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Maintaining the balance: coordinating excitation and inhibition in a
simple motor circuit

A Dissertation Presented

By

Hilary A. Petrash

Submitted to the faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 6th, 2012

Program in Neuroscience

Maintaining the balance: coordinating excitation and inhibition in a simple motor
circuit
A Dissertation Presented
By
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Program in Neuroscience
August 6th, 2012

Dedication

I would like to thank my parents, Howard and Doreen, for their support in my graduate school endeavor. Your support in my education and goals has been the foundation of my life's success. I love you both. I would like to thank my sister, Merideth, who has provided comic relief during the long days of science. To the rest of my extended family, thank you for your everlasting encouragement.

To my husband, Don Petrash, you are a great joy in my life. My successes are sweeter when shared with you. You have given me so much patience and love during this journey and you never once doubted I would succeed. I love you and cannot thank you enough for believing in me. I look forward to the following chapters in our life together.

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To the faculty of the Neurobiology Department at UMass Medical School I would like to thank you for your feedback and guidance in my scientific development. Lastly, I would like to thank all the members, past and present, of the Francis, Alkema, and Bénard laboratories for creating an environment that encourages discussions and development as a scientist. I have made lasting connections with many of these people, creating a second family that I hope to carry with me into the future. Thank you all.

Abstract

The generation of complex behaviors often requires the coordinated activity of diverse sets of neural circuits in the brain. Activation of neuronal circuits drives behavior. Inappropriate signaling can contribute to cognitive disorders such as epilepsy, Parkinson's, and addiction (Nordberg et al., 1992; Quirk and McIntosh, 2006; Steinlein et al., 2012). The molecular mechanisms by which the activity of neural circuits is coordinated remain unclear. What are the molecules that regulate the timing of neural circuit activation and how is signaling between various neural circuits achieved? While much work has attempted to address these points, answers to these questions have been difficult to ascertain, in part owing to the diversity of molecules involved and the complex connectivity patterns of neural circuits in the mammalian brain.

My thesis work addresses these questions in the context of the nervous system of an invertebrate model organism, the nematode *Caenorhabditis elegans*. The locomotory circuit contains two subsets of motor neurons, excitatory and inhibitory, and the body wall muscle. Dyadic synapses from excitatory neurons coordinate the simultaneous activation of inhibitory neurons and body wall muscle. Here I identify a distinct class of ionotropic acetylcholine receptors (ACR-12R) that are expressed in GABA neurons and contain the subunit ACR-12. ACR-12R localize to synapses of GABA neurons and facilitate consistent body bend amplitude across consecutive body bends. ACR-12Rs regulate GABA neuron activity under conditions of elevated ACh release. This is

in contrast to the diffuse and modulatory role of ACR-12 containing receptors expressed in cholinergic motor neurons (ACR-2R) (Barbagallo et al., 2010; Jospin et al., 2009). Additionally, I show transgenic animals expressing ACR-12 with a mutation in the second transmembrane domain [ACR-12(V/S)] results in spontaneous contractions. Unexpectedly, I found expression of ACR-12(V/S) results in the preferential toxicity of GABA neurons. Interestingly loss of presynaptic GABA neurons did not have any obvious effects on inhibitory NMJ receptor localization. Together, my thesis work demonstrates the diverse roles of nicotinic acetylcholine receptors (nAChRs) in the regulation of neuronal activity that underlies nematode movement. The findings presented here are broadly applicable to the mechanisms of cholinergic signaling in vertebrate models.

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List of Abbreviations or Nomenclature

ACh- acetylcholine

AChR- acetylcholine receptor

ACR- acetylcholine receptor (*C. elegans* specific)

ADNFLE- autosomal dominant nocturnal frontal lobe epilepsy

ALS- Amyotrophic lateral sclerosis

CNS- central nervous system

ER- endoplasmic reticulum

GABA- γ -amino butyric acid

iAChRs- ionotropic acetylcholine receptors

ICL- intracellular loop

iGluRs- ionotropic glutamate receptors

MNs- motor neurons

NAc- nucleus accumbens

nAChR- nicotinic acetylcholine receptor

NMJ- neuromuscular junction

L-AChRs- levamisole sensitive AChR

LDT- laterodorsal tegmentum

LGIC- ligand gated ion channel

PSC- postsynaptic current

TM- transmembrane

VTA- ventral tegmentum area

- containing (ex. $\alpha 7^$ means $\alpha 7$ containing)

A motor neurons- backward movement

B motor neurons- forward movement

Preface

All work described in this thesis was performed at University of Massachusetts Medical School in the lab of Michael M. Francis.

*In chapter II, Alison Philbrook built the cholinergic motor neuron specific channelrhodopsin in the *acr-2* mutant and cholinergic channelrhodopsin in the *acr-12* mutant and helped perform the channelrhodopsin experiments. Marian Habrucak contributed to the electrophysiology. Michael M. Francis supported this work in several ways including discussions, designing the experiments, writing the paper, and contributing the electrophysiological experiments. I designed and performed the remaining experiments, analyzed the data, and co-wrote the paper.*

In chapter III, Michael M. Francis supported this work with discussions and designing the experiments. I designed and performed the experiments and analyzed the data.

*In appendix I, I contributed cell specific rescue strains of *acr-12* for identifying neurons sufficient for *ufls25* paralysis, strain building, and coexpression of cholinergic and GABAergic neurons. The remaining authors were responsible for the rest of the work.*

Chapter 1:

Introduction

The nervous system is a complex and highly interconnected network of specialized cells called neurons. Groups of connecting neurons form circuits that modify behavior based on activity. These neurons are interconnected by two mechanisms: the electrical synapse, or gap junction, and a unique chemical junction called a synapse. Electrical coupling or gap junctions are a direct low resistance mechanism for transmitting electrical activity from one neuron to a neighboring neuron or other cell type. The gap junction is comprised of a hydrophilic intercellular channel, with one subunit of the channel contributed from each of the connecting cells (Flores et al., 2012; Pereda et al., 2012). In contrast, synapses utilize chemical transmission. An electrical signal from the presynapse triggers the release of a neurotransmitter into the synaptic cleft. After diffusing across the cleft, the neurotransmitter binds to receptors located on the postsynaptic neuron. Fast synaptic transmission is mediated by ionotropic receptors, also known as ligand-gated ion channels (LGICs). Binding of the chemical neurotransmitter triggers a conformational change in the receptor, opening a pore through the membrane that allows the flow of ions into or out of the cell, and converting the chemical signal into an electrical response (Holtmaat and Svoboda, 2009). Chemical signaling through a synapse translates into one of two simple outcomes: (1) the activation of the postsynaptic neuron and continued neuronal transmission or (2) the inhibition of the postsynaptic neuron that reduces downstream signaling. Signaling at synapses occurs on a time scale of milliseconds, allowing for rapid activation or inhibition of sequential neurons

within a circuit to appropriately drive circuit activity and ultimately determine behavior.

The generation of complex behaviors often requires the coordinated activity of diverse sets of neural circuits in the brain. The molecular mechanisms by which the activity of neural circuits is coordinated remain unclear. What are the molecules that regulate the timing of neural circuit activation and how is signaling between various neural circuits achieved? These are important questions, as disruption of coordinated circuit activity can have severe consequences (e.g., epilepsy). While scientific research has attempted to address these points, answers to these questions have been difficult to ascertain, in part owing to the diversity of molecules involved and the complex connectivity patterns of neural circuits in the mammalian brain. My thesis work addresses these questions in the context of the nervous system of an invertebrate model organism, the nematode *Caenorhabditis elegans*. The genetic tools available and well-established connectivity of this organism make it ideal for this type of study. As the major neurotransmitter in *C. elegans* is acetylcholine, a detailed description of cholinergic signaling and the molecules involved follows.

Ligand-gated ion channels and synaptic transmission

The ligand-gated ion channel (LGIC) super family includes receptors for the neurotransmitters glutamate, acetylcholine (ACh), γ amino butyric acid (GABA), glycine, and a single serotonin receptor (Corringer et al.,

2012; Millar and Harkness, 2008; Rosenberg et al., 2002). In mammals, LGIC family members can be broadly classified as either excitatory or inhibitory based on their permeability to either anions or cations. Ionotropic glutamate receptors (iGluR) and nicotinic acetylcholine receptors (nAChR) act as cation-selective channels and are excitatory, while GABA and glycine receptors act as anion-selective channels and are generally inhibitory in the adult nervous system. Over 40 distinct genes that encode subunits of LGICs have been identified in mammals (Baenziger and Corringer, 2011). These receptors are pentameric, with the exception of glutamate receptors which form tetramers, and are expressed in varying subunit combinations throughout the nervous system (Millar and Gotti, 2009). Pharmaceutical therapies designed to activate (agonists) or inhibit (antagonists) specific subunit combinations have been developed as potential treatments for diseases such as epilepsy and Parkinson's (Brooks-Kayal et al., 1998; Davies, 1995; Mulley et al., 2005; Quik and McIntosh, 2006). In Parkinson's disease stimulation of presynaptic nAChRs can increase dopamine release in the substantia nigra (Quik and McIntosh, 2006). Current treatments for Parkinson's disease synthetically compensate for a loss of dopamine using L-DOPA, a precursor of dopamine. However, as previously mentioned in Parkinson's models it is possible to achieve similar increases in dopamine levels by activating presynaptic nAChRs (Quik and McIntosh, 2006). nAChRs were the first members of the LGIC family to be isolated and studied *in*

vivo and much of our understanding of how LGICs function has come from studies of nAChRs (Unwin, 2005).

Structure, function and localization of nicotinic acetylcholine receptors

nAChRs were first isolated from the electric organ of the marine *Torpedo* ray and the *Electrophorus* eel (Changeux et al., 1970; Popot and Changeux, 1984). In vertebrates, there are 17 nAChR subunits: α 1-10, β 1-4, δ , γ , and ϵ (Lindstrom, 2003; Millar and Harkness, 2008). In general, nAChRs fall into two classes, receptors comprised of identical subunits (homomeric) or different subunits types (heteromeric) (Unwin, 2005). The heteromeric nAChR of the NMJ contains the subunits α 1₂ β 1 δ ϵ ; although, during development the subunit composition is slightly different (a γ subunit is substituted for an ϵ subunit) (Millar and Harkness, 2008). In the nervous system the subunit composition of nAChRs has more potential for variability, enabling functional diversity across specific subunit combinations. Receptors formed from the α 7 subunit (homologous to the ACR-16 subunit in *C. elegans*) are generally homomeric while the other subunits typically contribute to heteromeric receptors, the precise subunit combinations of which vary across neuronal classes.

Each subunit of the pentameric nAChR contains four membrane spanning domains (Lindstrom, 2003). Activation of the channel occurs when ACh attaches to a binding site on the external face of the receptor. Initially, the extracellular N-terminal region of α subunits was thought to be solely responsible for ligand

binding. However, more recent studies have shown that the ACh binding sites exist at the interface of adjacent subunits (Corringer et al., 2000; Luetje and Patrick, 1991). Proper formation of the ACh binding site is dependent on the presence of two adjacent cysteines located in the N-terminal region of α subunits (Figure 1-1) (Doyle, 2004; Lindstrom, 2003). Subunits without these adjacent cysteines are classified as non- α : β , δ , γ , and ϵ subunits. Each subunit has a large intracellular loop between the third and fourth transmembrane domains. The sequence of this intracellular loop does not appear to be highly conserved across other nAChR subunits of the same or different species (Doyle, 2004; Lindstrom, 2003). It is likely that sequence elements within the large intracellular loop are important for localization of the receptor.

The second transmembrane domain of each subunit contributes to the ion channel pore of the receptor (Imoto et al., 1988; Revah et al., 1991). Mutations in specific residues of the pore region and the area directly surrounding the pore can dramatically alter kinetics, prolonging channel open time (Figure 1-2). For example, mutations at amino acids 234, 237 and 258 convert a channel from cationic to anionic. Residues 234 and 237 are located just outside the second transmembrane domain in the extracellular space while residue 258 is located at the intracellular end of the second transmembrane domain (Revah et al., 1991). Others have identified residues 236 and 251 as contributing to ion selectivity (Corringer et al., 1999; Galzi et al., 1992; Imoto et al., 1988). Reconstitution studies have shown the kinetics of $\alpha 7$ homomers could be dramatically altered by

mutating a residue in the second transmembrane domain. Mutating residue 247 from a non-polar leucine to a polar serine has the most pronounced effect on mean channel open time (Revah et al., 1991). Hypersensitive nAChRs, or gain of function receptors, have since been used to examine the role of cholinergic signaling in the CNS. Using an $\alpha 4$ gain of function knock in mouse model, researchers were able to identify interactions of nAChRs and dopamine D_2 receptors in regulating cholinergic interneuron activity (Zhao-Shea et al., 2010). Mutations in the pore lining domain of $\alpha 6$ ($\alpha 6^{L9'S}$) nAChR subunit identified $\alpha 6\alpha 4\beta 2^*$ containing receptors as a key target for disorders associated with reduced dopamine release. The researchers were able to specifically link the behavioral deficits of $\alpha 6^{L9'S}$ animals to $\alpha 6\alpha 4\beta 2^*$ containing receptors in dopamine releasing neurons *in vivo* (Drenan et al., 2010). Additional work with gain of function $\alpha 6$ subunits has demonstrated a role for $\beta 3$ subunits in promoting the function of $\alpha 6$ containing receptors (Dash and Lukas, 2012).

Role of cholinergic signaling in the nervous system

In the CNS nAChRs are predominantly localized at presynaptic terminals, while postsynaptic nAChRs act in the peripheral nervous system to mediate transmission in the sympathetic ganglia (Rassadi et al., 2005). Cholinergic signaling coordinates neuronal activity in several manners. First, nAChRs located at presynaptic sites are important in modulating the release of neurotransmitter. For example, activation of presynaptic nAChRs on dopamine neurons increases

neurotransmitter release (Salminen et al., 2004). Second, activation of pre-synaptic nAChRs can trigger neurotransmitter release in the absence of a propagated signal; bypassing upstream signaling (Vizi and Lendvai, 1999). Third, cholinergic signaling is linked to changes in gene expression (Chang and Berg, 2001; Hu et al., 2002). Researchers have demonstrated that selective activation of nAChRs leads to the phosphorylation of CREB, a transcriptional coactivator, and changes in expression of the immediate early gene *c-Fos*. Fourth, nAChRs can directly activate neurons by localizing to postsynaptic sites. It is possible subunit diversity in nAChRs provides an opportunity for diverse roles for cholinergic signaling.

Altered cholinergic signaling is implicated in the pathophysiology of epilepsy, Parkinson's disease, Alzheimer's disease, and in addiction to nicotine (Dineley, 2007; Gotti and Clementi, 2004; Quik and McIntosh, 2006; Steinlein et al., 2012). Activation of nAChRs in the reward pathways of the brain facilitates nicotine addiction. There are at least three distinct classes of nAChRs involved in nicotine addiction $\alpha 4^*$, $\alpha 6^*$, and $\alpha 7$. Specifically, nicotine binds to presynaptic $\alpha 7$ receptors on glutamatergic neurons in the laterodorsal tegmentum (LTD) influencing neurotransmitter release onto dopaminergic neurons in the ventral tegmentum area (VTA). The dopaminergic neurons express two classes of nAChRs, $\alpha 4\beta 2^*$ - and $\beta 2^*$ - containing receptors, which modulate neurotransmitter release and increase in burst firing (Dani et al., 2001; Mansvelder and McGehee, 2002). Inhibitory GABA neurons also make synaptic contacts with dopaminergic

neurons of the VTA and express $\alpha 4\beta 2$ containing receptors (Dani and Harris, 2005). However, distinct nAChRs extend beyond the reward pathways of the brain. $\alpha 3\beta 4$ receptors were recently identified as directly mediating excitation of mitral cells from olfactory inputs. Activation of mitral cells filters the inputs from olfactory neurons (D'Souza and Vijayaraghavan, 2012). Additionally, impaired cholinergic signaling in Alzheimer's disease results in decreases in cognition (McGehee et al., 1995). Mutations in $\alpha 4$ and $\beta 2$ subunits have been linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Raggenbass and Bertrand, 2002). There are age and dementia associated changes in high and low affinity nAChRs in various regions of the brain (Nordberg et al., 1992). During development, loss of $\alpha 7$ containing receptors dramatically reduces the number of glutamatergic synapses in the adult brain. The reduction in glutamatergic synapses is associated with the behavioral changes in $\alpha 7$ knockout mice including attention deficits and impaired spatial discrimination (Lozada et al., 2012).

C. elegans as a model for studying nervous system function

C. elegans is an ideal invertebrate model for addressing fundamental principles of nervous system function. There are a host of cell specific drivers and the cell fate of all embryonic cells is known (Hobert, 2005). The genome of the organism has been fully sequenced and there is a myriad of genetic tools available, as well as readily available deletion mutants for many genes of

interest. Under laboratory conditions, *C. elegans* strains are maintained with a readily available bacterial food source so the animals do not have to move in order to feed. In addition, *C. elegans* are hermaphroditic so coordinated motor behaviors are not required for reproduction. These features allow one to propagate strains carrying severe mutations in genes required for nervous system function that would be lethal in most systems, and study the functional roles of these genes. In addition, the transparent body enables *in vivo* visualization of fluorescent markers in the intact animal. More recently developed techniques have made *C. elegans* amenable to electrophysiological experiments, which can be performed both *in vivo* and *in vitro* (Christensen et al., 2002; Francis et al., 2003; Richmond and Jorgensen, 1999). In addition, tools such as cell specific expression of light-activated ion channels, Channelrhodopsin-2 or Halorhodopsin, allow for the targeted activation of specific neuronal circuits *in vivo* and subsequent analysis using behavior and electrophysiology (Nagel et al., 2005; Zhang et al., 2007a). Qualities of the animal's characteristic sinusoidal movement can be measured under a variety of conditions. These tools allow researchers to tease apart how a single neuronal circuit contributes to a specific behavior.

The *C. elegans* nervous system uses many of the same neurotransmitters as mammals including acetylcholine, GABA, and glutamate. The genome is about 97 megabases and contains over 19,000 genes (Consortium, 1998). This genome has a high density of receptors important for synaptic function. In

addition, there are on the order of 90 neurotransmitter-gated ion channels of which there are more than 30 potential nAChR subunits (Bargmann, 1998; Jones et al., 2007; Jones and Sattelle, 2004). *C. elegans* possess the largest known family of AChRs (Mongan et al., 2002). These subunits fall into six different groups named for the first subunit identified in each group: *unc-29*, *unc-38*, *acr-8*, *acr-16*, *deg-3*, and orphan LGIC (Figure 1-3). Specifically, the *acr-8* and *deg-3* groups are unique to *C. elegans* (Jones and Sattelle, 2004). The *acr-8* group has a unique feature compared to the other groups with a basic (histidine) residue in place of a highly conserve acidic (glutamate) residue in the second transmembrane domain of the receptor. This alteration can alter the ion selectivity from cation to anion (Corringer et al., 1999; Imoto et al., 1988). The *C. elegans* nAChR subunits are classified as either α (ligand binding) or non- α (non-ligand binding) by the absence or presence of adjacent cysteines. Similar to mammalian subunits, *C. elegans* nAChRs show differential expression patterns and are not limited to a specific neuron class (Barbagallo et al., 2010; Cinar et al., 2005; Drenan et al., 2008; Fox et al., 2005; Jospin et al., 2009; Nordberg et al., 1992; Salminen et al., 2004).

Genes involved in the trafficking of AChRs include *Calnexin*, *BiP*, *unc-50*, *ric-3*, and *unc-74* (Boulin et al., 2008; Eimer et al., 2007; Forsayeth et al., 1992; Gelman et al., 1995). Both Calnexin and Bip are ER chaperones involved in the synthesis of nAChRs and are shed in the maturation process (Forsayeth et al., 1992; Gelman et al., 1995). Unfolded or misassembled subunits are retained in

the ER and degraded by ER-associated degradation (Christianson and Green, 2004). UNC-50 is specifically required for the trafficking of levamisole sensitive AChRs (L-AChRs). These proteins are involved in subunit specific trafficking of nAChRs, while the function of *unc-74* remains unknown (Eimer et al., 2007). RIC-3 is a protein important for the trafficking of nAChR, but not other LGIC, to the cell membrane and is localized to the endoplasmic reticulum (ER) (Halevi et al., 2002). This is similar to the human form of *ric-3* (*hric3*); however, the human form has further evolved to inhibit certain subunit combinations of nAChR and extend its regulation to other LGIC (Halevi et al., 2003). Taken together this evidence suggests factors involved in trafficking and localization are key regulators in preventing aberrant receptors from reaching the cell membrane. In addition, there are functional properties associated with distinct subunit compositions such as agonist sensitivity and calcium permeability (Changeux et al., 1970; Deneris et al., 1991; Drisdell and Green, 2000; Lipovsek et al., 2012). The *C. elegans* locomotory circuit is enriched for nAChRs making this circuit of alternating movement ideal for examining coordinated activity.

Generating alternating movements in C. elegans

Utilizing alternating movements as a model for identifying mechanisms that coordinate excitation and inhibition is common (Eklof-Ljunggren et al., 2012; Gabriel et al., 2011; Grillner and Jessell, 2009; Kupfermann and Weiss, 2001; Masino and Fetcho, 2005). One of the primary advantages to this model is a

completely defined network of neurons including the anatomical connections of all chemical and electrical synapses (White et al., 1986). The locomotory circuit is comprised of command interneurons, excitatory, and inhibitory motor neurons. Sensory information is relayed through command interneurons that synapse onto excitatory motor neurons. There are 5 sets of command interneurons: AVA, AVB, AVE, AVD, and PVC (White et al., 1986). AVA, AVE, and AVD regulate backwards movement through electrical and chemical synapses with the A motor neurons (backwards movement). AVB and PVC regulate forward movement via gap junctions and chemical synapses with B type motor neurons (forward movement) (Chalfie et al., 1985; Wicks et al., 1996; Zheng et al., 1999). Recent work has suggested gap junctions between AVA and A motor neurons function to bias the motor circuit towards forward versus backward movement. In addition electrical coupling may be modulatory in forward movement or there are alternative signaling mechanisms maintaining the communication between AVB and B type motor neurons in forward movement (Kawano et al., 2011). In turn, the excitatory motor neurons (A and B), form dyadic synapses with body wall muscle (BWM) initiating contraction while simultaneously activating inhibitory motor neurons. Activation of the inhibitory motor neurons initiates relaxation of the contralateral BWM. The simultaneous signaling of excitation and inhibition on opposing muscle groups is thought to be important for generation of the animal's characteristic sinusoidal wave form (Figure 1-4) (White et al., 1986).

In vertebrates there are fundamental differences in NMJ and CNS synapses. First NMJ synapses are far more stable than those of the CNS which have a higher degree of plasticity. During development, the half life of receptors at the NMJ are lengthened unlike the receptors of the CNS. Second, the formation of CNS synapses occurs on the time scale of hours whereas the time scale at the NMJ is weeks (Holtmaat and Svoboda, 2009). Third, proteins associated with NMJ formation (i.e. Agrin, MuSK, and LRP4) are not required for CNS synapse formation. Lastly, in stark contrast to the CNS synapses the NMJ is regulated primarily by one neurotransmitter and one class of nAChR (Lindstrom, 2003). In the CNS neurons are polyinnervated, receiving both inhibitory and excitatory neurotransmitters (Kim et al., 2008; Zhang et al., 2008). The *C. elegans* NMJ is similar in that it is also polyinnervated, expresses different classes of receptors, and forms through mechanisms independent of Agrin, MuSK, and LRP4 (Francis et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005) . Therefore, identifying molecular mechanisms involved in regulating the activity of the *C. elegans* locomotory circuit will be relevant for linking findings to mechanisms in the mammalian CNS.

Within the *C. elegans* locomotory circuit are previously characterized nAChRs. One receptor is a homomeric (contains one subunit type) receptor containing five Acetylcholine Receptor-16 (ACR-16) (Francis et al., 2005; Touroutine et al., 2005) subunits, and a second receptor is heteromeric comprised of UNC-29, LEV-1, LEV-8, UNC-38, and UNC-63 subunits (Culetto et

al., 2004; Fleming et al., 1997; Unwin, 2005). The UNC-29, UNC-38, and UNC-63 subunits are all essential for the function of the UNC-29 receptor. LEV-1 and LEV-8 are considered non-essential subunits. Similar to mammalian CNS synapses the inhibitory NMJ is mediated by a GABA receptor (UNC-49). This is in contrast to the mammalian NMJ which is regulated solely by nAChR. Formation of the *C. elegans* NMJ requires proteins such as CAM-1, a receptor tyrosine kinase, responsible for the proper trafficking of ACR-16 receptors (Fleming et al., 1997). CAM-1 is orthologous to the mammalian ROR1 and 2 (Francis et al., 2005; Jensen et al., 2012). Furthermore, LEV-10 is a single pass transmembrane protein with several CUB domains which has been implicated in receptor clustering at the *C. elegans* NMJ (Gally et al., 2004). The mechanisms introduced here generate the characteristic alternating movement of *C. elegans*. The locomotory circuit of *C. elegans* is one of many models of alternating movement being utilized for understanding nervous system function.

Alternating movement models in characterizing mechanisms of neuronal activity

Walking vertebrates or swimming fish are classic demonstrations of the precision required to maintain the balance of excitatory and inhibitory signaling in movement circuits. Alternating movement can be seen in a host of organisms ranging from humans to *C. elegans*. Opposing muscle groups coordinate their activation and relaxation in order to properly time the shortening of one group of muscles while simultaneously lengthening the contralateral muscle group.

Alternating movements are finely modulated to achieve a variety of speeds, direction, amplitude, and frequency. Similar to the neuronal circuits in the brain, alternating movements require molecular mechanisms to facilitate adaptation to stimuli with a high degree of precision while maintaining a coordinated balance of excitation and inhibition. The molecular mechanisms that regulate activity in alternating movements are easier to study than those of the mammalian central nervous system. This is due in part to the well defined circuitry providing a more controlled environment for identifying and characterizing mechanisms of neuronal activity. This model circuit is an ideal alternative to the complex neuronal circuitry of the mammalian brain.

Motor circuits have been characterized in several model systems including lamprey, zebrafish, mouse, and nematode (Goulding, 2009). The lamprey and zebrafish model systems have a unique feature of maintaining the complexity of highly connected interneuron populations while limiting the density of neurons (Eklof-Ljunggren et al., 2012; Masino and Fetcho, 2005). Comparing interneuron populations across species makes circuit analysis in vertebrate models more broadly relevant (Fetcho et al., 2008). Vertebrates allow us to address questions of how specific subsets of interneurons or motor neurons affect alternating movements and what kinds of molecular mechanisms are involved. However, invertebrate models present their own advantages for probing locomotory circuits. First, the circuitry is vastly simplified compared to vertebrate models (Altun, 2008; Jing and Weiss, 2002; Kupfermann and Weiss, 2001). Second, as

previously mentioned the connectivity of all neurons and synapses are identified and well characterized (Serrano et al., 2007; White et al., 1986). Third, the generation time of some invertebrate model organisms profoundly shorter than the generation time of vertebrate models. For example, the generation time of *C. elegans* is only 4 days. This allows researchers to perform large forward genetic screens and isolate mutants much faster than that of alternative model systems. Ultimately, the *C. elegans* locomotory circuit is ideally suited for linking mechanisms of neuronal activity to those of the mammalian CNS.

Dissertation Overview

My thesis work will focus on the chemical synapse, specifically how receptors regulate neuronal activity and directly influence behavior. I use the locomotory circuit of *C. elegans* as a model for identifying mechanisms that coordinate neuronal activity. The molecular mechanisms are critical to the balance of excitation and inhibition. The elucidation of these mechanisms will provide insights into mammalian nervous system function. In order to understand and study nervous system function it is important to be familiar with the types of molecular mechanisms that affect neuronal activity. Here in the first chapter I have introduced the structure and function of the nervous system including both chemical synapses and electrical coupling. I have also provided background on mechanisms that regulate neuronal activity and specifically the role of cholinergic

signaling in regulating neuronal activity. I have ended with establishing *C. elegans* as an informative model for studying nervous system function.

Chapter II identifies a mechanism important in directly coordinating excitatory and inhibitory signaling in the *C. elegans* locomotory circuit. Here, I provide evidence that a single nAChR subunit, ACR-12, forms two distinct classes of nAChRs. These distinct classes have differential localization, composition, and roles in regulating behavior. Our previous work with *acr-2* identified a heteromeric nAChR expressed in cholinergic motor neurons (ACR-2R) (Barbagallo et al., 2010). The ACR-2R has diffuse localization in the dendrites and a modulatory role in regulating neuron activity (Barbagallo et al., 2010; Jospin et al., 2009). Loss of ACR-2Rs results in subtle changes in behavior (see Appendix). In contrast the recently identified GABA neuron-specific ACR-12 (ACR-12R) localizes to discrete sites within the dendrites. Colocalization of these discrete sites opposed to a presynaptic reporter suggests ACR-12R is synaptic. Since two of the subunits from the ACR-2R are not expressed in GABA neurons, this observation suggests that GABA neuron-specific ACR-12 containing receptors are distinct from the heteromeric cholinergic receptor. In addition, the ACR-12R is implicated in regulating the consistency of the *C. elegans* waveform and mediating the activity of GABA neurons under conditions of elevated acetylcholine (ACh) release. Taken together, these results suggest diverse roles for receptor subtypes are important for coordinating neuronal activity.

Chapter III identifies the contributions of neuronal subsets to the generation of movement. Data in this chapter demonstrates that expression of a gain of function ACR-12 [ACR-12(V/S)] receptor has dramatic effects on behavior including highly reduced movement and spontaneous convulsions. In addition, expression of this transgene leads to the degeneration of the GABAergic nervous system, but not the cholinergic nervous system. These degenerative effects can be observed at early life stages and preliminary evidence suggests this degeneration is cell autonomous. Interestingly, the NMJ inhibitory receptor, UNC-49, remains unaffected by the degeneration of presynaptic GABA neurons. Our results indicate ACR-12(V/S) will be a useful tool in identifying how subsets of neurons contribute to the overall behavior of an animal. Lastly, expression of ACR-12(V/S) may prove a useful genetic tool for identifying proteins associated with receptor localization, subunit identification, and mechanisms of degeneration.

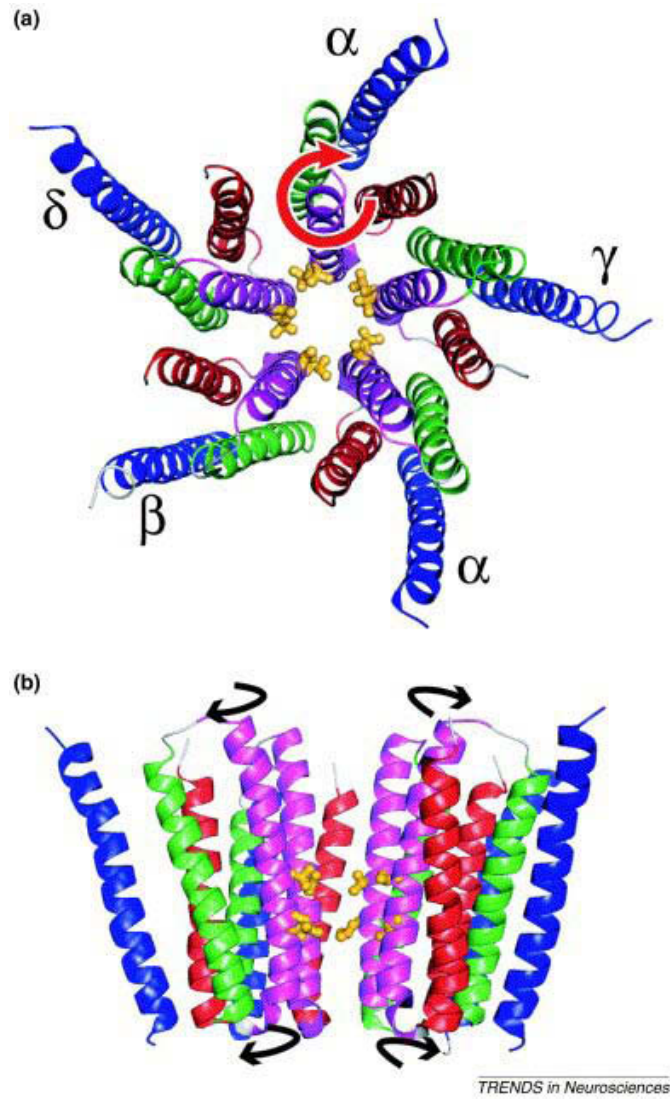


Figure 1-1

Figure 1-1 reprint from (Doyle, 2004). The structure of a nAChR.

(a) An extracellular perspective of the transmembrane helical topology in its closed state. **(b)**. A side view (γ subunit removed). For all subunits (2α , β , and δ), transmembrane (TM) TM1 (red), TM2 (magenta), TM3 (green) and TM4 (blue). Residues of the hydrophobic gate are modeled in yellow ball-and-stick. TM2 rotation direction is indicated by the arrows.

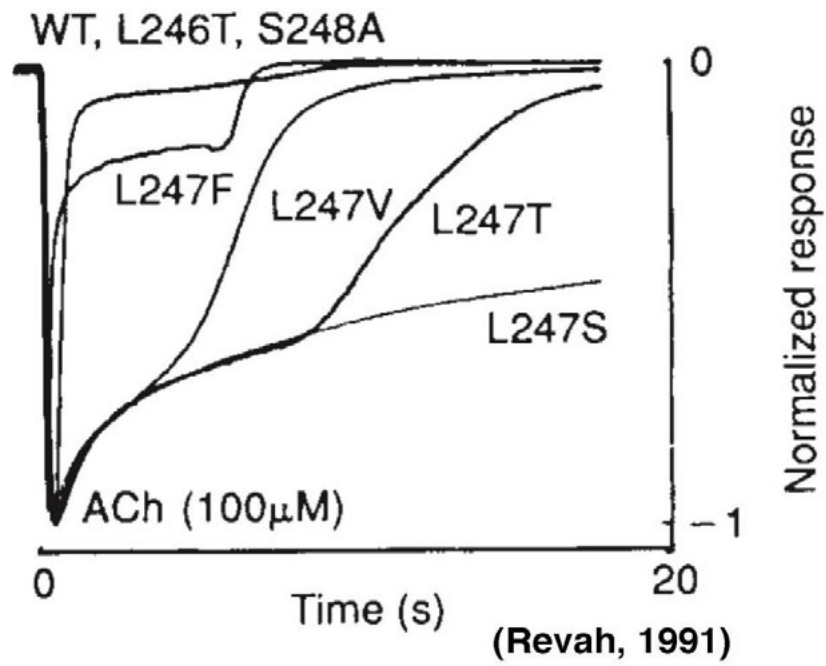


Figure 1-2

Figure 1-2 reprint from (Revah et al., 1991): “*b*, Functional properties of $\alpha 7$ receptor mutants. *b*, Normalized currents evoked by 100 μ M ACh in *Xenopus* oocytes expressing $\alpha 7$ wild type (WT) and $\alpha 7$ L247F, L247V, L247T, L247S, L246T, S248A receptors (where letters represent amino acids at positions given by the numbers) are superimposed. The normalized responses to ACh from WT, L246T and S248A were indistinguishable.”

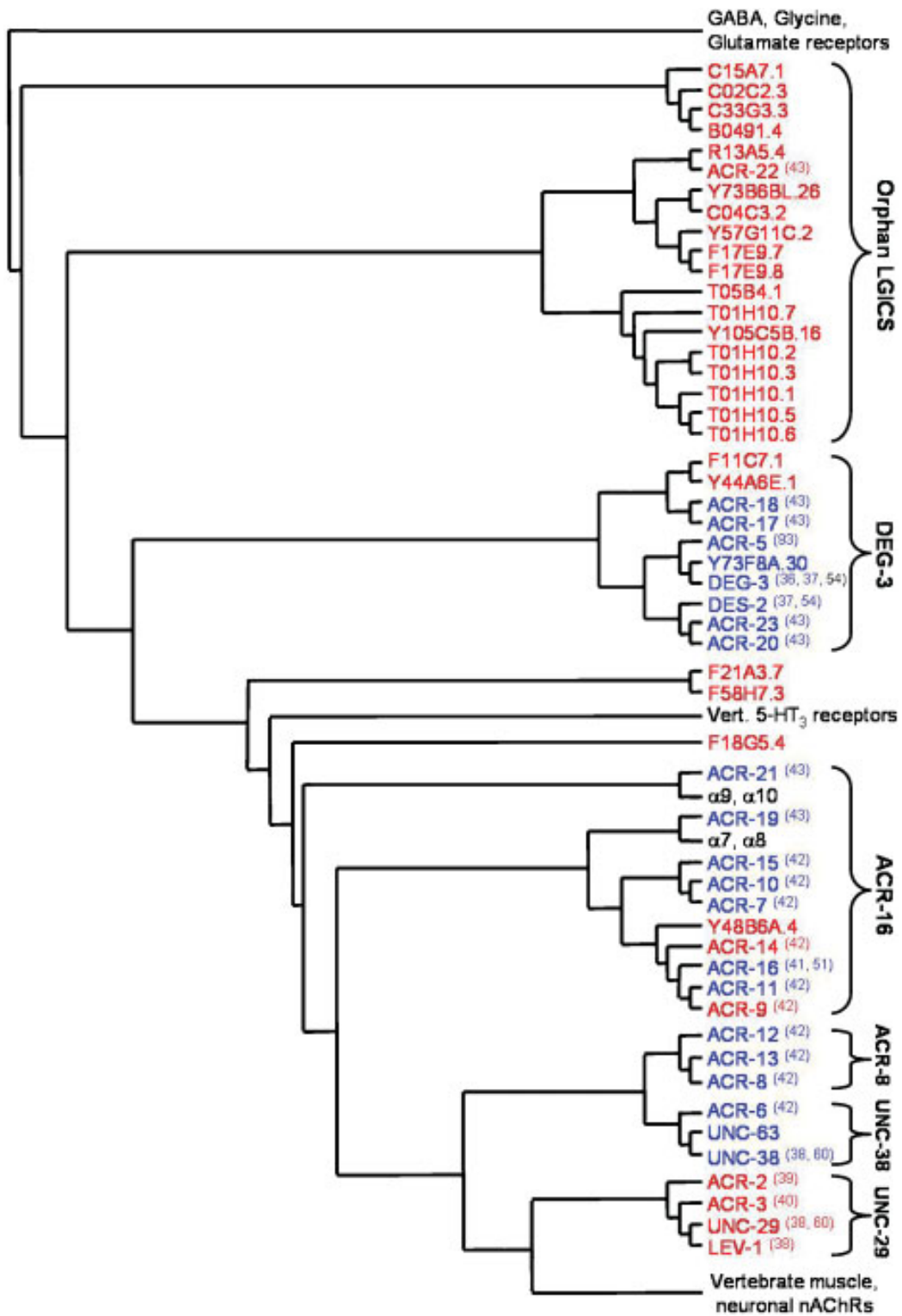


Figure 1-3

Figure 1-3 (Jones and Sattelle, 2004): Phylogeny of *C. elegans* nAChR subunits with vertebrate AChR subunits and other members of the LGIC superfamily. *C. elegans* α subunits (blue), non- α subunits (red), and GABA, glycine, glutamate, 5-HT3 and vertebrate nAChR subunits (black). Cosmid numbers are used for *C. elegans* sequences lacking gene names.

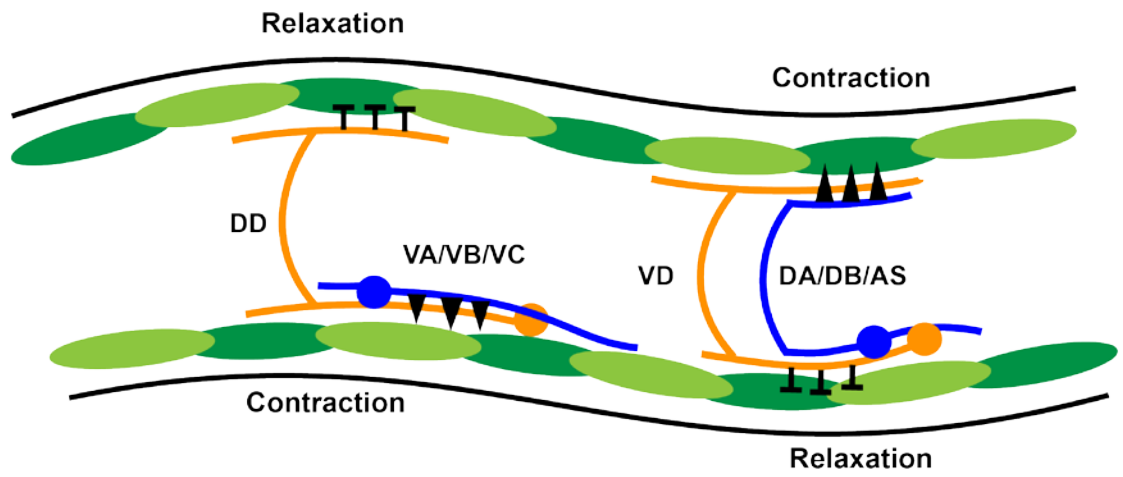


Figure 1-4

Figure 1-4 (Altun, 2011). Schematic of *C. elegans* locomotory circuit. Dark and light green shading indicates body wall muscles. In blue are the ACh MNs (VA, VB, DA, DB, AS, VC) and orange indicates GABA MNs (VD and DD). Axons and dendrites of ventrally directed cholinergic motor neurons (ventral A and ventral B classes) extend through the ventral nerve cord. Dorsally directed cholinergic motor neuron cell bodies (dorsal A class and dorsal B classes) are located adjacent to the ventral nerve cord and extend axons into the dorsal nerve cord, where they make dyadic synaptic contacts with body wall musculature and dendrites of ventrally directed GABA motor neurons (ventral D class).

Chapter II:

**Excitation and inhibition are coordinated
through multiple cholinergic (nicotinic)
signaling pathways in *C. elegans* motor
control**

ABSTRACT

Heterogeneity in the composition of neurotransmitter receptors is thought to provide functional diversity that may be important in patterning neural activity and shaping behavior (Dani and Bertrand, 2007; Sassoe-Pognetto, 2011). However, this idea has remained difficult to evaluate directly due to the complexity of neuronal connectivity patterns and uncertainty about the molecular composition of specific receptor types *in vivo*. Here we dissect how molecular diversity across receptor types contributes to the coordinated activity of excitatory and inhibitory motor neurons in the nematode *Caenorhabditis elegans*. We show that excitatory and inhibitory motor neurons express distinct populations of ionotropic acetylcholine receptors (iAChR) requiring the ACR-12 subunit. The activity level of excitatory motor neurons is influenced through activation of nonsynaptic iAChRs (Barbagallo et al., 2010; Jospin et al., 2009). In contrast, synaptic coupling of excitatory and inhibitory motor neurons is achieved through a second population of iAChRs specifically localized at postsynaptic sites on inhibitory motor neurons. Loss of ACR-12 iAChRs from inhibitory motor neurons leads to reduced synaptic drive, decreased inhibitory neuromuscular signaling and variability in the sinusoidal motor pattern. Our results provide new insights into mechanisms that establish appropriately balanced excitation and inhibition in the generation of a rhythmic motor behavior, and reveal functionally diverse roles for iAChR mediated signaling in this process.

INTRODUCTION

The pattern of activity in neuronal circuits is the basis for behavior, addition, learning and memory (Dani and Harris, 2005; Gotti and Clementi, 2004; Steinlein et al., 2012). Identifying the mechanisms responsible for this patterning will provide insights into how behaviors are readily adaptable with a constantly changing environment. The ligand-gated ion channel family is broadly expressed throughout the nervous system and is a major contributor to neuronal patterning (Doyle, 2004; Lindstrom, 2003). While many of the receptor families and individual subunits have been identified, the functional consequences for heterogeneity among individual receptor families remain unclear. Functional diversity remains difficult to evaluate directly due to the complexity of neuronal connectivity patterns and the uncertainty about the molecular composition of specific receptor types *in vivo*. Differences in subunit composition have been linked to changes in localization and kinetic properties of the receptors (Glykys and Mody, 2007; Imoto et al., 1988; Revah et al., 1991; Teichert et al., 2012; Zhang et al., 2007b). These changes can have profound effects on neuronal activity. Therefore, understanding how molecular diversity among individual receptor families gives rise to functional diversity and alters neuronal activity will be important for understanding normal brain physiology and the pathophysiology of disorders that affect post-synaptic receptors.

In *C. elegans*, excitatory cholinergic motor neurons (ACh MNs) make synaptic contacts onto both muscle cells and GABA MNs that, in turn, make

inhibitory synaptic contacts onto opposing musculature (Figure 2-3A) (White et al., 1986). Proper function of this circuit produces temporally coordinated and balanced excitatory and inhibitory signals that pattern movement. While the anatomical connectivity of this circuit has been well characterized, the signaling mechanisms that underlie coordinated motor neuron activity are not well understood. Expression studies have revealed that many of the 29 ionotropic acetylcholine receptor subunits encoded by the *C. elegans* genome are expressed in motor neurons, suggesting that cholinergic signaling may play a prominent role (Cinar et al., 2005; Fox et al., 2005; Jones et al., 2007; Rand, 2007). For example, cholinergic motor neurons express a class of heteromeric acetylcholine-gated ion channel complexes known as ACR-2R (Barbagallo et al., 2010; Jospin et al., 2009). ACR-2Rs are ionotropic receptors of the nicotinic acetylcholine receptor superfamily composed of five distinct subunits (ACR-2, ACR-3, UNC-38, UNC-63 and ACR-12), each of which is essential for function in heterologous expression studies. Loss of ACR-2R leads to relatively subtle changes in behavior; however, gain-of-function *acr-2* mutations (*acr-2(gf)*) have profound consequences including hyperactivation and, in extreme cases, death of ACh MNs (Barbagallo et al., 2010; Jospin et al., 2009). In a forward genetic screen to identify mutations that suppressed the toxic effects of *acr-2(gf)* (ACR-2^{L9S}), we isolated several loss-of-function alleles of a partnering acetylcholine receptor subunit, *acr-12* (Barbagallo et al., 2010). Specific expression of *acr-12* in

ACh MNs of animals coexpressing *acr-2(gf)* restored toxicity in *acr-12* mutants, demonstrating a cell autonomous role for ACR-12 in these neurons.

Here we identify and characterize a second class of ACR-12 containing receptors in GABA motor neurons (ACR-12R*). Animals that have lost GABA ACR-12Rs in GABA motor neurons exhibit inconsistency in body bend amplitude across consecutive body bends. Subcellular localization of GABA neurons ACR-12R show discrete sites of green fluorescent protein (GFP) fluorescence. Using electrophysiology we show loss of *acr-12* results in significant loss of inhibitory post synaptic events in the muscle that can be rescued with cell specific expression of ACR-12 in GABA neurons. Lastly, we demonstrate under conditions of elevated ACh release that *acr-12* mutants can suppress the paralyzing effects of channelrhodopsin in upstream cholinergic motor neurons. Taken together our results provide valuable insights into the mechanisms of cholinergic signaling in nervous system function.

MATERIALS AND METHODS

***C. elegans* strains**

C. elegans strains were grown under standard laboratory conditions at 22°C. All strains are derivatives of the N2 Bristol strain (wild type). Transgenic strains were obtained by microinjection to achieve germline transformation. Multiple independent extragenic lines were obtained for each transgenic strain and data presented are from a single representative transgenic line unless noted otherwise. In all cases, *lin-15(n765ts)* mutants were injected with the *lin-15*

rescuing plasmid (pL15Ek; 30ng/ul) and one or more of the following plasmids: pPRB5 [*Punc-47::mCherry*], pPRB6 [*Pacr-2::mCherry*], pPRB47 [*Pacr-2::mCherry-RAB-3*], pPRB53 [*ACR-12-GFP_{ICL}*], pPRB77 [*Pacr-2::ACR-12-GFP_{ICL}*], pHP7 [*Punc-47::ACR-12-GFP_{ICL}*], pAG21 [*ACR-12-GFP_{C-term}*]. Stably integrated lines were generated by X-ray integration and outcrossed at least four times to wild type. The following strains were used in this study: IZ914: *acr-12(ok367)X*; IZ853: *acr-12(ok367)X;ufls57[ACR-12-GFP_{ICL}]*; IZ984: *acr-12(ok367);ufls78[Pacr-2::ACR-12::GFP_{ICL}]*; IZ556: *acr-12(ok367);ufls92[Punc-47::ACR-12-GFP_{ICL}]*; IZ941: *unc-49(e382)III;acr-12(ok367)X*; IZ33: *unc-29(x29);acr-16(ok789)*; IZ514: *unc-29(x29);acr-16(ok789);acr-12(ok367)*; IZ519: *acr-12(ok367);ufls23[Pacr-2::ChR2-GFP]*; IZ801: *ufls23[Pacr-2::ChR2-GFP]*; IZ629: *ufls38[ACR-12-GFP_{C-term}];ufls34[Punc-47::mCherry]*; IZ651: *ufls38[ACR-12-GFP_{C-term}];ufls43[Pacr-2::mCherry]*; IZ632: *ufls26[Punc-4::mCherry];ufls38[ACR-12-GFP_{C-term}]*; IZ557: *ufls63[Pacr-2::mCherry-RAB-3];ufls92[Punc-47::ACR-12-GFP_{ICL}]*; CB382: *unc-49(e382)III*; IZ712: *acr-12(uf77)*; RB1559: *acr-2(ok1887)II*; IZ805: *ufls53[Punc-17::ChR2-mCherry]*; IZ1096: *acr-2(ok1887);ufls23[Pacr-2::ChR2-GFP]*; and IZ1095: *acr-12(ok367);ufls53[Punc17::ChR2-mCherry]*.

Molecular Biology

The ACR-12-GFP_{ICL} transgene (pPRB53) was generated by cloning the GFP coding sequence in-frame into the sequence of an *acr-12* genomic fragment (-

1514 to +4799 bp relative to the translational start site) encoding the intracellular loop (ICL) between transmembrane domains (TM)3 and TM4. ACR-12-GFP_{ICL} was localized in neuronal processes and expression of this construct was sufficient for rescue of *acr-12* mutants. pAG21 [ACR-12-GFP_{C-term}] was provided by Alexander Gottschalk and was used in cell identifications (Fig. 1). ACR-12-GFP_{C-term} fluorescence was largely confined to cell bodies. *Punc-47::ACR-12-GFP_{ICL}* (pHP7) and *Punc-47::ACR-12-GFP_{ICL}* (pPRB77) were generated by subcloning a 4.9 kb NruI/BglI fragment containing GFP from pPRB53 into constructs encoding the *acr-12* cDNA under control of the appropriate promoters (pBB25 and pHP3 respectively). *Punc-47::mCherry* (pPRB5) was generated by subcloning mCherry coding sequence downstream of a 1.3 kb promoter for the *unc-47* gene. *Pacr-2::mCherry* (pPRB6) was generated by subcloning a 3.3 kb promoter for the *acr-2* gene upstream of the mCherry coding sequence.

Microscopy

Confocal microscopy was performed using a Zeiss Axioskop 2 microscope system and LSM Pascal 5 imaging software (Zeiss). All images used animals 24 h after the L4 stage and were processed using ImageJ software. For all synapse quantification, a region of the dorsal cord directly across from the vulva was imaged. Synapses were quantified within a 50 μ M region of interest by thresholding fluorescence intensity and using the “analyze particles” module in ImageJ software. The percentage of overlap with pre-synaptic RAB-3 was

determined by quantifying the number of mCherry::RAB-3 puncta which were positioned either directly apposing or overlapping with ACR-12::GFP fluorescent signal.

Behavioral assays

All behavioral analyses were performed using staged populations of young adult animals (24 h following L4) at room temperature (22°C-24°C). Strains were scored in parallel, with the researcher blinded to the genotype during both experiment and analysis. For aldicarb assays, staged populations of adult animals (≥ 10) were transferred to NGM plates containing 1 mM aldicarb (ChemService), and movement was assessed every 15 min for 2 h. Movies and still images for behavioral analyses were obtained using an Olympus SZ61 upright microscope equipped with a FireWire camera (Imaging Source). For body bend measurements, amplitudes were determined using ImageJ software. The distance between the deepest point of the bend and a line tangent to the tip of the head and the body was measured. This measurement was then normalized to the length of each animal and either averaged across 3 consecutive body bends to generate a value for average body bend amplitude (Figure 2-6D) or 10 individual consecutive body bends (Figure 2-6B and C). For gross movement, individual worms were transferred to 100mm (large) unseeded plates. The number of body bends and spontaneous reversals were counted manually over 1-minute intervals for 3-minutes and averaged (Figure 2-6E). For optogenetic

experiments, animals were maintained on OP50 plates containing retinal and 450-490 nm light (2.5 mW/mm^2) was delivered using an Exfo X-cite series 120 light source and appropriate GFP excitation filters. On the day of the assay staged young adult animals were moved to a fresh plate for an equilibration period of 1 minute prior to filming for 40 s. After 10 s of filming the animals were exposed to blue light for 15 s and then filmed for an additional 10 s after blue light exposure. Responses were categorized using the following criteria: no movement impairment, tail bend $\leq 90^\circ$, tail bend $> 90^\circ$, or arrested movement.

Electrophysiology

Endogenous postsynaptic currents were recorded from body wall muscles as previously described (Francis et al., 2005). The extracellular solution consisted of 150 mM NaCl, 5 mM KCl, 4 mM MgCl_2 , 1 mM CaCl_2 , 15 mM HEPES, and 10 mM glucose (pH 7.4, osmolarity adjusted with 20 mM sucrose). The intracellular fluid (ICF) consisted of 115 mM K-gluconate, 25 mM KCl, 0.1 mM CaCl_2 , 50 mM HEPES, 5 mM Mg-ATP, 0.5 mM Na-GTP, 0.5 mM cGMP, 0.5 mM cAMP, and 1 mM BAPTA (pH 7.4, osmolarity adjusted with 10 mM sucrose). For some experiments measuring GABA-mediated currents, the intracellular solution contained 115 mM KCl and 25 mM K-gluconate. At least 60-90 s of continuous data were used in the analysis. Data analysis was performed using Igor Pro (WaveMetrics, Inc.) and Mini Analysis (Synaptosoft, Inc.) software. Statistical comparisons were made by Student's t test using GraphPad Prism.

RESULTS

Loss of ACR-12 leads to heightened excitability

acr-12 encodes a 573 amino acid iAChR alpha subunit (Figure 1) with significant homology to the ACR-8 like group of *C. elegans* subunits and more distant homology to mammalian neuronal heteromeric alpha subunits such as alpha6 (Jones et al., 2007). To investigate the contribution of ACR-12 receptor complexes to the excitability of neurons in the motor circuit, we evaluated independent strains carrying putative loss of function alleles of *acr-12* during acute exposure to the cholinesterase inhibitor aldicarb. Treatment of *C. elegans* with aldicarb leads to elevated levels of synaptic ACh and causes paralysis over time due to prolonged muscle contraction (Nguyen et al., 1995). The contractile state of muscles reflects the summed activity of excitatory ACh and inhibitory GABA synaptic inputs; genetic mutations that alter this balance will shift the time course over which aldicarb leads to paralysis. For example, manipulations that reduce or eliminate inhibitory GABA signaling cause enhanced muscle activation and more rapid paralysis (Loria et al., 2004; Vashlishan et al., 2008). *acr-12(uf77)* was isolated as a suppressor of *acr-2(gf)* and is a missense mutation affecting a splice acceptor site that results in premature termination prior to transmembrane domain 4 (aa 387) (Barbagallo et al., 2010). *acr-12(ok367)* is a deletion mutation that eliminates 1368 bp of chromosomal DNA, including sequence encoding transmembrane domains 1-3 (Figure 2-1). Strains carrying either of these alleles were viable and gross connectivity of the nervous system

was normal (not shown), indicating that ACR-12 is not required for normal development of the nervous system. We found the *acr-12(uf77)* and *acr-12(ok367)* mutations both accelerated the time course of aldicarb-induced paralysis (Figure 2-2A), suggesting loss of ACR-12 led to enhanced excitability. In contrast, previous work showed removal of ACR-2Rs slightly delayed paralysis in response to aldicarb treatment (Barbagallo et al., 2010; Jospin et al., 2009). The differential effects of aldicarb across *acr-2* and *acr-12* mutants suggested ACR-12 has additional roles in the nervous system independent of ACR-2. To better define potential functions for ACR-12 signaling, we constructed double mutants lacking both *acr-12* and *unc-49*. The *unc-49* gene encodes an essential subunit of ionotropic GABA_A-like receptors at inhibitory neuromuscular synapses and mutations in *unc-49* cause aldicarb hypersensitivity (Figure 2-2A) (Bamber et al., 1999; Vashlishan et al., 2008). *unc-49;acr-12* double mutants exhibited no additional hypersensitivity beyond that of *unc-49* single mutants, suggesting that *acr-12* and *unc-49* may act in the same pathway.

acr-12 is reported to have broad expression in the nervous system (Gottschalk et al., 2005) but a precise description of the motor neuron classes that express *acr-12* has remained unclear. To address this issue, we generated transgenic strains expressing ACR-12 tagged with GFP (green fluorescent protein) at the C-terminus together with a red fluorescent reporter (*Pacr-2::mCherry*) labeling ACh motor neurons, and examined the cellular distribution of ACR-12-GFP in the ventral nerve cord. ACR-12-GFP fluorescence was

broadly visible in motor neuron cell bodies and partially overlapped with *Pacr-2::mCherry* expression, confirming that *acr-12* is expressed in cholinergic motor neurons (Figure 2-2D). In addition we coexpressed ACR-12-GFP with a red fluorescent protein reporter for the A class and VC motor neurons (*Punc-4::mCherry*) and observed a partial overlap of ACR-12-GFP that did not include the VC neurons (Figure 2-2E). Fluorescence was also clearly visible in additional motor neurons along the ventral cord. To confirm the identity of these neurons, we imaged animals co-expressing ACR-12-GFP with a reporter labeling GABA neurons (*Punc-47::mCherry*) and observed overlapping fluorescent signals (Figure 2-2F). Our results indicate ACR-12 contributes to iAChRs expressed by both ACh and GABA motor neurons. Furthermore, the limited expression of some ACR-2R constituents to ACh MNs strongly suggests the iAChR populations expressed by ACh and GABA motor neurons are molecularly distinct (Barbagallo et al., 2010; Jospin et al., 2009).

To dissect functional roles for ACR-12 across motor neuron classes, we specifically expressed ACR-12 in either ACh or GABA motor neurons of *acr-12* mutants and tested the responses of these animals to aldicarb treatment (Figure 2-2B). Specific expression of *acr-12* in ACh MNs did not alter the aldicarb hypersensitivity and paralysis of *acr-12* mutants. In contrast, specific expression of *acr-12* in GABA neurons restored wild type sensitivity to aldicarb. Our results suggest that postsynaptic ACR-12 receptor complexes regulate GABA motor

neuron activity and inhibitory neuromuscular signaling under conditions when ACh levels are elevated.

iAChRs requiring ACR-12 have distinct patterns of localization and functional roles across motor neuron classes

We examined the subcellular distribution of ACR-12 in motor neurons by expressing a rescuing ACR-12-GFP fusion protein under control of the native promoter (Figure 3B). ACR-12-GFP expression was clearly visible in motor neuron processes of both the ventral and dorsal nerve cords of adult animals, and exhibited two contrasting patterns of fluorescence. In the ventral nerve cord we observed regions of punctate and diffuse localization while, in the dorsal cord, we observed punctate fluorescence almost exclusively (Figure 2-3B). In contrast, specific expression of ACR-12-GFP in ACh MNs produced only diffuse fluorescence in the ventral nerve cord and no detectable fluorescent signal in the dorsal nerve cord (Figure 2-3C). Axons and dendrites of ventrally directed cholinergic motor neurons (ventral A and B classes) extend through the ventral nerve cord. Cholinergic motor neurons innervating dorsal musculature (dorsal A and B classes) extend axons into the dorsal nerve cord, where they make dyadic synaptic contacts with body wall musculature and dendrites of ventrally directed GABA motor neurons (ventral D class) (Figure 2-3A) (White et al., 1986). The diffuse localization of ACR-12 in ACh MNs is consistent with our previous finding that ACR-12 contributes to heteromeric receptor complexes (e.g. ACR-2R)

without obvious postsynaptic localization in these neurons (Barbagallo et al., 2010). Specific expression of ACR-12-GFP in GABA MNs produced solely punctate fluorescence (Figure 2-3D). Many of these ACR-12-GFP puncta were located immediately opposed to regions of cholinergic motor neuron axons in which the synaptic vesicle marker mCherry-RAB-3 was concentrated (Figure 2-4A-C) (Klassen and Shen, 2007; Mahoney et al., 2006). Our results provide evidence that ACR-12 complexes in GABA MNs are clustered in receptor fields located opposite presynaptic specializations of ACh MNs.

GABA motor neuron expression of ACR-12 is required for normal levels of inhibitory synaptic activity

To directly test the requirement for ACR-12 in regulating motor neuron activity, we used standard electrophysiology recording techniques to measure the frequency of synaptic events at the NMJ *in vivo* (Figure 2-5) (Francis and Maricq, 2006). We employed two independent strategies to distinguish between GABA and ACh post synaptic currents (PSCs): 1) Recordings were made under ionic conditions where GABA and ACh mediated events were separable based on the directionality of the currents (see Experimental Procedures for details) and 2) We recorded from *unc-29;acr-16* double mutants that are devoid of functional iAChRs on body wall muscles and lack excitatory neurotransmission at the NMJ [18, 19]. In both cases, we observed a significant reduction in the rate of endogenous GABA mediated inhibitory PSCs in mutants lacking ACR-12

receptors (53%, $p < 0.001$ and 69 %, $p < 0.01$ respectively) (Figure 2-5). We did not observe a significant difference in the amplitude of endogenous inhibitory PSCs (wild type, 26 ± 1.8 pA; *acr-12*, 23.4 ± 2 pA, $p > 0.05$, Figure 5). To distinguish whether the reduction in inhibitory PSC frequency arose as a consequence of loss of ACR-12 from GABA MNs or arose due to reduced excitation levels in presynaptic ACh MNs, we recorded from animals that expressed *acr-12* specifically in ACh or GABA MNs (Figure 2-5). The reduced IPSC rate was partially rescued by expression of *acr-12* in GABA motor neurons, while expression in ACh MNs was not sufficient for rescue. Additionally, the *acr-12* deletion mutation did not significantly reduce the rate or amplitude of endogenous excitatory PSCs (wild type, 28.6 ± 1.8 pA; *acr-12*, 26.8 ± 1.3 pA, $p > 0.05$), although we did note a trend toward reduced frequency (33%, $p = 0.07$) (Figure 2-5E). Thus, the reduction in endogenous IPSC frequency cannot be explained by decreased excitation of presynaptic ACh MNs. Our results indicate that independent populations of ACR-12 receptors act cell autonomously in ACh and GABA MNs to regulate their activity.

ACR-12 mediated signaling onto motor neurons regulates locomotion

To determine how ACR-12 mediated signaling onto motor neurons contributes to normal locomotory behavior, we tracked animals during exploratory movements on agar plates. The sinusoidal locomotory wave appeared more erratic in *acr-12* animals (Figure 2-6A). To examine this in more

detail, we monitored consecutive body bends during extended periods of uninterrupted forward movement (Figure 2-6A and B). Wild type animals displayed remarkable consistency in their movement with only a few irregularities in their sinusoidal tracks. In the absence of ACR-12 receptors (*ok367* and *uf77*), the motor pattern was less stable, showing significantly increased variability in body bend amplitude from one body bend to the next (Figure 2-6C, $p < 0.001$). Furthermore, *acr-2(ok1887)* mutants do not have an irregular waveform suggesting loss of GABA-specific ACR-12 iAChRs is required for coordination of a consistent waveform. Animals expressing a genomic ACR-12-GFP reduced variability to wild type levels (Figure 2-6C green). In addition to an inconsistent waveform we observe a modest reduction in the average amplitude of body bends (~14% for both *uf77* and *ok367*, $p < 0.05$ and $p < 0.01$ respectively) (Figure 2-6D). Body bend amplitude was reversed by ACR-12 expression using the native promoter. In addition, specific expression of ACR-12 in GABA motor neurons, but not cholinergic motor neurons reversed the reduction in body bend amplitude. Moreover, *acr-2(ok1887)* mutants did not exhibit changes in body bend amplitude. These disruptions in body bend variability and amplitude likely contribute to the overall decrease *acr-12(ok367)* mutant movement (Figure 2-6E). In addition to changes in the number of body bends over time *acr-12(ok367)* mutants also exhibit an increase in the frequency of spontaneous reversals (Figure 2-6E). This increase may indicate important roles for ACR-12 iAChRs in the interneurons. Here we highlight how ACR-12 receptors under laboratory

conditions mediate subtle attributes of locomotory patterning leading to changes in the overall number as well as the quality of those body bends.

ACR-12 receptors mediate GABA neuron activity under elevated conditions of ACh release.

To investigate how synaptic activation of ACR-12 receptors on GABA MNs contributes to motor control under elevated levels of ACh release, we expressed the light-activated ion channel channelrhodopsin (ChR2) in A and B class ACh MNs (*ufIs23*) that make direct synaptic contacts onto GABA MNs. Wild type animals expressing ChR2 exhibited a behavioral response to light stimulation that was largely invariant from animal to animal (Figure 2-7A). Immediately following the onset of stimulation, animals typically slowed or paused forward movement and initiated muscle contraction in the posterior portion of the body. More prolonged exposure (>~5 s) led to a complete cessation of forward movement for the duration of the stimulation period and a characteristic bending of the tail (Figure 2-7B). Normal forward movement could be reinitiated immediately following optogenetic stimulation and these effects were completely dependent on the presence of exogenous retinal (Figure 2-7B and C). Head movements were unimpaired throughout the stimulation period, presumably because the *acr-2* promoter does not provide for efficient expression in motor neurons that synapse onto head and neck muscles (Barbagallo et al., 2010; Jospin et al., 2009). These observations suggest that photostimulation of wild

type animals impaired movement through synchronous depolarization of ACh MNs and simultaneous activation of both body wall muscles and GABA MNs along the length of the body. We found that loss of ACR-12 receptors ameliorated this effect (Figure 2-7C and D, ACh MN only (*Pacr-2*), $p < 0.0001$). *acr-12* mutants expressing ChR2 were able to execute sinusoidal body bends and maintain coordinated movement even during prolonged periods of light exposure (15-20 s). Thus, loss of ACR-12 receptors diminishes the intensity of the behavioral response to synchronous ACh MN depolarization. As ACR-12 receptors are located on GABA MNs that are directly postsynaptic to the photoresponsive ACh MNs, our results suggest that synaptic drive onto GABA MNs is reduced in *acr-12* mutants and that synchronous excitation of GABA MNs is critical for the behavioral response to optical stimulation. Furthermore, *acr-2(ok1887)* mutants showed no suppression of the ChR2 effect (Figure 2-7C and D). When we express ChR2 with a cell specific promoter for all cholinergic motor neurons (*ufls53*) we see dramatic changes in behavior that *acr-12(ok367)* mutants do not suppress (Figure 2-7C and E). However, simultaneously activating all cholinergic motor neurons may activate alternative neuronal circuits that feed back into GABAergic neurons through other signaling mechanisms such as neuropeptides or G-protein coupled receptors.

DISCUSSION

Characterizing functional diversity among receptor families will provide insights into signaling mechanisms that maintain a well balanced circuit.

Conditions that arise from a shift in the balance of excitation and inhibition have profound effects on behavior. For example changes in GABA_A receptor composition have been linked to patients with epilepsy, a seizure disorder. Specifically, $\alpha_1\beta_3\gamma_2$ receptors are replaced by $\alpha_4\beta_3\gamma_2$ receptors in the extrasynaptic regions of the thalamus and hippocampus (Lagrange et al., 2007). Additionally, this alteration in subunit composition augments the kinetics of the receptor which correlates with susceptibility to seizures (Lagrange et al., 2007). Furthermore, in some cases of temporal lobe epilepsy there is a down regulation of the δ subunit and an increase in α_4 and γ_2 subunits in peri-synaptic regions of the dentate gyrus. These altered receptors negatively affect tonic inhibitory currents (Zhang et al., 2007b).

We show here that subunit composition also affects receptor function in the *C. elegans* motor circuit. At least 2 classes of iAChRs requiring ACR-12 participate in shaping activity of this circuit. While receptors on cholinergic motor neurons appear diffusely distributed and may act to maintain appropriate levels of activation through an extrasynaptic mechanism (Barbagallo et al., 2010; Jospin et al., 2009), receptors located on GABA neurons appear positioned at synapses to mediate direct coupling of excitatory and inhibitory motor neurons. At least two subunits of iAChRs present on ACh MNs (ACR-2R*) are not expressed in GABA MNs (Jospin et al., 2009), implying that distinct receptor subunit combinations are expressed by each motor neuron class. The two localization patterns we observed may arise from differences in subunit

composition of the receptor or differential expression of localization factors unique to each motor neuron subtype. Further characterization of the iAChR constituents in GABA MNs will be key to addressing this important question, as well as understanding potential effects of differing biophysical properties across receptor types. Interestingly, iAChRs play similarly diverse roles in the mammalian nervous system where they are primarily postsynaptic at autonomic synapses and primarily presynaptic or extrasynaptic in the brain (Dani and Bertrand, 2007).

Our experiments also reveal the presence of additional signaling pathways onto GABA MNs that function independently of ACR-12 receptors. GABA IPSCs continued at a reduced rate in *acr-12* mutants, indicating GABA MN activity persists even when synaptic drive from ACh MNs is reduced. Consistent with this result, the absence of ACR-12 receptors led to variability in the locomotory wave but did not disrupt gross movement or lead to phenotypes similar to that of mutants in which GABA signaling is absent (McIntire et al., 1993). Finally, under conditions of elevated ACh release, as is the case with the photostimulation experiments, we showed the behavioral response to synchronous depolarization of ACh MNs was altered in *acr-12* mutants. This suggests that the reduced synaptic coupling of ACh and GABA MNs in *acr-12* mutants allows GABA MNs to function independently of synchronously active ACh MNs during photostimulation. Under these conditions, the timing of GABA MN activation may

be coordinated either by timing mechanisms intrinsic to GABA MNs or through activity of other neurons not expressing ChR2 that lie presynaptic to GABA MNs.

C. elegans locomotion is a dynamic and remarkably complex behavior. While the anatomical connectivity of the *C. elegans* nervous system was described over 30 years ago, we have only recently been able to reconcile this static picture of nervous system wiring with measures of functional connectivity (White et al., 1986). One of the barriers to progress in understanding the neural circuit mechanisms responsible for the generation of movement has been an inability to independently manipulate ACh and GABA MNs. For example, mutations that alter the activity of ACh MNs also impact synaptically coupled GABA MNs. The identification of signaling molecules, like ACR-12, responsible for mediating functional connectivity between these neuron classes will be essential in our continuing efforts to understand how coordinated excitation and inhibition in this circuit gives rise to a stable motor pattern. The presence of diverse signaling mechanisms in the control of motor neuron excitability may provide for behavioral flexibility in the face of changing levels of neuronal activity or environmental conditions. Support for this idea comes from the recent characterization of behaviorally important G-protein coupled receptor populations present on motor neurons, indicating additional levels of fine control (Chase et al., 2004; Dittman and Kaplan, 2008; Schultheis et al., 2011). Identification of the comprehensive set of relevant molecular signals and their physiological roles will enable detailed analyses of how functional differences across signaling

molecules contribute to signal integration by neurons, neural circuit function and motor control.

A

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1   MLYKKRLQMF SFNWALILLV AVGGTNAFKI KRTAKDLESQ LYEDLLFDYN
51  LIRIIDVDEK NQVLTTNLWL EMKWINDAKLT WTPEKYGGLK TLHIPSDFIW
101 TPDVLVYNNA AGDPDITILT DALVTFEGNV YWQPPAIYKS F*CPIDVTWFP
151 YDSQKCEMKF GTWYTGGRV DLKQLPQEEV VTITKDDNDV EFMQQGMDLS
201 FFYRSAEWDL LSLTSEHRSV LYASCCGPEK YVDITYYFGL RRKTLFFTCN
251 LILPCFLISI LTFVFYLSL HKITFSISIL VTLTVFFLVL IDLMEPTSLV
301 IPMFGRYLIT TMILVALSTV VSVITVNFRE RSGSAHKMSP WIRAVFLKFL
351 PKILLMSRPE KGEVTKQPPL VNAASLVGSN YATAAKANE YRNKAKGKNN
401 DLMSLRGKPS SQAVLNNTFD MRMFNNDSTV ENRLKPYLCA QNNNAPTVAF
451 RKTQRQKSKS VDDVVFVNLL NQVRFIAEHF RHNELEGEIS DDWTFVAMVL
501 DRLFLIIFSV LNVGTVFIIL ESPSLYDYSK PMNITVPNKP LGQANFYSSW
551 NLK

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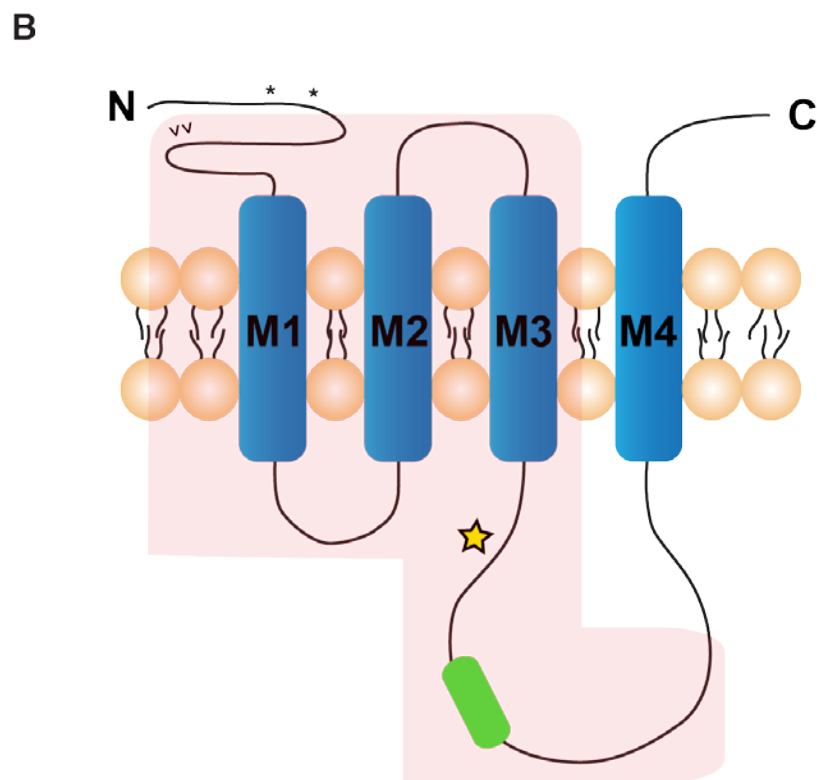


Figure 2-1

Figure 2-1 ACR-12 gene sequence. **(A)** *acr-12* amino acid sequence. cys-loop (*), vicinal cysteines present in alpha subunits (^), GFP insertion site (green box), transmembrane domains (blue boxes), *ok367* deletion (red lettering), *uf77* mutation (yellow box). Sequences are from GeneBank **(B)** Schematic of ACR-12 receptor subunit with approximate locations of annotations from A. cys-loop (*), vicinal cysteines present in alpha subunits (^), GFP insertion site (green box), transmembrane domains (M1-4), *ok367* deletion (red shading), *uf77* mutation (yellow star).

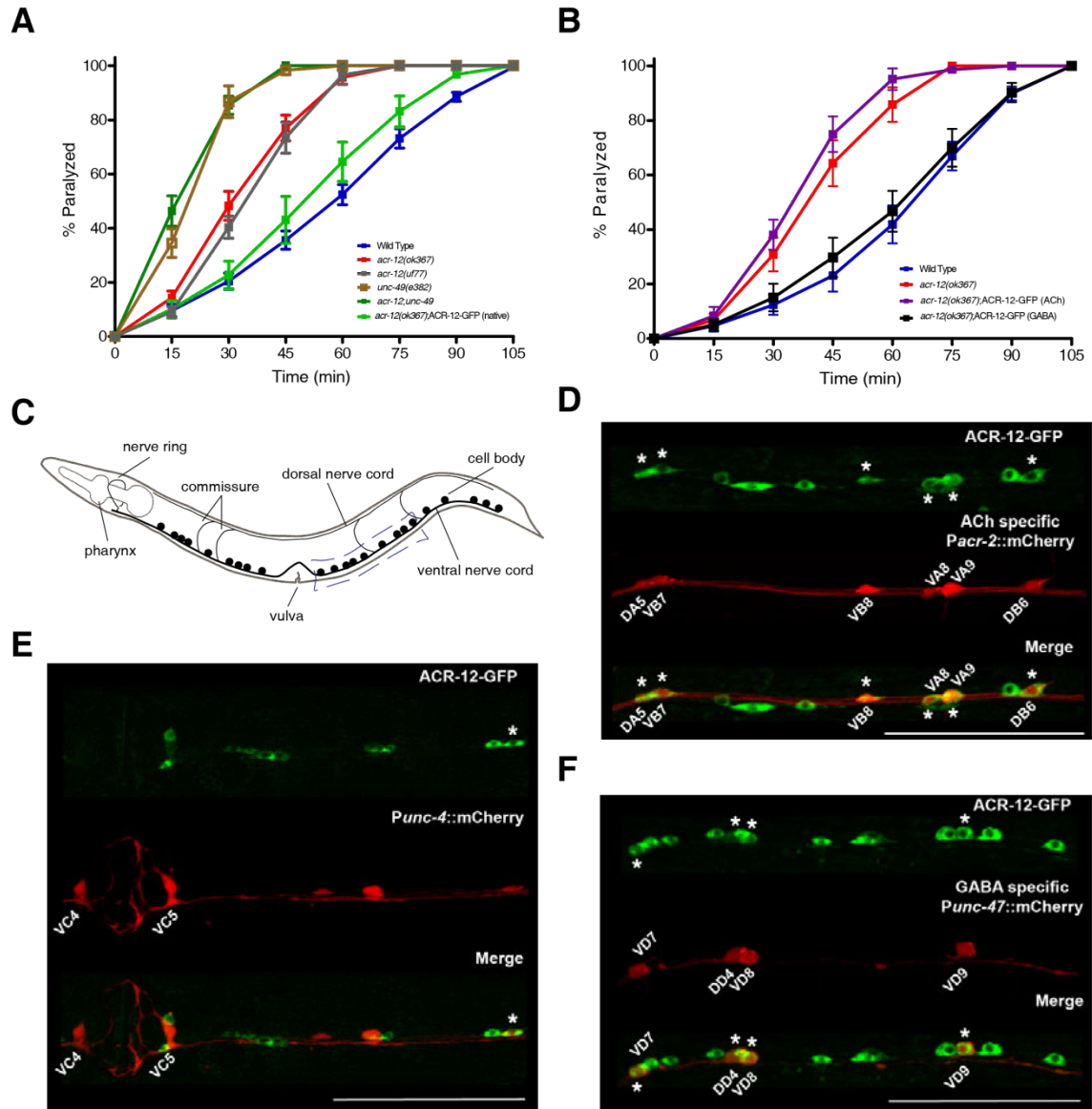


Figure 2-2

Figure 2-2 ACR-12 iAChRs regulate GABA motor neuron activity.

(A) Time course of paralysis in the presence of aldicarb (1 mM) for wild type (blue) [n=11], *acr-12(ok367)* mutants (red) [n=12], *acr-12(uf77)* mutants (gray) [n=8], *unc-49(e382)* mutants (brown)[n=11], and *acr-12(ok367);unc-49* double mutants (dark green) [n=10], and ACR-12-GFP rescue using the native promoter (green) [n=12]. Data represent mean \pm SEM. n=the number of experiments done with at least 10 animals per experiment. **(B)** Time course of aldicarb (1 mM) paralysis for wild type (blue) [n=13], *acr-12(ok367)* mutants (red) [n=14], ACh-specific (*Pacr-2*) ACR-12-GFP rescue (purple) [n=10], and GABA-specific (*Punc-47*) ACR-12-GFP rescue (black) [n=10]. **(C)** Diagram of *C. elegans* ventral nerve cord. Blue box indicates the region imaged in D-F. **(D)** Confocal image showing coexpression of ACR-12-GFP (*ufls38*) and a cholinergic motor neuron-specific marker (*ufls43*) in the posterior ventral nerve cord. For D-F, asterisks indicate cell bodies co-expressing reporters and scale bars indicate 50 μ m. **(E)** Confocal image showing coexpression of ACR-12-GFP (*ufls38*) and a GABA-specific marker (*ufls34*) in the posterior ventral nerve cord. **(F)** Confocal image showing coexpression of ACR-12-GFP (*ufls38*) and a *Punc-4::mCherry* (*ufls26*) in the posterior ventral nerve cord.

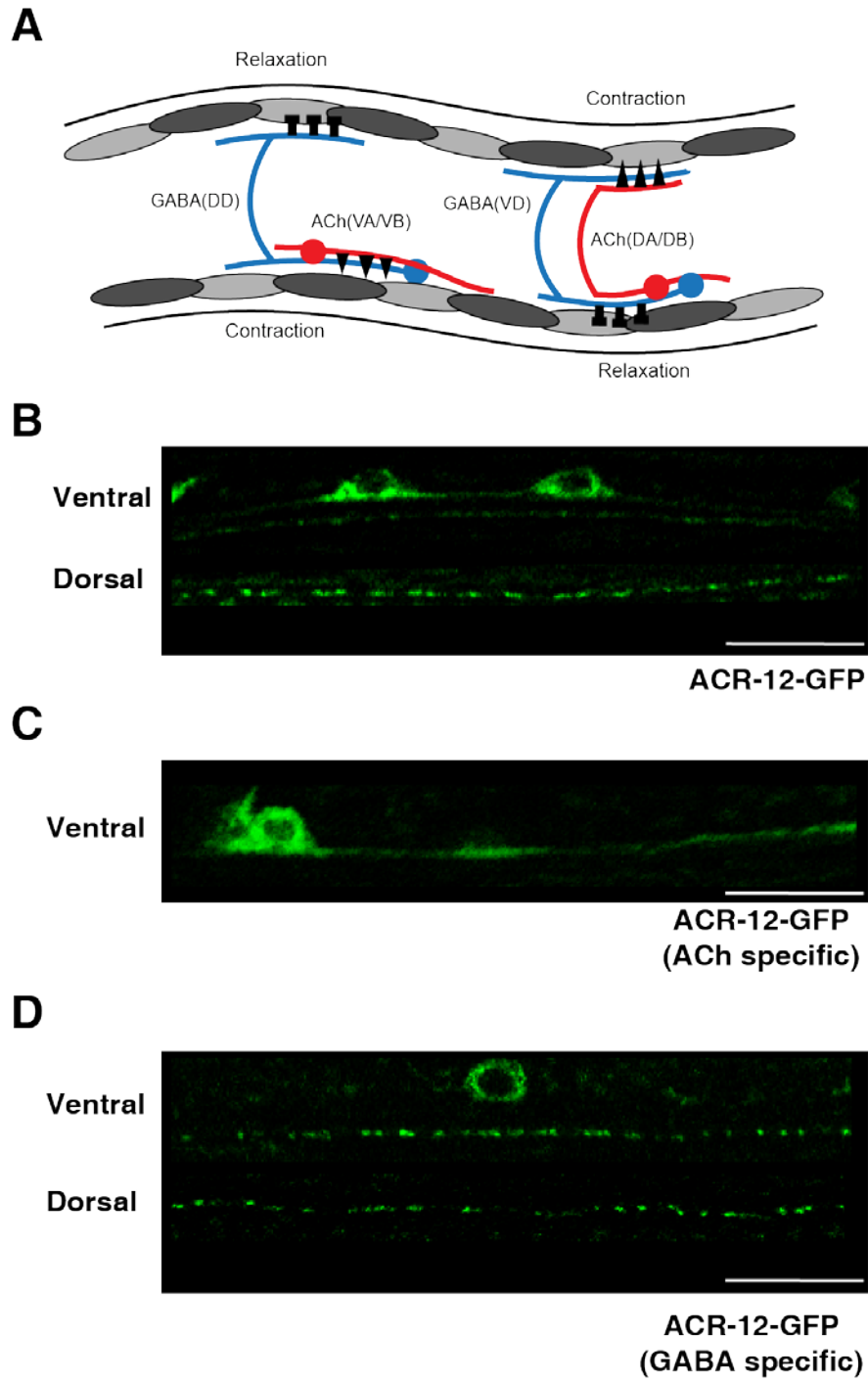


Figure 2-3

Figure 2-3 ACR-12 is differentially localized across motor neuron populations. **(A)** Schematic of *C. elegans* locomotory circuit. Dark green shading indicates ACh MNs (VA, VB, DA, DB) and light green indicates GABA MNs (DD and VD). Dark blue and light shading represent body wall muscles. Figure modified from WormAtlas. **(B)** Confocal images of ACR-12-GFP localization in ventral and dorsal nerve cords as indicated (*ufls57*). For B-D, scale bar indicates 10 μm . **(C)** Confocal image of ACR-12-GFP (*ufls78*) localization in ACh MNs of the posterior ventral nerve cord. **(D)** Confocal images ACR-12-GFP (*ufls92*) localization in GABA MNs of the posterior ventral and dorsal nerve cords as indicated.

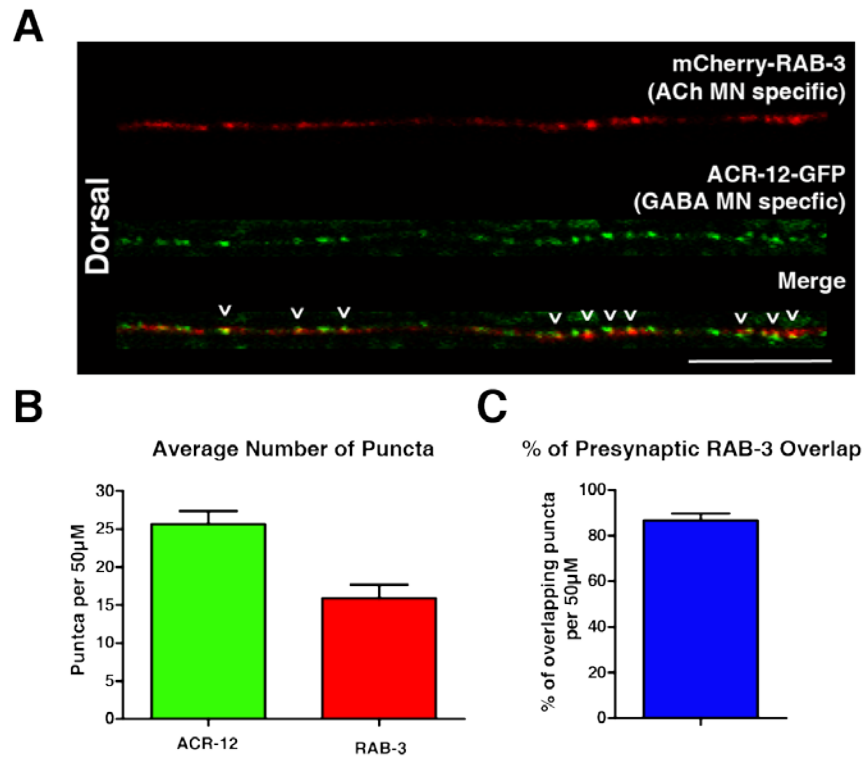


Figure 2-4

Figure 2-4 ACR-12 is localized at synapses on GABA motor neurons. **(A)** Confocal image of a 50 μm region of the dorsal nerve cord showing colocalization of mCherry-RAB-3 expressed in cholinergic motor neurons (*ufIs63*) with ACR-12::GFP expressed in GABA MNs (*ufIs92*). All images are oriented with the tail to the right and scale bar represents 10 μM . **(B, C)** Bar graphs showing the average number of ACR-12 and RAB-3 puncta and percentage overlapping puncta.

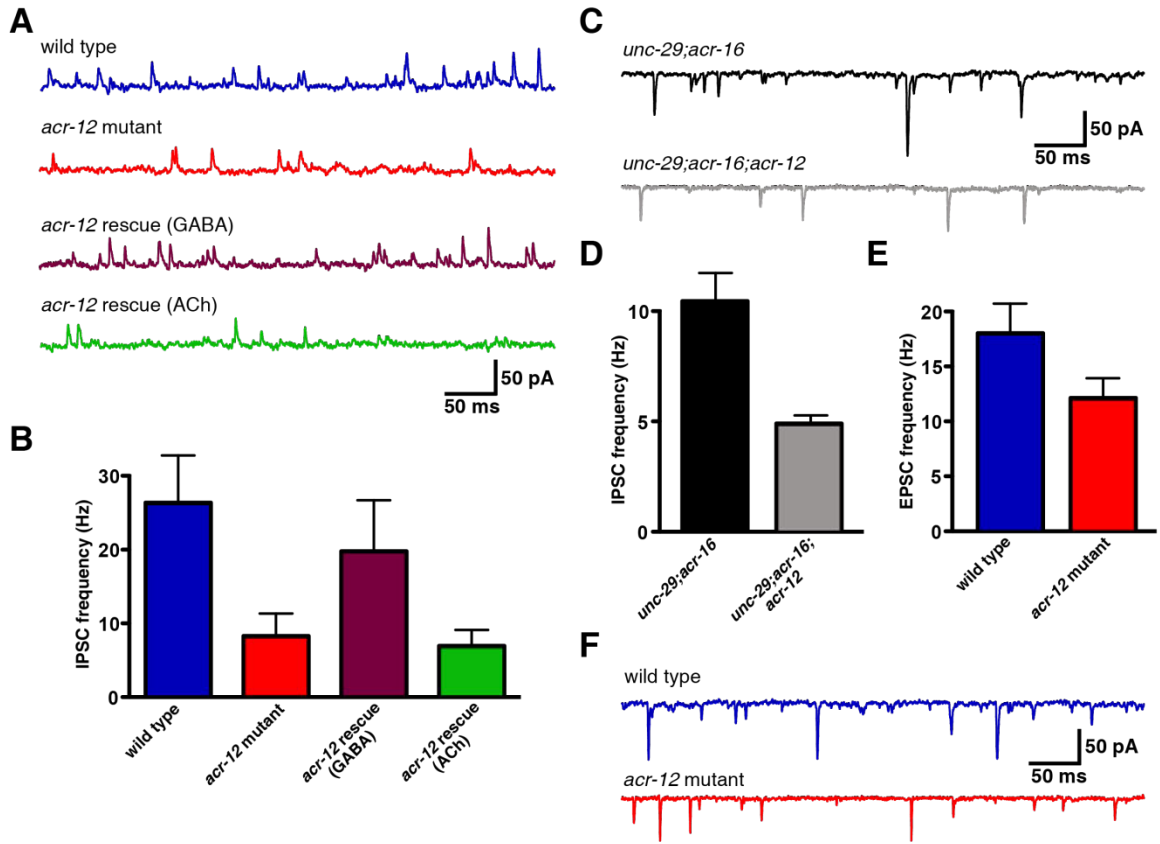


Figure 2-5

Figure 2-5. Loss of ACR-12 receptors reduces inhibitory signaling. **(A)** Representative recordings of endogenous inhibitory PSCs recorded at 0 mV for the indicated genotypes. Rescue refers to *acr-12* mutants expressing ACR-12 under control of either the *unc-47* (GABA) or *acr-2* (ACh) promoters. **(B)** Average endogenous IPSC frequency for the genotypes indicated. WT, n=7; *acr-12*, n=10; *acr-12* rescue (GABA), n=6; *acr-12* rescue (ACh), n=4. For (B) and (D-E), error bars show SEM. *p<0.01. **(C)** Representative recordings of endogenous inhibitory PSCs recorded at -60 mV for the indicated genotypes. Recordings were made using 115 mM KCl in the pipette. **(D)** Average endogenous IPSC frequency for the genotypes indicated. *unc-29;acr-16*, n=12; *unc-29;acr-16;acr-12*, n=11. **(E)** Average excitatory PSC frequency for the genotypes indicated. WT, n=17; *acr-12*, n=21. **(F)** Representative recordings of endogenous excitatory PSCs recorded at -60 mV for the indicated genotypes. Also refer to supplemental Figure S2 for PSC amplitude.

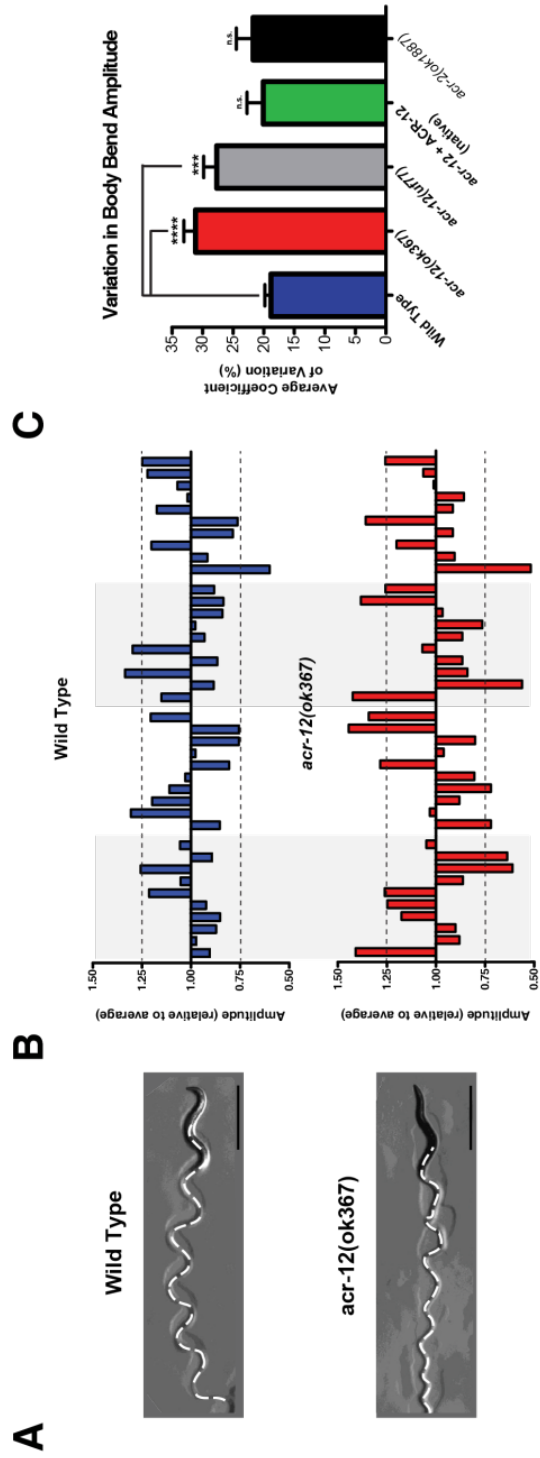
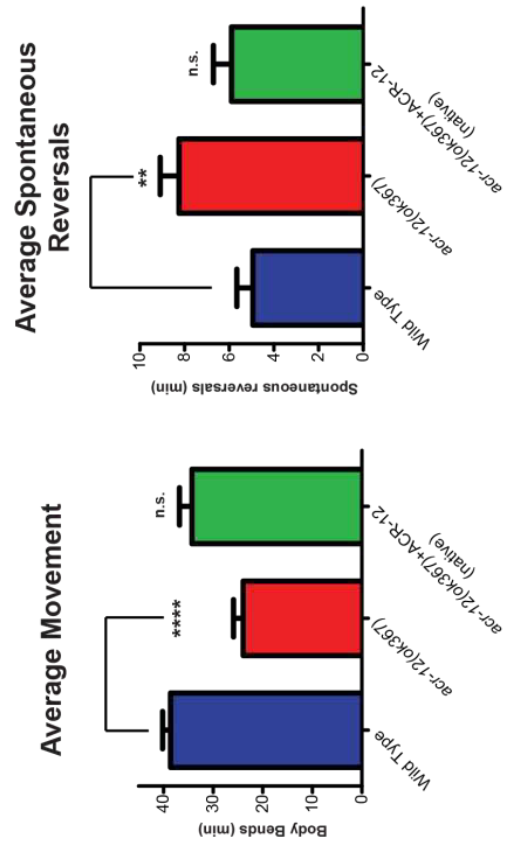


Figure 2-6

E



D

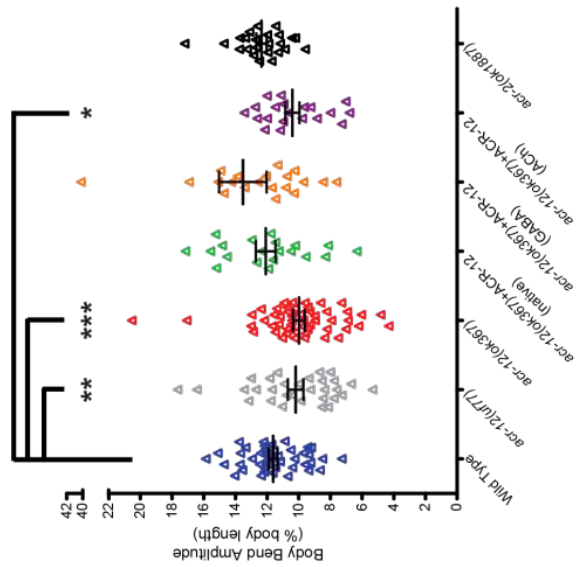


Figure 2-6 (continued)

Figure 2-6 ACR-12 receptors are required for consistent motor patterning. **(A)** Still images of locomotory paths for wild type and *acr-12(ok367)* mutants as indicated. Tracks are marked by white dashed lines. Scale bars indicate 2 mm. **(B)** Body bend amplitudes for 10 consecutive body bends of four representative animals of the indicated genotype plotted relative to average body bend amplitude. Gray shading indicate separate animals. **(C)** Coefficient of variance for body bend amplitude averaged over ten consecutive body bends for wild type (blue) [n=23] animals, *acr-12(ok367)* (red) [n=18], *acr-12(uf77)* (gray) [n=11], ACR-12-GFP rescue with native promoter (green) [n=11], *acr-2(ok1887)* (black) [n=13]. Asterisks indicate significant difference from wild type: *acr-12(ok367)* p<0.0001, *acr-12(uf77)* p<0.0001. **(D)** Scatter plot of body bend amplitudes for wild type, *acr-12(uf77)*, *acr-12(ok367)*, and rescue strains as indicated. Wild type (blue) [n=45], *acr-12(uf77)* mutants (gray) [n=31], *acr-12(ok367)* mutants (red) [n=52], ACh-specific (*Pacr-2*) ACR-12-GFP rescue (purple) [n=20], GABA-specific (*Punc-47*) ACR-12-GFP rescue (orange) [n=20], genomic rescue (green) [n=20], *acr-2(ok1887)* (black) [n=24]. Each point represents an average of three consecutive body bends for an individual animal. Mean \pm SEM is indicated by horizontal lines. Asterisks indicate significant difference from wild type: *acr-12(ok367)* p<0.01, *acr-12(uf77)* p<0.02, ACh specific rescue p<0.05. **(E)** Bar graphs of the average number of body bends and average number of spontaneous reversals of wild type (blue) [n=20], *acr-12(ok367)* (red) [n=20], and genomic rescue (green) [n=10] animals. Asterisks indicate significant difference from wild type: average movement *acr-12(ok367)* p<0.0001, average spontaneous reversal *acr-12(ok367)* p<0.005.

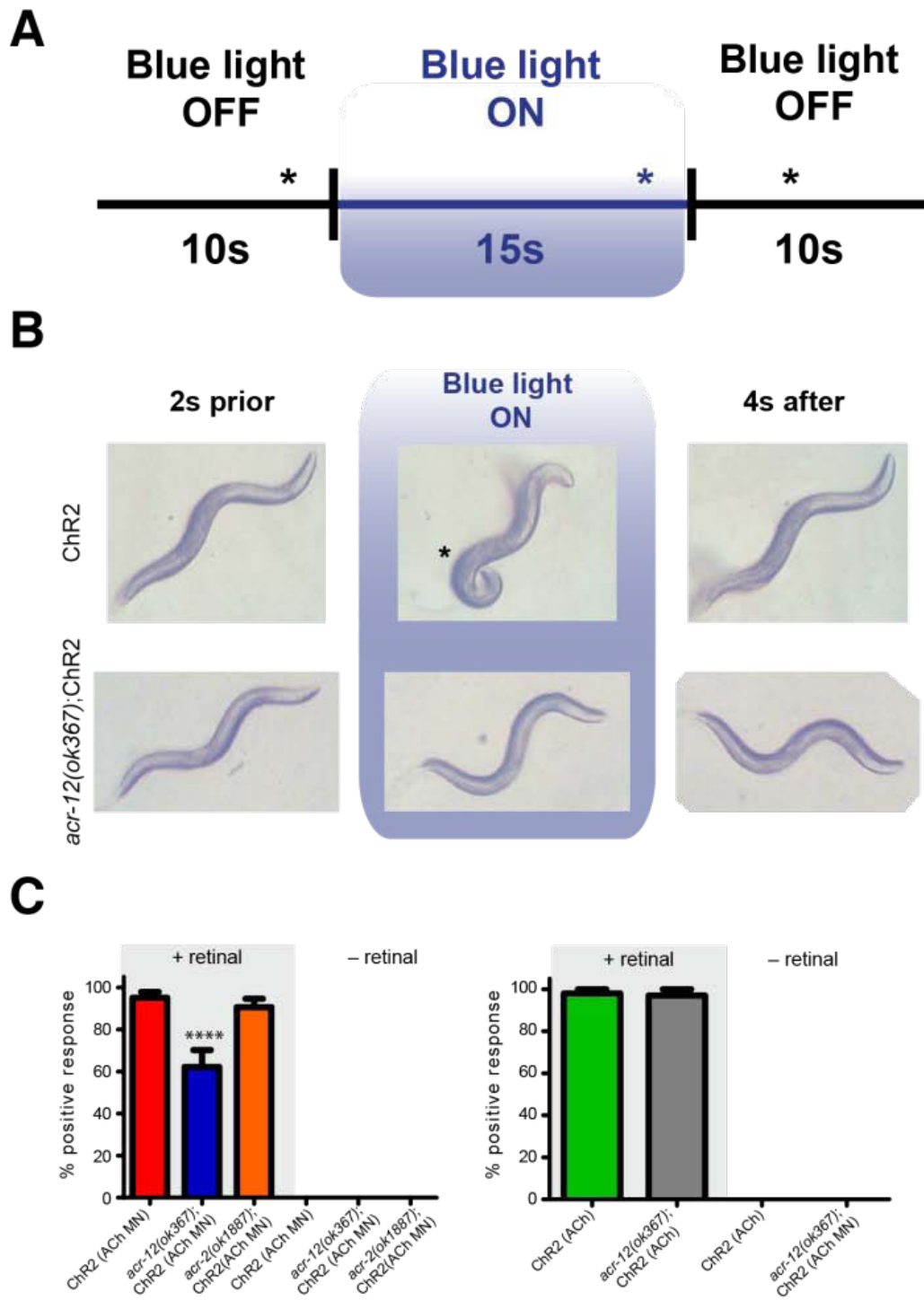


Figure 2-7

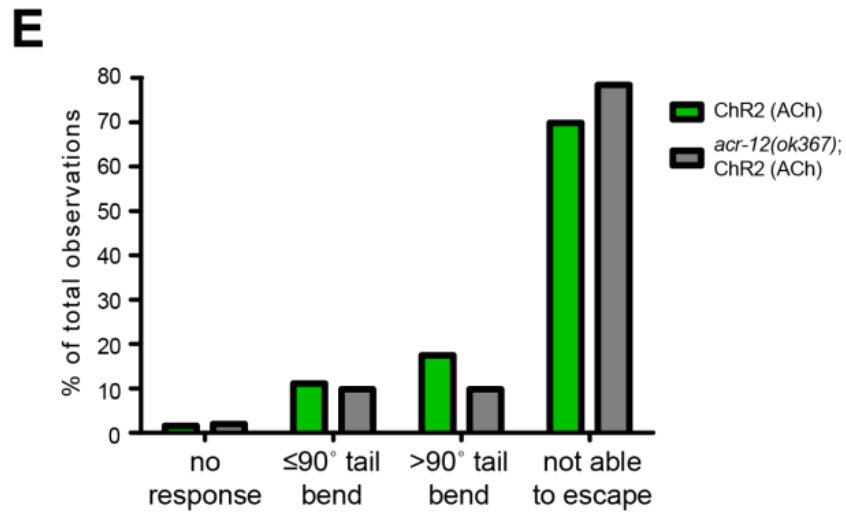
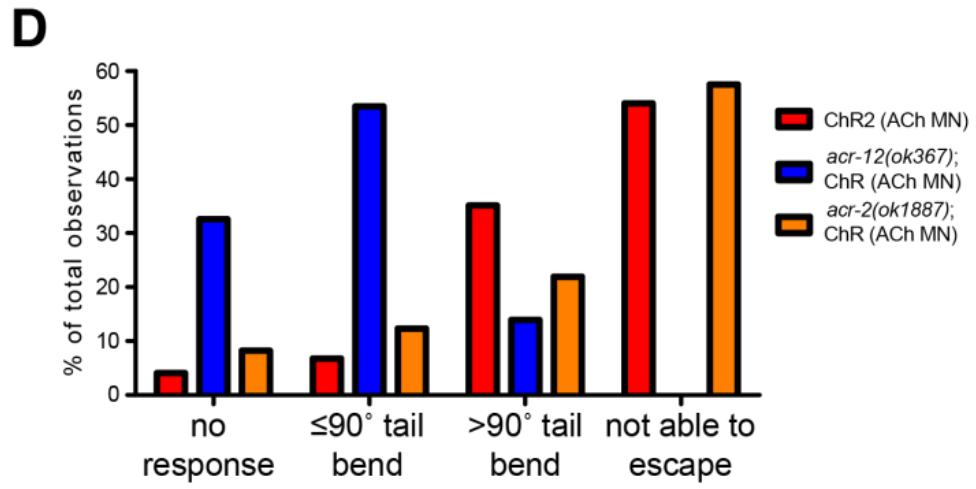


Figure 2-7 continued

Figure 2-7 GABA neuron-specific ACR-12 receptors mediate elevated levels ACh signaling. **(A)** Schematic depicting experimental protocol for photostimulation experiments. Asterisks indicate timing of still images shown in B. **(B)** Still images showing representative responses of wild type animals and *acr-12* mutants expressing ChR2 (*ufls23*) at the times indicated. Asterisks indicates posterior bend. **(C)** Quantification of percentage of animals responding to photostimulation of cholinergic motor neurons (ACh MN) (left) and all cholinergic neurons (ACh) (right). Asterisks indicate significant difference from ChR2 (ACh MN) $p < 0.0001$. **(D)** Distribution of response to photostimulation of cholinergic motor neurons (*ufls23*, ACh MN) types using the descriptive behavioral classifications indicated. ChR2+retinal [n=74], ChR2;*acr-12(ok367)*+retinal [n=43], ChR2;*acr-2(ok1887)*+retinal [n=73], ChR2 no retinal [n=36], ChR2;*acr-12(ok367)* no retinal [n=28], ChR2;*acr-2(ok1887)* no retinal [n=27]. **(E)** Distribution of response to photostimulation of all cholinergic neurons (*ufls53*) types using the descriptive behavioral classifications indicated. For **C-E** the sample sizes are as follows: ChR2+retinal [n=63], ChR2;*acr-12(ok367)*+retinal [n=51], ChR2 no retinal [27], ChR2;*acr-12(ok367)* no retinal [n=27]. For **D** and **E** no retinal controls are not shown as there were no responses to blue light.

Chapter III:

**A sensitized nAChR leads to spontaneous
contractions and GABA neuron toxicity in
the *C. elegans* locomotory circuit**

Abstract

Behavior is shaped by the precise coordination of activity in neuronal circuits. Recent findings utilizing sensitized nicotinic acetylcholine receptors (nAChRs) have provided valuable insights into mechanisms of regulating neuronal activity. Here we engineer a gain of function ACR-12 [ACR-12(V/S)] transgene to identify specific roles for neuronal subsets in generating the sinusoidal movement of *C. elegans*. We previously demonstrated a similar gain of function subunit in ACR-2R, specifically the *acr-2* subunit, resulted in neuronal toxicity and cell death (Barbagallo et al., 2010). However, transgenic ACR-12(V/S) expression resulted in the generation of spontaneous contractions and dramatically reduced movement. We were able to use cell specific drivers to identify the cholinergic motor neurons as sufficient to generate spontaneous contractions. Activation of DA and DB neurons did not have an obvious affect on movement or on the generation of contractions. Unexpectedly, we found expression of ACR-12(V/S) results in cell autonomous degeneration of GABA neurons. This degeneration has no obvious impact on downstream inhibitory NMJ receptors. Here we demonstrate sensitized nAChRs as a useful tool in identifying mechanisms of neuronal circuit activity in adapting behavior. Further development of ACR-12(V/S) and similar tools will enable genetic approaches to identify modifiers of nAChR signaling as well as investigation of the molecular pathways underlying differential susceptibility of neuronal populations to ion channel mediated toxicity.

Introduction

Subtle changes in the activity of neuronal circuits can profoundly shape behavior. The nervous system adapts behavior through the precise coordination of both excitatory and inhibitory signaling. Identifying the molecular mechanisms that regulate neuronal activity is important for understanding how balanced signaling is achieved and behavior is adapted. The inappropriate activation of neuronal circuits can have profound negative effects including disorders such as epilepsy, Parkinson's, and addiction (Dani and Harris, 2005; Drenan et al., 2008; Futatsugi and Riviello, 1998; Quik and McIntosh, 2006). Ionotropic receptors are a functionally diverse family of ion channels activated by neurotransmitters to facilitate neuronal activity. Much of our understanding of their structure and function is derived from the first receptor to be isolated and studied, nicotinic acetylcholine receptors (nAChR) (Changeux et al., 1970; Popot and Changeux, 1984).

Characterization of nAChRs identified the second transmembrane (TM2) domain as lining the ion channel pore (Gotti and Clementi, 2004; Unwin, 2005). Mutations in specific residues of the pore region and the area directly surrounding the pore can dramatically alter kinetics, prolonging channel open time, desensitization (Figure 1-3), and ion selectivity. Reconstitution studies revealed the kinetics of mammalian homomeric $\alpha 7$ nAChRs were dramatically altered by mutating a residue in the second transmembrane domain (Galzi et al.,

1991; Imoto et al., 1988; Revah et al., 1991). Mutating residue 247 from a non-polar leucine to a polar serine has the most pronounced effect on mean channel open time (Revah et al., 1991). Hypersensitive or gain-of-function nAChRs have since been used to examine the role of cholinergic signaling in the CNS. Using an $\alpha 4$ gain of function knock in mouse model, researchers were able to identify interactions of nAChRs and dopamine D_2 receptors in regulating cholinergic interneuron activity (Zhao-Shea et al., 2010). Mutations in the pore lining domain of $\alpha 6$ ($\alpha 6^{L9S}$) nAChR subunit identified $\alpha 6\alpha 4\beta 2^*$ receptors as a key target for disorders associated with reduced dopamine release. The researchers were able to specifically link the behavioral deficits of $\alpha 6^{L9S}$ animals to $\alpha 6\alpha 4\beta 2^*$ receptors in dopamine releasing neurons *in vivo* (Drenan et al., 2010). Additional work with gain of function $\alpha 6$ subunits has demonstrated a role for $\beta 3$ subunits in promoting the function of $\alpha 6$ containing receptors (Dash and Lukas, 2012).

Here we use a similar strategy to explore the function of nAChRs in the *C. elegans* locomotory circuit. The *C. elegans* locomotory circuit is an attractive model for probing mechanisms that regulating opposing signaling. In *C. elegans*, excitatory cholinergic motor neurons (ACh MNs) make synaptic contacts onto both muscle cells and GABA MNs that, in turn, make inhibitory synaptic contacts onto opposing musculature (Figure 1-4) (White et al., 1986). Proper function of this circuit produces temporally coordinated and balanced excitatory and inhibitory signals that pattern movement. While the anatomical connectivity of this circuit has been well characterized, the signaling mechanisms that underlie

coordinated motor neuron activity are not well understood. Expression studies have revealed that many of the 29 ionotropic acetylcholine receptor (iAChR) subunits encoded by the *C. elegans* genome are expressed in motor neurons, suggesting that cholinergic signaling may play a prominent role in regulating movement (Cinar et al., 2005; Fox et al., 2005; Jones et al., 2007; Rand, 2007). Finally, the nematode nervous system uses many of the same neurotransmitters as mammals including acetylcholine, GABA, and glutamate. <--This doesn't really fit with the rest

Previous studies have shown that cholinergic motor neurons express a class of heteromeric acetylcholine-gated ion channel complexes known as ACR-2R (Barbagallo et al., 2010; Jospin et al., 2009). ACR-2Rs are ionotropic receptors of the nicotinic acetylcholine receptor superfamily composed of five distinct subunits (ACR-2, ACR-3, UNC-38, UNC-63 and ACR-12), each subunit of which is essential for function in heterologous expression studies (Jospin et al., 2009). Loss of ACR-2R leads to relatively subtle changes in behavior; however, gain-of-function *acr-2* mutations have profound consequences including hyperactivation and, in extreme cases, death of ACh MNs. In addition to the ACR-2R we have identified the role of a second class of nAChRs containing the ACR-12 subunit (ACR-12R). These receptors are localized to synapses of GABA neurons and maintain consistency of body bend movements (see Chapter 2).

Here, using cell specific promoters and a gain of function approach we have isolated behaviors specific to subsets of motor neurons. In addition, we show that subsets of motor neurons appear selectively vulnerable to toxicity caused by expression of a gain of function ACR-12 receptor.

EXPERIMENTAL PROCEDURES

C. elegans strains

C. elegans strains were grown under standard laboratory conditions at 22°C. All strains are derivatives of the N2 Bristol strain (wild type). Transgenic strains were obtained by microinjection to achieve germline transformation. Multiple independent extragenic lines were obtained for each transgenic strain and data presented are from a single representative transgenic line unless noted otherwise. In all cases, *lin-15(n765ts)* mutants were injected with the *lin-15* rescuing plasmid (pL15Ek; 30ng/ul) and one or more of the following plasmids: pPRB5 [*Punc-47::mCherry*], pPRB53 [*ACR-12-GFP_{ICL}*] (intracellular loop), pPRB77 [*Pacr-2::ACR-12-GFP_{ICL}*], pHP7 [*Punc-47::ACR-12-GFP_{ICL}*], pPRB59 [*ACR-12(V/S)::GFP*], pPRB65 [*Punc-47::ACR-12(V/S)::GFP*], pPRB79 [*Pacr-2::ACR-12(V/S)::GFP*], and pHP8 [*Punc-129::ACR-12(V/S)::GFP*]. Stably integrated lines were generated by X-ray integration and outcrossed at least four times to wild type. The following strains were used in this study: IZ914: *acr-12(ok367)X*; IZ853: *acr-12(ok367)X;ufls57[ACR-12-GFP_{ICL}]*; IZ984: *acr-12(ok367);ufls78[Pacr-2::ACR-12::GFP_{ICL}]*; IZ556: *acr-12(ok367);ufls92[Punc-47::ACR-12-GFP_{ICL}]*; CB382: *unc-49(e382)III*; IZ712: *acr-12(uf77)*; IZ719: *acr-*

12(ok367);ufls90[ACR-12(V/S)::GFP]; IZ538: *acr-12(ok367);ufls74[Pacr-2::ACR-12(V/S)::GFP]*; IZ1058: *ufls102[Punc-47::ACR-12(V/S)::GFP]*; IZ1077: *acr-12(ok367);ufls111[Punc-129::ACR-12(V/S)::GFP]*; IX697: *acr-12(ok367);acr-2(ok1887);ufls90[ACR-12(V/S)::GFP]*; RB1559: *acr-2(ok1887)*; IZ324: *acr-2(ok1887);acr-12(ok367)*; LX949: *lin-15(n765ts);vsIs48[Punc-17::GFP]*; IZ829: *ufls34[Punc47::mCherry]*; IZ759: *ufls34[Punc-47::mCherry];vsIs48[Punc-17::GFP]*; IZ736: *acr-12(ok367);ufls34[Punc-47::mCherry];vsIs48[Punc-17::GFP];ufls90[ACR-12(V/S)::GFP]*; IZ1027: *ufis8[ACR-16::GFP];ufls90[ACR-12(V/S)::GFP]*; EG1653: *lin-15(n765ts);oxIs22[UNC-49::GFP]*; IZ758: *ufls34[Punc-47::mCherry];oxIs22[UNC-49::GFP]*; IZ766: *ufls34[Punc-47::mCherry];ufls90[ACR-12(V/S)::GFP];oxIs22[UNC-49::GFP]*.

Molecular Biology

The ACR-12-GFP_{ICL} transgene (pPRB53) was generated by cloning the GFP coding sequence in-frame into the sequence of an *acr-12* genomic fragment (-1514 to +4799 bp relative to the translational start site) encoding the intracellular loop (ICL) between transmembrane domains (TM) TM3 and TM4. ACR-12-GFP_{ICL} was localized in neuronal processes and expression of this construct was sufficient for rescue of *acr-12* mutants. *Punc-47::ACR-12-GFP_{ICL}* (pHP7) and *Punc-47::ACR-12-GFP_{ICL}* (pPRB77) were generated by subcloning a 4.9 kb NruI/BglI fragment containing GFP from pPRB53 into constructs encoding the *acr-12* cDNA under control of the appropriate promoters (pBB25 and pHP3,

respectively). The ACR-12(V/S)-GFP was generated using Qiagen's site directed mutagenesis kit. Point mutation was confirmed using enzymatic digest and subsequent sequencing. The cell specific versions were generating using the same cloning as the previously mentioned wild type versions. *P_{unc-47}::mCherry* (pPRB5) was generated by subcloning mCherry coding sequence downstream of a 1.3 kb promoter for the *unc-47* gene.

Microscopy

Confocal microscopy was performed using a Zeiss Axioskop 2 microscope system and LSM Pascal 5 imaging software (Zeiss). Epifluorescent imaging was performed using a Zeiss Axioimager M1 microscope and Axiovision software (Zeiss). All images of adult animals used staged animals 24 h after the L4 stage and were processed using ImageJ software. For the developmental timeline, synchronized populations were obtained by bleaching gravid animals on NGM plates seeded with OP50. The resulting progeny were allowed to mature at room temperature. Animals were imaged at 16, 28, 38, and 48 h after bleaching using wide-field epifluorescent microscopy.

Behavioral assays

All behavioral analyses were performed using staged populations of young adult animals (24 h following L4) at room temperature (22°C-24°C). Strains were scored in parallel, with the researcher blinded to the genotype during both experiment and analysis. For aldicarb assays, staged populations of adult animals (≥ 10) were transferred to NGM plates containing 1 mM aldicarb

(ChemService), and movement was assessed every 15 min for 2 h. Movies and still images for behavioral analyses were obtained using an Olympus SZ61 upright microscope equipped with a FireWire camera (Imaging Source). For gross movement, individual worms were transferred to 100mm (large) unseeded plates. The number of body bends were counted manually over 1-minute intervals for 3-minutes and averaged. Spontaneous contractions were assessed observing individual worms on large seeded plates. The number of contractions were counted manually over 1-minute intervals for 3-minutes and averaged. For stimulated contractions animals were individually assessed on large plates seeded with OP50. Animals were stimulated five times at 30s intervals using an eyelash on the tip of the nose and then the average was calculated as a percentage of response per five stimulations.

Results

ACR-12(V/S) has dramatic consequences for behavior

The *C. elegans* genome encodes 29 nAChR subunits that contribute to the formation of unique classes of homo- and hetero- pentameric receptors (Jones et al., 2007; Rand, 2007). Six of these nAChR subunits form two classes of nAChRs expressed in body wall muscle cells and are required for neuromuscular signaling (Francis et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005). Previous work from our lab and others has shown that the expression of several nicotinic acetylcholine receptor subunits, including the α subunit *acr-12*, is enriched in motor neurons of the ventral nerve cord (see

Chapter 2) (Barbagallo et al., 2010; Cinar et al., 2005; Hallam et al., 2000; Jospin et al., 2009).

To explore the role of ACR-12 in regulating motor neuron activity, we developed a gain-of-function strategy. The second transmembrane domains of Cys-loop family ligand-gated ion channel subunits line the ion channel pore and play a critical role in the properties of the channel (Galzi et al., 1991; Galzi et al., 1992; Gotti and Clementi, 2004; Imoto et al., 1988). Substitution of a polar amino acid (e.g., serine) for the non polar leucine at a highly conserved position within M2 (9', so called because it is the 9th residue from the beginning of the membrane-spanning segment) produces a gain-of-function effect, resulting in increased receptor activation and very slow inactivation (Labarca et al., 1995; Revah et al., 1991). In the case of *acr-12* this position also has a non polar residue, valine (Figure 3-1). We engineered a valine-to-serine point mutation into the sequence encoding the M2 9' position of an *acr-12* rescuing construct to generate ACR-12(V/S) (Figure 3-1). Transgenic animals expressing an integrated ACR-12(V/S) array (*ufls90*) in the *acr-12(ok367)* mutant background were used for all subsequent analyses. These animals are viable; however, are slightly delayed developmentally compared to wild type animals. Moreover, we noted obvious locomotory defects in transgenic ACR-12(V/S) animals (Figure 3-2). These defects were observed across all larval stages including adult animals. Transgenic ACR-12(V/S) animals have a 75% reduction in body bends per minute compared to their wild type counterparts and a 50% reduction when

compared to *acr-12* mutants (Figure 3-2B). ACR-12(V/S) animals exhibit a “shrinker” phenotype in response to touch along the body, similar to the response typical of GABA deficient mutants (Bamber et al., 1999). In addition to this phenotype, transgenic ACR-12(V/S) animals also exhibit spontaneous contractions (Figure 3-2A). These contractions are qualitatively similar to those previously described for a strain isolated in the Jin laboratory that carries a gain-of-function *acr-2* allele (Jospin et al., 2009). The dramatic changes in behavior as a result of ACR-12(V/S) expression suggest we can identify mechanisms for coordinating signaling and tease apart the contributions of subsets of motor neurons to movement.

Cell specific expression of ACR-12(V/S) identifies specific roles for subsets of motor neurons

Our previous work has shown ACR-12 to be broadly expressed in the nervous system (see Chapter 2). In order to tease apart mechanisms for coordinating movements and determine which subsets of motor neurons contribute to patterning movement, we used cell specific promoters to drive ACR-12(V/S) expression in *acr-12(ok367)* mutants. We quantified movement by counting the number of body bends/minute. We found that animals expressing ACR-12(V/S) in cholinergic (ACh) motor neurons (*Pacr-2*) had improved movement compared to animals expressing ACR-12(V/S) under the endogenous *acr-12* promoter (Figure 3-2). However this movement was not restored to wild

type levels, likely due to the presence of a secondary phenotype, spontaneous contractions. In contrast, movement in transgenic animals expressing ACR-12(V/S) specifically in GABA neurons (*Punc-47*) was similar to that of *acr-12(ok367)* mutants despite being in a wild type background (denoted with ‡ in Figure 3-2B). Movement of transgenic ACR-12(V/S) animals carrying a loss-of-function allele (*ok1887*) of *acr-2*, an obligate subunit of the cholinergic ACR-12* receptor, was similar to *acr-12* mutants. In this strain the ACR-12(V/S) is still present in GABA neurons with an *acr-12* mutant background. This suggests GABA specific expression of ACR-12(V/S) does not significantly alter the speed of movement relative to *acr-12* mutants. However, cell specific expression of ACR-12(V/S) in DA and DB neurons also reduces the movement to *acr-12(ok367)* levels (Figure 3-2B). As previously mentioned, the excitatory cholinergic motor neurons (ACh MNs) make synaptic contacts onto both muscle cells and GABA MNs. These contacts are patterned across the ventral and dorsal musculature. One might expect simultaneous activation all dorsal ACh neurons to alter the precise timing of activation in body wall muscle. As a result we would expect an additional reduction in movement beyond that seen in the *acr-12* mutant background.

As noted above, the stimulated and spontaneous contractions we observe in ACR-12(V/S) animals are similar to those observed previously in a strain carrying an alternative *acr-2(gf)* allele (Jospin et al., 2009). In fact cell specific expression of ACR-12(V/S) in ACh motor neurons recapitulates these

contractions; while mutations in *acr-2(ok1887)* mutants suppressed the contractions (Figure 3-2). These results suggest the bilateral contraction of the body wall muscle we see upon ACh motor neuron-specific expression of ACR-12(V/S) occurs when there is ACh build up along both dorsal and ventral body axis (Figure 3-2C and D). In contrast, cell-specific expression of ACR-12(V/S) solely in DA and DB motor neurons does not lead to spontaneous contractions. This may indicate that elevated levels of ACh directed along one axis of the body are not sufficient for the the generation of contractions. We did not see any spontaneous contractions with GABA specific expression of ACR-12(V/S). However there was a small increase (~20%) in the number of stimulated contractions (Figure 3-2C and D).

Expression of ACR-12(V/S) leads to GABA motor neuron specific toxicity

Since we observed dramatic changes in behavior we wanted to confirm the health of the nervous system. To address this issue, we generated a transgenic strain co-expressing GFP (green fluorescent protein) in all of the ACh neurons (*Punc-17::GFP*), a red fluorescent reporter for GABA neurons (*Punc-47::mCherry*), and the ACR-12(V/S) transgene under the endogenous *acr-12* promoter, and examined the ventral and dorsal cords. In wild type animals we observed clear separation of the two reporters and normal nervous system connectivity, including intact commissural projections that extended the length of the dorsal cord (Figure 3-3A). When we examined animals carrying the ACR-

12(V/S) transgene we observed that the cholinergic nervous system appeared intact with no obvious differences compared to the wild type strain. In contrast, we observed dramatic changes in the structure of the GABA nervous system, including decreased numbers of GABA motor neurons, migration defects in the commissures, defasciculation of the ventral nerve cord, and large gaps in the dorsal nerve cord (Figure 3-3B). The degeneration of the GABA nervous system can be observed as early as L1 (Figure 4). The degree of degeneration varied across populations of ACR-12(V/S) animals. A few animals exhibited no degeneration while others were missing several GABA neurons and had almost no dorsal cord coverage. Loss of GABA neurons may explain why we observed a small number of stimulated shrinking events in animals expressing ACR-12(V/S) specifically in GABA neurons (Figure 3-2D).

ACR-12(V/S) degeneration is a cell autonomous effect

The degeneration caused by ACR-12(V/S) expression could arise from three possibilities. First, the degeneration may be caused by over activation of the GABA neurons through elevated levels of ACh release from presynaptic cholinergic motor neurons. Second, ACR-12(V/S) expression in the GABA neurons may lead to prolonged activation of the channel resulting in tonic activation of GABA neurons and subsequent degeneration. Finally, toxicity of the neurons may arise from some combination of the first two scenarios. In order to identify the mechanism of toxicity, we expressed cell specific versions of ACR-

12(V/S) with the previously mentioned reporters for ACh (*Punc-17::GFP*) and GABA (*Punc-47::mCherry*) neurons. Animals expressing the GABA specific ACR-12(V/S) and a GABA neuron reporter exhibited degeneration (Figure 3-5A and B). Animals co-expressing ACR-12(V/S) specifically in ACh motor neurons with a GFP reporter that labeled the entire cholinergic nervous system had no obvious difference from wild type animals (Figure 3-5). Similarly, the GABA nervous system appeared normal with expression of ACR-12(V/S) in ACh motor neurons (data not shown). These results indicate that degeneration of the GABA nervous system arises from cell autonomous expression of ACR-12(V/S) in the GABA neurons.

UNC-49 receptors remain localized after GABA neuron degeneration

UNC-49 is the GABA receptor at the NMJ that mediates relaxation of contralateral body wall muscle. We wanted to investigate whether GABA motor neurons are required for maintenance of the post synaptic specialization. To test this we coexpressed a red fluorescent reporter (*Punc-47::mCherry*) for GABA neurons with a GFP reporter for UNC-49 receptors (Figure 3-5C). In wild type animals we observed a clear distribution of GFP puncta along the dorsal cord apposed to the GABA neuron reporter. Interestingly, in ACR-12(V/S) animals we saw no obvious changes in UNC-49 puncta even in cases where we noted substantial defects in the number and/or connectivity of GABA motor neurons (Figure 3-5C). There are two possibilities for the normal localization of UNC-49

receptors. First, the presence of the GABA motor neurons may only be required for establishing post synaptic sites and not for the maintenance of the UNC-49 receptor localization. Second, we cannot be sure how long presynaptic GABA neuron terminals have been absent from their normal site of innervation on body wall muscles, in which case there may not have been sufficient time for changes in UNC-49 receptor localization to take place.

Discussion

The development of sensitized nAChRs has provided researchers with new tools for identifying the mechanisms of cholinergic regulation of neuronal activity (Drenan et al., 2010; Galzi et al., 1991; Liu et al., 2012; Revah et al., 1991). Reconstitution studies using gain of function receptors have provided insights into the functional contributions of specific subunits in receptors. For example, $\beta 3$ subunits act to enhance the activity of $\alpha 6^*$ receptors (Dash and Lukas, 2012). Here we have engineered a gain of function mutation in the second transmembrane domain of ACR-12 containing receptors in order to identify mechanisms involved in coordinating the precise timing of alternating movements in *C. elegans*. Similar mutations in the pore lining domain of the mammalian $\alpha 6$ ($\alpha 6^{L9'S}$) nAChR subunit identified $\alpha 6\alpha 4\beta 2^*$ containing receptors as a key target for disorders associated with reduced dopamine release (Drenan et al., 2010). A $\alpha 4$ gain of function knock in mouse model identified interactions of nAChRs and dopamine D_2 receptors in regulating cholinergic interneuron activity

(Zhao-Shea et al., 2010). We have demonstrated expression of a similar sensitized nAChR in *C. elegans*, ACR-12(V/S), leads to spontaneous bilateral contractions of body wall muscles. Cell specific drivers identified the cholinergic motor neurons as sufficient to recapitulate these contractions. Transgenic ACR-12(V/S) animals carrying a deletion mutation in the *acr-2* gene suppressed the spontaneous contractions. These results suggest the spontaneous contractions of ACR-12(V/S) arise from the activity of a receptor containing both ACR-2 and ACR-12 (ACR-2R) and not the presence of an aberrant nAChR.

One might expect the spontaneous contractions to arise out of deregulated timing mechanisms in the circuit. Interference with the timed activation of cholinergic neurons, which drive the locomotory circuit, could be sufficient for generating locomotory phenotypes. However, animals expressing ACR-12(V/S) solely in dorsal cholinergic motor neurons did not exhibit spontaneous contractions. This suggests the bilateral excitation of body wall musculature from ACR-12(V/S) in all cholinergic motor neurons is required for spontaneous contractions. Furthermore, cell-specific expression of ACR-12(V/S) solely in dorsal cholinergic motor neurons did not lead to profound changes in movement (Figure 3-2). One reason for this could be the presence of the *acr-12(ok367)* mutant background. Previous behavior analysis has revealed *acr-12* mutants have reduced body bends/minute compared to wild type and an inconsistent waveform (Chapter II). This mutant background could be masking subtle changes in movement as a function of ACR-12(V/S) expression in DA and

DB motor neurons. Nervous system development is not obviously affected by specific expression of ACR-12(V/S) in DA and DB neurons, and increased aldicarb sensitivity suggests these animals have increased ACh release.

Unexpectedly we found cell specific expression of ACR-12(V/S) in GABA neurons produced toxicity and subsequent loss of GABA neurons. The toxicity was cell autonomous suggesting fundamental differences in the susceptibility of motor neuron populations to toxicity. This finding could provide valuable insights into the unique properties of GABA neurons. Perhaps GABA neurons express specific proteins that makes GABA neurons more sensitive to changes in activity. This toxicity was variable across animals and could be observed as early as L1 animals. The cholinergic nervous system remained intact with no obvious changes in connectivity, while the GABA nervous system exhibit neuronal degeneration which was severe in some animals. The variability in degeneration may arise from specific activity requirements of subsets of neurons. Perhaps the more heavily active neurons during development are more susceptible to toxicity while other neurons with less activity demands are able to compensate for excess signaling. It will be interesting to see if the GABA neurons that are degenerating are of a specific class, VD or DD. During the transition from the first larval stage to the second larval stage (L2) the number of ventral nerve cord motor neurons increases substantially, with the addition of >50 motor neuron cell bodies (Sulston and Horvitz, 1977). The loss of GABA neurons has surprising effects on the postsynaptic receptors.

We found that despite the dramatic loss of dorsal cord coverage of GABA neurons the UNC-49 receptor remained localized to discrete sites along the dorsal cord. One might expect in the absence of presynaptic motor neurons the inhibitory post-synaptic receptor field would not develop properly. Alternatively, the receptor field may develop normally, but not be maintained and slowly degenerate over time. One possibility is that internalization or delocalization of receptors requires more time. From these experiments it is difficult to assess how long the presynaptic GABA neurons have been absent. Another possibility is once the NMJ has formed the proper localization of the excitatory NMJ receptors, ACR-16 and UNC-29, aid in maintaining UNC-49 localization.

One might then expect these animals to exhibit phenotypes similar to the phenotypes of GABA deficient mutants. Similar to GABA deficit mutants GABA specific expression of ACR-12(V/S) resulted in aldicarb hypersensitivity (*data not shown*) and reduced movement. However, these transgenic animals exhibited a low occurrence of the shrinker phenotype associated with GABA deficient mutants such as *unc-49(e382)*. One possibility is that the remaining GABA neurons expressing sensitized ACR-12(V/S) are enough to compensate for the increased excitation in the locomotory circuit. Another possibility is the sensitized ACR-12(V/S) receptor actually inhibits GABA neuron activity but the neurons still have residual tonic activity.

In conclusion, ACR-12(V/S) has provided us with the opportunity for characterizing timing mechanisms of cholinergic motor neurons in generating spontaneous contractions. Additionally ACR-12(V/S) has shown the GABA neurons to be more susceptible to ion channel-mediated toxicity compared to their cholinergic motor neuron counterparts. Moving forward it will be interesting to identify the mechanism underlying degeneration of GABA neurons, examine mechanisms for regulation of the inhibitory NMJ in more detail, and investigate additional mechanisms underlying GABA neuron activity.

A

M2 Transmembrane Region

UNC-29	EKITLTISVLLSIVVFLLLVSKILP
ACR-16	EKITLQITVLLSICFFLSIVSEMSP
ACR-2	EKVSLTISLLLALVVFLLLVSKILP
ACR-12	EKITFSISILVTLTVFFLVLDLMP
ALPHA7	EKISLGITVLLSLTVFMLLVAEIMP
BETA4	EKMTLCISVLLALTFELLISKIVP

B

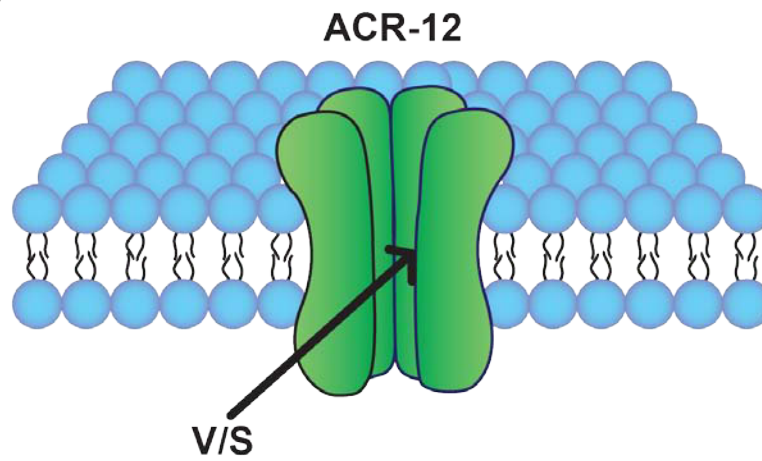


Figure 3-1

Figure 3-1 Mutating in a conserved residue of ACR-12. **(A)** Alignment of the second transmembrane domain of ACR-12 (green), other *C. elegans* nAChR (yellow), and various mammalian subunits (blue). The site of the 9' valine to serine gain-of-function mutation is indicated (red). Sequences from Genbank. **(B)** Schematic of ACR-12 receptor in cell membrane. Arrow indicates location of the valine to serine gain of function mutation.

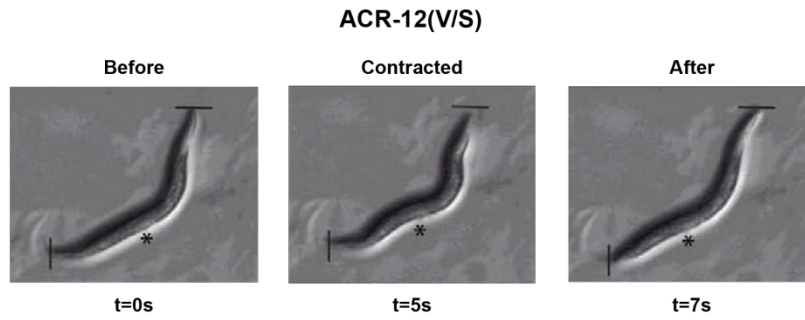
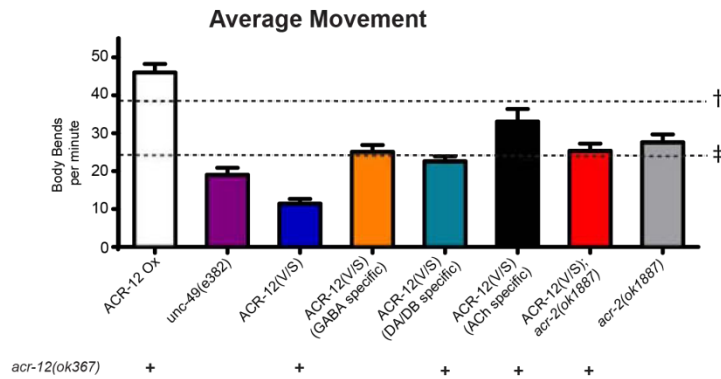
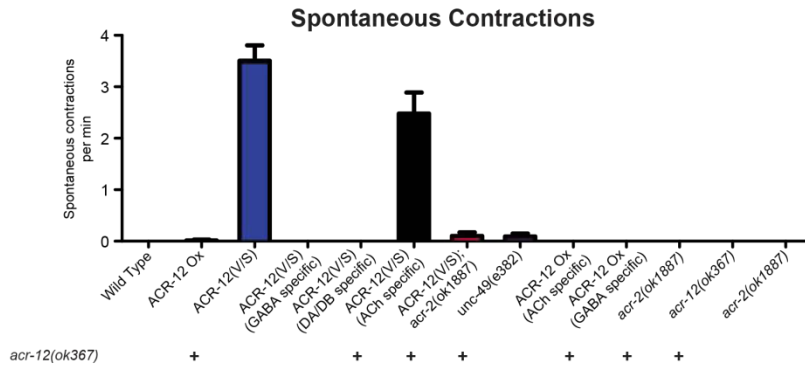
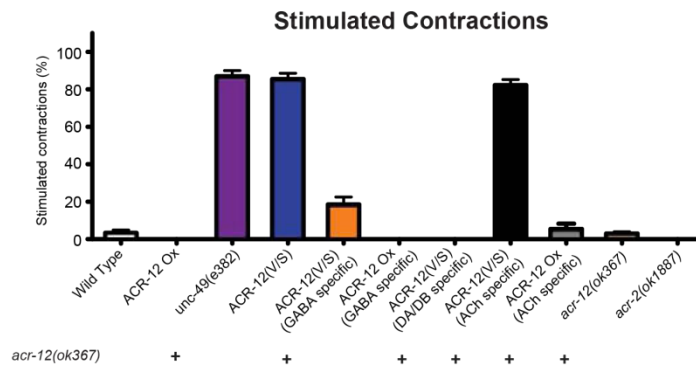
A**B****C****D****Figure 3-2**

Figure 3-2 ACR-12(V/S) animals exhibit dramatic behavioral deficits. **(A)** Still images showing representative spontaneous contractions of ACR-12(V/S) animals over a 7 second time interval. Black lines indicate original starting position of the nose and tail in t=0s still. Asterisks indicates posterior bend. **(B)** Bar graphs of the average number of body bends of ACR-12 Ox (genomic rescue) (white) [n=16], ACR-12(V/S);*acr-12(ok367)* (dark blue) [n=21], GABA-specific (*Punc-47*) ACR-12(V/S) (orange) [n=19], DA/DB-specific (*Punc-129*) ACR-12(V/S) (light blue) [n=10], ACh MN-specific (*Pacr-2*) ACR-12(V/S) (black) [n= 19], ACR-12(V/S);*acr-2(ok1887)*;*acr-12(ok367)* (red) [n=6], and *acr-2(ok1887)* (grey) [n=10]. For reference † indicates number of body bends for wild type animals and ‡ *acr-12(ok367)* mutants (see chapter 2) **(C, D)** Bar graphs of the average number of spontaneous contractions and average number of stimulated contractions of wild type (white) [n=21], [n=45], ACR-12 Ox (genomic rescue) (white) [n=22], [n=22], ACR-12(V/S);*acr-12(ok367)* (dark blue) [n=33], [n=22], GABA-specific (*Punc-47*) ACR-12(V/S) (orange) [n=11] [n=27], DA/DB-specific (*Punc-129*) ACR-12(V/S) (light blue) [n=11], [n=10], ACh MN-specific (*Pacr-2*) ACR-12(V/S) (black) [n= 23], [n=22], ACR-12(V/S);*acr-2(ok1887)*;*acr-12(ok367)* (red) [n=10], *unc-49(e382)* (purple) [n=25], [n=20], ACh-specific (*Pacr-2*) ACR-12-GFP rescue (grey) [n=11], [n=11], GABA-specific (*Punc-47*) ACR-12-GFP rescue (white) [n=11], [n=11], *acr-2(ok1887)*; *acr-2(ok1887)*;*acr-12(ok367)* (white [n=10]; *acr-12(ok367)* (white) [n=31], [n=43], and *acr-2(ok1887)* (white) [n=10], [n=10]. Sample sizes are listed where appropriate and respectively. For B-D genotypes in the *acr-12(ok367)* mutant background are indicated beneath each graph with a +.

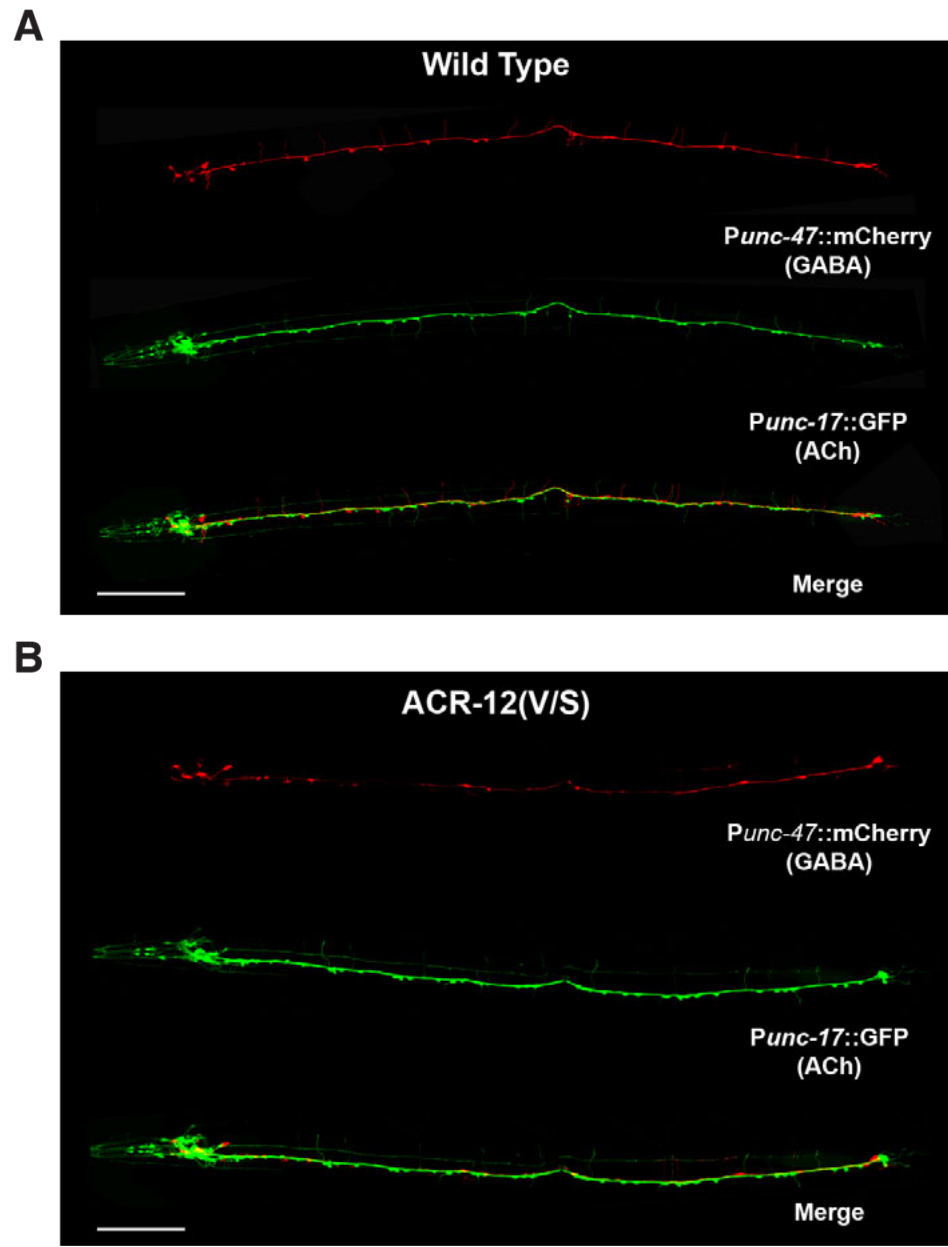


Figure 3-3

Figure 3-3 Expression of ACR-12(V/S) is toxic in GABA motor neurons. **(A)** Confocal image of a wild type animal showing coexpression of a cholinergic motor neuron-specific marker (*ufls48*, *Punc-17::GFP*), and a GABAergic specific marker (*ufls34*, *Punc-47::mCherry*) in the ventral nerve cord. **(B)** Confocal image of an ACR-12(V/S);*acr-12(ok367)* (*ufls90*) animal showing coexpression of a cholinergic motor neuron-specific marker (*ufls48*), and a GABAergic specific marker (*ufls34*) in the ventral nerve cord. Scale bars indicate 100 μ m and animals are oriented with their tail to the right.

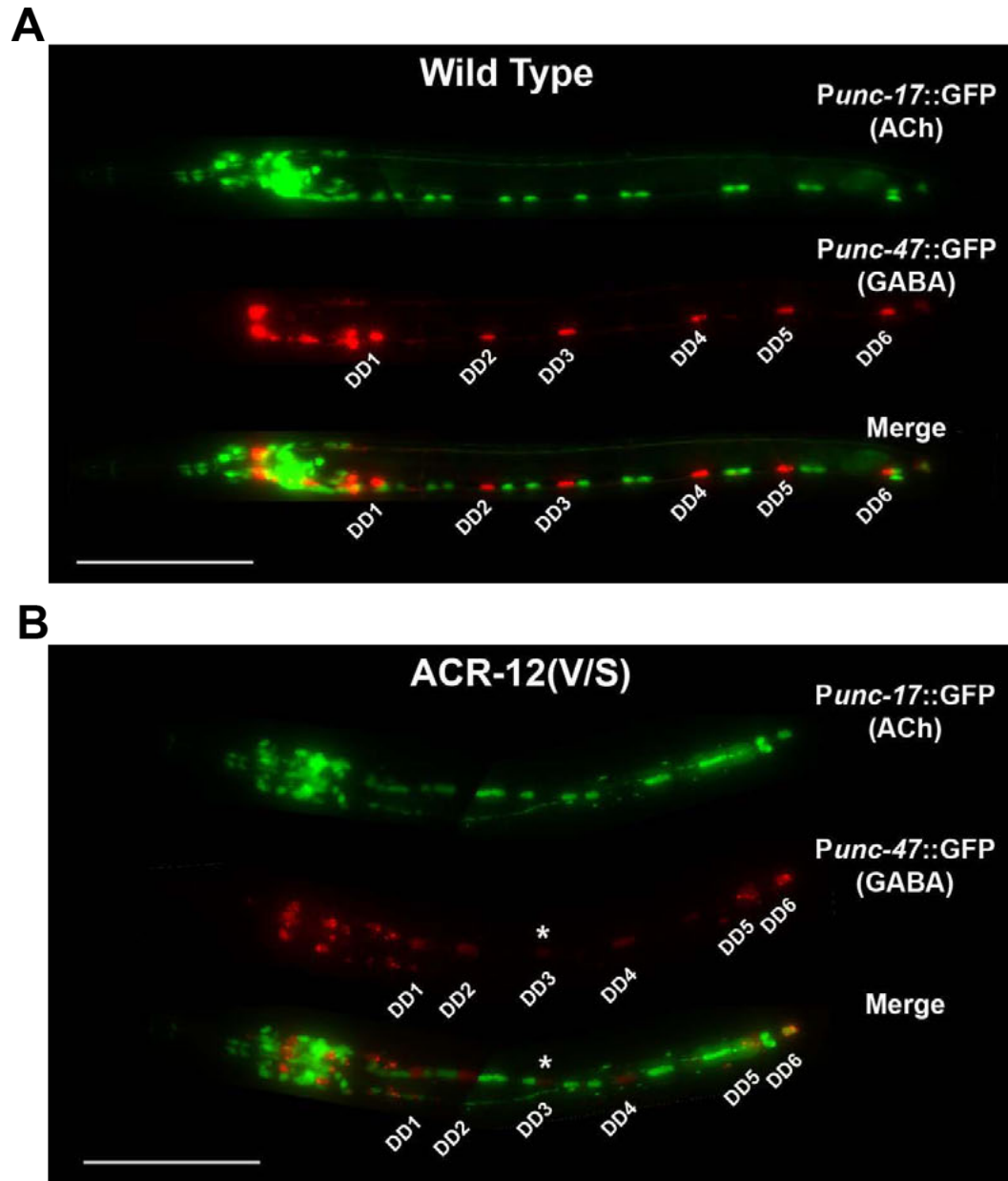


Figure 3-4

Figure 3-4 ACR-12(V/S) toxicity is observable in L1 larva. **(A)** Epifluorescence image of a wild type L1 animal (16hrs) showing coexpression of a cholinergic motor neuron-specific marker (*ufIs48*), and a GABAergic specific marker (*ufIs34*) in the ventral nerve cord. **(B)** Epifluorescence image of an ACR-12(V/S);*acr-12(ok367)* (*ufIs90*) L1 animal (16 hrs) showing coexpression of a cholinergic motor neuron-specific marker (*ufIs48*), and a GABAergic specific marker (*ufIs34*) in the ventral nerve cord. Scale bars indicate 50 μ m and animals are oriented with their tail to the right. Asterisk indicates weak expression.

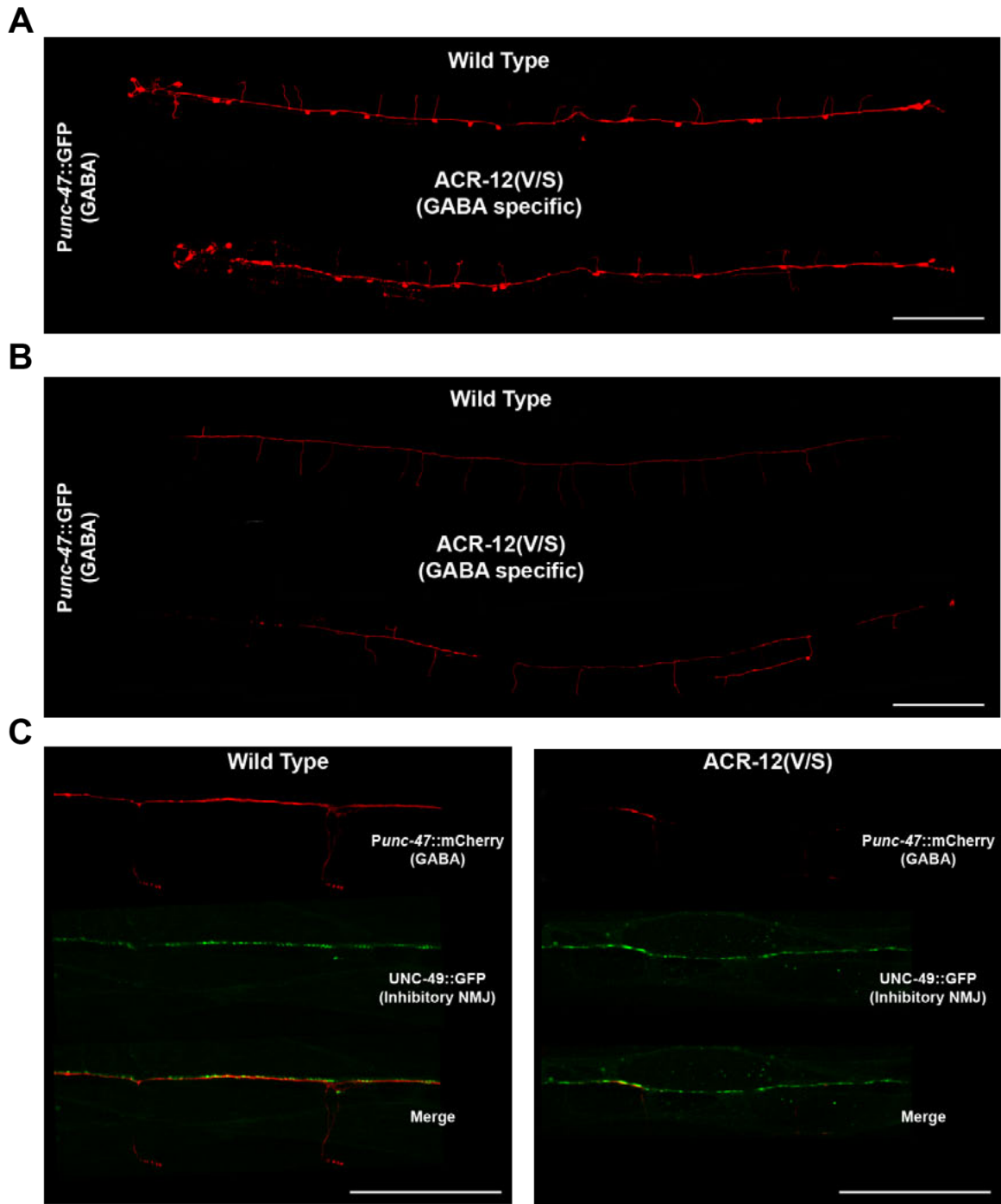


Figure 3-5

Figure 3-5 GABA-specific expression of ACR-12(V/S). **(A)** Confocal image of a wild type and GABA-specific (*Punc-47*) ACR-12(V/S) (*ufls102*) animals showing expression of a GABAergic specific marker (*ufls34*) in the ventral nerve cord. **(B)** Confocal image of a wild type and GABA-specific (*Punc-47*) ACR-12(V/S) (*ufls102*) animals showing expression of a GABAergic specific marker (*ufls34*) in the dorsal nerve cord. For A and B scale bar indicates 100 μ m. **(C)** Confocal image of a wild type and GABA-specific (*Punc-47*) ACR-12(V/S) (*ufls90*) animals showing coexpression of a GABAergic specific marker (*ufls34*) and UNC-49::GFP (*oxls22*) in the dorsal nerve cord. Scale bars indicate 50 μ m. For A-C animals are oriented with their tail to the right.

Chapter 4:

Discussion

In this thesis I have explored the mechanisms that drive neuronal activity. Using the *C. elegans* locomotory circuit I have made several interesting discoveries. First, similar to the mammalian CNS, I have identified diverse roles for distinct nAChRs subtypes in regulating neuronal activity. Two distinct classes of ACR-12 containing receptors are expressed in cholinergic motor neurons and GABA neurons. The cholinergic ACR-2R* is diffusely localized in dendrites and has a modulatory role in regulating neuronal activity, whereas the GABA neuron nAChR, ACR-12R* is localized to synapses, maintains consistency in movement, and mediates GABA neuron activity under conditions of elevated ACh release. Second, similar mutations in different subunits of the same receptor have dramatically different consequences for neuronal activity, suggesting receptor composition is crucial for function. Expression of ACR-2(L/S) resulted in toxicity of cholinergic motor neurons and subsequent degeneration, while expression of ACR-12(V/S) resulted in no obvious changes in neuronal architecture but profound behavioral consequences. Third, behavior can be adapted by altering the activity of subsets of neurons within a circuit. Over-activation of cholinergic motor neurons leads to the generation of spontaneous contractions while over-activation of GABA neurons resulted in decreased movement. Unexpectedly, I found that subsets of neurons within a circuit can exhibit differences in tolerances to over-active signaling. Interestingly, specific expression of ACR-12(V/S) in GABA neurons resulted in the loss of GABA neurons and commissural defects; in contrast, the integrity of the cholinergic nervous system was not obviously

affected. Lastly, I demonstrated that degeneration of subsets of neurons does not immediately affect postsynaptic receptors. Inhibitory NMJ UNC-49 receptors remained localized to discrete sites despite the absence of presynaptic GABA motor neurons. Taken together, this work provides exciting new insights into molecular mechanisms coordinating the activation of subsets of neurons in a circuit and the behavioral consequences.

Importance of regulating neuronal activity

The brain is capable of processing large amounts of stimuli with subtle changes in neuronal activity that translates into modified behavior. Maintaining tight coordination of circuit activity is critical for appropriately modifying behavior. When the patterned activation of neurons is deregulated there are profoundly adverse effects on behavior. For example, epilepsy, addiction, autism, dementia and depression are associated with deregulated or inappropriately modified brain signaling (Calabresi et al., 2007; Dani and Harris, 2005; Dzhala et al., 2012; Futatsugi and Riviello, 1998; Nordberg et al., 1992; Quik and McIntosh, 2006). Epilepsy is a clear example of the consequences associated with unregulated neuronal activity. Patients with epilepsy have what is called a “hyperexcitable” brain. Basically, patients suffer from one of two possibilities: (1) an excess of excitatory signaling or (2) a loss of inhibitory signaling which mimics an increase in excitation (Futatsugi and Riviello, 1998; Mulley et al., 2005; Raggenbass and Bertrand, 2002). As a result these patients suffer from convulsions (e.g., absence, grand mal, or partial) that have the potential to cause permanent brain

damage or, in extreme cases, death. Current treatments involve synthetically compensating for unregulated signaling to restore the brain to a more balanced state (Calabresi et al., 2007; Haut et al., 2006; Lee et al., 1995; Luszczki, 2009). Identifying the sources of inappropriate signaling will provide better therapeutic targets for maintaining a balanced brain state.

Advances in research have leaned on using models with dramatic cognitive defects to identify genes important for coordinating neuronal activity (Holmes and Zhao, 2008; Liu et al., 2012; Zhao-Shea et al., 2010). Yet, many fundamental questions about neuronal activity in physiological conditions remain unclear. For example, what are the molecules that regulate the timing of neural circuit activation and how is signaling between various neural circuits achieved? In order to adequately address how the nervous system functions, we have to focus on addressing these questions. It is critical that we identify the roles for ionotropic receptors in neuronal activity and how those changes in activity can affect signaling. We need to understand how activation of subsets of neurons is achieved and modifies behavior. Addressing these questions will provide valuable insights in our understanding of nervous system.

Identifying the versatile roles of nAChRs in neuronal function—are distinct receptor compositions functionally relevant?

A common feature of LGIC members such as GABA receptors or nAChRs is the diversity of subunits within the receptor family. This diversity suggests the subunit composition of fully formed receptors has functional significance. Within a

neuronal circuit individual neurons have specific roles in driving the activity of the circuit and the roles of each neuron can be very different. One possibility is diverse subunits fulfill the wide range of signaling requirements across the nervous system by forming distinct receptor types. Evidence of distinct receptor types suggests neuronal activity can be modified with a high degree of precision.

In vertebrates, nAChRs are localized predominantly at pre-synaptic terminals, although there are post-synaptic nAChRs in the sympathetic ganglia and muscle (Lindstrom et al., 1991; Rassadi et al., 2005). There are 17 nAChR subunits: α 1-10, β 1-4, δ , γ , and ϵ (Lindstrom, 2003; Millar and Harkness, 2008) which combine to form a myriad of distinct receptors. Nicotine's effects in the reward pathways are an excellent example of diverse roles for distinct nAChRs. Glutamatergic neurons that project to the ventral tegmentum area (VTA) contain pre-synaptic α 7 containing subunits (α 7*) (Millar and Gotti, 2009). These receptors act to increase excitation of post-synaptic dopamine neurons in the VTA. The dopamine neurons express α 4 β 2* receptors that regulate the activity of neurons, and β 2* containing receptors at presynaptic terminals to modulate release of neurotransmitter (Dani and Bertrand, 2007; Dani and Harris, 2005; Liu et al., 2012). In addition, inhibitory GABA neurons make synaptic contacts with the dopamine neurons in the VTA express α 4 β 2* receptors. Each of these nAChR classes has different localization patterns and affinity for nicotine (Dani and Harris, 2005). The actions of nicotine on nAChRs in this circuit result in nicotine dependence.

Similar to vertebrate models I have demonstrated nAChR have diverse functions in regulating the activity of locomotory neurons in *C. elegans*. ACR-2R receptors are diffusely localized in the upstream cholinergic motor neurons and modulate activity of the neurons (Barbagallo et al., 2010; Jospin et al., 2009). However, ACR-12R in GABA neurons localize to synapses and function to maintain the consistency in the amplitude of body bends over long forward runs. Under elevated levels of ACh release, ACR-12R can directly regulate GABA neurons activity (see Chapter II). Evidence for the importance of subunit composition in determining receptor functional properties can be found at the *C. elegans* NMJ as well. Previous work has identified nAChRs that mediate signaling at the excitatory NMJ. The homomeric ACR-16 receptor is responsible for nearly 90% of the excitatory signal yet deletion of this receptor subunit produces no obvious phenotype (Francis et al., 2005; Francis and Maricq, 2006). In contrast, UNC-29 receptors make up the remaining 10% of excitatory signal and have a more obvious uncoordinated movement (Culetto et al., 2004; Francis et al., 2005; Touroutine et al., 2005). Taken together, the distinct classes of nAChR in *C. elegans* with diverse roles reinforce the concept that subunit composition is an important factor in determining how receptors regulate neuronal activity.

It seems unlikely that subunit composition is established simply based on the expression profiles of the individual subunits. Expression profiling of neuronal subsets, including dopaminergic neurons in the substantia nigra and mitral cells,

shows expression of more than 5 nAChR genes within a population (D'Souza and Vijayaraghavan, 2012; Salminen et al., 2004). Dopamine neurons of the VTA express at least two distinct classes of nAChRs both of which contain the $\beta 2$ subunits (Dani and Harris, 2005). Similar studies in *C. elegans* indicate the presence of at least three additional nAChR subunits, *acr-9*, *acr-14*, and *lgc-11*, in GABA neurons (Cinar et al., 2005; Fox et al., 2005). In preliminary pharmacological experiments, I observed that mutation of any of these genes led to a different effects compared to mutation of *acr-12*, suggesting these subunits are unlikely to be obligate partners in a GABA neuron specific ACR-12R (*data not shown*). Some nAChR subunits preferentially interact with other specific subunits. For example, in vertebrates $\beta 3$ subunits preferentially associate with $\alpha 6$ subunits (Alkondon and Albuquerque, 2001; Drenan et al., 2008; Salminen et al., 2004). These data suggest there are mechanisms in place to determine the subunit composition of receptors. One possibility is that signaling motifs in the individual subunits regulate their interactions with other subunits. Another possibility is subsets of neurons express unique factors that establish appropriate subunit interactions. In the future these questions could be addressed by creating chimeric receptors. For example, it would be interesting to determine if swapping the large intracellular loop of a subunit that does not associate with ACR-12 with the intracellular loop of a subunit that does reconstitutes a functional receptor. Another approach would be to create knockouts of all the nAChRs expressed in

a subset of neurons and transgenically express the subunits of a receptor not found in that subset of neurons.

The punctate expression of GABA neuron specific ACR-12R* provides an opportunity to examine mechanisms of localization. Using GFP screens or a candidate gene approach we could determine proteins important for the localization and clustering of ACR-12R*s. Another possibility is subunit composition is important for the localization of receptors. This would suggest the subunits unique to the two distinct ACR-12Rs are driving their differential localization. Answers to these questions await determination of the precise subunit composition of the ACR-12 receptor in GABA motor neurons.

Identifying mechanisms that modify behavior—coordinating excitation and inhibition

Behavior is generated by the coordinated activation of neuronal circuits. However, the mechanisms that regulate the coordination of circuit activity remain unknown. The development of sensitized nAChRs has provided researchers with new tools for identifying the mechanisms of cholinergic regulation of neuronal activity. Reconstitution studies with gain of function receptors have provided insights into the functional contributions of specific subunits in receptors. For example, $\beta 3$ subunits act to enhance the activity of $\alpha 6^*$ containing receptors (Dash and Lukas, 2012). Here we have engineered a gain of function mutation in the second transmembrane domain of ACR-12 containing receptors in order to

identify additional mechanisms coordinating the precise timing of alternating movements in *C. elegans*. Similar mutations in the pore lining domain of mammalian $\alpha 6$ ($\alpha 6^{L9'S}$) nAChR subunit identified $\alpha 6\alpha 4\beta 2^*$ containing receptors as a key target for disorders associated with reduced dopamine release (Drenan et al., 2010). A $\alpha 4$ gain of function knock in mouse model identified interactions of nAChRs and dopamine D_2 receptors in regulating cholinergic interneuron activity (Zhao-Shea et al., 2010). We have demonstrated that ACR-12(V/S) animals exhibit spontaneous contractions. Cell specific drivers identified the cholinergic motor neurons as sufficient to recapitulate these contractions. Transgenic ACR-12(V/S) animals carrying a deletion for *acr-2* have suppressed spontaneous contractions. These results suggest the spontaneous contractions of ACR-12(V/S) arise from the activity of a previously characterized ACR-2R and not the presence of an aberrant nAChR.

One might expect the spontaneous contractions to arise out of deregulated timing mechanisms in the circuit. Interference with the timed activation of cholinergic neurons, which drive the locomotory circuit, could be sufficient for generating locomotory phenotypes. However, animals expressing ACR-12(V/S) in cholinergic motor neurons along the dorsal side with a cell specific promoter did not exhibit spontaneous contractions. This suggests the bilateral excitation of body wall musculature from ACR-12(V/S) in all cholinergic motor neurons is required for spontaneous contractions. Furthermore, these animals did not exhibit any profound changes in locomotory movement. One

reason for this could be the presence of the *acr-12(ok367)* mutant background. Previous behavior analysis has revealed *acr-12* mutants have reduced body bends/minute compared to wild type and an inconsistent waveform (Chapter II). This mutant background could be masking subtle changes in movement as a function of ACR-12(V/S) expression in DA and DB motor neurons. Nervous system development is not obviously affected by the expression of ACR-12(V/S) and increased aldicarb hypersensitivity suggests these animals have increased ACh release. Transgenic ACR-12(V/S) animals have provided insights into the diverse roles of cholinergic signaling in the *C. elegans* locomotory circuit. In the future we will be able to address additional questions about subunit composition, localization, and alternative degrees of excitability.

As previously mentioned in the introductory chapter of this thesis the activity of the locomotory circuit starts with command interneurons and is executed through excitatory and inhibitory motor neurons. Sensory information is relayed through command interneurons that synapse onto excitatory motor neurons. These excitatory motor neurons form dyadic synapses with body wall muscle and inhibitory GABA motor neurons(White et al., 1986). There are 5 sets of command interneurons: AVA, AVD, and AVE, for regulating backwards movement and AVB and PVC which regulate forward movement. The AVA, AVE, and AVD initiate backward movement through electrical and chemical contacts with the A motor neurons (backwards movement). AVB and PVC regulate forward movement via gap junctions and chemical synapses with B type motor

neurons (forward movement) (Chalfie et al., 1985; Wicks et al., 1996; Zheng et al., 1999). Specifically, AVA and AVB are directly connected with their respective motor neuron populations through gap junctions and function to drive forward and backward directionality of the animal. Animals mutant for innexins, UNC-7 and UNC-9, shortened body bends, or kinking, which arise from equal activation of both forward and backward motor neurons. Interestingly, experiments where the activity of either motor neuron population was reduced, the animals resumed movement biased by the shift in the activity equilibrium (Kawano et al., 2011).

One model for the diverse roles of ACR-12R* in cholinergic and GABAergic neurons would be support maintaining the activity equilibrium of the motor neurons. For example, during forward movement spill over of ACh from the synapses of B motor neurons activate ACR-2* receptors on the dendrites of B motor neurons. Activation of these receptors maintains a higher activity level in B motor neurons than that of A motor neurons and therefore maintains forward movement. Animals expressing the light activated ion channel, channelrhodopsin (ChR-2), in both A and B motor neurons exhibited a dramatic decrease in movement and ability to propagate a wave with blue light exposure. This further supports the idea that changes in this equilibrium will result in changes in directionality of the animal (Kawano et al., 2011). To address this one experiment would be to express ACR-12(V/S) using cell specific promoters in A or B motor neurons in order to hyperactivate specific classes of neurons in innexin mutants and look for a restoration in either directionality of movement. Further down the

neuronal circuit is the timed activation of GABA neurons and body wall muscle. We have identified a unique class of ACR-12R* that play a role in maintaining the coordination of body bends. Loss of these receptors leads to variability in long forward runs (Chapter II). Taken together these results suggest mechanisms dedicated to coupling each level of the locomotory circuit are important for the overall propagation of the sinusoidal wave. Interestingly, we found that loss of ACR-12R* in GABA motor neurons ameliorated the paralysis induced by the expression of ChR-2 in cholinergic motor neurons. The animals were able to move forward exhibiting only mild effects from blue light exposure. The presence of forward movement suggests that ACR-12R* in GABA motor neurons alters the motor circuit equilibrium to favor forward movement. One possibility is that a decrease in synaptic drive from photoresponsive ACh MNs allows for coordination of GABA MN activity through other pathways, enabling movement.

The many roles of nAChRs in nematode movement provides an opportunity to address additional questions surrounding cholinergic signaling that will be beneficial for our understanding of vertebrate CNS function. Are there changes in downstream NMJ receptor localization when cholinergic motor neurons are hyperactive? Are there obvious changes in behavior in a more challenging environment for movement? For example, in swimming behaviors do transgenic ACR-12(V/S) animals exhibit changes in body wall muscles due to over activation. Mutations to alternative amino acids have less profound effects in channel kinetics, using these mutations we can potentially identify alternative

phenotypes associated with a more mild increase in signaling (Imoto et al., 1988; Revah et al., 1991). For example, it would be interesting to see if alternative, less severe mutations in the second transmembrane domain of ACR-12 could affect the generation of spontaneous contractions.

Characterizing preferential neuronal degeneration—mechanisms of toxicity for GABA neurons

Unexpectedly, we found cell specific expression of ACR-12(V/S) in GABA neurons produced toxicity and subsequent loss of GABA neurons. The cholinergic nervous system remained intact with no obvious changes in connectivity, while the GABA nervous system exhibit neuronal degeneration that was severe in most animals. Toxicity was cell autonomous, suggesting fundamental differences in motor neuron populations. This finding could provide valuable insights into unique properties of GABA neurons. Perhaps GABA neurons express specific protein(s) that makes GABA neurons more sensitive to changes in activity. Alternatively, these neurons may lack a protein protective of toxicity. This toxicity was variable across animals and could be observed as early as L1 animals. The variability in degeneration may arise from specific activity requirements of subsets of neurons. Perhaps the more heavily active neurons during development are more susceptible to toxicity while other neurons with less activity demands are able to compensate for excess signaling. It will be interesting to see if the GABA neurons that are degenerating are of a specific

class, VD or DD. During the transition from the first larval stage to the second larval stage (L2) the number of ventral nerve cord motor neurons increases substantially, with the addition of >50 motor neuron cell bodies (Sulston and Horvitz, 1977). In the future we can identify the additional subunits of this receptor by looking for suppression of degeneration. Additionally, genetic screens could identify the mechanisms of degeneration or proteins important for the synthesis of nAChRs. The loss of GABA neurons has surprising effects on the postsynaptic receptors.

Despite dramatic defects in GABA motor neuron innervation of the dorsal musculature, we found the UNC-49 receptor remained localized to discrete sites along the dorsal cord. One might expect in the absence of presynaptic motor neurons there would be consequences for the downstream inhibitory receptors of the NMJ. One possibility is that the internalization or delocalization of receptors requires more time. While we observe defects as early as the L1 stage, we cannot be sure that degeneration does not also occur in later life stages. Therefore, at this time we cannot say the presynaptic neuron has been degenerated for a pre-determined amount of time. It is possible the gaps in coverage in the dorsal cord we see are from recent loss of presynaptic neurons. Another possibility is once the NMJ has formed the proper localization of the excitatory NMJ receptors, ACR-16 and UNC-29, aid in maintaining UNC-49 localization. In the future it will be interesting to examine the localization of UNC-

49 receptors under conditions where we can track degeneration of presynaptic GABA neurons.

One might then expect these animals to exhibit phenotypes similar to the phenotypes of GABA deficient mutants. Similar to GABA deficit mutants GABA specific expression of ACR-12(V/S) resulted in aldicarb hypersensitivity (*data not shown*) and reduced movement. However, these transgenic animals exhibited a low occurrence of the classical shrinker phenotype associated with GABA deficit mutants such as *unc-49(e382)* (Bamber et al., 1999). One possibility is that the remaining GABA neurons expressing sensitized ACR-12(V/S) are enough to compensate for the increased excitation in the locomotory circuit. Another possibility is the sensitized ACR-12(V/S) receptor actually inhibits GABA neuron activity but the neurons still have residual tonic activity. In this case, using the previously mentioned method of alternative second transmembrane domain mutations we may be able to examine behaviors associated with mild increases in GABA neuronal activity.

In conclusion, identification of a distinct class of nAChRs in driving the activation of subsets of motor neurons during nematode locomotion has provided us with insights into the mechanisms of vertebrate CNS functions. nAChRs can have dramatically different roles in regulating the activity of neuronal circuits depending on the amount of neurotransmitter present. ACR-12 mutants in low levels of ACh release were unable to maintain consistent amplitude across

consecutive body bends, while these mutants were able to suppress paralysis induced by elevated levels of ACh release induced by Channelrhodopsin-2. Interfering with the timed activation of cholinergic motor neurons activating the dorsal body muscle was not sufficient to generate spontaneous contraction. However, simultaneous activation of all the cholinergic motor neurons is sufficient for spontaneous contractions. In the process of this thesis work we have obtained several tools to examine new questions of subunit composition, localizations and mechanisms of activity. Moving forward we will also be able to characterize factors mediating the unique toxicity tolerance of GABA neurons compared to their cholinergic motor neurons counterparts. Additionally, it will be interesting to identify the mechanisms maintaining the inhibitory NMJ regulation, and additional mechanisms for GABA neuron activity.

Appendix I:

**A Dominant Mutation in a Neuronal
Acetylcholine Receptor Subunit Leads to
Motor Neuron Degeneration in
*Caenorhabditis elegans***

This manuscript has been modified from the published version: Figures have been renumbered and references have been removed and merged into the dissertation reference list. Minor typographical errors have been corrected: Belinda Barbagallo, Hilary A. Prescott,* Patrick Boyle*, Jason Climer, and Michael M. Francis. J Neuroscience, 20 October 2010: Vol. 30. No.42. pp 13932-13942.

Abstract

Inappropriate or excessive activation of ionotropic receptors can have dramatic consequences for neuronal function and, in many instances, leads to cell death. In *Caenorhabditis elegans*, nicotinic acetylcholine receptor (nAChR) subunits are highly expressed in a neural circuit that controls movement. Here, we show that heteromeric nAChRs containing the *acr-2* subunit are diffusely localized in the processes of excitatory motor neurons and act to modulate motor neuron activity. Excessive signaling through these receptors leads to cell-autonomous degeneration of cholinergic motor neurons and paralysis. *C. elegans* double mutants lacking calreticulin and calnexin—two genes previously implicated in the cellular events leading to necrotic-like cell death (Xu et al., 2001)—are resistant to nAChR mediated toxicity and possess normal numbers of motor neuron cell bodies. Nonetheless, excess nAChR activation leads to progressive destabilization of the motor neuron processes and, ultimately, paralysis in these animals. Our results provide new evidence that chronic activation of ionotropic receptors can have devastating degenerative effects in neurons and reveal that ion channel-mediated toxicity may have distinct consequences in neuronal cell bodies and processes.

Introduction

Roles for ionotropic receptor-mediated signaling in the nervous system extend far beyond a well characterized participation in cell– cell communication

at synapses. Ionotropic receptor activation is one of several key factors that influence cell survival in developing and mature nervous systems. For example, signaling through nicotinic acetylcholine receptors (nAChRs) promotes the elimination of neurons in the developing avian autonomic nervous system (Hruska et al., 2009; Hruska and Nishi, 2007), and inappropriate pharmacological activation of nAChRs in the *Caenorhabditis elegans* nervous system leads to developmental arrest (Ruaud and Bessereau, 2006). In the mature nervous system, inappropriate or excessive ion channel activation can have dramatic consequences. In mammals, hypoxic events, such as stroke, lead to excitotoxic cell death as a consequence of excess glutamate release and hyperexcitation of ionotropic glutamate receptors (iGluRs) (Sattler and Tymianski, 2001). Likewise, mutations that cause prolonged activation of nAChRs or iGluRs can lead to neurodegeneration and cell death in organisms ranging from nematodes to mammals (Heintz and Zoghbi, 2000; Labarca et al., 2001; Miwa et al., 2006; Orb et al., 2004; Orr-Urtreger et al., 2000; Treinin and Chalfie, 1995; Zuo et al., 1997). Excess ion channel activation is also a contributing factor in neurodegenerative diseases. For example, the selective vulnerability of motor neurons to cell death in amyotrophic lateral sclerosis (ALS) is believed to arise, at least in part, from hyperactivation of calcium-permeable AMPA type iGluRs (Grosskreutz et al., 2010; Kwak and Weiss, 2006). Interestingly, in various mouse models of motor neuron diseases, including ALS, genetic manipulations that prevent the death of motor neuron cell bodies are not successful in halting

disease progression (Gould et al., 2006; Sagot et al., 1995). This result implies that ion channel hyperactivation may contribute to degenerative events that persist even under conditions when neuronal cell death is attenuated. Shared features of ion channel-mediated degeneration across these diverse receptor types and systems suggest that aspects of this process may be broadly conserved across organisms (Driscoll and Gerstbrein, 2003). In most cases, however, a cohesive picture of the cellular events that influence the progression toward cell death as a consequence of ion channel hyperactivation remains unclear.

Ionotropic receptor signaling and its contribution to neurodegeneration can be dissected in detail in the compact nervous system of the nematode *Caenorhabditis elegans*. Here, we provide evidence that the non- α nAChR subunit ACR-2 contributes to a heteromeric receptor that is important for regulating the activity of excitatory motor neurons. A pore modification in ACR-2 leads to loss of motor neurons and paralysis of the animal. Genetic ablation of nAChR subunits that coassemble into a heteromeric receptor complex with ACR-2 suppresses ACR-2(L/S) (where L/S is leucine-to serine substitution) toxicity. In addition, *C. elegans* double mutants lacking two genes previously implicated in calcium homeostasis and necrotic cell death (*crt-1/calreticulin* and *cnx-1/calnexin*) are resistant to nAChR-mediated toxicity and possess normal numbers of motor neuron cell bodies. Nonetheless, we observe a progressive degeneration of the motor neuron processes that leads to paralysis in these

animals. Thus, ion channel hyperactivation has distinct consequences for neuronal cell bodies and processes.

Materials and Methods

Molecular biology

AChR subunit constructs. The full-length ACR-2::GFP transgene (pDM1232) was generated by introducing the green fluorescent protein (GFP) coding sequence in-frame into the sequence of an *acr-2* genomic fragment (-3353 to +7776 bp relative to the translational start site) encoding the intracellular loop between transmembrane domains TM3 and TM4. The full-length ACR-2(L/S) construct (pBB9) was generated by PCR-based site directed mutagenesis using mutant primers and pDM1232 as the template. The *Pacr-2*::ACR-12 cDNA (pHP3) and *Punc-47*::ACR-12 cDNA (pBB25) constructs were generated by amplifying the *acr-12* cDNA from the expressed sequence tag yk1093d12 (gift from Yuji Kohara, National Institute of Genetics, Mishima, Japan) using sequence-specific primers designed to the start and stop codons of *acr-12* and subcloning it into the *Nhe*I/*Sac*I sites of a plasmid containing an ~3.3 kb promoter for the *acr-2* gene or into a plasmid containing a 1.3 kb promoter for the *unc-47* gene.

Transcriptional reporters. The *Punc-47*::mCherry construct (pPRB5) was generated by subcloning an *Age*I/*Aat*II fragment that contained the full-length mCherry coding sequence into a vector containing a 1.3 kb promoter for the *unc-47* gene. The *Pacr-2*::GFP (pPRB19) construct was generated by subcloning an

AatII/BamHI fragment that contained an ~3.3 kb promoter for the *acr-2* gene into a vector containing the GFP coding sequence.

C. elegans strains

C. elegans strains were grown on nematode growth medium (NGM) plates with the OP50 strain of *Escherichia coli* at 22°C using standard laboratory procedures. Wild-type animals are the N2 Bristol strain. All transgenic strains were obtained by microinjection of plasmid DNA into the germ line and data presented are from a single representative transgenic line unless noted otherwise. In all cases, *lin-15(n765ts)* mutants were injected with the *lin-15* rescuing plasmid (pL15EK; 30 ng/μl) and one or more of the following plasmids (30 ng/μl): pBB9, pBB25, pDM1232, pHP3, pPRB4, pPRB5, pPRB14, pPRB19. Multiple independent extragenic lines were obtained for each transgenic strain. Stably integrated lines were generated as necessary by x-ray integration and outcrossed at least four times to wild type. The transgenic strain expressing the integrated ACR-2(L/S) transgene (*ufls25*) was outcrossed 10 times to wild type.

The following strains were used in this study: RB1559 *acr-2(ok1887)*, IZ421 *acr-12(ok367)*, RB2071 *ced-3(ok2734)*, VC1801 *cnx-1(ok2234)*, RB1021 *crt-1(ok948)*, IZ74 *unc-29(x29)*, CB904 *unc-38(e264)*, CB306 *unc-50(e306)*, VC731 *unc-63(ok1075)*, CB883 *unc-74(e883)*, IZ380 *ufls31*, IZ814 *ufls25*, IZ625 *ufls25;ufls31*, IZ790 *ufls49*, IZ627 *ufls42*, LX949 *vsIs48*, IZ924 *ufls25;vsIs48*, IZ950 *ced-3(ok2734); ufls25;vsIs48*, IZ877 *cnx-1(ok2234);ufls25;vsIs48*, IZ926 *crt-1(ok948); ufls25;vsIs48*, IZ976 *crt-1(ok948);cnx-1(ok2234);ufls25;vsIs48*,

IZ971 *crt-1(ok948);cnx-1(ok2234);vsIs48*, IZ927 *crt-1(ok948);cnx-1(ok2234);ufls42*, IZ787 *unc-29(x29);ufls25*, IZ928 *acr-3(ok2049);ufls265*, IZ929 *acr-5(ok180);ufls25*, IZ930 *acr-9(ok933);ufls25*, IZ931 *acr-19(ok967); ufls25*, IZ932 *acr-23(ok2840);ufls25*, IZ446 *unc-38(e264);ufls25*, IZ659 *unc-50(e306);ufls25*, IZ921 *unc-63(ok1075);ufls25*, IZ490 *unc-68(e540); ufls25*, IZ604 *unc-74(e883);ufls25*, IZ673 *acr-12(ok367);ufls25;ufEx148*, IZ937 *acr-12(ok367);ufls25;ufls60*, IZ861 *acr-12(ok367);ufls25;ufEx191*.

Genetic screen and identification of suppressors of ACR-2(L/S)-induced paralysis

Paralyzed animals expressing the integrated ACR-2(L/S) array (*ufls25*) were mutagenized with 50 mM ethyl methanesulfonate (Brenner, 1974). Young adult F2 progeny of ~20,000 mutagenized animals were washed twice with M9 buffer and transferred to fresh plates. After allowing time for the animals to disperse, moving animals were picked to single plates. Eighty one candidate suppressors were isolated. A secondary screen showed that fifty-one of these were resistant to the paralyzing effects of levamisole. For genetic complementation tests, males carrying a mutation in candidate levamisole-resistance genes were crossed with hermaphrodites carrying the ACR-2(L/S) transgene and a suppressor mutation. F1 progeny were scored for paralysis. A cross was performed in parallel using N2 males to identify X-linked suppressor mutations and determine dominance recessivity. The mapping of *acr-12* alleles was performed in the presence of the ACR-2(L/S) transgene. A strain carrying

the integrated ACR-2(L/S) transgene (*ufls25*) on linkage group I was backcrossed 7X to the CB4856 Hawaiian strain. *acr-12* alleles were mapped to a region the right of +8 on the X chromosome using the SNP mapping procedure as described previously (Davis et al., 2005; Wicks et al., 2001).

Behavioral assays

All behavioral analysis was performed with young adult animals (24 h post-L4) at room temperature (22°C–24°C); different genotypes were scored in parallel, with the researcher blinded to the genotype.

Aldicarb and levamisole assays. Staged populations of adult animals (~10) were transferred to NGM plates containing 1mM aldicarb (Chem-Service), and movement was assessed every 15 min for 2 h. Data represent the mean \pm SEM of at least four assays. For levamisole assays, staged populations of adult animals were scored for paralysis after 120 min on plates containing 200 μ M levamisole.

Body bend analysis. Body bends were scored on unseeded NGM agar. Animals were transferred from their culture plate to an unseeded plate and allowed to crawl away from any food that might have been transferred. The animals were then gently transferred without food to another unseeded plate and allowed to recover for 1 min. After the recovery period, the animals were filmed for 5 min using an ImagingSource DMK 21F04 FireWire camera and iMovie software.

Microscopy

Confocal microscopy was performed using a Zeiss Axioskop 2 microscope system and LSM Pascal 5 imaging software (Zeiss). Images were processed using ImageJ software (open source). Epifluorescent imaging was performed using a Zeiss Axioimager M1 microscope and Axiovision software (Zeiss). Movies and still images for behavioral analyses were obtained using an Olympus SZ61 upright microscope equipped with a FireWire camera (ImagingSource). For the developmental timeline, synchronized populations were obtained by bleaching gravid animals on NGM plates seeded with OP50. The resulting progeny were allowed to mature at room temperature. Animals were imaged at 16, 28, 38, and 48 h after bleaching using wide-field epifluorescent microscopy, and the number of surviving cell bodies were counted manually with the researcher blinded to genotype.

Results

***acr-2* encodes a nicotinic receptor subunit expressed in cholinergic motor neurons**

The *C. elegans* genome encodes 29 nAChR subunits that contribute to the formation of distinct classes of homopentameric and heteropentameric receptors (Jones et al., 2007; Rand, 2007). Two classes of nAChRs formed from six of these subunits are expressed in body wall muscle cells and required for neuromuscular signaling (Francis et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005). Previous studies have suggested that the expression of several nicotinic acetylcholine receptor subunits, including the non- α subunit *acr-*

2, is enriched in motor neurons of the ventral nerve cord (Cinar et al., 2005; Hallam et al., 2000; Jospin et al., 2009). To determine which ventral cord motor neurons express *acr-2*, we examined transgenic strains that expressed GFP (Chalfie et al., 1994) under the control of *acr-2* regulatory sequences. Expression of *Pacr-2::GFP* was limited to the nervous system and largely restricted to neurons located in the ventral nerve cord. Expression of *Pacr-2::GFP* did not overlap with expression of a GABAergic mCherry reporter (*Punc-47::mCherry*), indicating that expression of *acr-2* was limited to cholinergic motor neurons (DA, VA, DB, VB) in the ventral nerve cord of adult animals (Figure A1-1A). To determine the subcellular localization of ACR-2, we generated transgenic strains that expressed a full length ACR-2::GFP fusion protein in which GFP was inserted in frame in the intracellular loop region located between transmembrane domains three and four (Figure A1-S1, available at www.jneurosci.org as Supplemental Material). Expression of the GFP reporter construct could be observed at all larval stages and in the adult. We noted the onset of expression in late embryogenesis by the threefold stage (~550 min after fertilization). In first larval stage (L1) animals, when DA and DB motor neurons are the sole excitatory motor neurons present, ACR-2 expression was clearly visible in ventral nerve cord processes. DA and DB motor neuron dendrites receive synaptic input in the ventral cord, and these neurons extend commissural axons to the dorsal cord where they form neuromuscular synapses with the dorsal musculature. In adult animals, we found that the fusion protein was diffusely localized to neuronal

processes of the dorsal and ventral nerve cords. Our analysis suggests that ACR-2 is diffusely localized with enriched expression in the dendritic compartment of cholinergic motor neurons. The locomotory control interneurons provide synaptic input to the excitatory motor neurons; however, the role of acetylcholine (ACh) in this signaling remains unclear. To evaluate whether the locomotory control interneurons are cholinergic, we examined transgenic strains that coexpressed GFP under control of regulatory elements for the gene encoding the ACh vesicular transporter (*Punc-17::GFP*) together with the red fluorescent protein mCherry expressed under the control of the regulatory elements for the *nmr-1* gene (*Pnmr-1::mCherry*) (Figure A1-S2). *nmr-1* is expressed in the AVA, AVD, AVE, and PVC locomotory control interneurons, as well as the RIM and AVG neurons (Brockie et al., 2001). We noted no overlap in the pattern of the red and green fluorescent signals with the possible exception of the interneuron AVE, indicating that these two reporters labeled almost completely independent cell populations. These data are consistent with the idea that the locomotory control interneurons are not primarily cholinergic. The enriched expression of ACR-2 in the dendritic compartment of motor neurons may reflect the involvement of ACR-2 receptor complexes at synapses between AVE and cholinergic motor neurons; however, the lack of *Punc-17::GFP* expression in a majority of locomotory control interneurons and the diffuse distribution of ACR-2 are inconsistent with an exclusive role at synapses.

***acr-2* mutants have motor deficits**

The cholinergic motor neurons in the ventral nerve cord make synaptic contacts onto the body wall musculature that drives nematode locomotion. To evaluate the contribution of ACR-2 to cholinergic motor neuron excitability and motor output, we obtained a strain carrying a deletion mutation (*ok1887*) in the *acr-2* genomic locus. The *acr-2(ok1887)* eliminates ~2.8 kb of chromosomal DNA, including the transcriptional start, and is likely to be a null. Animals homozygous for the *ok1887* allele are healthy and viable. *acr-2(ok1887)* mutants are not obviously uncoordinated, though closer inspection revealed a modest decrease in locomotion rate (Figure A1-1B). Expression of the full-length ACR-2::GFP fusion protein in *acr-2(ok1887)* mutants was sufficient to restore normal movement.

The acetylcholinesterase inhibitor aldicarb has proven to be a useful tool for detecting alterations in neurotransmitter release from cholinergic motor neurons. To test whether ACR-2 receptor complexes may be important for regulating activity of the cholinergic motor neurons, we examined whether *acr-2* mutant worms exhibit altered sensitivity to the paralyzing effects of aldicarb. *acr-2* mutant animals were slightly resistant to paralysis by aldicarb, and this effect was normalized by expression of ACR-2::GFP (Figure A1-1C). These data are consistent with the notion that ACR-2 plays a role in modulating the activity of cholinergic motor neurons but suggest that ACR-2 is not absolutely required for motor neuron depolarization.

Introducing a dominant mutation in ACR-2 leads to profound motor deficits

The second transmembrane domains of Cys-loop family ligand-gated ion channel subunits are well known to line the ion channel pore and play a critical role in channel gating. In particular, a highly conserved nonpolar residue (typically leucine) in the M2 region has been shown to have profound effects on receptor activation properties (Figure A1-S3). Substitution of a polar amino acid (e.g., serine) for the leucine at this position produces a gain-of-function effect, resulting in increased receptor activation and very slow inactivation (Labarca et al., 1995; Revah et al., 1991).

We engineered the homologous leucine-to-serine point mutation into the sequence encoding the M2 9' position of an *acr-2* rescuing construct to generate ACR-2(L/S) (Figure A1-S3). Transgenic animals expressing an integrated ACR-2(L/S) array (*ufls25*) were used for all subsequent analyses. These animals are viable and have roughly normal brood sizes; however, adult animals are smaller than their wild-type counterparts (Figure A1-2). Moreover, we noted obvious locomotory defects in transgenic ACR-2(L/S) animals (Figure A1-2C). These defects were present in all larval stages as well as adult animals. Transgenic ACR-2(L/S) animals generated almost tenfold fewer body bends than the wild type and failed to propagate the sinusoidal wave that is typical of nematode movement, although animals remained capable of head foraging movements. The effects of ACR-2(L/S) were dominant, consistent with the notion that the phenotypes arose as a consequence of expression of a gain-of-function receptor.

Our results suggest that motor output to head muscles is unaffected, while control of body wall musculature is dramatically impaired in these animals.

ACR-2(L/S)-induced motor neuron degeneration

Examination of the ventral nerve cord region of transgenic ACR-2(L/S) animals by differential interference contrast (DIC) microscopy showed that a subset of the ventral nerve cord neurons that normally express *acr-2* swelled beyond their normal diameter and eventually disappeared, presumably as a result of cell death (Figures A1-3, 4). These results suggest that enhanced cholinergic signaling mediated by receptors incorporating ACR-2(L/S) leads to motor neuron toxicity. To characterize this in more detail, we examined the effects of ACR-2(L/S) expression in strains carrying fluorescent reporters that label populations of cholinergic neurons. We observed only dim *Pacr-2::GFP* fluorescence in the ventral nerve cord of ACR-2(L/S) animals, suggesting that many of the neurons labeled by this reporter were lost (data not shown). To evaluate the specificity of this effect for neurons that expressed *acr-2*, we examined a *Punc-17::GFP* reporter that is expressed in all cholinergic neurons (Figure A1-3) (Chase et al., 2004). While ACR-2(L/S) expression did not produce obvious differences in the number of head neurons labeled by *Punc-17::GFP*, we observed a dramatic decrease in the number of ventral nerve cord motor neuron cell bodies; yet, several motor neuron cell bodies remained present. The surviving neurons included the six VC motor neurons that do not normally express *acr-2* and a more variable group of 10–12 additional excitatory motor

neuron cell bodies (Figures A1-3; Figure A1-S4). Based on the position and number of cell bodies and commissural processes, the additional surviving neurons included both DA and DB motor neurons that normally express *acr-2*, as well as AS motor neurons that do not. Similar to our observations for ACR-2(L/S)-induced paralysis, the effects of ACR-2(L/S) on motor neurons were dominant. To evaluate the effects of ACR-2(L/S) on GABA motor neurons, we coexpressed ACR-2(L/S) together with a mCherry transcriptional reporter that labeled GABA neurons (*Punc-47::mCherry*) (Figure A1-3C,D). We observed that the full complement of GABA neurons was present and morphologically normal. These data suggest that ACR-2(L/S) acts cell autonomously to promote degeneration of motor neurons and that specific neurons are differentially susceptible to the effects of ACR-2(L/S) expression.

Motor neuron loss occurs soon after onset of ACR-2(L/S) expression

As noted above, we observed clear ACR-2::GFP fluorescence in late embryogenesis. We found that threefold embryos coexpressing ACR-2(L/S) with the *Pacr-2::GFP* transcriptional reporter possessed normal numbers of GFP-positive neurons, suggesting that ACR-2(L/S) toxicity occurred after hatch (Figures A1-S4). To precisely determine the onset of motor neuron cell death, we imaged transgenic ACR-2(L/S) animals that coexpressed the *Punc-17::GFP* transcriptional reporter at various time points ranging from newly hatched larvae to adults (Figure A1-4). We observed significant motor neuron loss in newly hatched larvae. Roughly 40% of the 16 cholinergic motor neurons present in L1

animals were lost within 16 h after hatch. During the transition from the first larval stage to the second larval stage (L2) the number of ventral nerve cord motor neurons increases substantially, with the addition of >50 motor neuron cell bodies (Sulston and Horvitz, 1977). In transgenic ACR-2(L/S) animals we observed only a slight increase in the number of cell bodies labeled by *Punc-17::GFP* over the course of development. Even by the time transgenic ACR-2(L/S) animals had clearly reached adulthood, the number of *Punc-17::GFP* labeled motor neuron cell bodies was roughly comparable to that of a wild-type L1 animal. The VC motor neurons do not express *acr-2* and are clearly present in transgenic ACR-2(L/S) animals (Figure A1-3; Figure A1-S5). Therefore, the small developmental increase in the number of motor neuron cell bodies that we observe in transgenic ACR-2(L/S) animals likely represents the postembryonic addition of VC neurons. These results suggest that the other classes of motor neurons that are born postembryonically and normally express *acr-2* (e.g., VA, VB) are almost completely absent in adult transgenic ACR-2(L/S) animals.

Mutations that suppress paralysis define the subunits of a neuronal nAChR

To identify genes required for the toxic effects of transgenic ACR-2(L/S) expression, we conducted a forward genetic screen for suppressors of ACR-2(L/S)-induced paralysis. We screened the F2 progeny of mutagenized hermaphrodites that expressed ACR-2(L/S) and selected animals that exhibited improved movement. A close examination of the mutants isolated from the screen showed that two phenotypic classes were easily distinguishable. One

class of animals phenocopied strains that lack a well characterized heteromeric nAChR that mediates excitatory signaling at the neuromuscular junction (NMJ) and is a principal target of the antihelminthic drug levamisole (L-AChR). We found that members of this class were strongly levamisole-resistant, and we isolated alleles of the levamisole resistance genes *unc-38*, *unc-63*, *unc-74*, and *unc-50* from these animals (Supplemental Table A1-S1). We also crossed available strains carrying single loss-of-function mutations in known levamisole resistance genes with transgenic ACR-2(L/S) animals (Table A1-1). Consistent with the results from our forward genetic approach, loss-of-function mutations in either *unc-38*, *unc-63*, *unc-74*, or *unc-50* were sufficient to suppress ACR-2(L/S) toxicity and restore movement (Figure A1-5A). Interestingly, we also found that *unc-29*, a non- α subunit required for L-AChR function, was not required for ACR-2(L/S)-induced paralysis. Likewise, *acr-16*, an essential subunit of homomeric nAChRs at the NMJ, was not required for ACR-2(L/S)-induced paralysis. *unc-38* and *unc-63* encode nAChR α subunits that are required for L-AChR function at the NMJ but are also expressed in the nervous system (Culetto et al., 2004; Eimer et al., 2007; Fleming et al., 1997). *unc-50* and *unc-74* encode genes previously implicated in L-AChR maturation and are broadly expressed in muscles and neurons. Our analysis suggests that each of these gene products may contribute in a cooperative fashion to the generation of functional ACR-2(L/S) receptors and subsequent toxicity.

A smaller number of animals isolated from the screen exhibited uncoordinated movement with deep body bends and showed normal sensitivity to the paralyzing effects of levamisole. We determined that the suppressor mutations in this second phenotypic class represented one complementation group and were X linked. Using single-nucleotide polymorphism mapping, we mapped one allele to the right of +8 on the X chromosome. The nAChR subunit gene *acr-12* is located in this genomic region. Sequence analysis revealed four nonsense mutations and two missense mutations in the *acr-12* coding sequence among our second class of suppressors (Figure A1-5B). We also found that a deletion mutation (*ok367*) in the *acr-12* gene suppressed paralysis in transgenic ACR- 2(L/S) animals and prevented the loss of motor neuron cell bodies. Expression of a full-length *acr-12* rescuing construct in *acr-12(ok367);ACR-2(L/S)* animals restored paralysis, verifying that *acr-12* is required (Figure A1-5C,F). In contrast, we found that several other nAChR subunits with restricted expression to the nervous system were not required for ACR-2(L/S)- induced paralysis (Table A1-1). *acr-12* encodes a nicotinic receptor α subunit that is broadly expressed in the nervous system, including many ventral cord motor neurons, but is not expressed in body wall muscles (Cinar et al. 2005; Gottschalk et al. 2005). *acr-12(ok367)* mutants have grossly normal movement and show normal sensitivity to the paralyzing effects of levamisole. To test whether *acr-12* expression solely in cholinergic motor neurons is sufficient for ACR-2(L/S)- induced toxicity, we specifically restored expression of *acr-12* to either ACh or

GABA motor neurons of *acr-12(ok367)* mutants that carried the ACR-2(L/S) transgene (Figure A1-5C– H). We found that-specific expression of the *acr-12* cDNA in cholinergic motor neurons of transgenic *acr-12(ok367); ACR-2(L/S)* mutants led to ACR-2(L/S) toxicity and paralysis. In contrast, specific expression in GABA neurons had no effect. Our results indicate that *acr-12* expression in cholinergic motor neurons is specifically required for ACR-2(L/S)-induced cell death. Furthermore, our results suggest that coassembly of ACR-2(L/S) into a heteromeric receptor complex with UNC-38, UNC-63, and ACR-12 is required to produce toxicity.

ACR-2(L/S)-induced motor neuron loss is suppressed in *cnx-1;crt-1* double mutants

At least two mechanistically distinct types of cell death have been described. Programmed cell death or apoptosis is a form of cell death common in development and tissue homeostasis and occurs by a genetic program that is broadly conserved across metazoans (Danial and Korsmeyer, 2004). Necrosis generally occurs following cellular injury and is often characterized by swelling of the dying cell (Festjens et al., 2006; Golstein and Kroemer, 2007). Our forward genetic screen did not identify genes previously implicated in the execution of either of these forms of cell death. To determine how ACR-2(L/S) expression leads to cell death, we introduced the ACR-2(L/S) transgene into genetic backgrounds deficient for genes essential for either apoptotic cell death or necrotic cell death (Figure A1-6). We found that a loss-of-function mutation in

proapoptotic *ced-3* (Ellis and Horvitz, 1986) or a gain-of-function mutation in anti-apoptotic *ced-9* (Hengartner et al., 1992) had no effect on ACR-2(L/S)-induced deaths. Thus, the programmed cell death machinery is not required for ACR-2(L/S)-induced neurodegeneration.

Dysregulation of intracellular calcium levels contributes to cell death under a variety of circumstances, including necrotic cell death (Mattson et al., 2000; Rao et al., 2004; Szydłowska and Tymianski, 2010). Calreticulin/CRT-1 and calnexin/CNX-1 are endoplasmic reticulum (ER) resident proteins that serve dual functions as Ca²⁺ binding proteins and molecular chaperones that facilitate glycoprotein folding (Ellgaard and Frickel, 2003). In mice, loss of either calnexin or calreticulin produces severe phenotypes: calnexin knock-out mice die within 4 months of birth, while knock-out of calreticulin results in embryonic lethality caused by defects in heart development (Denzel et al., 2002; Mesaeli et al., 1999). In *C. elegans*, *crt-1* and *cnx-1* single mutants are viable, and loss-of-function mutation in the *crt-1* gene or RNA interference knockdown of *cnx-1* expression suppresses several cases of ion channel mediated cell death (Xu et al., 2001). We found that the deletion mutation *cnx-1(ok2234)* had no significant effect on ACR-2(L/S)-induced cell death, whereas the deletion mutation *crt-1(ok948)* partially suppressed the loss of motor neurons observed in transgenic ACR-2(L/S) animals. Neither of these mutations led to significant locomotory improvement.

As calnexin and calreticulin perform similar cellular functions, we generated a strain carrying loss-of-function mutations in both genes to test whether they may act redundantly in ACR-2(L/S)-induced toxicity. *cnx-1;crt-1* double mutants were viable although smaller in size than wild-type animals, had grossly normal movement, and did not show obvious defects in nervous system connectivity. While ACR-2(L/S) expression in wild-type animals or *cnx-1* and *crt-1* single mutants caused paralysis across all developmental stages, we found that first larval stage *cnx-1;crt-1* double mutants expressing ACR-2(L/S) were often capable of grossly normal locomotion (Figure A1-7A,B,H). We also observed that the full complement of 16 cholinergic motor neuron cell bodies was present in L1 *cnx-1;crt-1;ACR-2(L/S)* animals, and we did not detect obvious defects in the connectivity of the cholinergic motor neurons (Figure A1-7C). These results indicate that embryonic born motor neurons developed normally and made appropriate synaptic contacts onto their partner muscle cells, suggesting that the combined loss of *crt-1* and *cnx-1* is strongly neuroprotective against ACR-2(L/S) toxicity in L1 animals. We observed that adult *cnx-1;crt-1* double mutants expressing ACR-2(L/S) also possessed normal numbers of cholinergic motor neuron cell bodies, providing additional evidence that the presence of either calnexin or calreticulin is required for the cellular events that lead to ACR-2(L/S)-induced cell deaths (Figures A1-6F,G, 7D). However, larvae that had progressed beyond L1 and adult *cnx-1;crt-1;ACR-2(L/S)* animals were unable to propagate sinusoidal body bends and move effectively. This observation suggested that

ACR-2(L/S) expression in *cnx-1;crt-1* double mutants led to a progressive disruption of motor function even when motor neuron death was attenuated. To determine whether altered motor neuron connectivity may underlie the paralysis of adult *cnx-1;crt-1;ACR-2(L/S)* animals, we made a close examination of the cholinergic motor neuron processes (Figure A1-7E–G,I). We found that defects in the motor neuron processes of control *cnx-1;crt-1* double mutants occurred only rarely (Figure A1-S6), and these animals exhibited grossly normal movement across all stages of development. In *cnx-1;crt-1* double mutants that expressed ACR-2(L/S), we observed defasciculation of the ventral nerve cord neuronal processes (in 96% of animals scored; $n=30$), as well as defects in the morphology of commissural axons (in 75% of axons) (Figure A1-7). We often observed several classes of defects within individual animals and, in some instances, individual commissural axons contained multiple defects. The defects were of several types. First, we observed abnormal axon branches that often terminated in growth cone like structures (54% of animals). Second, we observed ectopic sprouting with no clear single axonal process present (71% of animals). Finally, we observed axons with abnormal trajectories and wandering growth (83% of animals). These results suggest that the muscle targets of cholinergic motor neuron processes are not appropriately innervated in adult animals. We observed that the frequency of these defects increased dramatically after the first larval stage (Figure A1-7I), suggesting that a progressive deficiency in the

stabilization or maintenance of appropriate neuromuscular connectivity underlies the paralysis we observed in mature animals.

To better understand the mechanisms underlying the suppression of ACR-2(L/S)-mediated cell death in *cnx-1;crt-1* animals, we directly evaluated the role of intracellular calcium. We found that culturing transgenic ACR-2(L/S) animals in the presence of dantrolene, an inhibitor of ER calcium release, or the calcium chelator EGTA (Figure A1-8A,B) led to a modest but significant increase in the number of surviving motor neuron cell bodies. Similarly, we observed reduced ACR-2(L/S) toxicity in *unc-68* mutants lacking functional ryanodine receptors (Figure A1-8C). These results provide evidence that intracellular calcium signaling contributes to cell death in ACR-2(L/S) animals and support the idea that altered intracellular calcium in *cnx-1;crt-1* double mutants may likewise contribute to the neuroprotection we observed. To test whether altered expression of ACR-2(L/S) in *cnx-1;crt-1* double mutants may also be a contributing factor, we measured levels of ACR-2::GFP fluorescence. We observed an approximately twofold decrease in ACR-2::GFP fluorescence in both the cell bodies and ventral nerve cord of *cnx-1;crt-1* double mutant animals compared to wild-type animals (Figure A1-8D–G). This result suggests that a decrease in the levels of ACR-2(L/S) in *cnx-1;crt-1* animals also contributes to the neuroprotection we observe.

Discussion

Our analysis of ACR-2-containing nicotinic receptors in *C. elegans* neurons has revealed common features between the function of these receptors in the *C. elegans* nervous system and roles for heteromeric nAChRs in the vertebrate brain (Dani and Bertrand, 2007). First, heteromeric nAChRs in the mammalian brain are not primarily concentrated at postsynaptic sites; instead, they are more variably localized to presynaptic, preterminal, and nonsynaptic sites. Similarly, we find that ACR-2-containing nAChRs appear diffusely localized to the processes of excitatory motor neurons, suggesting that these receptors may function at extrasynaptic sites. Second, heteromeric brain nAChRs primarily function to modulate neurotransmitter release and neuronal excitability. Our studies of *acr-2* loss-of-function mutants indicate that heteromeric nAChRs containing ACR-2 modulate the excitability of cholinergic motor neurons but are not absolutely required for motor neuron depolarization or ACh release at neuromuscular synapses. Third, mouse studies have shown that knock-in expression of a heteromeric brain nAChR subunit bearing a L/S pore modification homologous to the one we describe here causes dramatic neuron loss and perinatal lethality (Labarca et al., 2001). Likewise, transgenic expression of ACR-2(L/S) leads to cell-autonomous neurodegeneration.

Importantly, our transgenic approach also enabled us to identify genes required for ACR-2(L/S) toxicity. Mutations that suppressed both the paralysis and neurodegeneration caused by ACR-2(L/S) expression defined the constituent subunits of a putative multimeric ACR-2 receptor complex as well as

genes required for receptor trafficking and assembly. Additionally, a single gene mutation in *crt-1*, previously shown to suppress other forms of ion channel-mediated cell death in *C. elegans*, partially suppressed ACR-2(L/S) toxicity. We found that the loss of motor neurons caused by ACR-2(L/S) expression was completely suppressed in adult *cnx-1;crt-1* double mutants; yet, these animals remained paralyzed. Interestingly, suppression of ACR-2(L/S)-induced cell death uncovered a secondary consequence of ACR-2(L/S) expression: the accumulation of morphological defects in the processes of surviving motor neurons. These axonal defects resemble outgrowth errors typically associated with secondary regrowth of axons (Hammarlund et al., 2007; Knobel et al., 2001). Therefore, the severe morphological defects we observed in adult animals may reflect inappropriate regrowth subsequent to destabilization. We propose that the necrotic-like cell death and destabilization of neuronal processes observed in our studies may represent genetically separable events and suggest that our transgenic approach may afford a powerful system to tease apart the molecular pathways that differentially contribute to these two processes.

ACR-2 is part of a heteromeric nAChR in cholinergic motor neurons

We have demonstrated that *acr-2* shows restricted expression to cholinergic motor neurons of the ventral nerve cord and appears diffusely localized in neuronal processes. These results suggest that the ACR-2 receptor complex may modulate motor neuron excitability by mediating signaling at extrasynaptic sites. Consistent with this notion, *acr-2* loss-of-function mutants are

not grossly uncoordinated and show only modest resistance to the paralyzing effects of the ACh esterase inhibitor aldicarb. Aldicarb-induced paralysis arises as a consequence of the prolonged action of ACh in the synaptic cleft; our analysis suggests that ACh release from motor neurons is decreased in *acr-2* mutants. Another recent study reached a similar conclusion based on electrophysiological analysis of *acr-2* loss-of-function mutants (Jospin et al., 2009). We also show that the locomotory control interneurons (with the possible exception of AVE)—the major source of synaptic inputs to excitatory motor neurons—do not express a reporter that labels cholinergic neurons, suggesting these neurons are unlikely to be cholinergic. Therefore, what is the source of ACh for activation of ACR-2 receptor complexes? The presynaptic ACh release sites of *en passant* neuromuscular synapses are highly intermingled and densely packed because of the intercalation of neuronal processes in the nerve cord. Thus, one possibility is that these receptors are activated by spillover of ACh from release sites at nearby neuromuscular synapses.

Our genetic analysis showed that mutations in three genes encoding AChR subunits can suppress the neurotoxic effects associated with expression of pore-modified ACR-2(L/S) receptors. *unc-38* and *unc-63* are highly expressed in ventral cord motor neurons and also contribute to a heteromeric receptor complex that mediates excitatory neurotransmission at the NMJ (Culetto et al., 2004; Fleming et al., 1997). *acr-12* is broadly expressed in the nervous system but is not expressed in body wall muscles (Cinar et al., 2005; Gottschalk et al.,

2005). Our data are consistent with the notion that UNC-38, UNC-63, and ACR-12 coassemble with the ACR-2 subunit [either native ACR-2 or transgenic ACR-2(L/S)] to form heteromeric nAChRs in cholinergic motor neurons (Jospin et al., 2009). Loss-of-function mutations in any of these genes impair assembly or function of ACR-2 receptor complexes in cholinergic motor neurons and suppress ACR-2(L/S)-induced cell death. Several pieces of evidence support this idea. First, mutations in *unc-29* and *acr-16*—genes that contribute to nAChRs at the NMJ and are essential for normal excitatory neurotransmission at neuromuscular synapses (Francis et al., 2005; Richmond and Jorgensen, 1999; Touroutine et al., 2005)—do not suppress ACR-2(L/S) neurotoxicity, indicating that reduced excitatory neuromuscular signaling alone is insufficient to suppress ACR-2(L/S)-induced toxicity. Second, specific expression of *acr-12* in the cholinergic motor neurons of transgenic *acr-12* mutants expressing ACR-2(L/S) was sufficient to produce paralysis, whereas specific expression of *acr-12* in other neuron classes was without effect. Third, it has recently been shown that coexpression of five subunits—ACR-2, ACR-12, UNC-38, UNC-63, and ACR-3—was required for reconstitution of ACR-2 receptor complexes in a heterologous system (Jospin et al., 2009).

Pore-modified ACR-2(L/S) receptors cause ion channel-mediated neurotoxicity

We have shown that expression of the ACR-2(L/S) transgene leads to degeneration of the cholinergic motor neurons and paralysis, reinforcing the

importance of these neurons in generating sinusoidal movement. The GABA motor neurons develop normally even in the absence of ACh motor neurons, their major source of synaptic input, indicating that the toxic effects of ACR-2(L/S) expression are cell autonomous. We found that mutations in genes that are essential for the formation of functional ACR-2 heteromeric receptors suppress this effect, consistent with the idea that excessive receptor activity leads to neurodegeneration. Our results suggest that the level of receptor activity is a critical determinant in the progression toward necrotic cell death. Consistent with this idea, a less severe gain-of-function *acr-2* allele leads to cellular hyperexcitability without obvious loss of motor neurons (Jospin et al., 2009). Interestingly, mouse studies using knock-in expression of similarly pore-modified heteromeric nAChR subunits have reported qualitatively similar degeneration as a consequence of excess receptor activation (Labarca et al., 2001; Orb et al., 2004; Orr-Urtreger et al., 2000). Knock-out of *Lynx1*, an endogenous negative regulator of nAChR function in the mouse brain, also leads to a similar form of vacuolating degeneration that is exacerbated by nicotine (Miwa et al., 2006).

Release of calcium from internal stores plays a major role in many forms of cell death, including some forms of ion channel mediated toxicity.

Pharmacological or genetic manipulation of intracellular calcium levels led to a modest suppression of ACR-2(L/S)-induced toxicity, providing evidence that calcium plays an important role. However, ACR-2::GFP fluorescence was decreased substantially in *cnx-1;crt-1* double mutants, suggesting that a

reduction in protein levels of the toxic transgene also contributes to suppression of cell death. Similar to the case for ACR- 2(L/S) expression, cell death because of a gain-of-function mutation in another *C. elegans* nAChR subunit, DEG-3, is not suppressed by a single gene mutation in *crt-1* (Syntichaki et al., 2002; Treinin and Chalfie, 1995; Xu et al., 2001). Our findings suggest that a requirement for genes additional to *crt-1* may be a common feature of nAChR-mediated neuronal death that is distinct from cell death caused by hyperactive Na⁺ channels such as MEC-4(d).

In mouse models of motor neuron disease, such as progressive motor neuropathy (*PMN*) or the transgenic SOD1 G93A model of amyotrophic lateral sclerosis, apoptosis of neuronal cell bodies was blocked by expression of the anti-apoptotic *Bcl-2* gene or knock-out of the proapoptotic *Bax* gene (Gould et al., 2006; Sagot et al., 1995). In each case, degeneration of the neuronal processes continued unimpeded and disease progression was unaffected. ACR-2(L/S)-induced cell death clearly occurs independently of the apoptotic pathway. However, it is interesting to note that we also observe a progressive destabilization of the motor neuron processes that leads to paralysis even under conditions when death of the cell body is attenuated. Therefore, NMJ denervation that occurs independently of the death of neuronal cell bodies is the dominant feature shared across each of these cases. In the future, it will be interesting to uncover the molecular events leading to degeneration of the neuronal processes

and determine whether elements of the degenerative process are conserved across these diverse models.

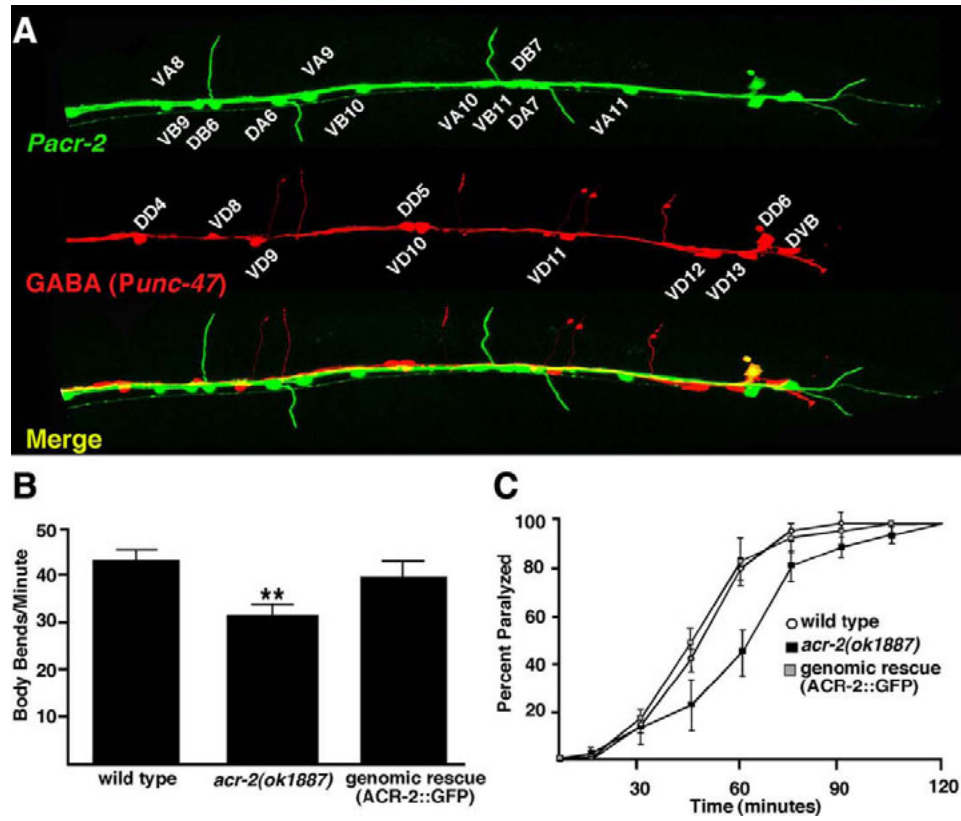


Figure A1-1

Figure A1-1. *acr-2* is expressed in cholinergic motor neurons and modulates motor neuron activity. **A**, Confocal image of the posterior ventral nerve cord of an adult animal coexpressing a GABA-specific marker (*Punc-47::mCherry*) and an ACR-2- specific marker (*Pacr-2::GFP*). No overlap is observed between GFP-expressing and mCherry-expressing neurons. The animal is oriented with the posterior (tail) on the right. **B**, Quantification of movement on a food-free agar plate. Average number of body bends per minute for wild type, *acr-2(ok1887)* mutants, and *acr-2(ok1887)* mutants expressing full length ACR-2::GFP (*ufls42*) over a 5 min period are shown. Data represents mean \pm SEM of at least 10 trials; ** $p < 0.01$ compared to wild type. **C**, Time course of paralysis in the presence of the cholinesterase inhibitor aldicarb (1 mM) for wild type, *acr-2(ok1887)* mutants, and *acr-2(ok1887)* mutants expressing full-length ACR-2::GFP (*ufls42*). The percentage of immobilized animals calculated every 15 min over a time course of 2 h is shown. Each data point represents the mean \pm SEM of at least four trials. * $p < 0.001$, two-way ANOVA.

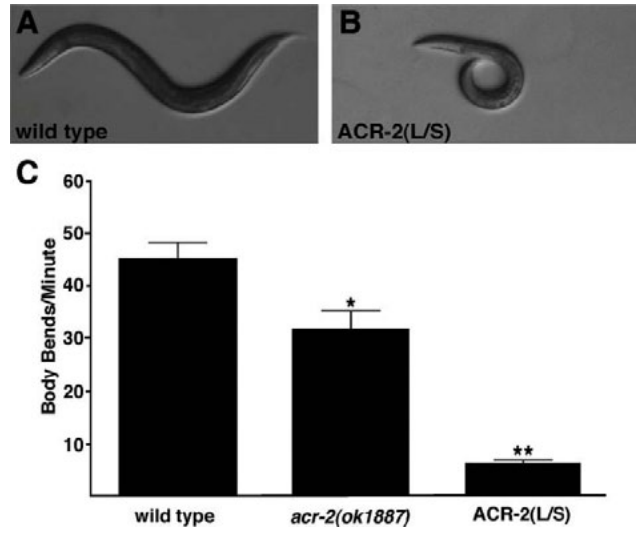


Figure A1-2

Figure A1-2. Transgenic animals expressing the dominant ACR-2(L/S) transgene are severely uncoordinated. **A, B**, Still image of a wild-type animal (**A**) and a transgenic animal expressing the ACR-2(L/S) transgene (**B**). Note the coiled posture and reduced size that occurs as a result of ACR-2(L/S) expression. **C**, Quantification of movement on a food-free agar plate. Average number of body bends per minute for wild type, *acr-2(ok1887)* mutants, and transgenic animals expressing full-length ACR-2(L/S) (*ufls25*) counted over a 5 min period are shown. Data represent mean \pm SEM of at least 10 trials; * $p < 0.02$; ** $p < 0.0001$.

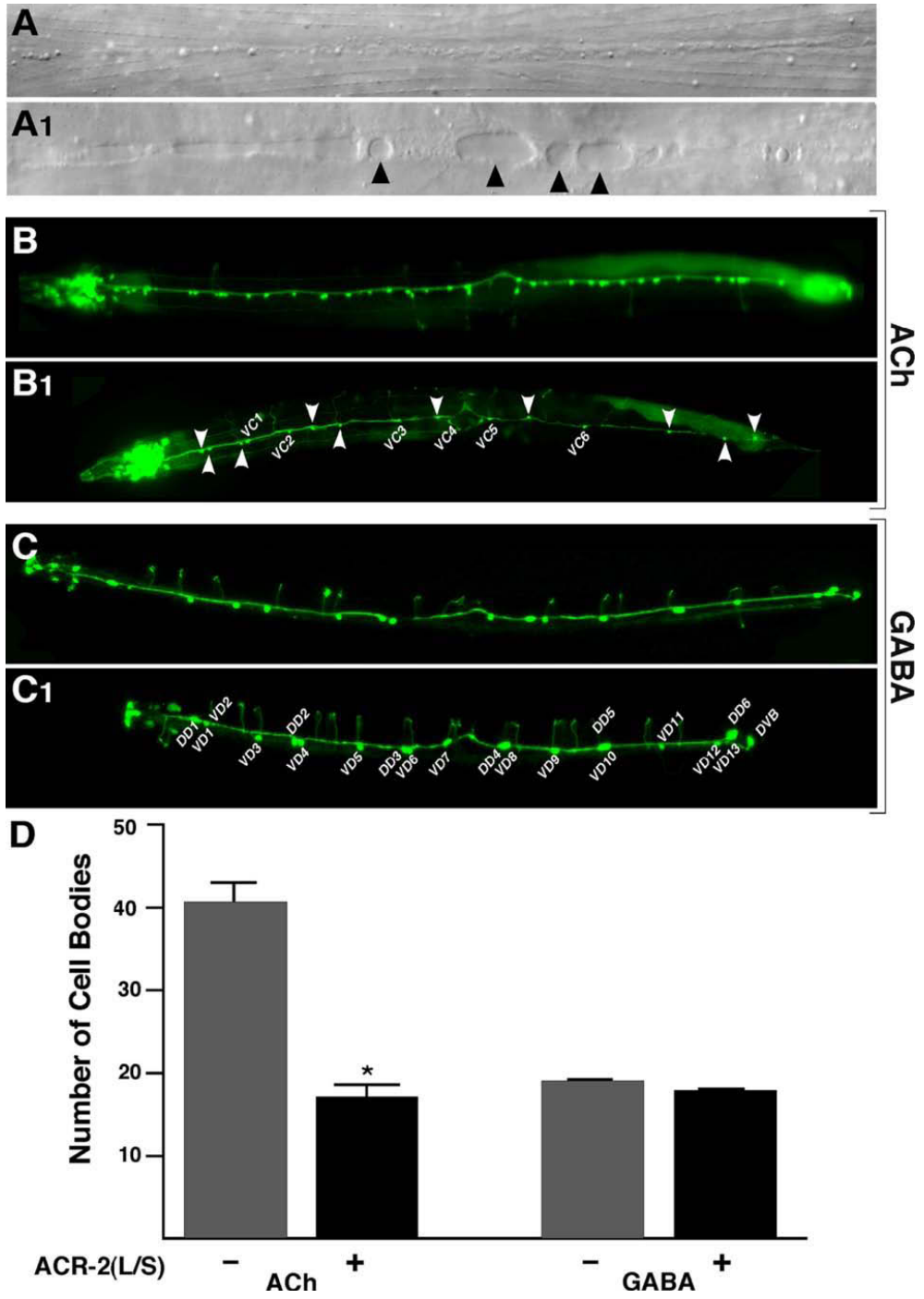


Figure A1-3

Figure A1-3. Transgenic expression of ACR-2(L/S) leads to a loss of cholinergic motor neurons. **A, A1**, DIC images of an adult wild type animal (**A**) and an adult transgenic animal expressing ACR-2(L/S) (**A1**). Triangles denote lesions observed along the ventral nerve cord of transgenic animals expressing ACR-2(L/S). Images show a region directly posterior of the vulva and are oriented with the ventral surface facing up and the anterior of the animal to the left. **B, B1**, Wide-field epifluorescent images of a transgenic animal expressing the cholinergic neuron marker *Punc-17::GFP* (*vsIs48*) (**B**) and a transgenic animal coexpressing ACR-2(L/S) with *Punc-17::GFP* (**B1**). The few *Punc-17::GFP*-labeled motor neurons that remain in transgenic ACR-2(L/S) animals include the six VCs (indicated) and a more variable group of ~10–12 neurons (arrowheads). **C, C1**, Wide-field epifluorescent images of a transgenic animal expressing *Punc-47::mCherry* (**C**) and a transgenic animal coexpressing *Punc-47::mCherry* with ACR-2(L/S) (**C1**). The full complement of *Punc-47::mCherry*-labeled motor neurons remains in transgenic ACR-2(L/S) animals and is indicated. **D**, Quantification of the total number of motor neurons in wild-type (gray) and ACR-2(L/S) animals (black); * $p < 0.01$. For all images, animals are positioned with the head on the left side of the image.

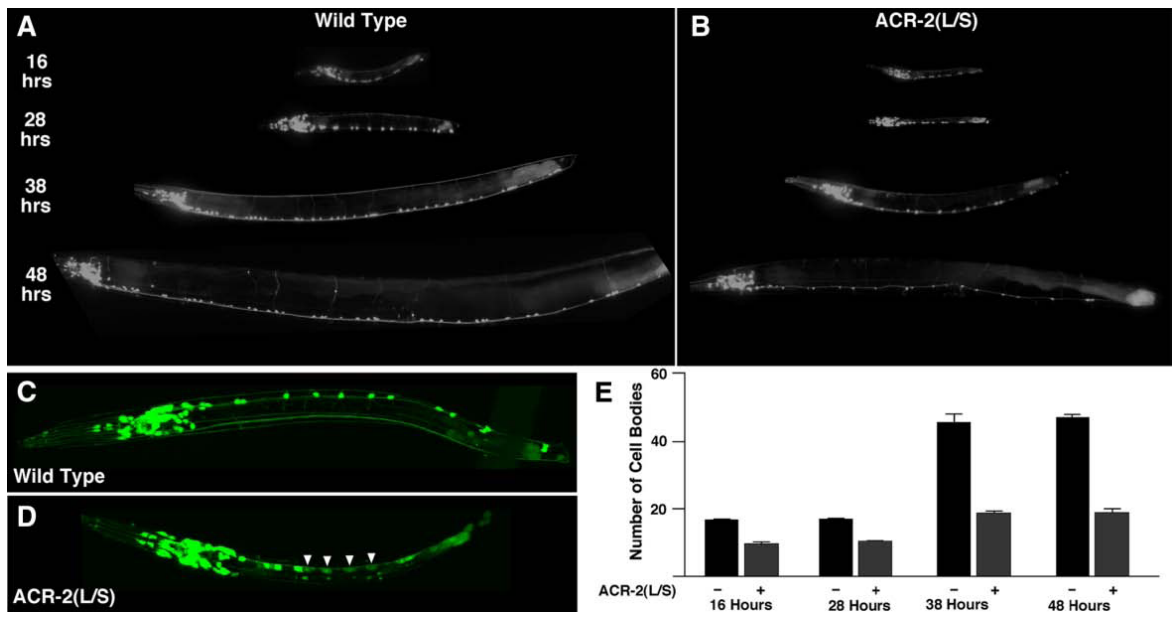


Figure A1-4

Figure A1-4. ACR-2(L/S)-induced motor neuron cell death is initiated before hatch. **A, B**, Wide-field epifluorescent images of wild-type (**A**) and transgenic ACR-2(L/S) (**B**) animals expressing *Punc-17::GFP* imaged 16, 28, 38, and 48 h after bleach synchronization of embryos. **C, D**, Confocal images of first larval stage wild-type (**C**) and transgenic ACR-2(L/S) (**D**) animals expressing *Punc-17::GFP*. Swollen or dying neuronal cell bodies are indicated (arrowheads). **E**, Quantification of the average number of cell bodies at the time points indicated for wild type (black bars) and transgenic ACR-2(L/S) animals (gray bars). Bars represent the mean \pm SEM for 5– 8 animals at each time point.

Table 1. Several nAChR subunits are required for ACR-2(L/S)-induced paralysis

Gene	ACR-2(L/S) suppression	Description
<i>acr-2(ok1887)</i>	—	nAChR non- α subunit
<i>acr-3(ok2049)</i>	—	nAChR non- α subunit
<i>acr-5(ok180)</i>	—	nAChR α subunit
<i>acr-9(ok933)</i>	—	nAChR non- α subunit
<i>acr-12(ok367)</i>	+	nAChR α subunit
<i>acr-14(ok1155)</i>	—	nAChR α subunit
<i>acr-15(ok1214)</i>	—	nAChR α subunit
<i>acr-16(ok789)</i>	—	nAChR α subunit
<i>acr-19(ok967)</i>	—	nAChR α subunit
<i>acr-23(ok2840)</i>	—	nAChR α subunit
<i>unc-63(x37)</i>	+	nAChR α subunit
<i>unc-38(e264)</i>	+	nAChR α subunit
<i>unc-29(x29)</i>	—	nAChR non- α subunit
<i>unc-50(e306)</i>	+	Golgi integral membrane protein
<i>unc-74(e883)</i>	+	Function unknown

Table A1-1

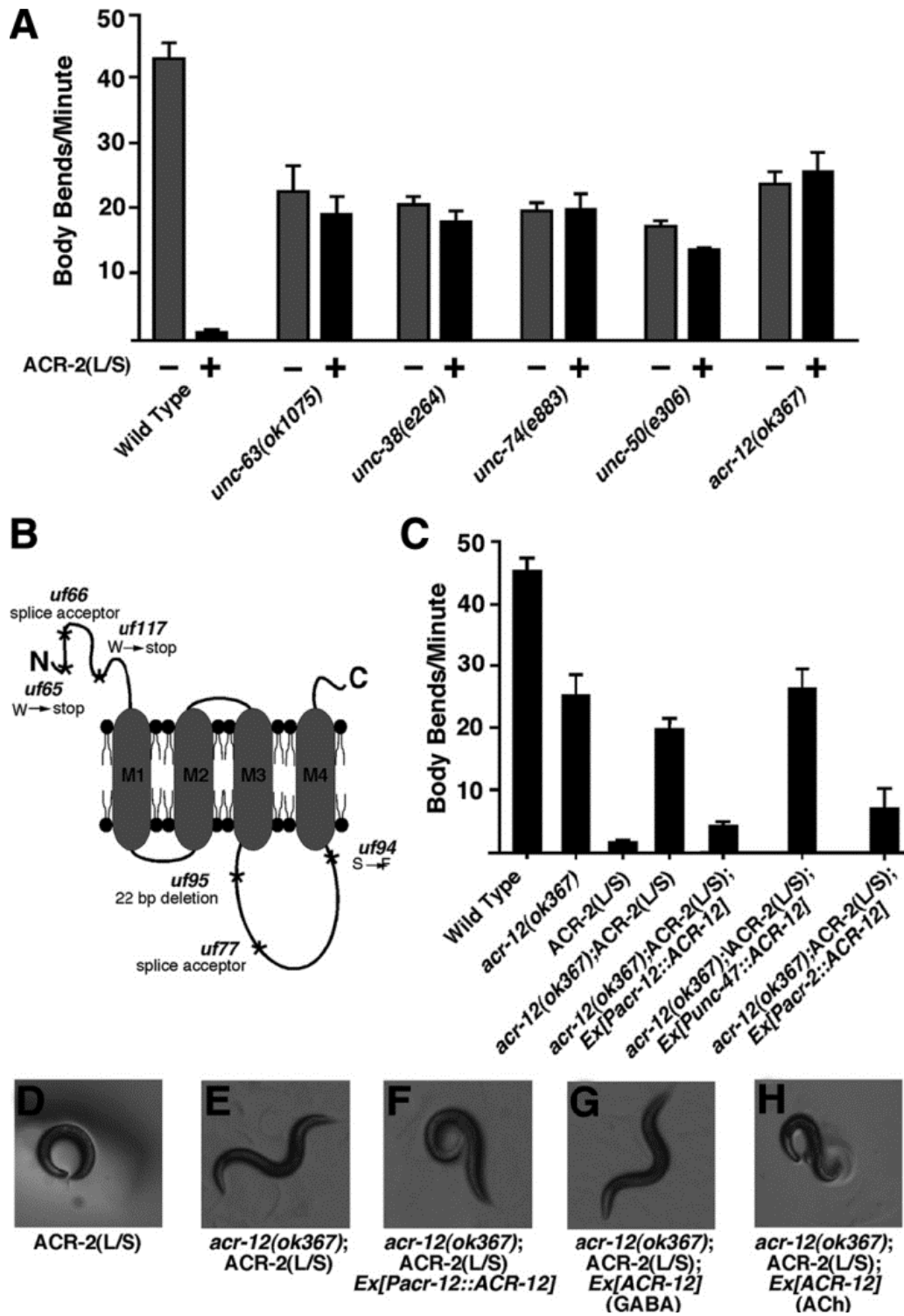


Figure A1-5

Figure A1-5. Mutations in nicotinic acetylcholine receptor subunits suppress ACR-2(L/S)-induced paralysis. **A**, Quantification of the average number of body bends per minute for wildtype animals, *acr-12(ok367)*, *unc-63(ok1075)*, *unc-38(e264)*, *unc-74(e883)*, and *unc-50(e306)* mutants in the absence (gray bars) or presence (black bars) of the ACR-2(L/S) transgene. Animals were placed on a food-free agar plate, and the average number of body bends per minute was quantified over a 5 min period. Data represent the mean \pm SEM of at least 10 animals for each genotype. **B**, Schematic of the membrane topology of ACR-12 with approximate location, allele names, and molecular nature of loss-of-function mutations that suppress ACR-2(L/S) toxicity indicated. **C**, Quantification of the average number of body bends per minute for the following genotypes: wild type, *acr-12(ok367)*, transgenic ACR-2(L/S), *acr-12* mutants expressing ACR-2(L/S), *acr-12* mutants expressing ACR-2(L/S) together with an extrachromosomal array containing *Punc-47::ACR-12*, and *acr-12* mutants expressing ACR-2(L/S) together with an extrachromosomal array containing the *Pacr-2::ACR-12* cDNA. Data represent the mean \pm SEM for 5–10 animals. **D–H**, Still images of adult animals on NGM plates without food for the genotypes indicated. “GABA” and “ACh” refer to specific expression of the *acr-12* cDNA in GABAergic or cholinergic neurons using the *unc-47* or *acr-2* promoters, respectively.

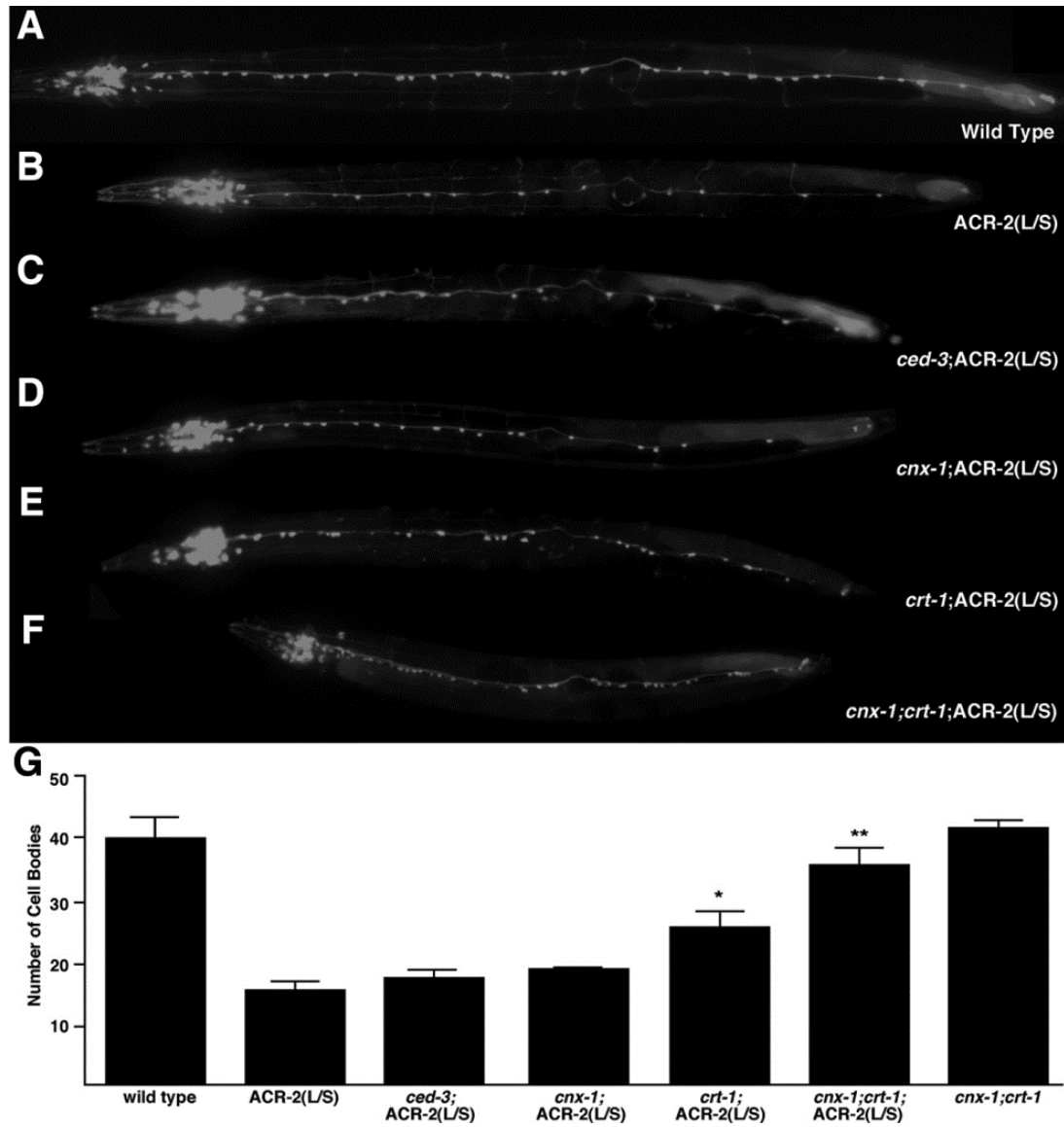


Figure A1-6

Figure A1-6. ACR-2(L/S)-mediated motor neuron loss is completely prevented in animals doubly mutant for calnexin and calreticulin. **A–F**, Representative wide-field images of *Punc-17::GFP* fluorescence in the ventral nerve cord of adult animals for the genotypes indicated. For each image the head is oriented to the left. The alleles used were *ced-3(ok2734)*, *cnx-1(ok2234)*, and *crt-1(ok948)*. **G**, Quantification of the average number of *Punc-17::GFP*-labeled cell bodies present in the ventral nerve cord for the genotypes indicated. Data represent the mean \pm SEM for at least 10 animals per genotype. * $p < 0.01$, ** $p < 0.01$, compared to transgenic ACR-2(L/S) animals.

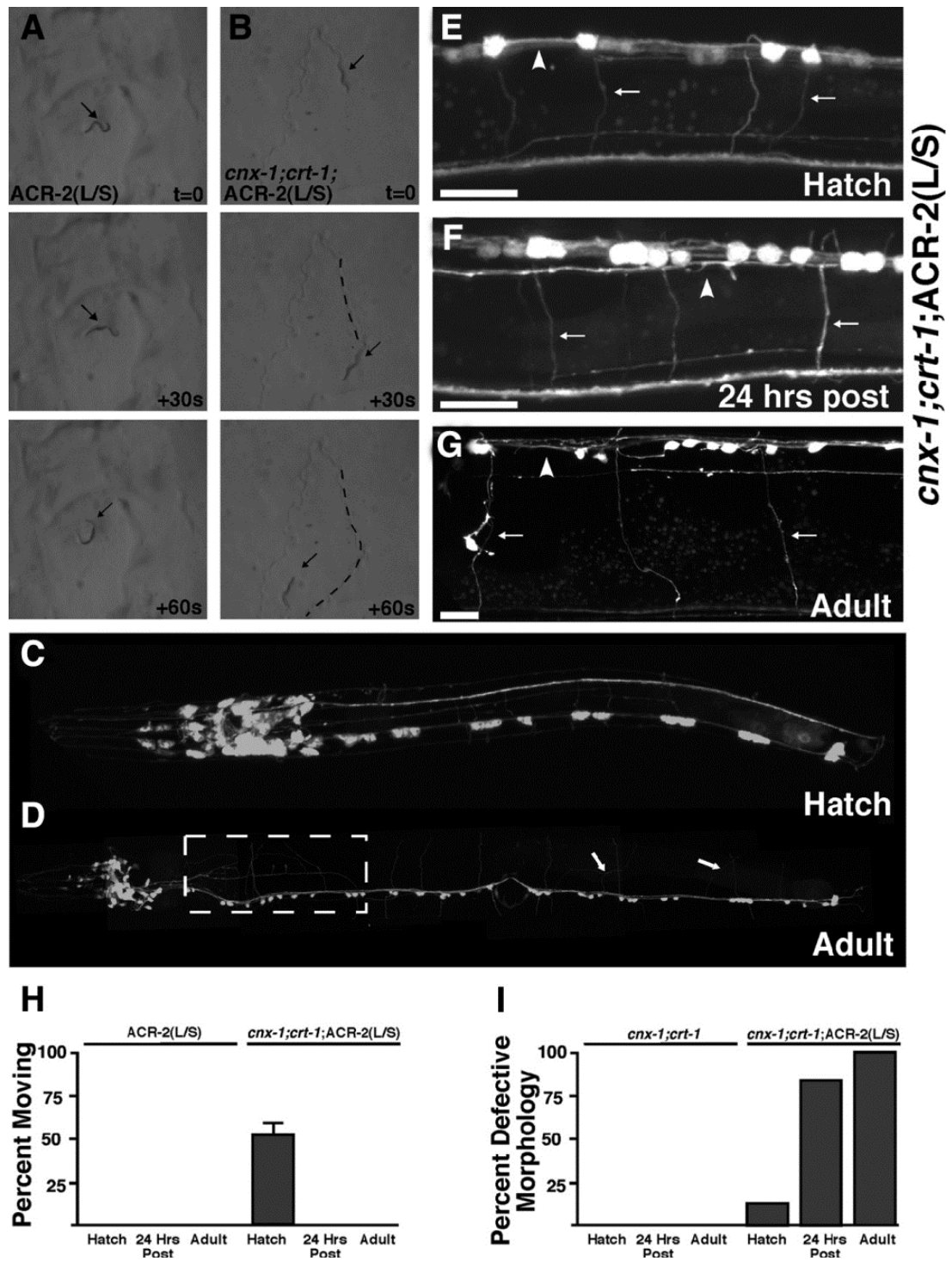


Figure A1-7

Figure A1-7. Progressive destabilization of motor neuron processes in *cnx-1;crt-1;ACR-2(L/S)* animals. **A, B**, Frames showing the movement of L1 transgenic ACR-2(L/S) (**A**) or *cnx-1;crt-1;ACR-2(L/S)* (**B**) animals 30 s (t_0), 60 s (+30), or 90 s (+60) after transfer to an agar plate. The black arrow marks the starting positions of the worms in each frame. The transgenic ACR-2(L/S) animal does not move from where it was placed on the plate. The dashed line shows the movement path of the L1 *cnx-1;crt-1;ACR-2(L/S)* animal over the course of 60 s. The additional tracks on the plate show the path of the animal in an ~30 s period before imaging began. **C, D**, Confocal images of a first larval stage (**C**) or adult *cnx-1;crt-1;ACR-2(L/S)* animals expressing an integrated *Punc-17::GFP* (**D**). Images show Z-projections of 15 confocal planes (0.5 μ m/slice) (**C**) or 16 confocal planes (0.5 μ m/slice) (**D**). Arrows (**D**) indicate positions of commissures. Dashed box (**D**) indicates area with multiple neuronal defects. **E–G**, Confocal images of *cnx-1;crt-1;ACR-2(L/S)* animals taken at hatch (**E**), 24 h post hatch (**F**), and adulthood (**G**). In each case, a region immediately posterior of the vulva was imaged and the ventral nerve cord is positioned at the top. Arrows indicate commissural processes; arrowheads indicate the ventral nerve cord. Images show Z-projections of 12 confocal planes (**E**), 23 confocal planes (**F**), or 33 confocal planes (**G**) (0.5 μ m/slice). Scale bars represent 10 μ m. **H**, Quantification of percentage of animals moving at hatch in ACR-2(L/S) and *cnx-1;crt-1;ACR-2(L/S)* animals. Data represent the mean number of animals \pm SEM making more than two consecutive body bends during a 3 min period. **I**, Quantification of the percentage of *cnx-1;crt-1* and *cnx-1;crt-1;ACR-2(L/S)* animals with defective neuronal process morphology. Observed defects include defasciculation of the ventral nerve cord, wandering commissural processes, or ectopic branching.

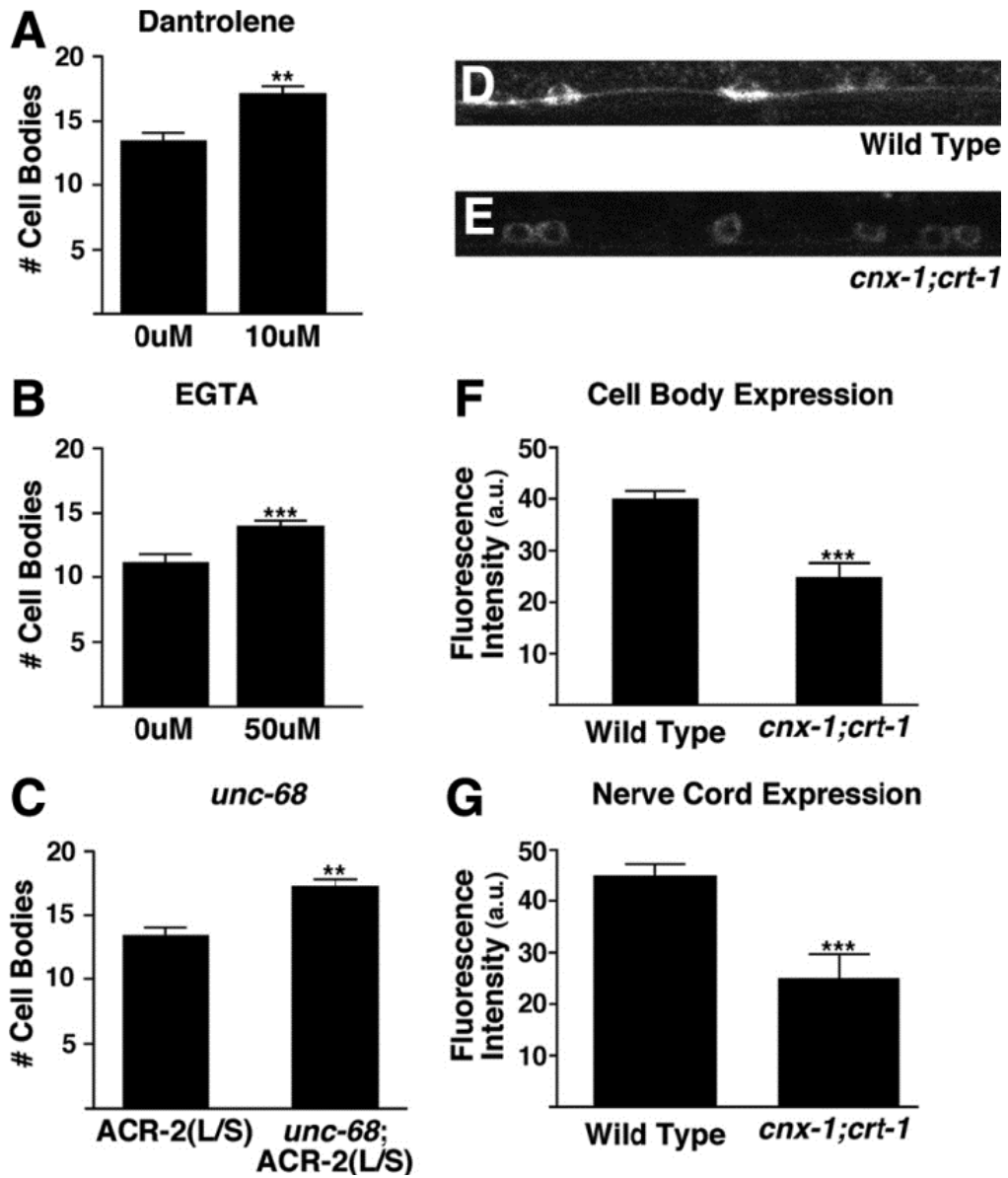


Figure A1-8

Figure A1-8. Perturbation of intracellular calcium and reduced ACR-2::GFP levels in *cnx-1;crt-1* double mutants suggest dual mechanisms for cell death suppression. **A–C**, Quantification of the average number of *Punc-17::GFP*-labeled cell bodies present in the ventral nerve cord in adult animals treated with dantrolene (**A**), L1 animals treated with EGTA (**B**), and adult *unc-68(e540)* mutant animals (**C**). Data represent the mean \pm SEM for at least eight animals. ** $p < 0.01$, *** $p < 0.001$, compared to untreated ACR-2(L/S) animals. **D, E**, Confocal images of adult wild type (**D**) or *cnx-1;crt-1* (**E**) animals expressing ACR-2::GFP. Images are taken immediately posterior to the vulva and show Z-projections of seven confocal planes (0.5 μ m/slice). **F, G**, Quantification of fluorescence levels in cell bodies (**F**) and ventral nerve cord processes (**G**) of wild-type and ACR-2(L/S) animals transgenically expressing ACR-2::GFP. Data represent the mean \pm SEM for at least 10 animals. *** $p < 0.001$, compared to ACR-2(L/S) animals.

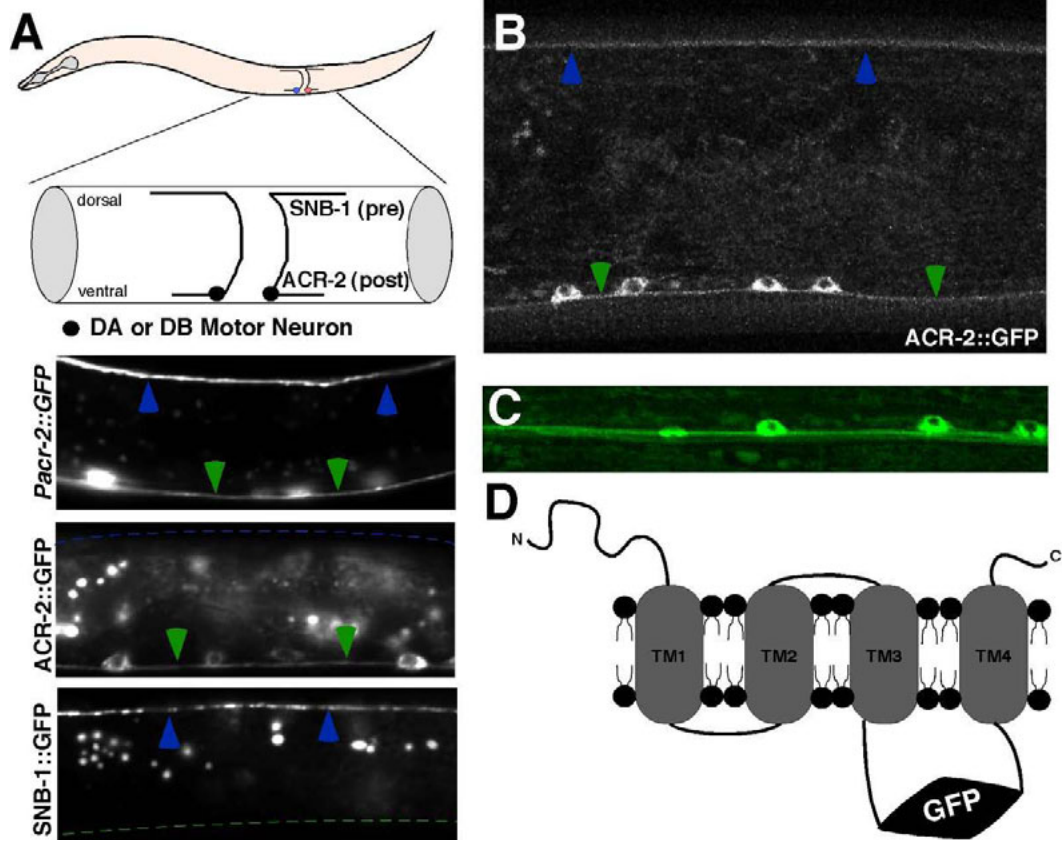


Figure A1-S1

Figure A1-S1. Expression of the non-alpha nAChR subunit ACR-2. **(A)** Schematic depicting morphology of cholinergic DA and DB motor neurons in an L1 animal. Wide-field epifluorescent projection images of the ventral (green) and dorsal (blue) nerve cords in L1 animals expressing integrated arrays containing either the transcriptional reporter *Pacr-2::GFP* (*ufls49*, top), full-length *Pacr-2::ACR-2::GFP* (*ufls42*, middle) or a presynaptic marker (*Punc-4::SNB-1::GFP*, lower). *Pacr-2::GFP* fluorescence is present in both ventral and dorsal nerve cords. Expression of the synaptic vesicle marker synaptobrevin (*SNB-1::GFP*) is limited to axons of the dorsal nerve cord. ACR-2::GFP fluorescence is limited to dendrites in the ventral nerve cord. **(B)** Projection of a confocal stack showing the dorsal and ventral nerve cords of an adult animal expressing full-length ACR-2::GFP. **(C)** Confocal image of ventral nerve cord showing diffuse ACR-2::GFP fluorescence. **(D)** Schematic of ACR-2 membrane topology and site of GFP insertion.

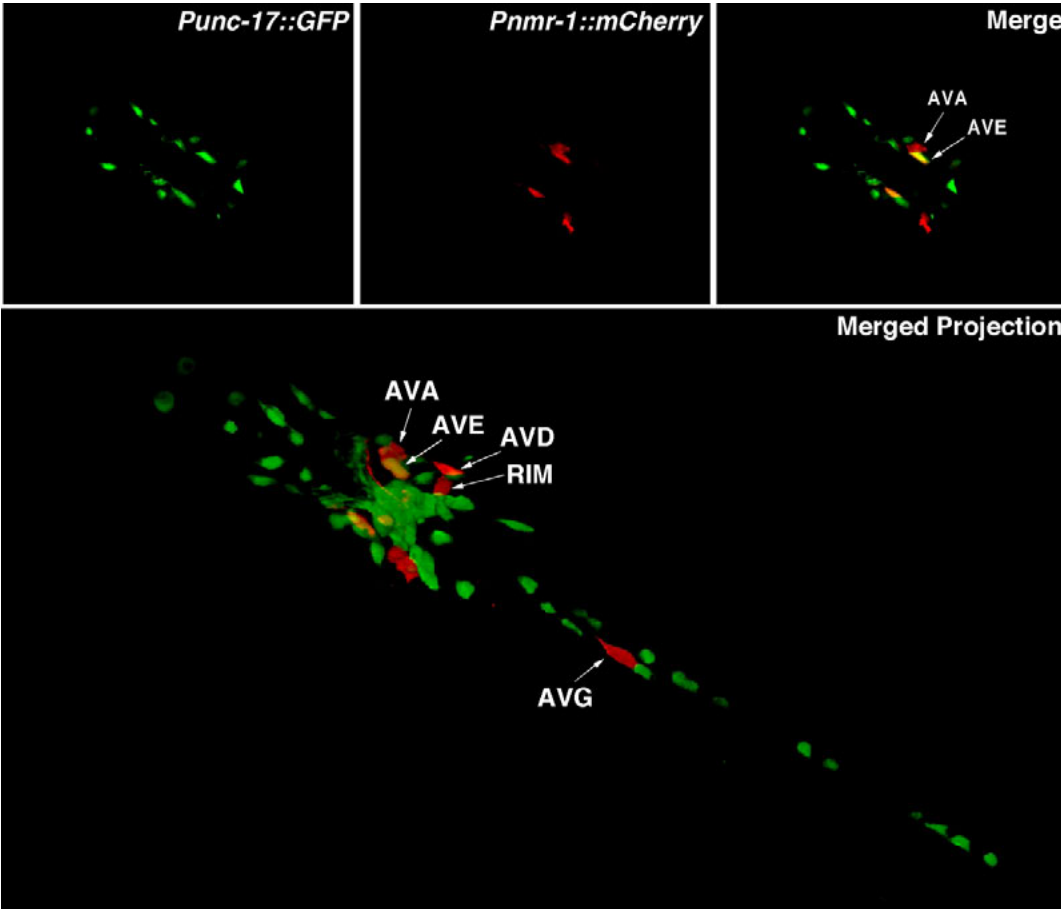


Figure A1-S2

Figure A1-S2. *C. elegans* command interneurons do not express *Punc-17::GFP*. (A-C) Single confocal images of an animal co-expressing an integrated array (*vs/s48*) containing the cholinergic neuron marker *Punc-17::GFP* together with an extrachromosomal array (*ufEx158*) containing *Pnmr-1::mCherry*. (D) A confocal projection showing the relative positions of neuronal cell bodies expressing *Punc-17::GFP* and *Pnmr-1::mCherry* in the head of the worm. Neurons expressing mCherry are indicated. Only a single AVA neuron expresses mCherry due to mosaic expression of the array.

A 1 MKKTVKILLILITVFLKVHCNGGHDDEAADFLSHTNIDDPNNSSDPNKNS
 51 DQGDTMGEDEDRLVIDLRFREYNFLIRPVKNVSSPPVVVDFGVAMILLIV
 101 DEKNQILQTNVWLTMKWNDQLAWNPAEYGNISNLHVPSDRVWLPDIVLF
 151 NNADGNYEVSFKSNVFDHHDGDTWVPPAMFKSSCRIDVEWFPPDEQCCT
 201 LVFGSWTYNSEEVRLHWYNNIQAVQLHDYSYSGIWDVIDVPGQLVHKPDL
 251 KENKMFVNVVIRRKTLFYTVILIIPTVLM AFLSVMAFYLPVDSGEKVSLT
 301 ISLLALVVFLLLVSKILPPTSNIPLMGKYLLLA FVLNITAVVGTVVIVN
 351 IYFRSALSHKMPTWVRKVFLEFLPHLLVMKRPERIPFNGYFVEEYCASE
 401 IFDASLVMPSTATMLPFLQVTTNLKAASSTSSGQSSEHHENCSKWKKRL
 451 SIRMSKRRAPRARLDDDDSEDIIDDTNGNHVDSLQEKISKEMKTTVEAIA Y
 501 IAEHMKREMSLKKMRDDWKYVAMVLDRLILLIFFGVTGGTLGIICSAPH
 551 VFDFVDQEAIIISKLNAKYLPSDMYS

B M2 Transmembrane Region

ACR-2	EKVSLTISLLALVVFLLLVSKILP
BETA1	EKMGLSIFALLTLTVFLLLLADKVP
BETA2	EKMTLCISVLLALTVFLLLLISKIVP
BETA4	EKMTLCISVLLALTFLLLLISKIVP
GAMMA	QKCTVATNVLLAQTVFLFLVAKKVP
DELTA	EKTSVAISVLLAQSVFLLLLISKRLP
EPSILON	QKCTVSINVLLAQTVFLFLIAQKIP

Figure A1-S2

Figure A1-S3. Sequence features of ACR-2. **(A)** Amino acid sequence of ACR-2. Predicted signal sequence (dashed box), transmembrane domains (underline), cys-loop (gray), gain-of-function mutation (box), and site of GFP insertion (asterisks) are indicated. **(B)** Alignment of the second transmembrane domain of ACR-2 and various mammalian non-alpha subunits. The site of the 9' leucine to serine gain-of-function mutation is indicated (gray shading).

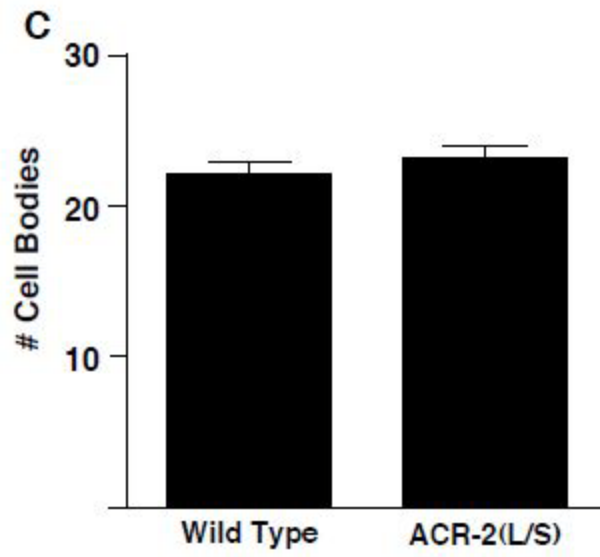
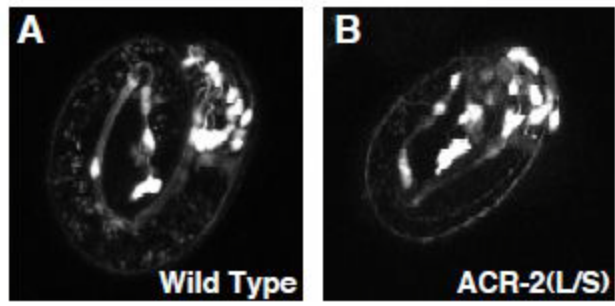


Figure A1-S4

Figure A1-S4. Wild type and ACR-2(L/S) animals have the same number of *acr-2* expressing cells at the 3-fold embryo stage. **(A and B)** Confocal images of wild type (A) and ACR-2(L/S) (B) 3-fold embryos expressing an integrated array containing *Pacr-2::GFP* (*ufls49*). Images represent Z-projections of 23 slices (0.5 μm /slice). **(C)** Quantification of the average number of *Pacr-2::GFP* labeled cell bodies present in wild type and ACR-2(L/S) 3-fold embryos. Data represents the mean \pm SEM of at least ten animals per genotype.

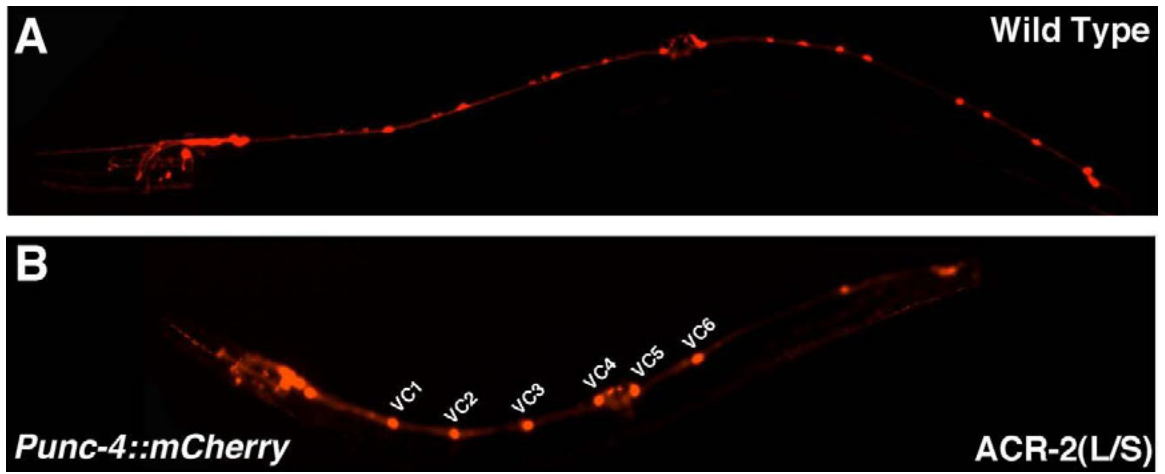


Figure A1-S5

Figure A1- S5. The VC neurons remain present in transgenic ACR-2(L/S) animals. Confocal images of wild type (A) and ACR-2(L/S) (B) animals expressing an integrated array (*ufls26*) that contains *P_{unc-4}::mCherry*.

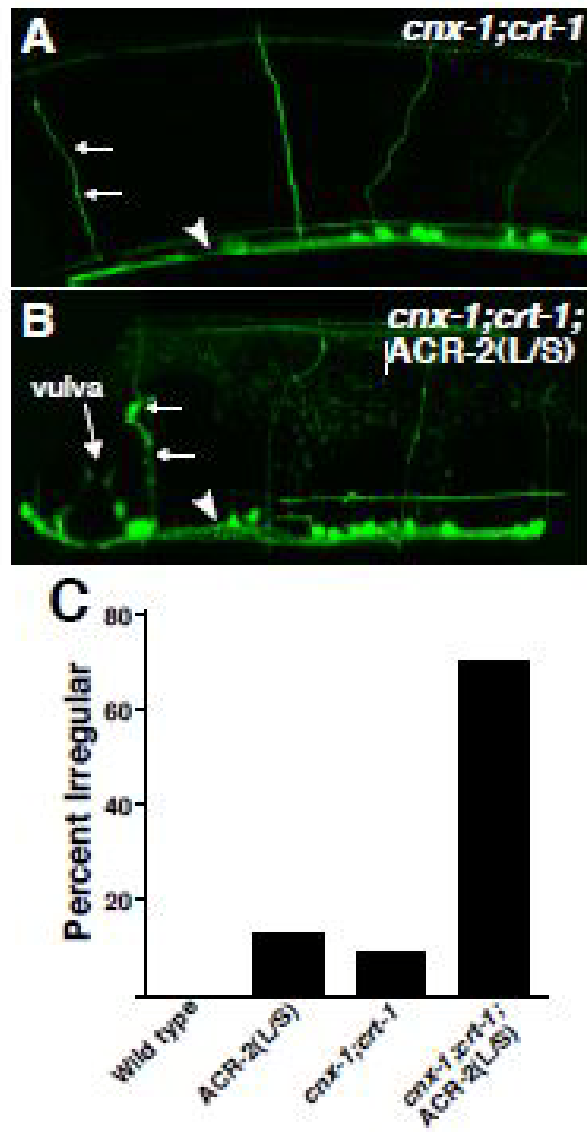


Figure A1-S6

Figure A1-S6. Motor neuron processes are irregular in *cnx-1;crt-1* double mutants expressing transgenic ACR-2(L/S). (A and B) Confocal images of commissural processes and ventral nerve cord processes in *cnx-1;crt-1* double mutants (A) and *cnx-1;crt-1* double mutants expressing the ACR-2(L/S) transgene (B). Arrowheads show normally fasciculated ventral nerve cord in *cnx-1;crt-1* double mutants (A) and defasciculation in *cnx-1;crt-1;ACR-2(L/S)* animals (B). Arrows show normal commissural process in (A) and ectopic sprouting in (B). In each case, a region immediately posterior of the vulva was imaged and the ventral nerve cord is positioned at the bottom. Images show Z-projections of 34 confocal planes (A) or 33 confocal planes (B) (0.5 μm /slice). For all fluorescent imaging, animals are expressing an integrated *Punc-17::GFP* transgene (*vs/s48*). (C) Quantification of the percentage of irregular commissural processes in wild type (15 commissures from 5 animals), transgenic ACR-2(L/S) (39 commissures from 10 animals), *cnx-1;crt-1* (75 commissures from 19 animals) and *cnx-1;crt-1;ACR-2(L/S)* (93 commissures from 17 animals). Since *Punc-17::GFP* also labels AS motor neurons that project commissural axons and do not express *acr-2*, the percentage of axonal defects in ACR-2(L/S) expressing neurons is likely higher.

Allele [†]	% Paralyzed* (levamisole)	Gene
Wild type	90±3	
<i>e264</i>	3±3	<i>unc-38</i>
<i>uf62</i>	0	<i>unc-38</i>
<i>uf67</i>	0	<i>unc-38</i>
<i>uf153</i>	0	<i>unc-38</i>
<i>E306</i>	0	<i>unc-50</i>
<i>uf86</i>	3±3	<i>unc-50</i>
<i>e883</i>	0	<i>unc-74</i>
<i>uf124</i>	0	<i>unc-74</i>
<i>uf72</i>	5±5	<i>unc-74</i>
<i>ok1075</i>	3±2	<i>unc-63</i>
<i>uf79</i>	5±5	<i>unc-63</i>
<i>ok367</i>	94±4	<i>acr-12</i>
<i>uf65</i>	95±5	<i>acr-12</i>
<i>uf65</i>	75±12	<i>acr-12</i>
<i>uf65</i>	85±5	<i>acr-12</i>
<i>uf65</i>	70±10	<i>acr-12</i>
<i>uf65</i>	100	<i>acr-12</i>
<i>uf66</i>	90±4	<i>acr-12</i>
<i>uf66</i>	98±3	<i>acr-12</i>
<i>uf66</i>	85±5	<i>acr-12</i>
<i>uf77</i>	95±5	<i>acr-12</i>
<i>uf77</i>	45±5	<i>acr-12</i>
<i>uf77</i>	93±5	<i>acr-12</i>
<i>uf77</i>	95±5	<i>acr-12</i>
<i>uf77</i>	75±5	<i>acr-12</i>
<i>uf77</i>	95±5	<i>acr-12</i>
<i>uf94</i>	78±6	<i>acr-12</i>
<i>uf94</i>	80±10	<i>acr-12</i>
<i>uf95</i>	100	<i>acr-12</i>
<i>uf95</i>	85±5	<i>acr-12</i>
<i>uf95</i>	100	<i>acr-12</i>
<i>uf117</i>	100	<i>acr-12</i>
<i>uf117</i>	85±5	<i>acr-12</i>
<i>uf117</i>	90±10	<i>acr-12</i>

Table A1-S1

Table A1-S1. Suppressors of ACR-2(L/S) induced paralysis isolated from a forward genetic screen.

Gray shading denotes available alleles used as reference

*denotes percentage of animals paralyzed after 120 minutes in the presence of 200 μ M levamisole

†Alleles listed multiple times indicated duplicate isolates of the same allele.

Forty-four additional levamisole-resistant mutants (<20% paralyzed) were isolated but not characterized further.

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