Investigating the role of a cation channel-like protein NCA-1 in regulating synaptic activity and development in *Caenorhabditis elegans*

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Molecular Genetics

University of Toronto

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**Abstract**

NCA-1 (putative nematode calcium channel) and NCA-2 are two cation channel-like proteins in *Caenorhabditis elegans* that function redundantly to regulate locomotion through unknown mechanisms. A recent study from our lab showed that *in vivo* Ca\(^{2+}\) imaging analyses of egg-laying neurons in *nca-1* loss- and gain-of-function mutants implicate that NCA channels regulate Ca\(^{2+}\) flux at synapses, without affecting Ca\(^{2+}\) dynamics in neuron somas. Furthermore, we observed that NCA-1 localizes to non-synaptic region along axons, strongly suggesting that NCA channels propagate electrical signals from cell bodies to synapses. To identify molecular components that function in the *nca-1* genetic pathway, I performed a genetic suppressor screen that led to the
identification of behavioral suppressors of \textit{nca-1} gain-of-function mutant. Possible NCA auxiliary subunits, UNC-79 (\textit{uncoordinated}) and UNC-80, were identified from this screen. Molecular characterization of other suppressors will help to identify other regulators and downstream signaling components through which NCA channels transmit electrical signals.
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<tr>
<td>C. elegans</td>
<td><em>Caenorhabditis elegans</em> (nematode)</td>
</tr>
<tr>
<td>NCA</td>
<td>putative nematode calcium channel</td>
</tr>
<tr>
<td><em>unc</em></td>
<td>uncoordinated locomotion</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>$I_{Na}$</td>
<td>sodium current</td>
</tr>
<tr>
<td>$I_K$</td>
<td>potassium current</td>
</tr>
<tr>
<td>BK channel</td>
<td>large conductance $\text{Ca}^{2+}$-activated $\text{K}^+$ channel</td>
</tr>
<tr>
<td>SK channel</td>
<td>small conductance $\text{Ca}^{2+}$-activated $\text{K}^+$ channel</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated $\text{Ca}^{2+}$ channel</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF-attachment protein receptor</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosome-associated protein of 25 kDa</td>
</tr>
<tr>
<td>VAMP</td>
<td>vesicle-associated membrane protein</td>
</tr>
<tr>
<td>CSP</td>
<td>cysteins string protein</td>
</tr>
<tr>
<td>Rim</td>
<td>Rab3A interacting molecule</td>
</tr>
<tr>
<td>ORL1</td>
<td>opioid receptor like 1</td>
</tr>
<tr>
<td>LVA</td>
<td>low-voltage-activated</td>
</tr>
<tr>
<td>HVA</td>
<td>high-voltage activated</td>
</tr>
<tr>
<td>DHP</td>
<td>dihydrophryidine</td>
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<tr>
<td>NALCN</td>
<td>$\text{Na}^+$ leak channel, nonselective</td>
</tr>
<tr>
<td>Dm$\alpha$1U</td>
<td><em>Drosophila melanogaster</em> $\alpha1$ subunit unique within the voltage-gate channel super family</td>
</tr>
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</table>
I current

V voltage

TTX tetrodotoxin

WT wild-type

AHP after hyperpolarization

Egl egg-laying defective

cca-1 Ca\(^{2+}\) channel alpha subunit 1

Vgcn1 voltage-gated ion channel 1

NMJ neuromuscular junction

lf loss-of-function

gf gain-of-function

mPSC miniature postsynaptic current

HSN hermaphrodite specific neuron

GFP green fluorescent protein

RFP red fluorescent protein

GABA gama-aminobutyric acid

N2 wild-type, Bristol strain

CB wild-type, Hawaiian strain

Bli blistered phenotype

PCR polymerase chain reaction

syd-2 synapse defective 2

sup suppressor

Dpy dumpy phenotype
SNP  single nucleotide polymorphism
Chapter 1 Introduction

1.1 Neuronal Excitation

Many excitable cells use electrical signals for intercellular communication and as stimuli for the generation of intra- and inter-cellular signaling messengers [1]. The electrical signals in the nervous system are transient and rapid, allowing the communication to be carried over long distances in a timely manner. In general, the generation of electrical signals involves the conductance of different ions through voltage-gated Na\(^+\), K\(^+\), and Ca\(^2+\) channels across the membrane [2]. These gated ion channels regulate temporal alterations in current flows by driving the electrical potentials of membranes away from their resting potentials [3]. The most common and well-studied form of neuronal excitation is the pulse-like electrical signals called action potentials.

1.1.1 Membrane Potential and Threshold

The lipid bilayer of the plasma membrane not only separates the cellular organelles from the extracellular environment, but also prevents the diffusion of ions across the membrane. The imbalanced concentration of ions across the membrane creates a difference of net charges, resulting in an electrical potential or voltage called the membrane potential [3].

The membrane potential of a cell at rest is called the resting potential. The excitability of a cell depends significantly on the resting membrane potential, which is determined by the relative distribution and permeability of different ions across the two sides of the cell membrane. The most abundant ion species in neurons are Ca\(^{2+}\), Na\(^+\) and Cl\(^-\), which are concentrated extracellularly, and K\(^+\) and organic anions such as amino
acids and proteins, which are concentrated intracellularly [3]. The intracellular concentrations of Na$^+$ and K$^+$ are established by the Na$^+$-K$^+$ pump which drives out three Na$^+$ ions and brings in two K$^+$ ions against the concentration gradient in an ATP dependent manner. In comparison, the distribution of Cl$^-$ is maintained passively in many neurons [3]. Among the abundant ion types, Ca$^{2+}$, Na$^+$, Cl$^-$, and K$^+$ are permeable at the resting membrane potential in neurons [4-6]. These permeant ions move passively in and out of the neuronal membrane through the resting (also called leak or persistent) ion channels. The two forces driving the flow of an ion are the concentration gradient-dependent chemical driving force, and the electrical driving force that depends on the electrical potential difference across the membrane [7]. Over time, the leakage of these ions will dissipate the ionic gradients, and reduce the resting membrane potential. Here, the Na$^+$-K$^+$ pump actively counter-balances the passive ion leaks to maintain a steady membrane potential. Ultimately, the relative proportion of different types of resting ion channels and active ion pumps determines the resting membrane potential of a neuron.

When a depolarizing electrical signal excites a neuron, voltage-gated ion channels open actively to generate an all-or-none action potential once the membrane potential reaches a critical level, called the threshold. There are three factors that contribute to the threshold value: the resting membrane resistance, the membrane capacitance, and the intracellular axial resistance along the axon [3]. The resting resistance depends on the density of resting ion channels and their conductance. The areas of the neuron with a higher density of resting ion channels and/or voltage-gated Na$^+$ channels (or other voltage sensitive cation channels) tend to have a lower threshold for generating action potentials, as the membrane is much more sensitive to voltage change. The membrane capacitance
increases with the size of the cell since larger cells have greater membrane areas which can store more charge. Thus, stronger current is required to change the net charge stored in the phospholipids bi-layers. The axial resistance depends on the intrinsic properties of the cytoplasm components and on the cross-sectional area of the neuronal process. A cytoplasmic core of larger diameter has a greater number of charge carriers and therefore the ions experience fewer collisions as they travel, leading to a lower resistance. In summary, the threshold for action potential generation is determined by the ion channel composition and the anatomy of individual neurons.

1.1.2 Action Potential: Generation and Propagation

Action potential is the rapid electrical impulse traveling along axons [2]. The all-or-none firing of action potentials is initiated by an excitatory stimulus that makes a supra threshold membrane depolarization [2, 8]. The membrane becomes depolarized (the rising phase) until it reaches a maximum velocity at a voltage near 0 mV, then repolarizes to the resting membrane potential (the decaying phase) (Fig. 1.1).

An action potentials were first described in the classical Hodgkin-Huxley analysis of the squid axon [9]. Using the voltage clamp developed by Marmont [10], Cole [11], and Hodgkin, Huxley and Katz [5, 12], Hodgkin and Huxley demonstrated that the action potential is mainly composed of two currents, $-I_{Na}$ and $I_{K}$ [13]. A combination of various approaches since 1970, from the molecular cloning of various channels, pharmacology studies, heterologous expression studies, to the development of patch-clamp technique for single channel recordings [14], revealed that action potentials in the squid axon are generated by voltage-gated Na$^+$ and K$^+$ channels.
The rising phase of the action potential is associated mainly with the opening of voltage-gated Na\(^+\) channels, which is triggered by the subthreshold depolarizing LVA (low-voltage-activated) Ca\(^{2+}\) current to bring the membrane potential closer to the threshold for a Na\(^+\) spike [2, 9] (Fig. 1.1). The inward Na\(^+\) current activates rapidly in hundreds of microseconds, and inactivates in less than a millisecond when membrane potential is above 0 mV [8]. The gating properties and kinetics of the Na\(^+\) current in mammalian CNS neurons [15-19] and squid axons [20, 21] are similar, and will be described in detail in a later section.

The repolarization of membranes is due mainly to voltage-gated K\(^+\) channels. This outward K\(^+\) current during the decaying phase of action potentials is mediated by the Kv3 family channels in mammalian neurons [22, 23]. In addition, two other K\(^+\) channels, the large conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK channels) [24-26] and the small conductance Ca\(^{2+}\)-activated K\(^+\) channels (SK channels) [27-34] also contributed to the decaying phase of action potentials in mammalian neurons. The activation of BK channels and SK channels are coupled to Ca\(^{2+}\) entry through different types of voltage-gated Ca\(^{2+}\) channels that open during the rising phase.

Before the membrane returns to its resting potential, the membrane permeability to K\(^+\) exceeds that during the resting state due to the delay in closing of the voltage-gated K\(^+\) channels. Hence, the membrane experiences a transient hyperpolarization stage, during which the membrane exhibits decreased excitability. During this absolute refractory period immediately following the firing of action potential, no action potential can be initiated. Directly after this phase is the relative refractory period, during which stronger supra-threshold stimuli are able to trigger an action potential.
The firing of an action potential behaves as an all-or-none event. This is because any subthreshold depolarization activates both voltage-gated Na\(^+\) and K\(^+\) channels, which increases not only the inward Na\(^+\) current, but also the outward K\(^+\) current as well as the electrochemical driven leakage current. However, depending on the strength of the stimulus and the specific property of the neuronal membrane, a supra-depolarization can eventually drive the inward Na\(^+\) current to surpass the outward potassium and leakage currents to induce membrane depolarization, hence the firing of action potential.

An action potential is regenerative, allowing it to propagate along the axon. After an action potential is fired, depolarization spreads electronically along the axon to depolarize the adjacent regions to approach the threshold, and generate another wave of action potential. The diameter of neuronal processes plays a significant role in the propagation of the action potential because lower axial resistance allows for more efficient and passive spread of voltage changes along the neuron. A big axon with large membrane areas and cytoplasmic volumes usually allows greater travel distance, and more rapid longitudinal flow of electrical conduction as the number of ion channels is higher and the resistance is lower.

1.1.3 Activation of Synaptic Transmission: Voltage-gated Ca\(^{2+}\) Channels at Synapses

When an action potential arrives at the presynaptic terminal, the voltage-gated Ca\(^{2+}\) channels (VGCCs) open and allow for Ca\(^{2+}\) influx. Together with the presynaptic intracellular Ca\(^{2+}\) buffers and pump systems, VGCCs regulate the release of vesicle-enclosed neurotransmitters through Ca\(^{2+}\)-dependent membrane fusion and exocytosis
[35]. There are different subtypes of VGCCs, and their classification will be discussed later (section 1.2.2). Among the multiple VGCCs, the presence of N-, P/Q- and R- type VGCCs have been implicated at the presynaptic termini, but most studies are carried out on N- and P/Q-type VGCCs [36].

Both N- and P/Q- type VGCCs bind to various synaptic proteins through the intracellular loop between domains II and III. These synaptic proteins include the SNARE (soluble NSF-attachment protein receptor) protein complex syntaxin, SNAP-25 (synaptosome-associated protein of 25 kDa), and VAMP (vesicle-associated membrane protein)/synaptobrevin [37], and other synaptic proteins such as CSP (cysteins string protein), Rim (Rab3A interacting molecule) and synaptotagmin 1 [38, 39]. These Ca\(^{2+}\) dependent [40] interactions are important for effective exocytosis through positioning synaptic vesicles near VGCCs. Consistently, injecting peptides to block the binding between these VGCCs and the synaptic proteins is sufficient to reduce neurotransmission [41, 42].

N- and P/Q- type VGCCs are also capable of integrating other signaling inputs to regulate Ca\(^{2+}\) homeostasis in a voltage-independent manner. When cultured tsA-201 cells are exposed to agonist for 15-30 minutes, the nociceptin receptor (also known as opioid receptor like 1 ORL1) mediates degradation of the N- type VGCCs, thereby reducing Ca\(^{2+}\) influx at the membrane [43, 44]. Another example of voltage independent regulation specific to P/Q type but not N type VGCC, is the calmodulin mediated Ca\(^{2+}\) dependent facilitation. After the P/Q type VGCCs are opened, Ca\(^{2+}\) binds to the high-affinity binding sites on calmodulin, which interacts with the channel C-terminal tails to further rapidly activate the channels [45].
1.2.1 Voltage-Gated Na\textsuperscript{+} Channels

The voltage-gated Na\textsuperscript{+} channel in the mammalian brain comprises the 260 kDa ion-conducting pore-forming α subunit and the auxiliary subunits β1, β2, and/or β3 [46-48] (Fig. 1.2A). The α subunit alone in the heterologous expression systems is sufficient to form voltage-sensitive Na\textsuperscript{+} channels that display rapid inactivation [49-51]. The β subunits are required for the modulation of channel kinetics and voltage range for channel gating [52, 53], as well as the localization and interactions between the channel and cell adhesion molecules, extracellular matrix, and intracellular cytoskeleton [54].

There are nine functionally characterized mammalian α subunit isoforms [54]. The neuronally enriched Na\textsubscript{V}1.1, Na\textsubscript{V}1.2, Na\textsubscript{V}1.3, and Na\textsubscript{V}1.7 are most closely related by phylogeny, similar in biophysical characteristics, and are highly sensitive to the neurotoxin tetrodotoxin (TTX). Na\textsubscript{V}1.5, Na\textsubscript{V}1.8, and Na\textsubscript{V}1.9 are also closely related by amino acid sequence, but display varying degrees of resistance to TTX. They are highly expressed in heart and dorsal root ganglion neurons [48]. Na\textsubscript{V}1.4 is highly expressed in the skeletal muscle and Na\textsubscript{V}1.6 is expressed in the central nervous system. Although both sensitive to TTX, they are phylogenetically distinct from each other and are evolutionarily distant from the other two groups [54].

1.2.1.1 The Structure of Voltage-Gated Na\textsuperscript{+} Channel Pore-Forming α-Subunit

The primary structure of the α subunit contains six α-helical transmembrane segments (S1-S6) in each of the four homologous domains (I-IV) [48] (Fig. 1.2A). An extracellular reentrant loop between S5 and S6 inserts back into the transmembrane...
region of the channel to form the outer pore and the ion selectivity filter. A pair of amino acid residues in analogous positions in each of the four domains forms the outer (EEDD) and inner (DEKA) rings that serve as the selectivity filter and as the receptor site for blockers TTX and saxitoxin [48]. The voltage sensor is located in S4. The intracellular loops are generally larger than the extracellular loops, except the relatively large extracellular loops connecting S5 or S6 to the reentrant loop. The N-terminal and C-terminal domains are also large and intracellular. The three-dimensions structure reveals a bell-shaped pore with the central pore splitting into four branches [55, 56], possibly allowing for channel gating. (Fig. 1.2B)

1.2.1.2 Voltage Dependent Activation

In response to voltage changes across the membrane, the movement of the gating charges in the voltage-sensing domains in voltage-gated Na\(^+\) and Ca\(^{2+}\) channels lead to their opening [9, 57]. In voltage-gated Na\(^+\) channels, the voltage sensing S4 segments contain repeated motifs of a positively charged amino acid residue followed by two hydrophobic residues that may form a cylindrical \(\alpha\) helix wrapped around by a spiral ribbon of positive charges [48]. At resting membrane potentials, the negative internal transmembrane electrical field pulls these positive charges into the cell in an inclined position. According to the sliding helix [58] or helical screw [59] model, the S4 helices are released to move outward in a spiral path upon depolarization. The outward rotation initiates a conformational change that opens the pore [48] to allow for Na\(^+\) influx.
1.2.1.3 Inactivation

The process of channel inactivation occurs within milliseconds following the channel opening, and abnormality in this process is associated with many channelopathies [48, 56]. The generally accepted model for fast inactivation involves the intracellular loop between domains III and IV. It is proposed that this loop can function as an inactivation gate that folds in and binds to the intracellular pore of the channel [57]. A tethered pore blocker contains a motif of a hydrophobic triad IFM in the inactivation gate, and binds to a specific region in the intracellular opening of the pore [60, 61]. The residue F1489 in the inactivation gate motif and the nearby T1491 seem to be particularly important for fast inactivation [62]. Mutagenesis experiments also revealed multiple hydrophobic residues near and within the intracellular mouth of the pore that contribute to the formation of the binding-region for the inactivation gate. These hydrophobic residues are located within the intracellular loops between S4 and S5 of domains III [63] and IV [64-67], and in the end of the intracellular segment of IVS6 [68].

Inactivation is also a voltage dependent process that couples with the activation of the channel. The end of the extracellular region of segment IVS4 is predicted to be involved in this coupling [69]. The present model is that fast inactivation of the channel is initiated by the outward movement of the IVS4 segment [48], but in depth molecular mechanism of this coupling still awaits investigation.

1.2.2. Voltage-gated Ca\(^{2+}\) Channels

Subtypes of voltage-gated Ca\(^{2+}\) channels are classified based on their electrophysiology and pharmacological properties. Two major categories are the high-
voltage-activated (HVA) and low-voltage-activated (LVA) Ca$^{2+}$-currents [70]. HVA Ca$^{2+}$-currents are further divided into the dihydropyridine (DHP) sensitive L-type and DHP insensitive non-L type Ca$^{2+}$ currents. The non-L-type currents include the snail toxin ω-conotoxin GIVA sensitive N-type current, the ω-conotoxin MVIIC and spider toxin ω-agatoxin IVA sensitive P/Q-type current, and the toxin resistant R-type current [70].

Strong depolarization signals are usually required for the activation of the L-type currents to initiate muscle contraction and secretion by endocrine cells [71]. There are also LVA-L-type currents that are predominantly confined to neurons and cardiac pacemaker cells. Most N-, P/Q-, and R- type currents are present in neurons for activation of fast synaptic transmission, and for mediating Ca$^{2+}$ entry into cell bodies and dendrites. The LVA- or T-type current that is resistant to both L-type antagonist and toxins that block N- and P/Q- type currents, is expressed in many cell types and is predicted to shape the action potential and to control patterns of repetitive firing [71].

1.2.2.1 Structures and Functions of Voltage-gated Ca$^{2+}$ Channel Subunits

The pore-forming α$_1$ subunit of VGCC is highly similar to that of the voltage-gated Na$^+$ channels. It too, is organized in four repeated domains I to IV, with each domain containing six transmembrane segments (S1 to S6) and a membrane-associated loop between the S5 and S6 segments (Fig. 1.3). The major difference between the α-subunits of VGCC and voltage-gated Na$^+$ channel resides at the amino acid sequence for the ion selectivity filter. In contrast to the DEKA sequence in voltage-gated Na$^+$ channels, the sequence is EEEE in HVA Ca$^{2+}$ channels, and EEDD in LVA Ca$^{2+}$ channels [70].
The VCGG contain several auxiliary subunits that differ from those for voltage-gated Na\(^+\) channels. The \(\beta\) subunit is intracellular and predicted to contain mainly \(\alpha\) helical structures [72]. The \(\gamma\) subunit is a glycoprotein with four transmembrane segments [73]. The \(\alpha_2\) is an extracellular protein with many glycosylation sites and is linked to the membrane through a disulfide bond with the \(\delta\) subunit [74], which is an \(\alpha_2\) subunit isoform [75]. In general, the co-expression of the \(\beta\) subunit enhances expression level of the \(\alpha_1\) subunit, as well as shifting the voltage dependent activation and inactivation to more negative membrane potentials [76]. In comparison, the \(\gamma\) and \(\alpha_2 \delta\) subunits have similar, yet, milder functional effects [76].

1.2.2.2 Activation of Voltage-gated Ca\(^{2+}\) Channel

The mode of activation of voltage-gated Na\(^+\) and Ca\(^{2+}\) channels is very similar. The voltage sensors in both channels are located in the S4 segments, and the repeated motifs of positive charges are also conserved in both channels [2]. According to the measurements by Hirschberg et al. [77], there are 12 elementary gating charges per channel in the skeletal muscle Na\(^+\) channel. In the case of VGCC, Noceti et al. [78] used the limiting slope method to obtain a consistent value of 8.6 in both cardiac and neuronal VGCC regardless of the presence and absence of \(\beta\)-subunits. This value was derived from the slope of current to voltage (I-V) curve in a negative potential range where the probability of channel opening was low and current conductance was mostly contributed by gating currents.
1.2.2.3 Inactivation

The mechanisms for inactivation differ significantly between different subtypes of VGCCs. The inactivation of the LVA-Ca$^{2+}$ channels is much more rapid than that of the HVA Ca$^{2+}$ channels. The rapid LVA-Ca$^{2+}$ channel inactivation appears similar to the typical voltage-dependent process displayed by voltage-gated Na$^+$ channels [2]. On the contrary, the HVA-Ca$^{2+}$ channel inactivation appears to be Ca$^{2+}$ dependent [79, 80]. According to this model, the function of HVA-Ca$^{2+}$ channels becomes self-limiting: their opening leads to not only the raise of local Ca$^{2+}$ concentration, but also its eventual inactivation. This Ca$^{2+}$ dependent inactivation is likely mediated by calmodulin in L-type VGCCs. In the presence of Ca$^{2+}$, calmodulin binds to different regions of the C terminal tail of the channel to modulate the inactivation kinetics [81, 82].

1.3 The NCA Family Channel

The NCA (putative nematode calcium channel) and NCA-related proteins comprise a class of channels that displays an overall structural similarity to both voltage-gated Na$^+$ and Ca$^{2+}$ channels, but differs in some specific residues that control the channel kinetics and ion selectivity. This class of channels contains some but not all of the positive charges in the voltage sensing S4 segments. Moreover, the key residues forming the ion selectivity filter in this channel family are EEKE, which differs from that in either voltage-gated Na$^+$ channels (DEKA), or voltage-gated Ca$^{2+}$ channels (EEEE or EEDD) [70]. Homologues of this channel family have been identified in many model organisms, including a mouse homologue NALCN (for Na$^+$ leak channel, nonselective) [83, 84], a Drosophila melanogaster homologue Dmα1U (for Drosophila melanogaster α1 subunit
unique within the voltage-gate channel super family) [85-87], and two C. elegans homologues NCA-1 and NCA-2 [88]. Their channel properties as well as physiological functions have only begun to be elucidated in this year [84, 89, 90].

1.3.1 Mouse NCA Regulates Membrane Potentials and Firing Frequency of Neurons

One major breakthrough in understanding this class of putative channels came from both in vivo and in vitro studies of mouse NALCN1 by Lu et al. [84]. Their study on channel properties leads to the hypothesis that NALCN, as a nonselective cation channel, forms the background Na\(^+\) leak conductance to regulate neuronal resting membrane potentials. Using a step voltage protocol, the authors detected a linear current-voltage (I-V) relationship in more than 80% of the NALCN-transfected HEK293 cells. This linear I-V relationship implies that NALCN opens constitutively regardless of the range of voltage applied. By changing the concentrations of various ions, they determined that NALCN is impermeable to anion Cl\(^-\), but permeable to cations in the following order: Na\(^+\)>K\(^+\)>Cs\(^+\)>Ca\(^{2+}\). Taken together, NALCN likely forms a voltage independent non-selective cation channel that is highly permeable to Na\(^+\) in vivo. These properties led the authors to postulate that NALCN is possibly the molecular entity responsible for the TTX and voltage insensitive background leak Na\(^+\) current in vivo [8, 91]. In support of this premise, the authors still detected Na\(^+\) dependent currents in the WT hippocampal neurons after blocking the two major Na\(^+\) background currents, the TTX sensitive depolarization activated persistent Na\(^+\) currents and the Cs\(^+\) sensitive
hyperpolarization-activated cation currents [8]. However, this Na$^+$ dependent leak conductance is absent in the hippocampal neurons isolated from NALCN deletion mice.

The resting membrane potentials of the NALCN mutant hippocampal neurons were decreased, consistent with NALCN contributing to the establishment of resting membrane potentials by conducting the constitutive Na$^+$ inward currents. Since neuronal excitability is determined by voltage difference between the resting membrane potentials and the membrane threshold, NALCN is important for controlling neuronal excitability. Indeed, the NALCN mutant neurons displayed a reduced frequency of continuous firing, which was manifested into disrupted respiratory rhythms in mutant pups and eventually led to neonatal lethality.

3.2 Role of NCA Channels in Biological Rhythmic Activity Regulation

The physiological role of the NALCN family seems to be associated with regulating rhythmic behaviors, such as respiration in mouse [84], circadian behaviors in Drosophila [85, 86], and the locomotory and egg-laying activities in C. elegans [89, 90]. All of these are neuronal outputs that involve precise control of behavioral patterns and frequencies. In the mouse model, Lu et al. demonstrated that the abnormal respiratory rhythms in NALCN mutant mice were likely due to the absence of robust electrical activity in the fourth cervical nerve root (C4) that innervates the diaphragm and activates rhythmic electrical signals to control breathing.

While respiration is one form of general biological rhythm, daily or circadian rhythm is another more specific form that uses light as the environment entraining stimulus to synchronize to the day-night cycle [92]. The general mechanism for
controlling the circadian rhythm is conserved, but the underlying neurons differ from species to species. In *Drosophila*, the clock genes for regulating circadian rhythms are expressed in a group of lateral neurons and dorsal neurons [93]. *Drosophila DmA1U* expression in these neurons was required for increased activity in anticipation to diurnal lighting under 12 hours light; 12 hours dark cycle conditions [86]. Moreover, *DmA1U* expression in ventral lateral neurons is necessary for robust locomotory rhythms during the constant dark condition. In addition to the circadian rhythm related phenotypes, the *DmA1U* mutant also displayed a hesitant walking phenotype [85]. This phenotype resembles the fainting locomotion phenotype displayed by the double loss of function mutant *nca-1; nca-2* in *C. elegans* [89, 90], and it can be rescued by neuronal expression of NCA-1 [90].

Although mutants of this channel family all displayed abnormal rhythmic behaviors, expression of family members is not exclusive to rhythm regulatory neurons, rather, these channels are more broadly expressed in the nervous system [84, 85, 90]. Thus, the behavioral rhythmicity may be secondary to a more global neuronal regulation of excitability that ensures efficient generation and/or propagation of electrical signals.

This channel family is dispensable for development in invertebrates, as reduced or abolished channel expressions in *C. elegans* and *Drosophila* mutants exhibit only arrhythmic or sporadic activities in some behaviors, without any gross anatomical defect [85, 89, 90]. In contrast, the mouse NALCN was required for neonatal survival [84], which may suggests a more important function in the vertebrate system. The second chapter of this thesis is a submitted manuscript that explores the cellular function of the
*C. elegans* NCA channel, and demonstrates that NCA, along with its auxiliary subunits UNC-79 and UNC-80, propagates calcium spikes along neuronal axons in *C. elegans*. 
Figure 1.1: Anatomy of an action potential

Typical shape of an action potential. The values shown here are taken from recording in the pyramidal neuron in the CA1 region of a rat hippocampus [94]. The action potential elicited by a suprathreshold current is shown in dark grey, and the response to a subthreshold current injection is shown in light grey. The resting potential ($V_{rest}$) is the membrane potential when the neuron is at rest. The voltage threshold ($V_{thresh}$) is the critical level that the membrane potential must reach to generate the action potential. The rising phase of the action potential reaches a maximum velocity near 0 mV. The peak relative to 0 mV is termed as overshoot, which represents the period during which the voltage-gated Na$^+$ channels are inactivating and the outward $I_K$ is slowly increasing to enter the decaying phase. AHP (after hyperpolarization) is the most negative voltage an action potential reaches during the refractory period. Spike height is defined as the peak relative to AHP. Figure modified and adapted from Ref. 8
Figure 1.2: Structure of voltage-gated Na\(^+\) channel

(A) Schematic representation of the pore forming \(\alpha\) subunit in association with \(\beta1\) and \(\beta2\) subunits. The domains of \(\alpha\) subunit are labeled with Roman numerals and the transmembrane segments are labeled with Arabic numbers in each domain. The pore lining S5 and S6 are highlighted in dark grey. The voltage sensors S4 are colored in light grey with plus signs. The ion selectivity filter is represented by the circles in the reentrant loops between S5 and S6 in each domain. The outer ring sequence is EEDD (dark grey circles), and inner ring sequence is DEKA (light grey colors). Dark grey circle with the label h indicates the inactivation gate IFM motif, and empty dark grey circles indicate the IFM binding sites.

(B) The three-dimensional structure of voltage-gated Na\(^+\) channel \(\alpha\) subunit at 20 Å resolution.

Figure modified and adapted from Ref. 56
Figure 1.3: Structure of voltage-gated Ca\textsuperscript{2+} channel

The pore forming \(\alpha_1\) subunit in association with auxiliary subunits \(\beta\), \(\alpha_2\), \(\gamma\), and \(\delta\). The \(\alpha_1\) subunit structure is similar to the \(\alpha\) subunit of voltage gated sodium channel. The major difference is the ion selectivity filter sequence is EEEE (grey circle), in contrast to DEKA in voltage gated Na\textsuperscript{+} channel. Figure modified and adapted from Ref. 76.
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Chapter 2 A Putative Cation Channel NCA-1 and a Novel Protein UNC-80 Transmit Neuronal Activity in C. elegans

Chapter 2 is a submitted manuscript that has just been accepted for publication in PLoS Biology, of which I am one of the three co-first authors. The author list includes Edward Yeh*, Sharon Ng*, Mi Zhang*, Magali Bouhours, Ying Wang, Min Wang, Wesley Hung, Kyota Aoyagi, Katya Melnik-Martinez, Michelle Li, Fang Liu, William R Schafer and Mei Zhen (* indicates equal contributions). The manuscript was written by Dr. Mei Zhen. I contributed to the mapping and cloning of nca-1(hp102), which were part of my undergraduate project. My contributions as a team member during my graduate study included: determining the NCA and UNC-80 expression pattern and their inter-dependent subcellular localization; sequencing of two unc-80 mutations; analyses of active zone phenotypes in different mutants. Moreover, I alone performed genetic analysis of the interactions between nca and unc-80, as well as nca and unc-80 with other calcium channel mutants. I also contributed to the preparation of Figure 1, 4, 5, 7, S4, and S6.
Summary

Voltage-gated cation channels regulate neuron excitability through selective ion flux. NALCN, a member of a protein family structurally related to the α1 subunits of voltage-gated sodium/calcium channels, was recently shown to regulate the resting membrane potentials by mediating sodium leak and the firing of mouse neurons. We identified a role for the *C. elegans* NALCN homologues NCA-1 and NCA-2 in the propagation of neuronal activity from cell bodies to synapses. Loss of NCA activities leads to reduced synaptic transmission at neuromuscular junctions and frequent halting in locomotion. *In vivo* calcium imaging experiments further indicate that while calcium influx in the cell bodies of egg-laying motorneurons is unaffected by altered NCA activity, synaptic calcium transients are significantly reduced in *nca* loss-of-function mutants and increased in *nca* gain-of-function mutants. NCA-1 localizes along axons and is enriched at non-synaptic regions. Its localization and function depend on UNC-80, which is a novel conserved protein also enriched at non-synaptic regions. We propose that NCA-1 and UNC-80 regulate neuronal activity at least in part by transmitting depolarization signals to synapses in *C. elegans* neurons.
Introduction

Neurons generate and propagate electrical signals along nerve processes, which are converted into chemical communication through neurotransmitter release at synapses. By allowing selective ion flux across the plasma membrane, cation channels regulate the excitation and function of neurons. In most nervous systems, action potentials, the traveling and rapidly reversing membrane potentials, are induced by the opening of voltage-gated sodium channels and are modulated by voltage-gated sodium, potassium and occasionally calcium (Ca\(^{2+}\)) channels [1,2]. Action potential-induced depolarization at presynaptic termini triggers the opening of voltage-gated calcium channels (VGCCs), leading to an influx of Ca\(^{2+}\) that allows for Ca\(^{2+}\)-dependent synaptic vesicle exocytosis and the release of neurotransmitters [3].

Voltage-gated sodium channels consist of a pore-forming \(\alpha_1\) subunit and variable numbers of auxiliary \(\beta\) subunits [4]. They display similar properties and possess similar functions in establishing membrane thresholds, generating and propagating action potentials. In contrast, multiple neuronal VGCCs differ in composition, property, localization and function. All known VGCCs are composed of a pore-forming \(\alpha_1\) subunit, which associates with various accessory \(\alpha_2\delta\), \(\beta\) and \(\gamma\) subunits that modulate the property of the channel [4-6]. Vertebrates have at least six subfamilies of VGCCs with different opening probabilities and kinetics [4-6]. Among them, P/Q- and N-type VGCCs are components of the active zone, the presynaptic subcellular structure where synaptic vesicles are released [7,8]. They mediate the Ca\(^{2+}\) influx that triggers the membrane fusion between synaptic vesicles and presynaptic termini [9]. Other VGCCs can also
participate in the modulation of neuronal excitation, affecting the duration of action potentials of specific neurons [2,10].

*C. elegans* does not encode voltage-gated sodium channel orthologues or display typical voltage-gated sodium currents [11-16]. Therefore, *C. elegans* cells either do not have action potentials, or generate and propagate atypical action potentials through alternative mechanisms such as VGCCs in muscles [13,15,17]. In *C. elegans* neurons, the nature of the excitation signals that lead to the depolarization at synapses, and how they are transmitted, are unknown. It was proposed that their membrane properties allow the passive spreading of electrical signals along axons in the sensory neurons [12]. Alternatively, they may also generate atypical action potentials.

*C. elegans* encodes a single P/Q-, N- and R-family VGCC α1 subunit (UNC-2), one L-type α1 subunit (EGL-19) and one T-type α1 subunit (CCA-1) [17-19]. UNC-2 is proposed to localize at presynaptic active zones and affects neurotransmitter release [20]. The loss of UNC-2 function leads to slow and abnormal locomotion, failure in neuronal migration and abnormal sensitivity to dopamine and serotonin [19-21]. In pharyngeal muscles, the excitability threshold is set by CCA-1, which initiates an atypical action potential in response to depolarization [15,17]. EGL-19 generates Ca^{2+} transients that define sarcomere excitability [13,15,18]. It also contributes to the Ca^{2+} transients in cultured mechanosensory neuron cell bodies [22].

A rat cDNA clone encoding a protein with homology to the α1 subunit of voltage-gated calcium and sodium channels was first isolated by degenerative oligo-based PCR screening [23]. Homologues of this protein are present in various animals, namely NCA-1 and NCA-2 in *C. elegans* [24], Dmα1U/CG1517 in *Drosophila* [25] and
Vgcnl1/NALCN [23,26] in mouse, rat and human. Unlike all known sodium and calcium channel $\alpha_1$ subunits, whose ion selectivity filter motifs are DEKA [27] and EEEE [28,29] respectively, these proteins contain EEKE at corresponding positions. They also display divergence from the known voltage-gated sodium/calcium channels by a reduction of charged amino acids in the voltage-sensing fourth transmembrane domains, suggesting that they may form channels with unique properties. Indeed a recent paper showed that the rat NALCN forms a voltage-insensitive and poorly selective cation leak channel in HEK293T cells [26].

*Drosophila Dm$\alpha_1U$* mutants are viable but display altered sensitivity to anesthetics and abnormal circadian rhythm [25]. *C. elegans nca-1; nca-2* double knockout mutants also display abnormal halothane sensitivity and more frequent pauses during locomotion, a phenotype termed fainter [24]. The physiological basis for these defects, however, is unknown. NALCN knockout mice are neonatal lethal due to a disrupted respiratory rhythm [26]. Mutant hippocampal neurons display reduced background Na$^+$ leak currents and decreased firing, suggesting that NALCN functions as a Na$^+$ leak channel and regulates neuronal excitability by affecting membrane potentials [26].

In this study, we describe the physiological and cell biological characterization of the NCA proteins in *C. elegans*. Our genetic and phenotypic analyses of nca loss- and gain-of function mutants show that NCA proteins affect synaptic function by modulating the transmission of depolarization signals. This function depends on two novel auxiliary proteins UNC-80 and UNC-79. Thus, a putative NCA channel regulates neuronal
activity in *C. elegans* neurons, at least in part by facilitating axonal conductance of depolarizing signals from the cell body to the synapse.

**Results**

**NCA is required for synaptic transmission at GABAergic and cholinergic NMJs**

To investigate the function of NCA channels in *C. elegans*, we identified and analyzed the phenotypes of animals carrying dominant and recessive mutations in *nca-1* and its homologue *nca-2*. Both dominant and recessive mutations in NCAs have clear effects on *C. elegans* behavior. *gk9* and *gk5*, the two deletion alleles for *nca-1* and *nca-2*, respectively, were generated by the *C. elegans* gene knockout consortium. Removing part of the essential pore-forming domain of NCA-1 and NCA-2, both mutations are predicted to cause severe losses of protein functions (Fig.1A). While either single deletion mutants display normal locomotion, *nca-1(gk9);nca-2(gk5)* double mutants are fainters that fail to sustain sinusoidal locomotion and succumb to long periods of halting ([24,30], Movie S1, S2). The fainter phenotype of *gk9;gk5* mutants is recessive and fully penetrant. This synergism, together with our results in later sections, suggest that the phenotypes of *gk9;gk5* mutants represent the physiological outcome of the complete loss of NCA activity, which we will henceforth refer to as *nca(lf)*.

We identified two gain-of-function alleles of *nca-1* (see Methods, Fig.1A). One of these mutants, *hp102*, was isolated in a screen for developmental defects in active zone markers in GABAergic neurons [31] (Fig.1A); whereas the other allele *e625*, was isolated as a locomotion-abnormal mutant originally named *unc-77* [32]. We identified a single missense mutations that alter residues at positions flanking IS6, the sixth transmembrane
domain in the first repeat (R403Q), or within IIS6, the sixth transmembrane domain of
the second repeat (A717V) of NCA-1 in hp102 and e625 mutants, respectively. Both
affected amino acids are conserved in the protein family (Fig.1B). Unlike the recessive
fainter phenotype of nca(lf) (movie S2), both hp102 and e625 showed semi-dominant,
uncoordinated and exaggerated body bends during either spontaneous or stimulated
locomotion (referred to as coiler phenotype henceforth) (movie S3). Moreover,
expression of nca-1 genomic fragment that harbors the hp102 mutation in wild-type
animals induced locomotion defects similar to that in hp102 mutants (movie S4,
Methods). In summary, both hp102 and e625 represent nca-1 gain-of-function alleles,
which may induce elevated, misregulated or altered NCA activities. They will henceforth
be referred to as nca(gf).

The locomotion defects of both nca(lf) and nca(gf) mutants suggest that NCA
activity regulates synapse function. To address this possibility, we recorded spontaneous
and evoked postsynaptic currents in body wall muscles as an indirect measure for
presynaptic activities of GABAergic and cholinergic neurons NMJs [33,34]. In the
presence of both high and low concentration of extracellular Ca\(^{2+}\), nca(lf) mutants
displayed a significant decrease in the frequency of spontaneous release (mPSC)
\((29.4 \pm 5.3 \text{ Hz, } p<0.01 \text{ at } 5 \text{mM Ca}^{2+} \text{ and } 11.8 \pm 2.5 \text{ Hz, } p<0.001 \text{ at } 1 \text{mM Ca}^{2+}) \) as compared
to wild-type animals \((55.6 \pm 5.3 \text{ Hz at } 5 \text{mM Ca}^{2+} \text{ and } 39.7 \pm 6.5 \text{ Hz at } 1 \text{mM Ca}^{2+}) \) (Fig.
2A,C). They also displayed significantly reduced evoked responses. Electric stimulation
of the ventral nerve cord in wild-type animals elicited currents (ePSC) of 1234.1 \pm 57.7 \text{pA}
in amplitude at 5mM Ca\(^{2+}\), and 1080 \pm 161.3 \text{ pA at } 1 \text{mM Ca}^{2+} \) (Fig.2B,D). In nca(lf)
mutants, the amplitude of ePSC was reduced by 60% at 5mM Ca\(^{2+}\) \((523.9 \pm 57.7 \text{ pA},\)
p<0.001), and by 75% at 1 mM Ca\(^{2+}\) (278.6±109.2 pA, p=0.01) (Fig. 2B,D). The decreased mPSC frequency and ePSC amplitude suggest a reduction of synaptic transmission at NMJs in nca(\textit{lf}) mutants.

We also examined how nca(\textit{gf}) mutations affect synaptic transmission. At 5 mM extracellular Ca\(^{2+}\), some nca(\textit{gf}) animals (Fig. 2E,G, Pop.1) displayed normal frequency of mPSC (59.1±6.0pA, N=7 versus 65.4±5.4pA, N=10 for wild-type), others (Fig. 2E,G, Pop.2) had no mPSC at all (7.2±1.4pA, N=6). No ePSC could be evoked in any of the two groups (Fig.2F,H). Although the cause of these abnormalities was not clear, these results indicate that nca(\textit{gf}) animals also show aberrant synaptic activity, and further establish the link between NCA channels and synaptic function.

**NCA activity regulates presynaptic activation at serotonergic NMJs**

To investigate how altered NCA activity regulates presynaptic function, we examined neuronal excitation directly with cameleon, a genetically-encoded Ca\(^{2+}\) sensor, in live \textit{C. elegans} [35]. We focused on the serotonergic HSN motoneurons, where we also observed both morphological (abnormal active zone marker distribution) and behavioral (constitutive egg-laying) defects associated with their synapses in nca(\textit{gf}) mutants (Fig. S1, D-F). Most importantly, the unusually large size of HSN synapses provided us the unique opportunity to perform \textit{in vivo} simultaneous Ca\(^{2+}\) imaging at both soma and the presynaptic regions (Fig.3A).

When \textit{C. elegans} is immersed in solutions that constitutively activate egg-laying (Methods), HSNs, the motoneurons that innervate the egg-laying vulval muscles, autonomously initiate periodic trains of Ca\(^{2+}\) transients in cell bodies that are independent
of presynaptic inputs (Zhang et al., unpublished, Fig.S2, S3, movie S5). These transients temporally correlated with the Ca\(^{2+}\) spikes in the presynaptic region (Fig.3A, blue and red traces; Fig.S2, S3). The Ca\(^{2+}\) transients at presynaptic regions and cell bodies displayed similar spike frequency (2.6±0.8 spikes/minute at synapses versus 3.2±0.6 spikes/minute at cell bodies, p>0.05, Fig.3C,F) and similar time intervals between spikes in the trains (7.5±0.5 seconds at synapses versus 8.6±0.2 seconds at cell bodies, P>0.05, Fig.3D,G), suggesting that the depolarization signals were generated at the cell bodies and quickly spread to the presynaptic regions.

Under the same conditions, in nca(1f) mutants, HSN cell bodies generated trains of Ca\(^{2+}\) spikes undistinguishable from those in wild-type soma for spike frequency (3.5±0.6 spikes/minute versus 3.2±0.6 spikes/minute for wild-type, p>0.05), interval (5.8±0.2 seconds versus 8.6±0.2 seconds for wild-type, p>0.05) and amplitude (5.3±0.6% versus 6.9±0.4% for wild-type, p>0.05) (Fig.3C-H). At synapses, while Ca\(^{2+}\) transients were present in all wild-type animals, half of the nca(1f) mutants showed no Ca\(^{2+}\) transients at all (Fig. 3B nca(1f), synapse, top trace). The rest of the nca(1f) mutants retained Ca\(^{2+}\) transient trains (Fig.3B, nca(1f), synapse, bottom trace). This resulted in an overall significant decrease of synaptic spike frequency (1.2±0.7 spike/minute in nca(1f)) compared to wild-type synapses (2.6±0.8 spike/minute, p=0.029), and to the spike frequency of nca(1f) cell bodies (3.5±0.6 spike/minute, p=0.005). Remarkably, the remaining trains of Ca\(^{2+}\) transients in the nca(1f) mutants maintained temporally correlated in spike interval (5.2±0.4 second) with the cell body of nca(1f) mutants (5.8±0.2 second, p>0.05). They also display comparable amplitude (6.8±0.7%) to those
in wild-type synapses (5.2±0.7%, p>0.05) (Fig.3C-H). Thus the loss of NCA function disrupts the initiation of Ca\(^{2+}\) transients at synapses.

In nca(gf) mutants, HSN cell bodies also displayed trains of calcium spikes similar to those in wild-type animals in their frequency (3.3±1.0 spikes/minute, versus 3.2±0.6 spikes/minute for wild-type, p>0.05), interval (5.3±0.3 seconds, versus 8.6±0.2 seconds for wild-type, p>0.05) and amplitude (9.2±1.4%, versus 6.9±0.4% for wild-type, p>0.05) (Fig.3B, 3C-E). At synapses, they all displayed trains of Ca\(^{2+}\) spikes that temporally correlated in frequency (2.7±0.8 spikes/minute versus 3.3±1.0 spikes/minute, p>0.05), and interval (6.4±0.3 seconds for synapses versus 5.3±0.3 seconds for cell bodies, p>0.05) with those in nca(gf) cell bodies. However, the amplitude of Ca\(^{2+}\) transients was significantly increased at synapses (9.5±1.3% for nca(gf) versus 5.2±0.7% for wild-type, p=0.029). Although the mean amplitude appears only moderately bigger than in wild-type animals, nca(gf) mutants exhibited a fraction of unusually large Ca\(^{2+}\) transients at synapses that were well above the range seen in wild-type animals (Fig.3H, red box).

In summary, both nca(\textit{lf}) and \textit{gf} mutants specifically altered Ca\(^{2+}\) transients at the presynaptic regions, indicating that under our assay conditions, NCA activity does not alter the excitation at HSN soma but affects presynaptic activity. The decrease of Ca\(^{2+}\) transients in nca(\textit{lf}) suggests that NCA is required to initiate presynaptic activation in response to depolarization signals. The elevated Ca\(^{2+}\) transients in nca(gf) mutants further suggests that the gain-of-function mutations enhance NCA’s activity in presynaptic activation.
NCA activity depends on UNC-80, a large novel protein

To identify proteins that modulate NCA activities, we performed a genetic suppressor screen for mutations that reverted locomotion defects of *nca(gf)* mutants (Methods). We identified two extragenic suppressors that reverted *nca(gf)* coilers to fainters, and fully suppressed their synaptic morphology defects (Fig.S1). One suppressor, *hp424*, corresponds to *unc-79*, a gene encoding a novel protein [24]. Another suppressor, *hp369*, failed to complement *unc-80*, an uncloned mutant previously isolated by its locomotion phenotype [32] and later shown to confer hypersensitivity to halothane [30]. *unc-80 (hp369)* as well as two previously identified *unc-80* alleles *e1272* and *e1069* exhibit recessive and fully penetrant fainter phenotypes identical to that of the *nca(lf)* double mutant (Movie S6). We found that *nca(lf);unc-80* triple mutants are indistinguishable from either *nca(lf)* double mutants or *unc-80* single mutants in behavior (Movie S7). Furthermore, all *nca(gf);unc-80* double mutants display the same fainter phenotype as *unc-80* single mutants (Movie S8). Therefore NCA and UNC-80 function in the same genetic pathway, with *unc-80* epistatic to *nca(gf)*, suggesting that NCA activity depends on UNC-80.

*unc-80* was recently cloned based on the observation that RNAi knock-down of an open reading frame *F25C8.3* in wild-type animals resulted in a fainter phenotype [36]. We confirmed that genomic fragments containing only *F25C8.3* rescued the fainter phenotype of *unc-80* mutants (Movie S9) and reverted the *unc-80;nca(gf)* mutants from fainters to *nca(gf)* locomotion patterns (Movie S10). Nonsense or splice junction mutations, all predicted to result in the loss of the protein function, were identified in three *unc-80* alleles (Fig.S4A), confirming that *unc-80* corresponds to *F25C8.3*. The *unc-
80 gene is predicted to encode multiple isoforms of a large protein that contain no known protein motifs. Uncharacterized UNC-80 homologues are present in *Drosophila*, mouse, rat and human (Fig.S4B), suggesting that UNC-80 is a member of a novel but conserved protein family.

We confirmed that *unc-80* also regulates calcium transients at synapses. The Ca$^{2+}$ dynamics of *unc-80* mutants were essentially identical to those observed in *ncatlf*. The HSN cell bodies displayed trains of Ca$^{2+}$ transients with normal frequency (5.1±1.3 spikes/minute for *unc-80* versus 3.2±0.6 spikes/minute for wild-type, p>0.05), interval (5.8±0.2 second for *unc-80* versus 8.6±0.2 second for wild-type, p>0.05) and amplitude (7.7±0.7% for *unc-80* versus 6.9±0.4% for wild-type, p>0.05) (Fig.3B, 3C-E). Likewise, half of these animals showed silencing of Ca$^{2+}$ transients at synapse regions (Fig.3B, *unc-80*, top trace), with an overall reduction in frequency (0.9±0.7 spikes/minute) when compared to *unc-80* cell body (5.1±1.3 spike/minute, p=0.037), and to wild-type synapses (2.6±0.8 spike/minute, p=0.032). The remaining trains of Ca$^{2+}$ transients at synapses maintained temporally correlated with cell body transients in spike interval (5.6±0.5 seconds for synapses versus 5.8±0.2 seconds for cell bodies, p>0.05). They were also comparable in amplitude with wild-type synaptic transients (4.9±0.5% versus 5.2±0.7% for wild-type, p>0.05) (Fig.3B, *unc-80*, bottom trace, Fig.3C-E). Therefore in addition to sharing behavioral phenotypes with *ncatlf* mutants, *unc-80* mutants also displayed identical changes in presynaptic activation. This indicates that UNC-80 either mediates, or functions together with the putative NCA channel to regulate presynaptic activation.
NCA-1 and UNC-80 are expressed and function in neuronal processes

To determine how UNC-80 regulates the NCA activity, we first examined if they are both expressed or function in the same tissue. GFP promoter reporter constructs, which contain their predicted upstream genomic sequences, revealed similar expression patterns in the nervous system, including many sensory neurons and all motoneurons, for both the unc-80 and nca-1 gene (Fig.4A). Expression of nca-1 or unc-80 by a pan-neural promoter (Supplemental Methods) was able to rescue the fainter phenotype of nca(lf) and unc-80 mutants, respectively (Movie S11 and S12). Therefore, consistent with their expression patterns, both NCA-1 and UNC-80 are required in neurons. Furthermore, specific expression of NCA-1 by a GABAergic promoter Punc-25 [37] rescued the active zone marker defects in GABAergic neurons of nca(gf) mutants (Fig. S5), suggesting that NCA-1 functions cell-autonomously. Hence both NCA-1 and UNC-80 function in neurons.

NCA and UNC-80 may regulate presynaptic activation through either conducting Ca^{2+} transients at synapses, or transmitting depolarization signals along axons. To investigate these possibilities, we further examined their subcellular localization. With an NCA-1 specific antibody, we observed dense and punctate staining in the nerve ring, a synapse rich region at CNS, and along the ventral and dorsal nerve cords that are comprised of inter- and motoneuron processes in wild-type animals (Fig.4B, upper left panel). These staining signals disappeared completely in nca-1(gk9) deletion mutants (Fig.4B, lower left panel). The punctate staining pattern suggests a subcellular enrichment of NCA-1 protein along axons. We therefore examined the localization of NCA-1 relative to the presynaptic termini using antibodies against a vesicle protein SNB-
1, an active zone protein UNC-10 and a presynaptic kinase SAD-1. Along both the dorsal and ventral nerve cords, we observed mostly non-colocalizing staining patterns between NCA-1 and all presynaptic proteins (Fig.4C, Fig. S6A), suggesting that NCA-1 is enriched at specific regions along motoneuron axons but not at synapses.

The subcellular localization of UNC-80 was examined using a functional Punc-80-UNC-80::RFP construct that rescued the fainter phenotype to the same degree as untagged genomic unc-80 (Supplemental method, data not shown). unc-80 mutants carrying hpIs98, an integrated transgenic array of Punc-80-UNC-80::mRFP, were stained with antibodies against RFP. We observed specific and punctate staining signals at the nerve ring and along the nerve processes (Fig.4B, wild-type as negative controls, Fig.4D) that do not co-localize with presynaptic proteins (Fig. 4D, Fig. S6B). This UNC-80::RFP staining pattern is highly reminiscent to that of NCA-1, suggesting that both NCA-1 and UNC-80 proteins are enriched at non-synaptic regions along nerve processes (Fig. 4C and D). This expression pattern is most consistent with NCA-1 and UNC-80 functioning together to transduce depolarization signals from neuronal cell bodies.

**UNC-80 and NCA-1 facilitate each other's localization**

To further determine how UNC-80 regulates NCA-1 activity, we examined the distribution of NCA-1 in unc-80 mutants, and vice versa. NCA-1 staining was eliminated or greatly reduced in multiple unc-80 alleles (Fig.5A, unc-80 panel). hpIs98 (Punc-80-UNC-80::RFP) restored NCA-1 expression at the nerve ring and along the nerve cords in unc-80 mutants (Fig. S7A), indicating that UNC-80 is both necessary and sufficient to localize NCA-1 along axons. While ample NCA-1 staining signals were present in
*nca*(gf) mutants, the staining was also eliminated or greatly reduced in *nca*(gf);*unc-80* mutants (Fig. 5A), suggesting that both wild-type and gain-of-function NCA-1 proteins depend on UNC-80 to localize along the nerve processes. *nca-1* transcripts were present at wild-type level in *unc-80* mutants (Fig. S7B). Together with the fact that no obvious UNC-80::RFP signal was detected in neuronal cell bodies (data not shown), these data indicate that UNC-80 regulates NCA-1 post-transcriptionally, perhaps through reduced translation of NCA-1 proteins or defective trafficking, clustering or stabilization of NCA along axons. In *nca*(lf) mutants, UNC-80::RFP staining was also significantly reduced (Fig. 5B, *nca*(lf);*hpIs98* panel), suggesting that UNC-80 localization along the axon is also dependent on the presence of NCA protein. This NCA-1-dependent localization of UNC-80::RFP, together with the fact that no transmembrane motifs are present in UNC-80, is consistent with the possibility that UNC-80 functions as an auxiliary subunit that regulates the transport, stability, or clustering of NCA at the membrane.

UNC-79 is another large protein with no known motif that has been implicated in the processes controlled by NCA-1, NCA-2 and UNC-80. *unc-79* loss of function mutants also have a fainter phenotype, and have been reported to contain lower than normal levels of NCA-1 protein by western blot analyses [24]. As for *unc-80* mutants, we observed reduced or completely diminished NCA-1 staining in the *unc-79* mutants (Fig. 5A, lower panels). Interestingly, UNC-80::RFP axonal staining was also absent in the *unc-79* mutants (Fig. 5B, lower panels), suggesting that UNC-79 is another auxiliary protein that facilitates NCA-1 localization along the axon. We generated an antibody against the UNC-79 protein, and observed punctate staining in ventral cord and nerve ring processes, consistent with the possibility of coexpression with UNC-79 and NCA-1.
(Fig. 4B, S8). When the same antibody was used to stain nca(lf), unc-80, and unc-80; nca(lf) mutants, no UNC-79 staining was detectable in neuronal processes (Fig. S9). Thus, NCA-1/2 and UNC-80 appear to also facilitate the localization of UNC-79 protein. These results are consistent with the possibility that UNC-79, like UNC-80, also functions as an accessory subunit or other regulatory interactor with the NCA channel.

**UNC-80 enhances the effect of NCA-1 in transfected HEK293T cells**

To further investigate whether NCA-1 and UNC-80 proteins might function together to promote NCA channel activity, we analyzed NCA function in a heterologous cell system. It was shown previously that expression of mammalian NALCN induced constitutive cation leak currents when transfected in HEK293T cells [26]. These currents were attributed to the NALCN channel activity, as they were inhibited by verapamil or gadolinium, two blockers for the endogenous, NALCN-mediated Na\(^+\) leak currents in hippocampal neurons [26]. In our experiments, these currents appeared to induce cell death in the transfected cells, as significantly increased cell death were observed 48 hours after HEK293T cells were transfected with constructs expressing NALCN (144.3±1.8%, normalized against untransfected cells, p<0.01). This effect was not induced by the expression of other channels (e.g. for Kv4.2, 113.7±13.9%, p>0.05), and was abolished when transfected cells were incubated with 100\(\mu\)M verapamil or 10\(\mu\)M gadolinium (Fig.6, Methods), suggesting that the cell death was indeed associated with the NALCN channel activity.

Using this same assay we examined whether *C. elegans* NCA-1, alone or together with UNC-80, exhibited similar activities in HEK293T cells (Fig. 6). cDNAs encoding
the longest isoform for NCA-1 and UNC-80 were maintained in a low copy number expression vector (Supplemental Method). Transfecting with either the NCA-1 or UNC-80 expression construct alone did not increase in the lethality of the host cells (NCA-1: 119.3±4.1%, and UNC-80: 114.3±6.7%, p>0.05). In contrast, co-transfection of NCA-1 and UNC-80 constructs induced significant cell death (159.3±2.9%, p<0.01). This effect was abolished when the UNC-80 expression vector was co-transfected with a NCA-1 clone carrying a deletion in the coding region (122.7±11.6%, p>0.05). Moreover, the increased cell death in NCA-1 and UNC-80 co-transfected cells was also blocked in the presence of 100µM verapamil (120.3±3.3%, p>0.05) or 10µM gadolinium (112.7±3.7%, p>0.05). Therefore the co-expression of UNC-80 and NCA-1 induced the same effect, with similar blocker responses as NALCN in HEK293T cells, suggesting that the putative NCA/UNC-80 channel complex likely shares similar ion leak properties.
Discussion

A putative NCA channel transmits depolarization signals in *C. elegans* neurons

We have shown here that the NCA-1 and NCA-2 proteins are required redundantly for synaptic activity. Both the reduction of postsynaptic currents at GABAergic and cholinergic NMJs, and the decrease of Ca\(^{2+}\) transients at serotonergic NMJs in *nca(lf)* mutants suggest a decreased presynaptic activity in the absence of the putative NCA channels. The calcium imaging analyses further suggest that this synaptic defect is related to a failure to initiate presynaptic activity. In wild-type animals, the calcium spikes at HSN cell bodies and synapses are temporally correlated. In both *nca(lf)* and *unc-80* mutants, at least under our assay conditions, despite the normal calcium dynamics in cell bodies, the number of Ca\(^{2+}\) transients was reduced at synapses. The NCA channel is unlikely to conduct Ca\(^{2+}\) transients at synapses, since the remaining transients in *nca(lf)* mutants were normal in amplitude and maintained temporal correlation with the depolarization signals in cell bodies. Together with their non-synaptic localization along nerve processes, these results strongly indicate that NCA channel activity is required to transmit depolarization signals to synapses (Fig.7).

Depolarization signals may propagate actively or spread passively along axons. Lacking typical voltage-gated sodium currents, the passive model, conceivable for neurons with short axons or axons with a high input resistance membrane property [38], was proposed for *C. elegans* sensory neurons [12]. Mouse NALCN mediates Na\(^+\) leak in hippocampal neurons [26]. A similar property for NCA channel would allow it to drive the membrane potential close to its excitation threshold at specific regions along *C. elegans* neurites, facilitating the activation of other channels along axons or around
synapses. This model is consistent with the presence of the active propagation of depolarization signals in *C. elegans* motoneurons.

Interestingly, the silencing of Ca$^{2+}$ transients in *nca(lf)* and *unc-80* mutants is incomplete; however, the molecular lesions in these mutants predict severe loss of protein functions. All *nca(lf)* and *unc-80* alleles are behaviorally indistinguishable from each other and fully penetrant for the fainter phenotype, which strongly argues against an allelic effect on phenotype penetrance. The partial loss of Ca$^{2+}$ transients and the variable degree of the decrease of mPSC frequency in *nca(lf)* mutants therefore more likely suggest that while some depolarization signals depend on NCA channels to induce presynaptic activation, other signals reach synapses independently of NCA activity (Fig. 7).

It is worth noticing that while we detected two distinct, active versus quiescent, populations in *nca(lf)* and *unc-80* mutants in our physiological analyses, there is little behavioral variability among individual animals. Since every animal alternates between a state of normal sinusoidal movement and quiescence, we speculate that *C. elegans* neurons alternately fire NCA/UNC-80-dependent and independent depolarization signals.

Perhaps due to the necessary experimental manipulation (such as immobilization of the animal) and the short assay time, we measured neuronal activity fixed in one ‘mode’, resulting in the appearance of two distinct populations.

**The effects of gain-of-function mutations on neuronal excitability**

In addition to the synaptic phenotypes observed in the loss-of-function mutants, we also observed behavioral and synaptic phenotypes in the *nca* gain-of-function
mutants. Specifically, we found that these mutant animals showed a coiler uncoordinated phenotype, and exhibited larger calcium transients at synaptic sites. Gain-of-function mutations in NCA-1 do not affect the temporal correlation of calcium transients between HSN cell bodies and synapses. Whole mount staining with antibodies against NCA-1 showed no obvious changes in the subcellular distribution or intensity of the staining signals in nca(gf) mutants, indicating that these mutations likely alter the activity rather than the abundance of the NCA-1 protein. The calcium imaging phenotype is consistent with the NCA(gf) channel further increasing the membrane excitability, which leads to enhanced activation of calcium channels at HSN synapses (Fig.7).

The hp102 mutation alters a conserved amino acid flanking the IS6 transmembrane domain. This coincided with a hot-spot region for identified gain-of-function alleles for several voltage-gated calcium channels. In several cases, these gain-of-function mutations lead to slowed inactivation, subsequently prolonging the duration of the corresponding currents [18,39,40]. If the hp102 mutation leads to further increase of the leak through the NCA channel, it could indeed bring the neuronal membrane to a hyper-excitible state. Expressing the mouse NALCN carrying the hp102 equivalent mutation was able to induce similar locomotion defects as NCA-1(gf) proteins in C. elegans, suggesting that hp102 mutation may induce similar property changes in all NCA family channels.

Both UNC-80 and UNC-79 regulate the putative NCA channel through localizing the pore-forming subunit
Another gene with a loss-of-function fainter phenotype, *unc-80*, encodes a novel protein with a critical role in NCA channel function. Based on behavioral and physiological characterization of mutants, UNC-80 appears exclusively to be involved in NCA-mediated functions. Not only do *unc-80* mutants show identical phenotypes as *nca(lf)* mutants, they do not enhance *nca(lf)* mutants, and suppress defects exhibited by *nca(gf)* mutant. By contrast, *unc-80* mutants do not phenocopy VGCC loss-of-function mutants or display obvious genetic epistasis with VGCC gain-of-function mutants (Table S1, Movie S13). These genetic interactions indicate that UNC-80 function is specifically required for NCA channels.

UNC-80 regulates NCA channel function at least in part by localizing the putative pore-forming NCA-1 subunit to the membrane. *nca-1* transcripts are present in normal levels in *unc-80* mutants, suggesting that UNC-80 regulates NCA-1 post-transcriptionally. The similar, and interdependent subcellular localization pattern of NCA-1 and UNC-80 implies that UNC-80 is a likely subunit of the NCA channel to transport, anchor or stabilize the pore-forming subunit NCA-1 along axons. With close homologues present in all animals, UNC-80 family proteins likely play a conserved role in regulating the localization of the NCA family channels.

We observed identical genetic interaction between *nca* and *unc-79* mutants, and interdependent localization of NCA-1 and UNC-79 proteins. *unc-79* encodes another large but evolutionarily conserved protein with no known protein motifs [24]. Similar to *unc-80*, loss of function mutations in the *unc-79* gene lead to not only the same fainter phenotype as *nca(lf)* mutants, but also a complete suppression of *nca(gf)* locomotion defects and the disappearance of NCA-1 and UNC-80 along nerve processes.
Furthermore, UNC-79 is dependent on the presence of both NCA and UNC-80 for its localization along neurites. Therefore, both UNC-80 and UNC-79 are likely conserved auxiliary components of the NCA family channels.

**Are the functions of NCA channels conserved in mammalian neurons?**

NCA-1 and NCA-2 have close sequence homologues in other vertebrate and invertebrate species, including humans. The mammalian member of this family, NALCN, has recently been characterized physiologically in HEK293 cells [26]. In spite of its sequence homology and similar topology to the pore-forming \( \alpha_1 \) subunits of voltage-gated cation channels, NALCN forms a voltage-insensitive and non-selective cation channel.

Two lines of indirect evidence support the hypothesis that *C. elegans* NCA and its mammalian homologues share common functional properties. First, *C. elegans* NCA proteins show at least similar properties to NALCN proteins when heterologously expressed in mammalian cell culture. In HEK293T cells, transfecting NALCN, or co-transfecting NCA-1 and UNC-80 induced cell death that was blocked by the NALCN blockers verapamil and gadolinium. Conversely, expressing mammalian NALCN proteins in *C. elegans* could substitute functionally for the NCA proteins. Specifically, wild-type *C. elegans* expressing a mouse cDNA that carries the *hp102* equivalent mutation (NALCN(R329Q)) in neurons exhibited a locomotion pattern with exaggerated body bends, reminiscent of the *nca(gf)* mutants (Supplemental Methods and Movie S14). Thus *C. elegans* NCA and its mammalian homologues can mediate similar physiological
functions, consistent with the possibility that NCA family proteins share similar channel properties.

Given the conservation in the functional properties of NCA family members, it is reasonable to speculate that these channels may also carry out similar functions in neurons. Our current studies suggest a specific function for the NCA channel in transmitting and regulating excitability along C. elegans neuronal processes, but do not rule out the possibility that NCA also controls neuronal firing. Since our calcium imaging analysis was performed under conditions that stimulated the constitutive firing of HSNs, an altered firing ability could be masked by the hyperactivation of neurons. While the enriched localization of NCA-1 and UNC-80 at non-synaptic regions along axons is consistent with the propagation role of the NCA channel, we do not exclude the possibility that the reduced synaptic transmission at GABAergic and cholinergic NMJs in nca(lf) mutants may result from a combination of deficits in the propagation of depolarization signals, neuronal firing, and even vesicle release. The mouse NALCN affects the resting potential and controls the excitability/firing rate of hippocampal neurons [26]; whether it is also involved in excitation propagation however is not examined. It is therefore interesting to determine the subcellular localization of mouse NALCN channels and examine whether they are also involved in such processes in mammalian neurons.
Figure 1: hp102 and e625 encode NCA-1, a cation channel-like subunit

A) Genetic mapping of nca(gf) mutants and a schematic representation of the nca-1 genetic locus (top) and the predicted NCA-1 protein structure (bottom). The positions of the nca(gf) (hp102 and e625) and nca(lf) mutations (gk9 and tm1851) are illustrated. The beginning and end of each ion transport motif are numbered. The amino acid residues that determine ion selectivity in related cation channels are labelled.

B) Similarity between NCA family members. The residues equivalent to hp102 and e625 are indicated. C.e.: C. elegans; D.m.: D. melanogaster; R.n.: R. norvagicus; M.m.: M. musculus; H.s.: H. sapiens.

Mapping of hp102 was performed by me during my undergraduate study. Isolation of hp102 was done by Dr. Edward Yeh. Cloning and non-complementation with nca-1 were performed by Dr. Mei Zhen and Dr. Edward Yeh. I also prepared Figure 1.
Figure 2: NCA-1 activity is required for normal synaptic transmission at NMJs

Representative traces of spontaneous activity (A for nca (lf) and E for nca(gf)) and responses evoked in muscle by an electric stimulation of the ventral nerve cord (B for nca(lf) and F for nca(gf)) are shown. A-D) The nca(lf) mutant mPSCs varied between wild-type (WT) values (nca(lf), upper trace) and decreased frequency and amplitude (nca(lf), lower trace). The overall mPSC frequency (C) and evoked response amplitude (D) were decreased in nca(lf) animals as compared to wild-type at either 5mM (nca(lf): N=15; WT: N=13) or 1mM (nca(lf): N=9; WT: N=6) extracellular Ca\(^{2+}\). No significant change in mPSC amplitude and distribution was observed between nca(lf) and WT animals. Individual results are shown in black, mean ± SEM in grey, for recordings at 5mM and 1mM extracellular Ca\(^{2+}\). E-H) Representative traces of spontaneous (E) and evoked (F) post-synaptic currents at the neuromuscular junction at 5mM Ca\(^{2+}\). The nca(gf) mutant mPSCs were either comparable to WT (Pop.1) or highly decreased in frequency and amplitude (Pop.2), while highly reduced (if any) responses could be evoked by electric stimulation of the nerve cord (F). The mPSC frequency (G) and evoked responses amplitude (H) are plotted for N2 and nca(gf) animals. Individual results are shown in black, mean±SEM in grey, when relevant. (WT, mPSCP, N=10, ePSP, N=7; nca(gf), mPSP, N=13, ePSP, N=8) Error bars: SEM, *p<0.05, **p<0.01. Statistic analysis was performed with student t test.

The preparation of Figure 2, and all electrophysiology recording and analyses were performed by Dr. Magali Bouhours.
Figure 3: Ca2+ transients at HSN cell bodies and synapses in nca(lf), nca(gf) and unc-80 mutants

A) Upper panel: Image of HSN neuron used in calcium imaging. The HSN cell body and the synapse where Ca²⁺ imaging were performed are circled in dots. Lower panel: Sample traces of simultaneous recording of calcium spikes of both cell body and synapse, showing the synchronicity of the calcium signals. B) Sample traces of yellow/cyan ratio that represent the relative Ca²⁺ concentration in HSN cell bodies (left panels) or their synapses (right panels). X-axis: time in seconds; Y-axis: yellow/cyan ratio in %. For nca(lf) and unc-80, animals displayed traces with either silent (top) or active (bottom) Ca²⁺ transients. C-E) Histograms for the total spike frequency, spike interval, and size of calcium spikes detected in each strain. Arrowheads: examples of calcium spikes. N, the number of animals examined (panel C) or the number of calcium spikes examined (panels D, E), was illustrated at the bottom of each bar. Error bar: SEM. * p<0.05, ** p<0.005. C) Average number of spikes/minute for each genotype. There is no statistically significant difference for HSN cell bodies (cell) among all strains, or between HSN cell bodies (cell) and synapses (syn) of the same strain except for nca(lf) and unc-80. D) Average time interval between two consecutive spikes within trains of calcium transients. There is no statistically significant difference between HSN cell bodies and corresponding synapses for all strains. E) Average spike size. Wild-type, nca(lf) and unc-80 neurons displayed no statistically significant difference in spike size, but for the nca-1(gf) neurons it was increased. F-H) Scatter plots for spike frequency, interspike time interval and spike size. Each cross represents a data point. Clear and filled triangles represent mean numbers for cell body and synapse calcium transients respectively. Populations of animals with silenced synapse calcium transients are circled. Rectangular box highlights a population of nca(gf) synapse spikes that were significantly larger than those seen in other genotypes. All statistic analysis was performed by Kolmogorov-Smirnov rank test.

The Ca²⁺ imagining experiments presented here were performed and analyzed by Dr. Mi Zhang. Dr. Magali Bouhours, Dr. Wesley Hung and Dr. Mi Zhang assembled Figure 3 together.
Figure 4

A

P\textsubscript{\textit{nca-1}}

P\textsubscript{\textit{unc-80}}

NR

VNC

HSN

VM

B

\textit{NR}

\textit{ns}

\textit{unc-80::RFP}

\textit{RFP}

\textit{wt}

\textit{\textit{nca-1}}

\textit{\textit{ns}}

\textit{\textit{unc-79}}

\textit{\textit{nca-1}}

\textit{\textit{wt}}

\textit{\textit{\textit{unc-79}}}

C

\textit{wt}

\textit{\textit{\textit{\textit{unc-10}}}}

\textit{\textit{nca-1}}

\textit{\textit{RFP}}

\textit{\textit{\textit{unc-79}}}

\textit{merge}

D

\textit{\textit{hpa99}}

\textit{\textit{\textit{unc-10}}}

\textit{\textit{\textit{unc-21}}}

\textit{merge}
Figure 4: NCA-1, UNC-80 and UNC-79 are expressed in the nervous system, enriched at non-synaptic regions along axons

A transcriptional GFP reporter driven by the nca-1 promoter (left panels) or unc-80 promoter (right panels) is active in neurons in the nerve ring (NR), and ventral nerve cord (VNC) motoneurons. Activity of the nca-1 promoter is also seen in the HSN neuron whereas the unc-80 promoter has activity in the vulval muscles (VM).

B) An antibody against NCA-1 shows specific staining (arrowheads) in the nerve ring (NR) and along nerve cords in wild-type (top left panel) animals that is absent in nca-1(tm1851) deletion mutants (bottom left panel). Similarly, anti-RFP antibody shows specific immunoreactivity in the nerve ring (NR) of UNC-80::RFP (hpIs98) expressing animal (top middle panel) but not in wild-type animals not carrying the transgene (bottom middle panel). * ns: non-specific staining persisted in negative controls where animals do not express NCA-1 or UNC-80::RFP protein (out of the focal plane for the nca-1 panel). An antibody against UNC-79 showed specific and similar staining at the nerve ring in wild-type animals (right top panel) that disappeared in unc-79 mutants (right bottom panel).

C) Wild-type animals were co-stained with anti-NCA-1 antibody (red) and anti-UNC-10 (green). D) unc-80;hpIs98 animals co-stained with anti-RFP antibody (red) and anti-UNC-10 (green) showed poor co-localization. Scale bar: 5µm.

Pnca-1 expression construct and αNCA-1 antibody were generated by Ying Wang and Dr. Edward Yeh, and expression pattern was analyzed by Dr. Edward Yeh, Ying Wang and me. The Punc-80 expression construct and Punc-stuUNC-80::RFP were generated by Dr. Mei Zhen, Dr. Ying Wang and Christine Hwang. The unc-80 expression pattern and localization were analyzed by Dr. Wesley Hung and me. The generation of αUNC-79 antibody examination of UNC-79 localization were performed by Dr. Kyota Aoyagi. I examined the localization of NCA-1 and UNC-80::RFP with respect to UNC-10. Figure 4 was assembled by Dr. Wesley Hung and me.
Figure 5

A

WT  αNCA-1

unc-80  αNCA-1

cmp(gf)  αNCA-1

unc(af); unc-80  αNCA-1

unc-79  αNCA-1

αUNC-10

αUNC-10

αUNC-10

αUNC-10

B

WT  αRFP

hpis98  αRFP

nca(af); hpis98  αRFP

unc-79; hpis98  αRFP

αUNC-10

αUNC-10

αUNC-10

αUNC-10
Figure 5: NCA-1, UNC-80 and UNC-79 depend on each other for localization

Wild-type (WT), hp102, unc-80(e1272), hp102;unc-80(e1272) and unc-79(e1279) animals co-stained with anti-NCA-1 (red) and anti-UNC-10 antibodies (green, as internal staining control). NCA-1 staining was present in wild-type and hp102 animals but disappeared in unc-80, unc-79, and hp102;unc-80 animals.

B) Staining with anti-RFP antibodies in wild-type (negative control), hpIs98 (UNC-80::RFP), nca(lf);hpIs98 and unc-79;hpIs98 animals (left panels). Specific nerve ring staining (arrow) of UNC-80::RFP disappeared in nca(lf) and unc-79 animals. UNC-10 staining was present in the same animals (right panels). Scale bar: 5 μm.

The localizations of NCA-1 in unc-80 and unc-79 were examined by Dr. Edward Yeh. The localizations of UNC-80::RFP in nca-1 and unc-79 were analyzed by Dr. Wesley Hung, Ying Wang and me. Figure 5 was prepared by Dr. Wesley Hung.
Figure 6

A

B

+100 μM verapamil

C

+10 μM Gd³⁺
**Figure 6: Co-transfecting NCA-1 and UNC-80 induces NALCN-like cell death in HEK293T cells**

The results of propidium iodide cell death assays in HEK293T cells are graphically represented. Assays were done on mock-transfected (untrans) HEK293T cells, or cells transfected various combination of DNA constructs that express UNC-80, NCA-1, truncated NCA-1 (NCA-1Δ), the rat (rNALCN) homologue of NCA-1, and the Kv4.2 potassium channel (as an additional control), either in the absence of any blockers (A), or in the presence of 100 µM Verapamil (B) or of 10 µM Gadolinium (C). The graph presented resulted from three independent sets of experiments. Statistically significance was analyzed by one-way ANOVA followed by *post-hoc* Student-Newman-Keuls tests. **P<0.01, significantly different from the mock-transfection control.**

The NALCN constructs were generated by Dr. Hang Li. The transfection of HEK293T cells and the cell death assays were performed by Min Wang. Figure 6 was prepared by Min Wang.
**Methods:**

A schematic representation of a *C. elegans* neuron with *en passant* synapses. Ca$^{2+}$ transients in the cell body are likely contributed by influx from L-type VGCCs and release from intracellular calcium pools. At the synapse, depolarization signals (red waves) initiate calcium influx through P/Q-type VGCCs at the active zone. NCA channels (NCA-1, NCA-2, UNC-80 and UNC-79) regulate membrane excitability along the axon to allow the propagation of some depolarization signals. Bottom - In *nca(lf)* mutants some depolarization signals fail to propagate along the axon while others can still reach synapses. In *nca(gf)* mutants the signals are amplified, resulting in increased synaptic activity, which indirectly regulates active zone distribution.

Figure 7 was prepared by me.
**Strains**

All strains were cultured at 22°C unless specified otherwise. *hp102* was originally identified in a genetic screen for *hpIs3* defective mutants [31] and was outcrossed 8 times against wild-type N2. *unc-80(hp369)* and *unc-79(hp424)* were identified in a *hp102* suppressor screen and outcrossed 3 times against N2. *e625, e1069* and *e1272* were identified through abnormal locomotion in previous *C. elegans* screens [32]. *gk9* and *gk5* were generated by the Gene Knockout Consortium and were outcrossed 3 times against N2. *tm1591* was generated by National Bioresource Project for the Nematode and was outcrossed once against N2.

**Identification, mapping and cloning of *hp102* and *unc-77(e625)*

*hp102* mutants were identified from an active zone marker *hpIs3* screen [31]. Based on both of its abnormal active zone marker distribution and locomotion defects, *hp102* was rough mapped to chromosome IV between E03H12 (1.40cM) and D2096 (3.74cM) by snip-SNP mapping against CB4856. During the mapping, we noticed that *unc(uncoordinated)-77(e625)*, an uncloned, previously identified locomotion defective mutant [32] that was linked to a similar region on chromosome IV, showed similar locomotion and active zone marker defects as *hp102* mutants (data not shown). We determined that *e625* and *hp102* were alleles of the same gene due to the genetic interactions displayed by these two mutants: while *hp102/e625* heterozygous animals showed fully penetrant coiling locomotion as either homozygous mutants, *hp102/+* or *e625/+* heterozygous mutants showed only slightly more exaggerated body bends compared to wild-type animals. This conclusion was confirmed when we mapped both
mutants to the same genetic locus, rescued both mutants with the same genetic fragments, and identified mutations in the same open reading frame (see below).

*hp102* and *e625* mutations were then further fine-mapped between B0273 (1.74cM) and C49A9 (3.08cM) based on the following data. From *dpy-13unc-77/CB4865*, 3 out of 20 Unc non Dpy animals had their recombination breakpoints between B0273 (1.74cM) and F38A5 (3.21cM), placing *unc-77* to the right of B0273 (1.74cM). From *unc-77bli-6/CB4856* animals, 3 out of 3 Unc non Bli recombinants and 2 out of 2 Bli non Unc recombinants had their recombination breakpoints between C49A9 (3.08cM) and F38A5 (3.21cM), placing *unc-77* to the left of C49A9 (3.08cM). From *unc-5unc-77/CB4856* heterozygous animals, 2 out of 2 *unc-77* non *unc-5* recombinants had their recombination breakpoints between C31H1 (2.56cM) and C49A9 (3.08cM), placing *unc-77* between C31H1 (2.56cM) and C49A9 (3.08cM). Cosmids and PCR fragments amplified from the genomic sequence covering this region (Details see Molecular biology) were injected into *hp102;hpIs3* and *e625;hpIs3* animals. Only DNA fragments covering the C11D2.6 (*nca-1*) genomic region rescued the locomotion and *hpIs3* marker defects. We further confirmed that *unc-77* corresponds to *nca-1* by sequencing the entire predicted genomic regions (all exons and introns) of *hp102* and *e625* mutants and identifying a single missense mutation in the coding region of each mutant (Figure 1).

Both *hp102* and *e625* encode gain-of-function mutation for *nca-1* because they both behaved as semi-dominant mutations. *hp102/+* or *e625/+* heterozygous mutants showed more exaggerated body bends compared to wild-type animals, but much less severe than homozygous or *hp102/e625* heterozygous animals. They also behaved
dominantly over \( nca-2 \) loss of function mutations, as \( hp102; nca-2(gk5) \) and \( e625; nca-2(gk5) \) mutants displayed the same behavior as \( hp102 \) and \( e625 \) homozygous mutants. Furthermore, PCR fragments amplified from the \( nca-1 \) genomic region from \( hp102 \) when expressed in wild-type animals induced the same coiling locomotory defects as \( hp102 \) mutants (Movie S4). Overexpression of the wild-type copy of \( nca-1 \) rescued phenotypes induced by a gain-of-function mutation likely by replacing the mutated NCA-1 protein from its putative channel complex.

**Identification, mapping and cloning of \( unc-80 \)**

\( hp102; hpIs3 \) mutants were mutagenized by EMS and F2 progeny displaying non-coiler locomotion patterns were recovered as candidate suppressors. Each candidate suppressor line was re-screened and confirmed by their rescuing of \( hpIs3 \) marker defects. We backcrossed each suppressor line to wild-type animals: if coilers could be recovered from the F2 generation, the suppressor was considered as extragenic. If the coilers could not be recovered, the suppressor line was then crossed into the \( nca-2(gk9) \) background. If they showed fainter phenotype, the suppressor was confirmed as intragenic revertants. From the progenies of 13,000 mutagenized F1 hermaphrodites (equivalent to 26,000 mutagenized haploid genomes), we identified four intragenic suppressors reverting \( hp102 \) mutants to wild-type locomotion, and multiple alleles of two different extragenic suppressors that reverted \( hp102 \) animals to fainters, and failed to complement \( unc-80 \) and \( unc-79 \) mutants, respectively.

\( unc-80 \) was first rough mapped between F21D9 (21.82cM) and F38A6 (27.08cM) on chromosome V through two-factor SNP mapping against CB4856. 2 out of 4 \( unc-80 \)
recombinants from *unc-51/unc-80/CB4856* broke between Y113G7A (24.71cM) and F38A6 (27.08cM), placing *unc-80* to the right of Y113G7A (24.71cM). 0/9 Rol non Unc recombinants from *unc-51rol-9/unc-80* animals segregated *unc-80*, placing it to the right of *rol-9* (25.12cM, pKP5057). In two-factor mapping against *pha-4* and CB4856, we could not find breakage between *unc-80* and *pha-4* (25.60cM), placing *unc-80* between 25.12cM and 27.08cM tentatively near 25.60cM. Clones that cover this region were generated by PCR to be tested for rescuing of *unc-80* mutants. Kim Schuske (University of Utah) observed that RNAi knockdown of *F25C8.3* (which lies within this region) in wild-type animals was able to induce a fainter phenotype (personal communication). We generated and shared with the Schuske group DNA fragments spanning *F25C8.3* (see Molecular Biology and Transgenic strains) that rescued the fainter phenotypes in *unc-80* and *unc-80;hp102* mutants.

**Molecular biology:** See Supplemental text.

**Immunocytochemistry:**

Antibodies against aa1731-1914 of the predicted NCA-1d isoform, and a combination of aa506-608 and aa1205-1851 of UNC-79 were generated in rat (*Covance*). Whole-mount immunofluorescent staining was carried out as previously described [41]. Antibodies against NCA-1, UNC-79, mRFP (*Clontech*), SAD-1, SNB-1 and UNC-10 (M. Nonet, Washington University, St. Louis) were used in 1:10, 1:10, 1:200, 1:200, 1:100 and 1:2000 dilutions, respectively.
Electrophysiology

Dissections on young adult *C. elegans* were performed as described [14,42]. The integrity of the anterior ventral medial body muscle and the ventral nerve cord was visually examined and muscle cells were then patched using fire-polished 4M\_ resistant borosilicate pipettes (*World Precision Instruments*). They were clamped at -60mV using a Axopatch 1D amplifier throughout experiments (*Molecular Devices*), and recorded using the whole-cell patch-clamp technique in previously described recording solutions [43] within 5 minutes following the dissection. Signals were filtered at 5kHz, and digitized via a Digidata 1322A acquisition card (*Molecular Devices*) The data were acquired and analysed using the pClamp software (*Molecular Devices*). After 10-60s of recording of spontaneous events, a highly resistant fire-polished electrode filled with 3M KCl was brought close to the ventral nerve cord region anterior to the recorded muscle cell, and a 1ms depolarizing current was applied to induce an evoked response.

Calcium imaging

*Pcat-1-cameleon* was used to reveal relative Ca\(^{2+}\) concentrations in HSN cell bodies and synapses corresponding to those on vm2 muscles. Adults 24 hour post L4 stage were immobilized by surgical glue on 2% agarose pads on microscope slides and covered with 1ml 10mM HEPES (pH 7.1), a condition that stimulates constitutive egg-laying thus spontaneous activation of HSN neurons. Recording was carried out as previously described [35]. All recordings started within 2 minutes after the animals were glued and lasted for 10 minutes. Data from HSN cell bodies and synapses were obtained simultaneously. Due to slight body movements during the recordings, some synapse data
sets were incomplete and were not included in analysis. Spike detection, data analysis and statistic analysis by Kolmogorov-Smirnov rank test (due to abnormal data distribution) were carried out as described previously ([44] and Supplemental text).

HEK293T cell death assay

HEK293T cells were grown in α-MEM (GIBCO) medium supplemented with 10% FBS (GIBCO) at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Lipofectamine 2000 was used to transfect the HEK293T cells following the standard procedure (Invitrogen). 0.4μg total DNA was used for each transfection experiment. Medium was replaced 4 hours after transfection, during which the culture was split into three sets with equal density, two sets were exposed to 100μM verapamil or 10μM Gd³⁺, respectively.

Cell death assays were performed 48 hours after transfection. Culture medium was replaced by extracellular solution containing 50 μg/ml of propidium iodide (PI) (Invitrogen). After 30 minute incubation at 37°C, fluorescence intensity in each well was measured with a plate reader (Victor3; PerkinElmer) as described previously [45,46]. The fraction of dead cells was normalized against the mock-transfected cultures.

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Supplemental text

Methods:

Molecular biology

The promoter reporter construct for nca-1, pJH859, was generated by inserting 6.3kb of upstream genomic sequence that includes the ATG start codon plus the first 8 amino acids of the predicted C11D2.6a isoform into the SpeI/BamHI sites of pPD95.79 Fire vector. A single nucleotide deletion (T5044) at the predicted ATG start codon for the C11D2.6d isoform was created in this clone to prevent early translation, which is essential to allow the strong GFP reporter expression. The promoter reporter ‘constructs’ for unc-80, pJH880 and pJH881, consist of PCR fragments containing 3.6kb of genomic sequence immediately upstream of and including the ATG start codon of the predicted F25C8.3a open reading frame, fused in frame with GFP and followed by universal unc-54 3’-UTR sequences. The GFP and unc-54 3’-UTR sequences for pJH880 and pJH881 were generated from pPD95.69 and pPD95.77 vectors, respectively.

The rescuing ‘construct’ for unc-77/nca-1 was a mixture of two DNA fragments, pJH986 and pJH901, PCR-amplified against C. elegans genomic sequences with primer pairs OZM390 (5’GCGAACTAGTGGATCGGCGAATTATCAAGTTGC3’)/OZM391 (5’ GCGAACTAGTCTCCGACATCTCGAACAACGG3’) and OZM392 (5’GCGAACT AGTGCGTCGCCGATTCTCGAAGAC3’)/OZM393 (5’GCGAACTAGTCTCGGCATTCTCGAAGAC3’), respectively. pJH986 contains 6.3kb of genomic sequence immediately upstream of the ATG start codon shared by the predicted C11D2.6a, b and c isoforms, followed by 7.7kb of N-terminal
genomic sequence of the predicted C11D2.6 open reading frames. pJH901 contains 5.8kb of C-terminal genomic sequences of the predicted C11D2.6 open reading frames and 472bp sequences immediately downstream of the shared predicted TAG stop codon of C11D2.6c and d isoforms. These two fragments overlap by 4.0kb. The same primer sets were used to amplify DNA fragments using hp102 genomic DNA as templates, sequencing confirmed for the hp102 mutation, and co-injected into wild-type (N2) animals for dominancy tests.

Pan-neuronal expression of NCA-1 was achieved through co-injection of a plasmid pJH975 and the PCR fragment pJH901. pJH975 was generated by inserting 8.99kb of the N-terminal portion of nca-1 genomic sequence (starting from the predicted ATG start codon of C11D2.6d isoform, PCR-amplified by an oligo pair OZM826 (5’GGGGATCCACATGT CGGAACGAAAAAAGAG3’)/OZM391 (5’GCAGACTAGTCTCCGACATCTCGAA CAACGG3’) against C. elegans genomic DNA, into the BamHI/KpnI sites of pJH625, a pan-neuronal expression vector driven by PF25B3.3 [1]. Expression of UNC-77/NCA-1 specifically in GABAergic motoneurons was achieved by co-injecting a DNA mix of pJH971 (which contains the same 8.99kb insert as pJH975 subcloned into the BamHI/KpnI sites of pJH387 (a GABAergic-specific expression vector driven by Punc-25) and the PCR fragment pJH901.

The rescuing ‘construct’ for unc-80 was a mixture of three overlapping PCR-amplified C. elegans genomic fragments (pJH983, pJH984 and pJH985). pJH983, amplified with an oligo pair OZM967 (5’CCTGTCGACGTAGGTCAGTCTCGAGTGCTGCA3’)/OZM973 (5’AGAGCTGATAGTCCCACATTTCGAAACGGA3’), contains 3.6kb sequence
immediately upstream of the predicted ATG start codon shared by F25C8.3a and b isoforms followed by 6.8kb of N-terminal genomic sequence for all predicted F25C8.3 open reading frames. pJH984, amplified with an oligo pair OZM594

(5’AAGGGCCCAATGCATGTT CGATACTTACAGCAC3’)/OZM679

(5’CGTGGTGAATCTTCTCTCTGTCAG3’), contains 9.8kb of genomic sequence starting from the predicted ATG start codon of F25C8.3 open reading frames. pJH985, amplified by an oligo pair OZM554 (5’TCCGTTTCGAAG

AGTGGGACTTACTTCTCTGTCAG3’)/OZM555 (5’GTTCGAATAGATTTGGTGGCCTTTACCC3’), contains 12.1kb of the C-terminal portion of the predicted F25C8.3 genomic sequence followed by 2.7kb of downstream sequence. Fragments pJH983-pJH984, as well as pJH984-pJH985, overlap by 3kb in genomic sequence. All three fragments were co-injected into unc-80(e1272) or unc-77(hp102); unc-80 mutants for rescuing experiments. The rescuing RFP-tagged unc-80 genomic ‘construct’ is a mixture of pJH983, pJH984 and pJH916. pJH916 is a PCR-generated DNA fragment containing the same last 12.1kb C-terminal genomic sequence as pJH985 with mRFP (cherry) sequence inserted immediately before the stop codon, followed by unc-54 3’-UTR sequence. This fragment was generated based on a PCR-based tagging method [2]. Pan-neuronal promoter driven UNC-80 was achieved through co-injection of pJH994, a plasmid with the same insert as pJH982 subcloned into the pan-neural expression vector pJH625, together with pJH984 and pJH985.

The protein expression construct for antibody generation against NCA-1/UNC-77 is pJH997. The C-terminal region of the predicted C11D2.6d isoform was PCR-amplified from a cDNA clone, yk1314e03, using the primer pair OZM1059
(5’GGGGATCCGGACATTCT TCAATTTCACATGAGG3’)/OZM 758
(5’CCAAGCTTCTAATCAACAAGGAATT CCACCA3’) and subcloned as a BamHI/HindIII fragment into the pMALC2X expression vector to generate pJH997.

Constructs for the expression of UNC-79 recombinant proteins for antibody production are pJH523 and pJH690. pJH523 was generated by PCR-amplifying against cosmid E03A3 with primers OZM480 (5’ AaaggatccGCTGTATTCATATCA AGTCTATGTGG 3’) and OZM481 (5’ AaagtcgacttaATAATCATCTCCACCAATTC TTCCAG 3’), and subcloned into the BamHI and SalI sites of pMal-c2x. pJH690 was generated by PCR-amplifying against yk1042g09 with primers OZM523 (5’ AAAGGATCCTCAGGAGGTTACGGTAGACTCCGTG 3’) and OZM524 (5’ CCCGTCGACTTATTCCGCCAACTTAACCTTCAGATCACCAC 3’), and subcloned into the BamHI-HindIII sites of pMal-c2x. YC2.12 [3] was placed under the Pcat-1 promoter to generate the cameleon construct used to measure activity in HSN neurons.

For heterologous expression experiments in HEK293T cells, yk1314e03 (NCA-1 cDNA) and yk1260h06 (UNC-80 cDNA) cDNA clones in pME18S-FL vector were used (generated and provided by Drs Kohara, Suzuki and Sugano, Institute of Medical Sciences, University of Tokyo). These clones express the cDNAs from the SRα promoter. An expression vector of a deleted NCA-1 cDNA (pJH1161) was created by removing the ClaI fragment from yk1314e03, which removes aa847-1610 of NCA-1. The rat NALCN cDNA used for the HEK cell expression experiment was cloned in pTracer-CMV2 vector and provided to us by Dr. Ren (University of Pennsylvania).

The construct for expressing the mouse NALCN cDNA carrying the hp102 mutation at the equivalent amino acid position under pan-neural promoter is pJH1160. 3
overlapping mouse NALCN partial cDNA fragments were amplified by RT-PCR, cloned
into the T-vector and sequence verified. R329Q mutation was introduced into the
Corresponding cDNA fragment and the three pieces were sequentially subcloned into the
BamHI site of the \( \text{C. elegans} \) expression vector pJH625. The complete insert for
pJH1160 was re-sequenced to confirm the presence of the single mutation in the open
reading frame.

**RT-PCR:**

Total RNA was isolated from mixed staged wild-type, \( \text{unc-80(e1272)} \) and \( \text{unc-}
80(hp369) \) animals using a Trizol Kit (Invitrogen). First strand synthesis was performed
using MMTV reverse transcriptase and \( 1\mu g \) of total RNA from each strain. The PCR
reaction to detect all predicted \( nca-1 \) transcripts was performed with oligos OZM684
(5’GGATC TGGCGATTCCTGATAC3’)/OZM685
(5’GTGATGAAGATTCGGCACCGA3’) which amplify a 1084bp region spanning
exons 8 and 9.

**Transgenic lines:**

Extrachromosomal arrays (\( \text{hpEx} \) lines) were generated by co-injecting various
DNA constructs with injection markers. The following are the strains and genotypes for
the \( \text{hpEx} \) lines generated in this study: promoter reporter lines for the \( nca-1 \)- ZM2154-
2158 (\( \text{lin-15(n765);hpEx528-532[pJH859+LIN-15]} \)); promoter reporter lines for \( \text{unc-80} \) -
ZM2159-2161 (\( \text{lin-15(n765);hpEx532-535[pJH880+LIN-15]} \)) and ZM2162-2164 (\( \text{lin-}
15(n765);hpEx536-538[pJH881+LIN-15]} \)); rescuing lines for \( nca-1/unc-77(hp102) \) were
ZM1115,1116 (nca-1/unc-77(hp102);juIs1; hpEx290,291[pJH986+pJH901+Pttx-3-GFP]); rescuing lines for nca-1(gk9);nca-2(gk5) mutants were ZM2393 (nca-1(gk9);nca-2(gk5); hpEx717[pJH986+pJH901+ Podr-1-GFP]); rescuing lines (3 out of 25 lines) for panneural-expression of nca-1 were ZM2429-2431 (nca-1(gk9);nca-2(gk5);hpEx738-740[pJH975+pJH901+Podr-1::GFP]); rescuing lines for Punc-25-driven NCA-1 were ZM2938-2985 (nca-1(hp102);hpIs3;hpEx1047-1049[pJH971+pJH901+pRF4]). Wild-type animals expressing nca(gf) genomic fragments were transgenic line ZM1658(hpEx408[PCR genomic fragment of nca-1(hp102)+ Podr-1-GFP]). Wild-type animals expressing Vgcnl1(gf) in neurons were transgenic lines, ZM3094 and 3095 (hpEx1099-1100[pJH1160+Podr-1-GFP]).

Rescuing lines for unc-80(e1272) were ZM2394 (unc-80(e1272); hpEx718[pJH983+pJH984+pJH985+ Podr-1-GFP]), ZM2389 (unc-80(e1272); hpEx630[pJH983+pJH984+pJH916+ Podr-1-GFP]), ZM2432 (unc-80(e1272) ;hpEx741[pJH994+pJH984+pJH985+Podr-1::GFP]) and ZM2275-2276 (unc-80(e1272) ;unc-77(hp102); hpEx617-696[pJH983+pJH984+pJH985+ Podr-1-GFP]). hpEx630 was integrated to generate a stable transgenic array: ZM2390 (unc-80(e1272); hpIs98 [pJH983+pJH984+pJH916+Podr-1-GFP]). ZM2390 was outcrossed against unc-80(e1272) mutants twice before crossing into other genetic backgrounds.

unc-77(hp102), nca-2(gk5); nca-1(gk9), unc-80(e1272) and unc-80(e1272);unc-77(hp102) mutant animals carrying the cameleon marker expressed in HSN neurons were generated by crossing the various mutations into an integrated cameleon transgenic array ljIs65[Pcat-1-iYC1.12].
Calcium imaging analysis:

General guidelines for image acquisition and data analysis were described previously [4]. Briefly, time-lapse image data were acquired using a 63X water lens and the Metavue program (Molecular Devices). The total intensity of light emitted from the two cameleon fluorophores was calculated by a JAVA program Jmalyze. Photo-bleaching derived baseline-decay was corrected by a MATLAB script, RA, and calcium spikes were identified and analyzed using a MATLAB script, SFS. All computer program-identified spikes were re-examined and confirmed individually and manually based on the total ratio of spike above baseline noise, and the asymmetric (fast rising and slow decay) shape of the spikes.

The total spike number divided by the total imaging time is referred to here as Spike Frequency (#/min). In most cases, calcium transients distribute as periodic trains of calcium spikes, with the time interval between two consecutive spikes within the train following a normal distribution curve by lillietest and jb tests. The Spike Interval presented here refers to the intervals between spikes within these periodic trains, therefore largely measures the periodicity of events within the trains. The Spike Size (ratio change) was calculated by dividing the difference of the yellow/cyan ratio between the high and low points, with that of the low point of a spike. Histograms refer to the mean and standard error of the mean (SEM); statistical significance was calculated using the non-parametric Kolmogorov-Smirnov rank test due to non-normal data distribution.
Supplemental Movies for *C. elegans* of various genetic backgrounds and calcium imaging:

**Supplemental movie S1:** a wild-type animal displaying sinusoidal forward and backward locomotion patterns, also called body bends.

**Supplemental movie S2:** a *nca-2*(gk5); *nca-1*(gk9) mutant displaying a locomotion deficit termed as ‘fainter’. The animal was capable of moving in a sinusoidal fashion upon stimulation (by probing, shown in this movie; or to survive, e.g. lack of food in their habitat, not shown), but stopped after only a couple of body bends.

**Supplemental movie S3:** *unc-77*(hp102) animals displaying constitutive and exaggerated body bends that make them appear ‘coiling’ during both forward and backward locomotion.

**Supplemental movie S4:** a wild type animal expressing *nca-1* genomic fragments harbouring the *hp102* mutation. They displayed the exaggerated body bends similar to those by *unc-77*(hp102) mutants (movie S3).

**Supplemental movie S5:** Calcium imaging of HSN cell body in a wild-type animal. 4X real time. The cameleon signal was pseudo-colored based on the YFP/CFP ratio value, with red representing the highest value. Each pink flash reflected the peak of one calcium spike.

**Supplemental movie S6:** a *unc-80*(e1272) mutant animal displaying the same ‘fainter’ locomotion deficit as *nca-2*(gk5);*nca-1*(gk9) mutants (movie S2).

**Supplemental movie S7:** a *nca-2*(gk5); *nca-1*(gk9); *unc-80*(e1272) mutant displaying the identical ‘fainter’ locomotion deficit as in *nca*(gk5);*nca-1*(gk9) (movie S2) and in *unc-80*(e1272) (movie S6) mutants.
Supplemental movie S8: A *unc-77(hp102); unc-80(e1272)* mutant displaying the same ‘fainter’ phenotype as *unc-80(e1272)* mutants (movie S6).

Supplemental movie S9: An *unc-80(e1272)* mutant animal carrying an extrachromosomal array that consisted of genomic fragments covering the *unc-80* locus. The transgenic animal displayed continuous locomotion, and did not ‘faint’.

Supplemental movie S10: An *unc-77(hp102); unc-80(e1272)* mutant animal carrying an extrachromosomal array that consisted of genomic fragments covering the *unc-80* locus. The transgenic animal displayed continuous and exaggerated sinusoidal (coiling) locomotion pattern, similar to that of *unc-77(hp102)* mutants (Movie S3).

Supplemental movie S11: A *nca-2(gk5); nca-1(gk9)* mutant animal carrying an extrachromosomal array expressing NCA-1 driven by a pan-neuronal promoter. The transgenic animal displayed continuous and sinusoidal locomotion, and did not ‘faint’.

Supplemental movie S12: A *unc-80(e1272)* mutant animal carrying an extrachromosomal array expressing UNC-80 driven by a pan-neuronal promoter. The transgenic animal displayed continuous and sinusoidal locomotion, and did not ‘faint’.

Supplemental movie S13: The locomotion phenotype of *egl-19(ad695)* gain-of-function mutants. The animals were hyperactive and egg-laying constitutive, but are behaviourally distinguishable from the ‘coiling’ *unc-77(hp102)* mutants (S3).

Supplemental movie S14: A wild-type animal carrying an extrachromosomal array that expresses mouse NALCN(R329Q) driven by a pan-neuronal promoter. The transgenic animals displayed exaggerated body bends, which is characteristic of *hp102* and *e625* animals.
Figure S1
**Figure S 1: hp102, a gain-of-function mutation in nca-1 affects active zone development**

*hp102* mutants affects locomotion, active zone marker distribution at NMJs and egg-laying behavior that were fully suppressed by *unc-80* mutations.

A) Images of the body morphology of *nca*(gf), *nca*(lf), *unc-80* and *nca*(gf);*unc-80* mutants. The coiling position of *nca*(gf) was fully restored in *nca*(gf);*unc-80* mutants.

B) Active zone marker morphology of DD GABAergic synapses in L2 larvae of wild-type, *hp102*, *unc-80* and *nca*-1(*hp102*); *unc-80* animals, visualized by SYD-2::GFP (*hpIs3*). Inserts are magnified views of regions marked by the dotted line. *nca*(gf) mutants showed abnormal clustering (arrowheads) and gaps between active zone marker puncta. This defect was fully suppressed in *nca*(gf);*unc-80* mutants.

C) Quantification of the average number (N=10 for each strain) for *hpIs3* (~1/3 of dorsal cord region), where *nca*(gf);*unc-80* mutants showed wild-type level puncta.

D) Morphology of HSN synapses visualized by an active zone marker *wyIs12* also showed abnormal clustering in *nca*(gf) *hp102* mutants (bottom panels) compared to wild-type animals (top panels). This phenotype was rescued in *nca*(gf);*unc-80* mutants.

E, F) *nca*(gf) *hp102* mutants display constitutive egg-laying, resulting in fewer eggs (E) and younger eggs (F, % of eggs 8 cells or younger) retained in uterus of *hp102* animals compared to wild-type animals. N=15, the number of eggs was counted from animals 24hr post L4 larval stage. Error bar: SEM. Statistic comparisons were performed against the wild-type dataset using the Tukey-Kramer multiple comparison test. * p<0.01, ** p<0.001, Scale bar: 5µm.

The double mutant *nca*(gf);*unc-80* was constructed by Dr. Edward Yeh. The genetic interaction between *nca* and *unc-80* was investigated by Dr. Edward Yeh and me. The qualitative analyses of *hpIs3* were performed by Dr. Wesley Hung, Dr. Edward Yeh and me. The quantification of *hpIs3* puncta, and examination of *wyIs12* were analyzed by Dr. Wesley Hung. The quantification of eggs was performed by me. Dr. Wesley Hung also assembled Figure S1.
Figure S2

A  Cell body

wt

unc-13(e51)

B  Spike Frequency

#/min

wt  unc-13(e51)
Figure S 2: HSN cell bodies autonomously generated calcium spikes independent of presynaptic input under the assay condition (10mM HEPES, pH7.1)

A manuscript describing this work is to be submitted. We presented here only one piece of supporting evidence. In *unc-13(e51)* mutants, where synaptic transmission is severely abolished, HSN cell bodies were capable of generating trains of calcium spikes. (A) Representative cameleon trace displayed by HSN cell bodies of *unc-13(e51)* mutants as in Fig.3. (B) Calcium spike frequencies of HSN cell bodies in wild-type (wt) and *unc-13(e51)* mutants showed no statistically difference by the Kolmogorov-Smirnov rank test (P > 0.1). The number at the bottom of each bar represented the number of animals examined.

The recording and analyzes of Ca²⁺ spikes, as well as the preparation of Figure S2 were done by Dr. Mi Zhang.
Figure S3: Raw traces of YFP and CFP recorded in HSN cell body and synapse

**Left panel:** Raw YFP and CFP traces for HSN cell body recordings shown in Fig. 3 are shown in the top two lines. The X axis represents the recording time in seconds, and the Y axis represents fluorescent intensity in arbitrary units of pixel intensity. The ratio between YFP and CFP fluorescent signal at each time point was plotted against the same X axis, resulting in a third trace that represents the YFP/CFP ratio change. Calcium spikes display a characteristic asymmetric shape, with a fast, linear rising phase followed by a slower, exponential decaying phase (arrows), while peaks due to noise, or random fluorescent ratio fluctuations, typically show a symmetric shape, with linear rising and decaying phases (arrowheads). Reciprocal changes in YFP and CFP intensity were observed for many ratio peaks (examples were shown by dashed lines), though reciprocity was sometimes obscured by motion artefacts caused by movement of the neurons during egg-laying. Despite of the fluctuations in the absolute YFP and CFP intensity levels over recording period, the YFP/CFP ratio-metric trace revealed similar ratio changes for most calcium spikes in wild-type animals, suggesting that the calcium spike size is insensitive to fluorescent baseline changes and accurately reflects changes in calcium concentration.

**Right panel:** Raw YFP and CFP traces for HSN synaptic trace shown in Fig. 3.

The Ca$^{2+}$ recording and the preparation of Figure S3 were performed by Dr. Mi Zhang.
Figure S 4: UNC-80 encodes a highly conserved novel protein.

A) A schematic representation of the gene structure of unc-80 (F25C8.3) adapted from Wormbase. The exons are shown as pink boxes. The genetic lesions of unc-80 alleles (e1069, e1272 and hp369) are shown. B) Protein structure and similarity of UNC-80 family members. C.e.: C. elegans; D.m.: D. melanogaster; R.n.: R. norvagicus; M.m.: M. musculus; H.s.: H. sapiens.

The mapping of unc-80 and sequencing of hp369 were performed by Michelle Li. Cloning of unc-80 was based on unpublished data shared by Dr. Kim Schuske. Sequencing of e1069 and e1272 was done by me. I also prepared Figure S4.
Figure S5: NCA-1 functions cell autonomously in neurons to regulate active zone morphology.

Active zone morphology was examined using the SYD-2::GFP marker hpIs3 in wild-type and nca(gf) respectively. In wild-type animals, SYD-2::GFP puncta are round and regularly spaced, while nca(gf) animals show regions lacking puncta, as well as clustering of puncta. Genomic DNA containing the nca-1 gene, or expression of NCA-1 from a GABAergic neuron-specific promoter, restored the hpIs3 phenotype of nca(gf) to wild-type morphology. Inserts show a higher magnification of the region underlined by the dotted line. Scale bar: 5 μm.

All nca-1-related constructs were generated by Ying Wang. hpIs3 analyses and preparation of Figure S5 were performed by Dr. Wesley Hung.
**Figure S6: NCA-1 and UNC-80 are enriched at non-synaptic regions.**

A) Wild-type animals were co-stained with anti-NCA-1 antibody (red) and either anti-SNB-1 or anti-SAD-1 antibodies (green). B) *unc-80; hpIs98* (UNC-80::RFP) animals were co-stained with anti-RFP antibody (red) and either anti-SNB-1 or anti-SAD-1 antibodies (green). Poor co-localization was observed in all cases. Scale bar = 5 µm.

Examinations of NCA-1 and UNC-80::RFP were performed by me. The preparation of Figure S6 was done by Dr. Wesley Hung and me.
Figure S7: UNC-80::RFP restores localization of NCA-1 in unc-80 mutants.

A) UNC-80 regulates NCA-1 protein localization. Wild-type and unc-80 animals were stained with anti-NCA-1 (red) and anti-UNC-10 (green) antibodies. Punctate NCA-1 staining pattern along the ventral and dorsal nerve cords was observed in wild-type but diminished in unc-80 animals. UNC-10 staining pattern was similar between wild-type and unc-80 mutant animals. Expression of UNC-80::RFP from its own promoter (hpIs98) restored the punctate expression pattern of NCA-1 along the nerve cords. B) unc-80 mutations do not affect the transcript level of nca-1. Total RNA was isolated from mixed staged wild-type, unc-80(e1069) and unc-80(e1272) animals and the transcript levels of nca-1 were analyzed by RT-PCR (with a control reaction containing no template). nca-1 transcript was present in all strains at similar levels. Scale bar: 5 µm.

The localization of NCA-1 in the rescued unc-80 mutant was examined by Dr. Wesley Hung. RT-PCR was performed by Michelle Li. Dr. Wesley Hung also assembled Figure S7.
Figure S8: Endogenous UNC-79 is expressed in nervous system.

A) An antibody against UNC-79 was used to stain wild-type (top panel) and \textit{unc-79} animals (bottom panel). Specific staining was observed in the nerve ring of wild-type but not in \textit{unc-79} mutant animals. *ns: non-specific staining. B) The dorsal (top panel) and ventral (bottom panel) nerve cords of wild-type animals stained with anti-UNC-79 antibody showed punctate staining pattern.

The UNC-79 localization and expression pattern were examined by Dr. Kyota Aoyagi. The preparation of Figure S8 was done by Dr. Wesley Hung.
Figure S9: UNC-79 localization depends on the presence of UNC-80 and NCA.

Wild-type, nca(gf), nca(lf), unc-80, nca(lf);unc-80 and nca(gf);unc-80 animals were simultaneously stained with anti-UNC-79 antibody. UNC-79 signals were detected in the nerve ring (NR) of wild-type and nca(gf) animals but disappeared in other mutants.

The localization of UNC-79 in nca and unc-80 mutants was investigated by Dr. Kyota Aoyagi. Dr. Wesley Hung assembled Figure S9.
Table S 1: Summary of phenotypes displayed by double mutants between nca and VGCCs

Table S1

<table>
<thead>
<tr>
<th>VGCC type</th>
<th>Subunit</th>
<th>Behavioral Phenyotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single Mutant</td>
</tr>
<tr>
<td>unc-2(na512) If</td>
<td>POG/N</td>
<td>α1</td>
</tr>
<tr>
<td>unc-36(ra612) If</td>
<td>L</td>
<td>α2/3</td>
</tr>
<tr>
<td>egl-19(n882) If</td>
<td>L</td>
<td>α1</td>
</tr>
<tr>
<td>egl-19(adf593) gf</td>
<td>L</td>
<td>α1</td>
</tr>
<tr>
<td>cca-1(adf1550) If</td>
<td>T</td>
<td>α1</td>
</tr>
</tbody>
</table>

- If: loss-of-function
- gf: gain-of-function
- N/A: not available
- Egld-c: egg-laying constitutive
- Egld: egg-laying defective

All genetic mutants described here were generated and analyzed by me. I also prepared Table S1.
Supplemental Reference


Chapter 3  Identification of Genetic Suppressors for nca-1 Gain of Function Mutation \textit{hp102}

Chapter 3 describes an \textit{nca-1} gain-of-function genetic suppressor screen, which was performed by Dr. Edward Yeh and me. Almost all of the suppressors presented here were isolated, characterized, and mapped by me, except for the isolation of \textit{hp459}, \textit{hp465}, \textit{hp468} and \textit{hp468}. These four suppressors were isolated by Dr. Mei Zhen, Dr. Edward Yeh and Michelle Li, and they were out-crossed by Dr. Mei Zhen. A former summer student, Parminder Duley, performed the initial mapping of suppressor \textit{hp420}. Dr. Wesley Hung suggested the idea of testing \textit{nca-1} loss-of-function revertant by generating the suppressor; \textit{nca-1(gk5)} double mutant. Otherwise, I solely completed the analyses of suppressors and the writing of Chapter 3.
3.1 Rationale and Objective

The gain of function effect of \textit{nca-1} was proposed to further increase membrane excitability, and thereby results in hyperactivated synapses and over-incorporation of active zone materials (Chapter 2). Therefore, a suppressor screen using the gain of function \textit{nca-1(\textit{hp102})} provides an unique opportunity to reveal the mechanisms that regulate properties of neuronal membrane, as well as mechanisms that correct or compensate for defects caused by synaptic hyperactivation. A suppressor screen using a gain of function allele can be very powerful in that it can potentially recover loss of function mutations in not only the \textit{nca} channel itself, but also in both upstream and downstream genes. Gain of function upstream negative regulators and gain of function downstream inhibitors may also be recovered, since it is possible that they can inhibit or bypass the \textit{hp102} gain of function effect.

The distinctive behavioral phenotype displayed by \textit{nca-1(\textit{hp102})} facilitated the identification of suppressors. \textit{nca-1(\textit{hp102})} animals alternate between periods of coiling (Fig. 3.5C) and steep sinusoidal (“loopy”) movements (Fig. 3.5B). The identification and selection of \textit{nca-1(\textit{hp102})} behavioral suppressors was based on reversal of the \textit{nca-1(\textit{hp102})} coiling phenotype back to wild-type, as well as the visible reduction of deep body bending that suppresses the loopy phenotype. The gain of function \textit{nca-1(\textit{hp102})} mutant will be referred to by its allele name, \textit{hp102}, hereafter.

Some of the \textit{hp102} related mutants mentioned previously will appear again in this chapter. I will recapitulate their behavioral phenotypes here to facilitate discussion of the \textit{hp102} suppressor screen. The \textit{nca-1} loss of function \textit{gk9}, as well as its close homologue \textit{nca-2} loss of function \textit{gk5}, do not show any behavioral defects on their own; but together
the double loss of function mutant \( nca-1(gk9); nca-2(gk5) \) shows a distinctive fainter phenotype. This fainter phenotype is also present in two \( hp102 \) suppressors, \( unc-79 \) and \( unc-80 \). Fainter animals move in alternating periods of active wild-type sine wave motion and quiescence (fainting). The distinctive \( hp102 \) coiling and loopy phenotypes are suppressed in the double mutants \( hp102; unc-79 \) and \( hp102; unc-80 \). As mentioned previously in Chapter 2, \( hp102 \) is a semi-dominant gain of function allele of \( nca-1 \).

Heterozygous \( hp102/+ \) animals display more loopy locomotion than wild-type, but less severe than homozygous \( hp102/hp102 \) animals that exhibit an additional coiling phenotype. Overexpression of \( nca-1 \) genomic PCR fragments containing the \( hp102 \) mutation also induced the coiling phenotype in wild-type animal. However, trans-heterozygote \( hp102/gk9 \) displays the same degree of coiling as homozygous \( hp102/hp102 \) animal, which argues against a simple neomorphic-\( hp102 \) proposition in which \( hp102/gk9 \) is expected to show the same degree of severity as \( hp102/+ \). \( hp102 \) is also dominant over \( nca-2 \), since \( nca-1(hp102); nca-2(gk5) \) double mutant shows the coiler phenotype as well. Two of the above genotypes, \( hp102/gk9 \) and \( nca-1(hp102); nca-2(gk5) \), will appear in later section 3.2.2.2.

### 3.2 Experimental Procedures and Results

#### 3.2.1 Suppressor Screen of \( hp102 \)

Mutagenesis is a random event. Therefore, the spermatocytes and the oocytes in \( hp102 \) \( P_0 \) hermaphrodites have equal opportunities to be mutated, and each of their F1 progeny will carry two mutagenized haploid genomes. For example, one sperm can be mutated for a hypothetical \( hp102 \) suppressor gene \( sup \), and any oocyte in the same
mutagenized hermaphrodite may still retain the wild-type copy of \textit{sup}. Fertilization of an oocyte by this mutated sperm will result in a heterozygous F1 individual. Assuming that \textit{sup} is recessive, the F1 individual will remain as a coiler as it is homozygous for \textit{hp102} (Fig. 3.1). All the germ cells in this F1 animal will be heterozygous for \textit{sup}, therefore after self-fertilization, approximately one quarter of its F2 progeny will be homozygous for \textit{sup} and result in a visible loopy or wild-type phenotype. This \textit{sup} suppressor will be isolated in the F2 screening process (Fig. 3.1).

\textit{hp102} animals were mutagenized following the standard protocol for ethyl methane sulfonate (EMS) mutagenesis in \textit{C. elegans} [1]. After 4 hours of incubation with 50mM EMS, healthy late larval stage 4 \textit{P}0 animals were recovered and transferred onto the fresh plates to allow for self-fertilization and generation of F1 progeny. Approximately 20 to 25 F1 progeny were selected randomly and transferred to large NG plates (3g/L NaCl, 2.5 g/L Bactopeptone and 17g/L Bacto-agar, 5mg/ml cholesterol in EtOH, 1mM/L CaCl2, 1mM/L MgSO4, and 25mM/ potassium phosphate buffer pH6.0) to allow for self-propagation into the F2 generation for screening. In total, 26,000 mutagenized haploid genomes were screened based on their behavioral suppressions of the \textit{hp102} coiler phenotype (Fig. 3.1).
3.2.2 Classification of Suppressors

3.2.2.1 Three Classes of hp102 Suppressors

A total of 31 hp102 suppressors were isolated. They were first divided into three groups based on their strengths for suppressing the coiler locomotion (Table 3.1).

The first class of strong suppressors does not exhibit additional locomotion defects. The second class of strong suppressors displays additional locomotion defects including fainters and twitchers. The fainter suppressors likely represent alleles of unc-79 and unc-80, which I have shown in chapter 2 to be epistatic to hp102. Indeed, non-complementation test showed that hp424 represents a genetic locus in unc-79. The twitcher phenotype in some of the suppressors are possibly conferred by mutations in the
unc-22 gene, whose role in muscle actomyosin contraction may suggest an indirect suppression event caused by hyper-activated NMJ activity [2, 3]. These suppressors together with the weak suppressors were not included for further analyses. Only the 14 strong suppressors in the first class (highlighted in red in Table 3.1) were subjected to further outcrosses and characterizations.

Table 3.1: Preliminary Behavioral Classification of All Suppressors

<table>
<thead>
<tr>
<th>Level of Behavioral Suppression</th>
<th>Description of Behavior</th>
<th>List of Suppressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Strong</td>
<td>loopy or wild-type movement</td>
<td>(1) hp102; hp411 (2) hp102; hp418</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) hp102; hp420 (4) hp102; hp421</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) hp102; hp423 (6) hp102; hp426</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) hp102; hp427 (8) hp102; hp428</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9) hp102; hp429 (10) hp102; hp430</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11) hp102; hp459‡ (12) hp102; hp465‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13) hp102; hp467‡ (14) hp102; hp468‡</td>
</tr>
<tr>
<td>2. Strong</td>
<td>fainter or twitcher</td>
<td>(1) hp102; hp402 (2) hp102; hp403</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) hp102; hp404 (4) hp102; hp407</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) hp102; hp408 (6) hp102; hp410</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(7) hp102; hp413 (8) hp102; hp415</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(9) hp102; hp416 (10) hp102; hp417</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11) hp102; hp419 (12) hp102; hp422</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13) hp102; hp424 (14) hp102; hp425</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(15) hp102; hp431</td>
</tr>
<tr>
<td>3. Weak</td>
<td>mild coiler or active UNC*</td>
<td>(1) hp102; hp404 (2) hp102; hp406</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) hp102; hp409 (4) hp102; hp412</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5) hp102; hp414 (6) hp102; hp433</td>
</tr>
</tbody>
</table>

*UNC stands for UNCoordinated animals that move differently from the wild-type sinusoidal motion.
‡ These suppressors were isolated by Dr. Mei Zhen, Dr. Edward Yeh and Michelle Li.
3.2.2.2 Intragenic versus Extrageneic *hp102* Suppressors

Out-crossing to the wild-type strain N2 not only serves to eliminate background mutations, but more importantly, can also reveal the nature of the genetic interaction between *hp102* and the suppressor loci. Genetic suppression can be either intragenic or extragenic. Figure 3.2 illustrates the out-crossing scheme for the 14 strong suppressors. The basic rationale is that any intragenic mutation or extragenic mutation that is closely linked to *nca-1* will segregate together with *hp102*, hence all progeny will display the suppressed coiler phenotype in all generations (Fig. 3.2 left column). Note that *nca-1* maps to chromosome IV (IV: 3.06), therefore this class of suppressors belongs to the chromosome IV linkage group. In contrast, unlinked extragenic suppressors should segregate independently of *hp102* mutation (Fig. 3.2 middle and right columns). In addition, recombination will occasionally separate the linked extragenic suppressor mutation away from *hp102*, so that the linked suppressor will segregate independently in the F2 and F3 generations like the unlinked suppressor.

Both dominant and recessive extragenic suppressors will segregate independently from *hp102* in the Mendelian ratio. The only difference is that the coilers (*hp102* animals) selected in F2 will segregate approximately 1/4 suppressed progeny in F3 if the suppressor is recessive (Fig. 3.2 middle column); while in the case of a dominant suppressor, coiler (*hp102*) F2s will only segregate coiler progeny (Fig. 3.2 right column). To facilitate the selection of crossed progeny and *hp102* homozygous animals, PCR oligos OZM 1071, 1072 and 1073 were used to genotype *hp102* by detecting the specific missense nucleotide change (Appendix A4).
Figure 3.2: Using an N2 out-cross to distinguish between intragenic, \( hp102 \)-linked, and extragenic recessive or extragenic dominant suppressor mutations for \( hp102 \).

\( hp102 \) mutation can be detected by oligo OZM1071-1073 (Appendix TableA2 and A4). \( sup \) stands for the suppressor gene. Plus sign represents wild-type copy of the gene.

Using this scheme, I determined that the 14 strong suppressors consist of 4 intragenic or \( hp102 \)-linked extragenic suppressors, 9 extragenic recessive suppressors and 1 extragenic semi-dominant suppressor (Summarized in Table 3.2). This semi-dominant suppressor, \( hp430 \), does not display any phenotype on its own, but exerts a semi-dominant suppression on \( hp102 \). The heterozygous \( hp430 \) in the background of
homozygous hp102 shows backward coiling but forward wild-type movement; whereas the homozygous hp102; hp430 exhibits wild-type backward and forward locomotion.

Table 3.2: Classification of Intragenic versus Extragenic Suppressors

<table>
<thead>
<tr>
<th>Nature of Suppression</th>
<th>Suppressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extragenic recessive suppressors</td>
<td>(1) hp102; hp411</td>
</tr>
<tr>
<td></td>
<td>(2) hp102; hp420</td>
</tr>
<tr>
<td></td>
<td>(3) hp102; hp421</td>
</tr>
<tr>
<td></td>
<td>(4) hp102; hp427</td>
</tr>
<tr>
<td></td>
<td>(5) hp102; hp428</td>
</tr>
<tr>
<td></td>
<td>(6) hp102; hp459‡</td>
</tr>
<tr>
<td></td>
<td>(7) hp102; hp465‡</td>
</tr>
<tr>
<td></td>
<td>(8) hp102; hp467‡</td>
</tr>
<tr>
<td></td>
<td>(9) hp102; hp468‡</td>
</tr>
<tr>
<td>Extragenic semi-dominant suppressors</td>
<td>(1) hp102; hp430</td>
</tr>
<tr>
<td>Intragenic loss-of-function revertants</td>
<td>(1) hp102 hp418</td>
</tr>
<tr>
<td></td>
<td>(2) hp102 hp423</td>
</tr>
<tr>
<td></td>
<td>(3) hp102 hp426</td>
</tr>
<tr>
<td>2nd site intragenic revertant or dominant hp102-linked</td>
<td>(1) hp102 hp429</td>
</tr>
<tr>
<td>extragenic suppressor</td>
<td></td>
</tr>
</tbody>
</table>

† These suppressors were isolated by Dr. Mei Zhen, Dr. Edward Yeh and Michelle Li.

The intragenic revertants can be distinguished from the hp102-linked extragenic suppressors by crossing hp102 suppressor males to nca-1(hp102); nca-2(gk5) coiler (Fig. 3.3). Use of this double mutant, nca-1(hp102); nca-2(gk5), offers the opportunities to backcross suppressors to hp102, and to generate the suppressor nca-2(gk5) genetic double mutant in one cross.

Backcross to hp102 is necessary to identify hp102-linked extragenic suppressors. If the suppressor is a recessive hp102-linked extragenic mutation, then the F1 cross progeny will behave like the homozygous hp102 coiler and segregate approximately 25%
of F2 suppressors in the gk5 homozygous background (Fig. 3.3). For a dominant hp102-linked suppressor, the same cross will result in F1s that behave like a loopy suppressor, and segregate about 25% F2 coilers that do not contain any paternal hp102 sup chromosome (Fig. 3.3). However, the same segregation pattern will appear if the suppressor is a second site intragenic revertant that corrects the original hp102 mutation, in which the hp102 sup chromosome will behave like the wild-type copy of nca-1 (Fig. 3.3). hp429 is one such suppressor that segregated loopy F1s (n >100) and roughly 25% F2 coilers (F2 n>10x100) in this cross. Therefore, hp429 is either a second site intragenic revertant, or a dominant hp102-linked extragenic suppressor. In this case, only sequencing can distinguish between these two possibilities.

As mentioned previously that nca-1 loss-of-function mutation can be revealed at the behavioral level as fainter only in the presence of the loss-of-function mutation of nca-2. The generation of double mutant of suppressor with nca-2(gk5) is therefore essential to identify the nca-1 loss-of-function revertant. Intragenic suppression can also be achieved by second site mutation that results in partial or complete loss of function of NCA-1. If this is the case, heterozygotes with one paternal copy of this allele in trans with the maternal hp102 chromosome are expected to display the coiler phenotype (Fig. 3.3), as hp102/nca-1(gk9) trans-heterozygous animals behave identically to hp102 homozygous animals. Moreover, homozygous alleles of this suppressor will display the fainter phenotype in the gk5 homozygous background, similar to the double loss of function mutant nca-1(gk9); nca-2(gk5). Although a recessive extragenic suppressor will also produce coilers in the F1, it should not behave as fainter in the gk5 homozygous
background. Using this approach, I determined that \textit{hp418}, \textit{hp423} and \textit{hp426} are the intragenic loss of function revertants (Table 3.2).

**Figure 3.3:** Crossing scheme to distinguish between intragenic loss-of-function revertant, intragenic 2nd site mutation revertant, \textit{hp102}-linked dominant and recessive extragenic mutations for \textit{hp102}

\textit{hp102} mutation can be detected by oligo OZM1071-1073 (Appendix TableA2 and A4). \textit{sup} stands for the suppressor gene. Plus sign represents wild-type copy of the gene. Oligos OZM 384-385 were used to detect \textit{nca-2(gk5)}, which maps to chromosome III position –2.29. For each suppressor, approximately 10x100 F2 animals were examined and the proportion of animals displaying different behaviors were roughly estimated.

### 3.2.3 Identification of Complementation Groups

To assess the total number of genetic loci that the extragenic recessive suppressors affected, the non-complementation test was used. The scheme was
illustrated in Figure 3.4. When one recessive suppressor is placed in trans with another recessive suppressor, the heterozygous animals are expected to exhibit suppression of the coiler phenotype in the hp102 mutant background if they are alleles of the same gene. This interaction is described as failing to complement, which places the two alleles to the same complementation group. On the other hand, the interaction between two alleles is termed ‘complementing’ if the trans-heterozygous animals fail to suppress the hp102 coiler phenotype; thereby placing them in two different complementation groups.

Males of one suppressor were crossed with each of the other suppressors so that complementation of any two alleles was tested for a second time in the reciprocal cross. The only exceptions were the male mating deficient hp102; hp427 and hp102; hp459 (Table 3.3). In summary, hp420, hp467 and hp468 failed to complement each other, but all the other suppressors complemented each other. Including the semi-dominant hp430, the 10 extragenic non hp102-linked suppressors were placed in 8 complementation groups that represented 8 different genetic loci.
Figure 3.4: A schematic depiction of complementation test

sup1 and sup2 represent two different recessive suppressor mutations. Plus sign represents the wild-type copy of the gene. Approximately 20 F1 animals were examined for each cross and the proportion of F2 coilers (if any) was roughly estimated.
Table 3.3: The Complementation Test Chart for All Extragenic Recessive Suppressors

<table>
<thead>
<tr>
<th></th>
<th>hp420</th>
<th>hp467</th>
<th>hp468</th>
<th>hp411</th>
<th>hp421</th>
<th>hp428</th>
<th>hp465</th>
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✓ = DO complement (highlighted in pink)  
X = DO NOT complement  
- = NOT AVAILABLE

3.2.4 Individual Extragenic Suppressor Displays Different Levels of Behavioral Suppression

Although all ten extragenic suppressors were classified as strong suppressors in the preliminary behavioral assessment (Table 3.1), they show different degrees of suppression to the hp102 locomotion pattern. Some of them also exhibit different phenotypes on their own.

hp102; hp411 and hp411 mutants display an Egg-laying defective (Egl) phenotype, with eggs retained within the uterus which makes their bodies appear as bloated (Fig. 3.5D). hp102; hp459 mutants are shorter than wild-type animals (Fig. 3.5K). hp102; hp427 are constitutive dauers (Fig. 3.5H) with a few escapers (Fig. 3.5G).
that permit propagation of this line. These dauers move actively in a wild-type locomotion pattern (Fig. 3.5H), whereas the wild-type dauers are inactive and the hp102 dauers remain as coilers. This therefore strongly argues against a possibility that the dauer stage is the direct cause of the suppression. In contrast, the hp102; hp427 escapers are short and fat, which is described as the Dumpy (Dpy) phenotype (Fig. 3.5G). The hp102 coiler phenotypes in hp102; hp411, hp102; hp427 escapers and hp102; hp459 are suppressed (Fig. 3.5 D, G, K). However, their short body length or wider body may indirectly reduce deep body bending and coiling, thereby may poses a indirect behavioral suppression on hp102.

hp420, hp421, hp428, hp430, hp465, hp467 and hp468 do not appear to show a behavioral phenotype on their own since no animals with distinctive morphological or behavioral features were observed during out-crossing. Thus, they can only be followed in the hp102 background. Most of these suppressors and the hp102; hp427 dauers exhibit close to 100% penetrance of wild-type sinusoidal movement with slightly more loopy body bending (Fig. 3.5E, F, H, I, J, L, M, N). The only exception is hp421, which shows approximately 50% penetrance of behavioral suppression.
**Figure 3.5: Behavioral phenotypes of hp102 suppressors**

Each image is a snapshot of the representative behavioral movement and body posture of the animals. (A) Wild-type N2. (B-C) hp102 moves in periods of exaggerated bending (B) and coiling (C). (D) Egl hp102; hp411. (E) hp102; hp420. (F) hp102; hp421. (G) Escaper of dauer constitutive hp102;hp427. (H) Constitutive dauer hp102; hp427 in dauer state. (I) hp102; hp428. (J) hp102; hp430. (K) Dpy hp102; hp459. (L) hp102; hp465. (M) hp102; hp467. (N) hp102; hp468.
3.2.5 Suppression of *hp102* Synaptic Phenotype by Extragenic Suppressors

In addition to the behavioral defects, *hp102* mutants displayed abnormal expression of an active zone marker, *hpIs3* (\(P_{unc-25}^{>25}\)SYD-2::GFP) (Fig. 3.6.1 A-B, 3.6.2 A-B). In fact, *hp102* was first identified in a genetic screen for mutants that affected this marker [4]. Two known genetic suppressors for *hp102*, *unc-79* and *unc-80*, fully reverted the *hpIs3* distribution defects of *hp102* animals (Chapter 2). This marker was then used to further distinguish the suppression strengths among different extragenic suppressors.

In *hp102* animals, *hpIs3* puncta become clustered in some areas but are absent in other regions (Fig. 3.6 1B, 3.6.2B). *hp411, hp420, hp421, hp428, hp430, hp465, hp467, and hp468* revert the *hpIs3* defects in *hp102* mutants close to the wild-type pattern (Fig. 3.6.1 C-J). Among these suppressors, the semi-dominant suppressor *hp430* and the recessive suppressor *hp465* both show fully penetrant suppression. The complementation group *hp420, hp467* and *hp468* shows 80% penetrance, and *hp411, hp421* and *hp428* only show 40%, 52% and 59% penetrance of suppression respectively. As these suppressors suppress both the locomotion and synaptic morphology defects of *hp102* animals, they are more likely encoding genes that directly function through, or with, the NCA channel activity.

Other extragenic suppressors do not revert the active zone marker defects in *hp102* mutants (Fig. 3.6.2 C-E). The SYD-2::GFP puncta remain clustered in *hp102; hp459, hp102; hp427* dauer and *hp102; hp427* escaper (Fig. 3.6.2C-E). The lack of synaptic morphology rescue suggests that the behavioral suppression displayed by some of these suppressors may not reflect a true genetic interaction, but are only anatomical defects that perturbed the body bending in *C. elegans.*
Figure 3.6.1: Suppression of SYD-2::GFP defects of *hp102* mutants by some extragenic suppressors

(A) Wild-type *hpl3* carrying the SYD-2::GFP marker expressed in GABAergic motoneurons. (B) A representative clustering SYD-2::GFP phenotype shown by *hp102*. (C-H) Wild-type distribution of SYD-2::GFP phenotype displayed by suppressors. (C) *hp102; hp411*. (D) *hp102; hp420*. (E) *hp102; hp421*. (F) *hp102; hp428*. (G) *hp102; hp430*. (H) *hp102; hp465*. (I) *hp102; hp467*. (J) *hp102; hp468*. Scale bar: 5 \( \mu \text{m} \).
3.2.6 Chromosome Linkage of Extragenic Suppressors

Both genetic two-factor mapping and snip-SNP mapping were used to assign chromosome linkage for the 10 extragenic, \textit{hp102}-unlinked suppressors. The principles for these two approaches are similar in that the linkage of a mutation to a chromosome is determined by non-Mendelian segregation ratio of the suppressors with a given chromosome marker. The difference is that snip-SNP mapping utilizes Single-
Nucleotide-Polymorphism (SNP) between the Bristol N2 strain and the Hawaiian CB strain as molecular markers [5], whereas genetic two-factor mapping uses GFP markers and morphological mutants that are associated with Chromosome I, II, II, V, and X.

For snip-SNP mapping, I first crossed the CB strain with the suppressor strains that were generated in the N2 background (Fig. 3.7). For all recessive suppressors, multiple hp102 suppressor lines were then recovered from the N2/CB hybrid parents. Crude genomic DNA lysate from these lines were pooled and used to examine the ratio of N2 and CB composition in each chromosome by distinct restriction enzyme digestion patterns resulting from N2 or CB chromosomes (Fig. 3.7). An oligo pairs were used to PCR amplify a small region of DNA fragment containing a chosen SNP. Some of the SNPs used here correspond to a specific restriction enzyme cut site in the N2 background but not in the CB background, and vice versa for some of the other SNPs. Therefore, restriction enzyme digest of the PCR products is expected to result in either uncut or digested smaller PCR products depending on whether the original lysate contains the N2 or CB chromosomes. Many different snip-SNPs exist in the C. elegans genome corresponding to various different restriction enzyme cut sites; but a set of DraI SNPs containing roughly 8 SNPs spanning each chromosome was used in this project (Appendix Table A1). Three SNP markers from each chromosome were chosen to examine linkage. The enrichment for N2 pattern associated with any markers suggests that these markers are linked to the suppressor loci (Fig. 3.7). Note that since all the suppressors still retain hp102 in the background, a N2 enrichment is expected to associate with SNPs that are close to where nca-1 maps to (IV: 3.06). For markers that are located on the same chromosome but further away from the suppressor mutation, a gradient
A decrease in N2/CB ratio is expected because the recombination frequency increases as the distance between the mutation and the marker increases.

For the semi-dominant suppressor *hp430*, a reverse approach was employed (Fig. 3.8). From N2/CB heterozygous animals, non-suppressed *hp102* coiler lines were recovered, and their DNA lysates were examined for CB/N2 ratio. Markers that show enrichment for CB chromosome are considered linked to the *hp430* locus.

**Figure 3.7: A schematic description for the generation of CB suppressor lines**

The black bar represents chromosome from the N2 Bristol strain. The red bar represents chromosome from the polymorphic CB Hawaiian strain. SNP1 is a hypothetical SNP marker that is closely linked to the recessive *hp102* suppressor *sup*. SNP2 is another hypothetical SNP marker that is on a different chromosome from *sup*. 
I performed both genetic mapping and snip-SNP mapping for some suppressors, as the genetic variation of CB strain in at least one case here appears to interfere with the penetrance of *hp102* suppressors thus prevents precise snip-SNP. Moreover, genetic...
mapping can further confirm the snip-SNP mapping data. The integrated GFP markers for chromosome I, II, III and V (ayIs4 I, juIs76 II, ncIs3 III and mIs10 V), and the body length mutant lon-2 (X) were used as visible markers for each chromosome. As described in Fig. 3.9, F2 coilers (hp102/hp102) were randomly picked from hp102/+; Marker/+ F1 animals, and scored for the presence of markers in F3 animals that display behavioral suppression (F1 n >100, F2 n >20x100, F3 n >8~39x100). The proportion of F3 suppressors that are marker-positive (Marker/+ or Marker/Marker) or marker-negative is denoted by S+ ratio or S- ratio respectively. Recessive suppressors will segregate away from a marker on the same chromosome, giving a high S- ratio. For markers that are on different chromosomes from the suppressor, an approximate 3:1 ratio of S+ versus S- is expected (Fig. 3.9).
Figure 3.9: Genetic mapping by examining the ratio of segregation of *hp102* suppressor with a chromosome marker

*sup* represents a *hp102* suppressor. *m* stands for a hypothetical chromosome marker. A chromosome marker was crossed with *hp102* suppressor. F2 coilers were picked blind to the presence of the chromosome markers. S+ stands for the ratio of F3 suppressors that segregated with the marker. S- stands for the ratio of F3 suppressors that are marker negative.

Genetic mapping by the loss of suppressor was utilized to identify chromosome linkage for the semi-dominant suppressor *hp430* (Fig. 3.10). In the F2 generation, *hp102* coilers should select against the presence of *hp430*, therefore enriching for markers that associate with the chromosome where *hp430* resides. Markers that are located on other chromosomes, on the other hand, should segregate independently of *hp102* coilers in the Mendelian ratio. Therefore, about 3/4 of the F3 coilers will be marker positive (C+), and
the rest will be marker negative (C-). If the marker is on the same chromosome as the suppressor, all the F3 coilers are expected to be marker positive.

Figure 3.10: Genetic mapping by examining the ratio of segregation of chromosome marker to the loss of hp102 suppressor

*sup* represents a *hp102 suppressor*. *m* stands for a hypothetical chromosome marker. A chromosome marker was crossed with *hp102 suppressor*. F2 coilers were picked blind to the presence of the chromosome markers. To confirm that these coilers contain no copy of *sup*, they are separated onto individual plate to verify that they throw 100% F3 coiler progeny. *C+* stands for the proportion of F2 coilers that segregated with the marker. *C-* stands for the proportion of F2 coilers that are marker negative.

Mapping scheme for the chromosome X marker *lon-2* mapping is different from other markers in that homozygous *lon-2* coilers were selected first in the F2 generation.
(n>20x100). The chromosome linkage to X was determined by high ratio of F2 lines that throw 100% long coilers (lc) in the F3 generation (n>100), verses a rough 1/3 lc ratio for non-X linked suppressor (Fig. 3.11).

Figure 3.11: Genetic mapping of hp102 suppressor with chromosome X marker lon-2

sup represents a hp102 suppressor. lon-2 males were crossed to hp102 suppressor. F2 long coilers were selected and segregation of lon-2 with suppressors was examined in F3 generation. Is stands for the ratio of F2 lon-2 coiler animals that segregated with the suppressor. Ic stands for the ratio of F2 lon-2 animals that did not segregate with the suppressor.

The genetic mapping schemes illustrated here assumes that the chosen marker and the suppressor are tightly linked. However, if the marker and the suppressor are on the same chromosome but are not tightly linked, they will appear to segregate independently from each other. To prepare for this shortcoming, snip-SNP mapping were used for most
of suppressor mapping here to confirm linkage, and more genetic markers were used if linkage was determined only by genetic mapping.

3.2.6.1 Determination of the Chromosome Linkage of Each Suppressor

3.2.6.1.1 hp411

snip-SNP mapping was used to determine the chromosome linkage for hp411 as described previously (Fig. 3.7). 11 CB suppressor lines were used. The results showed a single 100% N2 enrichment for SNP F36H9 located at V: -17, with a gradual decrease in N2 concentrations for the SNPs to the right (Fig. 3.12). Therefore, hp411 was mapped to a region close to or to the left of V: -17.
Figure 3.12: snip-SNP mapping data for *hp102; hp411*

11 CB lines were pooled together for mapping. Asterisks indicate that the DNA ladder was originally run on the same gel as the samples in the picture, but was later pasted right next to the sample for easier comparison of band size. The strong N2 enrichment is bolded. The high N2 ratio at SNP E03H12 (IV: 1) is a result of the *hp102* (IV: 3.06) background.
3.2.6.1.2 hp420, hp467 and hp468

Since hp420, hp467, and hp468 are alleles of the same complementation group representing the same genetic locus (Table 3.3), only hp420 was used for chromosome mapping. A total of 20 CB lines were combined for snip-SNP mapping. The first round of rough mapping showed strong linkage to SNP F56C11 located at I: -19 (Fig. 3.13.1). A second round of mapping was performed using 6 SNP markers to refine the hp420 residing region. Two 100% N2 enrichments were observed at SNP Y71G12A (I: -12) and F58D5 (I: 13) (Fig. 3.13.2), which suggests that hp420 possibly locates near to the region between I: -12 to +13, or the original hp102; hp420 strain contained two suppressor mutations on chromosome I. The generation of more CB lines and further mapping with more SNP or genetic markers can distinguish between these two possibilities. Most of the snip-SNP mapping data presented here was generated by a former summer student Parminder Dulay, but repeated and confirmed by me.
Figure 3.13.1: The rough snip-SNP mapping data for *hp102; hp420*

20 CB lines were pooled together for mapping. Asterisks indicate that the DNA ladder was originally run on the same gel as the samples in the picture, but was later pasted right next the sample for easier comparison of band size. The strong N2 enrichment is bolded. The high N2 ratio at SNP E03H12 (IV: 1) is a result of the *hp102* (IV: 3.06) background.
Figure 3.13.2

Figure 3. 13. 2: The chromosome I snip-SNP mapping data for hp102; hp420

20 CB lines were pooled together for mapping. Asterisks indicate that the DNA ladder was originally run on the same gel as the samples in the picture, but was later pasted right next the sample for easier comparison of band size. The strong N2 enrichment is bolded.

3.2.6.1.3 hp421 and hp428

Linkage of hp421 and hp428 to chromosome X was observed during out-crossing and complementation tests. In C. elegans, sex is determined by the dosage of X chromosomes: males only contain a single X chromosome but hermaphrodites contain two [6]. All the gametes produced by the hermaphrodites are haplo-X, and later give rise to XX hermaprodite and XO male progeny upon mating with males that provide haplo-X sperm or sperm lacking the X chromosome. Therefore, any male cross progeny obtain their single X chromosome from the mother, and any recessive mutation located on the X chromosome will be revealed in the male cross progeny.

I first tentatively assigned hp421 and hp428 to the X chromosome because the male progeny from hp102; hp421 and hp102; hp428 mothers always displayed the
suppressed *hp102* locomotion during out-crosses or complementation tests. Another indirect line of evidence was the linkage of *hp421* and *hp428* to the SYD-2::GFP marker *hpIs3*, which is located on X chromosome.

To further confirm the X chromosome linkage of these two suppressors, two-factor genetic mapping was performed using the *lon-2* marker (Fig. 3.11). For both suppressors, all the F2 long coilers segregated 100% coilers in the F3 (Table 3.4), suggesting a strong linkage to X chromosome.

Table 3.4: Summary of Segregation of *hp421* and *hp428* to Chromosome X Marker *lon-2*

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<th>ratio lc</th>
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<td>%</td>
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<td>0</td>
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</table>

Is and Ic represent the ratios of long suppressors and long coilers described in Figure 3.11.

3.2.6.1.4 *hp430*

Due to its semi-dominant suppressing nature, the chromosome linkage of *hp430* was determined by identifying chromosome markers that always associate with the non-suppressed animals. Using this strategy, my genetic mapping showed the strongest linkage of *hp430* to chromosome III, as 100% of the F2 coilers were homozygous for a GFP marker *ncIs3* that was integrated on chromosome III (Table 3.5).
Table 3.5: Summary of Segregation of *hp430* to Markers on Chromosome I, II, III, V and X

|        | Ratio C+ |  | Ratio C- |  | Linkage to marker |
|--------|----------|  |----------|  |-------------------|
|        | Ratio    | % | Ratio    | % |                    |
| I: *ayls4* | 4/9      | 44% | 5/9      | 54% | X                 |
| II: *juls76* | 34/39    | 87% | 4/39     | 13% | X                 |
| III: *ncls3* | 17/17    | 100% | 0        | 0%  | √                 |
| V: *mls10* | 5/20     | 25% | 15/20    | 75% | X                 |
| X: *lon-2* | 8/16     | 50% | 8/16     | 50% | X                 |

C+ and C- represent ratios of F2 coilers segregated with the markers, and those that did not (Fig. 3.10).

For snip-SNP mapping, I recovered 22 coiler F2 lines from the N2/CB hybrids. The *hp430* residing region was approximated by determining the interval that were enriched for CB SNP markers (Fig. 3.8). The snip-SNP results did not show any high CB concentration for markers on I, II, IV, V and X (Fig. 3.14.1), but consistently showed almost 100% CB for markers located at III: -7 to 21, suggesting that *hp430* possibly locates near III: -7 or to the right of –7 (Fig. 3.14.2). The high N2 enrichment for SNP T12B5 at III: -25 may be due to random recombination events since SNP Y39A3CL locating only 6 map units to the right showed a drastic 50% decrease in CB ratio.
**Figure 3.14.1**

*nca-1(hp102): hp430*

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<td>70% CB</td>
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<td>70% CB</td>
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**Figure 3.14.1: The rough snip-SNP mapping data for *hp102; hp430***

22 CB lines were pooled together for mapping. A strong linkage to any marker is indicated by high CB enrichment.
3.2.6.1.5 *hp459*

The first round of snip-SNP rough mapping was performed using 8 *hp102; hp459* CB lines that were recovered from N2/CB hybrids, and linkage was examined with 3 SNP markers on each chromosome. It suggested a strong linkage of *hp459* to both chromosome I and X (Fig. 3.15.1). To resolve this ambiguity, more SNP markers were examined for chromosome I and X. The restrictive enzyme diagnosis showed a clear 100% N2 enrichment for SNP Y71G12A (I: -12) and a gradient diminishment of N2 concentration for markers further to the right (Fig. 3.15.2). Therefore, *hp459* is more likely located near I: -12 or to the left of –12.
Figure 3.15.1: The rough snip-SNP mapping data for *hp102; hp459*

8 CB lines were pooled together for mapping. The strong N2 enrichments are bolded. The high N2 ratio at SNP E03H12 (IV: 1) is a result of the *hp102* (IV: 3.06) background.
Figure 3.15.2: The chromosome III and X snip-SNP mapping data for hp102; hp459

8 CB lines were pooled together for mapping. The strong N2 enrichment is bolded.

3.2.6.1.6 hp465

During the generation of hp102; hp465 lines from N2/CB hybrids, I noticed that very few F2 coilers segregated suppressed progeny in F3. In fact I only managed to
recover three lines. This suggests that the CB genetic background is interfering with the 

\textit{hp465} suppressive effect on \textit{hp102}.

Mapping of \textit{hp465} was then carried out using genetic two-factor mapping. The 
mapping results showed that \textit{hp465} segregated independently with markers on I, II, V 
and X, but showed a strong linkage to III (Table 3.6).

Table 3.6: Summary of Segregation of \textit{hp465} to Markers on Chromosome I, II, III, 
V, and X

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\textbf{Ratio S+} and \textbf{S-} represent the ratios of F3 suppressors lines that are marker positive and marker negative (Fig. 3.9). \textbf{C+} and \textbf{C-} represent the ratios of F2 coilers segregated with the markers, and those that did not (Fig. 3.10). \textbf{Is} and \textbf{Ic} represent the ratios of long suppressors and long coilers described in Figure 3.11. Also refer to section 3.2.6 for more details.

To further confirm the linkage of \textit{hp465} to chromosome III, 3 lines of \textit{hp102}; 

\textit{hp465} recovered from the N2/CB hybrids were subjected to snip-SNP mapping with 6 
SNPs spanning across the length of Chromosome III. Although SNP Y39A3CL showed 
a high N2/CB ratio, interpretation of the relative fraction of N2 DNA over CB DNA 
should be taken with caution. The little amount of Y30A3CL PCR product may create a 
lower estimation of CB DNA because the smaller CB-specific PCR fragments (272bp)
may be present in unobservable amount after digestion. Yet, two other SNPs, F45H7 (III: -7) and F56C9 (III: -1), showed unambiguous high N2 enrichments (Fig. 3.16). Therefore, both genetic and snip-SNP mapping suggest that *hp465* is strongly linked to chromosome III, and possibly locates near the region III: -7 to -1.

**Figure 3.16**

*nca-1(hp102): hp465*

<table>
<thead>
<tr>
<th>markers</th>
<th>T12B5</th>
<th>Y39A3CL</th>
<th>Y71H2D</th>
<th>F45H7</th>
<th>F56C9</th>
<th>Y39A1A</th>
<th>Y41C4A</th>
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</thead>
<tbody>
<tr>
<td>N2 digest</td>
<td>206, 189</td>
<td>342, 78, 76</td>
<td>388, 105</td>
<td>239, 65, 27</td>
<td>466</td>
<td>365, 143, 30</td>
<td>339, 156</td>
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<tr>
<td>CB digest</td>
<td>395</td>
<td>272, 78, 76</td>
<td>473</td>
<td>195, 85, 43, 27</td>
<td>354, 132</td>
<td>497, 30</td>
<td>495</td>
</tr>
<tr>
<td>N2/CB ratio</td>
<td>10% N2</td>
<td>100% N2</td>
<td>50% N2</td>
<td>80% N2</td>
<td>80% N2</td>
<td>50% N2</td>
<td>10% N2</td>
</tr>
</tbody>
</table>

Figure 3.16: The chromosome III and X snip-SNP mapping data for *hp102; hp465*

3 CB lines were pooled together for mapping. The strong N2 enrichments were bolded. The question marker besides Y39A3CL N2/CB ratio indicates this may be a false positive result.

### 3.3 Summary of the Mapping and Characterization of *hp102* Suppressors

The following Table 3.7 summarizes all the characterization and mapping data presented in section 3.2. In conclusion, 31 *hp102* suppressors were isolated from 26,000 EMS mutagenized haploid genomes. 14 strong suppressors were selected for further
analysis and mapping. Among them, 3 intragenic loss of function revertants, 1 second site intragenic revertant or dominant \(hp102\)-linked extragenic suppressor, and a total of 10 extragenic suppressors were identified. The 10 extragenic suppressors, including 9 \(hp102\) non-linked recessive suppressors and 1 \(hp102\) non-linked semi-dominant suppressor were assigned to 8 different genetic loci.

Table 3. 3.7: Summary of Phenotypic analysis and Genetic location of Suppressors

<table>
<thead>
<tr>
<th>Classification by suppressive effect</th>
<th>Genetic Locus</th>
<th>Morphological phenotype</th>
<th>Behavioral Suppression</th>
<th>SYD-2::GFP suppression</th>
<th>Chromosome Linkage</th>
<th>Genetic location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exogenous recessive</td>
<td>(hp411)</td>
<td>EGL</td>
<td>Intermediate</td>
<td>&gt;90%</td>
<td>Positive</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>(hp420/)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>Positive</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>(hp467/)</td>
<td>-</td>
<td>Strong</td>
<td>50%</td>
<td>Positive</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td>(hp427)</td>
<td>Dauer constitutive</td>
<td>Strong in dauer, intermediate in escapers</td>
<td>100%</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(hp429)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>Positive</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td>(hp459)</td>
<td>Dpy</td>
<td>Intermediate</td>
<td>100%</td>
<td>Negative</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(hp465)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>Positive</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>Exogenous semi-dominant</td>
<td>(hp430)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>Positive</td>
<td>100%</td>
</tr>
<tr>
<td>Intrinsic loss of function revertant</td>
<td>(hp419)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
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<td></td>
<td>(hp429)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>(hp429)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2nd site intrinsic revertant or (hp102)-linked extragenic dominant</td>
<td>(hp429)</td>
<td>-</td>
<td>Strong</td>
<td>100%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Strong behavioral suppression level indicates that the double \(hp102\) suppressor mutant moves similar to wild-type animals. Intermediate behavioral suppression level means that the double mutant is relatively more loopy than wild-type animals.
Reference
3.4 Future Perspective

NCA channel, a unique member within the four-repeated-domains ion channel family that also comprises voltage-gated Na\(^+\) and Ca\(^{2+}\) channels, has been a great interest to many electrophysiologists and neurobiologists. Despite the many intriguing phenotypes observed in different organisms, still little is known about how this protein functions as a channel in the nervous system. Until recently, reports by Lu et al. and our group begin to elucidate the roles of NCA in forming the background resting Na\(^+\) currents in mouse neurons, and in propagation of electrical signals in *C. elegans* neuronal processes. Nevertheless, information regarding regulators that modulate NCA functions, and how NCA cooperates with other channels and proteins to control electrical signal propagation and synaptic activation, is still lacking. The *nca-1* genetic suppressor screen described here is advantageous for quick and effective investigation of the *nca-1* pathway. Components of this pathway include proteins that directly control NCA channel kinetic or expression, such as UNC-79 and UNC-80. More intriguingly, a cohort of other players participating in electrical signal propagation and synaptic activation may also lays in the *nca-1* pathway. The rationale is that the NCA-1 gain-of-function channel was implicated to increase excitability of neuronal membrane that led to hyper-activated synapses (Chapter 2), so genetic suppressors that are able to restore neurons to the normal state may possibly represent proteins or mechanisms that also regulate these processes. Furthermore, study of axonal membrane property in *C. elegans* has always been a challenge due to the small size and compact bundling of most motoneuron processes, which create difficulties to electrophysiological recording *in vivo*. A genetic approach
utilizing \( nca-1 \) gain-of-function mutant that possesses hyper-excitable axonal membrane, is a much more effective way to analyze membrane properties in \( C. \ elegans \) neurons.

The suppressors for the gain of function \( hp102 \) can function through distinct mechanisms, with the most obvious one being affecting \( nca-1 \) expression, channel stabilization or localization, as in the case of the previously described NCA-1 auxiliary subunits UNC-79 and UNC-80 (Chapter 2). A simple experiment to test this possibility is to examine the endogenous NCA-1 localization in the \( hp102 \) suppressor animals by immuno-staining. If any extragenic suppressor described here indeed affects the expression or localization of NCA-1, its regulation will be specific to NCA-1 but will not affect the NCA-2 channels. Presumably, mutations in regulators, like UNC-79 and UNC-80 that affect both NCA-1 and NCA-2 should confer the \( nca-1(gk9); nca-2(gk5) \)–specific fainter phenotype to the mutants. However, the \( hp102 \) extragenic suppressors described here do not display the fainter phenotype. Therefore if a NCA-1 specific regulator is absent in any of these animals, NCA-2 can compensate for the loss of NCA-1 to confer the normal NCA channel activity to the animals.

Another potential mechanism by which these suppressors may function through down regulating or mutating an NCA-1 auxiliary subunit that normally controls the channel kinetics and/or function. Down regulation or mutation in this subunit may lead to an inactivated NCA-1 channel or a block in the pore opening of the channel. After the suppressors are cloned, this hypothesis can be examined by co-expressing a single suppressor with the wild-type NCA-1 channels in heterologous cells to analyze the channel kinetics.
The NCA channels are implicated in propagating depolarization signals to the synapses, possibly by driving the resting membrane potential closer to the membrane threshold (Chapter 2). Within the context of this model, the physiological gain of function effect of \textit{hp102} is proposed to augment the membrane excitability. Therefore, alteration in other ion channels that normally regulate the resting membrane potential or the membrane threshold may also suppress the \textit{hp102} mutants by restoring the membrane excitability near the normal levels. Indeed, a candidate for these ion channels is NCA-2, which presumably is also a non-selective cation channel like NCA-1. Mutation in NCA-2 potentially alters the ion selectivity of this channel and results in a change of the resting membrane potential. Since both \textit{hp430} and \textit{hp465} map to a region where \textit{nca-2} also resides (~III: -2), injection of wild-type \textit{nca-2} into those mutants should be performed to examine whether they encode mutations in \textit{nca-2}.

The molecular cloning of two \textit{hp102} suppressors (\textit{unc-79} and \textit{unc-80}) has resulted in the identification of auxiliary subunits of the novel NCA channels. Molecular cloning of other suppressors may further reveal mechanisms through which NCA ion channels regulate the excitability of neurons. These suppressors are undoubtedly a valuable resource to promote understanding of the electrical membrane properties, as well as the propagation of electrical signals, in \textit{C. elegans} neurons.
Conclusion

Here, I reported the characterization of a cation channel-like protein, - NCA-1, which belongs to a conserved but largely uncharacterized voltage-gated Na\(^+\)/Ca\(^{2+}\) channel super family. The mouse homologue, NALCN, was reported recently to form the resting Na\(^+\) leak currents that underlie basal neuronal excitability. Nonetheless, our investigation of NCA-1 in regulating synaptic development and activity in *C. elegans* revealed additional function of this channel family in propagation of electrical signals. I also described how two previously unknown NCA auxiliary subunits, UNC-79 and UNC-80, are required for NCA channel localization and activity. These subunits, together with 31 other suppressors, were identified in an *nca-1* genetic screen utilizing the distinctive behavioral phenotype of the *nca-1* gain-of-function allele. I characterized 14 of these 31 suppressors, and assigned chromosome linkage to all 14 of them. The recovery of UNC-79 and UNC-80 demonstrated the effectiveness of this genetic suppressor screen in identifying novel NCA channel regulators. Subsequent cloning of the other *nca-1* suppressors will surely reveal more novel mechanisms that will shed light to how the NCA channels function to regulate electrical signal propagation and synaptic activity in *C. elegans*. 
**Appendix**

Table A 1: SNP Oligos Used for Mapping.

**DraI SNP primers, locations and band sizes.** In each pair of primers, the left primer is listed first; all primer sequences are given 5' to 3'. Interpolated genetic positions are from [9] release WS143.

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<th>Genetic Location</th>
<th>Physical Location</th>
<th>Clone</th>
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<th>CB485 6 digest</th>
<th>Primers</th>
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<td>377, 126, 72</td>
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<td>I, -6</td>
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<td>I, -1</td>
<td>4,594,01</td>
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<td>I, 13</td>
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CCAGAAAATCTGCACAGAAGG
II, 16 13,235,5 F15D4 64 500 368, 132
TTCCAGGTAATACACATACAACTCC pkP2116
AAACAACACAAAGTTCAAAAACCC
II, 22 14,132,4 K09E4 66 365, 119 484
CCACTGGCTATAAGCTTTTCTAGG CE2-215
TAAGGATTTTCAGGCTTTTAGEG
III, -25 939,698 T12B5 206, 189 395
TATCATCGAAATCCCCGGAAA (rs3139227)**
TTCGACGCGGAGTAGAATTG uCE3-637
TTCAAATTTTCCCTCTAAAACCC uCE3-735
III, -19 1,827,73 Y39A3CL 2 342, 78, 76 272, 78, 76, 70
TTGAAATTGGACCATTTTTGGAGG
TTTCTCGAACAATTTTCTTTGC (rs3139272)
III, -12 2,599,69 Y71H2B 9 368, 105 473
GAGGAACCAAATCTGGCGTA snp_Y71H2B
TGAAAACCTTGGAAAAATCGGTTG
III, -7 3,359,03 F45H7 3 239, 85, 27 196, 85, 43, 27
TTTCTTGCAAAACATTTTCTTTGC CE3-127
AAAATACATGTCTACACACCGC snp_F56C9
III, -1 7,320,10 F56C9 7 486 354, 132
TTTCTTATCAGTGACTTTTGGC
III, 4 10,652,4 Y39A1A 76 355, 142, 30 497, 30
AGCGTTAAAGTATCGGTTATTTCG snp_Y39A1
III, 12 11,656,1 Y41C4A 88 339, 156 495
ATCAAGTTTCTGATTGCTCTTTCC snp_Y41C4
AAAACGTGATTTTTCAATTTTGC
III, +21 13,715,6 W06F12 22 273, 137, 78 200, 137, 78, 73
AGCAGGCTCACCATCATCATA snp_Y41C4
GACATTACGTTAGAGGAGATGGA uCE3-1426
III, -24 795,461 F56B3 71 301, 128, 71 429, 71
AAAATGGGAAGCGTACCAAA pkP4071
AGAGCTGGAGAGCACGGATA
IV, 1 4,991,85 E03H12 1 376 300, 76
AAAATGGGAAGCGTACCAAA pkP4071
TGCTTGTACGTTTCCAAGA
IV, 14 16,085,0 Y105C5B 85 241, 108, 78, 48 319, 108, 48
TCGAAATTGTGTGTGTTTCTTTTGA pkP4099
TTCCAATTTTCTCGGTTTG
IV, -17 1,773,46 F36H9 4 307, 87, 79 386, 87
TTCCGAAAATTCGACTGT pkP5076
CGCGTTTTGGAGAATTTGTTT
V, -13 2,726,66 C24B9 2 288, 167 455
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CGGTAATAATATGCTTTTGGGG
V, -17 1,373,28 F36H9 4 307, 87, 79 386, 87

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Table A 2: Oligos Used to identify *hp102* and *nca-2*(gk5).

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Table A 3: Strain List

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<td>ZM 2027</td>
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<td>ZM 2029</td>
<td><em>hp102</em> IV, <em>hp402</em></td>
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<td><em>hp102</em> IV, <em>hp405</em></td>
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<td><em>hp102</em> IV, <em>hp408</em></td>
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Figure A4: Design of *hp102* genotyping oligos.

OZM1071 is the *nca-1* wild-type sequence detecting oligo and OZM1072 is the *hp102* specific oligo. Two additional single nucleotide alternations (in blue) are introduced into OZM1071 and 1072 to prevent non-specific binding. The idea is that OZM1071 does not anneal to *hp102* sequence as there are three single nucleotide differences, and vice versa for OZM1072. Together with the reverse primer OZM1073, OZM1071 or OZM1072 can only generate a PCR product of about 800bp in size, in the presence of the wild-type template or *hp102* template, respectively.

(A) A schematic illustration of forward primer OZM1071 and reverse primer OZM1073 annealing to wild-type sequence. The wild-type nucleotide corresponding to *hp102* missense mutation is highlighted in red.

(B) A schematic illustration of forward primer OZM1072 and reverse primer OZM1073 annealing to *hp102* sequence. The nucleotide corresponding to *hp102* missense mutation is highlighted in red.

Oligos designed by Dr. Wesley Hung