Activity Regulates Neuronal Connectivity and Function in the C. elegans Motor Circuit: A Dissertation

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Activity regulates neuronal connectivity and function in the *C. elegans* motor circuit.

A Dissertation Presented

By

Belinda Barbagallo

Submitted to the Faculty of the

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July 15th 2014

Program in Neuroscience
Activity regulates neuronal connectivity and function in the 
*C. elegans* motor circuit.

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Abstract

Activity plays diverse roles in shaping neuronal development and function. These roles range from aiding in synaptic refinement to triggering cell death during traumatic brain injury. Though the importance of activity-dependent mechanisms is widely recognized, the genetic underpinnings of these processes have not been fully described. In this thesis, I use the motor circuit of *Caenorhabditis elegans* as a model system to explore the functional and morphological consequences of modulating neuronal activity.

First, I used a gain-of-function ionotropic receptor to hyperactivate motor neurons and asked how increased excitation affects neuronal function. Through this work, I identified a cell death pathway triggered by excess activation of motor neurons. I also showed that suppression of cell body death failed to block motor axon destabilization, providing evidence that death of the cell body and of motor axons can be genetically separated.

Secondly, I removed excitatory drive from a simple neural circuit and asked how loss of excitatory activity alters circuit development and function. I identified excitatory motor neurons as master regulators of inhibitory synaptic connectivity. Additionally, I was able to identify previously undescribed activity-dependent mechanisms for regulating inhibitory synapses in both developing and mature neural circuits.

Finally, I show data to implicate the highly conserved genes neurexin and neuroligin in determining inhibitory synapse connectivity. Collectively this work has lent insight into activity-dependent mechanisms in place to regulate neuronal development
and function, a core function of neurobiology that is relevant to the study of a wide range of neurological disorders.
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List of Abbreviations

ACh: acetylcholine
ALS: amyotrophic lateral sclerosis
ASD: autism spectrum disorder
BMP: bone morphogenic proteins
ChAT: choline acetyltransferase
CNS: central nervous system
DEG/ENaC: degenerin/epithelial sodium channel
DIC: differential interference contrast
dsRNA: double stranded RNA
ER: endoplasmic reticulum
E/I balance: excitation/inhibition balance
EPSC: excitatory postsynaptic current
FGF: fibroblast growth factor
GABA: gamma-aminobutyric acid
GABAR: GABA receptor
GAD: glutamic acid decarboxylase
iGLuR: ionotropic glutamate receptor
IPSC: inhibitory postsynaptic current
L1: first larval stage
L2: second larval stage
L-AChR/LevR: levamisole-sensitive acetylcholine receptor
LTD: long term depression
LTP: long term potentiation
mAChR: muscarinic acetylcholine receptor
MN: motor neuron
nAChR: nicotinic acetylcholine receptor
NMJ: Neuromuscular Junction
PMN: progressive motor neuronopathy
RNAi: RNA interference
RTK: receptor tyrosine kinase
SNP: single nucleotide polymorphism
TeTx: tetanus toxin
TM2: second transmembrane domain
vAChT: vesicular acetylcholine transporter
VNC: ventral nerve cord
CHAPTER I

General Introduction
Neuronal activity impacts a wide array of events in nervous system development and function. For example, patterned bursts of activity during early development are required for the refinement of neural networks (reviewed in Blankenship and Feller, 2010) and over activation of neurons during trauma, such as ischemic stroke, can lead to neurodegeneration (Arundine and Tymianski, 2004). In this thesis, I explore the functional consequences of altered excitatory activity within the nervous system in two ways: In Chapter II, I express a gain-of-function ionotropic receptor in cholinergic motor neurons and ask how increased excitation affects neuronal function. In Chapter III, I remove excitatory drive from a simple neural circuit and ask how loss of excitatory activity alters circuit development and function. In order to address these questions, I used the nematode Caenorhabditis elegans, which has a tractable motor circuit where activity can be easily modulated.

**Excitotoxic cell death**

Many studies have implicated hyperactivation of neurons through ionotropic receptors as a key factor that leads to excitotoxic cell death (Heintz, 2000). For example, hyperactivation of NMDA and AMPA receptors can lead to high levels of calcium in the cytoplasm, which triggers neurotoxic pathways within the cell (Szydlowska and Tymianski, 2010). This principle was explored in a series of knock-in mouse experiments where the pore spanning regions of various brain nicotinic acetylcholine receptor (nAChR) subunits were modified, leading to increased activation of the receptor (Orr-Urtreger et al., 2000); (Labarca et al., 1995); (Orb et al., 2004). This receptor activation
led to significant loss of neurons and perinatal lethality, suggesting that increasing receptor signaling is sufficient to trigger cell death. Work conducted on the rat α7 nAChR has shown that in cases of pharmacological activation, increased calcium levels occur prior to cell death, presumably due to a low level of calcium buffering capacity (Berger et al., 1998). These studies suggest that the hyperactivation of ionotropic receptors is ultimately sufficient to induce cell body death.

Ion channel-mediated cell death in *C. elegans* has been extensively characterized using a gain-of-function mutant of the degenerin/epithelial Na (DEG/ENaC) channel *mec-4*, which causes cell-autonomous death in all six mechanosensory neurons (Hong and Driscoll, 1994). Interestingly, *mec-4* is also capable of inducing cell death in motor neurons when ectopically expressed, suggesting that different neuronal types share common cell death pathways (Xu et al., 2001b). Morphological characterization of neuronal cell bodies in *mec-4*(*d*) mutants shows striking similarities to the changes observed in the organelles of mammalian neurons undergoing cell death. Electron microscopy in young animals shows subtle changes in the cellular morphology prior to the onset of cell death-associated behavioral changes (Hong and Driscoll, 1994) including the appearance of an electron dense ring around the nucleus, swollen mitochondria and disruption of the plasma membrane. By the onset of late stage degeneration, large vacuolar lesions within the cell body, chromatin aggregation and swelling of the ER occur, eventually leading to a complete breakdown of organelles and cell death (Tymianski et al., 1993). Similar phenotypes have been observed in mammalian models of excitotoxicity, where increased calcium levels lead to DNA fragmentation,
mitochondrial swelling and cellular membrane disruption (Szydlowska and Tymianski, 2010).

A forward genetic screen revealed a major role for calcium channels in the ER and the protein folding chaperone calreticulin in mediating cell body death in cases of *mec-4(d)*-induced degeneration (Flanagan-Steet et al., 2005). Calreticulin is primarily localized to the lumen of the endoplasmic reticulum and it acts to regulate calcium stores within the ER (Michalak et al., 1999). This genetic screen identified three other genes required for regulating calcium homeostasis in the ER: the calcium regulator calnexin (Wada et al., 1991), the calcium release channel inositol triphosphate receptor channel *itr-1* (Baylis et al., 1999) and the ER calcium release channel ryanodine receptor channel *unc-68* (Maryon et al., 1996). These studies support the hypothesis that calcium homeostasis plays an important role in regulating cell body death during excitotoxicity, but they do not address potential early events in excitotoxic damage such as axonal degeneration and synapse loss.

In this thesis, I use a gain-of-function ionotropic acetylcholine receptor in cholinergic motor neurons to carry out genetic analysis of genes required for motor neuron degeneration in cases of hyperactivation. Through this work, I show evidence that motor axon cell death is genetically separable from cell body death, lending potential insight into mechanism of axon dysfunction in neurodegenerative diseases.

**Ionotropic Acetylcholine Receptors**

Henry Hallett Dale first identified acetylcholine (ACh) in a 1914 study to determine the chemical nature of synaptic transmission. Otto Loewi later confirmed the
role of ACh as a neurotransmitter with his work on the vagus nerve (Fishman, 1972). This work resulted in a joint Nobel Prize in Physiology and Medicine in 1936 for their combined efforts to identify acetylcholine as the first known neurotransmitter.

Acetylcholine is primarily an excitatory neurotransmitter that depolarizes neurons through the activation of either ligand gated nicotinic receptors (nAChRs) or G-protein coupled muscarinic receptors (mAChRs) (Dani and Bertrand, 2007). nAChRs consist of five receptor subunits surrounding an aqueous ion pore that is permeable to cations upon activation of the channel (Figure 1-1) (McGehee and Role, 1995); (Jones et al., 1999); (Karlin, 2002). Channel activation is achieved by the binding of acetylcholine to one of the channel binding sites at the junction of two alpha subunits on the extracellular face of the receptor. Heteromeric receptors have two binding sites while homomeric receptors have five (Gay and Yakel, 2007).

The second transmembrane domain of each receptor subunit lines the ion pore forming the ion gate of the receptor (Albuquerque et al., 2009) (Figure 1-1). Upon acetylcholine binding, the receptor undergoes a conformational change leading to a repositioning of hydrophobic amino acid residues of the TM2 domain, which are replaced with more hydrophilic residues, allowing ions to pass through the pore. The TM2 domain is essential for establishing the channel gate, ion selectivity and channel conductance of the acetylcholine receptor (Albuquerque et al., 2009). Mutations in the amino acid sequence of the TM2 pore domain can alter properties of the ion gate resulting in an increase in channel permeability, a phenomenon seen in certain types of epilepsy (Kuryatov et al., 1997); (Lester et al., 2003).
A wide variety of nAChR subtypes arise from the combination of different receptor subunits, as the activation and desensitization kinetics of these receptors are directly influenced by the amino acid sequences within individual subunits (Jensen et al., 2005). The diversity of receptor subtypes, and their localization contribute to the variety of functions they perform in the nervous system. In the mammalian nervous system, synaptically localized AChRs are located primarily at the neuromuscular junction and within the autonomic ganglion but are rarely found in the CNS (Changeux, 2010). In the mammalian CNS, AChRs are primarily extrasynaptic and they modulate neuron excitability, regulate presynaptic release of neurotransmitters and coordinate the firing of neuron groups (Kawai et al., 2007); (Wonnacott, 1997); (Rice and Cragg, 2004); Zhang and Sulzer, 2004, #26132}. Through my work I have developed a novel method for the identification of AChR subunit composition and accessory proteins required for receptor localization.

Both nAChR and mAChRs have been linked to synaptic plasticity in the mammalian brain (Cobb and Davies, 2005); (Fujii and Sumikawa, 2001); (Ji et al., 2001); (Maylie and Adelman, 2010); (McGehee, 2002). For example, activation of alpha-7 containing receptors on presynaptic terminals can facilitate long term potentiation (LTP), the cellular basis of learning and memory, in pyramidal cells within the hippocampus (Ji et al., 2001).
Figure 1-1: The structure of nicotinic acetylcholine receptors

A: Homomeric receptor viewed from extracellular side. Homomeric receptors consist of five alpha subunits arranged around an aqueous ion pore. Acetylcholine binding sites are located at the junction between subunits. B: Heteromeric receptor viewed from extracellular side. Heteromeric receptors consist of two alpha subunits and one each of β, γ and δ subunits. Acetylcholine binding sites are located at the α-γ and α-δ junctions. C: Four transmembrane domain composition of a single nAChR subunit with the pore-spanning TM2 domain in green. D: Subunit arrangement around the ion pore. The pore-spanning TM2 subunit domains are indicated in green.
Defects in cholinergic signaling in the hippocampus have been linked to a variety of learning and memory disorders including Alzheimer’s disease, schizophrenia and epilepsy. (Dani and Bertrand, 2007); (Terry and Buccafusco, 2003).

**Activity dependent neural circuit assembly and E/I balance**

During development, a sculpting and refinement period occurs after axon guidance during which neurons hone their synaptic connections to form functional neural circuits (Katz and Shatz, 1996). Spontaneous activity within circuits play a critical role in this refinement period, with correlated spontaneous activity observed in areas of the brain including the retina, cochlea, spinal cord, cerebellum, hippocampus and neocortex (Blankenship and Feller, 2010); (Dehorter et al., 2012); (Feldt et al., 2011); (Moody and Bosma, 2005). It is proposed that activity during this time works to drive circuit refinement by strengthening and weakening connectivity between neurons (Balice-Gordon and Lichtman, 1994); (Goda and Davis, 2003). For example, repeated stimulation of postsynaptic cells by presynaptic activity causes strengthening of the synapse or long-term potentiation (LTP) while weak stimulation leads to depression of the synapse or long-term depression (LTD) in a process termed Hebbian Plasticity (Katz and Shatz, 1996). It is essential that excitatory and inhibitory signals within the network remain balanced (E/I balance) throughout these periods of refinement to enable appropriate neural circuit connectivity and brain function (Gatto and Broadie, 2010).

One of the most extensively studied family of disorders linked to E/I balance are the Autism Spectrum Disorders (ASD). This family of neurodevelopmental diseases is
characterized by compromised communication and social skills and reduced cognition. The early onset of these symptoms suggests that defects occur during fetal or early postnatal development. Studies on autism models have shown that the increased ratio of excitation/inhibition in the sensory, mnemonic social and emotional systems, leads to increased waves of excitation during development, which causes missed critical periods resulting in an unstable circuit in the mature brain (Rubenstein and Merzenich, 2003).

GABA interneurons comprise 10-25% of cortical neurons in the adult brain and play an essential role in regulating activity levels of pyramidal neurons (Le Magueresse and Monyer, 2013). In addition to controlling cortical activity in the adult brain, GABA neurons also help maintain E/I balance in the developing cortex. The development of the GABA nervous system begins prior to the establishment of release sites in glutamatergic neurons, and continues throughout normal synaptogenesis, suggesting a role for GABA in regulating synaptic development (Verhage et al., 2000). This is supported by work in rodent models, which shows that blocking GABA release during early development results in a permanent decrease in excitatory synaptic transmission and excitatory dendrite length in the adult nervous system (Wang and Kriegstein, 2008); (Wang and Kriegstein, 2011). Additionally, altering the timing of the switch between excitatory and inhibitory GABA signaling leads to changes in dendritic morphology, illustrating the importance of GABA signaling in the development of cortical circuits (Cancedda et al., 2007).

During postnatal development, GABA interneurons act as pacemakers for other cortical neurons, which is a function they maintain in the adult brain. Inhibitory drive
during this period is critical for the establishment of adult oscillation patterns, which control network refinement during postnatal critical periods. The best described critical period in activity-dependent circuit refinement is for ocular dominance, in which closure of one eye during periods of synaptic refinement leads to an increased field for the uncovered eye at the expense of the covered eye (Wiesel and Hubel, 1963). Blocking GABA activity during this ocular dominance paradigm either pharmacologically (Reiter and Stryker, 1988) or genetically (Hensch et al., 1998) blocks this change in territory, indicating that a threshold amount of GABA release is required for synaptic refinement. Synaptic wiring and neural circuit architecture are also directly shaped by GABAergic transmission in late cortical development (Hensch and Stryker, 2004); (Oray et al., 2004); (Mataga et al., 2004). Collectively, these studies show that GABA neuron function is essential for the establishment of nervous system connectivity.

Once initial nervous system connectivity is established, homeostatic mechanisms are in place to maintain circuit function within a tolerable range. These mechanisms prevent hyperexcitation of neurons, which maintains brain function. Homeostatic mechanisms that maintain circuit activity within a specific dynamic range have been described in many systems (Turrigiano and Nelson, 2004); (Davis, 2006); (Maffei and Fontanini, 2009), and disruption of these mechanisms has been linked to a large number of neuropsychiatric and neurodevelopmental disorders ranging from schizophrenia to autism (Gatto and Broadie, 2010); (Kehrer et al., 2008); (Wassef et al., 2003). Though many studies have identified disruption of E/I balance as a key component of
neurological disorders, many questions remain about the mechanisms required to maintain this balance in the developing nervous system.

Activity dependent remodeling of circuits

In mammals, mature neuromuscular junctions (NMJ) consist of muscle cells that receive input from a single motor neuron (Brown et al., 1976). Activity dependent synapse editing plays an important role in regulating the number of motor neurons synapsing onto each muscle cell. The mechanisms underlying this editing are not fully understood, but it is believed that retrograde signals from the post-synaptic muscle cells work to modulate the activity of pre-synaptic motor neurons (Balice-Gordon et al., 1993). One class of proteins that has been identified as an activity dependent messenger in NMJ formation is the TGFβ family of proteins. This family consists of TGFβ, bone morphogenic proteins (BMPs) and activins that are secreted by motor neurons, glia and muscle in mammals. Studies on BMP function at the Drosophila NMJ have shown that mutations in either the BMP ligand or the BMP receptors lead to presynaptic defects such as a reduced number of NMJs and impaired neurotransmitter release (Marques et al., 2002); (McCabe et al., 2004). Postsynaptic morphology is unaltered in these animals, suggesting that the TGFβ family act as retrograde messengers at the NMJ (Aberle et al., 2002).

A second class of proteins that have been implicated in retrograde signaling at the NMJ is the Wnt family of proteins. At the mammalian NMJ, Wnt3a functions through the canonical β-catenin pathway to decluster acetylcholine receptors (Henriquez et al.,
In addition to receptor declustering, activation of β-catenin leads to a retrograde signal that controls mechanisms of pre-synaptic assembly (Li et al., 2008). In *Drosophila*, the Wnt wingless and its receptor frizzled-2 play important roles in NMJ development. Wingless is secreted by motor neurons and activates signaling cascades, which work to form the neuromuscular synapse (Packard et al., 2003).

Evidence of activity-dependent synapse remodeling has also been demonstrated in *C. elegans*. During the transition from the first to second larval stage, the six GABAergic DD motor neurons switch polarity from receiving input dorsally and project ventrally to receiving input ventrally and project dorsally (White et al., 1978); (Thompson-Peer et al., 2012). During this switch, presynaptic terminals are disassembled on the ventral side and new synapses are formed on the dorsal processes (Hallam and Jin, 1998). The timing of this switch in innervation is directly tied to activity within the motor circuit, as mutations that increase or decrease circuit synaptic release alter the timing of remodeling (Thompson-Peer et al., 2012). Through the combined effort of multiple labs, some of the molecular mechanisms underlying this process have begun to be described.

During DD remodeling, the elimination of GABA presynaptic terminals in the ventral nerve cord and establishment of new release sites in the dorsal nerve cord occurs simultaneously. This process is mediated by expression of *cyy-1*, which encodes a cyclin box-containing protein that removes presynaptic proteins from the ventral nerve cord, and *cdk-5*, which encodes a cyclin-dependent kinase that facilitates trafficking to new synaptic sites (Park et al., 2011). Animals carrying single mutations in either of these genes show delayed, incomplete remodeling, while the double mutant animals fail to
remodel. Simultaneous reintroduction of cyy-1 and cdk-5 later in development triggers remodeling suggesting that remodeling is initiated by cyy-1 and cdk-5 expression (Park et al., 2011).

Unlike the DD motor neurons, the GABAergic VD motor neurons do not undergo a remodeling process. Remodeling in these neurons is inhibited by expression of the transcription factor unc-55 (Shan et al., 2005). unc-55 encodes the sole homolog of the COUP-TF nuclear hormone receptor family in C. elegans that has shown to play wide roles in the development of the mammalian nervous system (Zhou and Walthall, 1998); (Armentano et al., 2007); (Tomassy et al., 2010). This transcription factor is thought to function by regulating the activity of genes required for the remodeling of DD motor neurons. One of the downstream targets that has been characterized is the conserved homeodomain-transcription factor irx-1/Iroquois (Petersen et al., 2011). Loss of irx-1 results in delayed DD remodeling, but is unable to completely block the process. This suggests that parallel pathways must be in place to regulate the synaptic remodeling process.

DD remodeling has been linked to neuronal activity through hbl-1 (hunchback-like-1), a transcription factor, which is active in DD neurons but is repressed in VD neurons by unc-55 (Fay et al., 1999); (Thompson-Peer et al., 2012). Animals carrying loss of function mutations in hbl-1 show delayed DD remodeling, though the timing of related cell fates remains unchanged. Genetic analysis showed that changes in activity levels correlated to changes in hbl-1 expression in the DD neurons. Decreased circuit activity results in decreased hbl-1 expression, ultimately leading to delayed DD...
remodeling (Thompson-Peer et al., 2012). These results show that changes in hbl-1 expression are required to modulate activity-dependent changes in the timing of inhibitory synaptic remodeling. This system shows evidence for activity-dependent control of neuronal development in C. elegans but the role of activity in the initial establishment of synaptic fields remains uncharacterized.

**C. elegans as a model system to study nervous system function**

Sydney Brenner first proposed the use of the soil-dwelling nematode Caenorhabditis elegans as a model system for studying the nervous system in 1963. He published a paper detailing techniques for conducting forward genetic screens and identifying genetic mutations, which established C. elegans as a viable genetic model for studying nervous system function and behavior (Brenner, 1974a). The highly simplified nervous system of C. elegans consists of only 302 neurons with well-established connectivity (Ward et al., 1975; White et al., 1976), allowing for the study of neural networks. Additionally, the genome of C. elegans has been fully sequenced and highly annotated, and there are mutants of many genes available. The combination of well-defined connectivity and genetic tools makes C. elegans an ideal model for the study of neural circuit development and function.

The genome of C. elegans contains high levels of homology to mammalian genes, enabling the study of medically relevant genetic pathways in a convenient and tractable system. The ability of C. elegans to self-fertilize allows the study of genetic mutations that render the nervous system non-functional, many of which are lethal in other systems.
Furthermore, transgenic strains can be generated quickly and easily in this system (Mello et al., 1991a), allowing targeted genetic manipulation and tagging of specific proteins with genetically encoded fluorescent markers. These fluorescently marked strains are especially useful because the *C. elegans* cuticle is transparent, which allows the study of neuronal morphology and protein localization in a fully intact nervous system. *In vivo* electrophysiological techniques in *C. elegans* make it possible to directly monitor functional changes at synapses that occur as a result of genetic manipulations. This combination of tools has already been used to address fundamental questions in neurobiology and provided an ideal system for my study of the role of activity in nervous system development and dysfunction.

**The *C. elegans* motor circuit**

The structure of the *C. elegans* motor circuit was initially described in the Brenner Lab as part of an extensive electron microscopy reconstruction project spearheaded by John White. The motor neurons were organized into five classes and described based on neuronal morphology and connectivity within the circuit. These motor neuron classes have distinct, non-overlapping domains and are connected to members of their own class by gap junctions (White et al., 1976).

More recent work has characterized the five classes of motor neurons initially described by White as either inhibitory GABAergic motor neurons (VD/DD) or excitatory cholinergic motor neurons (VA, DA, VB, DB and VC), where V- indicates ventral innervation and D- indicates dorsal innervation. In the functional circuit,
interneurons activate excitatory cholinergic motor neurons that form synapses onto both body wall muscle and inhibitory GABA motor neurons. Depolarization of body wall muscle by cholinergic innervation results in muscle contraction. GABA motor neurons project to the contralateral musculature where they form synapses with body wall muscle, causing relaxation (Figure 1-2). The C. elegans genome does not encode voltage gated sodium channels, resulting in a lack of classical sodium action potentials in this process. Instead, motor neurons signal through graded release mechanisms, which are determined by the size of the depolarizing stimulus, resulting in high frequency bursts of postsynaptic currents lasting between 7s and 3min⁻¹ in wild type worms (Liu et al., 2009). The bursting activity of motor neurons results in action potential firing in body wall muscles (Liu et al., 2013) leading to muscle contractions. The balance of excitation and inhibition across muscle quadrants coordinates the characteristic sinusoidal body bend pattern of C. elegans.

The C. elegans Neuromuscular Junction

At the C. elegans neuromuscular junction (NMJ), muscles extend projections called muscle arms to the nerve cord where they form en passant synapses with motor neuron axons (Figure 1-3). Ionotropic receptors are clustered opposite release sites at the tips of muscle arms where they form neuromuscular junctions. Two receptor classes mediate excitatory cholinergic signaling in muscle, a group of nicotine sensitive acetylcholine receptors (N-AChRs), and a group of levamisole-sensitive acetylcholine receptors (L-AChRs). The N-AChR is homomeric, consisting of five subunits of
Acetylcholine receptor-16 (ACR-16) (Francis et al., 2005a); (Touroutine et al., 2005).

ACR-16 receptors are sensitive to activation by both nicotine and acetylcholine and have been shown to mediate the majority of cholinergic response at the NMJ ((Francis et al., 2005a)). L-AChRs are heteromeric, consisting of five receptor subunits (unc-29, unc-38, unc-63, lev-1, lev-8) (Fleming et al., 1997). These receptors are referred to as levamisole receptors (LevR) due to their sensitivity to activation by the anthelmintic drug levamisole. Functional responses to acetylcholine are completely abolished in mutant animals lacking essential subunits of these two receptor classes (unc-29; acr-16) (Francis et al., 2005a), suggesting that they are the sole mediators of ACh release onto body wall muscles.

Inhibitory transmission onto body wall muscle is mediated by a homomeric GABA receptor (GABAR) consisting of five subunits encoded by splice isoforms of the gene uncoordinated-49 (unc-49) (Bamber et al., 1999). The unc-49 gene contains a single N-terminus with three alternative c-terminal splice options, which results in three receptor subunits (UNC-49A, UNC-49B and UNC-49C). The UNC-49B and UNC-49C isoforms are highly expressed in muscle and co-localize to synaptic regions, suggesting that they form the functional inhibitory receptor at the C. elegans NMJ (Bamber et al., 1999).

During C. elegans locomotion, AChRs mediate contraction of the body wall muscle, while GABARs mediate inhibition of the contralateral muscle (Figure 1-2).
Figure 1-2: The *C. elegans* motor circuit

Schematic representation of the *C. elegans* motor circuit. Excitatory cholinergic motor neurons (gray) form dyadic synapses onto body wall muscles (brown) causing contraction and onto inhibitory GABA motor neurons (purple), which project to the contralateral muscle causing relaxation.
Maintaining proper balance of excitation and inhibition at the NMJ is essential for the characteristic sinusoidal locomotion pattern of *C. elegans* (Jospin et al., 2009); (Schuske et al., 2004). The well-characterized morphology of the NMJ enables the assessment of changes in synapse density or patterning caused by genetic manipulations of the circuit.

**Development of the *C. elegans* motor circuit and neuromuscular junction**

The *C. elegans* life cycle is short and temperature-sensitive. Animals develop from egg to fertile adult in approximately 2.5 days at room temperature (22°C). The life cycle consists of an embryonic stage followed by four larval stages before adulthood. During late embryogenesis, approximately 6-12 hours post-fertilization at room temperature, progenitor cells terminally differentiate into motor neurons of the locomotory circuit.

Immature muscle cells line up along the newly formed nerve cords then migrate away, leaving behind membrane extensions (Dixon and Roy, 2005a). These membrane extensions form the first muscle arms, which average 1-2 projections for each muscle cell (Dixon and Roy, 2005a). During the last phase of embryogenesis, the body plan of the animals is established and this basic organization remains largely unchanged throughout larval development. Larval development begins at hatch, approximately 14 hours post-fertilization at room temperature, and consists of four stages separated by cuticle molts. A burst of proliferation occurs between the first and second larval stages during which an additional 80 neurons and 14 body wall muscles are produced (Sulston and Horvitz, 1977).
**Figure 1-3: The *C. elegans* neuromuscular junction**
Cartoon representation of the *C. elegans* neuromuscular junction viewed from the ventral side. Cholinergic motor neurons (gray) and GABAergic motor neurons (purple) run along the ventral nerve cord where they form synapses onto body wall muscles. Body wall muscles send membrane projections, called muscle arms (brown), to the nerve cord. Two cholinergic receptors (ACR-16 and LevR) as well as one GABAergic receptor (UNC-49) are clustered along the tips of muscle arms where they form *en passant* synapses with motor neurons.
During this stage, body wall muscles actively extend additional muscle arms to the nerve cord where they form NMJs, resulting in an average of three or four muscle arm extensions for each body wall muscle (Dixon and Roy, 2005a). The outgrowth of muscle arms occurs through an active process by which motor neurons secrete a ligand (*madd-4*) that binds to the muscle receptor *unc-40/DCC* to control postsynaptic outgrowth via an *unc-73/trio* dependent pathway (Alexander et al., 2009; Seetharaman et al., 2011).

**Mechanisms of receptor clustering in *C. elegans***

How distinct synapses are organized remains a central question in neurobiology. Many studies have taken advantage of the high sequence homology and genetic tractability of the *C. elegans* system to better understand mechanisms underlying synaptic development. To date, most of this work has been focused on the clustering of excitatory AChRs at the NMJ and glutamate receptors in neurons. Two types of acetylcholine receptor are found at the *C. elegans* NMJ: the homomeric nicotine-sensitive receptor ACR-16 and the heteromeric levamisole-sensitive receptor Lev-R. Each class of receptor is localized by distinct mechanisms.

The localization and function of the homomeric ACR-16-containing receptor at the *C. elegans* NMJ is regulated by the gene *cam-1* (Francis et al., 2005a). *cam-1* encodes a ror-family receptor tyrosine kinase (RTK) with a high sequence homology to the mammalian RTK MuSK, which is associated with AChR clustering at the mammalian NMJ (Burden, 2002); (Ghazanfari et al., 2011). Initial observation of *cam-1* loss-of-function mutants showed uncoordinated locomotion patterns, suggesting that *cam-1* plays a role in
regulating synapses at the NMJ (Forrester and Garriga, 1997). CAM-1 is localized diffusely in the cell bodies of ACh motor neurons as well as in body wall muscle, with a higher concentration of protein at the tips of the muscle arms (Francis et al., 2005a).

The functional properties of the NMJ are altered in cam-1 mutants. Muscles are less responsive to ACh release and there is a reduction in the amplitude of both evoked and spontaneous release events, suggesting that the delivery or stabilization of ACR-16R is altered in the absence of CAM-1. Consistent with functional analysis, the localization of ACR-16-containing receptors, but not LevR or GABARs, is altered in cam-1 loss-of-function mutants. The combination of altered kinetics of ACh response and altered synaptic vesicle localization in cam-1 mutant animals suggests that cam-1 also regulates presynaptic release (Francis et al., 2005a).

The extracellular domain of CAM-1 contains a cysteine-rich domain that enables Wnt binding. Wnt proteins have conserved roles in synapse development across multiple systems and they play a role in a diverse set of developmental events (Budnik and Salinas, 2011). A recent study has linked cam-1 to Wnt signaling by identifying the C. elegans frizzled-like receptor lin-17 as the co-receptor for cam-1. Together these receptors bind the Wnt ligand cwn-2 to instruct ACR-16 localization in the synaptic membrane (Jensen et al., 2012). Expression of lin-17 is required throughout the lifespan of the animal to maintain appropriate localization of ACR-16 receptors. Changes in activity levels at the NMJ alter ACR-16 localization; this form of plasticity is cwn-2 dependent, suggesting that Wnts play an important role in synaptic plasticity at the C. elegans NMJ (Jensen et al., 2012).
Additional studies have identified multiple genes required for the localization of the levamisole-sensitive receptor at the *C. elegans* NMJ. Two of the genes that have been well characterized are *lev-9* and *lev-10*. *lev-9* encodes a 622 amino acid secreted protein consisting of a signal peptide followed by a single WAP domain and eight sushi domains (Gendrel et al., 2009). Localization studies indicate that LEV-9 is expressed only from body wall muscles and the secreted protein is localized in a punctate pattern along the ventral nerve cord and in the nerve ring. These puncta co-localize with LevR markers and are opposed to ACh release sites, suggesting that LEV-9 localizes to the synaptic cleft (Gendrel et al., 2009). *lev-10* encodes a type1 transmembrane protein with a large extracellular region (Gally et al., 2004). LEV-10 is localized to the tips of the body wall muscle arms and colocalizes with LevRs and LEV-9 at the NMJ.

LevRs are unable to cluster in animals carrying mutations in either *lev-9* or *lev-10*, though surface expression levels of the receptor and functional responses to levamisole application remain normal. Electrophysiological analysis shows normal responses to pressure-applied levamisole but large reductions in evoked responses in *lev-9* mutants, further supporting the idea that receptors are expressed at the surface normally but are unable to cluster at synapses in the absence of LEV-9 and LEV-10. The localization of both ACR-16R and GABARs at the NMJ and cholinergic presynaptic boutons remain unaltered in the absence of LEV-9. These data indicate that LEV-9 and LEV-10 are specifically required to localize LevRs to synapses at the NMJ (Gendrel et al., 2009).

Though genetic pathways required to cluster cholinergic receptors have been identified, less is known about genetic pathways underlying GABA synapse clustering.
and localization in *C. elegans*. Most of the studies conducted have focused on the role of presynaptic release in clustering postsynaptic GABAergic receptors. In wild type animals, GABA receptors form puncta along the tips of muscle arms where they cluster opposed to presynaptic release sites (Gally and Bessereau, 2003a). Removing GABA motor neuron innervation of muscle results in a loss of these puncta and a diffusion of GABAR in the body wall muscle cell surface (Rowland et al., 2006). This result suggests that presynaptic neurons provide a cue for the trafficking of GABA receptors. This cue is not related to neurotransmission, as presynaptic GABA release is not required for the localization of UNC-49 or for the differentiation of GABA presynaptic terminals (Jin et al., 1999a); (Gally and Bessereau, 2003a). GABA receptor clustering remains wild type in the absence of cholinergic innervation and complete denervation of muscle results in the accumulation of receptor GFP in autophagosomes (Gally and Bessereau, 2003a). The cue that stimulates GABA receptor clustering remains unknown, but some speculate that a secreted growth factor, an extracellular matrix molecule or other cell surface receptor in the presynaptic neuron fulfills this function.
Significance of this work

Excitotoxicity in the nervous system

It is widely recognized that the hyperactivation of ion channels can have devastating consequences for the nervous system, often resulting in cell death. Ion channel-mediated neuronal death is the result of the excitotoxic events that occur during ischemic stroke (Simon et al., 1984), and has also been observed in a variety of human neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) (Grosskreutz et al., 2010). Despite overwhelming evidence documenting the importance of excess ion channel activation in triggering neuronal degeneration, a mechanistic understanding of the downstream cellular events that lead to neuronal damage and death remains largely unclear.

In this work, I developed a C. elegans model of ionotropic receptor-mediated excitotoxicity in motor neurons. I used this model to identify a calcium-mediated cell death pathway that triggered necrotic-like cell death in response to hyperactivation. Additionally, I showed evidence that necrotic-like death of cell bodies is genetically separable from the mechanisms that control destabilization of motor axons. These findings provide mechanistic insights into ion-channel mediated cell death and establish a genetic model for further study of pathways underlying excitotoxicity.

Activity-dependent mechanism instructing inhibitory network assembly and maintenance

The balance of excitation and inhibition within neural circuits is required for proper brain function. Loss of the ability to maintain this balance leads to cases of hyper-
and hypo-activation within the brain that can lead to dysfunction. Many common neurological diseases ranging from autism to epilepsy are thought to have an E/I balance component. Though these studies establish the importance of maintaining E/I balance for brain function, the genetic pathways that regulate activity levels during development have not been completely characterized.

Through my work, I have shown a mechanism by which excitatory drive instructs the patterning and strength of inhibitory motor neurons during development. I have also demonstrated differential roles for excitatory drive in the developing versus the mature nervous system. I observed a rapid scaling of inhibitory synapse size in response to changes in excitatory activity, which supports the existence of acute activity-dependent plasticity, a phenomenon that has not been thoroughly characterized in *C. elegans*. Exploratory experiments have linked the conserved genes *nrx-1/neurexin* and *nlg-1/neuroligin* to this activity-dependent process providing evidence that my model can be used to identify genes required for synapse development and plasticity.
CHAPTER II

A dominant mutation in a neuronal acetylcholine receptor subunit leads to motor neuron degeneration in *Caenorhabditis elegans*

Belinda Barbagallo, Hilary A. Prescott, Patrick Boyle, Jason Climber and Michael M. Francis

Contribution Summary

The following work was carried out in the lab of Dr. Michael M. Francis at the University of Massachusetts Medical School. In this chapter, Hilary A. Prescott provided the co-expression strain and image in Figure 2-1A and the *Pacr-12::acr-12* rescuing construct in Figure 2-5; Patrick Boyle provided strains and constructs; Jason Climber provided strains and provided advice for the SNP mapping of candidate suppressors. Michael Francis supported the project by discussing experimental design, providing advice on data analysis and co-writing the paper. I designed the experiments, analyzed data and co-wrote the paper.

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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 receptors (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., 2001a)- 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 and Nishi, 2007; Hruska et al., 2009). 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 (Treinin and Chalfie, 1995; Zuo et al., 1997; Heintz and Zoghbi, 2000; Orr-Urtreger et al., 2000; Labarca et al., 2001; Orb et al., 2004; Miwa et al., 2006). 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 (Kwak and Weiss, 2006; Grosskreutz et al., 2010). Interestingly, in various mouse models of motor neuron disease, including ALS, genetic manipulations that prevent the death of motor neuron cell bodies are not successful in halting disease progression (Sagot et al., 1995; Gould et al., 2006). 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 details 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.

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 are required for neuromuscular signaling (Richmond and Jorgensen, 1999);(Francis et al., 2005a);(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 (Hallam et al., 2000);(Cinar et al., 2005a);(Jospin et al., 2009, #12023).

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 the cholinergic motor neurons (DA, VA, DB, VB) in the ventral nerve cord of adult animals (Figure 2-1A). 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 2-S1). 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 co-expressed GFP under the 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 2-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 in 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 defects

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 ~2.8kb 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 2-1B). 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 2-1C). 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 defects
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 TM2 region has been shown to have profound effects on receptor activation properties (Figure 2-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 activation and very slow inactivation (Revah et al., 1991);(Labarca et al., 1995).

We engineered the homologous leucine-to-serine point mutation into the sequence encoding the TM2 9’ position of an acr-2 rescuing construct to generate ACR-2(L/S) (Figure 2-S3). Transgenic animals expressing and integrated ACR-2(L/S) array (ufIs25) were used for all subsequent analysis. These animals are viable and have roughly normal brood sized; however, adult animals are smaller than their wild type counterparts (Figure 2-2). Moreover, we noted obvious locomotory defects in transgenic ACR-2(L/S) animals (Figure 2-2C). These defects were present in all larval stages as well as adult animals. Transgenic ACR-2(L/S) animals generated almost ten-fold 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 in 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, presumable as a result of cell death (Figures 2-3, 2-4). 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 2-3) (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 that included 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 (Figure 2-3, Figure 2-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 was dominant. To evaluate the effects of ACR-2(L/S) on GABA motor neurons, we co-expressed ACR-2(L/S) together with an mCherry transcriptional reporter that labeled GABA neurons (Punc-47::mCherry) (Figure 2-3C,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 promoter 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 co-expressing 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 (Figure 2-S4). To precisely determine the onset of motor neurons cell death, we imaged transgenic ACR-2(L/S) animals that co-expressed the Punc-17::GFP transcriptional reporter at various time points ranging from newly hatched larvae to adults (Figure 2-4). 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 16h after hatch. During that 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 2-3, Figure 2-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 that postembryonic addition of VC neurons. These results suggest that the other classes of motor neurons that are born post embryonically 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 AChR

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 2-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 2-1). 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 2-5A). 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 (Fleming et al., 1997); (Culetto et al., 2004); (Eimer et al., 2007). 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 2-5B). 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 2-5C,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 2-1). 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., 2005b);(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 2-5C–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, 2007a). 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 2-6). We found that a loss-of-function mutation in pro-apoptotic *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); (Szydlowska 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 four months of birth, while knock-out of calreticulin results in embryonic lethality caused by defects in heart development.
(Mesaeli et al., 1999); (Denzel et al., 2002). 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., 2001a). 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 2-7A,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 2-7C*). 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 2-6F,G, 2-7D). 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 2-7E–G,I). We found that defects in the
motor neuron processes of control cnx-1;crt-1 double mutants occurred only rarely
(Figure 2-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 2-7). 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 2-7I), 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 \textit{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 2-8A,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 \textit{unc-68} mutants lacking functional ryanodine receptors (Figure 2-8C). 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 \textit{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 \textit{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 \textit{cnx-1;crt-1} double mutant animals compared to wild-type animals (Figure 2-8D–G). This result suggests that a decrease in the levels of ACR-2(L/S) in \textit{cnx-1;crt-1} animals also contributes to the neuroprotection we observe.

Discussion

Our analysis of ACR-2-containing nicotinic receptors in \textit{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 \textit{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 (Knobel et al., 2001);(Hammarlund et al., 2007a). 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.

\textit{ACR-2 is part of a heteromeric nAChR in cholinergic motor neurons}

We have demonstrated that \textit{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, \textit{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 \textit{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 (Fleming et al., 1997);(Culetto et al., 2004). acr-12 is broadly expressed in the nervous system but is not expressed in body wall muscles (Cinar et al., 2005b; 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 (Richmond and Jorgensen, 1999); (Francis et al., 2005a); (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 \textit{acr-12} in the cholinergic motor neurons of transgenic \textit{acr-12} mutants expressing ACR-2(L/S) was sufficient to produce paralysis, whereas specific expression of \textit{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).

\textit{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 \textit{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 (Orr-Urtreger et al., 2000);(Labarca et al., 2001);(Orb et al., 2004). 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 (Treinin and Chalfie, 1995);(Xu et al., 2001a);(Syntichaki et al., 2002). 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 neuronopathy (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 pro-apoptotic Bax gene (Sagot et al., 1995); (Gould et al., 2006). 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.

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 of *acr-12* and subcloning it into the NheI/SacI 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 AgeI/AatII 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, pH3, 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 (ufIs25) 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 ufIs31, IZ814 ufIs25, IZ625 ufIs25;ufIs31, IZ790 ufIs49, IZ627 ufIs42, LX949 vsIs48, IZ924 ufIs25;vsIs48, IZ950 ced-3(ok2734); ufIs25;vsIs48, IZ877 cnx-1(ok2234);ufIs25;vsIs48, IZ926 crt-1(ok948);ufIs25;vsIs48, IZ976 crt-1(ok948);cnx-1(ok2234);ufIs25;vsIs48, IZ971 crt-1(ok948);cnx-1(ok2234);vsIs48, IZ927 crt-1(ok948);cnx-1(ok2234);ufIs25, IZ787 unc-29(x29);ufIs25, IZ928 acr-3(ok2049);ufIs265, IZ929 acr-5(ok180);ufIs25, IZ930 acr-9(ok933);ufIs25, IZ931 acr-19(ok967); ufIs25, IZ932 acr-23(ok2840);ufIs25,IZ446 unc-38(e264);ufIs25, IZ659 unc-50(e306);ufIs25, IZ921 unc-63(ok1075);ufIs25, IZ490 unc-68(e540);ufIs25, IZ604 unc-74(e883);ufIs25, IZ673 acr-12(ok367);ufIs25;ufEx148,IZ937 acr-12(ok367);ufIs25;ufIs60, IZ861 acr-12(ok367);ufIs25;ufEx191.

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

Paralyzed animals expressing the integrated ACR-2(L/S) array (ufIs25) were mutagenized with 50 mM ethyl methanesulfonate ((Brenner, 1974a)). Young adult F2 progeny of ~20,000 mutagenized animals were washed twice with M9 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 (ufIs25) 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 described as previously ((Wicks et al., 2001); (Davis et al., 2005)).

**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 1 mM aldicarb (Chem-Service), and movement was assessed every 15 min for 2 h. Data represent the mean ±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 µM 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 Imaging Source DMK21F04 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 (Imaging Source). 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 was counted manually with the researcher blinded to genotype.
Figure 2-1: *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 orientated 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 (*ufIs42*) over a 5 minute period are shown. Data represents mean ±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 (1mM) for wild type, *acr-2(ok1887)* mutants and *acr-2(ok1887)* mutants expressing full-length ACR-2::GFP (*ufIs42*). The percentage of immobilized animals calculated every 15 minutes over a time course of 2hr is shown. Each data point represents the mean ±SEM for at least four trials. *p,0.001, two-way ANOVA.*
Figure 2-2: 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). Not the coiled posture and reduced size that occurs as a result of ACR-2(L/S) expression. C. Quantification of the 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) (*ufIs25*) counted over a 5 min period are shown. Data represents the mean ±SEM of at least 10 trials; *p<0.02, **p<0.0001.
A: Wild type
B: ACR-2(L/S)

C: Graph showing body bends per minute for wild type, acr-2(ok1887), and ACR-2(L/S) with statistical significance indicated by asterisks (*) and double asterisks (**).
Figure 2-3: 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 to 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 motor 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 (ufIs53)* (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 (grey) and ACR-2(L/S) animals (black); *p<0.01 student t-test. For all images, animals are positioned with the head on the left side of the image.
Figure 2-4: 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) animals (B) expressing Punc-17::GFP images 16, 28, 38 and 48 hours after bleach synchronization of embryos. C, D: Confocal images of first larval stage wild type (C) and transgenic ACR-2(L/S) animals (D) 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 ±SEM for 5-8 animals at each time point.
Figure 2-5: 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 wild type animals, *acr-12(ok367), unc-63(ok1075), unc-38(e246), unc-74(e883)* and *unc-50(e306)* mutants in the absence (grey bars) 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 five minute period. Data represent the mean ±SEM of at least ten animals for each genotype. **B.** Schematic of the membrane topology of ACR-12 with the 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 *Pacr-2::ACR-12*. Data represent the mean ±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 2-6: ACR-2(L/S)-mediated motor neuron loss is completely prevented in animals doubly mutant in 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 orientated to the left. The alleles used were \textit{ced-3(ok2734), cnx-1(ok2234)}, and \textit{crt-1(ok948)}. G. Quantification of the average number of \textit{Punc-17::GFP} labeled cell bodies present in the ventral nerve cord for the genotypes indicated. Data represents the mean ±SEM for at least ten animals per genotype. *p<0.01, **p<0.001, compared to transgenic ACR-2(L/S) animals.
Figure 2-7: 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) animals (A) or *cnx-1;crt-1*;ACR-2(L/S) animals (B) 30s (t=0), 60s (+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) animals over the course of 60 s. The additional tracks on the plate show the path of the animals 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 (D) expressing *Punc-17:*GFP. Images show Z-projections of 15 confocal planes (0.5 µM/slice) (C) or 16 confocal planes (0.5 µM/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 to the vulva was images 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 µM/slice). Scale bars represent 10µM. **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 ±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 2-8: 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)* mutants animals (C). Data represent the mean ±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 (0.5µM/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 ±SEM for at least 10 animals/genotype, compared to ACR-2(L/S) animals.
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<tr>
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Table 2-1: Several nAChR subunits are required for ACR-2(L/S)-induced paralysis.
Figure 2-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 (ufIs49, top), full-length Pacr-2::ACR-2::GFP (ufIs42, 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 2-S2: *C. elegans* command interneurons do not express *Punc-17::GFP*.

**A-C:** Single confocal images of an animal co-expressing an integrated array (*vsIs48*) 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.
Figure 2-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 (box).
A

MKKTVKILLILITVFLKVCNGGHDEAADFLSHTNIDDPNNSSDPNKNS
DQGDMGEDEDRLVIDLFREYNFLRVPKVNVSSPPVVVDGIVAMILLIV
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Figure 2-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,B: Confocal images of wild type (A) and ACR-2(L/S) (B) 3-fold embryos expressing an integrated array containing *Pacr-2::GFP (ufIs49)*. 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 ± SEM of at least ten animals per genotype.
Figure 2-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 (ufIs26) that contains Punc-4::mCherry.
Figure 2-S6: Motor neuron processes are irregular in cnx-1;crt-1 double mutants expressing transgenic ACR-2(L/S).

A,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 (vsIs48). 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.
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Supplemental Table 2-1: Suppressors of ACR-2(L/S) induced paralysis isolated from a forward genetic screen.
CHAPTER III

Excitatory neurons sculpt inhibitory synaptic connections in the

\textit{C. elegans motor circuit.}

Belinda Barbagallo, Denis Turutin and Michael M. Francis

\textbf{Contribution Summary}

The following work was carried out in the lab of Dr. Michael M. Francis at the University of Massachusetts Medical School. Denis Turutin carried out the electrophysiology experiments and data analysis. Michael Francis supported the project by discussing experimental design, providing advice on data analysis and co-writing the paper. I designed the experiments, analyzed data and co-wrote the paper.
Abstract

Neuronal activity is a major driving force behind the development and maintenance of synapses within neural circuits. In this study, we use the motor circuit of the nematode *Caenorhabditis elegans* as a model for circuit development. We find that when neural circuits develop in the absence of excitatory drive there is a net increase in inhibitory signaling onto postsynaptic cells. This increase in signaling is reflected in an increase in the size of both presynaptic release sites and postsynaptic receptors. This change in circuit function occurs only after loss of excitatory, but not inhibitory function suggesting that excitatory neurons act as master regulators of circuit functional properties. Global reductions in activity are insufficient to alter inhibitory synapse patterning, though specifically disrupting excitatory drive onto GABA neurons leads to altered inhibitory synapses. Loss of cholinergic function specifically during development leads to irreversible changes in inhibitory synapse development suggesting that an activity-dependent critical period occurs to pattern inhibitory synapses. Loss of excitatory drive in the established nervous system leads to scaling of inhibitory synapses suggesting that homeostatic mechanisms act in the adult nervous system to maintain circuit activity levels.
Introduction

Neurons in the brain are organized into neural circuits that work together to control specific functions. The regulation of brain function requires the appropriate balance of excitation and inhibition (E/I balance) within these circuits during developmental time windows, termed critical periods, during which synaptic connections are specified. The number of synapses formed, as well as the maturation of synaptic fields, is directly tied to circuit activity levels, highlighting the importance of regulating E/I balance during development (Katz and Shatz, 1996); (Moody and Bosma, 2005); (Blankenship and Feller, 2010); (Dehorter et al., 2012); (Feldt et al., 2011).

Shifting of E/I balance beyond the acceptable functional range can lead to hyper and hypo excitable states within neural circuits resulting in misregulated circuit activity. Many studies have identified E/I balance dysfunction components in neurological disorders ranging from schizophrenia to autism, though the mechanism regulating activity have not been fully described (Wassef et al., 2003); (Rubenstein and Merzenich, 2003); (Kehrer et al., 2008); (Gatto and Broadie, 2010).

E/I balance also plays a critical role in the mature nervous system. Once networks are established, neural circuits must be able to adapt to changes in activity levels and maintain circuit output within a functional range. This is achieved through the activation of negative feedback mechanisms that are able to adjust synaptic strengths and maintain the balance of activity in a process termed homeostatic plasticity (Reviewed in (Pozo and Goda, 2010)). To date, a majority of the studies investigating mechanisms of homeostatic plasticity have been carried out in cell culture systems and show synaptic scaling in
response to modulation in circuit activity (Turrigiano et al., 1998). More recent studies have shown that these mechanisms are more complex than originally though, with in vivo studies showing non-uniform scaling of synapses, suggesting that not all neuron types are equally affected by changes in activity levels (Cingolani and Goda, 2008); (Echegoyen et al., 2007); (Goel and Lee, 2007). The complexities of circuit development highlight the need to better understand the mechanisms of activity-dependent circuit development and homeostatic mechanisms in intact neural networks.

Here we investigate the role of E/I balance in the development and maintenance of inhibitory synaptic fields. Using electrophysiological and genetic approaches in the C. elegans motor circuit, we have shown that the loss of excitatory input throughout development leads to increased inhibitory signaling in the adult nervous system. This increased signaling is reflected as an enlargement of both GABA presynaptic release sites and postsynaptic receptor clusters, suggesting that loss of excitatory drive leads to increased GABA synaptic efficacy. Global reduction of neurotransmission is insufficient to reproduce this enhanced inhibitory phenotype, while selective disruption of cholinergic release onto GABA neurons resulted in altered GABA synapse size and patterning.

We also show that ACh release is required during a developmental critical period to establish appropriate patterning of GABA synapses. In the adult nervous system, loss of ACh release leads to compensatory scaling of GABA synapse size, suggesting that homeostatic mechanisms are in place to modulate activity within the mature motor circuit.

Taken together, our results support a model where ACh release plays a duel role in regulating inhibitory synapse function. During development, ACh instructs GABA
synapse patterning while in the mature circuit, ACh levels trigger compensatory mechanisms that scale inhibitory synapses to maintain E/I balance in the circuit.

**Results**

**Reduced cholinergic innervation alters inhibitory transmission**

In order to investigate the role of excitatory signaling in shaping neural circuit connectivity and function, our studies focused on the anatomically well-defined *C. elegans* motor circuit. In this system, body wall muscles receive synaptic inputs from both excitatory and inhibitory motor neurons and the balance of this excitatory and inhibitory signaling shapes muscle activity and patterns movement (Figure 3-1A). Cholinergic motor neurons provide excitatory synaptic drive onto inhibitory GABA motor neurons and onto body wall muscles. GABA motor neurons make inhibitory synaptic connections onto opposing musculature, reinforcing a sinusoidal locomotory pattern.

To address whether excitatory motor neurons are important for shaping functional connectivity in this circuit, we first examined *unc-3* mutants, in which cholinergic motor neurons fail to terminally differentiate (Kratsios et al., 2012). *unc-3* encodes the sole *C. elegans* homolog of the COE family of transcription factors and is essential for the terminal differentiation of A and B cholinergic motor neurons (Kratsios et al., 2012). In *unc-3* mutants, these neurons are born and express generic neuronal markers, but fail to express cholinergic-specific genes. Thus, cholinergic innervation of both body wall muscles and GABA motor neurons should be severely disrupted in *unc-3* animals. To test this idea, we made whole-cell patch electrophysiology recordings of synaptic events from
body wall muscles of adult *unc-3* mutants and found significant defects in excitatory transmission. Specifically, the frequency of endogenous excitatory post-synaptic currents (EPSCs) was significantly reduced compared to the wild type (data not shown). Nonetheless, a low level of cholinergic transmission remained detectable. This residual cholinergic transmission may reflect activity of cholinergic VC motor neurons, which do not require *unc-3* for expression of cholinergic markers (Kratsios et al., 2012). Similar to the A and B classes, VC motor neurons innervate both GABA neurons and muscle, but by comparison, account for far fewer synapses. Thus, our results are consistent with the idea that mutation of *unc-3* reduces cholinergic drive in the motor circuit by decreasing the number of cholinergic synaptic contacts onto body wall muscles and GABA motor neurons.

We next sought to determine whether the reduced excitatory drive associated with mutation of *unc-3* affected inhibitory transmission onto muscles. Using conditions that isolated inhibitory transmission, we measured both endogenous and evoked inhibitory synaptic currents (IPSCs) from *unc-3* mutants. Remarkably, inhibitory events were almost completely eliminated under our standard recording conditions (1 mM Ca²⁺). For both wild type and *unc-3*, the rate of inhibitory events increased with higher levels of extracellular Ca²⁺ (5 mM); nonetheless, the endogenous IPSC rate in *unc-3* mutants was dramatically reduced compared to the wild type recorded under the same conditions (Figure 3-1 B, C).

The amplitude of spontaneous release events rose from 31.30±0.21 pA in wild type animals to 35.74±0.33 pA in *unc-3* mutants reflecting an increase in muscle
response to GABA release (Figure 3-1 D). The frequency of spontaneous GABA release is decreased in *unc-3* animals (14.78±2.1 Hz compared to 38.61±3.9 Hz in wild type), presumably due to the lack of direct synaptic input from the cholinergic motor neurons (Figure 3-1 E). The kinetics of evoked response were also altered in these animals, with an increase decay time for body wall muscle response to GABA motor neuron activation suggesting an expanded postsynaptic receptor field in *unc-3* mutant animals. These data show that cholinergic motor neuron activity influences the functional properties of GABA synapses.

Changes in GABA functional properties in *unc-3* mutant animals suggest that these neurons are able to alter properties of other synapse types within the circuit. In order to determine if GABA motor neurons are able to alter cholinergic functional properties, we recorded evoked responses from *unc-30* mutant animals. *unc-30* encodes a homeodomain transcription factor that is required for the terminal differentiation of GABA motor neurons (Jin et al., 1994; Eastman et al., 1999). We did not observe any differences in cholinergic evoked responses in *unc-30* mutant animals compared to wild type (Figure 3-S1). These data suggest that GABA motor neuron activity is unable to alter cholinergic synapse function leading to a model where cholinergic motor neuron activity is the master regulator of circuit activity.

**Cholinergic input patterns GABA synaptic outputs**

Our electrophysiological studies suggested that reduced excitatory signaling altered inhibitory transmission. In order to address whether these functional changes might
reflect alterations in circuit development or assembly, we examined the structure and synaptic connectivity of the motor circuit in \textit{unc-3} mutants. We first sought to determine whether body wall muscles and GABA motor neurons developed normally by examining their gross morphology. In wild type animals, body wall muscles project membrane extensions, called muscle arms, to the nerve cords where muscle arm termini bifurcate and project lengthwise along the nerve cord, forming \textit{en passant} synaptic connections with motor neurons (Dixon and Roy, 2005b). We used muscle-specific expression of membrane bound mCD8-GFP (\textit{him-4p::mCD8-GFP})(Collins and Koelle, 2013);(Lee and Luo, 1999) (Dixon and Roy, 2005b) to visualize muscle cell bodies and muscle arms (\textbf{Figure 3-S2 A’-C’}). Despite a dramatic reduction in synaptic innervation from cholinergic motor neurons, neither gross muscle morphology nor the extent of muscle membrane contact with the nerve cord were appreciably altered in \textit{unc-3} mutants (\textbf{Figure 3-S2 D}).

In addition to forming neuromuscular synapses with body wall muscles, cholinergic motor neurons also synapse directly onto inhibitory GABA motor neurons. We used cell-specific expression of mCherry to visualize GABA motor neurons (\textit{Punc-47::mCherry}) in \textit{unc-3} mutants. Similar to the case for muscle cells, disruption of cholinergic innervation did not produce obvious defects in GABA motor neuron number, positioning or morphology (\textbf{Figure 3-S2 A-C}). Thus, our results suggest that both body wall muscles and GABA motor neurons develop normally in the absence of cholinergic inputs from A and B motor neurons.
The above results indicated that the functional changes in inhibitory transmission we observed did not arise due to defects in the gross morphological development of either muscles or GABA motor neurons. Therefore, we hypothesized that altered inhibitory function may have been caused by structural changes at inhibitory synapses. To address this possibility, we examined the structure and distribution of GABA synapses in a transgenic strain co-expressing the synaptic vesicle marker mCherry-RAB-3 in GABA motor neurons together with the GFP-tagged GABA₆-like receptor UNC-49 in muscles. Interestingly, we found that mutation of unc-3 produced significant changes in the size and positioning of GABA synapses. In wild type animals, GABA synapses are evenly spaced along the ventral nerve cord, with presynaptic vesicles and post-synaptic GABA receptor clusters in close apposition (Figure 3-2 A, D). With disruption of cholinergic innervation (unc-3), we observed an altered distribution of GABA synaptic clusters as well as a significant increase in their size (Figure 3-2 B, E, F and 3-S3). Specifically, the average size of both presynaptic mCherry-RAB-3 puncta and postsynaptic UNC-49-GFP puncta increased significantly, while the number of pre- and post-synaptic puncta was significantly decreased (Figure 3-2 E, F). Localization of the active zone marker SYD-2-GFP and UNC-10::GFP as well as the synaptic vesicle marker SNB-1::GFP were similarly affected (Figure 3-S4). We did not observe obvious changes in the apposition of presynaptic mCherry-RAB-3 and postsynaptic UNC-49-GFP fluorescent signals (Figure 3-2 D); however, we noted large regions of the ventral nerve cord lacked detectable fluorescence in unc-3 mutants (56 ± 4%, compared to 20 ± 2% in the wild type, p<0.01), indicating that the patterning of GABA synaptic outputs onto muscles was
disrupted (Figure S3 D-G). Our analysis of unc-3 mutants suggests that cholinergic innervation within the motor circuit may shape inhibitory connectivity at the NMJ.

To gain further support for the notion that the defects in inhibitory connectivity we observed arose due to a disruption in cholinergic innervation, we examined inhibitory synapse connectivity following genetic ablation of ACh motor neurons. To ablate ACh motor neurons, we expressed a transgene engineered to encode a putative gain-of-function variant of the acetylcholine receptor subunit ACR-2, ACR-2(L/S) (Barbagallo et al., 2010). Expression of ACR-2(L/S) produces cell-autonomous death of cholinergic motor neurons during late embryogenesis. We found that genetic ablation of ACh motor neurons by ACR-2(L/S) expression caused defects in inhibitory synapses that were similar to those observed for unc-3 mutants. Specifically, we observed a significant increase in the size of both mCherry-RAB-3 presynaptic structures and postsynaptic UNC-49-GFP clusters, as well as a significant decrease in their number (Figure 3-2C-F). Further, the proportion of nerve cord lacking pre- or post-synaptic fluorescence was significantly increased. As was the case for unc-3, we did not observe gross morphological changes in the muscles or GABA neurons of ACR-2(L/S) animals, indicating that the altered distribution of RAB-3 and UNC-49 was not caused by defects in cell morphology (Figure 3-S2).

As our findings above indicated that the area of membrane contacts between motor neurons and muscles was unaffected by disrupting cholinergic innervation, we next sought to determine whether the altered distribution of GABA synaptic clusters reflected a failure of synapse assembly associated with specific muscle arm termini or an altered
distribution within individual muscle arms. We analyzed the localization of GABA receptors in individual muscle arm termini in the wild type, unc-3 mutants and transgenic ACR-2(L/S) animals. When examined at the level of individual muscle arms in wild type animals, GABA receptors are distributed evenly across the full extent of the muscle arm terminus (Figure 3-S5). In contrast, disruption of presynaptic cholinergic innervation produced irregularly distributed, larger aggregates of UNC-49-GFP clusters, leading to extended regions of muscle membrane that lack GABA receptor clusters (Figure S5). Taken together, our analysis of inhibitory synapses in unc-3 mutants and ACR-2(L/S) transgenic animals supports the hypothesis that presynaptic innervation from cholinergic motor neurons play a central role in shaping GABA synaptic outputs.

GABA synapse patterning is unaffected by global reductions in synaptic activity

To investigate whether the effects of cholinergic denervation on GABA synapses arose due to a reduction in neurotransmission within the circuit, we next examined GABA synapse patterning in unc-64 and unc-13 mutants. unc-64 encodes a C. elegans ortholog of syntaxin, a SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor) family member, while unc-13/Munc13 encodes a conserved synapse-associated diacylglycerol binding protein (Saifee et al., 1998), (Maruyama and Brenner, 1991), (Brose et al., 1995). Both UNC-64/syntaxin and UNC-13/Munc13 are essential components of vesicular release machinery and mutation of either of these genes in C. elegans impairs synaptic vesicle fusion at the plasma membrane, resulting in a near-complete disruption of neurotransmitter release (Richmond et al., 1999; Hammarlund et
Consistent with previous work, we found that mutations in *unc-13* and *unc-64* did not alter the size or distribution of GABA synapses (Figure 3-3). Additionally, we evaluated GABA synapse localization in animals lacking vesicular release due to expression of tetanus toxin in both cholinergic and GABAergic motor neurons (Figure 3-3D). The size and number of GABA presynaptic release sites and postsynaptic receptor clusters were not appreciably altered by the simultaneous loss of synaptic vesicle release in GABA and ACh motor neurons (Figure 3-3 E,F). Thus, our analysis indicates that global reductions in neurotransmission are insufficient to disrupt GABA synapses, while specific disruption of ACh motor neurons alters the size and positioning of GABA synapses.

**Vesicular release from cholinergic neurons shapes GABA synapses**

We envisioned two alternative models that could account for the above findings. One possibility is that there is a requirement for ACh neurons that occurs independently of transmission onto GABA neurons, for example through interactions between cell surface proteins expressed by ACh and GABA neurons. Alternatively, GABA synapse formation may be sensitive to relative levels of excitatory and inhibitory signaling. To distinguish between these possibilities, we selectively disrupted vesicular release from either excitatory or inhibitory motor neurons using neuron-specific expression of tetanus toxin light chain (TeTx). Cell-specific expression of TeTx in cholinergic and GABAergic motor neurons led to reduced motor neuron function but was insufficient to alter motor neuron morphology or outgrowth (Figure 3-S6).
Specific TeTx expression in ACh motor neurons produced severe abnormalities in GABA synapses (Figure 3-4A, B), including enlargement of both presynaptic mCherry-RAB-3 and postsynaptic receptor clusters, as well as gaps in fluorescence that were similar to those observed for unc-3 mutants and acr-2(L/S) transgenic animals (Figure 3-4E, F). In contrast, specific expression of TeTx in GABA motor neurons produced no discernible changes in the size or distribution of mCherry-RAB-3 clusters in GABA neurons (Figure 3-4C, E, F). We did note diffuse post-synaptic labeling, consistent with previous reports that a vesicle associated signal may be important for clustering of GABA receptors (Gally and Bessereau, 2003b), complicating analysis of individual puncta. However, the percentage of the cord devoid of post-synaptic signal was unchanged in GABA specific TeTx strains compared to wild type (Figure 3-S7), consistent with the idea that patterning of GABA synapses was unchanged by specific disruption of vesicle release from GABA neurons.

Additionally, we found that the size and distribution of GABA synapses in unc-25/glutamic acid decarboxylase (GAD) mutants that are defective in GABA biosynthesis were indistinguishable from the wild type, consistent with previous reports (Jin et al., 1999b); (Gally and Bessereau, 2003b) and (Figure 3-4D, E, F). Taken together, our results suggest that vesicle release from ACh motor neurons is required for the regulation of GABA synapse size and distribution while vesicle release from GABA neurons is not essential.
Acetylcholine release onto GABA motor neurons instructs inhibitory synaptic patterning

Cholinergic motor neurons synapse directly onto GABA motor neurons and body wall muscles. Thus, our above findings may indicate that cholinergic motor neurons regulate GABA synapse patterning through direct synaptic activation of GABA motor neurons. *unc-17(e245)* mutants are defective in a vesicular ACh transporter (vAChT) that loads ACh into synaptic vesicles, and have strong locomotory defects as well as numerous other phenotypes consistent with decreased cholinergic transmission (Alfonso et al., 1993); (Brenner, 1974b); (Rand and Russell, 1984). The *e345* allele produces strong reduction-of-function effects and we selected it for our studies as an experimentally tractable alternative to *unc-17* null alleles, which are lethal (Alfonso et al., 1993). Mutation of *unc-17* produced significant defects in GABA synapse patterning, including enlarged presynaptic release sites and postsynaptic receptor clusters, as well as gaps in the distribution of synapses (*Figure 3-5A, B*). These effects were less pronounced compared to those observed for specific expression of TeTx in cholinergic motor neurons, suggesting ACh release is less strongly impaired by the hypomorphic *unc-17* mutation or involvement of additional ACh-independent signals important for synapse patterning.

To address whether the effects of decreased ACh release reflected a requirement for ACh signaling onto GABA motor neurons or onto muscles, we examined GABA synapses in animals lacking functional iAChRs at the NMJ (*unc-29;acr-16* double mutants). *unc-29* and *acr-16* encode essential subunits of the two AChR classes that
mediate cholinergic neurotransmission onto muscles. Combined mutation of these genes produces a complete loss of muscle responses to ACh application (Francis et al., 2005b). Eliminating synaptic activation of muscles did not alter the density or positioning of GABA release sites, suggesting that GABA synapse patterning was unaffected (Figure 3-5C-E). We did note a slight reduction in the number of postsynaptic receptor clusters in \textit{unc-29;acr-16} double mutants compared to wild type. These specific effects on the postsynapse are most consistent with homeostatic changes in muscles in the absence of cholinergic innervation. Our findings support a model where direct activation of GABA motor neurons by acetylcholine release instructs GABA synapse patterning.

**Acetylcholine release is required during a developmental critical period to instruct GABA synapse localization**

Our previous genetic analysis showed that cholinergic release onto GABA motor neurons is required for GABA synapse patterning, but did not indicate whether these effects are due to deficits in synaptic development or maintenance. To investigate whether the alterations in GABA synapse size and patterning we observed reflected a requirement for ACh signaling during specific developmental periods, we reversibly disrupted acetylcholine signaling using a temperature sensitive allele (y226) of the \textit{C. elegans} choline acetyltransferase gene (ChAT), \textit{cha-1}, leading to decreased ACh biosynthesis (Rand and Russell, 1984);(Zhang et al., 2008). At the permissive temperature (15°C), temperature-sensitive \textit{cha-1}/ChAT mutants proceed normally through development, with the full complement of motor neurons developing similarly to
wild type (Figure 3-6A, B). Inhibitory synapse development also proceeds normally at 15°C in both wild type and cha-1/ChAT mutants, forming a uniform punctate pattern along the ventral nerve cord with proper alignment of pre- and postsynapse (Figure 3-6C, D). cha-1(y226) mutants become uncoordinated within minutes (<10) when switched to a non-permissive temperature (25°C), consistent with rapid impairment of cholinergic transmission, and these effects are completely reversible upon shifting back to the permissive temperature (data not shown).

We first assessed the requirement for ACh signaling during development by blocking ACh release for a 24-hour period of time spanning the highly synaptogenic window between hatch and the second larval stage. We then allowed animals to grow to adulthood at a permissive temperature and assessed GABA synapse morphology in young adult animals (24 hours post-L4). This manipulation resulted in a disruption in GABA synapse patterning, including gaps in the distribution of both pre- and postsynaptic puncta and a mild increase in puncta size.

To precisely define a developmental time window in which ACh signaling was required, we examined the effects of removing acetylcholine release for 8hr and 4hr windows beginning during the first larval stage. We identified a four-hour time window beginning 12 hours after hatch at 15°C (late L1) during which a shift to the restrictive temperature was sufficient to produce significant defects in GABA synapse patterning (Figure 3-6 F-J). In particular, we found that a 4-hour shift to the restrictive temperature produced a significant increase in areas devoid of pre- or post-synaptic signal though puncta size only increased marginally compared to wild type (Figure 3-6G-J).
Importantly, we did not observe these effects in *cha-1* mutants grown at 15°C or in wild type animals that experienced the same shift in temperature (*Figure 3-6D, E*). Our findings indicate that ACh release is critical during the end of the first larval stage to instruct GABA synaptic patterning, defining a critical developmental period during which cholinergic synaptic activity regulates the development of inhibitory synapses.

**Acute loss of acetylcholine release in the adult nervous system leads to a decrease in GABA synapse size.**

Our analysis of GABA synapses in the adult nervous system suggested that while the patterning of established synaptic fields was unaffected by changes in ACh transmission the area of both presynaptic release sites and postsynaptic receptor clusters are significantly reduced upon removing ACh release in adult animals. To explore whether this represented a form of plasticity at adult GABA synapses, we reduced acetylcholine release by shifting animals to the restrictive temperature for a four-hour period in early adulthood, after the nervous system has been fully established (*Figure 3-7*). These animals were grown at the permissive temperature and staged as young adults, 24 hours after the fourth larval stage. These young adult animals were then shifted to the non-permissive temperature for four hours and inhibitory synapses were immediately images. Restricting cholinergic transmission produced a significant decrease in the size of presynaptic mCherry-RAB-3 clusters and post-synaptic UNC-49-GFP puncta without significant effects on the distribution of synaptic clusters (*Figure 3-7A-D, I-L*). The same temperature shift did not produce significant changes at GABA synapses of wild
type animals, consistent with this being a specific result of acute reductions in ACh release in \textit{cha-1}/ChAT mutants. Interestingly, increasing the time that \textit{cha-1}/ChAT mutants were exposed to the restrictive temperature produced further decreases in the size of GABA pre- and postsynaptic clusters, suggesting that inhibitory synapse size decreases proportionally with the length of time that excitatory ACh transmission is impaired (\textbf{Figure 3-7 E, F, I-L}). We were unable to test more prolonged temperature shifts because adult animals left at 25°C longer than eight hours became unhealthy leading to nonspecific effects on nervous system structure. These results suggest that inhibitory synapse size is scaled proportionately to the amount of excitatory release within the circuit hinting at the presence of a compensatory mechanism for balancing excitation and inhibition within the circuit.

In order to further characterize the relationship between the levels of excitatory release and inhibitory synapse size, we reintroduced ACh to the circuit after deprivation and assessed GABA synapse size for recovery. A four-hour recovery period was sufficient to restore wild type body posture and locomotion in \textit{cha-1} mutant animals (data not shown). Based on this behavioral observation, adult animals were shifted to the non-permissive temperature as above then immediately placed back at the permissive temperature and allowed to recover for four hours (\textbf{Figure 3-7 G, H}). Decreases in the size of inhibitory synaptic clusters were completely reversed following a four hour recovery period at 15°C, indicating that these changes in GABA synapses were reversible (\textbf{Figure 3-7 I, J}) and could be normalized by restoring wild type levels of excitatory ACh transmission. Our findings indicate that a temporally restricted period of reduced ACh
release at adult synapses leads to reductions in the size of GABA synapses without obvious effects on synapse distribution. The reversible effects of decreased ACh transmission on GABA synapses suggest that compensatory mechanisms are present in the adult nervous system, and act by scaling inhibitory synapse size in response to decreases in excitatory transmission, providing a mechanism balancing levels of excitatory and inhibitory neuromuscular signaling in adults.

**Discussion**

The activity of the GABAergic nervous system plays an essential role in the establishment and refinement of neuronal networks within the mammalian brain (Hensch and Fagiolini, 2005b); (Ben-Ari, 2002b). Though the importance of inhibitory activity during development is well understood, little is known about how the synaptic patterning and the functional range of activity in the inhibitory nervous system are established during network development in vivo. In this study, we use *C. elegans* as a model system to show that excitatory drive onto GABAergic neurons during early development is an essential driving force behind inhibitory synapse function and patterning.

In the *C. elegans* motor circuit, cholinergic motor neurons provide the primary excitatory input onto both body wall muscles and GABAergic motor neurons. We found that in the absence of functional cholinergic neurons, both the body wall muscle and the GABA motor neurons were able to establish normal morphology and outgrowth. This result suggests that cell intrinsic mechanisms are in place to facilitate the gross
morphology of the motor circuit while activity-dependent mechanisms dictate synaptic connectivity.

Electrophysiological analysis of inhibitory signaling showed that GABA motor neurons remain functional in the absence of excitatory input. This data implies that presynaptic activation of GABA motor neurons is not essential for neuronal function, providing evidence for the existence of intrinsic activity within the GABA motor neurons. Functional analysis of inhibitory activity in \textit{unc-3(e151)} mutant animals show an increase in the amplitude of spontaneous release in animals chronically lacking excitatory drive. This data suggests that excitatory drive onto the GABA motor neurons is required for the refinement of inhibitory synapses. Interestingly, we found that changes in inhibitory neuron function (\textit{unc-30} mutants) were unable to alter excitatory synapse function leading us to hypothesize that excitatory neurons act as master regulators of activity during development to control synapse patterning and functional properties.

In addition to functional changes, we also observed an increase in GABA synapse size and patterning in the absence of functional cholinergic motor neurons. Studies in the mammalian cortex have reported a correlation between excitatory synapse size and synaptic efficacy. For example, at glutamatergic synapses, stronger synapses have larger dendritic spines and an increased number of glutamate receptors at the postsynaptic density (Matsuzaki et al., 2004b). When combined with functional analysis, our results suggest that synapse size is also an indicator of synapse strength for inhibitory synapses.

We found that global reductions in neurotransmission were insufficient to alter inhibitory synapse patterning, leading us to hypothesize that the balance of excitation and
inhibition acted as the driving force behind synapse patterning. Our finding that selective
disruption of cholinergic release phenocopied animals chronically lacking functional
cholinergic neurons, while disruption of inhibitory neurons had no effect on presynaptic
patterning, further supported this hypothesis. Additionally, we show that loss of ACh
release, but not loss of cholinergic neurotransmission directly onto body wall muscle, led
to altered GABA receptors. These results support our model that cholinergic
neurotransmission triggers activity-dependent mechanisms, perhaps transcriptional or
translational programs, within the GABA motor neurons to instruct GABA synapse
patterning during development.

In the mammalian brain, the inhibitory nervous system has been implicated as a
key player in determining the timing of critical period plasticity (Fagiolini and Hensch,
2000b); (Huang et al., 2007). Multiple studies have shown the ability of GABA
interneurons to control key aspects of cortical circuits including neuronal excitability,
integration and the temporal oscillations of excitatory networks (Swadlow, 2003b);
(Pouille and Scanziani, 2001b); (Somogyi and Klausberger, 2005). Though many studies
have focused on roles for inhibitory signaling in critical period plasticity, little is known
about factors that define critical periods in inhibitory synapse development. In this study
we identified a critical period during which excitatory drive onto GABA motor neurons
set inhibitory activity levels and instruct inhibitory patterning. The circuit is unable to
recover if activity is disrupted during this critical period suggesting that specific activity-
dependent programs must occur in the immature circuit in order to produce a functional
circuit.
In contrast, decreased excitatory drive onto the established circuit leads to compensatory scaling of inhibitory synapses hinting at the presence of homeostatic mechanisms that work to maintain circuit activity within a functional range. The synaptic scaling we observe in the established nervous system correlates to what is observed in circuits where homeostatic plasticity is functioning appropriately to maintain circuit function. This data implies that distinct activity-dependent mechanisms function in GABA motor neurons during development and in the adult nervous system to regulate inhibitory signaling.

In this study we provide evidence that the *C. elegans* motor circuit is a viable genetic system for studying activity-dependent regulation of circuit development *in vivo*. Our model provides an ideal platform to carry out further genetic studies to identify the molecular mechanisms underlying the opening and closing of critical period windows. Understanding the differences between highly plastic brain during early developmental critical periods and less plastic adult brains may lead to the ability to aid circuit rewiring alter brain trauma such as stroke or for pharmacological ways to correct neurodevelopmental disorders such as autism spectrum disorders and epilepsy.

**Methods**

**C. elegans Strains**

*C. elegans* strains were grown at room temperature (22-24°C) on nematode growth media (NGM) plates seeded with the *Escherichia Coli* strain OP50. Wild type represents the N2 Bristol strain. Mutant lines were obtained from the *Caenorhabditis*
Genetics Center (GCG), funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). All transgenic strains presented were made by injecting plasmid DNA (30-50ng/ul) into the germ line using standard methods (Mello et al., 1991b). Data presented represents a single transgenic line unless otherwise indicated. Strains were made using either a lin-15 co-injection marker for rescue (lin-15(n765ts)) or using a fluorescent co-injection marker located in the pharynx of the animal (lgc-11::GFP, lgc-11::mCherry), details for each strain can be found in the strain list. All co-injection markers were injected at 30ng/ul and multiple transgenic lines were obtained for each transgene. Selected transgenes were integrated using x-ray bombardment and outcrossed to wild type animals.

Molecular Biology

\textit{Him-4p::mCD8::GFP}. Plasmid was constructed using the two-slot Gateway Cloning system. Entry Vector: the \textit{him-4p} promoter fragment (2164bp) was amplified from pPRZ138.2 (a gift from Peter Roy) and cloned into the d-TOPO Vector (Invitrogen). Destination Vector: mCD8-GFP (1586bp) was amplified from plasmid PKMC72 by PCR using a forward primer ATTAGGTACCAGGCCTACCCGTTGACCCG (OMF735) and a reverse primer GCTAGCCGGCTTTATTGTATAGTTCCATCATCCATG (OMF736). Respectively, each primer contained a restriction site KpnI and NgoMIV. The mCD8-GFP PCR product and a destination vector (pDest-16) were digested with NgoMIV and
KpnI for 2 hours at 37°C. All digested products were size separated using agarose gel electrophoresis, purified, and ligated overnight at 4°C with T4 DNA ligase.

**Tetanus toxin constructs:** All tetanus toxin plasmids were constructed using the two-slot gateway system. Entry Vectors: Cholinergic-specific tetanus toxin: A 3394bp fragment of *Pacr-2* was amplified from pBB67 and subcloned into the d-TOPO vector (Invitrogen). GABAergic-specific tetanus toxin: A 1166bp fragment of *Punc-47* was amplified from pPRB65 and subcloned into the d-TOPO vector (Invitrogen). Destination Vector: Tetanus Toxin Light Chain (1394bp) was amplified from plasmid TetX-pWD157 (Alkema Lab) by PCR using a forward primer ATTAACCGGTATGCGATCACCATCAACAC (OMF883) and a reverse primer GCTAGCGGCTTAAAGCGGTACGTTGTACA(OMF884). Respectively, each primer contained a restriction site AgeI and NgoMIV.

The final expression clones were generated by recombining the respective entry vectors with the tetanus toxin destination vector using an LR clonase reaction (Invitrogen). The Tetanus Toxin PCR product and a destination vector (pDest-8) were digested with AgeI and NgoMIV for 2 hours at 37°C. All digested products were size separated using agarose gel electrophoresis, purified, and ligated overnight at 4°C with T4 DNA ligase.

**Electrophysiology**

Endogenous postsynaptic currents (PSCs) were recorded from body wall muscles as described previously (Francis et al., 2005b). The extracellular solution consisted of the following (in mm): 150 NaCl, 5 KCl, 4 MgCl₂, 1 CaCl₂, 15 HEPES, and 10 glucose, pH
7.4, osmolarity adjusted with 20 sucrose. The intracellular fluid consisted of the following (in mm): 115 K-gluconate, 25 KCl, 0.1 CaCl₂, 50 HEPES, 5 Mg-ATP, 0.5 Na-GTP, 0.5 cGMP, 0.5 cAMP, and 1 BAPTA, pH 7.4, osmolarity adjusted with 10 sucrose. For some experiments measuring GABA-mediated currents, the intracellular solution contained 115 mm KCl and 25 mm K-gluconate, enabling measurement of inward inhibitory current responses (chloride efflux). At least 60–90 s of continuous data were used in the analysis. Data analysis was performed using Igor Pro (Wavemetrics) and Mini Analysis (Synaptosoft) software. Statistical comparisons were made by Student's t test using GraphPad Prism.

**Aldicarb Assay**

Aldicarb assays were performed on adult animals (24 hours post L4) at room temperature (22°C - 24°C) with the researcher blind to the genotypes of the plates. Staged animals (10/plate) were transferred to NGM plates containing 1mM Aldicarb (ChemService) and accessed for movement every 15 minutes for two hours. Data represents the mean ±SEM for a minimum of ten assays for each genotype.

**Microscopy and Image analysis**

Confocal microscopy was performed using a Zeiss LSM510 microscope with LSM Pascal 5 imaging software (Zeiss). Images were analyzed using ImageJ software (open source). Full worm images were obtained using a 63x oil lens and piecing together the resulting images using Photoshop software (Adobe).
Synapse density was calculated from a line-scan analysis of a 50uM section of the ventral nerve cord directly posterior to the vulva of the animal. A minimum fluorescence threshold was set to identify synaptic peaks (1500a.u. for cholinergic synapses and 500a.u. for GABAergic synapses). Peaks were identified as two or more consecutive data points above the predetermined threshold and spaces were defined as two or more data points below threshold. Synapse area was calculated by thresholding a 50uM span of the ventral nerve cord using standardized parameters, separating particles using the ImageJ watershed algorithm and performing the ImageJ analyze particles function. Particles below a defined threshold ($\leq 0.2uM^2$) were excluded as background.

Muscle arm number was counted manually. Muscle arms from ventral muscle cells 17 and 19 were included in the analysis. Percent of nerve cord covered was measured using ImageJ software (open source) to measure the length of the ventral midline of the posterior body wall muscles. The total length of muscle arm termini were then measured and used to calculate the percent of the midline covered.

**cha-1 time course**

*Developmental Shift:* Embryos were staged at the 21+ cell stage and grown at 15°C for 12 hours when they were shifted to 25°C for four hours and then returned to 15°C until adulthood. Animals were imaged at young adulthood, staged at L4 and allowed to grow up at 15°C for 12 hours.

*Adult Shifts and Recovery:* Wild type and cha-1 animals were cultured at 15°C using standard methods. Animals were staged at L4 and allowed to develop to adulthood (12
additional hours at 15°C. Young adult animals were then shifted to 25°C for either four or eight hours and imaged immediately. Young adult recovery animals were shifted to 25°C for four hours, then placed back at 15°C for four hours before imaging.
Figure 3-1: GABA activity increases in the absence of functional cholinergic neurons

A. Diagram of the *C. elegans* motor circuit with excitatory cholinergic motor neurons (Gray) synapsing onto inhibitory GABAergic motor neurons (purple) and onto body wall muscles (brown).  

B, C: Examples of GABA spontaneous release events in wild type (B) and *unc-3(e151)* mutant animals (C).  

D. Quantification of the frequency of spontaneous GABA events. Student t-test, ***p<0.0001.  

E. Cumulative distribution of amplitude of spontaneous IPSCs in wild type and *unc-3* mutant animals. Kolmogorov-Smirnov test. ***p=0.0007.
Figure 3-2: GABA synapse patterning requires functional cholinergic motor neurons.

A-C. Confocal images of the ventral nerve cord in adult animals co-expressing a GABA presynaptic marker (*Punc-47::mCherry::RAB-3*) and a GABA postsynaptic marker (*UNC-49::GFP*) in wild type (A), *unc-3(e151)* (B) and ACR-2(L/S) (C) animals. Alterations in synapse localization are characterized as gaps in synapse distribution (bracket) and enlarged puncta (^). Asterisks indicate GABA motor neuron cell bodies. Scale bar: 20μm.  D. Quantification of the ratio of the number of presynaptic puncta to postsynaptic puncta. No statistical significance is observed. E. Quantification of synapse cluster number per 50μm area of the ventral nerve cord. All data has been normalized to wild type. F. Quantification of synaptic cluster area for GABAergic synapses. Each graph represents the mean ± SEM of at least ten animals/genotype. Student T-test *p<0.05, **p<0.01, ***p<0.0001
Figure 3-3: Global reductions in neurotransmission are insufficient to alter GABA synapse localization.

A-D. Confocal images of the ventral nerve cord adult animals co-expressing a GABA presynaptic marker (*Punc-47::mCherry::RAB-3*) with a GABA postsynaptic marker (*UNC-49::GFP*) in genotypes indicated. Carrots mark enlarged puncta and brackets indicate gaps in synapse distribution. Scale bar: 20um.  

E. Quantification of presynaptic (black bars) and postsynaptic (gray bars) cluster density for all genotypes, normalized to wild type.  

F. Quantification of presynaptic (black bars) and postsynaptic (gray bars) area for genotypes indicated. Each graph represents the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01, ***p<0.0001.
Figure 3-4: Cell specific silencing of cholinergic motor neurons leads to abnormal GABA synapse localization.

A-C. Confocal images of adult animals expressing GABA synapse markers in wild type animals (A) and transgenic animals cell-specifically expressing tetanus toxin light chain in cholinergic (B) or GABAergic (C) motor neurons. Large accumulations of receptor (^) and gaps in synapse coverage (bracket) are observed in cholinergic-specific tetanus toxin strains. Scale bar: 20uM. D. Quantification of number of presynaptic (black bars) and postsynaptic clusters (gray bars) in 50uM spans of the ventral nerve cord for all genotypes, normalized to wild type. E. Quantification of presynaptic area (black bars) and postsynaptic area (gray bars) for genotypes indicated. For all quantification, graphs represent the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01, ***p<0.0001
A: Wild Type
B: ACh TetTx
C: GABA TetTx
D: unc-25(e156)

E: Synapse Cluster Number Normalized
Pre: Wild Type, ACh TetTx, GABA TetTx, unc-25(e156)
Post: Wild Type, ACh TetTx, GABA TetTx, unc-25(e156)

F: Synapse Cluster Total (nm)
Pre: Wild Type, ACh TetTx, GABA TetTx, unc-25(e156)
Post: Wild Type, ACh TetTx, GABA TetTx, unc-25(e156)
Figure 3-5: Cholinergic neurotransmission onto body wall muscle is dispensable for GABA synapse patterning

A-D. Confocal images of the ventral nerve cord in adult animals co-expressing a GABA synaptic markers (Punc-47::mCherry::RAB-3,UNC-49::GFP) in wild type (A), animals with reduced GABA release (unc-25(e156)) (B), animals with reductions in acetylcholine release (unc-17(e245)) (C) and animals lacking cholinergic receptors at the neuromuscular junction (acr-16(ok789);unc-29(x29)) (D). Large accumulations of receptor (^) are seen in unc-17(e245) mutant animals. GABA motor neuron cell bodies are marked with asterisks. Scale bar: 20um.  

E. Quantification of presynaptic (black bars) and postsynaptic cluster density (gray bars) for all genotypes, normalized to wild type.  

F. Quantification of presynaptic area (black bars) and postsynaptic area (gray bars) for genotypes indicated. Graphs represent the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01, ***p<0.0001.
Figure 3-6: Excitatory input is required during synaptogenesis to instruct GABA synapse localization.

A-D: Confocal images of the ventral nerve cord in adult wild type and cha-1(y226) mutant animals co-expressing GABA pre- and postsynaptic markers (Punc-47::mCherry::RAB-3 and UNC-49::GFP) grown at the permissive temperature (A,B) and after a four hour shift to the non-permissive temperature (C,D) during the first larval stage. Carrots indicate accumulations of synaptic markers and brackets indicate gap in synapse distribution. Asterisks mark GABA motor neuron cell bodies. Scale bar: 20uM. 

E-F: Quantification of the presynaptic (E) and postsynaptic (F) cluster density for wild type (black bars) and cha-1(y226) mutant animals (gray bar).

G-H: Quantification of presynaptic (G) and postsynaptic (H) cluster area for wild type (black bars) and cha-1(y226) mutant animals (gray bar). Graphs represent the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01, ***p<0.0001.
Figure 3-7: Acute reductions in acetylcholine release in the mature nervous system leads to scaling of GABA synapse size.

**A-H:** Confocal images of the ventral nerve cord in adult wild type and *cha-1(y226)* mutant animals co-expressing a GABA pre- and postsynaptic markers (*Punc-47::mCherry::RAB-3* and *UNC-49::GFP*) grown to young adulthood at the permissive temperature (A,B), following a four hour shift to the non-permissive temperature during adulthood (C,D), an eight hour shift (E,F) to the non-permissive temperature and after a four hour shift and a four hour recovery period (G,H). Asterisks mark GABA motor neuron cell bodies. Scale bar: 20μM. **I-J:** Quantification of the presynaptic (I) and postsynaptic (J) cluster density for wild type (black bars) and *cha-1(y226)* mutant animals (gray bar). **K-L:** Quantification of presynaptic (K) and postsynaptic (L) cluster area for wild type (black bars) and *cha-1(y226)* mutant animals (gray bar). Graphs represent the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01, ***p<0.0001.
Figure 3-S1: Excitatory motor neuron function does not require GABA motor neuron function.

A,B. Example cholinergic evoked responses from wild type (A) and *unc-30(e191)* mutant animals.  C. Quantification of amplitude of cholinergic evoked response in wild type and *unc-30* mutant animals. Quantification shows no significant difference between genotypes.
Figure 3-S2: Gross motor circuit development occurs independently of excitatory inputs.

A-C. Confocal image of the ventral nerve cord and body wall muscles posterior to the vulva of an adult wild type (A), *unc-3(e151)* mutant (B) and ACR-2(L/S) (C) animals expressing mCherry in the GABA nervous system (*punc-47::mCherry*) (A-C) or a membrane bound GFP in the distal row of body wall muscles (*him4p::mCD8::GFP*) (A’-C’). GABA motor neuron cell bodies were identified by cell body position and asterisks indicate GABA commissural extensions. Arrows indicate muscle arm extensions and arrowheads indicate muscle arm termini. D. Quantification of the percentage of the ventral nerve cord covered by muscle membrane. Graphs show the mean ±SEM for at least ten animals/genotype. ns: not significant, Student t-test.
Figure 3-S3: Gaps in inhibitory synapse distribution occur in the absence of cholinergic input.

A-C. Confocal images of the ventral nerve cord of adult wild type (A), unc-3 (B) and ACR-2(L/S) (C) animals expressing GABA pre- and postsynaptic markers (Punc-47::mCherry::RAB-3, UNC-49::GFP). Large gaps in synapse distribution are indicated with brackets in unc-3 and ACR-2(L/S) animals. D-F. Confocal images of the ventral nerve cord of adult animals expressing GFP tagged GABA receptors at the NMJ (UNC-49::GFP). Brackets indicate gaps in receptor cluster distribution. G. Quantification of the percent of the ventral nerve cord devoid of GABA receptor clusters. Each graph represents the mean ± SEM of at least ten animals/genotype. Student T-test ***p<0.0001
Figure 3-S4: Presynaptic density localization is altered in the absence of cholinergic input.

A-F. Confocal images of the ventral nerve cord of adult wild type and unc-3(e151) mutant animals expressing a GFP tagged SNB-1 (A-B), SYD-2 (C-D) and UNC-10 (E-F) in GABAergic motor neurons. Carrot indicates enlarged presynaptic puncta and asterisk indicates GABA motor neuron cell body. Scale bar: 20um. G. Quantification of synapse cluster number per 50um area of the ventral nerve cord. All data has been normalized to wild type. H. Quantification of synaptic cluster area for GABAergic synapses. Each graph represents the mean ± SEM of at least ten animals/genotype. Student T-test

**p<0.01, ***p<0.0001
Figure 3-S5: Cholinergic and GABAergic receptors are mislocalized within the muscle membrane.

A-C. Confocal images of individual muscle arms in animals expressing GFP tagged GABAergic receptors (UNC-49::GFP) in wild type (A), *unc-3(e151) (B)* or ACR-2(L/S) (C). Muscle membrane boundaries are outlined with white dotted line. Carrots indicate accumulations of postsynaptic receptor.
GABA

Wild Type

ACR-2(L/S)

unc-3(e151)
Figure 3-S6: Cell specific expression of tetanus toxin alters neuronal activity without altering cell outgrowth or morphology.

A. Schematic representation of the motor circuit of *C. elegans* showing the wiring of cholinergic neurons (green) and GABAergic neurons (red). Sites of action for tetanus toxin is denoted by black Xs. B. Time course of paralysis of adult animals on 1mM Aldicarb, an inhibitor of cholinesterase. The percentage of animals paralyzed was calculated every 15 minutes for two hours. Each data point represents the average ±SEM of at least ten drug assays, ten animals/assay. C-E. Confocal images of the posterior ventral nerve cord in adult animals co-expressing a transcriptional markers labeling cholinergic neurons (*Punc-17::GFP*) and GABA neurons (*Punc-47::GFP*) in indicated tetanus toxin backgrounds. Cell body identities are labeled in red (GABAergic neurons) and green (cholinergic neurons).
Figure 3-S7: The balance of excitatory and inhibitory signaling instructs GABA synapse patterning.

A-D. Confocal images of the ventral nerve cord in adult animals expressing a GFP tagged GABA receptor at the neuromuscular junction (UNC-49::GFP). Cell-specific expression of tetanus toxin light chain in cholinergic motor neurons leads to large accumulations of receptor (^) and gaps in synapse coverage (bracket). Cell-specific expression of tetanus toxin light chain in GABA motor neurons results in diffusion of postsynaptic receptor clusters (dotted line). E. Quantification of the percentage of the ventral nerve cord lacking GABA receptor clusters. Each graph represents the mean ± SEM of at least ten animals/genotype. Student t-test *p<0.05, **p<0.01
CHAPTER IV

General Discussion
In this thesis I have explored the consequences of modulating activity levels within a simple neural circuit. Through this work, I have made several discoveries about the impact of activity on neural circuits; I showed that expression of a gain-of-function nicotinic acetylcholine receptor leads to the hyperactivation of cholinergic motor neurons. Hyperactivation of these neurons triggered calcium-induced calcium release from the ER activating a necrotic-like cell death program. Interestingly, I observed that in cases where cell body death is suppressed, axons continue to deteriorate leading to paralysis. This work supports a model where degeneration of the cell body and the axon are genetically separable and provides a genetic model for the study of motor axon degeneration.

In later work I focused on the consequences of decreased excitation on neural circuit development. I identified excitatory presynaptic input onto GABAergic motor neurons as a driving force behind inhibitory synapse development. While cellular morphology and outgrowth are normal in the absence of excitatory drive, synaptic connectivity is highly sensitive to changes in circuit activity. I was able to elucidate differential roles for activity in regulating inhibitory synapse morphology during different developmental periods. I identified a critical period window in early development during which excitatory drive is required for the establishment of inhibitory synapses. After this critical period, I observed synaptic alterations consistent with scaling of inhibitory synapses in responses to changes in excitation. These observations suggest the

Taken together, my work has provided new insights into the cellular biology of activity-dependent circuit changes in an \textit{in vivo} system. Additionally, this work has
established a genetic model for further study of critical neurological functions such as mechanisms of axonal death and critical period plasticity. Disruption of these processes in mammalian systems has devastating effects on brain function leading to neurodevelopmental and neurodegenerative diseases.

**Part 1: Excess activation of cholinergic neurons results in necrotic-like cell death**

Excess ion channel activation plays a role in a variety of human neurological diseases from ischemic stroke to ALS. In Chapter II of this thesis, I carried out a study to understand mechanisms underlying cell death in cases of excess ion channel function. Through this work I was able to characterize an extrasynaptic ionotropic acetylcholine receptor (ACR-2R) expressed in cholinergic neurons. Introduction of a gain-of-function point mutation to the TM2 domain of ACR-2 led to cell-autonomous death of the cholinergic motor neurons through a necrotic-like pathway (Figure 4-1). Mutations in genes essential for calcium-induced calcium release from the ER blocked cell body death but were insufficient to protect against axon destabilization that resulted in paralysis, suggesting that cell body death and axon degeneration are genetically separable.

**ACR-2R as a model for extrasynaptic modulatory receptors**

The mammalian genome contains 17 nicotinic acetylcholine receptor subunits (Graham et al., 2002). These subunits are arranged into pentamers, and the differential amino acid sequence of each subunit dictates the function and localization of the receptor (Jensen et al., 2005). A detailed understanding of how individual subunits influence
receptor function and localization is required to better understand the roles of AChRs in
the central nervous system. *C. elegans* provides an ideal model to explore ACh receptor
composition with the genome expressing 29 putative acetylcholine receptor subunits by
sequence homology. The receptor composition of nAChRs at the neuromuscular junction
has been well characterized but little is known about the composition or function of
neuronal nAChRs. (Francis et al., 2005a);(Fleming et al., 1997);(Richmond and
Jorgensen, 1999);(Culetto et al., 2004).

In my work, I have characterized the first neuronal AChR identified in *C. elegans*,
which contains the non-alpha acetylcholine receptor subunit: ACR-2. ACR-2R shows
restricted expression within cholinergic motor neurons where it is diffusely localized in
the dendritic compartment. Behavioral analysis revealed a slight yet significant decrease
in the number of body bends per minute made by *acr-2* mutant animals, while
pharmacological analysis showed a decrease in cholinergic motor neuron activity in *acr-2*
mutants. These results suggest that ACR-2 is participating in an extrasynaptic modulatory
receptor within cholinergic motor neurons, where it provides small changes in excitability.
Similarly, a majority of acetylcholine receptors in the mammalian CNS function as
extrasynaptic modulators of neural circuit excitability, highlighting the conserved nature
of cholinergic signaling in governing nervous system function.
**Figure 4-1: Excitotoxic cell death in cholinergic motor neurons summary model**

ACR-2 forms a functional heteromeric receptor with four additional subunits: ACR-3, ACR-12, UNC-38 and UNC-63. Introduction of a putative gain-of-function point mutation into the second transmembrane domain of the ACR-2 subunit leads to an increased influx of calcium into the cytoplasm. Calcium-induced calcium release from the ER is mediated by calnexin and calreticulin and triggers downstream events resulting in necrotic-like cell death.
ACR-2R
ACR-2  UNC-38
ACR-3  UNC-63
ACR-12

In the Cell Body

Calreticulin

Ca^{2+}

Calnexin

ER

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

Necrotic Cell Death
Using gain-of-function receptors as a tool to identify receptor composition

Due to their modulatory role, the specific function of ACR-2 containing receptors in controlling motor neuron excitability was unclear. To further elucidate its role in controlling cholinergic motor neuron excitability, I introduced a gain-of-function mutation into the TM2 pore-spanning region of ACR-2. A wide array of point mutations within the TM2 domain were originally characterized using mammalian α7 subunits, and these mutants were shown to increase the open time and sensitivity of the receptor (Orr-Urtreger et al., 2000); (Labarca et al., 1995); (Orb et al., 2004). We introduced the strongest mutation characterized in this study, a leucine to serine substitution at the 9th position of the TM2 domain. Expression of this subunit, called ACR-2(L/S), resulted in paralysis, suggesting that hyperactivation of cholinergic neurons results in loss of cholinergic motor neuron function.

ACR-2R is the first neuronal nAChR that has been characterized in C. elegans, providing evidence that this gain-of-function strain provided an ideal genetic background to identify AChR subunit composition. Using a combination of forward genetic screens and candidate mutant screens for suppressors of ACR-2(L/S)-induced paralysis, we identified the subunit composition of the ACR-2 receptor, as well as accessory subunits required for the assembly and trafficking of the receptor. The success of this gain-of-function receptor method validates our technique as a viable way to characterize receptor subunit composition. This allows us to pursue studies of the functional roles of the wide array of nAChR subunits encoded by the mammalian and C. elegans genomes, advancing our understanding of how specific receptor populations may regulate neuronal classes
that are more prone to dysfunction in neurological disease.

Despite sharing three subunits with the synaptically localized levamisole receptor, ACR-2-GFP has a diffuse localization. This suggests that specific features of the ACR-2 and/or ACR-3 subunits are key to understanding mechanisms of extrasynaptic receptor localization. In the mammalian nervous system, nAChRs can be located synaptically or extrasynaptically resulting in differential effects on neuronal function. Closer analysis of the amino acid composition of these receptor subunits could be used to identify residues responsible for dictating synaptic versus extrasynaptic localization. The identification of accessory proteins from the ACR-2(L/S) suppressor screen suggests that this method can also be used to identify proteins required for the assembly and trafficking of synaptic AChRs. The high level of homology between C. elegans and mammalian genes suggests that critical amino acids and accessory proteins identified through these experiments will be directly applicable to understanding how AChRs function in the human brain.

Pore-modified ACR-2(L/S) receptors provide insight into the molecular mechanisms of necrotic cell death

Expression of pore-modified ACR-2(L/S) resulted in the paralysis of animals from hatch leading me to question how hyperactivation of cholinergic neurons effects cell survival. Further investigation revealed vacuolar lesions along the ventral nerve cord and a reduction in the number of cholinergic cell bodies in the adult animal. Genetic analysis confirmed that ACR-2(L/S) expression occurred through a necrotic-like mechanism around the time of hatch. Necrotic-like cell death is a common result of hyperactivation in the brain after ischemic stroke (Hou and MacManus, 2002). Understanding how
ionotropic receptor activation contributes to cell death is an important factor in mapping out mechanisms triggered in stroke patients, leading to potential therapeutic targets.

Necrosis was originally thought of as a passive form of cell death that occurred as a consequence of cellular injury without requiring the activation of a specific genetic mechanism (Golstein and Kroemer, 2007b);(Kitanaka and Kuchino, 1999). More recent work has shown evidence that this process can be induced through the activation of specific cell death receptors, though the cellular mechanisms controlling necrosis remain poorly understood (Galluzzi et al., 2011). My work has shown direct evidence for a specific cellular mechanism underlying necrosis in motor neurons, contributing to the understanding of mechanisms that link AChR hyperactivation to necrotic cell death.

Our model shows that hyperexcitation of the cell through expression of ACR-2(L/S) leads to calcium-induced calcium release from the ER triggering downstream mechanisms that result in necrotic cell death of cholinergic motor neurons. The necrotic-like cell body death observed in ACR-2(L/S) animals was suppressed by eliminating function of calreticulin and calnexin, proteins which sequester calcium within the ER. This work provides a pathway through which excess excitatory signaling can cause necrotic-like cell death in motor neurons, providing a new model to study the genetic underpinnings of excitotoxicity.

The levels of excitation required to trigger necrotic cell death have not been fully described. In addition to ACR-2(L/S), a spontaneous gain-of-function in the ACR-2 subunit has been identified that does not cause excitotoxicity. This strain has a spontaneously occurring valine to methionine substitution at the 13th position of the
second transmembrane domain of the ACR-2 subunit phenotype (Jospin et al., 2009). Unlike ACR-2(L/S) which causes necrotic cell death, expression of ACR-2(V/M) leads to a spontaneous convulsion phenotype. The significant differences in the functional results of these two gain-of-function receptors lends insight into the amount of excitation that motor neurons can tolerate before the necrotic cell death program is initiated. Comparing the relative strength of these gain-of-function mutations in this model system will allow us to further these initial studies by characterizing the biological consequences of altering neuronal excitability.

*Excitotoxic cell death occurs through differential mechanisms in the axon and cell body*

Suppression of cell body death failed to prevent the paralysis caused by ACR-2(L/S) expression, suggesting that neuronal function was altered by a secondary mechanism independent of necrosis. In lines carrying deletion mutations in both the calnexin and calreticulin genes, adult animals maintained a full complement of motor neuron cell bodies, though the animals remained paralyzed. Further analysis revealed that these animals were able to move at hatch and became paralyzed because of the progressive degeneration of cholinergic motor neuron axons. This finding suggested that the dying back of axons and cell soma death seen as a result of excitotoxicity may be genetically separable.

Many neurodegenerative diseases feature a dying back phenomenon where degeneration begins at the distal tip of the axon and moves towards the soma (Fischer et al., 2004). For example, analysis of the neuromuscular junction of ALS mouse models shows evidence for weakening of cholinergic synapses before the behavioral onset of
disease symptoms (Frey et al., 2000). Similar progressive destabilization of cholinergic motor neuron axons was observed in ACR-2(L/S) animals with blocked cell body death. This provides evidence that this dying back phenomenon may be directly related to the hyperactivation of neurons by excess ionotropic receptor activation. Recent studies have begun to characterize the molecular underpinnings of axonal degeneration, though the genetics remain poorly understood. My model for axon degeneration in a genetically tractable organism provides a model to study the molecular mechanism underlying axonal death. The high level of homology between the *C. elegans* and mammalian genome suggests that these mechanisms will be conserved across species providing potential therapeutic targets for neurodegenerative disease.

**Future Directions**

Though this work has lent insight into the mechanism underlying ion channel-mediated cell death in motor neurons, further work is required to fully characterize the axon destabilization that occurs independently of cell body death. I would propose a two pronged approach to this project: first I would characterize the progression of axon destabilization using morphological and behavioral analysis, and second, I would identify the genetic pathways regulating axon destabilization by performing a forward genetic screen for suppressors of axon destabilization in *crt-1;cnx-1;ACR-2(L/S)* mutant animals. I would begin by carrying out live-imaging experiments to document the progression of axon destabilization. These experiments will provide a closer time course of destabilization and give us a better understanding of how axon branching occurs. Using
the axon destabilization experiments as a reference, I would also conduct synapse imaging experiments, which will allow me to document the progression of synapse loss in response to neuronal hyperactivity.

In human neuromuscular diseases, synapse loss is thought to begin even before the onset of behavioral symptoms, a theory that can be easily tested in this *C. elegans* model. My previous work has shown that *crt-1;cnx-1;ACR-2(L/S)* animals are able to move normally at hatch but become paralyzed before the onset of the second larval stage. New worm tracking software available in the lab will allow for a more comprehensive study of this behavioral change by filming animals over a prolonged period of time and tracking the progression of paralysis. Pairing this detailed time course of paralysis with a microscopic analysis of synapses will lead to the establishment of a genetic model system to identify the molecular mechanism controlling axon destabilization and synapse loss in cases of hyperactivation.

These molecular mechanisms can be identified by carrying out a forward genetic screen using *crt-1;cnx-1;ACR-2(L/S)* animals as a sensitized background. A primary screen for suppression of adult paralysis followed by a secondary screen examining axon morphology would lead to two major categories: animals with wild type movement and morphology and animals with branched axons and wild type movement. I expect this first group to contain many of the same genes identified in my original screen for suppressors of ACR-2(L/S)-induced paralysis, including components of the receptor and receptor trafficking proteins. The second group has the potential to identify interesting neuroprotective genes that are able to at least partially rescue axons from the effects of
hyperexcitation. This screen was not pursued originally due to the highly complicated genetic background that would make identification of suppressors using SNP mapping very difficult. With recent advances in deep sequencing technology, including wide availability and lower cost, I believe that this screen could be revisited and yield interesting results.

**Part 2: Inactivation of cholinergic neurons disrupts neural circuit development**

Disruptions in the balance of excitation and inhibition (E/I balance) in the developing mammalian brain has been linked to many neurological disorders including epilepsy and autism (Fritschy, 2008);(Rubenstein and Merzenich, 2003). In Chapter III, I characterized the roles of E/I balance in establishing and maintaining inhibitory synapses in the *C. elegans* motor circuit. In this work, I show that loss of excitatory drive results in altered function and patterning of GABA synapses, though gross circuit morphology remained intact. Global reductions in neurotransmission are not sufficient to alter inhibitory synapse morphology but selective disruption of cholinergic neurons results in altered inhibitory synapses, establishing ACh motor neurons as master regulators of inhibitory synapse patterning. I identified a four-hour critical period during the highly synaptogenic first larval stage when acetylcholine release onto GABA motor neurons is required to establish inhibitory synapse distribution. Finally, loss of excitatory drive in the mature circuit resulted in a homeostatic decrease in inhibitory synapse size, which was reversed upon reintroduction of acetylcholine into the system (see Figure 4-2). My work implicates excitatory motor neurons as master regulators of inhibitory synapse
development and provides evidence for activity-dependent synaptic plasticity in *C. elegans* neurons, a previously debated phenomenon. This work established a genetic model to study critical period development and homeostatic plasticity in an intact neural circuit. Gaining a better understanding of these basic biological processes will lead to insights into the specifics of critical period plasticity disorders which are thought to contribute to many human neurological diseases.

**Activity-dependent control of neural circuit development**

In my work, I have shown that body wall muscle morphology is slightly altered in animals lacking cholinergic drive, with muscles extending 2-3 muscle arms per cell body rather than the 4-5 observed in wild type animals (Dixon and Roy, 2005a). Despite this reduction in muscle arm number, there was no change in the area of the ventral nerve cord covered by muscle membrane. These results show a previously undescribed mechanism where muscle arms expand their domains through a possible homeostatic mechanism to cover the entire ventral nerve cord. This compensatory mechanism is reminiscent to a phenomenon observed in the tiling of neurons where loss of a neuron results in the invasion of its area by surrounding neurons of the same class. These results uncover a novel mechanism for compensatory plasticity in the development of body wall muscles of *C. elegans*, a system that is classically thought to contain an invariable blueprint for the establishment of body architecture.

In contrast, GABA motor neurons develop independently of excitatory presynaptic input. Neuronal cell body positioning in animals lacking functional ACh
MNs (unc-3) or in animals where ACh MNs are killed early in development (ACR-2(L/S)) are indistinguishable from wild type. Commissural projections extend normally and travel to the dorsal musculature to form an intact dorsal nerve cord. These results suggest that cell-intrinsic mechanisms are in place to instruct the birth and outgrowth of GABA motor neurons. Comparing the results in motor neuron versus body wall muscle shows that activity modulation has differential effects depending on cell types. These differences in response to altered activity in different cell types further adds to the evidence supporting the model that C. elegans nervous system development is more plastic than originally thought.

Roles for E/I balance in inhibitory synapse development

The activity of the GABAergic nervous system plays an essential role in the establishment and refinement of neuronal networks within the mammalian brain (Ben-Ari, 2002a);(Hensch and Fagiolini, 2005a). Though the importance of inhibitory activity during development is well established, little is known about inhibitory nervous development in vivo. Through this work I show that excitatory drive onto GABAergic motor neurons during early development is an essential driving force behind inhibitory synapse patterning and function.

Through electrophysiological analysis of inhibitory signaling, we observed that GABA motor neurons remain functional in the absence of excitatory input. This result implies that presynaptic activation of GABA motor neurons is not essential for neuronal function providing evidence for the existence of intrinsic activity within GABA motor
Figure 4-2: Model- Excitatory input regulates inhibitory synapse development and function

A. Loss of excitatory drive in the developing motor circuit leads to altered patterning of inhibitory synapses characterized by large gaps in synapse distribution and increased pre- and post-synaptic area. These changes lead to the model that excitatory drive onto GABA motor neurons triggers an activity-dependent transcriptional or translational mechanism within these neurons that regulates the patterning or refinement of inhibitory synapses. B. Reduced excitatory drive onto GABA motor neurons in the mature nervous system results in decreased synapse size, without altering synaptic patterning. In this model, homeostatic mechanisms in the adult nervous system react to decreased activity levels by reducing inhibitory synapse size, functionally maintaining the balance of excitation and inhibition within the circuit.
neurons. The frequency of spontaneous release events in these animals is reduced to approximately 1/3 of wild type, which reflected either a loss of presynaptic drive or alterations in synapse function. Additionally, the amplitude of spontaneous release events was significantly increased, suggesting that while gross neuronal morphology remains intact, functional changes occur at the synapse as a result of decreased excitatory innervation. Consistent with our recordings of spontaneous release, we found that the amplitude of evoked GABA events was significantly increased in animals chronically lacking cholinergic function. Taken together, functional analysis shows that while GABA motor neurons remain active in the absence of cholinergic input, the functional properties of the synapses are altered. This data shows that while motor neuron development is activity dependent, synaptic connectivity is highly influenced by changes in presynaptic input.

Spontaneous activity has been observed in the developing nervous systems of many species and across multiple brain regions during periods when neural circuits undergo significant refinement (Katz and Shatz, 1996); (Blankenship and Feller, 2010); (Dehотор et al., 2012); (Feldt et al., 2011); (Moody and Bosma, 2005) leading to the theory that correlated spontaneous activity is responsible for circuit refinement. The mammalian visual system has been widely used to study this theory, though many questions remain due to the limitation of anesthetizing animals, which inhibits patterns of spontaneous activity in the cortex (Hanganu et al., 2006); (Siegel et al., 2012). In my studies, I used the genetic tools available in C. elegans to modulate the activity of a specific neuronal class to determine how neural circuit function and morphology were altered. We
observed an increase in GABA synapse size in the absence of functional cholinergic motor neurons, a result that was consistent with the increased GABA synaptic function we observed in our electrophysiology experiments. We also observed a decrease in synapse density in animals lacking cholinergic innervation. These results support the hypothesis that the circuit is not able to refine synapses in the absence of excitatory activity. My work has provided a new model for the study of activity-dependent circuit refinement in a motor circuit where the genetic pathways underlying activity-dependent refinement can be directly studied.

Studies in the mammalian cortex have shown a correlation between excitatory synapse size and synaptic efficacy. For example, at glutamatergic synapses, stronger synapses have larger dendritic spines and an increase in the number of glutamate receptors at the PSD (Matsuzaki et al., 2004a). Our observation that increased GABA function in the absence of cholinergic innervation correlates to an increase in synapse size suggests that synapse size is also an indicator of synapse strength for inhibitory synapses. This result was particularly interesting due to the fact that GABA motor neuron morphology remained unaltered in these mutants, suggesting that there may be an activity-dependent mechanism that is required to establish inhibitory synaptic connectivity independently of neural wiring.

Global reductions in neurotransmission were insufficient to alter inhibitory synapse size or patterning. This result led me to the hypothesis that the balance of excitation and inhibition may regulate inhibitory synapse size and patterning. In contrast, mutant animals with selective disruption of GABAergic release failed to phenocopy the
synaptic patterning defects. This result was further supported by evoked recordings from animals carrying a mutation in the transcription factor *unc-30*, which leads to loss of expression of essential GABAergic genes. We observed no change in the amplitude of evoked cholinergic responses in these animals, further supporting the hypothesis that excitatory activity acts as a driving force for inhibitory synapse patterning.

Cholinergic motor neurons synapse onto both body wall muscles and onto GABA motor neurons leading to two potential sites of action for the cholinergic instruction of inhibitory synapse patterning. Blocking cholinergic neurotransmission onto the body wall muscle using animals lacking functional cholinergic receptors at the NMJ, *unc-29;acr-16*, did not alter inhibitory synapse patterning. This result eliminated cholinergic signals onto the muscle as a potential mechanism for inhibitory synapse patterning. I was unable to directly test the consequence of removing cholinergic neurotransmission onto GABA motor neurons because the full complement of receptors at this synapse has yet to be identified. To circumvent this problem, I used a mutant in the vascular acetylcholine transporter (vAChT) *unc-17* to block all cholinergic neurotransmission in the motor circuit. The loss of acetylcholine release resulted in disruption of inhibitory synapses. This result supports the hypothesis that cholinergic release onto the GABA motor neurons is required to pattern inhibitory synapses. Together, these results suggest a potential model where an activity dependent transcriptional or translational mechanism within the GABA motor neurons instructs GABA synapse patterning.

*Critical period control of neural circuit development*
In the mammalian brain, the function of the inhibitory nervous system has been implicated as a key player in determining the timing of critical period plasticity (Fagiolini and Hensch, 2000a). Multiple studies have shown the ability of GABA interneurons to control key aspects of cortical circuits including neuronal excitability, integration and the temporal oscillations of excitatory networks (Swadlow, 2003a);(Pouille and Scanziani, 2001a);(Somogyi and Klausberger, 2005).

Through my work, I have identified a four-hour critical period window during which cholinergic drive onto inhibitory motor neurons is required for the appropriate patterning of inhibitory synapses. Loss of excitatory drive during this window leads to changes in inhibitory synaptic patterning that phenocopy the defects observed in animals chronically lacking functional cholinergic neurons. Interestingly, there is no increase in the size of synapses in these animals, though there is a significant increase in synapse size in animals chronically lacking cholinergic release. This suggests that the increase in synapse size may be a separate phenotype from changes in patterning and a second critical period window could exist to determine synapse size.

In addition to providing new insights into the development of the inhibitory nervous system, these results also provide evidence for a new genetic model to study critical period windows during development. Identification of these pathways is the first step in understanding potential methods for reintroducing plasticity into the adult brain as a therapy for patients suffering from brain injury.

*Activity-dependent plasticity in modulating mature neural circuits*
The primary function of a neuron is to receive information from presynaptic partners, integrate that information and provide appropriate synaptic output. The ability of the neurons to dynamically change synaptic strength is a key component in neural circuit function. Strong, repeated stimulation from a presynaptic cell strengthens synaptic connections, termed Hebbian plasticity, and has been studied as a cellular basis of learning and memory (Neves et al., 2008); (Sjostrom et al., 2008). In order to prevent this process from leading to runaway excitation, homeostatic feedback mechanisms are thought to maintain network stability. Studies of homeostatic mechanisms in mammalian cell culture have led to the identification of molecular properties of homeostatic plasticity but the lack of intact neural network connectivity may obscure specific aspects of control that are dependent on precise connectivity (Pozo and Goda, 2010). These studies highlight the need for established in vivo systems for the study of homeostatic mechanisms in intact neural networks.

My work has shown evidence of homeostatic plasticity in the motor circuit of C. elegans. I observed that when acetylcholine release is removed from the established motor circuit, a proportionate decrease in the size of GABA presynaptic release sites and postsynaptic clusters occurred. Further, adding acetylcholine release back into the circuit led to a recovery of inhibitory synapse size back to wild type levels. The synaptic scaling we observe in the established nervous system correlates to what is observed in circuits where homeostatic plasticity is functioning appropriately to maintain circuit function. For example, in hippocampal slices, reduction of network activity by TTX treatment leads to reduced mIPSCs in mature cultures, suggesting a decrease in inhibitory synapse
size (Hartman et al., 2006). This data implies that activity-dependent mechanisms functioning in GABA motor neurons are different in the developing compared to the adult nervous system.

Homeostatic plasticity is not only important in cases of hyperexcitation, it may also play a role in conditions of chronic reduced activity to increase synapse strength, preventing unnecessary synapse loss within the circuit (Vitureira and Goda, 2013). In cases where excitatory drive was chronically lost, I observed an increase in both the size of inhibitory synapses and in the amplitude of spontaneous release events from GABA neurons. These data suggest that homeostatic mechanisms may be in place to maintain GABA synaptic efficacy in cases of chronic activity loss.

**Future Directions**

The clear next step in this project is to use an array of complementary genetic approaches to explore activity-dependent mechanisms regulating inhibitory synapses. I have begun this analysis using an RNAi screen to evaluate the function of previously characterized synaptogenic genes at inhibitory synapses. The highly conserved gene neurexin was identified in this pilot screen as a regulator of inhibitory synapse formation in *C. elegans* validating this approach for screening synaptogenic genes (see Appendix).

I would also like to compare gene expression levels in wild type animals versus animals lacking cholinergic innervation (*unc-3*). Using deep sequencing and/or microarray analysis, hundreds of genes can be compared between these two groups, shedding insights into the role activity plays on gene expression. Any genes that show dramatic changes in expression provide us targets for further analysis, ultimately
resulting in the identification of activity-dependent pathways that regulate inhibitory
synapse patterning.
Appendix

Identification of genetic mechanisms required for the patterning of inhibitory synapses: Neurexin and Neuroligin.

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Contribution Summary

The following work was carried out in the lab of Dr. Michael M. Francis at the University of Massachusetts Medical School. In this chapter, Michael Francis provided advice on experimental design and data analysis. I designed and carried out experiments and analyzed data.
Introduction

Initial synapse formation begins when axons reach their target postsynaptic cell and requires a number of developmental steps, many of which are thought to be mediated by cell adhesion molecules (Gerrow and El-Husseini, 2006); (Dalva et al., 2007). Defects in genes responsible for the formation, stabilization and maintenance of synapses have been linked to many neurological disorders ranging from schizophrenia to autism, emphasizing the need to better understand the underlying molecular mechanisms of synapse development (Banerjee et al., 2014); (Jenkins et al., 2014).

Two molecules that have been implicated in synapse development are the single pass transmembrane protein neurexin and its binding partner neuroligin. Recent genome sequencing studies have identified mutations in neurexin and neuroligin in patients with autism spectrum disorders, highlighting the importance of investigating roles for these genes in synapse development and function (Liu et al., 2012);(Camacho-Garcia et al., 2012);(Camacho-Garcia et al., 2013). Neurexin and neuroligin form a trans-synaptic complex that interacts with the pre- and postsynaptic scaffolding networks and have been shown to recruit synaptic proteins in cell culture studies (Rao et al., 2000);(Scheiffele et al., 2000);(Graf et al., 2004); (Nam and Chen, 2005). In contrast to cell culture studies, neurexin and neuroligin knock out mouse models do not show alterations in synapse morphology; instead they show defects in calcium mediated synaptic release structure (Missler et al., 2003);(Varoqueaux et al., 2006). These results lead to conflicting models for neurexin and neuroligin function at synapses.
The conflicting results of these cell culture and mouse knock out models have been clarified by *Drosophila* studies at excitatory synapses though the function of these proteins at inhibitory synapses within an intact circuit remain uncharacterized. Recent studies have linked mutations in neurexin and neuroligin to autism spectrum disorders, highlighting the importance of elucidating roles for neurexin in the CNS (Liu et al., 2012); (Camacho-Garcia et al., 2012); (Camacho-Garcia et al., 2013). This link to neurological disorders highlights the need for further *in vivo* studies of gene function to better understand the contradictions in cell culture versus mouse knockout models. Because the functional redundancy of splice isoforms complicates the study of neurexin-neuroligin interactions in mammalian models, I turned to and invertebrate model to better understand neurexin and neuroligin function in the development of synapses.

The *C. elegans* genome encodes four neurexin-like genes by sequence homology, with the gene *nrx-1* encoding the most highly related homolog of mammalian neurexin (Tabuchi and Sudhof, 2002). NRX-1 is expressed almost pan-neuronally and is localized specifically at presynaptic release sites, suggesting a role in synapse function (Haklai-Topper et al., 2011). Neurexin has been shown to interact with a retrograde signaling pathway at cholinergic neurons in *C. elegans* that controls presynaptic vesicle release and postsynaptic receptor density (Hu et al., 2012). Roles for neurexin in the development and function of inhibitory synapses in *C. elegans* have not been identified.

Here, we demonstrate that neurexin and neuroligin play an instructive role in the patterning of inhibitory GABA synapses. We identified neurexin from an RNAi screen for modulators of GABA receptor patterning. Neurexin loss of function mutant animals
show altered patterning of both pre and postsynaptic markers, characterized by large gaps in synapse distribution along the nerve cord. In contrast to neurexin mutant phenotypes, neuroligin mutant animals show an increased coverage of inhibitory synapses at the ventral nerve cord. This suggests that neuroligin and neurexin do not have the same function during the development of inhibitory synapses. Finally, genetic analysis suggests that neuroligin functions downstream of cholinergic motor neuron function while neurexin functions in a parallel pathway, further supporting the hypothesis that neurexin and neuroligin function in independent pathways to regulate the patterning of inhibitory synapses.

Results

A candidate mutant screen for genes required for synapse development

My previous work has shown that loss of excitatory drive during early development results in altered patterning of inhibitory synapses within the *C. elegans* motor circuit, providing evidence for the existence of activity-dependent synaptic mechanisms. In order to characterize the mechanisms underlying these activity-dependent changes, I carried out a pilot RNAi screen for genes that are required for inhibitory synapse patterning.

In *C. elegans*, RNAi is delivered as dsRNA that can be administered to the animals by feeding, soaking or microinjection (Conte and Mello, 2003). For initial screening, I chose to use a feeding method where animals were grown up for two generations on NGM plates seeded with bacteria expressing dsRNA for the gene of interest. GABA receptor localization in adult F2 animals was imaged and the percent coverage of the
ventral nerve cord was calculated for each gene knockdown tested. My initial screen included genes previously characterized as playing essential roles in nervous system development. Of the genes screened, two RNAi lines produced alterations in GABA receptor localization: egl-15 and nrx-1 (Figure A-1).

The gene egl-15 encodes the sole C. elegans fibroblast growth factor (FGF) receptor, which is orthologous to the vertebrate FGFR1-4 (DeVore et al., 1995)). FGF receptors have been shown to be involved in presynaptic differentiation in the mouse hippocampus (Terauchi et al., 2010), leading to the hypothesis that the FGF receptor egl-15 in C. elegans mediates a similar effect. Work done in C. elegans has shown that egl-15 function is required for the development and outgrowth of muscle arm extensions (Dixon et al., 2006). This result led me to believe that the observed disruption in GABA receptors may be a secondary effect of altered muscle arm membranes, not a synapse-specific phenotype. Due to these findings, I did not pursue elg-15 as a candidate for further analysis.

The second candidate that was identified from my pilot screen was nrx-1. nrx-1 encodes C. elegans neurexin, a single-pass transmembrane domain protein that has been linked to synapse development. Neurexin interacts directly with its binding partner neuroligin and has been shown to be sufficient to recruit synaptic proteins in cell culture studies (Rao et al., 2000);(Scheiffele et al., 2000);(Graf et al., 2004); (Nam and Chen, 2005). Animals cultured on RNAi against nrx-1 showed a significant reduction in the amount of the muscle membrane extensions expressing postsynaptic receptors, with nrx-1 RNAi strains showing 65% coverage compared to 90% in wild type (Figure A1-A). The
requirement for neurexin at GABAergic synapses was not previously explored in *C. elegans* making it an interesting candidate for further study.

**Characterization of inhibitory synapses in neurexin mutants**

To further characterize the role of neurexin in inhibitory synapse development, I began by confirming the results from my RNAi screen by assessing inhibitory synapse patterning in animals carrying a deletion mutation in *nrx-1*. *nrx-1(ok1649)* contains a 861bp deletion in the *nrx-1* gene that removes the C-terminus of all four splice isoforms of NRX-1, which encodes the PDZ binding motif. The PDZ binding motif has been shown to be required for the exit of neurexins from the golgi and transport to synapses leading us to believe that this allele is acting as a null mutation (Fairless et al., 2008).

I expressed genetically encoded markers for both the inhibitory presynaptic release sites (*Punc-47::mCherry::RAB-3*) and postsynaptic receptors (*UNC-49::GFP*) and found that inhibitory synapse patterning was also disrupted in *nrx-1(ok1649)* mutant animals. This disruption included large gaps in synaptic distribution resulting in 40% of the nerve cord lacking synapses in *nrx-1* mutants, compared to 10% in wild type (Figure A2). Presynaptic release sites and postsynaptic receptors remained aligned in *nrx-1* mutants suggesting that the localization of entire synapses, not synaptic registration, is altered. These data further support the model that neurexin is required to localize inhibitory synapses at the *C. elegans* neuromuscular junction.
Neurexin and neuroligin work in opposing pathways to regulate inhibitory synapse density

Cell culture studies have shown that neurexin interacts directly with another single pass transmembrane protein neuroligin to initiate synaptogenesis in cultured cells (Scheiffele et al., 2000); (Graf et al., 2004); (Nam and Chen, 2005). This leads to the hypothesis that the interaction between neurexin and neuroligin may also be mediating inhibitory synapse distribution in C. elegans. In order to test this hypothesis, I began by fluorescently tagging inhibitory synapses in nlg-1(ok259) mutant animals and assessing synaptic patterning. An increased area of both presynaptic release sites and postsynaptic receptor clusters is seen in these animals resulting in a near-complete coverage of the ventral nerve cord with synapses (Figure A-3C). Quantification of this result shows a decrease in the percent of the nerve cord lacking postsynaptic markers from 20% in wild type to 5% in nlg-1 mutant animals indicating an increase proliferation of inhibitory synapse proteins (Figure A-3E). These results suggest that neuroligin plays a role in inhibiting GABA synapse formation in a wild type circuit.

We used pharmacological analysis to determine whether the physical changes we observed in neurexin and neuroligin mutant animals reflected functional changes in inhibitory synapses. Aldicarb is an acetylcholinesterase inhibitor that prevents acetylcholine breakdown in the synaptic cleft, leading to a buildup of ACh and paralysis of the animals, providing a behavioral readout for synapse function. nrx-1(ok1649) animals are hypersensitive to the paralytic effect of aldicarb suggesting that inhibitory synaptic function is decreased in these animals (Figure A-3F). This is consistent with the
decrease in the number of inhibitory synapses observed in these animals (Figure A-3B,E). In contrast, nlg-1(ok259) animals are resistant to aldicarb-induced paralysis suggesting increased inhibitory synapse function. This result is again consistent with the morphological analysis which showed increased coverage of inhibitory synapses in nlg-1 mutant animals (Figure A-3C, E, F). Taken as a whole, these experiments show that inhibitory synapse morphology and function are dependent on neurexin and neuroligin.

Neuroligin has been shown to directly interact with neurexin to facilitate synaptogenesis. Interestingly, alterations in inhibitory synapse patterning in neuroligin mutant animals fail to phenocopy patterning defects observed in neurexin mutant animals suggesting that they do not always work in the same pathway. In order to characterize the genetic interactions between neurexin and neuroligin in the C. elegans motor circuit, I evaluated inhibitory synapse patterning in nrx-1(ok1649);nlg-1(ok259) double mutant animals. In these animals, inhibitory synapse patterning returns to near wild type distribution, which is reflected in the quantification of coverage (Figure A-3D, E). In contrast, double mutant animals are hypersensitive to aldicarb-induced paralysis, phenocopying nrx-1 single mutants. These differences in synapse patterning and synaptic function suggest that synaptic morphology and strength are not directly correlated.

Excitatory activity works upstream of neurexin and neuroligin to pattern inhibitory synapses.

My previous work has shown that cholinergic motor neuron function is required for inhibitory synaptic patterning. In order to determine if neurexin is working
downstream of excitatory activity to pattern inhibitory synapses, we compared phenotypes in *nrx-1* and *unc-3* mutant animals. *unc-3* encodes a transcription factor that is required for the terminal differentiation of cholinergic motor neurons. Inhibitory synapse patterning is disrupted in *unc-3* mutant animals showing large gaps in the distribution of synapses (Figure A-4B). These animals phenocopy the patterning defects in *nrx-1* mutant animals, suggesting that neurexin may work downstream of cholinergic activity to drive inhibitory synapse development. We tested this hypothesis by generating *unc-3; nrx-1* double mutant animals and assessing changes in inhibitory synapse patterning to identify any genetic interactions. Inhibitory synapses are altered in *nrx-1;unc-3* double mutants showing gaps in distribution as well as enlarged puncta size. Quantification of the percent of the nerve cord lacking postsynaptic receptors shows that the *nrx-1;unc-3* double mutants show a more severe loss of synapse phenotype than either single mutant. This additive effect suggests that neurexin and *unc-3* work in parallel pathways to regulate inhibitory synapse distribution.

In order to determine if neuroligin function was dependent on cholinergic activity, I also fluorescently tagged inhibitory synapses in *unc-3; nlg-1* double mutant animals and assessed synapse morphology. Synapses in *nlg-1;unc-3* double mutant animals show gaps in distribution and enlarged puncta. Synaptic morphology in these animals phenocopies the observed defects in *nlg-1;nrx-1* double mutants (Figure A-4D, Figure A3). The percentage of nerve cord lacking synaptic markers increases significantly from 5% in *nlg-1* single mutants to 30% in *nlg-1;unc-3* double mutants. These data suggest that *nlg-1* is working downstream of both activity and neurexin to pattern inhibitory synapses.
Discussion and Future Directions

Cell culture and mouse model studies paint different pictures of neurexin and neuroligin function at synapses, making it critical to carry out further studies in intact neural circuits. My work has used the *C. elegans* motor circuit to provide mechanistic insights into the function of neurexin and neuroligin in the development of inhibitory synapses and revealed potential links between gene function and the activity of upstream neurons.

My data suggest that neurexin and neuroligin work in opposing pathways to instruct inhibitory synapse density, with neurexin promoting diffusion of inhibitory synapses and neuroligin working to cluster synapses in wild type animals. These opposing functions differ from the more traditional pathway where neurexin directly binds neuroligin to recruit synaptic proteins. Neurexin; neuroligin double mutant animals show a near wild type distribution of synapses, further supporting the model where neurexin and neuroligin function opposite each other to balance inhibitory synapse density. Functional analysis of these double mutant animals show that they phenocopy the aldicarb hypersensitivity seen in neurexin single mutant animals. The differences in the severity of the physical and functional changes seen at inhibitory synapses suggests that synaptic morphology is not always a direct indicator of the level of functional changes at a synapse. Further analysis to identify the site of action for neurexin and neuroligin would be instrumental in understanding the mechanism regulating inhibitory synapse function in this circuit.
My previous work has produced a model where cholinergic motor neuron activity triggers an activity-dependent pathway in the GABA motor neurons that regulates inhibitory synapse patterning. Based on inhibitory synapse phenotypes, neurexin was thought to play a role in this activity-dependent mechanism. The double mutant analysis for \textit{unc-3;nrx-1} showed an additive phenotype where gaps in synapse distribution were larger in either single mutant. This suggests that neurexin is working in a parallel pathway to cholinergic activity to regulate inhibitory synapses. These experiments also showed that \textit{nlg-1;unc-3} mutants closely phenocopied \textit{nlg-1;nrx-1} double mutants. These results suggested that neuroligin works downstream of both neurexin and activity, though the mechanisms linking neuroligin function to activity have yet to be identified.

These experiments bring up many questions about the function of neurexin and neuroligin at inhibitory synapses. What is the mechanism linking neuroligin to activity? How do activity levels alter transcriptional and/or translational levels of neurexin and neuroligin? Determining the site of action for \textit{nrx-1} and \textit{nlg-1} function at inhibitory synapses and looking for differences in protein levels in wild type and \textit{unc-3} mutant animals could further address these questions. Additionally, a forward genetic screen for suppressors of inhibitory synapse patterning defects in a \textit{nlg-1} or \textit{nrx-1} mutant background can be used to determine other players in these pathways. The results of these experiments will lead to a better mechanistic understanding of neurexin and neuroligin function in an \textit{in vivo} system.
Using *C. elegans* as a genetic model to identify genes required for inhibitory synapse development.

Identification of neurexin, a previously identified synaptogenic gene in mammalian cells, validates our approach for using *C. elegans* to identify key mechanisms underlying inhibitory synapse development. This leads to a two-pronged approach to broaden our knowledge of inhibitory synapse development. Further RNAi screens will allow us to characterize previously identified genes in a simple genetic system. Many genes are highly conserved between *C. elegans* and the mammalian genome allowing us to test them in our model system. *C. elegans* is amenable to many genetic manipulations that are lethal in other systems, allowing for the study of essential synaptogenic genes.

In addition to RNAi screens, we are able to carry out forward genetic screens for genes required for inhibitory synapse development. This non-biased approach allows us to identify previously uncharacterized synaptogenic genes. Using the genetically tractable *C. elegans* system to quickly identify and characterize molecular pathways that can be further studied in mammalian systems will quickly push the field forward.
Figure A-1: RNAi knockdown of neurexin disrupts the patterning of GABA receptor clusters.

A. Quantification of the percent of the ventral nerve cord of adult animals expressing GABA postsynaptic receptor clusters (UNC-49::GFP) in wild type animals grown for two generations on the RNAi lines indicated. The graph shows data for control strains (black bars), positive hits (*nrx-1* in red, *egl-15* in white) and negative results (gray bars). Bars represent the mean ±SEM of at least ten animals /RNAi line. Student t-test.

***p<0.0001, **p<0.01. B-D: Confocal images of UNC-49::GFP expressing animals grown on empty vector (B), *nrx-1(c29A12.5)* (C) or beta integrin(*C05D9.3*) (D) RNAi plates. Brackets indicate gaps in receptor clusters in the *nrx-1* animals. Scale bar: 20uM.
Percent of nerve cord expressing UNC-49::GFP

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UNC-49::GFP (%)</th>
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<tbody>
<tr>
<td>Wild Type</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>ACR-2(L/S) Empty Vector</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>nrx-1(C29A12.5)</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>egl-15(F58A3.2)</td>
<td>60 ± 5</td>
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<tr>
<td>ncam-1(F02G3.1)</td>
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<tr>
<td>cdh-1(C45G7.5)</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>apr-1(K04G2.8a)</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>β integrin(C05D9.3)</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

A) Bar graph showing the percent of nerve cord expressing UNC-49::GFP for different treatments. Error bars represent ± standard error.

B) Image of neuronal processes with expression of UNC-49::GFP in Empty Vector condition.

C) Image of neuronal processes with expression of UNC-49::GFP in nrx-1(c29a12.5) condition.

D) Image of neuronal processes with expression of UNC-49::GFP in β integrin (C05D9.3) condition.
Figure A-2: Inhibitory synapse patterning is disrupted in neurexin mutant animals.

A-C: Confocal images of the posterior ventral nerve cord of adult animals co-expressing a GABA presynaptic marker (*Punc-47::mCherry::RAB-3*) with a GABA postsynaptic receptor marker (UNC-49::GFP). Brackets indicate gaps in synapse distribution. Scale bar: 20uM. D. Quantification of the percent of the ventral nerve cord lacking presynaptic release sites. Graph represents the mean ±SEM for at least ten animals/genotype. Student t-test: *p<0.01, ***p<0.0001.
Wild Type

nrx-1(ok1649)

ACR-2(L/S)

% of nerve cord lacking release sites

Wild Type  ACR-2(L/S)  nrx-1(ok1649)

D

% of nerve cord lacking release sites

Wild Type  ACR-2(L/S)  nrx-1(ok1649)

***  ****  *
Figure A-3: Neurexin and neuroligin have differential effects on inhibitory synapse distribution.

A-D: Confocal images of the posterior ventral nerve cord of adult animals co-expressing a GABA presynaptic marker (*Punc-47::mCherry::RAB-3*) and a GABA postsynaptic receptor marker (UNC-49::GFP). Brackets indicate gaps in synapse distribution; dotted lines indicate areas of diffuse synapse clustering. Scale bar: 20\mu M. E. Quantification of the percent of the ventral nerve cord lacking presynaptic release sites. Graph represents the mean ±SEM for at least ten animals/genotype. Student t-test: **p<0.001. F. Paralysis time course for animals in the presence of the cholinesterase inhibitor aldicarb (1mM). The percentage of immobilized animals was calculated every 15 minutes; the graph represents the mean ±SEM of at least ten trials/genotype, with populations of 10-15 animals/trial.
**Wild Type**

**nrx-1(ok1649)**

**nlg-1(ok259)**

**nlg-1(ok259);nrx-1(ok1649)**

% of nerve cord lacking receptor clusters

10

20

30

40

**Wild Type**

**nrx-1(ok1649)nlg-1(ok259)nrx-1(ok1649);nlg-1(ok259)**

**ns**

A

B

C

D

E

F

E: % of nerve cord lacking receptor clusters

Wild Type  nrx-1(ok1649)  nlg-1(ok259)  nrx-1(ok1649);nlg-1(ok259)

ns

F: Graph showing percentage of paralysis over time.
Figure A-4: Neurexin and neuroligin work downstream of cholinergic motor neuron activity to pattern inhibitory synapses.

A-F: Confocal images of the posterior ventral nerve cord of adult animals co-expressing GABA synapse markers (Punc-47::mCherry::RAB-3 and UNC-49::GFP) in the genotypes indicated. Brackets indicate gaps in synapse distribution. Scale bar: 20μM. G: Quantification of the percent of the ventral nerve cord lacking postsynaptic receptor clusters in control animals (black bars), neurexin mutant animals (gray bars) and neuroligin mutant animals (white bars). The graphs represent the mean ±SEM for at least ten animals/genotype. Student t-test: **p<0.01, ***p<0.000.
Wild Type

nrx-1(ok1649) nlg-1(ok259) unc-3(e151)
nrx-1(ok1649);unc-3(e151)

% of nerve cord lacking receptor clusters

A B C D
e 10 20 30 40
G

ns

185
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