.3% ± 3.3 of *Ptag-168::unc-2(zf35gf)*, 27.1% ± 7.3 of *unc-2(lf)* animals, 54.8% ± 2.9 of *Ptag-168::unc-2(R192Q)* transgene in *unc-2(lf)* background, 100% of *Ptag-168::unc-2(zf35gf)* transgene in *unc-2(lf)* background, 100% of *Ptag-168::unc-2(S218L)* transgene in *unc-2(lf)* background and 100%
*Ptag-168::unc-2(S218L)* transgene in *unc-2(lf)* background. Three independent trials with totaling 50 animals for each genotype. ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons test.
**Cholinergic frequency**

wild type
unc-2(zf35gf)
unc-49(e407)
unc-49(e407); unc-2(zf35gf)

**GABAergic frequency**

wild type
unc-2(zf35gf)

**Total mPSC frequency**

wild type
unc-2(zf35gf)
unc-49(e407); unc-2(zf35gf)

**Total mPSC amplitude**

wild type
unc-2(zf35gf)
unc-49(e407); unc-2(zf35gf)

**Cholinergic amplitude**

wild type
unc-2(zf35gf)
unc-49(e407)
unc-49(e407); unc-2(zf35gf)

**GABAergic amplitude**

wild type
unc-2(zf35gf)
Figure II-9. *unc-2(zf35gf)* mutants have increased cholinergic and decreased GABAergic transmission. (a) Representative traces of total endogenous postsynaptic currents recorded (mPSCs) from ventral body wall muscles in wild-type and *unc-2(zf35gf)* mutants. (b and c) Average endogenous mPSCs frequency and amplitude of wild-type and *unc-2(zf35gf)* mutants. (d) Representative traces of endogenous cholinergic mEPSCs in wild-type and *unc-2(zf35gf)* mutants. (e and f) Average mEPSCs frequency and amplitude of wild-type and *unc-2(zf35gf)* mutants. (g) Representative traces of endogenous GABAergic mIPSCs in wild-type and *unc-2(zf35gf)* mutants. (h and i) Average mIPSCs frequency and amplitude of wild-type and *unc-2(zf35gf)* mutants. Error bars depict SEM. *p<0.05, **p<0.01, two-tailed Student’s t test.
a

Cell Body

Ventral Cord

presynaptic sites

Muscle

postsynaptic receptors

b

b

wild type

unc-2(zf35gf)

Cholinergic synapses

UNC-29: RAB-3::mCherry

C

Presynaptic RAB-3

Normalized Fluorescence

0 1 2 3 4

wild type

unc-2(zf35gf)

 UNC-29::GFP

d

ACh receptor UNC-29

Normalized Fluorescence

0.0 0.5 1.0 1.5 2.0 2.5

wild type

unc-2(zf35gf)

e

GABAergic synapses

UNC-49::mCherry

f

Presynaptic RAB-3

Normalized Fluorescence

0.0 0.5 1.0 1.5 2.0 2.5

wild type

unc-2(zf35gf)

 UNC-49::GFP

g

GABA receptor UNC-49

Normalized Fluorescence

0.0 0.5 1.0 1.5 2.0

wild type

unc-2(zf35gf)
**Figure II-10.** *unc-2(zf35gf)* mutants have decreased GABA receptor expression at the NMJ. (a) Schematic of the neuromuscular junction (NMJ) of the ventral nerve cord (VNC) motorneurons. *C. elegans* forms en passant synapses, with presynaptic regions along the length of the ventral nerve cord, indicated as red. Postsynaptic receptors expressed in muscle cells represented as green. (b) Representative images of cholinergic synapses in wild type and *unc-2(zf35gf)* mutants. Presynaptic sites are labeled with synaptic vesicle marker RAB-3::mCherry while postsynaptic nicotinic acetylcholine receptors are labeled by UNC-29::GFP. Scale bar represents 10 µm. (c and d) Quantification of the fluorescence intensity of RAB-3::mcherry and UNC-29::GFP along the nerve cord at cholinergic synapses in wild type and *unc-2(zf35gf)* mutants. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized fluorescence of cholinergic RAB-3::mCherry: 0.99 ± 0.76, n=21 in wild type and 1.36 ± 0.18, n=14 in *unc-2(zf35gf)* mutants. UNC-29::GFP: 0.97 ± 0.05, n=35 in wild type and 1.19 ± 0.06, n=38 in *unc-2(zf35gf)* mutants. (e) Representative images of GABAergic synapses in wild type and *unc-2(zf35gf)* mutants. Presynaptic sites are labeled with synaptic vesicle marker RAB-3::mCherry while postsynaptic GABA receptors are labeled by UNC-49::GFP. Scale bar represents 10 µm. (f and g) Quantification of the fluorescence intensity of RAB-3::mcherry and UNC-49::GFP along the nerve cord at GABAergic synapses in wild type and *unc-2(zf35gf)* mutants. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized
fluorescence of GABAergic RAB-3::mCherry: 1 ± 0.07, n=15 in wild type and 1.25 ± 0.08, n=20 in unc-2(zf35gf) mutants. UNC-49::GFP: 1 ± 0.09, n=15 in wild type and 0.75 ± 0.06, n=20 in unc-2(zf35gf) mutants. For all the quantification above, error bars depict SEM. *p<0.05, **p<0.01, two-tailed Student’s t test.
Figure II-11. *unc-2(zf35gf)* mutants are resistant to the GABA agonist **mucimol**. (a) Quantification of movement on 0.5 mM levamisole. Each data point represents the mean ± SEM of the percentage of animals paralyzed by levamisole every 15 minutes for at least three trials, totaling a minimum of 50 animals. (b) *unc-2(zf35gf)* mutants are resistant to the muscimol-induced muscle relaxation compared with the wild type in rubberband phenotype. Shown is the percentage of animals that displayed the indicated phenotype on 1 mM muscimol after 60 min. Wild type animals: category 0: 0%, category 1: 3 ± 2.9%, category 2: 17 ± 5.8%, category 3: 62 ± 2.7% and category 4: 32 ± 13.7%. *unc-2(zf35gf) mutants*: category 0: 12 ± 3.3%, category 1: 33 ± 2.2%, category 2: 26 ± 2.4%, category 3: 25 ± 4.5% and category 4 and 3 ± 1%. Severity of muscimol-induced phenotype is increased from 0 (normal locomotion) to 4 (complete flaccid paralysis) (see Materials and Methods for scoring details). * p<0.05, ***p<0.001 between wild type and *unc-2(zf35gf)* mutants, Chi-square.
Figure II-12. The reduction of GABA receptor in *unc-2(zf35gf)* mutants is dependent on nicotinic acetylcholine receptor mediated signaling. (a)

Representative images of GABAergic presynaptic sites labeled with RAB-3::mCherry and UNC-49::GFP.
3::mcherry of indicated genotypes. Scale bar represents 10 µm (b) Quantification of the fluorescence intensity of RAB-3::mcherry along the nerve cord of indicated genotypes. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized RAB-3::mCherry fluorescence: wild type (1 ± 0.05, n=42), unc-2(zf35gf) (1.5 ± 0.09, n=15), $P_{GABA::unc-2(zf35gf)}$ (1.3 ± 0.08, n=14), acr-12; unc-2(zf35gf) (1.2 ± 0.06, n=15) and unc-29; unc-2(zf35gf) (1.21 ± 0.08, n=14). (c) Representative images of GABA receptor UNC-49::GFP of indicated genotypes. Scale bar represents 10 µm. (d) Quantification of the fluorescence intensity of UNC-49::GFP along the nerve cord of indicated genotypes. Arbitrary fluorescence units of individual animals are normalized to the mean value of wild type. Normalized UNC-49::GFP fluorescence: wild type (1 ± 0.06, n=41), unc-2(zf35gf) (0.4 ± 0.07, n=15), $P_{GABA::unc-2(zf35gf)}$ (1.5 ± 0.12, n=13), acr-12; unc-2(zf35gf) (1 ± 0.11, n=15) and unc-29; unc-2(zf35gf) (0.9 ± 0.07, n=14). For all the quantification above, error bars depict SEM. *p<0.05, *p<0.01, ***p<0.001, ****p<0.0001, one way ANOVA with Dunnett’s multiple comparisons test.
Wild-type animals:

![Diagram showing Wild-type animals with wild-type and mutant calcium channels.]

Ca$_y$2a1/unc-2(zf35gf) mutants:

![Diagram showing mutants with altered calcium channel activity.]

**Figure II-13.** Proposed model of the imbalance between excitatory and inhibitory neurotransmission in *unc-2(zf35gf)* mutants.
CHAPTER III

A Forward Genetic Screen for Regulators of Calcium Channel Function
in Synaptic Transmission
Introduction

Presynaptic Ca\textsubscript{\textit{V}}2 channels play a central role in the function of the nervous system including calcium-dependent gene expression, neuronal migration, and neurotransmitter release (Catterall 2000; West et al. 2001). Inherited gene mutations disrupting presynaptic Ca\textsubscript{\textit{V}}2 function can result in human epilepsy, migraine, autism-spectrum disorders and bipolar disease, underscoring the importance of these channels in human physiology (Bidaud et al., 2006; Pietrobon, 2010).

Presynaptic Ca\textsubscript{\textit{V}}2 channels are particularly crucial for synaptic transmission, which is a major mechanism of communication between neurons. Biochemical and molecular studies have identified and elucidated some proteins and their roles in modulating Ca\textsubscript{\textit{V}}2 channel function (Catterall 2011; Simms and Zamponi 2014). Genetic approaches using \textit{C. elegans} can further complement these studies not only by assessing the functional importance of individual proteins in living animals, but also by identifying additional modulatory components (Miller et al., 1996). The isolation of the \textit{Ca\textsubscript{\textit{V}}2a1/unc-2(zf35gf)} mutants opened a unique and powerful opportunity to elucidate regulatory mechanisms underlying Cav2 channel function, modulation and localization \textit{in vivo}. Therefore, we carried out an unbiased forward genetic suppressor screen, and identified new alleles of genes that genetically interact and modulate Ca\textsubscript{\textit{V}}2 channel function in synaptic transmission.
Through this suppressor screen of \( \text{CaV}_2\alpha_1/\text{unc}-2(\text{zf}35\text{gf}) \) mutants, we isolated alleles of genes that have been shown to play a role in synaptic vesicle release, Cav2 channel trafficking and localization as well as components of the channel complex, suggesting our screening approach was successful. Ca\(_{v}\)2 channels are heteromultimeric membrane complexes that are composed of a pore-forming Ca\(_{v}\)\(\alpha\)1 subunit together with two indispensable auxiliary subunits, Ca\(_{v}\)\(\alpha\)2\(\delta\) and Ca\(_{v}\)\(\beta\). Loss of Ca\(_{v}\)\(\beta\) subunit function is detrimental and can lead to lethality from worms to mice (Ball et al., 2002; Burgess et al., 1999; Lainé et al., 2011; Strube et al., 1996). \(\text{ccb}-1\) encodes the Ca\(_{v}\)\(\beta\) subunit in \(\text{C. elegans}\) and \(\text{ccb}-1\) null mutants are embryonic lethal (Lainé et al., 2011). We isolated a previously unavailable viable hypomorphic allele of \(\text{ccb}-1\), and showed that Ca\(_{v}\)\(\beta\)/CCB-1 functions in neurons and muscles to generate coordinated behaviors. In addition to genes previously implicated in Cav2 channel function, we recovered alleles of genes that are less characterized with their roles in modulating Cav2 channels. Uncovering their roles will potentially illuminate new mechanisms for regulating calcium dependent synaptic transmission.

**Results**

**Isolation and identification of presynaptic Ca\(_{v}\)2 channel regulators**

The \(\text{C. elegans}\) genome encodes a single presynaptic Ca\(_{v}\)2\(\alpha\)1 subunit, UNC-2. We have previously reported \(\text{CaV}_2\alpha_1/\text{unc}-2(\text{zf}35\text{gf})\) mutants have increased acetylcholine (ACh) transmission and are hypersensitive to the
cholinesterase inhibitor, aldicarb. Aldicarb causes hypercontracted paralysis as well as the cessation of growth and reproduction (Miller et al., 1996). To identify genes that are required for expression, trafficking, and composition of CaV2 channels, we performed a forward genetic screen looking for mutants that suppress the aldicarb hypersensitivity in the unc-2(zf35gf) mutant background. To focus on mutations that affect presynaptic function, a secondary screen with the cholinergic agonist, levamisole, was performed. Since levamisole directly activates postsynaptic receptors, mutants that are resistant to this drug in addition to aldicarb have a postsynaptic defect rather than a defect at the presynapse. We isolated 29 alleles from the screen in total and identified causal mutations for 28 alleles using genetic analysis and single nucleotide polymorphisms (SNPs) mapping combined with whole genome sequencing.

Table III-1 lists the genes that were identified in the screen and the number of alleles for each gene. Two gene classes account for the majority of the suppressors: CaV2 complex components and presynaptic active zone proteins. We obtained seven intragenic alleles of the CaV2α1 subunit unc-2 as well as alleles of other auxiliary subunit, CaVα2γ/ccb-1 and CaVβ/unc-36. Interestingly, we isolated one allele of the CaV1α1 subunit egl-19, suggesting a presynaptic interaction between these two types of CaV channels. Core active zone proteins are crucial for CaV2 channel function in synaptic transmission. We isolated alleles of unc-10/Rim which are essential for synaptic vesicle docking and priming, as well as for recruiting CaV2 channels to active zones (Gracheva...
et al., 2008; Han et al., 2011; Kaeser et al., 2011) and alleles of \textit{unc-13/Munc13}\ and \textit{unc-18/Munc18} (Südhof, 2012). In addition to components of the \textit{CaV2} complex and active zone proteins, we isolated one allele of \textit{calf-1}, a nematode-specific single transmembrane ER protein required to traffic UNC-2 from the ER to the surface of the membrane (Saheki and Bargmann, 2009). We also isolated mutations in two genes with no implicated roles in synaptic transmission so far: \textit{unc-31/CAPS} and \textit{mec-9}. \textit{unc-31/CAPS} is required for dense core vesicle fusion and \textit{mec-9} is a predicted extracellular matrix protein (Du et al., 1996; Speese et al., 2007). Genes from the suppressor screen are summarized in Figure III-1.

These results show that our screening approach was highly successful in identifying genes that regulate the \textit{CaV2} channel function as well as opening new avenues for understanding the \textit{CaV2} channel mediated neurotransmission.

**A mutation in the \textit{CaVβ} subunit \textit{ccb-1} suppresses the aldicarb hypersensitivity of \textit{CaV2a1/unc-2(zf35gf)} mutants**

Despite the biophysical and pharmacological properties of \textit{CaV} channels are largely determined by the \textit{CaVα1} subunit, proper surface expression and function of these channels require the auxiliary subunits. From the suppressor screen, we isolated an allele, \textit{zf121}, of the \textit{CaVβ} subunit \textit{ccb-1} gene, which partially suppresses the aldicarb hypersensitivity of \textit{CaV2a1/unc-2(zf35gf)} mutants (Figure III-2a). \textit{CaVβ} subunits have a common structure consisting of the N and C termini, which are relatively variable and subject to alternative splicing,
and a highly conserved core region comprised of an Src homology 3 (SH3) domain and an enzymatic inactive guanylate kinase (GK)-like domain which are connected by the HOOK domain (Chen et al. 2004). Sequence analysis revealed the zf121 allele contains a single amino acid substitution (isoleucine to threonine) in the highly conserved SH3 domain (Figure III-2b).

Two putative CaVβ subunits, ccb-1 and ccb-2, are present in the C. elegans genome based on sequence similarity with mammalian β subunits. Research has shown that CCB-1 is indispensible for voltage-dependent calcium currents in C. elegans muscle cells (Lainé et al., 2011). The identification of the ccb-1(zf121) allele from our Cav2a1/unc-2(zf35gf) suppressor screen shows that CCB-1 function is also crucial in neurons.

**CCB-1 localizes to the presynaptic release sites**

Expression of a biologically active, GFP-tagged CCB-1 protein driven by its native promoter is detected in most neurons as well as pharyngeal and body wall muscles (Figure III-2c). To achieve better spatial resolution of the subcellular localization of CCB-1, we expressed the GFP::CCB-1 only in GABAergic motor neurons. We were able to observe the GFP::CCB-1 signal colocalized with the mCherry-labeled synaptic vesicle marker RAB-3 along the axon (Figure III-2d), indicating CCB-1 localizes to the presynaptic release sites where CaV2 channels resided. One intriguing observation was that in addition to being localized to both the axon and the cytoplasm, CCB-1 was also found in nuclei of motor neurons.
As the Ca\(\text{V}\beta\) subunit is the only non-membrane anchored subunit in the Ca\(\text{V}\)2 channel complex, this CCB-1 nuclear localization may suggest a novel role of Ca\(\text{V}\beta\) subunits independent of their role in synaptic transmission.

**ccb-1(zf121) mutants are sluggish and defective in egg-laying**

The *C. elegans* genome encodes three Ca\(\text{V}\alpha1\) subunits: *egl-19* (Ca\(\text{V}\alpha1\)), *unc-2* (Ca\(\text{V}\alpha1\)) and *cca-1* (Ca\(\text{V}\alpha3\)) (Jeziorski et al., 2000). Among them, *egl-19* (Ca\(\text{V}\alpha1\)) and *unc-2* (Ca\(\text{V}\alpha2\)) are high-voltage activated Ca\(\text{V}\) channels and require auxiliary subunits, Ca\(\text{V}\beta\) and Ca\(\text{V}\alpha2\gamma\) subunits for proper function (Perez-Reyes, 2006; Perez-Reyes et al., 1998; Takahashi et al., 1987). Ca\(\text{V}\alpha1/EGL-19\) is expressed in muscle cells and some neurons, and *egl-19(lf)* mutants are slow, defective in egg-laying, and accumulate fertilized eggs in the uterus (Figure III-3a and b). Ca\(\text{V}\alpha1/UNC-2\) is exclusively expressed in neurons, and *unc-2(lf)* mutants are sluggish and uncoordinated (Figure. III-3a). *ccb-1(zf121)* mutants, similar to *egl-19(lf)* and *unc-2(lf)*, exhibit decreased locomotor velocity and an increased number of eggs in the uterus (Figure III-3a and b), suggesting it plays an important role in both EGL-19 and UNC-2 function. The locomotor and egg-laying phenotypes of *ccb-1(zf121)* mutants were rescued by wild-type *ccb-1* transgene driven by its native promoter. Expression of *ccb-1* specifically in the body wall muscles were able to rescue the egg-laying defect, but not the sluggishness of *ccb-1(zf121)* mutants, while expression of *ccb-1* specifically in the nervous system partially rescued the locomotion but not the egg-laying
defects of *ccb-1(zf121)* mutants. Our results strongly suggest that CCB-1 is the main, if not the only, CaVβ subunit required for both EGL-19 and UNC-2 function. The results of tissue-specific rescue of *ccb-1* support the notion that CaV1α1/EGL-19 functions in vulva muscles to regulate egg-laying behavior (Raymond et al., 1997; Trent et al., 1983). On the other hand, coordination between neurons and muscles is required for proper locomotion behavior (Gjorgjieva et al., 2014) and we found that expression of CCB-1 only in neurons or muscles is not sufficient to rescue the locomotor defect, strongly suggesting that CCB-1 is the main, if not the only, CaVβ subunit required for both EGL-19 and UNC-2 function.

**ccb-1(zf121) mutants are hypersensitive to the cholinergic agonist, levamisole**

*ccb-1(zf121)* mutants are behaviorally defective in locomotion and egg-laying, suggesting a reduced function of both CaV1/EGL-19 and CaV2/UNC-2 channels, which function mainly in muscles and presynaptic neurons, respectively. To test whether these observed locomotor and egg-laying phenotypes of *ccb-1(zf121)* mutants are the result of impacted synaptic transmission, we used pharmacological assays to examine the functional consequences of the reduction of function of CaVβ subunits using *ccb-1(zf121)* mutant animals. We placed *ccb-1(zf121)* mutants on plates containing 1 mM aldicarb and analyzed their rate of paralysis compared to wild type. Although we
expected the \textit{ccb-1(zf121)} mutants to be resistant to aldicarb, we unexpectedly observed that \textit{ccb-1(zf121)} mutants showed a wild-type response, suggesting normal presynaptic release of acetylcholine (Figure III-4a). We next tested the responses of \textit{ccb-1(zf121)} mutants to the cholinergic agonist, levamisole, and found that \textit{ccb-1(zf121)} mutants were hypersensitive, which suggests an up-regulation of acetylcholine receptor function (Figure III-4b). The hypersensitivity to levamisole of \textit{ccb-1(zf121)} mutants can be rescued by expression of the wild-type \textit{ccb-1} transgene driven by its native promoter. Because of the increase in acetylcholine receptor function, we cannot rule out the possibly that the decreased presynaptic release was masked by the increased postsynaptic signaling in the aldicarb assay.

\textbf{The GK domain of Ca\textsubscript{V}\textbeta/CCB-1 is essential for its function}

Ca\textsubscript{V}\textbeta subunits share a common structure with a highly conserved core region containing an Src homology 3 (SH3) domain and an enzymatically inactive guanylate kinase (GK)-like domain, which are connected by a less conserved HOOK region (Chen et al. 2004). While GK domain has been well demonstrated with its importance in the direct interaction with the Ca\textsubscript{V}\alpha\textsubscript{1} subunit, and regulating Ca\textsubscript{V}\alpha\textsubscript{1} subunit gating properties and surface expression (Opatowsky et al., 2004), the roles of SH3 and HOOK domains are less clear. Unlike mammals which have four genes that encode Ca\textsubscript{V}\textbeta subunits, \textit{ccb-1} is likely the only Ca\textsubscript{V}\textbeta gene in \textit{C. elegans}. This provides an opportunity to investigate the functional
significance of different domains in living animals without the complications of redundancy. To test which domains of CCB-1 are required for its function, we expressed GFP-tagged forms of CCB-1 lacking specific domains under its native promoter in the ccb-1(zf121) mutant background (Figure III-5a). We detected normal expression and localization of these mutant forms of CCB-1 to neurons and muscles (Figure III-5b). At subcellular level, we noticed that the fluorescence signal of CCB-1(ΔSH3) and CCB-1(ΔHOOK) was nuclear excluded and appeared clumpier compared with the wild type. We also observed that CCB-1(ΔGK) have fewer puncta along the VNC.

Functionally, CCB-1(ΔSH3) fully rescued the uncoordinated locomotion of ccb-1(zf121) mutants and partially rescued the egg-laying defect. CCB-1(ΔHOOK) partially rescued both locomotion or egg-laying behaviors and CCB-1(ΔGK) failed to rescue either of these phenotypes (Figure III-5c and d). These results suggest the GK domain of CaVβ/CCB-1 is essential for its function. To exclude the possibility that the rescue we observed in ccb-1(zf121) mutants was due to an interaction between our deletion constructs with the mutant CCB-1(I84T) produced in the ccb-1(zf121) mutant background, we performed a rescue assay of lethality using a null allele of ccb-1. ccb-1(gk18) allele contains a large deletion of the ccb-1 gene and thus is thought to be a null allele. ccb-1(gk18) mutants are embryonic arrested and have to be maintained in the balancer strain by the translocation szT1 (Lainé et al., 2011). We injected the GFP-tagged domain-deleted CCB-1 constructs into ccb-1(gk18) adult animals (P0) and picked
the progeny (F₁) carrying the transgenes singly to individual plates by selecting GFP. We then examined if any of the plates have progenies (F₂) that were able to reach the adulthood and have lose the balancer. We found that transgenic animals carrying CCB-1(ΔSH3) or CCB-1(ΔHOOK) transgenes were able to reach the adulthood and produce progeny in the non-balancer background, while animals carrying CCB-1(ΔGK) transgene remained embryonic lethal after losing the balancer. Taken together, our data show that the GK domain is indispensible for the function of CaVβ/CCB-1, and the SH3 and HOOK domain play more modulatory roles.

**Discussion**

Presynaptic CaV2 channel function is crucial for cell excitability and synaptic vesicle release. From a forward genetic screen, we isolated new alleles of genes that genetically interact with the CaV2 channel and regulate its function in neurotransmission. These genes encode proteins in active zone and synaptic vesicle release machinery (unc-10, unc-13 and unc-18), calcium channel trafficking and surface expression (calf-1, unc-10, unc-36 and ccb-1), and calcium channel auxiliary subunits (unc-36 and ccb-1). This screen was highly successful in that many of the genes we recovered have been implicated in regulating CaV2 channel function (Gracheva et al., 2008; Kaeser et al., 2011; Saheki and Bargmann, 2009).
Three suppressors isolated from our screen have not been previously implicated in Cav2 channel mediated synaptic transmission, and therefore are novel regulators of CaV2 channel function. These include *egl-19(zf89)*, *unc-31(zf107)* and *mec-9(zf132)*. *egl-19* encodes for the only CaV1α1 subunit in *C. elegans* and is the sole CaV channels in body wall muscles (Lainé et al., 2011). EGL-19 is required for muscle contraction during late embryogenesis, locomotion, egg-laying, mating and feeding (Garcia et al., 2001). CaV1α1/EGL-19 is expressed in all muscles as well as some neurons (Raymond et al., 1997), but its role in synaptic transmission is less clear compared with presynaptic CaV2α1/UNC-2 (Mathews et al., 2003). *egl-19(zf89)* mutation is sufficient to suppress the aldicarb hypersensitivity of *CaV2α1/unc-2(zf35gf)* mutants without altering the postsynaptic levamisole response, indicating this mutation mainly affects the EGL-19 function in neurons. Our *egl-19(zf89)* allele has an amber mutation in exon 16 which only affects the *egl-19* b isoform, suggesting that *egl-19* b isoform may potentially be the neuronal-specific isoform. *C. elegans* body wall muscles fire all-or-none, calcium-dependent action potentials, and the frequency can be regulated by EGL-19 activity in motor neurons (Gao and Zhen, 2011). The identification of *egl-19(zf89)* allele from the suppressor screen provides a line of evidence that different CaV channel types functionally interact in neurons.

*unc-31* encodes the *C. elegans* ortholog of human calcium-dependent activator protein for secretion/CAPS, which has been shown to be required for
the dense core vesicle exocytosis (Speese et al., 2007). unc-31(zf107) has a single nucleotide transition that disrupts the 5' splice donor of exon 9 and affects alternative splicing of certain isoforms. Notably, we tested the canonical null allele unc-31(e928), which has a large deletion in the unc-31 gene, and found this allele failed to suppress the elevated cholinergic neurotransmission in \textit{Ca}_{v}2a1/unc-2(zf35gf) mutants. This allele–specific effect is likely due to the molecular differences between these two alleles. Strains with the unc-31(e928) allele lacked UNC-31 protein (Speese et al., 2007), whereas our unc-31(zf107) mutation may lead to alternative splicing of the unc-31 transcript. Munc-13/UNC-13 is required for synaptic vesicle fusion (Betz et al., 1998). CAPS/UNC-31 and Munc13/UNC-13 proteins have related C- terminal SNARE protein–binding domains, and both need to translocate to membrane PIP2-rich domains to mediate vesicle fusion (Kabachinski et al., 2014). It is possible that our unc-31(zf107) allele produces mutated UNC-31 that competes with UNC-13 to interact with membrane PIP2 and thus suppresses the elevated acetylcholine release in \textit{Ca}_{v}2a1/unc-2(zf35gf) mutants. The other gene we isolated is \textit{mec}-9, which encodes the predicted extracellular matrix protein containing EGF repeats, Kunitz domains and a glutamate-rich region (Du et al., 1996). While the long isoform of MEC-9 is crucial for mechanosensation in \textit{C. elegans} through its role in organizing the mechanosensory channel complex, little is known about the function of the short isoform of MEC-9 (Du et al., 1996; Emtage et al., 2004). While MEC-9(L) has a narrow expression pattern and is detected only in the six
touch neurons, MEC-9(S) is widely distributed in the nervous system including neurons in the head and ventral nerve cord (Du et al., 1996), suggesting that the MEC-9(S) may play a universal role in all neurons, such as organizing active zones. Our allele mec-9(zf132) has an amber mutation in exon 18 which affects both isoforms. This may provide a new perspective on mechanistic basis of regulation of CaV2 channel function.

CaVβ auxiliary subunits are crucial for CaV channel function and regulation. However, due to complex expression patterns of each subtype and specific pairing of different CaVα1 subunit in mammals, most studies of the CaVβ subunit have been performed in vitro or in heterologous expression systems. In this study, we provided the genetic evidence that CaVβ/CCB-1 regulates CaV2/UNC-2 channel function through isolating a hypomorphic ccb-1 allele from the CaV2α1/unc-2(zf35gf) suppressor screen (Figure III-1a and b). In addition to uncoordinated locomotion, ccb-1(zf121) mutants are defective in egg-laying behavior, which is a classical phenotype exhibited by the CaV1/EGL-19 channel loss-of-function egl-19 mutants (Raymond et al., 1997) (Figure III-2), suggesting CCB-1 function is required by both CaV channel types in C. elegans. ccb-1(zf121) mutants are hypersensitive to levamisole (Figure III-3b). This suggests the occurrence of a compensatory mechanism in response to the decreased presynaptic neurotransmission and decreased calcium currents in body wall muscles seen in ccb-1(zf121) mutants. Further investigation into whether this increased postsynaptic response result from increased expression or binding
efficacy of receptors, or from the signaling factors downstream of ligand binding is likely to shed light on homeostatic mechanisms that affect synapse formation during development.

The ccb-1(zf121) allele has an isoleucine to threonine amino acid substitution in the SH3 domain of Ca\(\gamma\)\(\beta\)/CCB-1 (Figure III-2b). We tested the functional significance of each domain of CCB-1 in vivo by rescuing the ccb-1 mutants with forms of ccb-1 cDNA with individual domains deleted (Figure III-4a). The ccb-1(\(\Delta\)GK) cDNA construct failed to rescue the lethality of ccb-1 null mutants, or behavioral defects of ccb-1(zf121) mutants (Figure III-4c and d). Previous work has shown the GK domain of the Ca\(\gamma\)\(\beta\) subunit is necessary and sufficient for Ca\(\gamma\) channel surface expression through direct interaction with Ca\(\gamma\)\(\alpha\)1 subunits (He et al. 2007). Therefore, we speculate that Ca\(\gamma\)\(\alpha\)1 subunits were retained in the ER in the ccb-1 mutants expressing the ccb-1(\(\Delta\)GK) rescuing construct. ccb-1(\(\Delta\)SH3) and ccb-1(\(\Delta\)HOOK) cDNA constructs rescued the lethality of ccb-1 null allele and only partially rescued the behavioral defects of ccb-1(zf121) mutants, suggesting the SH3 and HOOK domains play roles in modulation and regulation of Ca\(\gamma\) channel kinetics. While the HOOK domain has been shown to be critical for regulating Ca\(\gamma\) channel inactivation (He et al. 2007; Richards et al. 2007), the functional significance of the SH3 domain remains elusive. Studies have shown that the SH3 domain has GK domain-dependent low binding affinity with the Ca\(\gamma\)\(\alpha\)1 subunits in vitro (Dubuis et al., 2006), and can interact with GK domains intramolecularly (Opatowsky et al. 2004; Chen et al.
Mutations that weaken this intracellular interaction severely compromises the gating effects of the Ca\textsubscript{v}\beta subunits (Chen et al. 2009). Since our ccb-1(zf121) mutation affects a residue within the SH3 domain, perhaps its ability to suppress the Ca\textsubscript{v}2\alpha1/unc-2(zf35gf) mutants could be a result of changes to the 3D folding structure of Ca\textsubscript{v}\beta subunits, thus impacting critical intramolecular interactions.

Taken together, our screen for suppressors of the Ca\textsubscript{v}2\alpha1/unc-2(zf35gf) mutants isolated a collection of suppressors including Ca\textsubscript{v}2\alpha1 interacting molecules that not found in previous aldicarb-resistance screens (Miller et al., 1996). Further genetic and molecular analysis of these new alleles and genes will continue to yield insight into the regulation and functioning of the presynaptic Ca\textsubscript{v}2 channels, particularly in synaptic transmission. Additionally, through isolating a previously unavailable viable hypomorphic allele of Ca\textsubscript{v}\beta/ccb-1, we show for the first time in C. elegans that the CCB-1 is crucial for presynaptic Ca\textsubscript{v}2 channel function, as well as the functional significance of the GK domain of Ca\textsubscript{v}\beta/CCB-1 in vivo. Overall this work has contributed to the elucidation of regulatory mechanisms underlying Cav2 function, modulation, and localization using an in vivo system, and has provided many avenues for future investigations.
Materials and Methods

Strains

All strains were cultured at room temperature (22-24°C) on nematode growth media (NGM) agar plates with the E. coli strain OP50 as a food source. Experiments were performed on young adult animals at room temperature (22-24°C). The wild-type strain was Bristol N2. Transgenic strains were obtained by microinjection of plasmid DNA into the germline with coinjection marker lin-15 rescuing plasmid indicated. At least three independent transgenic lines were obtained. The data presented is from a single representative line. The following strains were utilized in this study:

QW37 unc-2(zf35gf)
QW1092 ccb-1(zf121)
QW1131 lin-15(n765ts); ccb-1(zf121); zfex469[pccb-1::CCB-1]
QW1137 lin-15(n765ts); ccb-1(zf121); zfex473[prgef-1::CCB-1]
QW1140 lin-15(n765ts); ccb-1(zf121); zfex476[pmyo-3::CCB-1]
QW1181 lin-15(n765ts); zfex489 [pccb-1::gfp::CCB-1]
QW1305 lin-15(n765ts); ccb-1(zf121); zfex567[pccb-1::CCB-1(deltaGK)]
QW1309 lin-15(n765ts); ccb-1(zf121); zfex570[pccb-1::CCB-1(deltaHOOK)]
QW1311 lin-15(n765ts); ccb-1(zf121); zfex572[pccb-1::CCB-1(deltaSH3)]
QW1400 zfis136 [pccb-1::CCB-1::GFP]
QW1407 zfIs136[pccb-1::CCB-1::GFP];otIs355 [rab-3::NLS::tagRFP]
QW1491 ufls58[Punc-47::mcherry::RAB-3]; zfex687[Punc-47::GFP::CCB-1]
Isolation and identification of Ca\textsubscript{v}2\alpha1/unc-2(zf35gf) suppressors

Ca\textsubscript{v}2\alpha1/unc-2(zf35gf) L4 animals (P0) were mutagenized with 0.5 mM N-ethyl-N-nitrosourea (ENU) for 4 hr. Approximately 10,000 F1 animals were bleached to obtain F2 eggs. F2 eggs were plated on NGM plates containing 0.25 mM aldicarb and examined for viable progeny after 7 and 14 days. Aldicarb resistant animals were individually transferred to fresh NGM plates, and their progeny were retested for aldicarb resistance and levamisole sensitivity. Suppressors that were aldicarb resistant and had either wild type or sensitive responses to levamisole were kept further characterization. All suppressors isolated from the screen were first backcrossed with wild-type N2. Suppressors that showed linkage to the X chromosome were tested for complementation with unc-2(lf) mutants. Molecular identities of unc-2 intragenic suppressors were identified by DNA sequencing of the unc-2 gene. Other suppressors were first mapped using single nucleotide polymorphisms (SNPs) (Wicks et al., 2001) to narrow down the chromosomal region and combined with whole genome sequencing to identify possible gene candidates and causal mutations. The causative mutations were confirmed by complementation test.

Behavioral and pharmacological assays
To quantify the instantaneous velocity and average forward velocity, animals were transferred from their culture plate to a new NGM plate seeded with a thin bacterial lawn and allowed to recover for 5 min. After the recovery period the animals were tracked for 90 sec using a multiple worm tracker (MWT) (Swierczek et al., 2011). Videos from the MWT were analyzed with MWT-associated software to calculate the average forward velocity. Egg-laying assays were performed as described by Koelle and Horvitz (1996). In brief, L4 larvae were isolated and allowed to develop for 40 hr. Adults were then transferred into individual wells of 96-well plates containing 1% sodium hypochlorite and incubated until the bodies were dissolved. The number of unlaid eggs accumulated inside of adult animals was then counted.

Aldicarb and levamisole assays were performed with young adults. Animals were transferred to NGM plates supplemented with 1 mM aldicarb and 200µM levamisole respectively. The percentage of paralyzed animals was scored at 15 min intervals until every assayed animal was paralyzed. Animals were scored as paralyzed when they did not move when prodded with a platinum wire.

Microscopy
Animals were mounted on 2% agarose pads containing 60 mM sodium azide for 5 minutes before being examined for fluorescent protein expression and localization. Images were captured under consistent detector settings with a
Hamamatsu Photonics C2400 CCD camera on a Zeiss Axioplan2 Imaging System. Images were projected into a single plane using NIH ImageJ software.
Table III-1. Forward suppressor screen identifies new alleles of genes required for CaV2 trafficking, localization and function. Shown is a list of alleles identified in the *unc-2(zf35gf)* suppressor screens grouped according to function. We isolated 29 alleles that were resistant to the effects of aldicarb but not resistant to levamisole. Genetic analysis and whole genome sequencing lead us to identify 28 of the 29 mutations, all of which occur in genes known to play a role in CaV2 trafficking, localization and function. One allele, *zf136*, still needs to be identified.

<table>
<thead>
<tr>
<th>Gene class</th>
<th>Gene</th>
<th>No. of alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pore forming subunit (α1)</td>
<td><em>unc-2/CaV2</em></td>
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<tr>
<td></td>
<td><em>egl-19/CaV1</em></td>
<td>1</td>
</tr>
<tr>
<td>Auxiliary subunit (α2δ)</td>
<td><em>unc-36</em></td>
<td>4</td>
</tr>
<tr>
<td>Auxiliary subunit (β)</td>
<td><em>ccb-1</em></td>
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</tr>
<tr>
<td>Channel trafficking</td>
<td><em>colf-1</em></td>
<td>1</td>
</tr>
<tr>
<td>Synaptic vesicle docking</td>
<td><em>unc-13/Munc-13</em></td>
<td>7</td>
</tr>
<tr>
<td>and fusion</td>
<td>*unc-10/RIM,</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>unc-18/Munc-18</em></td>
<td>2</td>
</tr>
<tr>
<td>Dense core vesicle fusion</td>
<td><em>unc-31/CAPS</em></td>
<td>1</td>
</tr>
<tr>
<td>Extracellular matrix</td>
<td><em>mec-9</em></td>
<td>1</td>
</tr>
<tr>
<td>Undetermined</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>
Figure III-1. Genes required for CaV2 channel trafficking, localization and function in synaptic transmission
Figure III-2. *zf121* is a novel allele of the CaVβ subunit gene *ccb-1*. (a)
Quantification of % animals paralyzed on 1 mM aldicarb over time for indicated genotypes. Each data point represents the mean ± SEM of the percentage of animals paralyzed by aldicarb every 15 minutes for two trials, totaling a minimum
of 30 animals. (b) Gene structure and protein domains of ccb-1. Coding sequences are represented as black boxes. The zf121 allele is a single nucleotide transition resulting in an isoleucine to threonine (I -> T) amino acid substitution at position 84 in the SH3 domain. (c) Expression of the Cavβ/CCB-1. GFP::CCB-1 driven by its native promoter is detected in neurons and muscle cells of the pharynx (upper panel) and body wall (lower panel). Subcellular localization of the GFP::CCB-1 is distributed in both nuclei and cytoplasm. (d) GFP::CCB-1 is localized to presynaptic sites and colocalized with the RAB-3::mCherry vesicle marker in the GABAergic motor neurons in the ventral nerve cord (VNC). Asterisk indicates soma and arrows point to the pre-synaptic sites. (e) CCB-1 is distributed in both the nuclei and cytoplasm. Single slice of confocal images showing that GFP::CCB-1 colocalizes with the NLS::RFP in nuclei along the VNC. All images, if not otherwise specified, represent Z projections with 0.3 µm interval between individual slices and were taken with confocal microscopes. Scale bars represent 10 µm.
Figure III-3. *ccb-1*(*zf121*) mutants are defective in locomotion and egg-laying behaviors. (a) Average instant forward velocity analysis for the wild type (0.10 ± 0.0 wormlength/sec), *ccb-1*(*zf121*) (0.05 ± 0.00 wormlength/sec), *unc-2*(e55lf) (0.03 ± 0.00 wormlength/sec), *egl-19* (n582lf) (0.02 ± 0.00 wormlength/sec), *Pccb-1::CCB-1; ccb-1*(*zf121*) (0.11 ± 0.01 wormlength/sec), *Prgef-1::CCB-1; ccb-1*(*zf121*) (0.07 ± 0.01 wormlength/sec), *Pmyo-3::CCB-1; ccb-1*(*zf121*) (0.05 ± 0.00 wormlength/sec). Data obtained from 3 independent trials totaling more than 20 animals. (b) Numbers of unlaid eggs in the uterus of indicated genotypes. Wild type (17.65 ± 0.68, n=43), *unc-2*(e55lf) (8.09 ± 1.33, n=85), *egl-19* (n582lf) (39.14 ± 1.58, n=14), *ccb-1*(*zf121*) (31.5 ± 1.52, n=28), *Pccb-1::CCB-1; ccb-1*(*zf121*) (19.78 ± 0.86, n=36); *Prgef-1::CCB-1; ccb-1*(*zf121*) (24.42 ± 0.98, n=33); *Pmyo-3::CCB-1; ccb-1*(*zf121*) (18.67 ± 1.26, n=12). Error bars represent S.E.M. * represents statistical difference from wild type, * p<0.05, ****p<0.0001, one way ANOVA with Dunnett’s tests.
Figure III-4. *ccb-1(zf121)* mutants are hypersensitive to the cholinergic agonist, levamisole. (a) Quantification % animals paralyzed on 1 mM aldicarb over time for indicated genotypes. Each data point represents the mean ± SEM of the percentage of animals paralyzed by aldicarb every 15 minutes for at least three trials, totaling a minimum of 50 animals. (b) Quantification % animals paralyzed on 200 µM levamisole over time for indicated genotypes. Each data point represents the mean ± SEM of the percentage of animals paralyzed by aldicarb every 15 minutes for at least three trials, totaling a minimum of 50 animals.
### a

<table>
<thead>
<tr>
<th>Structure</th>
<th>GFP</th>
<th>SH3</th>
<th>HOOK</th>
<th>GK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔSH3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ΔHOOK</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>ΔGK</td>
<td></td>
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</table>

### b

**Pccb-1::GFP::CCB-1**

- **Pccb-1::GFP::CCB-1**
- **Pccb-1::GFP::CCB-1 (ΔSH3)**
- **Pccb-1::GFP::CCB-1 (ΔHOOK)**
- **Pccb-1::GFP::CCB-1 (ΔGK)**

### c

![Graph](image)

**Wormlength/sec**

- **ccb-1(zf121)**
- **wild type**
- **Pccb-1::GFP::CCB-1**
- **Pccb-1::GFP::CCB-1 (ΔSH3)**
- **Pccb-1::GFP::CCB-1 (ΔHOOK)**
- **Pccb-1::GFP::CCB-1 (ΔGK)**

### d

![Graph](image)

**Unlaid eggs in uterus**

- **ccb-1(zf121)**
- **wild type**
- **Pccb-1::GFP::CCB-1**
- **Pccb-1::GFP::CCB-1 (ΔSH3)**
- **Pccb-1::GFP::CCB-1 (ΔHOOK)**
- **Pccb-1::GFP::CCB-1 (ΔGK)**
Figure III-5. GK domain is essential for the function of Ca_{v}\beta/CCB-1. (a) Schematic of different domain-deleted CCB-1 constructs (b) Representative images of the localization of domain-deleted GFP::CCB-1 in young adult animals. All images represent Z projections with 0.3 \( \mu m \) interval between individual slices and were taken with a spinning scan confocal microscope. Scale bar represents 10 \( \mu m \). (c) Average instant velocity for the wild type (0.13 ± 0.01 wormlength/sec, n=12), ccb-1(zf121) (0.06 wormlength/sec, n=12), ccb-1(zf121); Pccb-1::GFP::CCB-1 (0.11 ± 0.01 wormlength/sec, n=11), Pccb-1::GFP::CCB-1(\( \Delta \)SH3) (0.12 wormlength/sec, n=12), Pccb-1::GFP::CCB-1(\( \Delta \)HOOK) (0.11 wormlength/sec, n=12) and Pccb-1::GFP::CCB-1(\( \Delta \)GK) (0.08 ± 0.01 wormlength/sec, n=12). (d) Numbers of unladen eggs in the uterus of indicated genotypes. Wild type (19.63 ± 1.07, n=24), ccb-1(zf121) (37.75 ± 2.103, n=24), ccb-1(zf121); Pccb-1::GFP::CCB-1 (19.88 ± 1.18, n=24), Pccb-1::GFP::CCB-1(\( \Delta \)SH3) (26.46 ± 1.15, n=24), Pccb-1::GFP::CCB-1(\( \Delta \)HOOK) (27.54 ± 1.9, n=24) and Pccb-1::GFP::CCB-1(\( \Delta \)GK) (32.13 ± 1.55, n=24). Error bars represent S.E.M. * represents statistical difference from wild type unless otherwise indicated, ** p<0.01, **** p<0.0001, one way ANOVA with Dunnett’s tests.
CHAPTER IV

Discussion and Future Directions
Most neuronal communications in the brain involve rapid neurotransmission. Synaptic transmission is initiated when an action potential arrives presynaptic nerve terminals and triggers the opening of presynaptic Ca\textsubscript{v}2 channels. Calcium entry into presynaptic active zones then stimulates the synaptic vesicle exocytosis (Katz, 1970; Südhof, 2004). Consequently, Ca\textsubscript{v}2 channel activity is crucial for precise synaptic transmission, and disruption of normal channel activity can be physiologically detrimental. Studies in the nematode \textit{Caenorhabditis elegans} have provided fundamental insights into neural development and synaptic function that are highly conserved from worms to humans. In this dissertation, I have used \textit{C. elegans} as a model to show how the dysregulation of Ca\textsubscript{v}2 channel activity affects synaptic transmission, circuit function and behavioral consequences.

\textbf{A potential invertebrate model for human FHM1}

Three subtypes of Ca\textsubscript{v}2 channels are encoded by the mammalian genome, P/Q-type (Ca\textsubscript{v}2.1 channels), N-type (Ca\textsubscript{v}2.2 channels) and R-type (Ca\textsubscript{v}2.3 channels), which are abundantly expressed calcium channel subtypes in the central nervous system. Among them, P/Q-type (Ca\textsubscript{v}2.1) have a dominant role in controlling neurotransmitter release, partly because of a more efficient coupling to the exocytosis machinery (Mintz et al., 1995; Wu et al., 1999). Mutations in the human Ca\textsubscript{v}2.1\textsubscript{α1} gene, \textit{CACNA1A}, have been associated with several calcium channelopathies such as Familial Hemiplegic Migraine (FHM1),
Episodic Ataxia type 2 (EA2) and Spinocerebellar Ataxia type 6 (SCA6) (Pietrobon, 2010). While EA2 and SCA6 mutations have been shown to cause a reduction in Cav2.1 activity, the effects of FHM1 mutations yield varying results in heterologous expression systems with regards to changes in channel expression and altered biophysical properties (Cain and Snutch, 2011). FHM1 knockin mice have provided the opportunity to study the functional consequences of FHM1 mutations in physiological relevant conditions and at their endogenous level of expression. Studies from cortical neuronal microculture and brain slices of FHM1 knockin mice suggested these mutations have gain-of-function effects including increased Cav2.1 current density in cerebellar neurons and enhanced neurotransmission at the neuromuscular junction (Van Den Maagdenberg et al., 2004; Tottene et al., 2009). However, direct genetic characterization of FHM1 mutations and molecular mechanisms of how elevated Cav2.1 channel activity affects synaptic transmission still need further investigation.

In chapter II, I identified a novel allele of the sole C. elegans Cav2α1 subunit gene, unc-2. This allele contains a Gly to Arg substitution in the highly conserved intracellular linker between domain III and IV. While unc-2(1f) mutants are sluggish, our unc-2(zf35) mutants display behavioral hyperactivity in locomotion and egg-laying behaviors. In addition, direct recording of spontaneous release events at the neuromuscular junction showed that unc-2(zf35) mutants had increased neurotransmission. Electrophysiology recordings from HEK cells transfected with the mutant Cav2 channels (G/R) showed a shift
in the voltage dependence to more hyperpolarized potentials and increased calcium current density, which is similar to Ca\textsubscript{v}2.1 channels with FHM1 mutations. Identification of intragenic \textit{unc-2}(lf) suppressors confirmed the zf35 allele is a gain-of-function allele of \textit{unc-2}. Excitingly, animals that express \textit{unc-2} transgene carrying corresponding FHM mutations recapitulate both the behavioral and pharmacological phenotypes of \textit{unc-2}(zf35gf) mutants. Our results provide strong genetic evidence showing that FHM1 mutations are gain-of-function mutations of the Ca\textsubscript{v}2.1\textalpha subunit and an important proof-of-principle that the molecular function of UNC-2 and CACANA1A are highly conserved from worms to humans.

\textbf{Ca\textsubscript{v}2(gf) channels leads to an excitation-dominant neurotransmission}

What are the functional consequences of the gain-of-function mutation in Ca\textsubscript{v}2\textalpha subunit at the level of synaptic transmission? \textit{unc-2}(zf35gf) mutants are hypersensitive to the acetylcholine esterase inhibitor, aldicarb, and show an increased frequency of endogenous cholinergic mEPSCs. Surprisingly, \textit{unc-2}(zf35gf) mutants have a marked reduction of endogenous GABAergic mIPSCs resulting in a raised excitation-inhibition ratio. Analysis of synaptic markers showed that \textit{unc-2}(zf35gf) mutants display enlarged pre-synaptic domains in both cholinergic and GABAergic neurons, indicating that elevated Ca\textsubscript{v}2 channel activity recruits increased numbers of synaptic vesicles to presynaptic release sites. However, \textit{unc-2}(zf35gf) mutants have an increased level of the
acetylcholine receptor, UNC-29, at synapses but a significant decrease in the level of the GABA receptor, UNC-49. The increased UNC-29 expression is likely due to the Hebbian synaptic plasticity in response to the elevated cholinergic release. In general, neuronal activity induces a calcium influx into the postsynaptic sites. Mammalian literatures have shown that a low calcium level leads to a decrease of the number of AMPA receptors, while a high calcium level yields an insertion of new ones (Beattie et al., 2000; Fauth and Tetzlaff, 2016; Kauer et al., 1988; Mulkey and Malenka, 1992; Shi et al., 1999). On the other hand, the reduction in GABA receptor expression is dependent on the acetylcholine receptor function, suggesting a crosstalk between excitatory and inhibitory receptors at postsynaptic sites.

Brain function relies on the ability of neural network to maintain its activity within a bounded range throughout developmental stages and different experiences. Altered excitability of the network has been associated with many neurodevelopment and neuropsychiatric disorders, such as epilepsy, Rett syndrome and autism spectrum disorder. While most studies have focused on mechanisms regulating excitatory or inhibitory transmission independently, a growing body of literature has suggested the possibility of crosstalk between excitatory and inhibitory signaling pathways (Bannai et al., 2015; Hirono et al., 2001; Wang and Maffei, 2014). This is particularly relevant for circuits that have excitatory and inhibitory synapses that occupy overlapping postsynaptic targets (Chiu et al., 2013; Miller, 1996). My data support this model, illustrating crosstalk
between excitatory and inhibitory neurotransmission and showed a functional interaction between excitatory and inhibitory receptors in postsynaptic cells. Enhanced excitatory synaptic activity has been reported to decrease the cluster size of GABA_ARs through NMDA receptor-dependent calcium influx and calcineurin dephosphorylation (Bannai et al., 2009, 2015). Investigating if the activation of nicotinic acetylcholine receptors, increased calcium influx in muscle cells or simply the expression level of the nicotinic acetylcholine receptors are required for the reduction of GABA receptor clustering in unc-2(zf35gf) mutants will be important to understand this crosstalk. Elevated excitatory transmission at cortical synapses has also been reported in FHM1 knockin mice (Tottene et al., 2009). The enhanced glutamate release contributes to the cortical spreading depression (CSD) facilitation, a key player in the pathogenesis of migraine (Vecchia and Pietrobon, 2012). Therefore, deeper understanding of the underlying molecular signaling pathways contributing to reduction in GABA receptors in unc-2(zf35gf) mutants may lead to the identification of new targets for therapeutic interventions.

**Molecular machinery regulating CaV2 function**

Presynaptic CaV2 channels are crucial regulators for many biophysical processes in the nervous system, from calcium-dependent gene expression, synaptic transmission to the activation of various calcium-dependent enzymes (Simms and Zamponi, 2014). Thus, understanding regulatory mechanisms
modulating Cav2 channel function is of profound importance in neuroscience. Forward genetic screens in C. elegans have been instrumental in the identification of new and highly conserved genes that regulate fundamental processes, such as programmed cell death and neural transmission (Ellis and Horvitz, 1986; Horvitz and Sulston, 1980; Miller et al., 1996). Unbiased genetic screens for suppressors of gain-of-function mutants (e.g. in apoptosis and Ras signaling) have been particularly important to unravel unsuspected components in these pathways (Hengartner et al., 1992), providing a tremendous drive for advancements in the field. Our identification of a unique gain-of-function mutation in Cav2α1/UNC-2 opens up an exciting opportunity to powerfully elucidate regulatory mechanisms underlying Cav2 channel function, modulation and localization in vivo.

In chapter III, I performed a genetic screen to identify Cav2 channel signaling components. unc-2(zf35gf) mutants are hypersensitive to the acetylcholine esterase inhibitor, aldicarb, as this mutation induces enhanced cholinergic transmission. In this suppressor screen, I searched for mutants that are resistant to the paralytic effects of aldicarb in the unc-2(zf35gf) mutant background. Secondary screen was performed with levamisole to exclude post-synaptic mutations. I isolated 29 alleles from the screen in total including new alleles of genes that have been implicated in regulating Cav2 channels mediated synaptic transmission. For example I isolated alleles of genes that have been shown to play a role in synaptic vesicle release (unc-10, unc-13 and unc-18),
calcium channel trafficking and surface expression (calf-1, unc-10, unc-36 and ccb-1), and calcium channel auxiliary subunits (unc-36 and ccb-1). These results suggested our screening approach was successful in isolating genes required for CaV2 channel function. In addition, I recovered three genes that are less characterized with their roles in modulating CaV2 channel function: Ca1/egl-19, CAPS/unc-31 and a extracellular protein mec-9, as well as one allele that still needs to be identified. Uncovering their roles in CaV2 channel function will potentially illuminate new mechanisms for regulating calcium dependent synaptic transmission.

One limitation of this screening approach is that it also pulls out genes that function in both pre- and post-synapses. For instance, the CaV1 α 1 subunit, egl-19, which functions in both neurons and muscles (Gao and Zhen, 2011), was isolated from the screen and showed levamisole hypersensitivity. In addition, the calcium channel auxiliary subunits, unc-36 and ccb-1, which are required for the function of both CaV1 and CaV2 channels were also recovered from the screen (Catterall, 2011). In these cases, we have to also consider their roles in post-synaptic muscles. To dissect if these genes suppress the unc-2(zf35gf) mutant aldicarb hypersensitivity through pre- or post-synaptic function, neuronal- and muscle-specific rescue of these genes are necessary. In addition, further investigations on why these suppressors are hypersensitive to levamisole despite having defects in muscle CaV1 channels will likely further advance our understanding of homeostatic compensation plasticity.
In addition to aldicarb hypersensitivity, in the future, using other phenotypes of \textit{unc-2(zf35gf)} mutants may lead to identification of molecules in synaptic organization and function in different neuron types. It is less clear how synaptic plasticity applies to different classes of neurons. By searching for mutants that have reduced reversal frequency in the \textit{unc-2(zf35gf)} mutant background, we may isolate potential regulators for glutamate signaling in interneurons controlling directional movements. In addition, looking for mutants that have rescued GABA transmission in the \textit{unc-2(zf35gf)} mutant background will shed light on synaptic plasticity during circuit perturbations.

**Functional significance of the CaVβ SH3 domain**

CaV2 channels are heteromultimeric membrane complexes that are composed of a pore-forming CaVα1 subunit together with auxiliary subunits, CaVα2δ and CaVβ. Despite the fact that the CaVα1 subunit determines the major biophysical and pharmacological properties of the channel, the auxiliary subunits are indispensible and dramatically influence the gating properties and surface expression of these channels (Karunasekara et al., 2009). The CaVβ subunit has been reported to traffic the CaVα1 subunit from the ER to plasma membrane (Bichet et al., 2000) and enhance both the activation and inactivation of CaV2 channels in an isoform-specific manner (He et al. 2007).

The \textit{ccb-1} gene encodes the putative CaVβ subunit in \textit{C. elegans}, and \textit{ccb-1} null allele, \textit{ccb-1 (gk18)} that contains a large deletion of \textit{ccb-1} gene, leads
to embryonic lethality, suggesting the importance of the CaVβ subunit function (Lainé et al., 2011). In chapter III, I isolated a hypomorphic allele, zf121, of ccb-1 from the suppressor screen. ccb-1(zf121) mutants are viable with defects in locomotion and egg-laying behaviors. The zf121 allele has an isoleucine to threonine amino acid substitution in the highly conserved SH3 domain. CaVβ subunits share a common structure with a highly conserved core region, containing an Src homology 3 (SH3) domain and an enzymatic inactive guanylate kinase (GK) like domain, which are connected by a less conserved HOOK region (Chen et al. 2004). GK domain has been well demonstrated with its importance in the direct interaction with the CaVα1 subunit, and this interaction is required for regulating CaVα1 subunit gating properties and surface expression (Opatowsky et al., 2004). My in vivo experiments support these findings as ccb-1(ΔGK) cDNA failed to rescue either ccb-1(zf121) or ccb-1(gk18) mutants.

On the other hand, the functional significance of SH3 domain and HOOK domain remains to be elucidated. Our suppressor ccb-1(zf121) mutants provide a line of evidence for the functional interaction between the CaVβ SH3 domain and CaV2α1 subunits. The SH3 domain mediates specific protein-protein interactions with proline-rich recognition sites, such as PXXP (McPherson, 1999). In general, CaVβ SH3 domain has well conserved sequence and structure consisting of five β strands. However, crystallographic studies have shown that the fifth β strand is separated by the HOOK domain which creates a “split architecture” for the SH3 domain of the CaVβ subunit, which might allow CaVβ subunits to have more
diverse interacting partners (Karunasekara et al., 2009; Opatowsky et al., 2004; Van Petegem et al., 2004). Potential binding partners range from protein kinases, GTPase, membrane receptors and ion channels to synaptic proteins (Rima et al., 2016). Performing pull down assays in transgenic animals carrying GFP-tagged wild-type CCB-1, CCB-1(ΔSH3) and CCB-1(zf121) and cross reference the interacting proteins will likely identify SH3 domain specific binding partners. Further mechanistic investigation on these binding partners will potentially provide insight into the roles of the SH3 domain in modulating CaV2 channel function.

**CaVβ subunits in regulating transcriptional activity**

For many years, the main functions associated with CaVβ subunits are trafficking and modulating the gating properties of CaVα1 subunits. However, several studies have shown its role in regulating transcription (Hibino et al., 2003; Xu et al., 2011), emphasizing the multifunctional role of this versatile protein. The CaVβ subunit is the only cytosolic auxiliary subunit of CaV channels and multiple lines of evidence have suggested that CaVβ subunits are able to translocate to nuclei in an activity and calcium dependent manner (Subramanyam et al., 2009; Tadmouri et al., 2012; Taylor et al., 2014). This translocation couples electrical activity to gene expression, and defects in this process are associated with juvenile epilepsy (Tadmouri et al., 2012). Excitingly, CaVβ function in transcription activity is believed to be independent of the GK domain (Hibino et al., 2003), which is
consistent with our finding that CaVβ/CCB-1 localizes to the nucleus in a SH3 and HOOK domain dependent manner. In addition, preliminary data suggest that mutant CaVβ/CCB-1(zf121) subunits, with a isoleucine to threonine mutation in the SH3 domain, were also largely excluded from the nucleus. This raised the possibility that the zf121 mutation suppresses the gain-of-function CaV2α1 through a mechanism independent of the CaV2 gating properties.

With the power of CRISPR technique, I can now label the endogenous CaVβ/CCB-1 gene with a fluorescence tag to observe the localization of the endogenous CCB-1 in vivo and avoid overexpression effects (Paix et al., 2015). Optogenetic manipulation of neuronal activity provides a way to precisely deliver the stimulation to specific neuron subsets (Leifer et al., 2011). Together, I will be able to examine if the CCB-1 localization is neuronal activity dependent in living animals. ccb-1 cDNA does not contain any conventional nuclear localization signal (NLS) sequences, suggesting the nuclear localization of CCB-1 require at least one protein partner. I showed that this nuclear localization is SH3 domain dependent. Thus, the identification of specific binding partners of the SH3 domain will provide potential gene candidates that are required for this process. Lastly, comparing the RNA seq profiling between the transgenic strains carrying wild-type CCB-1 and CCB-1(ΔSH3) will offer promising gene candidates that are regulated transcriptionally regulated by the CaVβ subunits.

Calcium is one of the most important second messengers in neurons and plays a key role in various cell signaling pathways. Calcium entry into neurons
occurs mainly via presynaptic CaV2 channels, therefore, understanding the functional consequences of CaV2 dysfunction and determining the functional interacting proteins of CaV2 channels is crucial to comprehend the regulation of these physiological events. My doctoral thesis work has shown gain-of-function CaV2 channels result in increased calcium influx and synaptic release. At the neural circuit level, elevated CaV2 channel activity leads to an imbalance between excitatory and inhibitory neurotransmission, which has been implicated in several neurological disorders. Suppressors from the genetic screen have provided new potential modulators of CaV2 channels as well as a novel pathway for Cavβ subunit function. Together, the work has provided new insights into the pivotal role of CaV2 channel activity in the integration of neural circuits and lays the groundwork for future investigators to explore the regulation of CaV2 channels at molecular, cellular and circuit levels.


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