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Dissecting the Molecular Mechanisms Underlying Synpase Development and Neuronal Functions in Caenorhabditis elegans

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DISSECTING THE MOLECULAR MECHANISMS UNDERLYING SYNAPSE DEVELOPMENT AND NEURONAL FUNCTIONS IN CAENORHABDITIS ELEGANS

by

Shuo Luo

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ABSTRACT OF THE DISSERTATION

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Dr. Michael L. Nonet, Chairperson

The development and function of the nervous system is under delicate regulation of diverse tissue-derived signals in multi-cellular organisms. In Dr. Nonet's lab, I am using the model organism *Caenorhabditis elegans* to ask two basic questions: 1) How do different tissues in an organism coordinate to regulate neural functions and behaviors? 2) What controls the development of synapse, the basic unit of the nervous system? These questions divide my dissertation into three parts, with the first two parts related to the first question and the third part to the second question.

In the first part of this dissertation, I present work that demonstrates the role of the *C. elegans* intestine as an endocrine organ in regulating the rhythmic defecation behavior (Chapter 2). The *C. elegans* defecation behavior consists of three well-coordinated

muscle contractions that enable the nematode to expel intestinal contents out to the environment. Genetic and cell biology analyses showed that the early and late muscle contractions involve activities in the intestine and GABAergic neurons (AVL and DVB), respectively, while it remains unclear how the intestinal event is coordinated with later activation of GABAergic neurons. Using molecular genetics and cell biology approaches, we demonstrate that the exocytic protein AEX-4 and proprotein convertase AEX-5 function in the worm intestine to control the defecation motor program. When expressed in the intestine, AEX-5 is secreted into the pseudocoelom, and this secretion is blocked by AEX-4 disruption. Moreover, we show that the G-protein coupled receptor (GPCR) AEX-2 functions in GABAergic neurons to regulate defecation behavior, and it is genetically downstream of intestinal AEX-4 and AEX-5 signals. We also demonstrate that the stimulatory Gα pathway relays the AEX-2 signal in GABAergic neurons. Together, our results provide evidence that the *C. elegans* intestine is able to modulate neuronal function by secretory signals.

In the second part of this dissertation, I present work that demonstrates the role of the *C. elegans* intestine in modulating the cholinergic neurotransmission (Chapter 3). *C. elegans* utilizes acetyl choline as a neurotransmitter at its neuromuscular junctions (NMJs) to control muscle contractions and locomotion related behaviors. Using molecular genetics, pharmacological, and physiological approaches, we show that the proprotein convertase AEX-5 is required in the intestine to maintain normal cholinergic transmission in the nematode. In addition, we find that the GPCR AEX-2 functions in the GABAergic neurons to maintain cholinergic transmission level, and the stimulatory

Gα pathway is genetically downstream of AEX-2. Interestingly, we find that although both the defecation motor program and the cholinergic transmission modulation involve intestinal signals and neuronal G-protein pathways, they depend on different downstream molecules: while the defecation requires GABA to activate the enteric muscle contraction in the last step of the defecation, the modulation of cholinergic transmission depends on neuropeptide processing enzymes EGL-3 and EGL-21. As GABAergic neurons do not directly synapse on cholinergic neurons in *C. elegans*, we speculate that the peptide signals act in a paracrine manner on cholinergic neurons. This suggests the *C. elegans* intestine could function as an endocrine organ to modulate multiple aspects of neuronal functions.

In the last part of this dissertation, I focus on the early neural development of *C. elegans* and I present the preliminary work on the focal adhesion complex molecule ZYX-1 for its role in mechanosensory synapse development (Chapter 4). We cloned the *zyx-1* allele from the genetic screen that looked for worms defective in PLM synaptic patch formation. Using time course imaging analysis of fluorescence labeled PLM neurons, we show that *zyx-1* mutants are able to form synapses during early development, while they fail to maintain the synapse to adulthood. In addition, we demonstrate that ZYX-1 acts cell-autonomously in mechanosensory neurons to regulate PLM synapse maintenance. We are currently working to dissect the molecular mechanisms that underlie ZYX-1's function in synapse maintenance. I expect the study will shed light on our understanding of the molecular mechanisms underlying neural development.

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the neuroscience field and encouraged me to apply U.S. graduate schools. Without her, it is impossible for me to come to today's accomplishments. I will try my best to keep learning and moving forward.

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Chapter 1

Introduction to the Defecation Behavior in *C. elegans* And the Modulation of Neuronal Functions

1. The *C. elegans* Defecation Behavior

1.1. *C. elegans* As A Model Organism to Study Behaviors

C. elegans is a ~1mm long, free-living soil nematode that was first isolated and settled as a genetic model organism by Sydney Brenner in 1960's (Wood, 1988). With as few as 959 somatic cells, *C. elegans* exhibits surprisingly diversified behaviors from feeding, locomotion, chemotaxis, to more complex behaviors such as mating and egg-laying, making this small animal a desirable model to dissect the cellular and molecular mechanisms underlying behaviors (Riddle et al., 1997). In addition, with the completion of the 302-cell nervous system circuitry that discloses the complete connecting patterns of all neurons in a single adult hermaphrodite (White et al., 1988; White, 1986), people started looking forward to understanding how different tissues (including neurons) in a single animal coordinate to regulate complex behaviors.

1.2. Defecation Motor Program (DMP)

Among various behaviors observed in *C. elegans*, the defecation behavior attracts our attentions because it is rhythmic and it involves the communication among different tissues including neuronal and non-neuronal cells (which will be discussed in more details in following sections). The *C. elegans* defecation motor program (DMP) consists of three well-coordinated muscle contractions that happen about every 45 seconds in healthy, feeding animals (Fig 1A) (Thomas, 1990). This program first starts with a posterior body-wall muscle contraction (pBoc), which squeezes and pushes intestinal

contents forward. The posterior body-wall muscles then relax. About 1-2 seconds later, the anterior body-wall muscles contract (aBoc), pressurizing intestinal contents around the anus. Almost at the same time, the set of enteric muscles that wrap the posterior intestine and the anus of the animal contract, opening the anus and expulsing the intestinal contents out to the environment (Exp) (Fig 1A) (Thomas, 1990). This completes the defecation cycle and the whole program repeats itself in precision about 45 seconds later. As this behavior is highly invariant in wild type animals, and as the disruption of the defecation motor program leads to phenotypes that are easily observable under dissecting microscopes (such as constipation), people adopted a variety of molecular genetics and cell biology strategies to identify the cells and genes that are involved in defecation regulation.

1.3. GABAergic Neurons in the Defecation Regulation

The first immediate speculation on the identity of the cells that are involved in controlling the serial muscle contractions during the defecation cycle involved neurons. Indeed, in a systematic study of the GABAergic nervous system in the nematode, people found that two GABAergic neurons, AVL and DVB, are required for the execution of aBoc and Exp steps in the defecation motor program (McIntire et al., 1993a; McIntire et al., 1993b). The *C. elegans* GABAergic nervous system consists of 26 GABAergic neurons, with 19 type-D motor neurons that synapse on body wall muscles, 4 RME motor neurons that control worm foraging behaviors, one RIS interneuron with unknown functions, and AVL and DVB motor neurons (Fig 1B) (McIntire et al., 1993b). Serial

electron microscopy analysis shows that DVB (and likely AVL) forms a neuromuscular synapse on the anal depressor, an enteric muscle that regulates anus opening during the expulsion (Fig 1B) (White, 1986). Interestingly, when AVL and DVB neurons are killed with a laser microbeam, the worm exhibits strong expulsion defects and becomes constipated (McIntire et al., 1993b), suggesting the two GABAergic neurons are involved in direct enteric muscle activations. In support of this observation, in mutant animals that lack the functional GABA synthase UNC-25 (the worm homologue of the glutamic acid decarboxylase, GAD), most of the expulsion step of the defecation cycle is absent and the animal becomes constipated (McIntire et al., 1993a; Thomas, 1990). These data strongly suggest that GABAergic neurons AVL and DVB directly control enteric muscle contractions by releasing GABA. But how can the conventionally inhibitory neurotransmitter GABA excite muscles? With the cloning of expulsion defective gene *exp-1*, the mystery was resolved: *exp-1* encodes an excitatory GABA-gated ion channel and it is expressed in enteric muscles (Beg and Jorgensen, 2003). Unlike most other ionotropic GABA receptors that are permeable to chloride ions, EXP-1 is mainly selective for sodium ions (Beg and Jorgensen, 2003). Thus EXP-1 is the downstream receptor for the expulsion-inducing GABA signals during the defecation cycle.

1.4. Intestines in the Defecation Regulation

In contrast to the studies on GABAergic neurons, the disclosure of the intestine as a regulator of the nematode defecation motor program is less straightforward. One of the first insights into the involvement of the intestine in the defecation regulation came from

the study on oscillatory intestinal Ca^{2+} waves during rhythmic defecation cycles (Fig 1C). The *C. elegans* intestine is a tube-like structure that is made up of single-layer epithelial cells joined by gap junctions (McGhee, 2007; Peters et al., 2007; Sulston et al., 1983). Using Ca^{2+} sensors that detect intracellular Ca^{2+} concentrations in the intestine, several groups reported the occurrence of periodic Ca^{2+} spikes in the posterior intestine immediately prior to pBoc, the first defecation muscle contraction in the worm (Fig 1C) (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006). Further analysis showed that the posterior Ca^{2+} wave propagates anteriorly throughout the intestine, and the timing of Ca^{2+} peaks at the anterior intestine correlates well with that of aBoc, the second defecation muscle contraction (Fig 1C) (Teramoto and Iwasaki, 2006). Interestingly, an intracellular Ca^{2+} channel inositol trisphosphate (IP3) receptor ITR-1 and a gap junction subunit INX-16 are shown to be required for mediating the Ca^{2+} wave propagation. In the absence of functional ITR-1 or INX-16, the propagation of Ca^{2+} waves in the intestine is slowed down or eliminated, resulting in reduced or abolished aBoc and Exp (Dal Santo et al., 1999; Peters et al., 2007). This is similar to the observation in the worms where aBoc and Exp are disrupted by the intestinal application of IP3 receptor inhibitor heparin during the Ca^{2+} wave propagation (Teramoto and Iwasaki, 2006). Importantly, intestinal Ca^{2+} waves persist in the AVL- and DVB-neurons-ablated worms, even though these animals exhibit strong aBoc and Exp defects (Teramoto and Iwasaki, 2006). This suggests that the intestinal Ca^{2+} oscillation is not only crucial for the execution of defecation related muscle contractions, but it also precedes the AVL and DVB

GABAergic neurons controlled muscle contractions, the expulsion, during defecation cycles.

Then how does the intestine, a non-neuronal tissue, regulate neuron-controlled cellular processes in *C. elegans*? The recent interesting discovery of the intestine as a proton gradient generator and the activation of the posterior body wall muscle by H^+ may give us some hints. Using molecular genetics and physiological approaches, Beg et al. showed that the nematode intestine is able to acidify the pseudocoelom (the worm body cavity to which body wall muscles are exposed) by pumping protons out of intestinal epithelial cells via a Na^+/H^+ exchanger (Beg et al., 2008). The body wall muscles express a H^+ gated ion channel, and when the proton gets to the threshold concentration, it activates posterior body wall muscles and triggers pBoc (Beg et al., 2008). Interestingly, the H^+ gradient is generated in a cyclic pattern that coincides with defecation cycles (Beg et al., 2008). This indicates that the intestine might utilize a diffusible signal to control cellular processes in other tissues. This hypothesis is further supported by the observation that Ca^{2+} oscillations are normally associated with electric excitation and exocytosis events in excitable cells (such as neurons). In the following chapter (Chapter 2), I am going to present evidence to show that this is indeed the case.

1.5. *aex* Genes in the Defecation Regulation

To understand the molecular basis of the defecation behavior, in 1990 James Thomas performed a mutagenesis screen using the chemical mutagen ethyl methane sulfonate (EMS) to look for mutant worms that had altered defecation motor programs

(Thomas, 1990). He identified a variety of defecation mutants that either have one or more coordinated muscle contractions missing or have the cycle time altered (Fig 2). Based on the specific muscle contractions affected by the mutation, the defecation mutants were classified into 4 groups: *pbo* mutants, which have weak or missing pBoc; *abo* mutants, which have defective aBoc; *exp* mutants, which have normal pBoc and aBoc but frequently miss Exp; and *aex* mutants, which have defective aBoc and Exp (Fig 2) (Thomas, 1990). Other defecation mutants that have either prolonged or shorted defecation period are designated as *dec* mutants, while to a large extent they have the three muscle contractions unaffected and intact (Fig 2) (Thomas, 1990). The subsequent studies on these mutated genes have led to discoveries of several novel mechanisms that underlie tissue communications and behavior regulation. This includes the aforementioned excitatory GABA ionotropic receptor EXP-1 in regulating enteric muscle contractions (Beg and Jorgensen, 2003) and the intestinal Na^+/H^+ exchanger PBO-4 in establishing the proton gradient that activates posterior body wall muscles (Beg et al., 2008). As an additional example, the intestinal IP3 receptor ITR-1 is encoded by *dec-4*, and the extended defecation period in the *itr-1*/*dec-4* mutant results from the frequent absence of Ca^{2+} waves in the intestine (Dal Santo et al., 1999). Together, these studies show that molecular genetics are a powerful means to dissect not only the molecular basis but also the cellular basis underlying behaviors.

Among all the defecation mutants isolated, we are particularly interested in *aex* mutants because the *aex* animal exhibits aBoc and Exp defects very similar to those of AVL- and DVB-GABAergic neuron-killed worms (Liu and Thomas, 1994; McIntire et

al., 1993b; Thomas, 1990). This suggests that by studying these genes, we may be able to gain valuable insights into the molecular mechanisms that control GABAergic neuron activation during the defecation cycle. The *aex* gene family consists of 6 members, from *aex-1* to *aex-6* (Thomas, 1990). Prior to our studies, only *aex-1*, *aex-3* and *aex-6* have been cloned and studied in details, while the molecular identities and functions of *aex-2*, *aex-4* and *aex-5* remained unclear (although there are limited data on *aex-5* showing it encodes a proprotein convertase). The *aex-1* gene encodes an exocytic factor that is homologous to MUNC-13 and evidence suggests it functions in the intestine to regulate enteric muscle contractions (i.e. Exp) during the defecation (Doi and Iwasaki, 2002). In contrast, *aex-3* and *aex-6* encode a guanine exchange factor (GEF) and small GTPase RAB-27, respectively, and both are widely expressed in neurons (Iwasaki et al., 1997; Mahoney et al., 2006). Genetic, biochemical and physiological analyses showed that AEX-3 acts as an AEX-6/RAB-27 GEF to activate AEX-6/RAB-27, and both proteins are required for the normal presynaptic transmission in the nervous system (Iwasaki et al., 1997; Mahoney et al., 2006). Therefore, the studies on these *aex* genes support the notion that both intestines and neurons are involved in executing the defecation motor program. Nevertheless, the signal(s) involved in the neuronal regulation and its receptor(s), if there is any, need to be identified. This prompts us to study the uncharacterized or less well characterized *aex* genes *aex-2*, *aex-4*, and *aex-5* to see how different *aex* genes may regulate the defecation behavior in a coordinated manner.

1.6. Overview of Chapter 2: Intestinal Signaling to GABAergic Neurons Regulates A Rhythmic Behavior in *Caenorhabditis elegans*

In Chapter 2, we describe our work on *aex-2*, *aex-4* and *aex-5* genes and we present our model whereby intestinal *aex* genes regulate GABAergic neuron activation during the defecation by controlling the release of intestinal peptidergic signals. Using molecular genetics and cell biology approaches, we demonstrate that *aex-4* encodes an exocytic protein SNAP25 homologue and it is expressed and functions in the intestine. The proprotein convertase AEX-5 functions in the intestine, too; and when expressed in the intestine it is secreted into the pseudocoelom in an AEX-4-dependent manner. Moreover, we show that *aex-2* encodes a G-protein coupled receptor (GPCR) and it is expressed and functions in GABAergic neurons to regulate the defecation behavior. Epistatic analysis reveals that *aex-2* is genetically downstream of intestinal *aex-4* and *aex-5*, and the stimulatory Gα pathway relays the AEX-2 signal in GABAergic neurons. Together, our results provide evidence that the *C. elegans* intestine functions as an endocrine organ to regulate neuronal functions and behaviors.

2. The Modulation of Neuronal Functions

2.1. Neurons and Plasticity

The nervous system probably is the system that experiences the most dramatic changes in morphologies and functions in an organism throughout its life: from massive synaptogenesis during early development to the learning and memory triggered synaptic

alterations in the adulthood. To adapt to these changes and to ensure neurons constantly generate proper electric signals in response to environmental stimuli, neurons must develop mechanisms to stabilize their electric gains (Turrigiano and Nelson, 2000; Turrigiano and Nelson, 2004). This cellular process is known as neural plasticity. In general, the neural plasticity could lie in morphological changes or functional changes in the nervous system. In following sections, I will mainly focus on the plasticity of neuronal functions and I will discuss the current understanding of molecules involved in modulating synaptic transmission in the nervous system.

2.2. Local and Global Plasticity

The plasticity that regulates synaptic strength could take place at different spatial scales, either locally or globally, and both play important roles in developing and adult nervous systems. One of the extensively studied mechanisms of local plasticity is the Hebbian plasticity, where the pre- and postsynaptic partners that are activated together get wired together, while a non-correlated activation results in the synaptic depression (Fig 3A) (Bi and Poo, 2001; Turrigiano and Nelson, 2000). In the developing nervous system, the Hebbian plasticity plays a fundamental role in shaping neuronal connections. This is especially important for higher animals such as mammals, as in these organisms the developing neurons frequently over-sprout and innervate incorrect targets (Bi and Poo, 2001). Furthermore, the Hebbian plasticity also forms the basis for long-term potentiation (LTP) and long-term depression (LTD), suggesting it is likely involved in higher brain functions such as learning and memory (Bi and Poo, 2001). Therefore, the

local plastic regulation of synaptic strength provides an important mechanism to selectively refine neural connections during the development and to encode synapse-specific information in neural networks.

In contrast to local plasticity, global plasticity involves the global modulation of a large number of synapses in neural networks. One of the well-characterized global plastic mechanisms is the homeostatic plasticity, which was first described in neuronal cultures where neural activities are constantly elevated or dampened by drug treatments. Over prolonged time windows, the homeostatic mechanism scales neural activities in the opposite direction to that induced by the drug treatment, and it re-stabilizes the global neural firing rate (Fig 3B) (Turrigiano and Nelson, 2004). Interestingly, careful physiological analysis disclosed that the strength of all excitatory synapses on a single neuron is modulated in a proportional manner, as the cumulative plot of AMPA type glutamate receptor mediated miniature excitatory postsynaptic currents (mEPSC) form a continuous shifted distribution (Fig 3B, 3C) (Turrigiano and Nelson, 2004). These observations suggest that the global homeostatic plasticity is important for the nervous system development and functions in at least two ways: 1) It helps stabilize neuronal gains in specified neural networks. During neural development, the rapid increase in synapse numbers and the selective facilitation of stronger connections by the Hebbian plasticity potentially de-stabilize the nervous system. With the homeostatic mechanism, neurons are able to globally scale their synaptic strength in proper directions to compensate the change in neuronal gains brought by the synaptogenesis and local wirings, ensuring they are in the optimal range to respond to stimuli inputs; 2) It

preserves the synaptic codes introduced by the Hebbian plasticity. With the multiplicative scaling during the homeostatic plasticity, neurons are able to maintain the differences in synaptic strength that are introduced by the Hebbian mechanism, thus making the information storage at synapses possible (Turrigiano and Nelson, 2004).

To summarize, the nervous system adopts both local and global plastic mechanisms to modulate neuronal functions. While local plasticity (like the Hebbian plasticity) selectively shapes synaptic strength and is likely involved in information coding, global plasticity (such as the homeostatic plasticity) acts to maintain system stability in neural networks. It is worth noting that people have identified new forms of synaptic plasticity in recent years, including synapse-specific homeostatic plasticity and anti-homeostatic plasticity in both cultured and physiologically relevant neuronal preparations (Carrasco et al., 2007; Kim and Tsien, 2008; Moulder et al., 2006). Nevertheless, in terms of local and global mechanisms, based on our understandings so far the Hebbian and homeostatic plasticity probably represents two essential and best-characterized forms of plasticity in the nervous system.

2.3. Molecules Involved in Synaptic Plasticity

A great variety of molecules have been identified in the past decade to play roles in modulating synaptic functions, from voltage-gated ion channels (Beck and Yaari, 2008; Catterall and Few, 2008; Wang, 2008), ionotropic and metabotropic neurotransmitter receptors (Cho and Bashir, 2002; Malinow and Malenka, 2002; Turrigiano, 2008), scaffolding proteins (Renner et al., 2008), signaling enzymes (such as

kinases and ubiquitin ligases) (Cho and Bashir, 2002; Schwartz, 2003; Wang, 2008), transcription factors (Alberini, 2009), to proteins involved in transcriptional and translational regulation (Alberini, 2009; Richter and Klann, 2009). Among them, membrane bound molecules and secretory molecules are two types of molecules of great interest. This is mainly because they are able to act non-cell-autonomously on neighboring synaptic partners or distant cells to modulate their functions (Fig 4). In addition, since their expressions / secretions are frequently dependent on synaptic activities (as discussed below), they provide an important feedback mechanism to modulate synaptic strength in both developing and adult nervous systems (Tao and Poo, 2001). In following sections, I will briefly review examples of cell adhesion molecules as well as secretory molecules that are extensively studied and are established for their roles in synaptic plasticity.

2.3.1. Cell Adhesion Molecules in Synaptic Modulations

2.3.1.1. Cadherins

Cadherins are a class of cell adhesion molecules that is composed of over 80 members and is divided into several subfamilies (Yagi and Takeichi, 2000). The extensively studied 'classic' cadherins, including N- (neural) and E- (epithelial) cadherins, are one of the subclasses that share a common molecular structure including 5 extracellular tandem repeats, a single transmembrane domain, and a cytoplasmic tail (Yagi and Takeichi, 2000). Cadherins are able to form homophilic complexes in a Ca^{2+} dependent manner. In the developing nervous system, N- and E-cadherins are initially

diffusely expressed at synapses, whereas in mature synapses they become clustered at transmitter-release zones (such as peri-active regions) at apposed synaptic sides (Elste and Benson, 2006; Rubio et al., 2005; Uchida et al., 1996). This suggests that cadherins may be involved in modulating synaptic properties, like synaptic plasticity.

In line with this hypothesis, a collection of studies showed that cadherins are involved in modulating synaptic functions and plasticity. Interestingly, cadherins likely function as sensors of synaptic activity, and several lines of evidence suggest that cadherins modulate synaptic plasticity through trans-synaptic signaling (Arikkath and Reichardt, 2008; Gottmann, 2008; Tai et al., 2008). The extracellular adhesion domains of cadherins bind Ca^{2+} and adopt different conformations based on extracellular Ca^{2+} concentrations (Boggon et al., 2002; Heupel et al., 2008; Pertz et al., 1999). At high Ca^{2+} levels, the adhesive ectodomains form a rigid rod-like structure that is required for trans- (on opposite cells) or cis- (on the same cell) cadherin adhesions, whereas low Ca^{2+} concentrations loosen up adhesive domains and dissociate cadherin dimers (Heupel et al., 2008; Pertz et al., 1999). In cultured hippocampal neurons, disrupting N- or E-cadherin function by blocking peptides blocks the induction of long-term potentiation (LTP) (Tang et al., 1998). Interestingly, blocking peptides interfere with LTP only when neurons are repeatedly depolarized and the extracellular Ca^{2+} is lowered (and presumably cadherins are open for peptide binding), while supplementing blocking peptides at resting state or after LTP is established has no effects (Tang et al., 1998). This suggests that cadherins are dynamically regulated by synaptic activity, and this may underlie their contributions to synaptic plasticity. Further analyses revealed that cadherins modulate synaptic

functions by a trans-synaptic mechanism. In rat hippocampal cultures and at fly neuromuscular junctions, the overexpression or knockout of β-catenin, a cytoplasmic signaling protein that interacts with cadherins, at postsynapses induces morphological and functional changes in presynaptic sides (Li et al., 2008; Murase et al., 2002). Moreover, when the postsynapse is deprived of N-cadherin by using either RNAi-mediated gene silencing or using an N-cadherin-knockout mice embryonic-stem-cell-derived neuron line, defects are observed in presynaptic neurotransmissions (Jungling et al., 2006; Saglietti et al., 2007). Together, these observations strongly suggest that cadherins modulate synaptic functions and plasticity, and they function through a trans-synaptic mechanism that is probably mediated through trans-interactions between cadherins on apposing synapses.

N- and E-cadherins probably represent the best-characterized cadherin molecules in the cadherin superfamily. In the nervous system, central neurons also express other types of cadherins, while their functional roles in modulating synaptic functions and plasticity are less extensively explored (Arikkath and Reichardt, 2008). As currently much is still unknown about the signaling components downstream of cadherins, in the near future the focus will be to elucidate the molecular mechanisms underlying cadherin-mediated changes in synaptic plasticity.

2.3.1.2. Neurexins / Neuroligins

Neurexins and neuroligins are types of Ca^{2+} dependent cell adhesion molecules. In mammals, there are three neurexin genes and at least three (humans have five)

neuroligin genes, and both molecular families possess an extracellular domain of varying length, a single transmembrane domain, and a single cytoplasmic tail followed by a protein-protein interaction PDZ domain-binding site at the C-terminus (Craig and Kang, 2007; Dalva et al., 2007). Although limited in numbers of genes, neurexins and neuroligins have their transcripts under intensive splicing processing, resulting in a surprisingly large number of splice isoforms that may help generate cell specificity for their functions (Ichtchenko et al., 1996; Ullrich et al., 1995). Both neurexins and neuroligins are highly enriched in the central nervous system (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Ullrich et al., 1995). Antibody labeling reveals that neurexins localize to the presynaptic terminus, consistent with its roles in α-latrotoxin (a component in the black widow spider venom) binding and the toxin-induced massive neurotransmitter release (Dean et al., 2003; Graf et al., 2004; Ushkaryov et al., 1992). In contrast, neuroligins interact with a battery of postsynaptic density proteins, and neuroligin-1 and -2 localize specifically to the excitatory and inhibitory postsynapses, respectively (Craig and Kang, 2007; Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004). This indicates that neurexins and neuroligins may regulate synaptic development and function through trans-synaptic interactions.

So far, the function of neurexins and neuroligins in synapse formation and structure regulation has been extensively studied (Craig and Kang, 2007; Dalva et al., 2007). Intriguingly, emerging evidence suggested that neurexins and neuroligins could also modulate synaptic functions and plasticity in mature neurons (Gottmann, 2008; Huang and Scheiffele, 2008). In cultured hippocampal slices, overexpressing neuroligin-

1 in excitatory postsynapses induces alterations in vesicle release probability and short-term plasticity at presynaptic sides (Futai et al., 2007). This effect is likely mediated through trans-synaptic interactions between neuroligins and neurexins, as blocking neurexin functions in the presynapse by a dominant-negative form of neurexin induces decreases in the release probability similar to that induced by postsynaptic downregulation of neuroligin-1 (Futai et al., 2007). In addition, overexpression of neuroligin-2 at hippocampal inhibitory postsynapses selectively enhances the amplitude of miniature inhibitory postsynaptic currents (mIPSC), and this effect appears to depend on synaptic activities as the pharmacological blockade of network firing eliminates this phenomenon (Chubykin et al., 2007). Currently, it remains largely unknown how neurexins and neuroligins may modulate synaptic transmissions through trans-synaptic signaling, and people are actively exploring their downstream molecules. It would be interesting to examine the molecular mechanisms that underlie the neurexin-neuroligin mediated synaptic plasticity and see if synaptic activity plays a role in this process.

2.3.1.3. NCAM

Neural cell adhesion molecule (NCAM) is a member of the immunoglobulin (Ig) superfamily that contains variable numbers of extracellular globular cysteine-looped domains (Vaughn and Bjorkman, 1996). In mammals, a single NCAM gene encodes three alternatively spliced NCAM isoforms, and they contain five IgG domains as well as two fibronectin III (FNIII) domains in the extracellular region, followed by a single membrane-spanning region and isoform specific cytoplasmic tails (Cunningham et al.,

1987; Vaughn and Bjorkman, 1996). In the developing nervous system, NCAM expression is localized to synaptic regions (Aaron and Chesselet, 1989; Szele et al., 1994; Uryu et al., 1999). Overexpressing NCAM in cultured neurons induces preferable formation of synapses in transfected cells, and similar phenomenon is observed in wild-type neurons co-cultured with $NCAM^{-/-}$ cells (Dityatev et al., 2000; Dityatev et al., 2004). Thus NCAM likely possesses synaptogenic activities in early developing neurons.

Other than its role in early synapse development, a number of studies disclosed that NCAM actively participates in modulating synaptic functions (Bisaz et al., 2009; Dalva et al., 2007). One well-characterized role of NCAM in synaptic plasticity is its involvement in the induction of long-term potentiation (LTP). Applying antibodies or synthetic peptides that block NCAM mediated adhesion blocks LTP formation in hippocampal CA1 regions, while the basal transmissions remain unaltered (Luthl et al., 1994; Ronn et al., 1995). Consistent with the observations, deletion of NCAM in NCAM knockout mice results in impaired LTP induction in hippocampal CA1 and CA3 regions (Cremer et al., 1998; Muller et al., 2000). Interestingly, the modulation of NCAM in experimental animals triggers a series of behavioral changes including cognitive impairment and emotional alterations (Bisaz et al., 2009). This suggests that an appropriate NCAM level in the nervous system is required for maintaining normal behaviors.

The NCAM molecule undergoes a post-translational modification known as polysialylation, with chains of polysialic-acid (PSA) being attached to its glycosol groups (Finne et al., 1983). This is probably the most important modification identified for

NCAM, as many lines of evidence suggest that PSA-NCAM is responsible for numerous NCAM-mediated effects on synaptic functions (Bisaz et al., 2009; Dalva et al., 2007). For example, blocking antibodies that inhibit PSA function or enzymes that remove PSA from PSA-NCAM prevent LTP and LTD formation in hippocampus (Becker et al., 1996; Muller et al., 1996). Moreover, when polysialyltransferase, the enzyme that adds PSA to NCAM, is deleted, hippocampal regions in knockout mice show specific loss of PSA-NCAM expressions and both LTP and LTD (but not basal transmissions) are impaired (Eckhardt et al., 2000). As PSA weakens homo- and heterophilic NCAM interactions (Rutishauser, 1996), one possible mechanism that may underlie PSA's function is that PSA weakens NCAM mediated synaptic adhesions to allow plastic modifications. The clarification of signaling events that lie downstream of PSA-NCAM mediated synaptic plasticity will be the future's focus.

2.3.1.4. Eph Receptors / Ephrins

Eph receptors comprise by far the largest known receptor tyrosine kinase (RTK) family, with 9 EphA and 5 EphB members identified so far (Flanagan and Vanderhaeghen, 1998; Klein, 2009). All Eph receptors are single membrane-spanning molecules, which contain an extracellular region that bares cysteine-rich and fibronectin III domains and an intracellular tail that bares the protein tyrosine kinase domain (Flanagan and Vanderhaeghen, 1998). Like Eph receptors, ephrins are all membrane-associated, while they are classified into A and B subtypes based on the presence of either a glycosylphosphatidylinositol (GPI) moiety (ephrinA) or a single transmembrane

domain (ephrinB) (Flanagan and Vanderhaeghen, 1998; Klein, 2009). All Eph receptors preferably bind to ephrins of their own class (A to A, B to B), whereas EphA4 serves as an exception to bind both ephrinA and ephrinB subclasses (Flanagan and Vanderhaeghen, 1998). The high expression level of Eph receptors and ephrins in the developing nervous system as well as their localization at the synapse is consistent with their functions in synapse development (Buchert et al., 1999; Dalva et al., 2000; Torres et al., 1998).

One noticeable feature of Ephs and ephrins is their ability to initiate bidirectional signaling upon the receptor-ligand binding by triggering signaling events such as tyrosine phosphorylation on both cytoplasmic tails (Kullander and Klein, 2002). In the developing nervous system, extensive studies have established functional roles of the bidirectional signaling in axon pathfinding, cell boundary formation, and synaptogenesis, and multiple lines of evidence suggested that such signaling also underlies the Eph-ephrin mediated modulation of synaptic plasticity in mature neurons (Dalva et al., 2007; Klein, 2009; Kullander and Klein, 2002). One of the well-studied examples is the hippocampal mossy fiber-CA3 connection, where both EphB2 and ephrinB ligands are highly enriched at synapses (Armstrong et al., 2006; Grunwald et al., 2001). When blocking peptides and soluble ephrinBs are used to disrupt the postsynaptic EphB2-PDZ protein interactions and the cross-synaptic EphB2-ephrinB interactions, respectively, the induction of LTP in the presynaptic mossy fibers is prevented (Contractor et al., 2002). Interestingly, removing the cytoplasmic tail from ephrinBs blocks LTP formation in the same region, indicating that postsynaptic EphB receptors regulate synaptic plasticity through a presynaptic signaling cascade that is dependent on ephrinB cytoplasmic tails (Armstrong

et al., 2006). Similar involvements of Eph and ephrins in synaptic plasticity have also been documented in hippocampal CA1 regions, whereas in CA1 ephrinBs act in the postsynapse rather than in the presynapse to modulate the plasticity (Grunwald et al., 2004). Together, these studies strongly support that Ephs and ephrins play important roles during the plastic modulation of synaptic functions, and they likely mediate the modulation through a trans-synaptic mechanism. A careful examination of the molecular pathways downstream of the bidirectional Eph-ephrin signaling will shed light onto the molecular mechanisms that underlie the trans-synaptic plasticity.

2.3.2. Secreted Molecules in Synaptic Modulations

2.3.2.1. Neurotrophins

Neurotrophins are a family of secreted proteins that were originally identified for their functions in supporting nerve growth. The mammals encode at least four neurotrophins in the genome, including the founding member nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Lewin and Barde, 1996). In cells, neurotrophins are synthesized as precursors, and they are processed by proteolytic cleavage before being secreted as dimers (Lewin and Barde, 1996; Seidah et al., 1996). The expression of neurotrophins is not only widely detected in the nervous system, but also in targets of innervating peripheral neurons, suggesting a broad impact neurotrophins have on developing nervous systems (Lewin and Barde, 1996; Snider, 1994).

The involvement of neurotrophins in modulating synaptic functions was first proposed by the neurotrophin hypothesis, which states that neurotrophins participate in activity-induced modification of synaptic transmissions (Schinder and Poo, 2000). Indeed, extensive studies revealed that the expression of neurotrophins in mature neurons is regulated by neuronal activities. In cultured rat hippocampal slices, repeated activation of neurons upregulates mRNA levels for both NGF and BDNF, whereas blocking the network electric activity by drug treatment or GABA neuron stimulation decreases NGF and BDNF transcripts (Ernfors et al., 1991; Gall and Isackson, 1989; Zafra et al., 1991; Zafra et al., 1990). In addition, depolarization triggers the release of BDNF from cultured neurons, suggesting BDNF is secreted in an activity-dependent manner (Mowla et al., 1999). The role of neurotrophins in synaptic plasticity is subsequently demonstrated by a series of LTP studies in hippocampus. During LTP induction in cultured hippocampal slices, the removal of endogenous BDNF by antibodies or soluble TrkB-IgG (TrkB is the BDNF receptor and binds BDNF) impairs LTP formation (Chen et al., 1999; Figurov et al., 1996; Kang et al., 1997). This is supported by genetic studies where the deletion of BDNF gene results in disrupted LTP in hippocampus (Korte et al., 1995; Patterson et al., 1996). Interestingly, BDNF appears to act as a retrograde trans-synaptic signal, as its functions on LTP induction are mediated through presynaptic, but not postsynaptic, TrkB receptors (Li et al., 1998; Xu et al., 2000). These studies, together with the work on the direct enhancement of presynaptic transmissions by neurotrophins, provided a strong support for the neurotrophin hypothesis (Schinder and Poo, 2000). It will be interesting to determine if neurotrophins play a more instructive or a more

permissive role in synaptic plasticity by examining their downstream signaling components.

2.3.2.2. Nitric Oxide

Nitric oxide (NO) belongs to a group of membrane permeable molecules that include nitric monoxide (NO), carbon monoxide (CO), arachidonic acid (AA), and platelet-activating factor (PAF) whose roles in synaptic plasticity has been established or suggested by a number of studies (Fitzsimonds and Poo, 1998; Hawkins et al., 1998). In mammals, NO is synthesized from L-arginine by three different NO synthase isoforms (i.e. neuronal nNOS, endothelial eNOS, and inducible iNOS), and in the nervous system several lines of evidence suggest that NO is released from synthesizing neurons in an activity-dependent manner (Dawson and Snyder, 1994; Tao and Poo, 2001). For example, in cerebellar cells, activating NMDA-type glutamate receptors induces a Ca^{2+}-dependent release of NO (Garthwaite et al., 1988; Garthwaite et al., 1989). In a more recent study, antibody staining reveals the localization of nNOS in the postsynaptic cytoplasm of excitatory auditory synapses, and the stimulation of neuronal activity elicits NO release (Steinert et al., 2008). The role of NO in modulating synaptic functions has been demonstrated by a collection of studies, and the experiments on the hippocampal LTP are of the most interest, as these studies strongly suggest NO modulates synaptic plasticity through a retrograde mechanism. In hippocampal cultures, bath application of NO scavenger hemoglobin blocks LTP formation in the CA1 region (O'Dell et al., 1991; Schuman and Madison, 1991). The NO generated during LTP appears to be released

from postsynapses, as the extracellular application of oxymyoglobin (another NO scavenger) inhibits the LTP induction in cultures that are postsynaptically injected with NO donors, while it fails to block LTP when NO donors are supplied presynaptically (Arancio et al., 1996). Interestingly, injecting nNOS inhibitors into postsynapses specifically prevents LTP, suggesting NO is synthesized in postsynaptic cells and is released as a retrograde messenger to modulate presynapses during LTP induction (Arancio et al., 1996; O'Dell et al., 1991; Schuman and Madison, 1991). Together, with other studies on CO, AA, and PAF (Fitzsimonds and Poo, 1998), these observations strongly suggest that the nervous system utilizes small membrane permeable molecules to modulate synaptic functions.

2.3.2.3. Other Diffusible Molecules Involved in Synaptic Modulations

So far, all the molecules discussed have established or suggested roles in long-term synaptic plasticity (i.e. LTP / LTD), which is of special interest to neuroscientists as LTP / LTD is thought to underlie the information coding process during learning and memory. Nevertheless, synaptic transmission is a delicate cellular process that is under precise spatial and temporal regulation. This means that synaptic modulations require the functions of a complex of proteins, which include, but are not limited to, the molecular families discussed above. There are examples of other diffusible factors that are involved in modulating synaptic transmissions, including endocannabinoids (Harkany et al., 2008), small nucleotides (Barnstable et al., 2004; Pankratov et al., 2009), lipids (Yang and Chen, 2008), and unexpectedly, reactive oxygen species (ROS) (Kamsler and Segal, 2004).

With the fast progressions of scientific researches today, it will not be surprising to see more molecular players in the modulation of synaptic transmissions and higher brain functions in the near future.

2.4. Open Questions and Hormonal Signaling

Although great progress has been made in the past decade, many questions still remain unanswered in the field of synaptic modulations. One interesting question that needs to be elucidated is: what are the molecules that globally modulate synaptic functions? As discussed earlier, the nervous system has local plasticity and global plasticity, both of which need to be tightly regulated. Within the two types of trans-synaptic signals discussed, cell adhesion molecules are able to induce non-cell autonomous changes in neighboring synapses, thus transmitting information to other neurons (Fig 4). However, due to their associations with the plasma membrane, their actions are limited to local neural connections. Secretory molecules, on the other hand, are diffusible in nature and can signal to more distant cells from the releasing site (Fig 4). However, as synaptically secreted molecules are often not at high concentrations under physiological conditions, and as the extracellular matrix (ECM) frequently immobilizes molecules after their secretion, the molecules secreted from a population of neurons are unlikely to act on a global scale to affect the whole neural network. Therefore, the nervous system must have adopted other mechanisms to realize the global modulation.

Three mechanisms, including experience-based stimuli (which activate coordinated neural networks), intracellular signaling (such as intracellular Ca^{2+}

signaling), and hormonal signaling (which is often carried by the circulation system), are likely involved, as all three can transmit signals across a large number of synapses. Interestingly, recent studies on several non-neuronal derived hormonal factors suggest they likely play roles in modulating synaptic functions and plasticity in central neurons (McNay, 2007; Moult and Harvey, 2008). For example, the adipose cell derived hormonal factor leptin is able to cross the blood brain barrier, and the expression of leptin receptors is detected in many brain regions including hippocampus (Moult and Harvey, 2008). Deleting leptin receptor gene in knockout mice impairs hippocampal LTP and LTD induction, whereas supplying leptin to hippocampal slice cultures and live animals facilitates LTP formation and memory retention in learning-related tasks (Farr et al., 2006; Li et al., 2002; Oomura et al., 2006). Similarly, the 6KD hormone insulin that is mainly synthesized in and secreted from pancreatic β-cells can also cross the blood brain barrier, and it modulates hippocampal long-term plasticity by acting on insulin receptors, whose expressions in many brain regions have been well-characterized (McNay, 2007; Moult and Harvey, 2008). One prominent feature of hormonal signaling mediated by circulating hormonal factors is their potential ability to globally modulate nervous system functions. In addition, as many hormonal factors are synthesized in non-neuronal tissues, their signaling provides an important approach by which central neurons and peripheral tissues can communicate. This has been well demonstrated by the modulatory effects of leptin and insulin on animal behaviors (McNay, 2007; Moult and Harvey, 2008). In the future, the identification of other synaptic modulating hormonal factors will provide

additional insights into the nervous system function and its ability to maintain homeostasis through plastic modulations.

2.5. The *C. elegans* Intestine As An Endocrine Organ

In *C. elegans*, the intestine serves as an important organ to regulate multiple biological processes such as food digestion, defecation, stress response, and host-pathogen interactions (McGhee, 2007). Composed of polarized epithelial cells, the intestine is involved in intensive cross-membrane trafficking, and a number of exocytic and endocytic factors have been shown expressed and functioning in the intestine (Fig 5) (Chen et al., 2006; Doi and Iwasaki, 2002; Mahoney et al., 2006; Parker et al., 2009; Yamashita et al., 2009). Interestingly, several recent observations suggest that the intestine may secret signals to modulate neuronal functions. For example, during rhythmic defecation cycles, a Ca^{2+} wave is initiated near the posterior end of the intestine and it propagates through the intestine (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006). Blocking the Ca^{2+} oscillation in the intestine by drugs or by disrupting an intestinal gap junction not only prevents the execution of subsequent neuron-controlled muscle contractions, but also leads to phenotypes indicative of altered synaptic transmissions (Peters et al., 2007; Teramoto and Iwasaki, 2006). Thus, the *C. elegans* intestine may function as an endocrine organ (the organ that secretes hormonal factors) to modulate synaptic functions. It would be interesting to identify such intestine-derived signals, and if there are any, the characterization of their downstream signaling

mechanisms will shed light onto our understanding of neuronal plasticity as well as the interactions between neurons and non-neuronal tissues in multicellular organisms.

2.6. Overview of Chapter 3: Intestine-Derived Signals Regulate Synaptic Transmissions in *Caenorhabditis elegans*

In Chapter 3, I present the identification of an intestinal signaling system that regulates cholinergic neurotransmissions in the *C. elegans*. *C. elegans* utilizes acetylcholine as a neurotransmitter at its neuromuscular junctions (NMJs) to control muscle contractions and locomotion-related behaviors. Using molecular genetics, pharmacological, and physiological approaches, we show that the proprotein convertase AEX-5 is required in the intestine to maintain normal cholinergic transmissions in the nematode. In addition, we find that the GPCR AEX-2 functions in the GABAergic neurons to maintain cholinergic transmission level, and the stimulatory Gα pathway is genetically downstream of AEX-2. Interestingly, we find that although both the defecation motor program and the cholinergic transmission modulation involve intestinal signals and neuronal G-protein pathways, they depend on different downstream molecules: while the defecation requires GABA to activate the enteric muscle contraction, the modulation of cholinergic transmissions partially depends on neuropeptide processing enzymes EGL-3 and EGL-21. As GABAergic neurons do not directly synapse on cholinergic neurons in *C. elegans*, we speculate that the peptide signals act in a hormonal manner on cholinergic neurons. This suggests that the *C.*

elegans intestine could function as an endocrine organ to control rhythmic behaviors as well as to modulate neuronal functions.

3. Synapse Development and Overview of Chapter 4: A Homolog of the LIM Domain Focal Adhesion Protein ZYX-1 Regulates Synaptic Development in *C. elegans*

Besides the studies on the modulations of behaviors and neuronal functions in *C. elegans*, I have also briefly examined the molecular mechanisms that control synaptic development. As a continuation of Dr. Schaefer's project on the molecular genetic analysis of synaptogenesis in the lab (Schaefer, 2001), I have focused on the early development of the *C. elegans* mechanosensory nervous system. In Chapter 4, I present the preliminary evidence from my work on mechanosensory PLM synapses that supports a role of the LIM domain focal adhesion molecule ZYX-1 in the PLM synapse development. We cloned the *zyx-1* allele and demonstrated that ZYX-1 is widely expressed in neurons and non-neuronal tissues such as muscles and spermatheca. Using time-course imaging analysis of fluorescence labeled PLM neurons, we show that *zyx-1* mutants are able to form synapses during early development, while they fail to maintain the synapse to adulthood. In addition, we demonstrate that ZYX-1 acts cell-autonomously in mechanosensory neurons to regulate PLM synapse maintenance. I expect the identification of additional molecular players involved in the ZYX-1 signaling will shed light onto our understanding of synapse development and the maintenance of neuronal functions in mature nervous systems.

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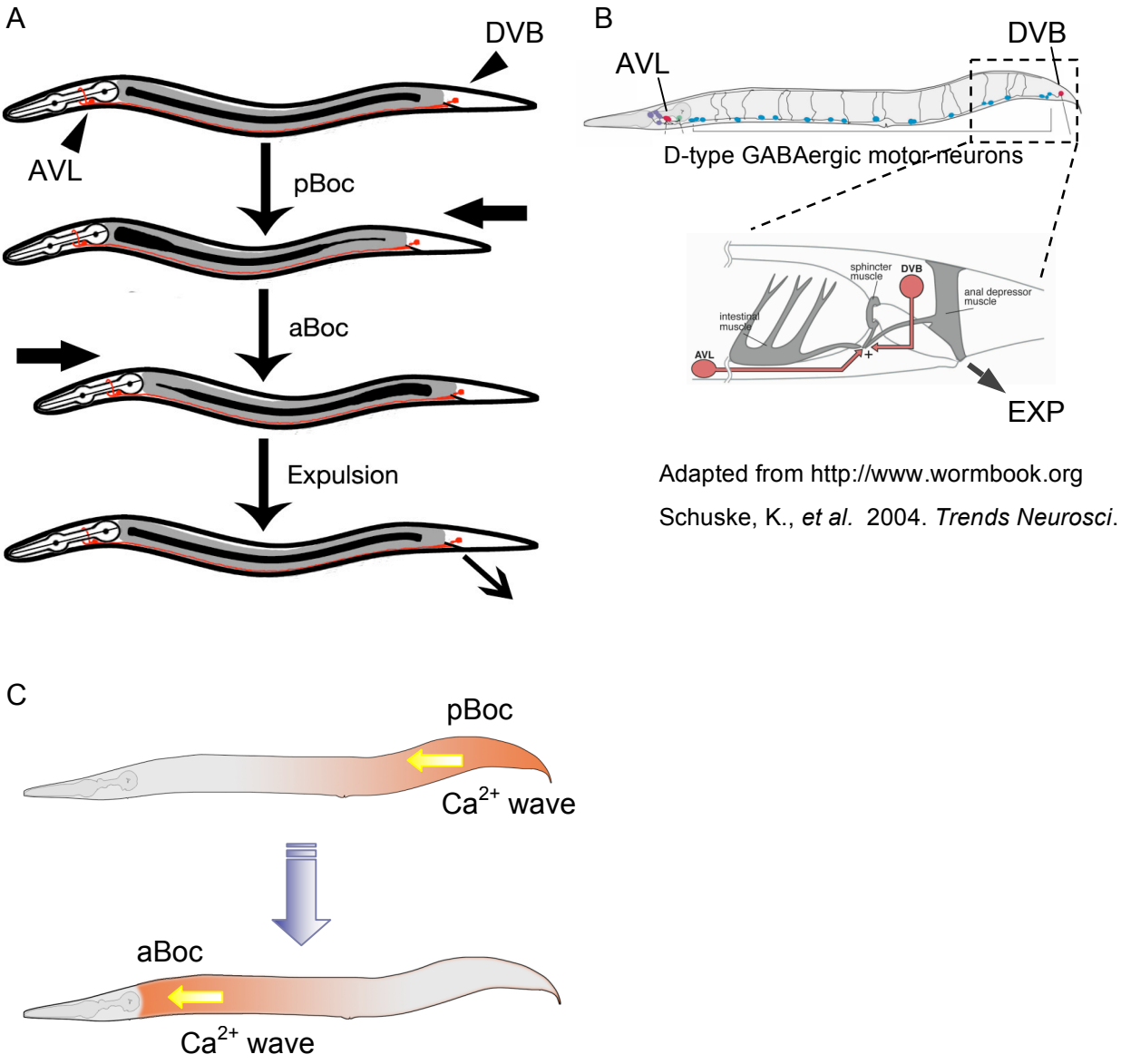
Figures and figure legends

Figure 1. Multiple tissues are involved in regulating the *C. elegans* defecation behavior. A) The *C. elegans* defecation motor program (DMP) consists of three temporally coordinated muscle contractions. In the schematic diagram, the worm head is to the left, and the tail is to the right. The intestine and the intestinal lumen are labeled in grey and black, respectively. Arrowheads denote GABAergic neurons AVL and DVB that control defecation cycles (see text). In feeding animals, the DMP starts with posterior body-wall muscle contractions (pBoc) that push intestinal contents anteriorly. Around 2-3 seconds later, anterior body-wall muscles contract (aBoc), pressurizing intestinal contents at the anus region at the posterior intestine. This is followed almost immediately by enteric muscle contractions (EMC) that open the anus and allow the pressurized intestinal contents being expulsed from the intestine (Exp). The whole cycle repeats itself with little variance in about 45 seconds. B) GABAergic neurons AVL and DVB are required for the execution of DMP. The *C. elegans* GABAergic system consists of 26 GABAergic neurons, with GABAergic motor neurons shown in blue, and AVL and DVB shown in red. The magnified view of the posterior intestine shows the axons of DVB and AVL (red) and enteric muscles (grey) that control the expulsion. The neurotransmitter GABA excites enteric muscles, which leads to muscle contractions and expulsion (see text). C) The rhythmic intestinal Ca^{2+} oscillations are required for the execution of DMP. In the *C. elegans* intestine, the timing of calcium spikes (orange) in posterior and anterior intestines correlates well with that of pBoc and aBoc, and the disruption of Ca^{2+} wave propagation in the intestine eliminates aBoc and expulsion.

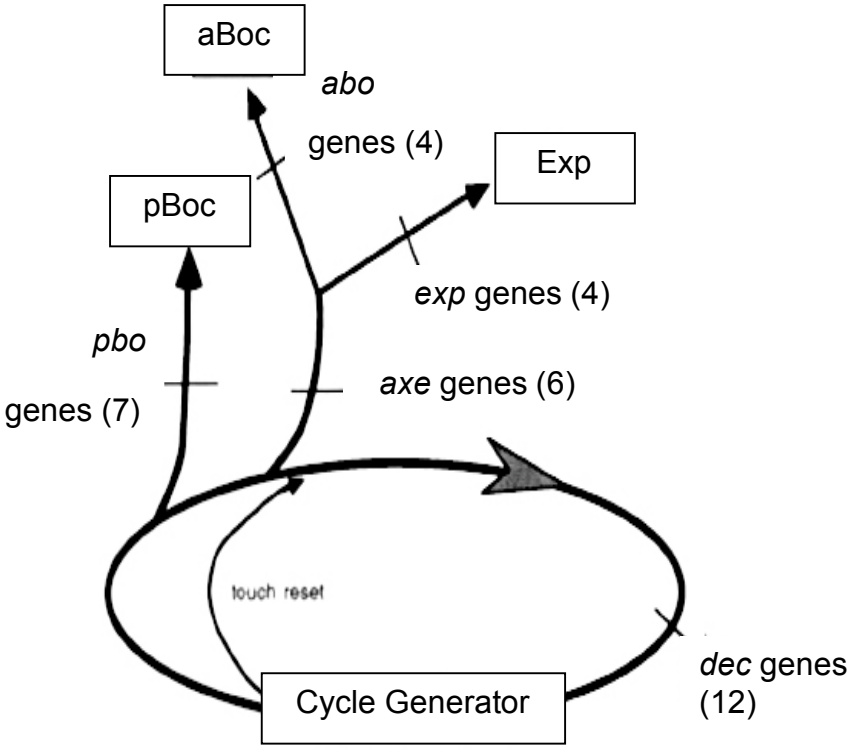
Figure 1. Multiple tissues are involved in regulating the *C. elegans* defecation behavior.



Adapted from http://www.wormbook.org

Schuske, K., *et al.* 2004. *Trends Neurosci*.

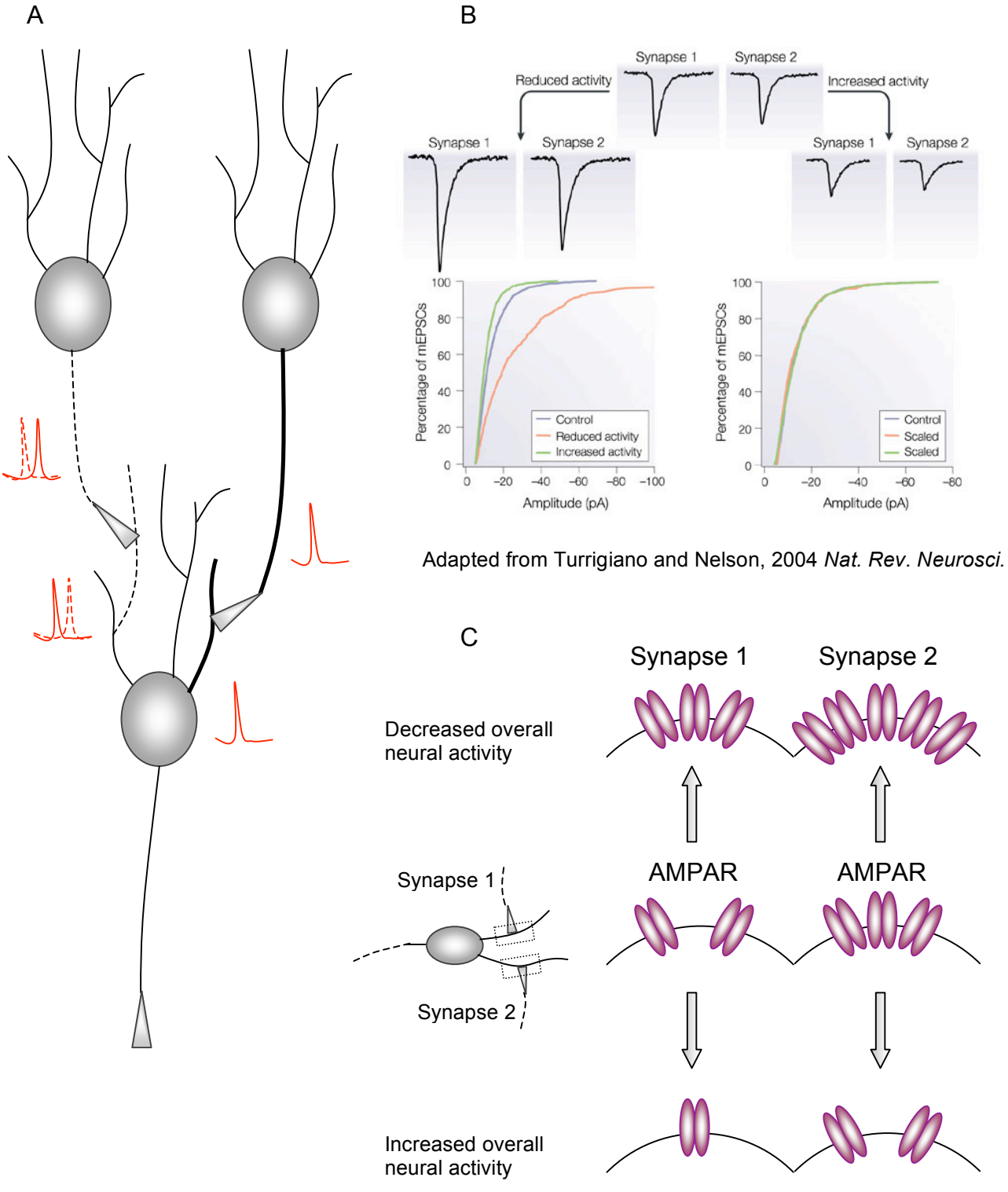
Figure 2. Multiple genes are involved in regulating the *C. elegans* defecation behavior. The mutagenesis screen that looked for altered defecation behaviors in *C. elegans* has identified 5 classes of genes that regulate different steps of DMP. They are: *pbo* (for the regulation of pBoc), *abo* (for the regulation of aBoc), *exp* (for the regulation of expulsion), *aex* (for the regulation of aBoc and Exp), and *dec* (for the defecation cycle abnormal). The *aex* mutants share a similar defecation phenotype as the AVL- and DVB-GABAergic-neuron-killed animals, suggesting a possible link between *aex* genes and GABAergic neuron functions.



Adapted from Thomas J, 1990 *Genetics*

Figure 3. Local and global plasticity in the nervous system. A) The schematic diagram showing the Hebbian plasticity that locally modulates neuronal functions. The dendrites and axons are drawn on the apical and basal sides of neurons, respectively, and the strength of synaptic connections is represented by the thickness of lines. Thicker lines denote stronger connections. The insets show the timing of spikes in pre- and postsynaptic neurons. Correlated firings in pre- and postsynaptic compartments strengthen the synaptic connections, while non-correlated firings weaken the synaptic strength. B) The schematic diagram showing the global regulation of synaptic strength in neuronal cultures where neuronal activities are chronically enhanced or suppressed by drug treatments. Chronic enhancement of neural activities leads to global decrease in the amplitudes of miniature excitatory postsynaptic currents (mEPSC), while long-term suppression increases mEPSC amplitudes. One of the possible mechanisms that underlie the global synaptic modulation is the regulation of AMPA receptor levels on postsynaptic sides, which is depicted in C).

Figure 3. Local and global plasticity in the nervous system.



Adapted from Turrigiano and Nelson, 2004 *Nat. Rev. Neurosci.*

Figure 4. Trans-synaptic signals and hormonal factors are involved in modulating synapse strength in the nervous system. The schematic diagram shows three types of synaptic signals that can modulate synaptic functions through a trans-synaptic mechanism: 1) neurotransmitters, which are released from presynaptic terminals and function on postsynapses; 2) cell adhesion molecules, which can be expressed on both pre- and postsynaptic compartments and regulate synaptic functions from both sides, and 3) secretory molecules, which can be released from both pre- and postsynaptic compartments in an activity-dependent manner, and they can also act on both pre- and postsynapses to modulate synaptic functions. Other than those synaptically generated signals, a fourth class of molecules, hormonal factors, can modulate synaptic functions from a distance. Either neurons or non-neuronal cells are able to produce hormonal factors, which are released from synthesizing cells and transported by the circulation system to their target cells. This is different from the local regulation by synaptically produced signals, and we refer this as endocrine regulation.

Figure 4. Trans-synaptic signals and hormonal factors are involved in modulating synapse strength in the nervous system.

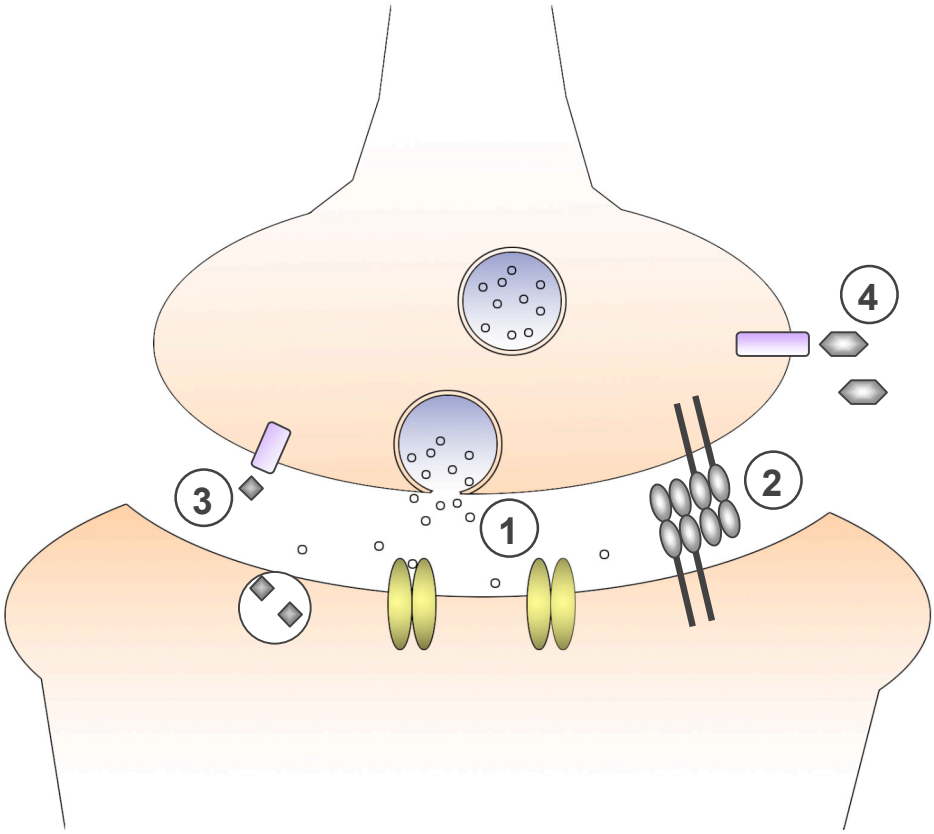
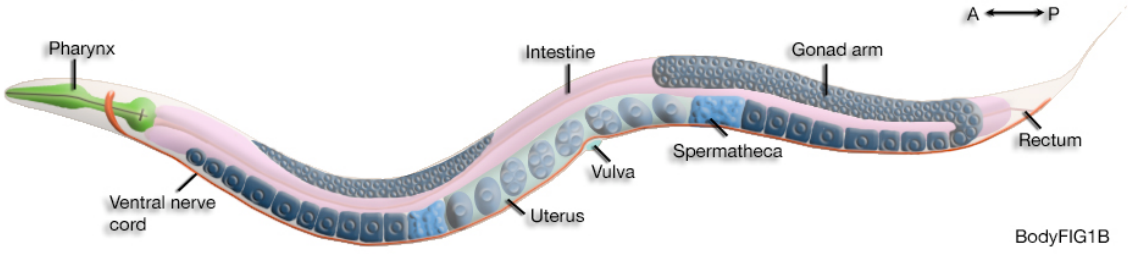


Figure 5. The *C. elegans* intestine is a tube-like organ made up of interconnected single-layer epithelial cells. The cells int1 through int9 form the main part of the intestine. Anterior to int1, the pharyngeal-intestinal valve cells vpi1 through vpi3 connect int1 to the posterior pharyngeal muscle m8. Posterior to int9, the intestinal-rectal valve cell vir connects int9 to the rectal epithelial cells rep through hyp7. The intestinal lumen runs through the middle of the intestinal cells and connects posterior pharynx with anus. The Z2 and Z3 denote germ line cells.



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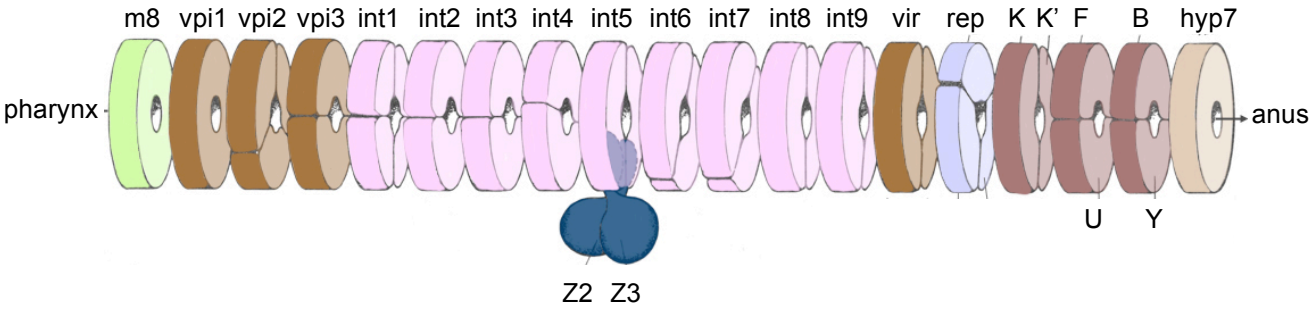

Adapted from Sulston *et al.*, 1983, *Dev. Biol.*

Chapter 2

Intestinal Signaling to GABAergic Neurons Regulate A Rhythmic Behavior in *C. elegans*

Intestinal Signaling to GABAergic Neurons Regulates a Rhythmic Behavior in *C. elegans*

Classification: Biological Sciences - Neuroscience

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Abbreviations: DMP: defecation motor program, pBoc: posterior body wall muscle contraction, aBoc: anterior body wall muscle contractions, Exp: expulsion, *aex*: aBoc and expulsion defective

Abstract

The *C. elegans* defecation motor program (DMP) is a highly coordinated rhythmic behavior that requires two GABAergic neurons that synapse onto the enteric muscles. One class of DMP mutants, called *aex* mutants, exhibits similar defects to those caused by the loss of these two neurons. Here we demonstrate that *aex-2* encodes a G-protein coupled receptor (GPCR) and *aex-4* encodes an exocytic SNAP25 homolog. We found that *aex-2* functions in the nervous system and activates a $G_s\alpha$ signaling pathway to regulate defecation. *aex-4*, on the other hand, functions in the intestinal epithelial cells. Furthermore, we show that *aex-5*, which encodes a pro-protein convertase, functions in the intestine to regulate the DMP, and its secretion from the intestine is impaired in *aex-4* mutants. Activation of the $G_s\alpha$ GPCR pathway in GABAergic neurons can suppress the defecation defect of the intestinal mutants *aex-4* and *aex-5*. Lastly, we demonstrate that activation of GABAergic neurons using the light-gated cation channel channelrhodopsin-2 (ChR2) is sufficient to suppress the behavioral defects of *aex-2*, *-4*, and *-5*. These results genetically place intestinal genes *aex-4* and *aex-5* upstream of GABAergic GPCR signaling. We propose a model whereby the intestinal genes, *aex-4* and *aex-5*, control the DMP by regulating the secretion of a signal, which activates the neuronal receptor *aex-2*.

Introduction

The *C. elegans* defecation motor program (DMP) is a highly coordinated series of three muscle contractions that are executed every 45 seconds (Fig. 1A and SI Movie 1). The cycle is initiated by a posterior body wall muscle contraction (pBoc), followed 2-3 seconds later by an anterior body wall muscle contraction (aBoc). About 1 second after the aBoc, enteric muscles contract, thus causing the expulsion of intestinal contents (Exp). Approximately 45 seconds later the process repeats itself with little variability in the timing of contractions (Thomas 1990). A genetic screen for mutants that displayed defects in the DMP isolated mutants defective in each of the three muscle contractions, known as *pbo*, *abo*, and *exp* (Thomas 1990). The screen also recovered mutants defective in the last two muscle contractions (aBoc and Exp, *aex*), and mutants defective in the cycle periodicity (i.e., longer or shorter than normal DMP cycling times, *dec*) (Thomas 1990). Molecular studies of these mutants have suggested the behavior is orchestrated through the communication between the intestine, GABAergic neurons and muscle.

The periodicity of the DMP is regulated by the *C. elegans* intestine, a single-cell layer tube of polarized epithelial cells joined by gap junctions (McGhee 2007; Peters, Teramoto et al. 2007). Intestinal Ca^{2+} oscillations with approximately 45-second periodicity appear to play a central role in this timing. They consist of a posterior to anterior Ca^{2+} wave whose levels peak in the posterior and anterior intestinal cells just prior to the pBoc and aBoc contractions, respectively (Dal Santo, Logan et al. 1999; Teramoto and Iwasaki 2006; Peters, Teramoto et al. 2007). Mutations in genes involved

in the maintenance of Ca^{2+} oscillations or in the propagation of Ca^{2+} waves between cells affect the periodicity of the DMP (Dal Santo, Logan et al. 1999; Teramoto and Iwasaki 2006; Peters, Teramoto et al. 2007). These studies suggest that the intestine may control the timing of the DMP via Ca^{2+} dependent process, such as Ca^{2+} induced exocytosis.

Furthermore, Recent work demonstrates that the intestine induces the pBoc by releasing protons (through a Na^{+}/H^{+} exchanger) onto posterior body wall muscle cells (Beg, Ernstrom et al. 2008). The posterior body wall muscle cells contract in response to the change in pH because they express a proton-gated cation channel (Beg, Ernstrom et al. 2008). By contrast, the expulsion step of the DMP is regulated by the GABAergic neurons AVL and $DVB^{||}$ (McIntire, Jorgensen et al. 1993; Liu and Thomas 1994). These neurons secret GABA onto enteric muscles that express the excitatory GABA receptor EXP-1 and cause them to contract (Beg and Jorgensen 2003).

If the intestine is the cycle timer and initiates the pBoc step, and neurons initiate the Exp step, then how are the intestinal and neuronal mediated behaviors synchronized? It seems likely that studies of *aex* genes will give some insight into how the AVL and DVB neurons are activated, since the behavioral defects of *aex* mutants are reminiscent of animals whose AVL and DVB GABAergic neurons are laser ablated (McIntire, Jorgensen et al. 1993; Liu and Thomas 1994). *aex-3* and *aex-6* regulate synaptic transmission, probably by regulating exocytosis of neurotransmitter: *aex-3* is a guanine nucleotide exchange factor that regulates Rab small GTPase function, and *aex-6* (also known as *rab-27*) is a Rab small GTPase that regulates secretory vesicle exocytosis (Iwasaki, Staunton et al. 1997; Mahoney, Liu et al. 2006). *aex-5* encodes a pro-protein

convertase, an enzyme that is co-packaged with pro-peptides and processes them to make mature secretory molecules (Doi and Iwasaki 2002; Husson, Clynen et al. 2006). Lastly, Doi and Iwasaki (Doi and Iwasaki 2002) demonstrated that *aex-1* is a distant homolog of the synaptic gene *unc-13* (or Munc13) which acts in the intestine to regulate the DMP (Doi and Iwasaki 2002). Thus, prior molecular characterization of *aex* genes implicates a secretory event is in control of aBoc and Exp.

Here we uncover how the intestinal cells regulate the activity of GABAergic neuronal function during the DMP. We cloned *aex-4*, which encodes a SNAP25 SNARE homolog, and *aex-2*, which encodes a G-protein coupled receptor (GPCR). We demonstrate that while *aex-4* and *aex-5* act in the intestine to regulate defecation, *aex-2* functions in GABAergic neurons to control this behavior. Disruption of *aex-4* function blocks AEX-5 secretion from the intestine. Moreover, GABAergic expression of either activated adenylyl cyclase or photoactivatable channelrhodopsin rescues the defecation defects of *aex-2*, *aex-4* and *-5*. We propose a model where intestinal *aex* genes, *aex-4* and *aex-5*, regulate secretion of a signal that activates the GPCR *aex-2* in AVL and DVB, which in turn activates these neurons to complete the DMP.

Results

aex mutants are primarily defective in expulsion and only mildly defective in aBoc

In order to better understand how the DMP operates, we carefully characterized the defecation defects of each of the *aex* mutants. *aex* mutants are primarily defective in the expulsion step (SI Fig. 1 and 2). Surprisingly, *aex* mutants have only slightly fewer aBoc contractions per defecation cycle than wild type; however, those aBoc contractions are usually significantly later in the cycle than in a wild-type DMP (SI Fig. 1 and 2). Therefore, the aBoc defects of most *aex* mutants are relatively mild when compared to the expulsion defects. Most *aex* mutants have a relatively normal cycle length (or period), although *aex-5* did exhibit a slightly longer defecation cycle period (SI Fig. 1). The normal cycle periodicity suggests these mutants do not have a defect in cycle time generation. Taken together, these results suggest *aex* mutants are primarily defective in the expulsion step.

aex-4 encodes a SNARE Protein

We predicted *aex-4* would encode a protein involved in exocytosis, neuropeptide production, or neuropeptide signaling, since all other *aex* strains have mutations in genes regulating these pathways (Iwasaki, Staunton et al. 1997; Thacker and Rose 2000; Doi and Iwasaki 2002). By searching the genomic interval in which aex-4 had been mapped (Thomas 1990), we identified a candidate gene for the *aex-4* locus. All *aex-4* alleles sequenced have mutations in the gene T14G12.2, and a 4kb genomic clone of this gene is sufficient to rescue the defecation defect of *aex-4* (SI Fig. 3 and 4A). T14G12.2 encodes a homolog of the SNARE (soluble N-ethylmaleimide sensitive factor attachment

receptor) protein SNAP25 (SI Fig. 3)(Bock, Matern et al. 2001; Kloepper, Kienle et al. 2007). These findings implicate *aex-4* in exocytosis.

AEX-4 Regulates the Defecation Motor Program from the Intestine

To dissect how AEX-4 regulates defecation behavior, we first determined the expression pattern of *aex-4* by building a transgenic animal that expresses GFP fused to a nuclear localization signal under the *aex-4* promoter. Surprisingly, *aex-4* expresses only in intestinal cells (Fig. 1B). To determine the subcellular localization of AEX-4, we built a transgene expressing a functional GFP-tagged AEX-4 fusion under its native promoter. GFP–tagged AEX-4 localizes along the plasma membrane of intestinal cells (Fig. 1C and D). Therefore, AEX-4 likely acts at the surface of intestinal cells to regulate the DMP.

We expressed the *aex-4* gene under an intestinal, a muscle, and a neuronal specific promoter to determine in what tissue *aex*-4 regulates the DMP. Intestinal expression of *aex-4* fully rescues the defecation defects of *aex-4* mutant animals (Fig. 2A and SI Fig. 4B and C), while muscle and neuronal specific promoters only partially rescue the defecation defect of *aex-4* mutants (Fig. 2A).

To verify our tissue specific rescue experiments, we tested whether RNAi of *aex-4* in a strain that is only sensitive to RNAi in the intestine would result in a defecation defect (Espelt, Estevez et al. 2005). Indeed, intestinal-specific RNAi of *aex-4* results in a strong expulsion defect consistent with *aex-4* functioning in the intestine (Fig. 2C). In addition to *aex-4*, RNAi of *aex-5* and *aex-6* in the intestinal-specific RNAi strain causes a strong expulsion defect. RNAi of *aex-3* causes a moderate expulsion defect similar to that of the *aex-3* loss of function (SI Fig. 1). These results are consistent with *aex-3*, *-4*, -

5, and *-6* functioning in the intestine. RNAi of *aex-1*, in contrast, causes only a mild defect in expulsion, although tissue specific rescue experiments suggest *aex-1* functions in the intestine (Doi and Iwasaki 2002). We suspect that *aex-1* levels were not reduced enough in the intestinal-specific RNAi strain since RNAi of *aex-1* in wild type strains causes a strong expulsion defect (data not shown). Combined together, these results strongly suggest that a cohort of exocytic *aex* genes including aex-*4* all function in the intestine to regulate the DMP.

AEX-5 Regulates the Defecation Motor Program from the Intestine

aex-5 encodes a pro-protein convertase and was identified in the same screen that isolated *aex-4* (Thomas 1990; Doi and Iwasaki 2002; Husson, Clynen et al. 2006). We confirmed that the pro-protein convertase gene is mutated in *aex-5(sa23)* by sequencing (lesion is C453W) and by genomic fosmid rescue (SI Fig. 4A). Since *aex-4* likely regulates secretion of a signal from the intestine, and pro-protein convertases are typically packaged into secretory vesicles (Thacker and Rose 2000), we wished to determine whether AEX-5 is secreted from the intestine. Intestinal specific expression of AEX-5 fused to VENUS, a variant of GFP (Nagai, Ibata et al. 2002), fully rescues the defecation defects of *aex-5* mutant animals, while muscle and neuronal specific expression only partially rescues (Fig. 2A and SI Fig. 4B and C). AEX-5::VENUS is secreted from the intestine and subsequently endocytosed by coelomocytes, specialized endocytic cells in *C. elegans* (Fig. 1E-F). These findings suggest AEX-5 is secreted from the intestine where it regulates the DMP.

AEX-4 Regulates the Secretion of AEX-5 from the Intestine

We hypothesized that AEX-4 regulates the secretion of AEX-5 from the intestine. To test this, we determined if *aex-4* mutants are defective in AEX-5::VENUS secretion. Wild type animals that express AEX-5::VENUS in the intestine secrete AEX-5::VENUS, which accumulates in coelomocytes. In contrast, *aex-4* mutants accumulate AEX-5::VENUS in intestinal cells and accumulate significantly less AEX-5::VENUS in coelomocytes (Fig. 3 and SI Fig. 5). There is no defect in the secretion of AEX-5::VENUS in wild type or *aex-2* mutants. Therefore, AEX-4 likely regulates the secretion of AEX-5 from the intestine during the DMP. These results lead us to the question: what receives this signal?

aex-2 Encodes a G-Protein Coupled Receptor

We mapped *aex-2* to a region on the X chromosome and revealed the gene T14B1.2 encodes *aex-2*. All sequenced *aex-2* alleles have mutations in T14B1.2, and a transgene that contains the T14B1.2 gene rescues the *aex-2* mutant phenotype (SI Fig. 4A and 6). T14B1.2 encodes a protein that shares homology with the A class of G-protein coupled receptors (GPCR), some of which mediate peptide signaling (SI Fig. 6) (Gether 2000). When expressing a *mCherry* tagged *aex-2* genomic fusion construct (which fully rescues the *aex-2* defecation defects), we detected AEX-2::mCherry signal in the nerve ring, ventral nerve cord, and in the enteric muscles (Fig. 1G-I). So unlike *aex-4*, which is exclusively expressed in the intestine, *aex-2* is expressed in both neuronal and non-neuronal tissues.

aex-2 Regulates the Defecation Motor Program from the Nervous System

To determine what tissue *aex-2* functions in, we expressed an *aex-2* cDNA::GFP fusion under a neuronal, a muscular, and an intestinal promoter. While neuronal expression of AEX-2::GFP fully rescues the defecation defects of *aex-2* mutants, muscular and intestinal *aex-2* do not (Fig. 2A). Consistent with this observation, intestinal specific RNAi of *aex-2* does not cause a robust defecation defect (Fig. 2C). Thus unlike *aex-4* and -*5*, *aex-2* acts in neurons to regulate the DMP.

As previous studies have indicated that the GABAergic neurons AVL and DVB are required for expulsion (McIntire, Jorgensen et al. 1993), we analyzed *aex-2*'s expression pattern in various neuronal subtypes. We co-expressed mCherry protein under the *aex-2* promoter with the GFP under either an *unc-17* (drives expression in cholinergic neurons), a *glr-1* (drives expression in subsets of interneurons), or an *unc-47* (drives expression in GABAergic neurons) promoter to assess if any of the *aex-2* positive neurons are co-labeled by these neuronal subtype specific markers. We found that the *aex-2* reporter is detected in all three neuronal cell types (Fig. 1J-L and SI Fig. 7). Briefly, at least one glutamatergic interneuron (likely AVD), several head and pharyngeal cholinergic neurons, and two GABAergic neurons (AVL and DVB) express mCherry under the control of the *aex-2* promoter (Fig. 1J-L and SI Fig. 7). None of the ventral nerve cord motor neurons (neither cholinergic nor GABAergic) are labeled by P*aex-2*::mCherry. Therefore, *aex-2* is expressed in the defecation regulating GABAergic neurons (AVL and DVB) as well as other types of non-motor neurons.

In order to determine which neuronal subtype *aex-2* functions in, we expressed an *aex-2*::GFP cDNA fusion under neuronal subtype specific promoters. GABAergic

expression of *aex-2* significantly rescues the expulsion defect and partially rescues the aBoc defect of *aex-2* mutants, while the other two promoters do not (Fig. 2B and SI Fig. 4B and C). Taken together, these results suggest that *aex-2* acts in GABAergic neurons (i.e., AVL and DVB) to control the expulsion, and perhaps the aBoc.

***aex-2* Functions Through the G_sa and Adenylyl Cyclase Signaling Pathway**

As *aex-2* encodes a GPCR, we examined which Ga subunit might act downstream of the *aex-2* receptor signaling. To determine this, we genetically tested a variety of candidate Gα subunits for their ability to suppress *aex-2* mutant phenotype. We built double mutants between an *aex-2* loss-of-function mutant and a *gsa-1* ($G_s\alpha$) gain-of-function mutant (Schade, Reynolds et al. 2005), and an *egl-30* ($G_q\alpha$) gain-of-function mutant (Jansen, Thijssen et al. 1999; Lackner, Nurrish et al. 1999; Miller, Emerson et al. 1999). We also built an *aex-2* and *dgk-1* (which encodes a diacylglycerol kinase) double loss-of-function mutant, as *dgk-1* normally antagonizes *egl-30* signaling and its loss-of-function phenocopies *egl-30* gain-of-function (Jansen, Thijssen et al. 1999; Lackner, Nurrish et al. 1999; Miller, Emerson et al. 1999). G_sa gain-of-function mutation causes ectopic expulsion in both wild type and *aex-2* mutant animals, and it suppresses the expulsion defect in *aex-2* mutants (Fig. 4A). On the other hand, the G_qa pathway mutants do not have a strong effect on the *aex-2* phenotype (Fig. 4A). Interestingly, gain-of-function in *gsa-1* does not rescue the aBoc defect (SI Fig. 4B and C). The irregular timing of expulsions in *gsa-1* mutants (i.e., some expulsions do not occur at the proper time, which is 3 seconds after pBoc) may account for the lack of rescue of aBoc in *aex-2* mutants. To assess if the $G_s\alpha$ rescue is specifically mediated by GABAergic neurons, we

tested whether the GABAergic expression of a gain-of-function adenylyl cyclase (*acy-1*) (Saifee 2003), a downstream effector of G_sa, would suppress the defecation defects in *aex-2* mutants. Similar to the *gsa-1* gain-of-function mutant, over expression of a gain-of-function *acy-1* gene in *aex-2* mutants causes ectopic expulsions, and this expression significantly rescues the expulsion defects in *aex-2* mutants (Fig. 4A). Taken together, these results support a model where the *aex-2* GPCR acts in GABAergic neurons to regulate the DMP via a downstream G_sa and adenylyl cyclase pathway.

Neuronal Adenylyl Cyclase Acts Downstream of AEX-4 and AEX-5 to Control Defecation

While *aex-2* acts in AVL and DVB GABAergic neurons, *aex-4* and *aex-5* function in the intestine to regulate expulsion. We wished to determine if *aex-2* signaling acts downstream of intestinal *aex-4* and *aex-5*. Since we have not identified the ligand for AEX-2, we asked whether gain-of-function mutations in the *aex-2* pathway could suppress the *aex-4* and *aex-5* expulsion defects. GABAergic expression of the gain-of-function *acy-1* gene is sufficient to rescue the expulsion defects seen in *aex-4* and *aex-5* mutants (Fig. 4A). As seen in *aex-2* mutants, over expression of gain-of-function *acy-1* in *aex-4* and *aex-5* mutants also causes ectopic expulsions. These data suggest that GABAergic signaling of *aex-2* acts downstream of intestinal signaling of *aex-4* and *aex-5*.

Activation of GABAergic Neurons by a Light-Activatable Channel can Bypass the Requirement of AEX-2, AEX-4, and AEX-5 for Defecation

We wished to demonstrate that the role of *aex-2*, *aex-4*, and *aex-5* is to specifically activate GABAergic neurons controlling the Exp step. We used channelrhodopsin-2 (ChR2) under a GABAergic promoter to bypass the loss of these *aex* genes. ChR2, from the green alga *Chlamydomonas reinhardtii*, is a light-activatable non-selective cation channel that in the presence of all-*trans*-retinal will depolarize excitable cells (Nagel, Brauner et al. 2005). We hypothesized that the activation of GABAergic neurons using the ChR2 transgene would suppress the behavioral defect of each of the *aex* mutants. Indeed, activation of ChR2 in GABAergic neurons by brief (~1 second) pulses of blue-light ~2 seconds after pBoc is sufficient to completely rescue the expulsion defects of *aex-2*, *aex-4*, and *aex-5* mutants (Fig. 4B and SI Movie 2). These results strongly suggest that *aex-2*, *aex-4*, and *aex-5* regulate the activity of GABAergic neurons AVL and DVB to induce expulsion during the DMP.

Discussion

Several lines of evidence suggest a group of exocytic genes function in the *C. elegans* intestine to control secretion of a signal to regulate the aBoc and expulsion step of the DMP. *aex-1*, *aex-3*, *aex-4*, and *aex-6* are each homologous to the genes that regulate exocytosis in secretory cells (Doi and Iwasaki 2002; Mahoney, Liu et al. 2006). Here we present data that these genes regulate the secretion of a signal from the intestine to induce aBoc and expulsion. This model is further supported by the observation that *aex-4* mutants prevent the secretion of an intestinal AEX-5::VENUS into the pseudocoelom and its subsequent endocytosis by coelomocytes. Therefore, *aex-4* (and likely *aex-1*, *aex-3*, and *aex-6*) regulates the secretion of AEX-5, and arguably its substrate, from the intestine. Although our data indicate *aex-3* and *aex-6* function in intestine, it does not exclude the possibility that these genes also function in AVL and DVB to regulate the DMP (see SI Fig. 8 for model).

The model that the intestine secretes a signal to regulate expulsion prompted us to search for the receptor for the signal. *aex-2* encodes a putative GPCR, and our data suggest this receptor likely functions in AVL and DVB GABAergic neurons to regulate expulsion. Interestingly, when using either a gain-of-function G_sa or an activated adenylyl cyclase that is expressed in GABAergic neurons, we suppressed the expulsion defect not only in *aex-2*, but also in the intestinal *aex-4* and *aex-5* mutants. These results are consistent with *aex-2* encoding the receptor of the intestinal signal and acting downstream of intestinal *aex* genes.

We speculate that the G_sa and adenylyl cyclase act downstream of *aex-2* to excite

AVL and DVB during the expulsion. In support of this model, some *gsa-1* loss-of-function animals, with a mosaic rescuing transgene, were reported to exhibit a defecation defect (Korswagen, Park et al. 1997). We also observe a robust defect in the Exp step in these animals (11+/-3%, $p<0.0005$). In contrast, *acy-1* loss-of-function mutants, with a rescuing transgene expressing in muscle (Reynolds, Schade et al. 2005), do not have a significant defect in the Exp step (81+/-7%, $p>0.05$). This may be due to redundancy of the 3 other adenylyl cyclase genes in *C. elegans* (Bastiani and Mendel 2006) and/or the action of other *gsa-1* effectors.

Activation of ChR2 in GABAergic neurons is sufficient to suppress the expulsion defects of *aex-2*, *aex-4*, and *aex-5* mutants. The aBoc defect of these *aex* mutants, however, was not rescued. Activation of GABAergic ChR2 causes the worm to become paralyzed due to muscle relaxation; therefore it may be difficult to observe the aBoc contractions under these conditions. These findings indicate that the *aex* genes likely act to activate AVL and DVB GABAergic neurons through *aex-2*.

Although *aex-2* is likely involved in AVL and DVB activation, it remains unclear how *aex-2* is activated. As *aex-5* encodes a pro-protein convertase and *aex-5* mutants are defective in neuropeptide production (Husson, Clynen et al. 2006), we suspect *aex-2* encodes a neuropeptide-like receptor. There are approximately 100 genes in the *C. elegans* genome encoding over 250 peptides (Nathoo, Moeller et al. 2001; Husson, Clynen et al. 2005). The identification of *aex-2* ligand(s) will shed new light on our understanding of this GPCR's signaling and the regulation of neuronal functions by non-neuronal tissues.

Several of our observations suggest the expulsion step may be regulated by more than one signal. First, we noticed that activation of ChR2 in either wild type or *aex* mutant animals does not induce ectopic expulsions. Only temporally correct light-activation of AVL and DVB rescues the Exp defect of *aex-2*, *-4*, and *-5*. This observation indicates that activation of AVL and DVB (at least via ChR2) is permissive, but not completely instructive, to drive the Exp step of the DMP. Second, while constitutive activation of the $G_s\alpha$ pathway causes ectopic expulsions in *aex* mutants, there is a strong tendency for Exp's to occur at the proper time (~3 seconds after pBoc). If *aex* genes were the sole connection between the intestinal pacemaker and activation of expulsion, then one would expect to see a weaker tendency for Exp's to occur at the proper time. These observations suggest that a second signal may function to regulate the Exp step. This signal could, for example, consist of a permissive signal that allows enteric muscles to be excited at the right time point in the DMP.

Upon first glance, one cannot help notice the similarities between the intestinal *aex* genes and the neuronal secretory apparatus. SNARE proteins (AEX-4) are thought to create a membrane fusion structure at the nerve terminal, with SNARE regulators (AEX-1) playing a critical role in exocytosis (Sudhof 2004). Although the precise function of Rabs (AEX-6) in exocytosis *per se* is unclear, they play an important role in synaptic transmission (Mahoney, Liu et al. 2006). Interestingly, while the regulator for AEX-6, AEX-3, has a similar defecation phenotype when mutated, the effector of AEX-6, RBF-1, does not (Mahoney, Liu et al. 2006). Perhaps AEX-6, also known as Rab27, functions through a novel effector to regulate the DMP. Given the similarities between the genes

involved in the DMP and those involved in synaptic transmission, the *C. elegans* intestine could be seen as an alternative means for investigating the mechanisms governing regulated exocytosis and by analogy synaptic transmission. When one adds on the observation that RNAi is highly ineffective in *C. elegans* neurons (Winston, Molodowitch et al. 2002), but very effective in the intestine (Espelt, Estevez et al. 2005), the intestine becomes an attractive model for discovering genes and genetic pathways regulating exocytosis.

Our work shows that the intestine may secrete a signal to activate the AVL and DVB neurons to induce the DMP (SI Fig. 8). This might explain why the Ca^{2+} oscillations are necessary for the timing of the DMP, since excitable cells use increased Ca^{2+} to induce secretion. Our work provides the first explanation of how the intestine may regulate this behavior by activating the AVL and DVB neurons.

Materials and Methods

Refer to SI Text for Materials and Methods

Behavioral Assay

For detailed methods refer to SI Text for Materials and Methods. Briefly, 8-20 one-day-old adults were scored for 10 defecation cycles (i.e., 10 pBocs). Statistical significance was determined by using unpaired two-tailed student t tests, with unequal variance.

Channelrhodopsin-2 Experiments

For detailed methods refer to SI Text for Materials and Methods. Briefly, L4 larval staged animals were grown in the presence or absence of 500μM all-*trans* retinal (Sigma) overnight at 22°C. The next day, defecation was scored. During the DMP animals were stimulated with a brief, ~1 second pulse of blue light, ~2 seconds after pBoc. These experiments were performed on a Leica MZ16F fluorescent stereomicroscope with an x-Cite 120 excitation light source (EXFO) and a standard GFP filter.

Footnotes

|| AVL and DVB are not abbreviations; they are the actual names of two specific GABAergic neurons in *C. elegans* (White, Southgate et al. 1986).

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Figure Legends

Fig. 1. *aex* genes are expressed in both neuronal and non-neuronal tissues of *C. elegans*. (A) Diagram of the *C. elegans* defecation motor program (DMP). First, a posterior contraction (pBoc) forces the intestinal contents to the anterior end of the worm. About 3 seconds later, an anterior contraction (aBoc) forces intestinal contents to the posterior end. Within about 1 second of the aBoc, an enteric muscle contraction in the tail leads to excretion of intestinal contents (expulsion). Arrowheads indicate the location of AVL and DVB GABAergic neurons. (B-D) *aex-4* is expressed in the intestine. (B) GFP detected solely in the intestinal nuclei in a transgenic animal that expresses nuclear localized GFP under the *aex-4* promoter. (C, D) A functional AEX-4::GFP fusion is expressed in intestinal cells and enriched at the cell surface (arrows) (red, auto-fluorescence). (C) A confocal slice through the middle of the intestine. (D) Cell surface view of the posterior intestinal cell. Bright field images are provided for orientation (E, F) Intestinal AEX-5::VENUS (which is driven by the intestinal promoter P*vha-6*) is secreted from the intestine and taken up by coelomocytes (CC, arrows; red, auto-fluorescence). (G-L") *aex-2* is expressed in the GABAergic neurons AVL and DVB as well as in enteric muscles. (G, I) AEX-2::mCherry is detected in the nerve ring (NR, arrow), ciliary sensory processes (CA, open arrow), nerve cord (I), and head mesodermal cell (HMC, arrow head). (H) AEX-2::mCherry is expressed in the intestinal muscle (IM, arrow) and anal depressor (AD, arrowhead). (J-L") mCherry expressed under the *aex-2* promoter is detected in both AVL (J, K, L, arrow) and DVB (J', K', L', arrow)

GABAergic neurons. AEX-2::mCherry signal does not significantly overlap with GABAergic GFP in the ventral Nerve cord (J”-L”). Scale bar, 20μm.

Fig. 1. *aex* genes are expressed in both neuronal and non-neuronal tissues of *C. elegans*.

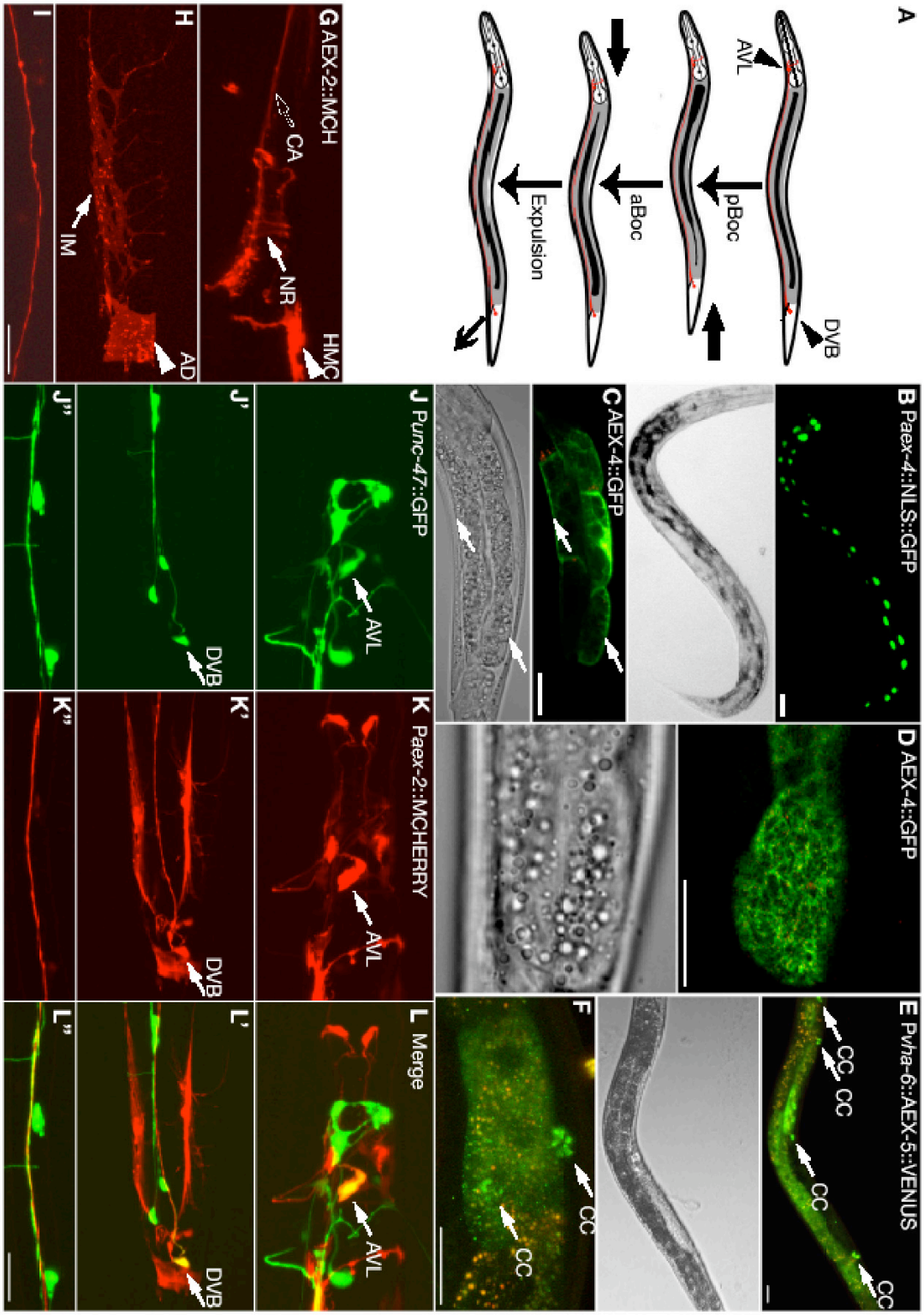


Fig. 2. *aex-4* and *aex-5* function in the intestine, while *aex-2* acts in GABAergic neurons to regulate defecation. (A) A neuronal (P*rab-3*), a muscular (P*myo-3*) and an intestinal (P*ges-1* or P*vha-6*) promoter was used to drive the expression of individual *aex* genes. Intestinal *aex-4* and *aex-5* fully rescue the *aex-4* and *aex-5* expulsion defect, respectively, and neuronal *aex-2* rescues the *aex-2* expulsion defect. Muscular and neuronal expressions of *aex-4* and *aex-5* only partially rescue the *aex-4* and *aex-5* expulsion defect. (B) P*unc-47* (GABAergic neurons), but not P*unc-17* (cholinergic neurons) or P*glr-1* (subset of interneurons) expression of *aex-2* rescues the *aex-2* expulsion defect. DMP function was assayed by observation of 8-20 animals for 10 cycles and plotted as the ratio of expulsions to pBocs. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significantly different from the respective mutant. +$p>0.05$ not significantly different from wild type. (C) Intestinal RNAi of *aex-4*, *aex-5*, and *aex-6* induces strong expulsion defects. RNAi of *aex-3* induces a moderate defecation defect, while the RNAi on *aex-1* and *aex-2* induces only mild defecation defects. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significantly different from the vector control RNAi. Error bars represent standard error of the mean (SEM).

Fig. 2. *aex-4* and *aex-5* function in the intestine, while *aex-2* acts in GABAergic neurons to regulate defecation.

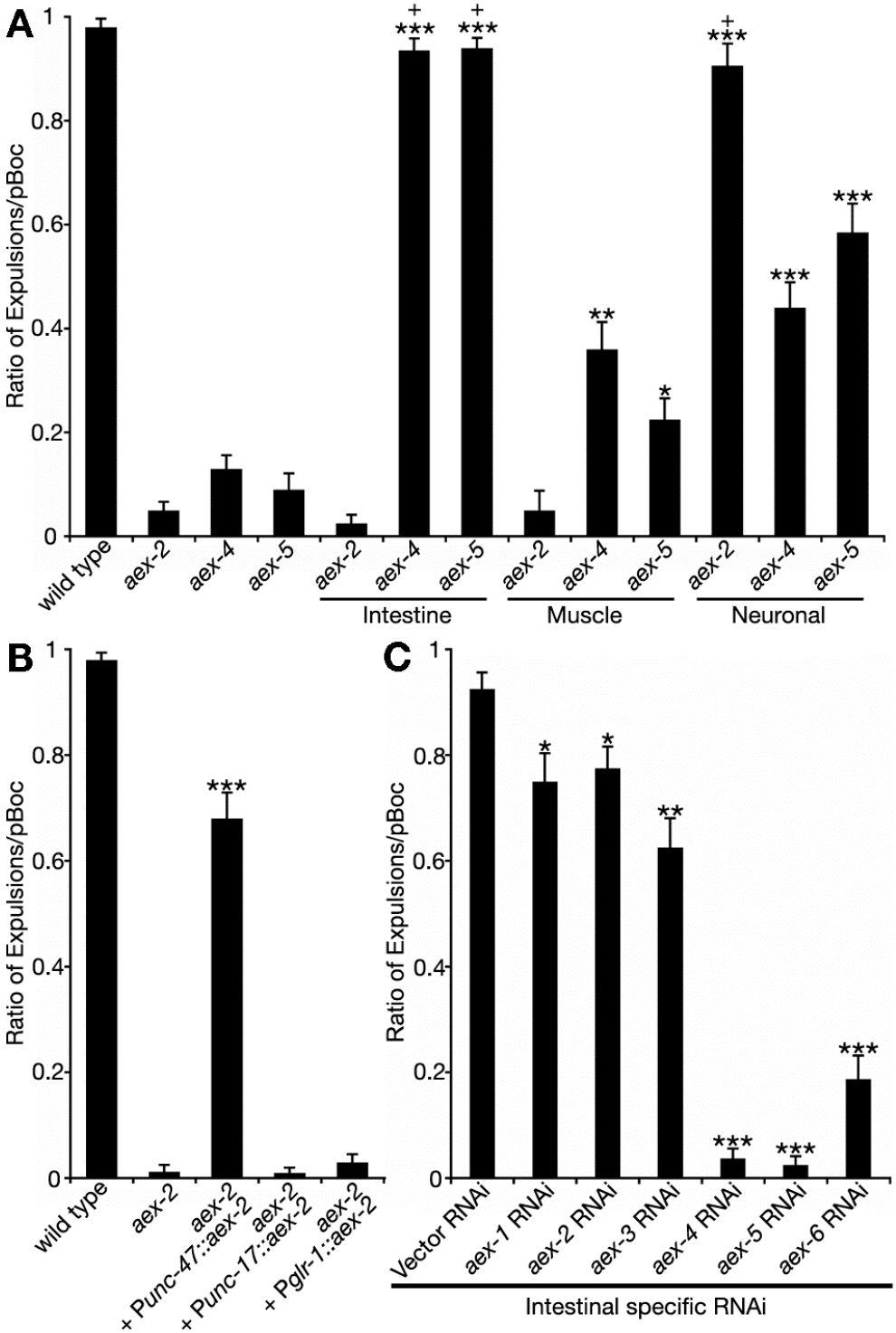


Fig. 3. Intestinal *aex-4* regulates the secretion of AEX-5. (A) In *aex-4* mutants, the total fluorescence, normalized to wild type, of intestinal AEX-5::VENUS in coelomocytes is significantly less than wild type. There is no significant change in coelomocyte fluorescence in *aex-2* mutants. ***$p<0.0005$ significantly different from the respective mutant. +$p>0.05$ not significantly different from wild type. Error bars represent SEM. (B) Representative photographs of anterior coelomocytes in wild type, *aex-2*, and *aex-4*. Left image is a Nomarski image. Right is AEX-5::VENUS fluorescence. Scale bar, 5μm.

Fig. 3. Intestinal *aex-4* regulates the secretion of AEX-5.

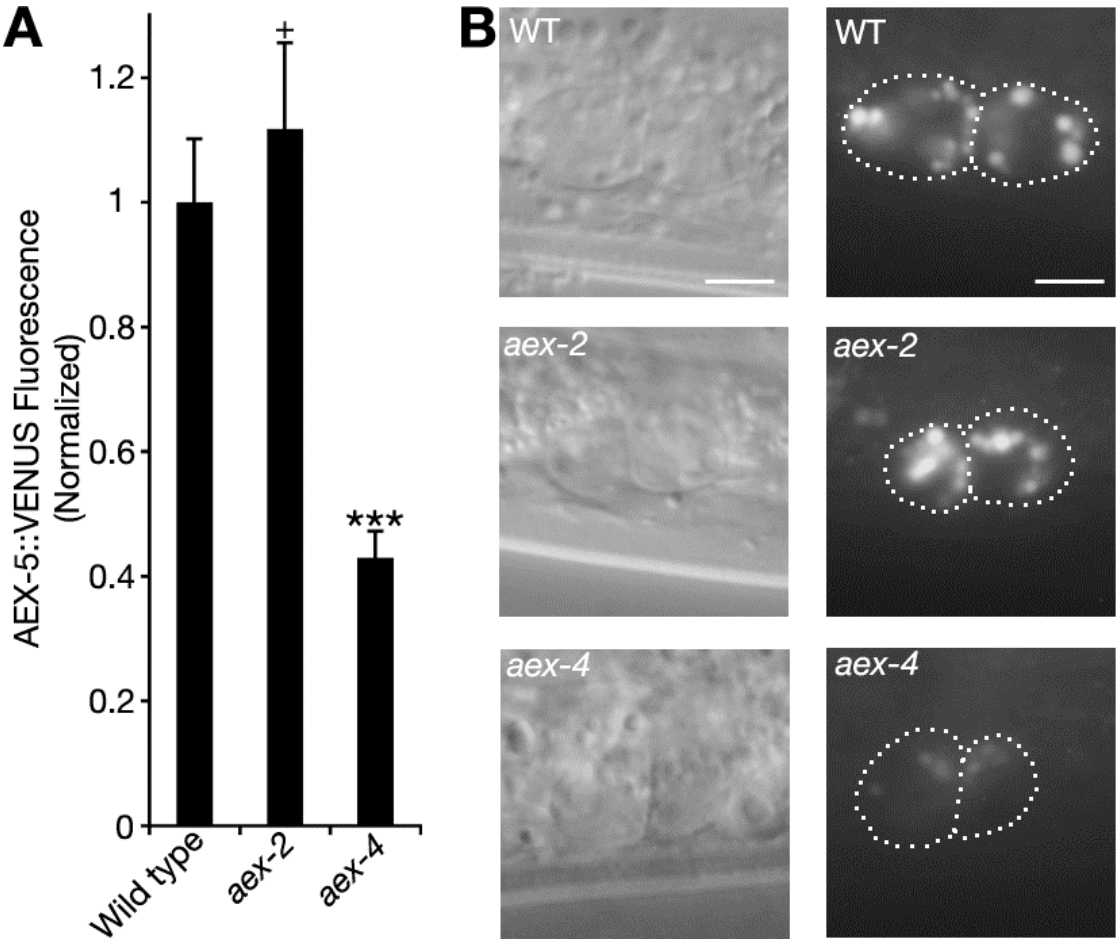
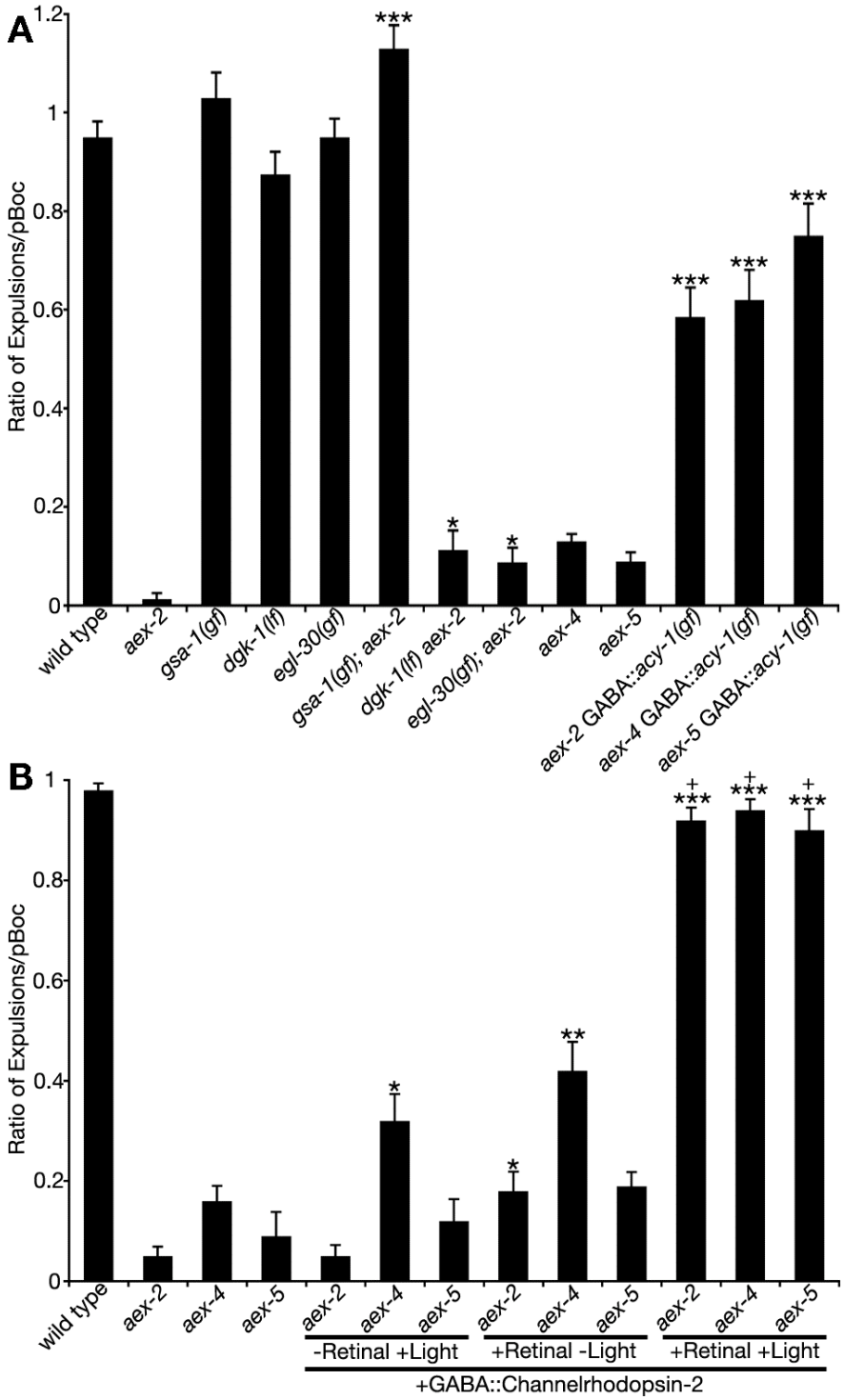


Fig. 4. *aex* genes likely regulate the defecation through downstream G_{α}s and adenylyl cyclase signaling and GABAergic neuron activation. (A) A gain-of-function allele of G_{α}s (*gsa-1(ce81)*) completely suppresses the expulsion defects in *aex-2* mutants. In contrast, a gain-of-function in G_{α}q (*egl-30(js126)*) and a loss-of-function *dgk-1(sy428)*, which phenocopies *egl-30* gain-of-function, have only mild effects on the *aex-2* expulsion defects. When an activated adenylyl cyclase gene (*acy-1(js127)*) is specifically expressed in the GABAergic neurons, it significantly suppresses the expulsion defects of *aex-2*, *aex-4* and *aex-5* mutants. (B) Activation of GABAergic neurons by photoactivatable channelrhodopsin-2 (ChR2) suppresses the expulsion defects in *aex* mutants. The photoactivatable cation channel ChR2 was expressed specifically in GABAergic neurons under the *Punc-47* promoter in *aex-2*, *aex-4* and *aex-5* mutants. In the presence of all-*trans* retinal and blue light activation, the ChR2 fully suppresses the expulsion defects in all the *aex* mutants. In contrast, in the absence of either all-*trans* retinal or blue light, the ChR2 transgene does not rescue the defecation mutant phenotypes to wild type levels. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significantly different from the respective mutant. +$p>0.05$ not significantly different from wild type. Error bars represent SEM.

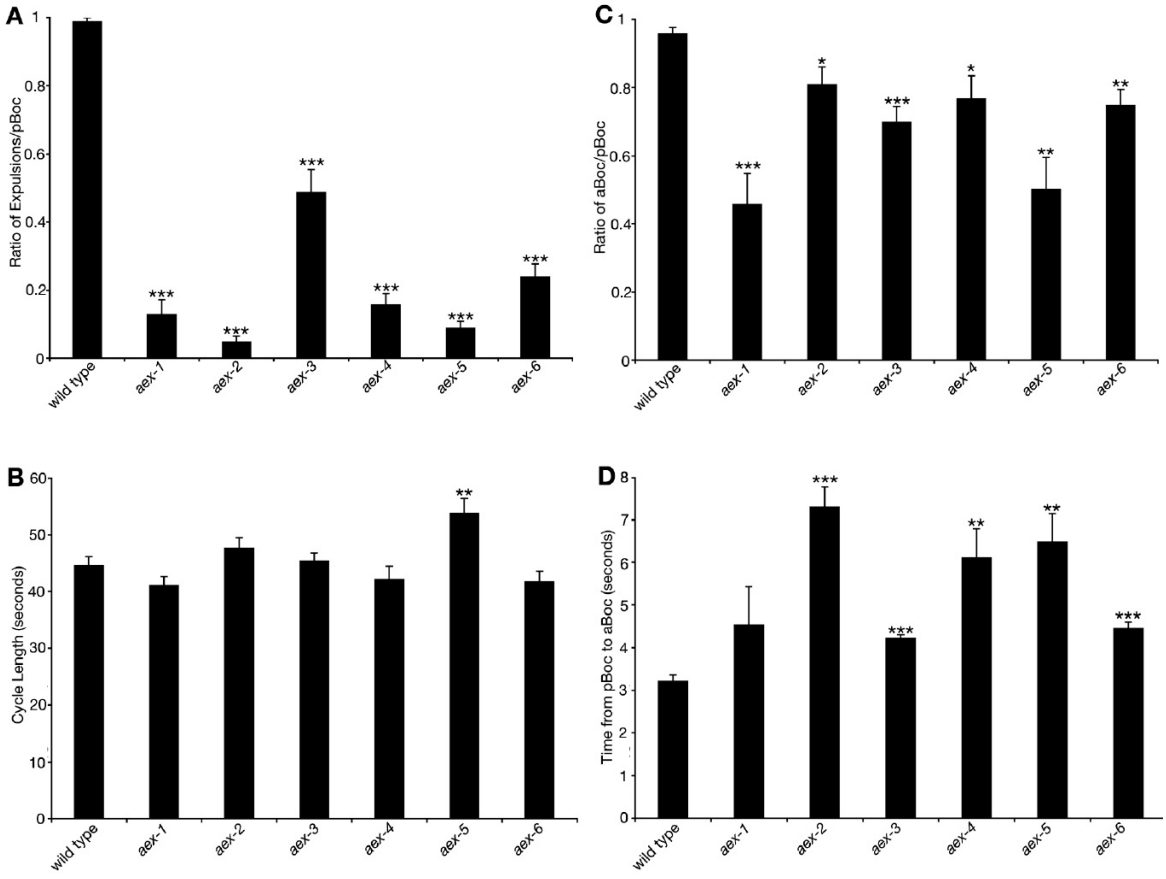
Fig. 4. *aex* genes likely regulate the defecation through downstream G_{α}s and adenylyl cyclase signaling and GABAergic neuron activation.



SI Figure Legend

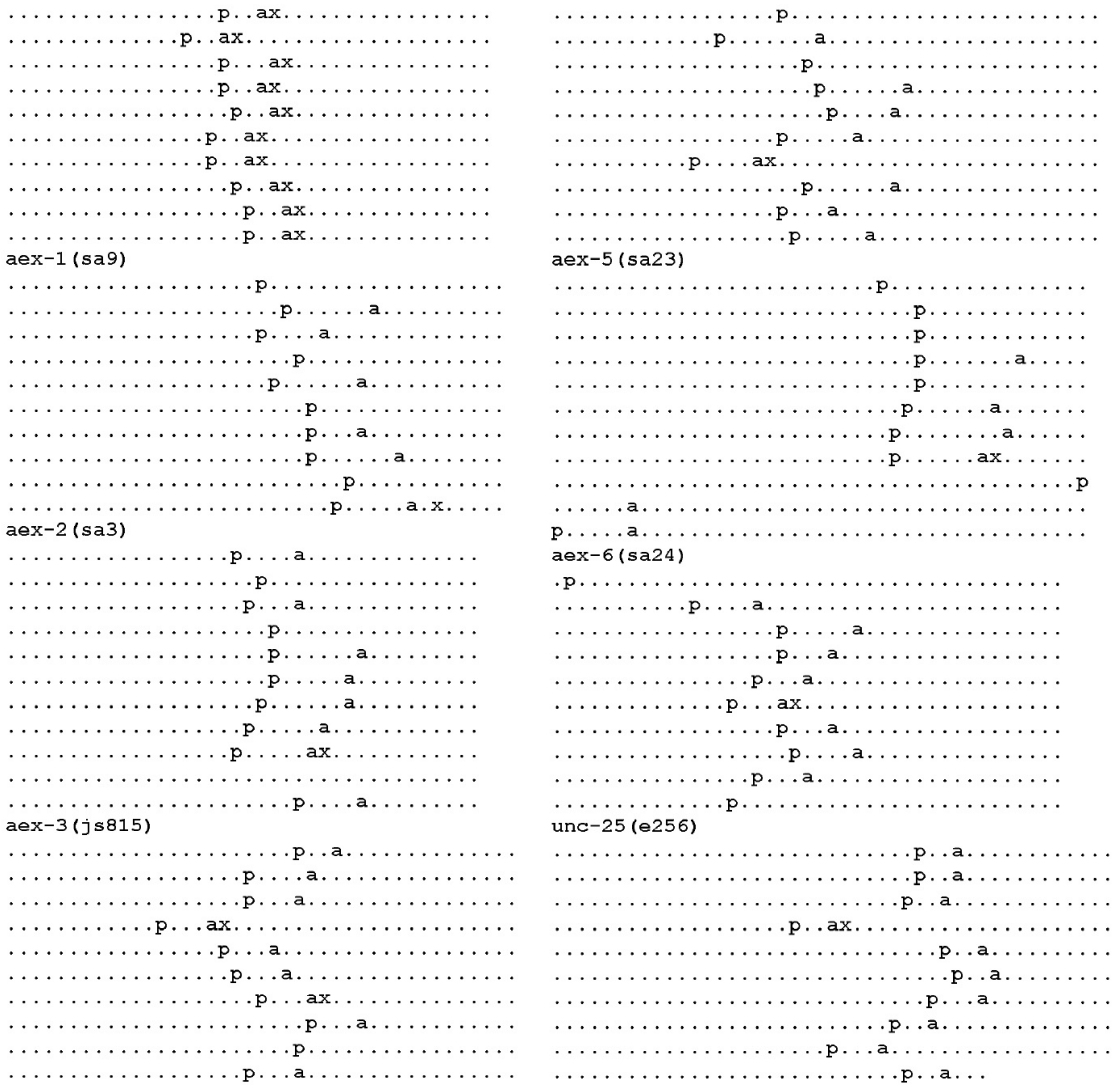
SI Fig. 1. *aex* mutants are primarily defective in expulsion while only mildly defective in aBoc. (A) *aex* mutants are defective in expulsion. (B) *aex* mutants do not have defective cycle time length, except for *aex-5*. (C) *aex* mutants have a defect in aBoc frequency. (D) *aex* mutants have defects in the timing of aBoc. *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significantly different from wild type. Error bars represent SEM.

SI Fig. 1. *aex* mutants are primarily defective in expulsion while only mildly defective in aBoc.



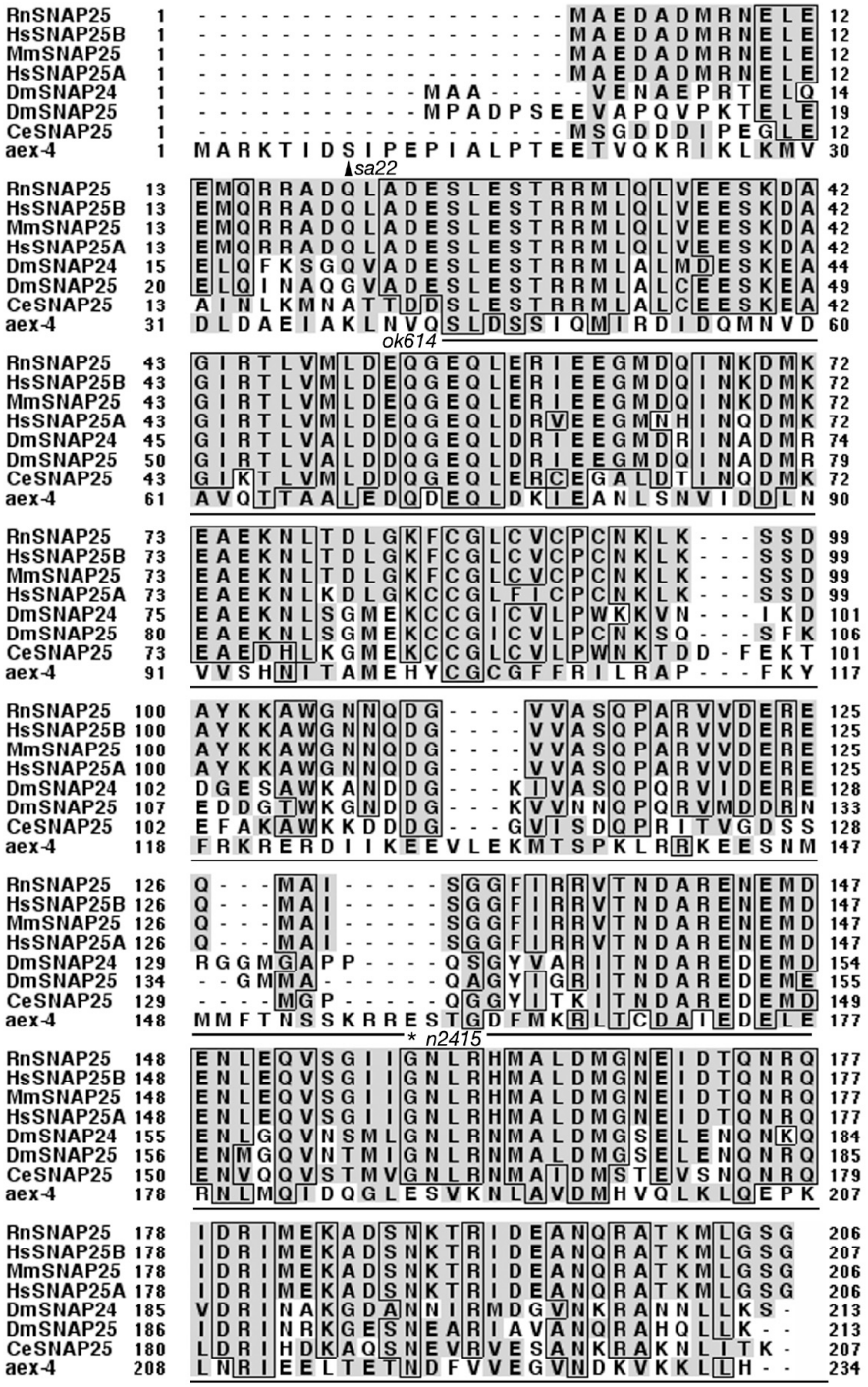
SI Fig. 2. Behavioral recordings of *aex* mutants. Each dot (.) represents 1 second, while (p) indicates pBoc, (a) indicates aBoc, and (x) indicates expulsion. Most *aex* mutants have regular cycle periodicity. The primary defect is a decrease in the number of expulsions. They also have a decrease in the number of aBoc contractions per cycle and have delayed timing of the aBoc contraction. *unc-25(e256)* is a loss-of-function mutation in a gene required for GABA biosynthesis. *unc-25* mutants do not have a defect in aBoc, therefore aBoc is likely regulated by a GABA-independent process.

SI Fig. 2. Behavioral recordings of *aex* mutants.



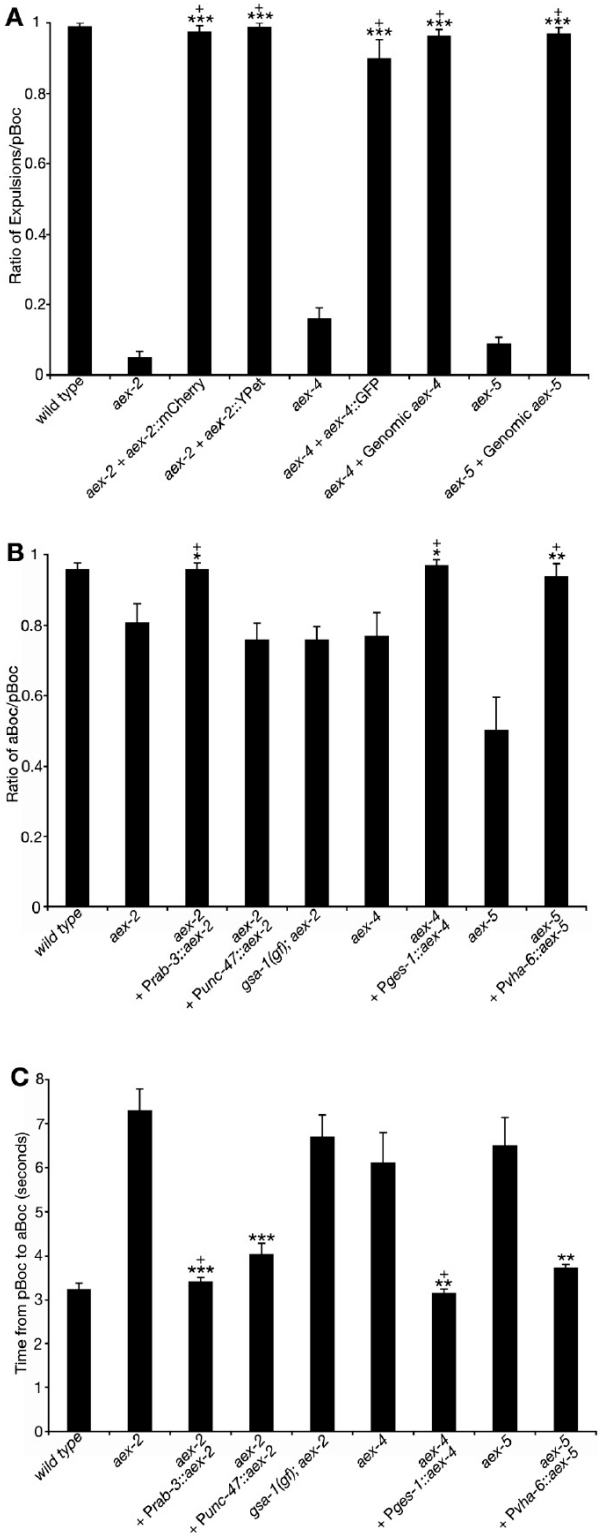
SI Fig. 3. *aex-4* encodes a SNAP25 homolog. Alignment of AEX-4 (T14G12.2) with SNAP25 shows 47% similarity and 15% identity with other SNAP25 genes. There is 46% similarity / 23% identity with drosophila SNAP24 (DmSNAP24) and 51% similarity / 19% identity with RIC-4 *C. elegans* SNAP25 (CeSNAP25). Mutations in various alleles used are indicated. *aex-4(sa22)* contains a splice site mutation at S8. *aex-4(ok614)* (also called *tag-81(ok614)*) contains a deletion marked by a line from S43 past the end, which deletes part of a neighboring gene *tag-18* (or T14G12.3). *aex-4(n2415)* encodes a stop codon at E158.

SI Fig. 3. *aex-4* encodes a SNAP25 homolog.



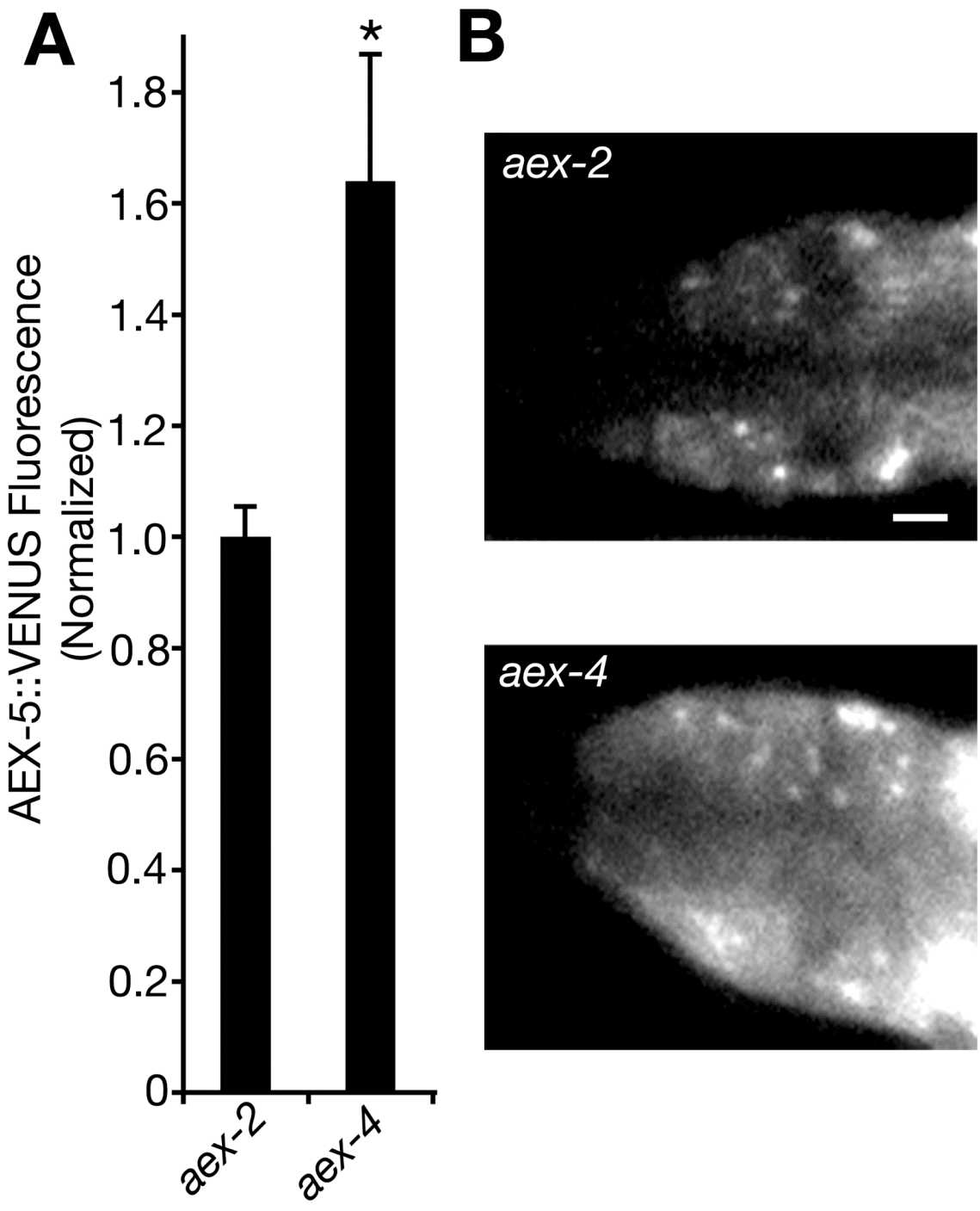
SI Fig. 4. Rescue of *aex* mutants with genomic and fluorescent protein tagged clones and rescue of aBoc defects with tissue specific constructs. (A) AEX-2::mCherry and AEX-2::YPet expressed under the *aex-2* promoter are sufficient to rescue *aex-2* expulsion defect. A genomic clone of *aex-4* and a fosmid containing the *aex-5* gene are sufficient to rescue their respective mutant phenotypes. AEX-4::GFP expressed under an *aex-4* promoter is sufficient to rescue *aex-4* expulsion defect. (B) Neuronal (P*rab-3*) expression of *aex-2* rescues the decreased frequency of aBoc in *aex-2* mutants. Intestinal (P*ges-1* or P*vha-6*) expression of *aex-4* and *aex-5* rescues their respective mutants decrease in aBoc frequency. GABAergic expression of *aex-2* did not suppress this particular aBoc defect of *aex-2* mutants (C) The timing of aBoc is restored by neuronal (P*rab-3*) or GABAergic (P*unc-47*) expression of *aex-2* in *aex-2* mutants. The aBoc timing defects of *aex-4* and *aex-5* are rescued by intestinal (P*ges-1* or P*vha-6*) expression of these genes. *gsa-1* gain-of-function mutants do not suppress the aBoc defects of *aex-2* mutants (B and C). *$p<0.05$, **$p<0.005$, ***$p<0.0005$ significantly different from the respective mutant. +$p>0.05$ not significantly different from wild type. Error bars represent SEM.

SI Fig. 4. Rescue of *aex* mutants with genomic and fluorescent protein tagged clones and rescue of aBoc defects with tissue specific constructs.



SI Fig. 5. *aex-4* mutants accumulate AEX-5::VENUS in the intestine. (A) In *aex-4* mutants, the average fluorescence, normalized to *aex-2*, of intestinal AEX-5::VENUS is significantly greater than *aex-2* mutants. *$p<0.05$ significantly different from *aex-2*. (B) Representative photographs of anterior intestinal cells of *aex-2* and *aex-4* expressing AEX-5::VENUS. Scale bar, 5μm.

SI Fig. 5. *aex-4* mutants accumulate AEX-5::VENUS in the intestine.

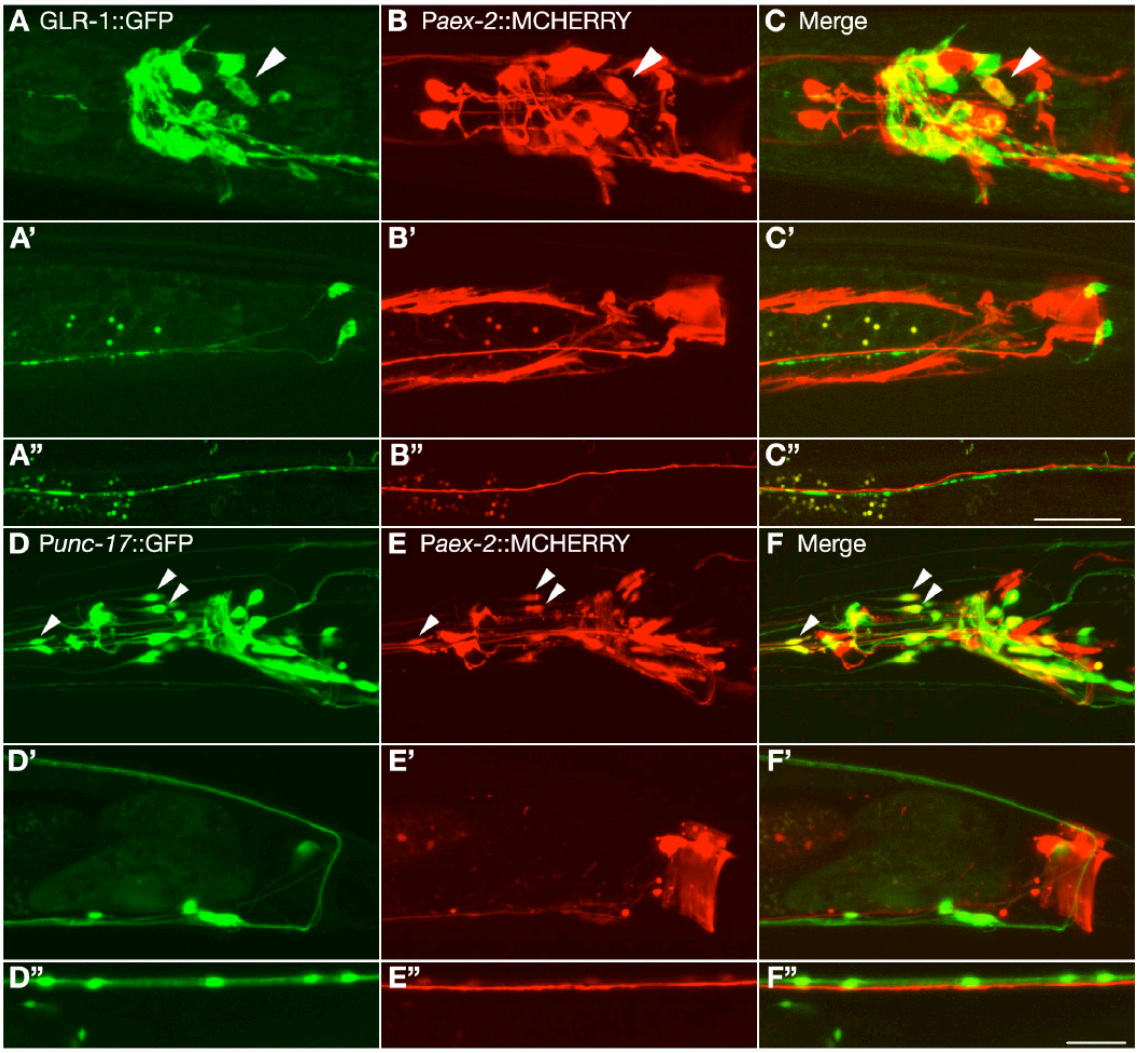


SI Fig. 6. *aex-2* encodes a GPCR. The transmembrane domains of AEX-2 (T14B1.2) are predicted by the free online tool SMART (http://smart.embl-heidelberg.de/) and marked by thick lines. The changes in specific residues in various *aex-2* alleles are indicated. Light grey italic letters mark residues that are conserved in class A GPCRs. *aex-2(sa21)* encodes a stop codon at Y144, *aex-2(sa1040)* encodes a S174G mutation, and *aex-2(sa3)* encodes an R232Q mutation.

SI Fig. 6. *aex-2* encodes a GPCR.

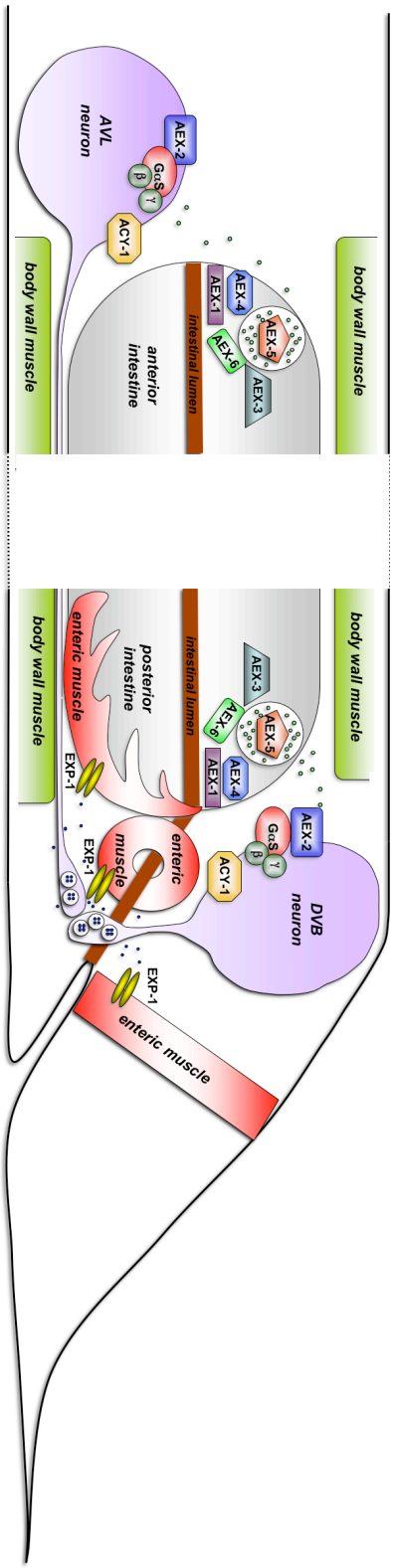
SI Fig. 7. *aex-2* is likely expressed in subsets of glutamatergic and cholinergic neurons. Fluorescence images show transgenic animals that express (A-C") mCherry and GLR-1::GFP under the *aex-2* and glutamate receptor *glr-1* promoter, respectively, and (D-F") mCherry and GFP under the *aex-2* and vesicular acetylcholine transporter *unc-17* promoter, respectively. The individual panel shows the fluorescence expression in (A-F) head ganglia, (A'-F') tail ganglia, and (A"-F") ventral nerve cord. At least one glutamatergic interneuron, possibly AVD, and several cholinergic neurons in the head and pharynx are co-labeled by P*aex-2*::mCherry (arrow heads). Scale bar, 20mm.

SI Fig. 7. *aex-2* is likely expressed in subsets of glutamatergic and cholinergic neurons.



SI Fig. 8. Model of intestinal to neuronal signaling in the defecation motor program. Diagrammed are the posterior and anterior intestinal regions in *C. elegans* showing the position of body wall muscle, enteric muscle, the intestine, and the AVL and DVB GABAergic motor neurons. In the anterior and posterior intestine AEX-1, AEX-3, AEX-4 and AEX-6 regulate the release of AEX-5 containing vesicles that we propose also release the AEX-2 ligand. The ligand diffuses to activate AVL and DVB via activation of $G_{\alpha}s$ (GSA-1) and adenylyl cyclase (ACY-1) coupled to the GPCR AEX-2. This in turn causes release of GABA onto enteric muscles that are activated via the excitatory GABA receptor EXP-1. Activation of the enteric muscle results in expulsion of gut content via the anus. Not depicted are muscle arms of the enteric muscles that contact the presynaptic specializations of DVB and perhaps AVL. The detailed anatomy of these structures is available at www.wormatlas.org.

SI Fig. 8. Model of Intestinal to neuronal signaling in the defecation motor program.



SI Movie 1. Defecation Motor Program (DMP) movie. The DMP is described in Fig. 1A. *rbf-1(js232)* was used because these animals move very little when unstimulated.

SI Movie 2. Activation of channelrhodopsin-2 by blue light suppresses expulsion defects in *aex-5(sa23)*. The first DMP is in the absence of light. The animal has a clear pBoc with no expulsion in the first cycle. In the subsequent defecation cycle, a brief pulse of blue-light following pBoc induces expulsion.

SI Materials and Methods

Growth and Culture of *C. elegans* and General Methods

Caenorhabditis elegans were grown at 22.5°C as described (Wood 1988). Standard cloning and molecular biology methods were used, unless otherwise described (Sambrook and Russell 2001).

Strains Used

N2(wild type) (Brenner 1974), *acy-1(js127)* (Saifee 2003), *aex-1(sa9)* (Thomas 1990; Doi and Iwasaki 2002), *aex-2(sa3)* (Thomas 1990), *aex-3(js815)* (Iwasaki, Staunton et al. 1997; Mahoney, Liu et al. 2006), *aex-4(n2415)*, *aex-4(sa22)*, *aex-4(ok614)*, *aex-5(sa23)* (Thomas 1990), *aex-6(sa24)* (Thomas 1990; Mahoney, Liu et al. 2006), *lin-15(n765) zxEx51*[P*unc-47::chop-2(H134R)::yfp; lin-15*$^{+}$], *unc-25(e156)* (Jin, Jorgensen et al. 1999), *rde-1(ne219); kbIs7[*P*nhx-2::rde-1; rol-6]* (Espelt, Estevez et al. 2005), *gsa-1(ce81)* (Schade, Reynolds et al. 2005), *dgk-1(sy428)* (Nurrish, Segalat et al. 1999; Miller and Rand 2000), *egl-30(js126)* (Saifee 2003), *gsa-1(ce81); aex-2(sa3), dgk-1(sy428); aex-2(sa3), egl-30(js126) ; aex-2(sa3), rbf-1(js232)* (Staunton, Ganetzky et al. 2001; Mahoney, Liu et al. 2006), *unc-119(ed3); jsIs1072*[NM2072 (P*vha-6*::AEX-5::VENUS CbUNC-119)], *gsa-1(pk75)*; *pkEx270*[*rol-6(su1006) gsa-1*(+)](Korswagen, Park et al. 1997), and *acy-1(pk1279);* ceEx108(myo-3::acy-1) (Reynolds, Schade et al. 2005).

aex-4(n2415) jsEx904[NM1427(P*aex-4::*NLS::GFP) + pRF4(rol-6)], *aex-4(n2415) jsEx905*[NM1471(genomic *aex-4*) + pRF4(rol-6) line1], *aex-4(n2415) jsEx906*[NM1471(genomic *aex-4*) + pRF4(rol-6) line2], *aex-4(n2415)*

jsEx960[NM1726(P*ges-1::aex-4*) + pRF4(rol-6) line1], *aex-4(n2415)* *jsEx964*[NM1726(P*ges-1::aex-4*) + pRF4(rol-6) line2], *aex-4(n2415)* *jsEx961*[NM1727(P*myo-3::aex-4*) + pRF4(rol-6) line1], *aex-4(n2415)* *jsEx967*[NM1727(P*myo-3::aex-4*) + pRF4(rol-6) line2], *aex-4(n2415)* *jsEx969*[NM1742(P*rab-3::aex-4*) + pRF4(rol-6) line1], *aex-4(n2415)* *jsEx970*[NM1742(P*rab-3::aex-4*) + pRF4(rol-6) line2], *aex-4(n2415)* *jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(rol-6) line1], *aex-4(n2415)* *jsEx994*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(rol-6) line2], *aex-4(n2415)* *jsEx1028*[NM1609(P*aex-4::*GFP*::aex-4*) + pRF4(rol-6) line1], *aex-4(n2415* *zxEx51*[P*unc-47::chop-2(H134R)::yfp; lin-15*$^{+}$], *aex-4(n2415); jsIs1072*[NM2072 (P*vha-6*::AEX-5::VENUS CbUNC-119)]

aex-5(sa23) jsEx1057 [WRM068bG11[aex-5 fosmid]; pPD118.25(plet-858::NLS-GFP); pRF4(rol-6)], *aex-5(sa23) jsEx1061*[NM2059(P*vha-6::aex-5::*VENUS) + pRF4(rol-6) line1], *aex-5(sa23) jsEx1062*[NM2059(P*vha-6::aex-5::*VENUS) + pRF4(rol-6) line2], *aex-5(sa23) jsEx1063*[NM2061(P*rab-3:: aex-5::*VENUS) + pRF4(rol-6) line1], *aex-5(sa23) jsEx1064*[NM2061(P*rab-3:: aex-5::*VENUS) + pRF4(rol-6) line2], *aex-5(sa23) jsEx1066*[NM2062(P*myo-3:: aex-5::*VENUS) + pRF4(rol-6) line1], *aex-5(sa23) jsEx1067*[NM2062(P*myo-3:: aex-5::*VENUS) + pRF4(rol-6) line2], *aex-5(sa23) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(rol-6) line1], *aex-5(sa23) jsEx994*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(rol-6) line2], *aex-5(sa23) zxEx51*[P*unc-47::chop-2(H134R)::yfp; lin-15*$^{+}$], *aex-5(sa23); jsIs1072*[NM2072 (P*vha-6*::AEX-5::VENUS CbUNC-119)]

aex-2(sa3) jsEx937[NM2099(P*aex-2*::*aex-2*::*mCherry*) + pPD118.33(P*myo-2*::*gfp*) line1], *aex-2(sa3) jsEx938*[NM2099(P*aex-2::aex-2::mCherry*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx924*[NM2100(P*aex-2::aex-2::YPet*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx921*[NM2101(P*rab-3::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx922*[NM2101(P*rab-3::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx925*[NM2102(P*myo-3::aex-2::yfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx928*[NM2102(P*myo-3::aex-2::yfp*) + pPD118.33 (P*myo-2::gfp*) line2], *aex-2(sa3) jsEx929*[NM2103(P*ges-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx930*[NM2103(P*ges-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *nuIs25*[P*glr-1::glr-1::gfp*] *jsEx976*[NM1736(P*aex-2::mCherry*) line1], *oxIs12*[P*unc-47::gfp*] *jsEx978*[NM1736(P*aex-2::mCherry*) line1], *mdIs135*[P*unc-17::gfp*] *jsEx977*[NM1736(P*aex-2::mCherry*) line1], *aex-2(sa3) jsEx999*[NM1707(P*glr-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1000*[NM1707(P*glr-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx1007*[NM1841(P*unc-17::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1008*[NM1841(P*unc-17::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx1003*[NM1843(P*unc-47::aex-2::gfp*) + PD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1004*[NM1843(P*unc-47::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx1026*[NM1778(P*unc-25::acy-1(js127))* + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1027*[NM1778(P*unc-25::acy-1(js127))* + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3); zxEx51*[P*unc-47::chop-2(H134R)::yfp; lin-15*$^{+}$], *aex-2(sa3); jsIs1072*[NM2072 (P*vha-6*::AEX-5::VENUS CbUNC-119)]

Promoters used in this study

Neuronal (P*rab-3*, *rab-3* promoter) (Nonet, Staunton et al. 1997), intestinal (P*ges-1*, *ges-1* promoter) (Marshall and McGhee 2001), intestinal (P*vha-6*, *vha-6* promoter) (Oka, Toyomura et al. 2001), muscle (P*myo-3*, *myo-3* promoter) (Okkema, Harrison et al. 1993), cholinergic neurons (P*unc-17*, *unc-17* promoter) (Sieburth, Ch'ng et al. 2005), subsets of interneurons (P*glr-1*, *glr-1* promoter) (Hart, Sims et al. 1995; Maricq, Peckol et al. 1995), GABAergic neurons (P*unc-47*, *unc-47 promoter*) (McIntire, Reimer et al. 1997), and GABAergic neurons (P*unc-25*, *unc-25* promoter) (Jin, Jorgensen et al. 1999). P*aex-4* and P*aex-2* (*aex-4* and *aex-2* promoters) are described in this study (see below for details).

Transgenic Animals

Transgenic animals were generated as previously described (Mello, Kramer et al. 1991). Briefly, QIAGEN (Valencia, CA) purified plasmids (5-50ng/µl) were coinjected with either 150ng/µl pRF4 (*rol-6* dominant marker) or 5ng/µl pPD118.33 (*myo-2::GFP* a gift of A. Fire) and 100ng/µl pBluescript into wild type or mutant animals. Transgenic progeny were identified in the following generation by the presence of the dominant *rol-6* or *myo-2::GFP* marker,. *unc-119(ed3); jsIs1072* [NM2072 (P*vha-6*::AEX-5::VENUS CbUNC-119)] was created by ballistic transformation of *unc-119(ed3)* using standard methods (Praitis, Casey et al. 2001).

Generation of the *C. elegans* strain expressing ChR2 in GABAergic neurons

The *punc-47::chop-2(H134R)::yfp* construct was generated by amplifying a 1.44 kb genomic fragment upstream of the *unc-47* start codon via PCR, using primers 5'-

CCCCGCAAGCTTGTTGTCATCACTTCAAACTTTTCAATG-3' and 5'-CCCCGCTGATCACTGTAATGAAATAAATGTGACGCTGT-3'. AfterHindIII/BclI cleavage, the fragment was ligated into a *pmec-4::chop-2(H134R)::yfp* vector (Nagel, Brauner et al. 2005), where *pmec-4* was removed using HindIII and BamHI. *punc-47::chop-2(H134R)::yfp* was injected together with a *lin-15* rescuing construct into *lin-15(n765ts)* animals at a concentration of 80 ng/ml each, to yield strain ZX416 (*lin-15(n765ts); zxEx51[punc-47::chop-2(H134R)::yfp; lin-15^{+}]*).

Plasmid Construction

NM1427- *aex-4* promoter driving GFP tagged to a nuclear localization signal. A 1.1kbp *aex-4* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-CAACGTTCTAGAACTCCAGCGAACATCAAGCTAC-3' and 5'-CTAGTGCCCGGGTGAATCAATGGTTTTTCTAGCCAT-3' digested with XbaI and XmaI, and inserted into XbaI-XmaI pPD95.70 (A. Fire).

NM1471- genomic clone of *aex-4* containing promoter, coding region, and 3'UTR. A 4kbp *aex-4* genomic sequence was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-GAACGTCCCGGGACTCCAGCGAACATCAAGCTAC-3' and 5'-CTCTATGGGCCCCATCGCTACTTCTACTCTTTCTC-3' digested with XmaI and ApaI, and inserted into XmaI-ApaI pBluescript KS(-).

NM1526- genomic clone of *aex-4* containing promoter, N-terminal multiple cloning site, coding region, and 3'UTR. Performed quick change PCR reaction of NM1427 using

oligonucleotides 5'-GTTTCATCAAAAATCAAAAAATTGCAAAACCTAGGTTAATTAATCCATGG ATGGCTAGAAAAACCATTGATTCGTAAG-3' and 5'-CTTACGAATCAATGGTTTTTCTAGCCATCCATGGATTAATTAACCTAGGT TTTGCAATTTTTTGATTTTTGATGAAAC -3' digested with DpnI and transformed into DH5α chemically competent cells.

NM1609- *aex-4* promoter driving eGFP fused to the N-terminus of *aex-4* coding region. A 700bp eGFP sequence was amplified in a PCR from NM1090(pRAB100) using oligonucleotides 5'-CATGTCCCTAGGATGGTGAGCAAGGGCGAGGAG-3' and 5'-GAATCCATGGTCCGCGGCCGTCCTTGTACAG-3' digested with AvrII and NcoI, and inserted into AvrII-NcoI NM1526.

NM1404- *ges-1* (intestinal) promoter driving eGFP. A 2.1kbp *ges-1* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-TAACCCGGGAATCGCATTTCAAACTG-3' and 5'-TAACCATGGTCATCTGAATTCAAAGATAAG-3' digested with XmaI and NcoI, and inserted into XmaI-NcoI NM1019 (Mahoney, Liu et al. 2006).

NM1726- *ges-1* (intestinal) promoter driving *aex-4* coding region. A 2.1kbp *ges-1* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-TAACCCGGGAATCGCATTTCAAACTG-3' and 5'-

TAACCATGGCATCTGAATTCAAAGATAAG-3' digested with XmaI and NcoI, and inserted into XmaI-NcoI NM1526.

NM1727- *myo-3* (muscle) promoter driving *aex-4* coding region. A 2.6kbp *myo-3* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-GAATGGATCCTCTGTTTTCGTTAATTTTGAATTTTG-3' and 5'-GATTTCCCATGGCATTTCTAGATGGATCTAGTGGTCGTG-3' digested with BamHI and NcoI, and inserted into BamHI-NcoI NM1526.

NM1742- *rab-3* (neuronal) promoter driving *aex-4* coding region. A 1.2kbp *rab-3* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-CTGCAGCCCGGGGGATCTTCAGATGGGAGCAGTG-3' and 5'-GTGGCGACCCATGGCATCTGAAAATAGGGCTACTGTAG-3' digested with XmaI and NcoI, and inserted into XmaI-NcoI NM1526.

NM1610- *ges-1* (intestinal) promoter driving GFP with an N-terminal multiple cloning site. Performed quick change PCR reaction of NM1404 using oligonucleotides 5'-CATATCTTATCTTTGAATTCAGCCTAGGTGGTACCACTCGAGATGACCAT GGTGAGCAAGGGCGAG-3' and 5'-CTCGCCCTTGCTCACCATGGTCATCTCGAGTGGTACCACCTAGGCTGAAT TCAAAGATAAGATATG-3' digested with DpnI and transformed into DH5α chemically competent cells. Resulting clone (NM1528) had mutations in GFP. A 1.1kbp

sequence containing GFP and the rim3' UTR was amplified in a PCR from NM1090(pRAB100) using oligonucleotides 5'-CTAGCACTCGAGATGACCATGGTGAGCAAGGGCGAGGAG-3' and 5'-CAGCTATGACCATGATTACGCCAAGCGCGCAATTAAC-3' digested with XhoI and BssHI, and inserted into XhoI-BssHI NM1528. The reading frame was shifted just before the GFP ATG (CCTAGGTGGTACCACTCGAGATGACC was converted to CCTAGGTGGTACCACTCGAGCATGACC) during the subcloning of the new GFP, which adds a C before the ATG of GFP and after the XhoI site shifting the reading frame 1+.

NM1728- *ges-1* (intestinal) promoter driving *aex-5* cDNA fused to out-of-frame GFP. A 1.6kbp sequence containing AEX-5 cDNA was amplified in a PCR using oligonucleotides 5'-CTCCAACCTAGGATGAAATTAATTTTCCTGCTTTTGCTTTTTG-3' and 5'-GTGAAGCTCGAGTATGACATTGTTCCCACCACTTTGAAC-3' digested with AvrII and XhoI, and inserted into AvrII-XhoI NM1610. The resulting clone (NM1728) has a base pair deletion that causes GFP to be out of frame. The following sequence TCATACTCGAGGATGA was converted TCTACTCGAGGATGA.

NM1977- *ges-1* (intestinal) promoter driving *aex-5* cDNA fused to VENUS. A 900bp sequence containing the VENUS variant of GFP with artificial introns was amplified in a PCR from NM1358(pRab3VENUSrim3') using oligonucleotides 5'-

GAGAGTCTCGAGTAATGAGTAAAGGAGAAGAAC-3' and 5'-GATCTACCGCGGCTATTTGTATAGTTCATCCATGCCAAG-3' digested with XhoI and SacII, and inserted into XhoI-SacII NM1728.

NM2059- *vha-6* (intestinal) promoter driving *aex-5* cDNA fused to VENUS. A 1.2kbp *vha-6* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-GTCGATCCCGGGCATGTACCTTTATAG-3' and 5'-GTCAGTCCTAGGGGGTAGGTTTTAG-3' digested with XmaI and AvrII, and inserted into XmaI-AvrII NM1977.

NM2061- *rab-3* (neuronal) promoter driving *aex-5* cDNA fused to VENUS. A 1.2kbp *rab-3* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-CTGCAGCCCGGGGGATCTTCAGATGGGAGCAGTG-3' and 5'-TCAGCACCTAGGCTGAAAATAGGGCTACTGTAG-3' digested with XmaI and AvrII, and inserted into XmaI-AvrII NM2059.

NM2062- *myo-3* (muscle) promoter driving *aex-5* cDNA fused to VENUS. A 2.5kbp *myo-3* promoter was amplified in a PCR from wild type genomic DNA using oligonucleotides 5'-GAATACCGGTTCTGTTTTCGTTAATTTTGAATTTTG-3' and 5'-GATTTCGCTAGCTTCTAGATGGATCTAGTGGTCGTG-3' digested with AgeI and NheI, and inserted into XmaI-AvrII NM2059.

NM2099- genomic clone of *aex-2* containing promoter, coding region, *mCherry* at the C'-terminus of coding region, and 3'UTR. A homologous recombination method was used to construct *aex-2* genomic plasmid (Warming, Costantino et al. 2005). Briefly, fosmid T14B1.2 containing aex-2 sequence was transformed into recombineering strain SW102. A *galK* gene was inserted into the C'-terminus of *aex-2* coding region with following oligonucleotides: forward GACGAGCATCTGAAAGGCCGCCGGAGCACACCCCCTTACGGTGTGATATGCC TGTTGACAATTAATCATCGGCA, reverse CATTTTTTCCACAAGTTTTACTTACATACATTGCGAATTACTACGATCTATCAG CACTGTCCTGCTCCTT. The galK gene was subsequently replaced by mCherry gene by homologous recombination with following oligonucleotides: forward GACGAGCATCTGAAAGGCCGCCGGAGCACACCCCCTTACGGTGTGATATGAT GGTGAGCAAGGGCGAGGAG, reverse CATTTTTTCCACAAGTTTTACTTACATACATTGCGAATTACTACGATCTACTTG TACAGCTCGTCCATGCC. In the final step, the whole fragment that contains *aex-2* promoter, *aex-2* coding sequence, mCherry coding sequence and 3'-UTR was gap repaired into an Amp containing vector backbone using oligonucleotides forward CATTGATCTGCCGCATGATGAAGTACCAAGTCTGAATGATGAAGAATTTCATT CGTTATGCATTATGGGTAC and reverse AATCAAACGACATTAACGATTTCTCAAAAAAAAAAAACTTTAGGAAAACATA CCAATCTAAGTCTGTGCTCC.

NM2100- genomic clone of *aex-2* containing promoter, coding region, *YPet* at the C'-terminus of coding region, and 3'UTR. NM2100 was constructed similarly to NM2099, except that the following oligonucleotides were used to replace *galK* with YPet rather than mCherry: forward, GACGAGCATCTGAAAGGCCGCCGGAGCACACCCCCTTACGGTGTGATATG ATGTCTAAAGGTGAAGAATTATTC, reverse CATTTTTTCCACAAGTTTTACTTACATACATTGCGAATTACTACGATCTA TTTGTACAATTCATTCATACCC.

NM2101- *rab-3* (neuronal) promoter driving *aex-2* cDNA fused to GFP at the C'-terminus. The 1kb *aex-2* cDNA was amplified using oligonucleotides 5'-AAGTCAGGATCCTCCACCATGAACTCAACGGACATTATTG-3' and 5'-ACTCATACTAGTCATATCACACCGTAAGGG-3'. The GFP containing vector backbone was amplified from the plasmid NM1019 (prab3GFPrim3') using oligonucleotides 5'-ACTCATACTAGTATGGCACCGGTCGCCAC-3' and 5'-AAGTCAGGATCCCTGAAAATAGGGCTACTGTAG-3'. The PCR fragments were purified, digested with BamHI and SpeI and ligated.

NM2102- *myo-3* (muscular) promoter driving *aex-2* cDNA fused to YFP at the C'-terminus. The 1kb *aex-2* cDNA was amplified using oligonucleotides 5'-AAGTCAGCTAGCTCCACCATGAACTCAACGGACATTATTG -3' and 5'-ACTCATAGGCCTCATATCACACCGTAAGGG -3'. The YFP containing vector

backbone was amplified from the plasmid NM783 (pPD132.112, Pmyo-3::YFP, A. Fire) using oligonucleotides 5'- ACTCATAGGCCTATGACTGCTCCAAAGAAGA -3' and 5' -AAGTCAGCTAGCTTTTTCTGAGCTCGGTACC -3'. The PCR fragments were purified, digested with NheI and StuI and ligated.

NM2103- *ges-1* (intestinal) promoter driving *aex-2* cDNA fused to GFP at the C'-terminus. The 1kb *aex-2* cDNA was amplified using oligonucleotides 5'-AAGTCAGGATCCTCCACCATGAACTCAACGGACATTATTG -3' and 5'-ACTCATACTAGTCATATCACACCGTAAGGG -3'. The GFP containing vector backbone was amplified from the plasmid NM1019 (Prab3GFPrim3') using oligonucleotides 5'- ACTCATACTAGTATGACCATGGTGAGCAAG -3' and 5' -AAGTCAGGATCCCTGAATTCAAAGATAAGATATG -3'. The PCR fragments were purified, digested with BamHI and SpeI and ligated.

NM1736- 10kb *aex-2* promoter driving *mCherry* cDNA. The vector backbone was amplified from the plasmid NM1019 (prab3GFPrim3') using oligonucleotides 5'-AACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGT AAGCCGCGGATAACAAATTTCATA -3' and 5'-AATCAAACGACATTAACGATTTCTCAAAAAAAAAAAACTTTAGGAAAACATA CCAATCTAAGTCTGTGCTCC -3'. The final plasmid was made by gap repair from the intermediate fosmid T14B1.2 that contains *mCherry::aex-2*.

NM1707- *glr-1* (subsets of interneurons) promoter driving *aex-2* cDNA fused to GFP at the C'-terminus. The 2.5kb *glr-1* promoter was amplified using oligonucleotides 5'-GAAGTCCCCGGGCGTGCTCTGAAAATTCTTTTAT -3' and 5'-TACAGTGGATCCTGTGAATGTGTCAGATTGGG -3'. Then the PCR product and plasmid NM 2101 (Prab-3::aex-2::gfp) were digested with XmaI and BamHI and ligated.

NM1841- *unc-17* (cholinergic) promoter driving *aex-2* cDNA fused to GFP at the C'-terminus. The 3.5kb *unc-17* promoter was amplified using oligonucleotides 5'-TAGAAGTCCCCGGGTAGACCCAAAATGGTCCAAAA -3' and 5'-TACAGTGGATCCCTCTCTCTCTCCCCCTG -3'. Then the PCR product and plasmid NM2101 (Prab-3::aex-2::gfp) were digested with XmaI (partial digest for Punc-17) and BamHI and ligated.

NM1843- *unc-47* (GABAergic) promoter driving *aex-2* cDNA fused to GFP at the C'-terminus. The 1.45kb *unc-47* promoter was amplified using oligonucleotides 5'-TAGAAGTCCCCGGGATGTTGTCATCACTTCAAACTT -3' and 5'-TACAGTGGATCCCTGTAATGAAATAAATGTGACG -3'. Then the PCR product and plasmid NM2101 (Prab-3::aex-2::gfp) were digested with XmaI and BamHI (partial digest for Punc-47) and ligated.

NM1778- *unc-25* (GABAergic) promoter driving *acy-1 (js127, gain of function allele)*. The 1.5kb *unc-25* promoter was amplified using oligonucleotides 5'-

GAAGTCCCGCGGGCCGAAATTTAAAGCTAGTTT -3' and 5'-TACAGTGGATCCTTTTGGCGGTGAACTGAGC -3'. Then the PCR product and plasmid NM964 pAC5 (psnb-1::acy-1(js127)) with SacII and BamHI and ligated.

NM2072- *vha-6* (intestinal) promoter driving *aex-5* cDNA fused to VENUS vector includes a copy of the *C. briggsae unc-119* gene (also called P*vha-6*::AEX-5-VENUScbUNC-119). Plasmid pDONR221-CBunc-119 (which contains a 2.0 kb C. briggsae unc-119 gene fragment in pDONR 221, a gift of M. Driscoll) was digested with PvuII and XbaI and inserted into SmaI-XbaI NM2059.

Imaging and *C. elegans* Anatomy

All images are shown with the anterior end to the left and dorsal side on the top. For a guide to *C. elegans* anatomy we suggest the following sources (Altun and Hall 2002-2006; Jorgensen 2005; McGhee 2007). Images were taken on an Olympus FluoView FV500 scanning confocal microscope. Since the *C. elegans* intestine has higher auto-fluorescence than other tissue, an image was taken in the red channel (RFP filter sets) while imaging GFP or VENUS in the intestine in order to distinguish auto-fluorescence from fluorescent protein signal. Images were analyzed using either MetaMorph imaging software or NIH ImageJ and subsequently processed in Adobe Photoshop.

AEX-5::VENUS images for quantification were taken on an Olymous BX60 with using a 60x objective with a 1.25x Optivar. Images were taken on a Retiga 2000R 12-bit

RGB camera (Q-imaging). Photos were acquired using Openlab software (Improvision), and subsequently analyzed using NIH ImageJ. Regions of interest were selected by outlining the two anterior coelomocytes of L4 larval stage animals. The total amount of fluorescence was determined by subtracting background fluorescence from the integrated density (which is the total amount of fluorescence in a given area). The normalized fluorescence was determined by dividing each value by the average wild type fluorescence. AEX-5::VENUS accumulation in the intestine was determined by imagin the anterior intestinal cells. Average fluorescene, with background subtraction, was determined using NIH ImageJ. Statistical significance was determined by using unpaired two-tailed student t tests, with unequal variance.

RNAi experiments

The RNAi experiments were performed based on a modified feeding protocol (Espelt, Estevez et al. 2005). Briefly, bacterial strains that express double stranded RNA targeting individual *aex* genes were inoculated from single colonies on freshly streaked plates and allowed to grow to $O.D._{600}$>1.0. About 50ml of the liquid culture was spotted onto each well of 24 well plates, which was supplemented with 100mg/ml Ampicillin and 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG), and the plates were incubated at RT for 12 hours to induce double stranded RNA expression. Two L4 intestinal RNAi-sensitive animals (*rde-1(ne219); kbIs7*[P*nhx-2::rde-1*]) (Espelt, Estevez et al. 2005) were then picked onto each well and allowed to give progeny at 22°C. Two to three days later,

8 F1 young adults per well were scored for their expulsion defects. Statistical significance was determined by using unpaired two-tailed student t tests, with unequal variance.

Behavioral Assay

Defecation was assayed as previously described (Thomas 1990). Briefly, the 10 cycles of the defecation motor program of 8-20 one-day old (24hrs after the L4 larval stage) adults was observed on a dissecting stereomicroscope. Expulsions were recorded at all times, this included release events and ectopic expulsions seen in some gain-of-function mutants. When appropriate defecation cycles were recorded using the program Etho Java event recording software as described in (Thomas 1990). Statistical significance was determined by using unpaired two-tailed student t tests, with unequal variance.

Channelrhodopsin-2 Experiments

The assay was modified from (Nagel, Brauner et al. 2005). NGM agar plates were spotted with OP50 bacterial cultures with or without 500μM all-*trans* retinal (Sigma). 10 animals expressing the *zxEx51* transgene were picked onto the 500μM all-*trans* retinal spotted plates and grown overnight at 22°C. Young adult animals were observed on a Leica MZ16F fluorescent stereomicroscope with an x-Cite 120 excitation light source (EXFO). The defecation motor program was recorded for 5 cycles in the absence of blue light. The next 10 cycles were recorded with a brief (1-5 sec) pulse of blue light (using a standard GFP filter) just after (about 1-3 sec) the pBoc contraction. 5 more cycles were recorded in the absence of blue light. We did not detect a significant difference between expulsion frequencies recorded before the blue light activation

procedure and the expulsion frequencies recorded after the blue light activation procedure. The blue light flashes were repeated on *zxEx51* positive animals in the absence of all-*trans* retinal with no noticeable change in the frequency of expulsions from mutant strains, demonstrating the specificity of the activation of ChR2. Blue light was not left on as this generally left the *zxEx51* positive animals paralyzed. Blue light on wild type worms caused an increase in locomotion and sometimes inhibited defecation. Statistical significance was determined by using unpaired two-tailed student t tests, with unequal variance.

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Chapter 3

Intestine-Derived Signals Regulate Synaptic Transmissions in *Caenorhabditis elegans*

Intestine-Derived Signals Regulate Synaptic Transmissions in *Caenorhabditis elegans*

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Abstract

The ability of animals to modulate their behaviors based on environmental cues critically depends on the modulation of nervous system functions. Here we describe a pathway whereby *C. elegans* intestinal epithelial cells secret a signal(s) to regulate cholinergic synaptic transmission. Using genetic and pharmacological analyses, we demonstrate that the prohormone converting enzyme AEX-5 modulates cholinergic transmission from the intestine. The intestine derived signals appear to act on GABAergic neurons through AEX-2, a G-protein coupled receptor that is coupled with downstream Gsα and adenylate cyclase signaling. Interestingly, the AEX-5/AEX-2 signaling has been previously shown to control the rhythmic defecation motor program by activating GABA release from GABAergic neurons. In contrast, we find that for the modulation cholinergic transmission, AEX-5/AEX-2 depends at least partially on neuronal peptide signals, while GABA is dispensable for the process. We further show

that in the absence of food, the decrease in the defecation frequency of *C. elegans* is accompanied by reduced cholinergic transmission, and such decrease in cholinergic transmission is not seen when the adenylate cyclase is activated in GABAergic neurons. We hypothesize that the *C. elegans* intestine coordinates the rhythmic behavior and neuronal functions by controlling the secretion of neural modulatory signals.

Introduction

The proper modulation of synaptic strength is essential for the nervous system function and neuronal plasticity. At central synapses, the modulation of synaptic strength involves alterations in pre- and/or postsynapses, and many lines of evidence suggest that trans-synaptic secretory molecules play important roles in inducing and coordinating pre- and postsynaptic changes. For example, at 'silent' hippocampal synapses, activation of the postsynapse by glutamate neurotransmission triggers the insertion of AMPA type glutamate receptors into the postsynaptic site (Durand et al., 1996; Isaac et al., 1995; Renger et al., 2001). In contrast, at hippocampal and cerebellar synapses, depolarization of postsynaptic neurons induces the release of endocannabinoids that acts on presynaptic cannabinoid receptors, resulting in presynaptic suppression (Alger and Pitler, 1995; Kreitzer and Regehr, 2001a; Kreitzer and Regehr, 2001b; Wilson and Nicoll, 2001). Interestingly, increasing evidence suggests that such synaptic modulatory signals are also present in non-neuronal tissues, and they may modulate neuronal functions through a secretory pathway. For example, at fly neuromuscular junctions (NMJs), the exocytic protein synaptotagmin 4 (Syt 4) functions in the muscle to regulate the induction of presynaptic plasticity (Yoshihara et al., 2005). Similarly, a *C. elegans* Munc-13 homologue protein AEX-1 is expressed in muscles, and it functions in the muscle to maintain normal synaptic transmission (Doi and Iwasaki, 2002). These observations imply an interesting interaction between neuronal and non-neuronal tissues. However, as so far our knowledge on the regulation of neuronal functions by non-neuronal tissues is

still limited, in many situations the identities of such elusive secretory molecules and their signaling mechanisms underlying the synaptic modulation remain unknown.

The *C. elegans* intestine serves as an important organ to regulate multiple biological processes in the nematode such as food digestion, defecation, stress response, and host-pathogen interactions (McGhee, 2007). Composed of polarized epithelial cells, the intestine is involved in intensive cross-membrane trafficking, and a number of exocytic and endocytic factors have been shown expressed and functioning in the intestine (Chen et al., 2006; Doi and Iwasaki, 2002; Mahoney et al., 2006a; Parker et al., 2009; Yamashita et al., 2009). Interestingly, several recent observations suggest that the intestine may secret signals to modulate neuronal functions. For example, during rhythmic defecation cycles, a Ca^{2+} wave is initiated near the posterior end of the intestine and it propagates through the intestine (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006). Blocking the Ca^{2+} oscillation in the intestine by drugs or by disrupting an intestinal gap junction not only prevents the execution of subsequent neuron-controlled muscle contractions, but also leads to phenotypes indicative of altered synaptic transmissions (Peters et al., 2007; Teramoto and Iwasaki, 2006). Thus, the *C. elegans* intestine may function as an endocrine organ to modulate synaptic functions. It would be interesting to identify the molecules that are involved in synaptic modulations from the intestine.

Here we report the identification of a peptidergic pathway that acts in the intestine to modulate cholinergic transmissions in *C. elegans*. We find that the prohormone converting enzyme AEX-5 is required in the intestine to maintain normal cholinergic

functions. To our surprise, GABAergic neurons appear to be downstream of the intestinal signal, as epistatic analysis genetically places GABAergic GPCR AEX-2 downstream of AEX-5. The stimulatory Gsα subunit and adenylate cyclase are involved in relaying AEX-2 signals, and the activation of adenylate cyclase in GABAergic neurons specifically suppresses *aex-2* and *aex-5* cholinergic defects. Previously, we have shown that AEX-5/2 signaling acts on GABAergic neurons to activate enteric muscle contractions, a defecation process that depends on neurotransmitter GABA (Mahoney et al., 2008). We show here that GABA is dispensable for modulating cholinergic functions, while two neuronal prohormone processing enzymes, EGL-3 and EGL-21, are required at least in part downstream of AEX-2. Interestingly, the stimulation of worm defecation by expressing an IP3 receptor in the intestine also enhances its aldicarb sensitivity, a phenotype indicative of enhanced cholinergic transmissions. Such enhancement is not observed when the functional AEX-5 is absent. Together, our results support a model where the *C. elegans* intestine acts as an endocrine organ to coordinate the synaptic transmission and the defecation motor program by secreting neural modulatory signals.

Results

aex-5 mutants are resistant to cholinesterase inhibitor aldicarb

We have previously shown that the prohormone converting enzyme AEX-5 functions in the intestine to regulate GABAergic functions during the defecation (Mahoney et al., 2008). As intestine may also modulates cholinergic functions, we decided to examine if AEX-5 is involved in such modulatory signaling from the intestine. We used aldicarb-induced paralysis assays to assess the status of cholinergic transmission in *aex-5* mutants. Interestingly, *aex-5* worms show delayed paralysis in the presence of 1mM aldicarb, indicating reduced cholinergic transmissions in these animals (Fig 1A).

aex-5 animals have apparently normal acetylcholine receptor (AchR) surface expression and gross neural anatomy

The altered cholinergic transmissions seen in *aex-5* animals could be caused by a combination of changes in presynaptic structures, functions, and postsynaptic receptors. To distinguish between these possibilities, we performed levamisole assays on *aex-5* animals. Levamisole is an acetylcholine receptor (AChR) agonist that paralyzes worm by causing muscle over-contractions. In the presence of 200μM levamisole, *aex-5* worms are paralyzed in a way comparable to wild type animals, while the acetylcholine receptor *unc-29* mutant remains completely resistant to the treatment (Fig 1B). This suggests that the surface expression of postsynaptic AChRs in *aex-5* worms remains generally unaffected. To assess whether *aex-5* mutants might exhibit cholinergic defects as a result of a general alteration in presynaptic structures, we performed RAB-3 (a synaptic vesicle

protein) and RIM (a presynaptic active-zone protein) immunostaining. We failed to detect any noticeable changes in general synaptic organizations in *aex-5* animals (Fig 1C, 1D). Together, these results suggest that the altered cholinergic transmission in *aex-5* animals likely results from reduced presynaptic functions.

aex-5 mutation does not affect presynaptic UNC-13 accumulations or glutamate receptor expressions in command interneurons

Several groups have previously managed to correlate synaptic activities with the synaptic localization of a specific UNC-13 isoform (a presynaptic vesicle priming protein) at presynaptic neurons (Doi and Iwasaki, 2002; Metz et al., 2007; Nurrish et al., 1999). To assess if AEX-5 directly regulates presynaptic functions, we crossed *aex-5* mutants into a UNC-13S::GFP expressing line and examined UNC-13S::GFP levels in the mutant dorsal cord, a region consisting of mainly neuromuscular synapses. As shown in Fig 2A-2C, both the wild type and *aex-5* dorsal cords exhibit comparable UNC-13S::GFP expressions, and the quantification of puncta numbers and puncta intensities reveals no significant difference (Fig 2A-2C). This suggests that AEX-5 does not affect cholinergic transmission by directly modulating presynaptic UNC-13 localizations. We then asked if the mutation in *aex-5* affects the ability of cholinergic motor neurons to receive input from presynaptic command interneurons. Both *C. elegans* command interneurons and motor neurons express ionotropic glutamate receptors (Brockie et al., 2001). We examined the expression of a glutamate receptor GLR-1 in *aex-5* animals using a GLR-1::GFP line, as GLR-1 is known to function in regulating locomotion (Hart

et al., 1995; Maricq et al., 1995; Zheng et al., 1999). As shown in Fig 2D-2E, we failed to detect any significant changes in GLR-1::GFP puncta number and intensity in *aex-5* mutants. This suggests that the interneuron-motor neuron circuits in *aex-5* mutants are roughly normal at least in terms of their ability to receive inputs through GLR-1 receptors.

AEX-5 acts in the intestine to regulate synaptic transmission

To assess where AEX-5 acts to regulate synaptic transmission, we directed the expression of AEX-5::VENUS fusion proteins to neurons (P*rab-3* driven), muscles (P*myo-3* driven), or intestines (P*vha-6* driven) and examined which expression rescues *aex-5* defects. As shown in Fig 3A, intestinal expression of AEX-5::VENUS fully restores the aldicarb sensitivity in *aex-5* mutants (Fig 3A). In comparison, neuronal and muscular AEX-5::VENUS also confers noticeable rescue activities, which could be caused by leaky expressions of AEX-5 under high expression levels or by the ubiquitous presence of AEX-5 in multiple tissues (Fig 3A). To figure out if AEX-5 is required only in the intestine for its function, we performed intestine-specific *aex-5* gene silencing using a transgenic line that is sensitive to RNAi only in the intestine (Espelt et al., 2005). As shown in Fig 3B, intestine specific knockdown of *aex-5* expression induces strong aldicarb resistance comparable to that observed in *aex-5* mutants. As a control, the intestine knockdown of *aex-2* (a neuronal GPCR) and other *aex* genes do not have similar effects (Fig 3B and data not shown). This suggests that AEX-5 is required in the intestine to maintain normal synaptic functions. Interestingly, consistent with our

previous findings, the intestinal RNAi of *aex-5* also induces strong defecation defects (Mahoney et al., 2008). This suggests that AEX-5 may regulate multiple aspects of neuronal functions from the intestine.

The GPCR AEX-2 regulates cholinergic functions from GABAergic neurons

Our previous study has shown that AEX-5 genetically acts on the neuronal GPCR AEX-2 to regulate defecation motor program in *C. elegans* (Mahoney et al., 2008). We then asked if AEX-2 is also involved in regulating cholinergic functions by performing aldicarb assays on the *aex-2* mutant. As shown in Fig 1A, *aex-2* mutants are resistant to 1mM aldicarb treatment, suggesting a reduced cholinergic transmission in these animals. Using levamisole assays and RAB-3/RIM immunohistochemistry, we found no obvious changes in postsynaptic AChR levels or presynaptic organizations in *aex-2* animals, suggesting AEX-2 mainly affects the efficacy of presynaptic transmissions (Fig 1B, 1C, 1D). Interestingly, like *aex-5* animals, *aex-2* mutants are normal in both UNC-13S::GFP and GLR-1::GFP expressions in nerve cords (Fig 2A-2E). These results support the notion that like AEX-5, AEX-2 modulates cholinergic functions through an indirect action on presynaptic cholinergic neurons.

The expression of AEX-2 has been previously detected in cholinergic neurons, GABAergic neurons, command interneurons, and enteric muscles (Mahoney et al., 2008). To assess where AEX-2 acts to regulate synaptic transmission, we then directed the expression of AEX-2::GFP fusion proteins to neurons (P*rab-3* driven), muscles (P*myo-3* driven), or intestines (P*ges-1* driven) and examined which expression rescues *aex-2*

defects. As shown in Fig 4A, neuronal expression of AEX-2::GFP fully restores the aldicarb sensitivity in *aex-2* mutants, while muscular and intestinal expressions do not, suggesting AEX-2 acts in neurons to regulate cholinergic function (Fig 4A). To further assess what neurons may underlie AEX-2's functions, we directed AEX-2::GFP expressions specifically to cholinergic neurons (P*unc-17* driven), GABAergic neurons (P*unc-47* driven), or subsets of command interneurons (P*glr-1* driven), and we examined their rescue activities on *aex-2* mutants. To our surprise, GABAergic expression of AEX-2::GFP best restores the aldicarb sensitivity in *aex-2* animals, while the cholinergic expression confers a partial rescue (Fig 4B). The expression of AEX-2 in interneurons fails to rescue aldicarb phenotypes (Fig 4B). In conclusion, these results suggest that AEX-2 mainly acts through GABAergic neurons to modulate synaptic transmissions, whereas we cannot fully rule out an involvement of cholinergic expression in AEX-2 mediated functions.

Gsα pathway is genetically downstream of AEX-2 and AEX-5 signaling

We next examined which Gα pathway may be involved in AEX-2 mediated synaptic modulations. We have previously shown that AEX-2 genetically acts on the stimulatory Gsα pathway (but not on Gqα or Goα pathways) to regulate defecation behaviors, and a Gsα gain-of-function allele fully rescues defecation defects in both *aex-2* and *aex-5* mutants (Mahoney et al., 2008). To find out if Gsα acts downstream of AEX-2 to modulate cholinergic functions, we constructed *aex-2; gsa-1(gf)* double mutants and examined the mutant for its aldicarb sensitivity. As shown in Fig 5A, both

gsa-1(gf) and *aex-2; gsa-1(gf)* animals exhibit similar level of hypersensitivity to 1mM aldicarb treatment, suggesting Gsα is downstream of AEX-2 (Fig 5A). In addition, *aex-5; gsa-1(gf)* animals also show similar level of aldicarb hypersensitivity as *gsa-1(gf)* mutants (Fig 5A). This indicates that the Gsα pathway likely acts downstream of AEX-2 and AEX-5 to regulate synaptic transmissions.

One caveat of the above experiments is that over-activating Gsα non-specifically (Gsα is widely expressed in *C. elegans*) may lead to non-specific suppression of synaptic transmission phenotypes. To address this issue, we made a transgenic line that specifically expresses an activated Gsα pathway component adenylate cyclase ACY-1 in GABAergic neurons using an *unc-25* promoter. We tested the aldicarb sensitivity of the transgenic line in both wild type and *aex-2*/*aex-5* mutant backgrounds. As shown in Fig 5B, expression of activated ACY-1 in GABAergic neurons alone induces robust aldicarb hypersensitivity in the transgenic worms (Fig 5B). Interestingly, when combined with an *aex-2* or *aex-5* mutant background, the transgene strongly suppresses the aldicarb resistant phenotype in *aex* mutants, making them comparable to hypersensitive ACY-1 transgenic worms (Fig 5B). This strongly supports the conclusion that the Gsα-adenylate cyclase pathway acts downstream of AEX-2 and intestinal AEX-5 to regulate synaptic transmissions.

AEX-2 acts at least in part through neural peptide signals to modulate cholinergic transmission

We then asked what molecules may act downstream of Gsα following AEX-2-Gsα activation. We first examined if neurotransmitter GABA is involved, as GABA is released from GABAergic neurons to activate defecation behaviors, and disrupting GABA synthesis in the nematode leads to defecation defects reminiscent of those observed in *aex-2* and *aex-5* mutants (Beg and Jorgensen, 2003; McIntire et al., 1993). We thus performed aldicarb assays on a GABA synthase *unc-25* mutant in which GABA is depleted. We found that unlike *aex-2* and *aex-5* mutants, *unc-25* animals are hypersensitive to aldicarb treatment, while all of them exhibit similar defecation defects (data not shown). This suggests that GABA is unlikely downstream of AEX-2 mediated synaptic modulations.

We thus considered if neuropeptides could be involved in the AEX-2 downstream signaling. The worm genome encodes four prohormone convertases to process premature peptides: KPC-1, EGL-3/ KPC-2, AEX-5/ KPC-3, and BLI-4/KPC-4 (Thacker and Rose, 2000). Among KPC-1, EGL-3, and BLI-4, we were especially interested in EGL-3, as EGL-3 is broadly expressed in the nervous system, and similar to *aex-5*, mutations in *egl-3* result in massive peptide processing defects (Husson et al., 2006; Kass et al., 2001). We thus performed aldicarb assays on *egl-3* mutants to see if cholinergic functions are altered in these animals. As shown in Fig 6A, *egl-3* mutants are resistant to 1mM aldicarb treatment, suggesting these animals have decreased cholinergic transmissions (Fig 6A). In addition, crossing *egl-3* animals into either *aex-2* or *aex-5* mutants does not enhance the aldicarb phenotype. This indicates that EGL-3 may act in the same genetic pathway as AEX-2 and AEX-5 (Fig 6A).

We next assessed where EGL-3 acts to regulate cholinergic functions. For this purpose, we drove EGL-3::VENUS expressions in neurons (using P*rab-3*), muscles (using P*myo-3*), or intestines (using P*vha-6*) and examined which expression rescues *egl-3* mutant phenotypes. As shown in Fig 6B, only neuronal expression of EGL-3::VENUS completely restores aldicarb sensitivity in *egl-3* mutants, indicating EGL-3 functions in the nervous system (Fig 6B). We then further asked if EGL-3 acts in certain neuronal subtypes to regulate synaptic transmission by driving EGL-3::VENUS expressions in cholinergic neurons (using P*unc-17*), GABAergic neurons (using P*unc-47*), or command interneurons (using P*glr-1*) of *egl-3* mutants. As shown in Table 1, all three expressions lead to partial rescues of the aldicarb sensitivity in *egl-3* animals (Table 1). This suggests that EGL-3 likely functions broadly in the nervous system rather than in specific neuronal subtypes to regulate cholinergic functions.

We then asked if the AEX-2/Gsα function requires the presence of neuropeptide signaling. For this purpose, we crossed the *egl-3* mutant into the GABAergic *acy-1(gf)* transgenic line, and we asked if the absence of neuropeptide processing blocks the *acy-1(gf)* induced aldicarb hypersensitivity. As shown in Fig 6C, GABAergic expression of activated ACY-1 induces aldicarb hypersensitivity in transgenic animals (Fig 6C). In contrast, disruption of *egl-3* in these worms significantly suppresses ACY-1 induced aldicarb hypersensitivity, suggesting the AEX-2/Gsα signaling requires functional EGL-3 at least in part to regulate cholinergic transmission (Fig 6C). We further tested if EGL-21, a carboxypeptidase E broadly expressed in neurons and involved in neuropeptide processing, is also required for the AEX-2/Gsα signaling (Jacob and Kaplan, 2003).

Consistent with previous findings, *egl-21* animals exhibit strong resistance to aldicarb treatment, indicating a decreased cholinergic transmission in these animals (Fig 6C). Noticeably, EGL-21 dysfunction nearly completely blocks ACY-1 induced aldicarb hypersensitivity, suggesting a neuropeptide signaling is required for the AEX-2/Gsα function (Fig 6C). Together, our results genetically place EGL-3 and EGL-21 downstream of AEX-2/Gsα pathway, and based on the available data they are at least partially required for the AEX-2/Gsa mediated synaptic modulations.

The *C. elegans* intestine likely coordinates the defecation behavior and synaptic transmission by secreting neural modulatory signals

We were curious on the ability of the *C. elegans* intestine to modulate both defecation behaviors and cholinergic functions. As the secretion of intestinal signals is likely triggered by the excitation of intestinal cells (as supported by Ca^{2+} oscillations), one working hypothesis is that, the nematode intestine secrets peptidergic signals in an activity-dependent manner to coordinately regulate defecation and synaptic transmission. If this were true, by modulating intestinal activities, we would expect to see coordinated changes in both defecation patterns and cholinergic functions. For this purpose, we first decreased the intestinal activity by starving animals, as food deprivation is known to trigger the defecation to cease (Liu and Thomas, 1994). As shown in Fig 7A, food deprivation in wild type young adults significantly reduces defecation frequencies, supporting an accompanied decrease in intestinal activities (Fig 7A). All the defecation-related muscle contractions are normal in these worms, suggesting they do not have

defecation defects (Fig 7B). Interestingly, the starved animals exhibit strong resistance to aldicarb treatment (Fig 7C). This suggests that they have reduced cholinergic transmission when food is scarce and the intestine is in a less active state. To test if the altered aldicarb sensitivity is specific to the GABAergic Gsα pathway, we further examined GABAergic *acy-1(gf)* transgenic animals. As shown in Fig 7A, starvation similarly reduces defecation frequencies in these transgenic worms (Fig 7A). In contrast, GABAergic specific expression of ACY-1(gf) completely suppresses starvation induced aldicarb resistance, and transgenic animals remain aldicarb hypersensitive even without food (Fig 7C). This suggests that GABAergic Gsα signaling may lie downstream of starvation induced changes to mediate the modulation on synaptic functions. Together, our results show that in *C. elegans*, both defecation patterns and cholinergic functions may rely on intestinal activities, and the nematode intestine likely functions as an endocrine organ to modulate multiple aspects of neuronal functions (Fig 8).

Discussion

The identification of neural modulatory signals has long been the focus of extensive studies in the neuroscience field, as they are believed to form the molecular basis for neural plasticity and normal brain functions. In this study, we have provided evidence that the *C. elegans* intestine is able to modulate cholinergic functions through a prohormone convertase pathway. Unexpectedly, the intestinal signals appear to act on GABAergic neurons, and the GABAergic signaling appears to partially depend on neuropeptide processing. Thus, we propose a novel mechanism through which non-neuronal tissues modulate neuronal functions via secretory peptides.

The *C. elegans* intestine likely uses exocytic proteins to control the secretion of AEX-5 and its peptide substrates. In our previous study, we showed a SNAP25 protein AEX-4 functions in the intestine to regulate *C. elegans* defecation, and it appears to regulate the secretion of AEX-5 from the intestine (Mahoney et al., 2008). Interestingly, we constantly detected medium-to-mild level of aldicarb resistance in *aex-4* mutants using three different *aex-4* alleles (data not shown). As AEX-4 is solely expressed in the intestine, this observation further supports an involvement of the *C. elegans* intestine in modulating synaptic functions. The milder aldicarb phenotype in *aex-4* animals (compared with *aex-5* mutants) also suggests the presence of redundant exocytic factors, which is supported by recent identification of an intestinally functioning exocytic factor in *C. elegans* (Yamashita et al., 2009). Therefore, we propose that the *C. elegans* intestine expresses a set of redundant exocytic factors to regulate the secretion of AEX-5

processed peptides. It would be interesting to identify additional exocytic proteins involved in intestine-specific secretions and neuronal modulations.

It is a bit surprising to see that GABAergic neurons could positively modulate cholinergic functions downstream of the intestine. We propose that neuropeptides may act downstream of GABAergic neurons to mediate the modulatory function, and this is supported by three observations: 1) Depleting GABA synthesis in *C. elegans* results in aldicarb hypersensitivity, which argues against an involvement of GABA in the modulation. 2) Major neuropeptide processing enzymes EGL-3 and EGL-21 are expressed and function in neurons. 3) Disrupting EGL-3 and EGL-21 functions strongly suppresses aldicarb hypersensitivity induced by GABAergic expression of activated Gsα signaling components. Consistent with the observations, GABAerigc neurons have been suggested to process and secret peptides. This includes AVL and DVB, two GABAergic neurons that express AEX-2 and are required for defecation (Li, 2005; Schinkmann and Li, 1992). Therefore, the AEX-2 expressing GABAergic neurons may modulate defecation and cholinergic transmission in a coordinated manner through GABA and neuropeptides. As GABAergic neurons do not directly synapse onto cholinergic neurons, we propose that the EGL-3/EGL-21 processed neuropeptides act on the cholinergic system in a hormonal manner. Nevertheless, we cannot fully exclude the involvement of other factors in the GABAergic neuron-mediated synaptic modulations.

Several recent studies suggest that non-neuronal tissues can secret small hormonal factors to regulate physiological processes and neuronal functions. One example, leptin, is secreted from adipose tissues, and it is shown to regulate food intakes as well as

nervous system functions such as learning and memory (Moult and Harvey, 2008). Thus, with the multi-functional modulatory signals, the organism may be able to couple their behaviors (such as food intake) with their neuronal functions as a feedback to the environment. Similarly, our identification of a common pathway that underlies the controls over defecation and cholinergic transmission in *C. elegans* suggests it may confer survival advantages to the nematode. For example, in the absence of food, the worm may reduce the defecation frequency and cholinergic functions simultaneously by secreting fewer intestinal signals (resulting from less intestinal activations), and this will allow the animal to increase food retention in the intestine while decreasing energy expenditures. On the other hand, the identification of such dual regulation of the defecation and cholinergic transmission by the *C. elegans* intestine may also facilitate the screen for neural modulatory molecules. As mutations in any components of this synaptic modulation pathway may lead to defecation defects at the same time, one could simply perform a saturated screen for synaptic transmission mutants by easily scoring constipation phenotypes. This greatly facilitates the screening process. In the future, it will be interesting to use this approach to identify the neuropeptides that are involved in synaptic modulations.

Materials and methods

For detailed information on transgenic strains and plasmid clones, see supplemental information.

Pharmacological assay

The time-course analysis of aldicarb or levamisole induced paralysis in *C. elegans* was performed as previously described (Mahoney et al., 2006b). Briefly, 25-30 L4 stage larvae of various genetic backgrounds were picked to fresh OP50 spotted NGM plates and allowed to grow at 25°C for overnight. The young adults were then transferred to aldicarb (1mM) or levamisole (200μM) containing plates and the drug-induced paralysis was scored as a function of time. Each set of experiments was repeated at least three times independently and the mean value and standard error of mean (SEM) were reported.

Immunohistochemistry

The immunohistochemistry was performed as previously described (Mahoney et al., 2006a). Briefly, worms were collected, washed, and fixed in Bouin fixative. A 1:10,000 mouse monoclonal α-RAB-3 antibody and a 1:5,000 chicken polyclonal α-RIM-1 antibody were used for RAB-3 and RIM-1 immunostaining, respectively. Fixed worms were incubated with primary antibodies at 4°C for overnight, washed, and then incubated with appropriate Alexa-conjugated secondary antibodies at 1:1000 dilution at RT for 1 hour. Images were captured by a Retiga 2000R 12-bit RGB camera (Q-

imaging) on an Olympus BX60 epifluorescence microscope using X60 objective and X1 optivar, which were then processed in Adobe Photoshop.

Fluorescence imaging and quantification

The images of UNC-13S::GFP and GLR-1::GFP labeled nerve cords were captured under Olympus BX60 epifluorescence microscope as stated above. For fluorescence quantification, a 100μm fragment of dorsal nerve cord anterior to the anus (UNC-13S::GFP) or ventral nerve cord posterior to the vulva (GLR-1::GFP) was selected and analyzed in NIH ImageJ. Line scan was performed to identify puncta on the nerve cords with a fixed threshold. For coelomocyte AEX-5::VENUS fluorescence quantification, images were taken on an Olympus FluoView FV500 scanning confocal microscope. The total coelomocyte fluorescence was then analyzed and quantified in ImageJ as described previously (Mahoney et al., 2008).

RNAi

The bacterial feeding RNAi was carried out essentially as described before (Mahoney et al., 2008). Briefly, bacterial strains expressing dsRNAs that target various *aex* genes were spotted onto NGM plates supplemented with 1mM IPTG and 100μg/ml Ampicillin to induce dsRNA expression. Twelve hours after the induction, two L4 intestinal RNAi-sensitive animals were picked onto the plates and were allowed to give progenies for two generations. The L4 larvae of the second generation were picked for subsequent aldicarb assay. The experiment was repeated three times and mean and

standard error of mean were reported.

Germ line transformation

The transgenic lines were constructed using standard germ line transformation procedures (Mello et al., 1991). Typically plasmids were injected at 30ng/µl final concentration. For co-injection marker P*myo-2::gfp*, 5ng/µl final concentration was used, while for pRF4 (*rol-6*) the plasmid was injected at 100ng/µl. At least two independent lines of each injection were analyzed.

Supplemental materials and methods

Strain maintenance

All *C. elegans* strains were maintained on *E. coli* OP50 spotted NGM plates at RT using standard procedures unless otherwise stated (Wood, 1988).

Strains

N2 (wild type) REF(Brenner, 1974), *aex-1(sa9)* REF(Doi and Iwasaki, 2002; Thomas, 1990), *aex-2(sa3)* REF(Thomas, 1990), *aex-4(n2415)*, *aex-4(sa22)*, *aex-4(ok614)*, *aex-5(sa23)* REF(Thomas, 1990), *aex-2(sa3);aex-5(sa23), aex-2(sa3);egl-3(n150); aex-5(sa23);egl-3(n150), nuIs46[unc-13s::gfp], aex-1(sa9);nuIs46[unc-13s::gfp], aex-2(sa3);nuIs46[unc-13s::gfp], aex-5(sa23);nuIs46[unc-13s::gfp], nuIs25[glr-1::gfp], aex-2(sa3);nuIs25[glr-1::gfp], aex-5(sa23);nuIs25[glr-1::gfp], unc-25(e156)* REF(Jin et al., 1999), *egl-3(n150), egl-3(ok979), egl-21(n476), rde-*

*1(ne219);kbIs7[*P*nhx-2::rde-1; rol-6]* REF(Espelt et al., 2005)*, gsa-1(ce81)* REF(Schade et al., 2005)*, gsa-1(ce81);aex-2(sa3), gsa-1(ce81);aex-5(sa23), acy-1(js127)* REF(Saifee, 2003)*, unc-119(ed3);jsIs1072*[NM2072 (P*vha-6::aex-5::Venus* Cb*unc-119*)], *acy-1(pk1279);ceEx108(myo-3::acy-1)* REF(Reynolds et al., 2005).

aex-2(sa3) jsEx921[NM2101(P*rab-3::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx922*[NM2101(P*rab-3::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx925*[NM2102(P*myo-3::aex-2::yfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx928*[NM2102(P*myo-3::aex-2::yfp*) + pPD118.33 (P*myo-2::gfp*) line2], *aex-2(sa3) jsEx929*[NM2103(P*ges-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx930*[NM2103(P*ges-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx999*[NM1707(P*glr-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1000*[NM1707(P*glr-1::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx1007*[NM1841(P*unc-17::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1008*[NM1841(P*unc-17::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx1003*[NM1843(P*unc-47::aex-2::gfp*) + PD118.33(P*myo-2::gfp*) line1], *aex-2(sa3) jsEx1004*[NM1843(P*unc-47::aex-2::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *aex-2(sa3) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line1], *aex-2(sa3) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line2]

aex-5(sa23) jsEx1061[NM2059(P*vha-6::aex-5::Venus*) + pRF4(*rol-6*) line1], *aex-5(sa23) jsEx1062*[NM2059(P*vha-6::aex-5::Venus*) + pRF4(*rol-6*) line2], *aex-5(sa23)*

jsEx1063[NM2061(P*rab-3:: aex-5::Venus*) + pRF4(*rol-6*) line1], *aex-5(sa23) jsEx1064*[NM2061(P*rab-3:: aex-5::Venus*) + pRF4(*rol-6*) line2], *aex-5(sa23) jsEx1066*[NM2062(P*myo-3:: aex-5::Venus*) + pRF4(*rol-6*) line1], *aex-5(sa23) jsEx1067*[NM2062(P*myo-3:: aex-5::Venus*) + pRF4(*rol-6*) line2], *aex-5(sa23) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line1], *aex-5(sa23) jsEx994*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line2]

egl-3(n150) jsEx1115[NM2242(P*rab-3::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1116*[NM2242(P*rab-3::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1118*[NM2248(P*vha-6::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1119*[NM2248(P*vha-6::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1121*[NM2182(P*myo-3::egl-3::yfp*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1122*[NM2182(P*myo-3::egl-3::yfp*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1142*[NM2249(P*glr-1::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1143*[NM2249(P*glr-1::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1144*[NM2250(P*unc-17::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1146*[NM2250(P*unc-17::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1149*[NM2251(P*unc-25::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1150*[NM2251(P*unc-25::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx1152*[NM2252(P*unc-47::egl-3::Venus*) + pPD118.33(P*myo-2::gfp*) line1], *egl-3(n150) jsEx1153*[NM2252(P*unc-47::egl-3::Venus*)

+ pPD118.33(P*myo-2::gfp*) line2], *egl-3(n150) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line1], *egl-3(ok979) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line1], *egl-21(n476) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line1], *egl-21(n476) jsEx987*[NM1778(P*unc-25::acy-1(js127)*) + pRF4(*rol-6*) line2]

DNA constructs

The construction of following plasmids has been previously described: NM1707, NM1778, NM1841, NM1843, NM2059, NM2061, NM2062, NM2101, NM2102, NM2103 (Mahoney et al., 2008). See reference for oligos and detailed cloning procedures.

NM2242-*egl-3* cDNA fused with *Venus* at the C-terminus driven by neuronal *rab-3* promoter. The 2kb full-length *egl-3* cDNA was amplified from *C. elegans* cDNA library (kindly provided by Dr. Robert Barstead) using oligos 5'-TAGA AGTC GGA TCC ATGAAAAACACACATGTCGACCT-3' and 5'-TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3'. The PCR product was fully digested by SpeI and partially digested by BamHI, with the full length digestion product ligated into BamHI and SpeI digested plasmid NM2101 (P*rab-3::aex-2::gfp*). The 2kb *egl-3* cDNA swapped *aex-2* coding region, resulting in P*rab-3::egl-3::gfp* construct. This plasmid was then digested with SpeI and SacII to remove *gfp* coding region. The 900bp *Venus* coding region was subsequently amplified from NM1358 (P*rab-3::Venus*) using oligos 5'-TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA

AGTC CCG CGG CTATTTGTATAGTTCATCCATGCCAAG-3', digested with SpeI and SacII and ligated into above P*rab-3::egl-3* vector. This gave the plasmid P*rab-3::egl-3::Venus*.

NM2248-*egl-3* cDNA fused with *Venus* at the C-terminus driven by intestinal *vha-6* promoter. An intermediate plasmid NM2244 (P*unc-25::egl-3::gfp*) was first made to facilitate the construction of NM2248 (P*vha-6::egl-3::Venus*). The 1.5kb *unc-25* promoter and 2.0kb full-length *egl-3* cDNA were fused together by overlap PCR using oligos 5'- TAGA AGTC GCA TGC GCCGAAATTTAAAGCTAGTTTTTTTG-3', 5'-AGGTCGACATGTGTGTTTTTCAT GCT AGC TTTTGGCGGTGAACTGAGCT-3' (amplifying 1.5kb P*unc-25*) and 5'- AGCTCAGTTCACCGCCAAAA GCT AGC ATGAAAAACACACATGTCGACCT-3', 5'- TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3' (amplifying 2.0kb *egl-3* cDNA). The P*unc-25::egl-3* fusion fragment was digested with SphI and SpeI and ligated with SphI and SpeI digested 5.0kb *gfp* vector backbone amplified from NM1019 (P*rab-3::gfp::rim3'*) using oligos 5'- ACT CAT ACT AGT ATGGCACCGGTCGCCAC-3' and 5'- TAGA AGTC GCA TGC ACTGCTCCCATCTGAAGATC-3'. The plasmid NM2244 (P*unc-25::egl-3::gfp*) was then digested with SphI and NheI to remove P*unc-25*, followed by ligation with SphI (partial) and NheI digested 1.2kb *vha-6* intestinal promoter amplified from NM1352 (VHA-6mch) using oligos 5'- TCTA CGAT GCA TGC GCATGTACCTTTATAGGTGCG-3', 5'- TCTA CGAT GCT AGC GGGTAGGTTTTAGTCGCCCT-3'. This gives the plasmid NM2247 (P*vha-6::egl-*

3::gfp). Finally, the *gfp* in this plasmid was digested out with SpeI and SacII and replaced with SpeI and SacII digested 900bp *Venus* coding sequence, which was amplified from NM1358 (P*rab-3::Venus*) using oligos 5'- TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA AGTC CCG CGG CTATTTGTATAGTTCATCCATGCCAAG-3'. This gave the plasmid P*vha-6::egl-3::Venus*.

NM2182-*egl-3* cDNA fused with *yfp* at the C-terminus driven by muscular *myo-3* promoter. The 2kb full-length *egl-3* cDNA was amplified from *C. elegans* cDNA library using oligos 5'- TAGA AGTC GCT AGC ATGAAAAACACACATGTCGACCT-3' and 5'- TAGA AGTC AGG CCT GTGGCTGCGTTTGTGGGCTT-3'. The PCR product was then digested with NheI and StuI and ligated with NheI, StuI double digested plasmid NM2102 (P*myo-3::aex-2::yfp*). This replaced *aex-2* with *egl-3* coding sequence and gave the plasmid P*myo-3::egl-3::yfp*.

NM2249-*egl-3* cDNA fused with *Venus* at the C-terminus driven by glutamate receptor *glr-1* promoter. An intermediated plasmid NM2184 (P*glr-1::egl-3::gfp*) was first made to facilitate the construction of NM2249 (P*glr-1::egl-3::Venus*). The 2kb full-length *egl-3* cDNA was amplified from the *C. elegans* cDNA library using oligos 5'-TAGA AGTC GGA TCC ATGAAAAACACACATGTCGACCT-3' and 5'-TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3'. The PCR product was fully digested by SpeI and partially digested by BamHI, with the full length digestion product

ligated into BamHI and SpeI digested plasmid NM1707 (P*glr-1::aex-2::gfp*). The 2kb *egl-3* cDNA replaced *aex-2* coding region, giving NM2184 (P*glr-1::egl-3::*gfp). This plasmid was then digested with SpeI and SacII to remove the *gfp* coding sequence and ligated with SpeI and SacII double digested 900bp *Venus* coding region amplified from plasmid NM1358 (P*rab-3::Venus*) using oligos 5'- TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA AGTC CCG CGG CTATTTGTATAGTTCATCCATGCCAAG-3'. This gave the plasmid P*glr-1::egl-3::Venus*.

NM2250-*egl-3* cDNA fused with *Venus* at the C-terminus driven by cholinergic *unc-17* promoter. An intermediated plasmid NM2185 (P*unc-17::egl-3::gfp*) was first made to facilitate the construction of NM2250 (P*unc-17::egl-3::Venus*). The 2kb full-length *egl-3* cDNA was amplified from *C. elegans* cDNA library using oligos 5'-TAGA AGTC GGA TCC ATGAAAAACACACATGTCGACCT-3' and 5'-TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3'. The PCR product was fully digested by SpeI and partially digested by BamHI, with the full length digestion product ligated into BamHI and SpeI digested plasmid NM1841 (P*unc-17::aex-2::gfp*). The 2kb *egl-3* cDNA replaced *aex-2* coding region, giving NM2185 (P*unc-17::egl-3::*gfp). This plasmid was then digested with SpeI and SacII to remove the *gfp* coding sequence and ligated with SpeI and SacII double digested 900bp *Venus* coding region amplified from plasmid NM1358 (P*rab-3::Venus*) using oligos 5'- TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA AGTC CCG CGG

CTATTTGTATAGTTCATCCATGCCAAG-3'. This gave the plasmid P*unc-17::egl-3::Venus*.

NM2251-*egl-3* cDNA fused with *Venus* at the C-terminus driven by GABA synthase *unc-25* promoter. An intermediated plasmid NM2244 (P*unc-25::egl-3::gfp*) was first made to facilitate the construction of NM2251 (P*unc-25::egl-3::Venus*). The 1.5kb *unc-25* promoter and 2.0kb full-length *egl-3* cDNA were fused together by overlap PCR using oligos 5'- TAGA AGTC GCA TGC GCCGAAATTTAAAGCTAGTTTTTTTG-3', 5'- AGGTCGACATGTGTGTTTTTCAT GCT AGC TTTTGGCGGTGAACTGAGCT-3' (amplifying 1.5kb P*unc-25*) and 5'- AGCTCAGTTCACCGCCAAAA GCT AGC ATGAAAAACACACATGTCGACCT-3', 5'- TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3' (amplifying 2.0kb *egl-3* cDNA). The P*unc-25::egl-3* fusion fragment was digested with SphI and SpeI and ligated with SphI and SpeI digested 5.0kb *gfp* vector backbone amplified from NM1019 (P*rab-3::gfp::rim3'*) using oligos 5'- ACT CAT ACT AGT ATGGCACCGGTCGCCAC-3' and 5'- TAGA AGTC GCA TGC ACTGCTCCCATCTGAAGATC-3'. The resulting plasmid NM2244 (P*unc-25::egl-3::gfp*) was then digested with SpeI and SacII to remove the *gfp* coding sequence and ligated with SpeI and SacII double digested 900bp *Venus* coding region amplified from plasmid NM1358 (P*rab-3::Venus*) using oligos 5'- TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA AGTC CCG CGG

CTATTTGTATAGTTCATCCATGCCAAG-3'. This gave the plasmid P*unc-25::egl-3::Venus*.

NM2252-*egl-3* cDNA fused with *Venus* at the C-terminus driven by GABAergic *unc-47* promoter. An intermediated plasmid NM2245 (P*unc-47::egl-3::gfp*) was first made to facilitate the construction of NM2252 (P*unc-47::egl-3::Venus*). The 1.45kb *unc-47* promoter and 2.0kb full-length *egl-3* cDNA were fused together by overlap PCR using oligos 5'- TAGA AGTC GCA TGC ATGTTGTCATCACTTCAAACTTTTC-3', 5'-AGGTCGACATGTGTGTTTTTCAT GCT AGC CTGTAATGAAATAAATGTGACGCTG-3' (amplifying 1.45kb P*unc-47*) and 5'-CAGCGTCACATTTATTTCATTACAG GCT AGC ATGAAAAACACACATGTCGACCT-3', 5'-TAGA AGTC ACT AGT GTGGCTGCGTTTGTGGGCTT-3' (amplifying 2.0kb *egl-3* cDNA). The P*unc-47::egl-3* fusion fragment was digested with SphI and SpeI and ligated with SphI and SpeI digested 5.0kb *gfp* vector backbone amplified from NM1019 (P*rab-3::gfp::rim3'*) using oligos 5'-ACT CAT ACT AGT ATGGCACCGGTCGCCAC-3' and 5'-TAGA AGTC GCA TGC ACTGCTCCCATCTGAAGATC-3'. The resulting plasmid NM2245 (P*unc-47::egl-3::gfp*) was then digested with SpeI and SacII to remove the *gfp* coding sequence and ligated with SpeI and SacII double digested 900bp *Venus* coding region amplified from plasmid NM1358 (P*rab-3::Venus*) using oligos 5'- TAGA AGTC ACT AGT ATGAGTAAAGGAGAAGAACTTTTC-3' and 5'- TAGA AGTC CCG CGG

CTATTTGTATAGTTCATCCATGCCAAG-3'. This gave the plasmid P*unc-47::egl-3::Venus*.

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Figure legends

Figure 1. *aex-2* and *aex-5* mutants are defective in presynaptic cholinergic transmission. A) Time-course analysis of 1mM aldicarb induced paralysis in *aex* mutants. Both *aex-2* and *aex-5* animals show enhanced resistance to aldicarb treatment. B) Time-course analysis of 200μM Ach agonist levamisole induced paralysis. *aex-2* and *aex-5* mutants show similar sensitivity to levamisole as wild type animals. As a control, AchR mutant *unc-29(e1072)* is completely resistant to levamisole induced paralysis. C) RAB-3 antibody staining of wild type and *aex* animals. All three lines show comparable staining at nerve ring (NR), dorsal nerve cord (DNC), and SAB head cholinergic motor axons (SAB). D) RIM-1 antibody staining of wild type and *aex* animals. All three lines show comparable staining at nerve ring (NR), dorsal nerve cord (DNC), and ventral nerve cord (VNC). Scale bar, 20μm.

Figure 1. *aex-2* and *aex-5* mutants are defective in presynaptic cholinergic transmission.

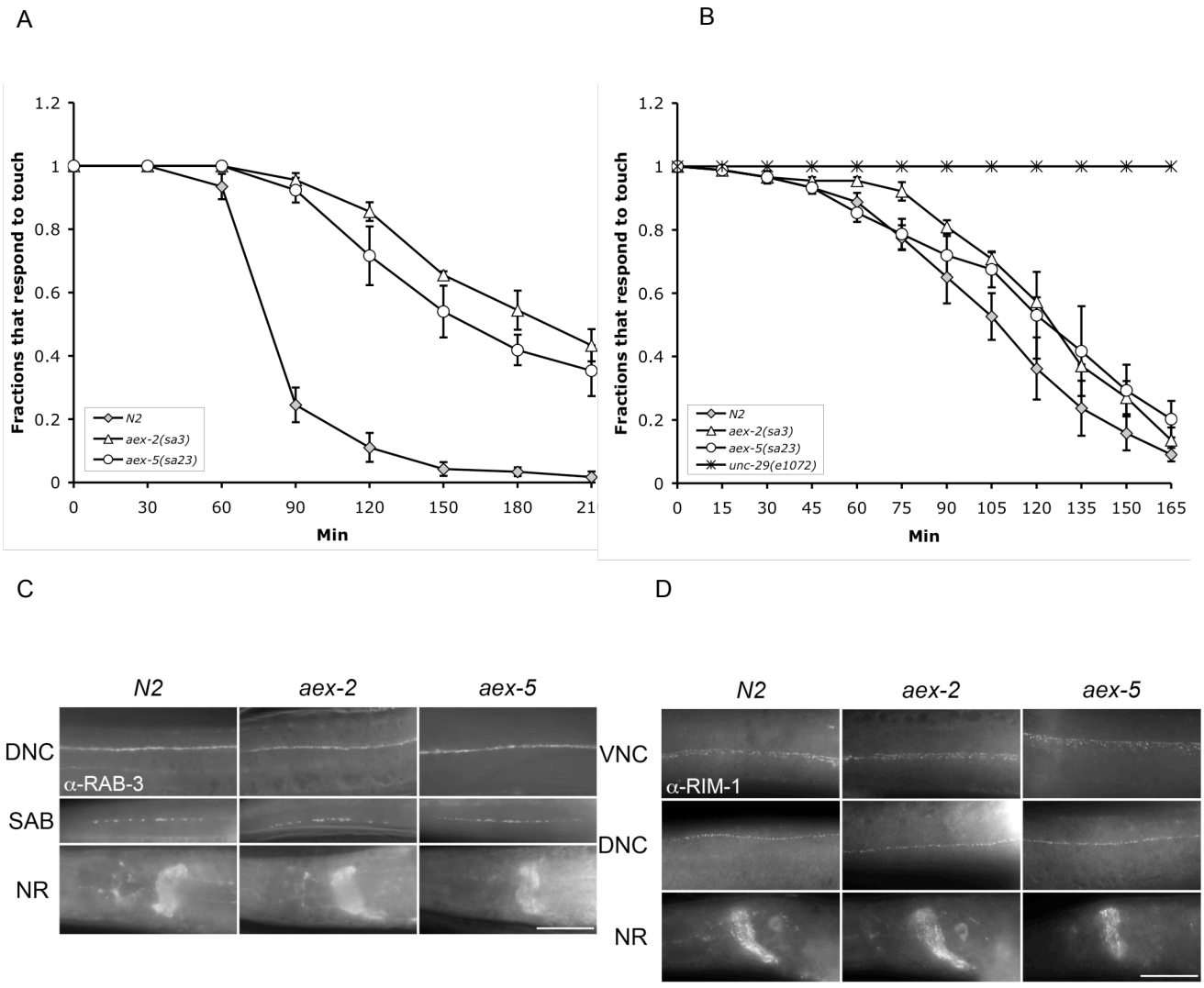

Figure 2. *aex-2* and *aex-5* mutants have normal UNC-13S::GFP and GLR-1::GFP expressions in the nerve cords. A) Fluorescence images of UNC-13S::GFP expressions in the dorsal nerve cord of wild type and *aex* animals. No obvious changes in the expression are seen in the *aex* mutants. The cord fluorescence and number of UNC-13S::GFP puncta are quantified in B) and C), respectively. D) Fluorescence images of GLR-1::GFP expressions the ventral nerve cord of wild type and *aex* animals. No obvious changes in the expression are seen in the *aex* mutants. The number of GLR-1::GFP puncta is quantified in E). Scale bar, 20μm.

Figure 2. *aex-2* and *aex-5* mutants have normal UNC-13S::GFP and GLR-1::GFP expressions in the nerve cords.

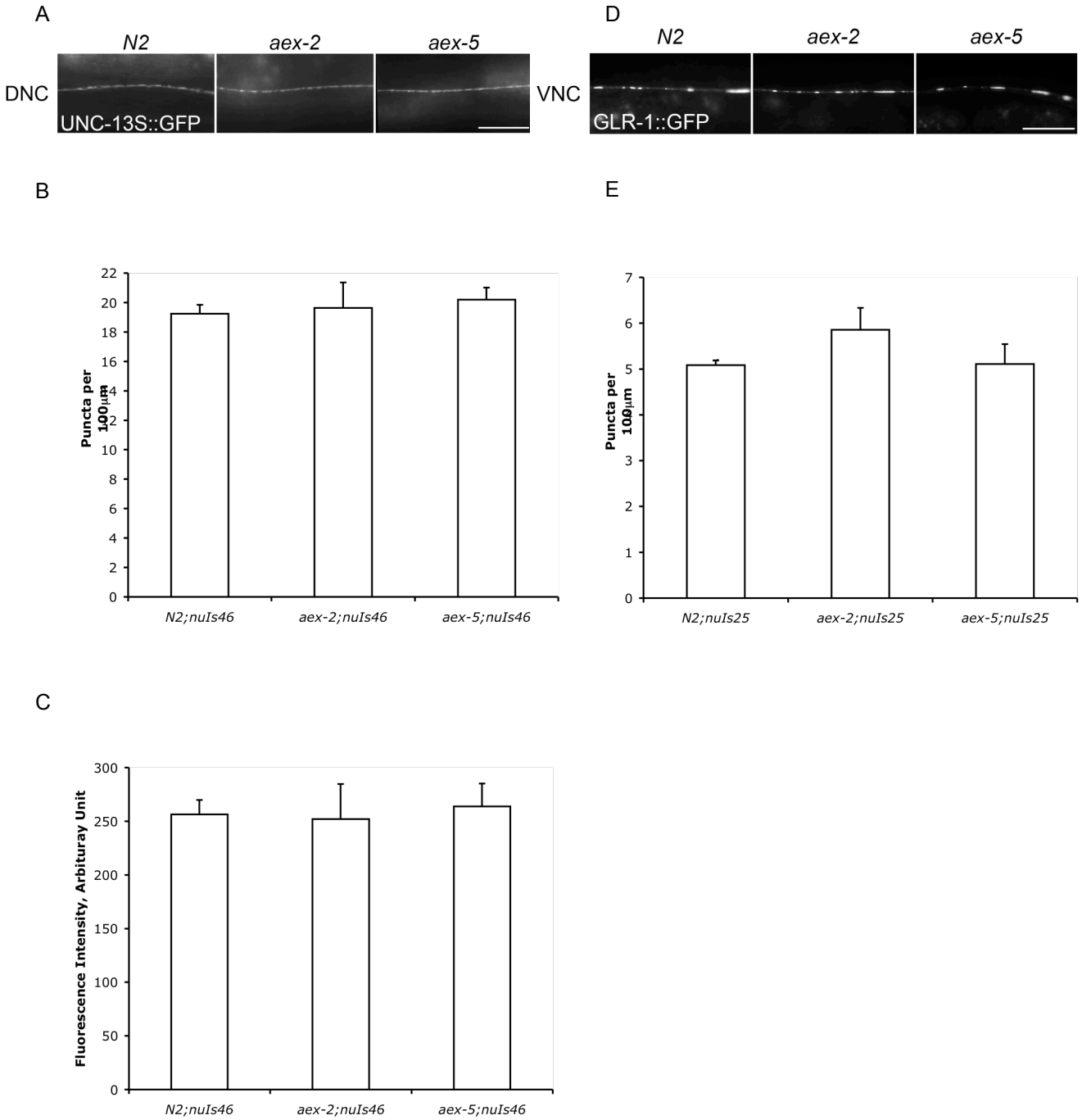

Figure 3. AEX-5 functions in the intestine to regulation synaptic transmission. A) The expression of AEX-5::VENUS in the intestine rescues aldicarb phenotype in the *aex-5* mutant. In contrast, both neuronal and muscular expressions of AEX-5::VENUS partially restores aldicarb sensitivity in *aex-5* animals. B) Intestinal specific RNAi mediated knockdown of endogenous AEX-5 expression induces strong resistance of the animal to 1mM aldicarb treatment. As a control, neither AEX-1 nor AEX-2 RNAi affects aldicarb sensitivity in the strain.

Figure 3. AEX-5 functions in the intestine to regulation synaptic transmission.

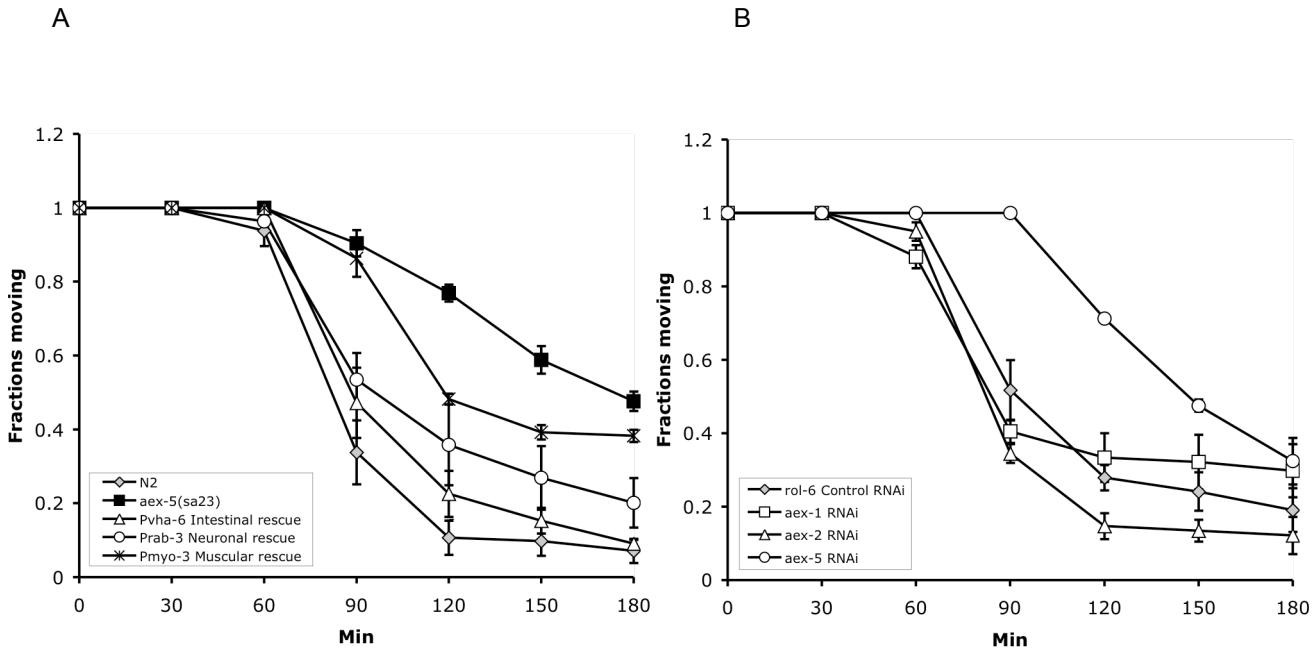

Figure 4. AEX-2 functions in GABAergic neurons to regulate synaptic transmission. A) Neuronal expression of AEX-2::GFP completely restores aldicarb sensitivity in *aex-2* mutants, while muscular and intestinal expressions do not. B) GABAergic expression of AEX-2::GFP restores aldicarb sensitivity in *aex-2* mutants. In contrast, cholinergic AEX-2::GFP expression partially rescues *aex-2* aldicarb phenotype, while interneuronal expression fails to do so.

Figure 4. AEX-2 functions in GABAergic neurons to regulate synaptic transmission.

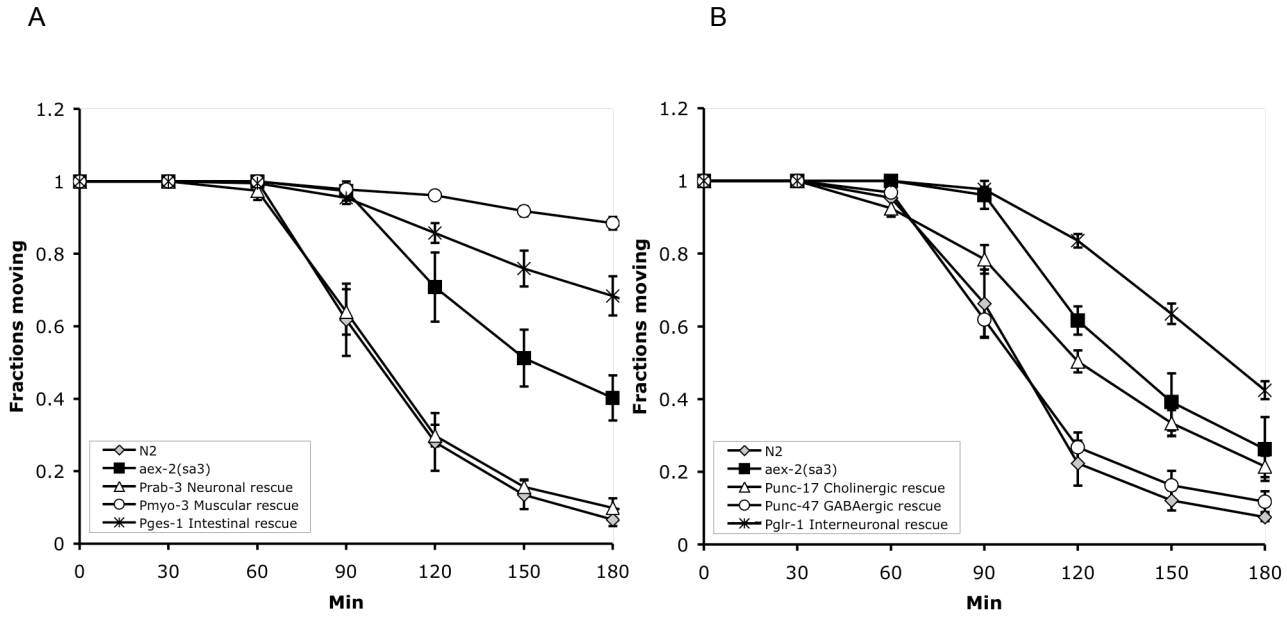

Figure 5. Gsα pathway acts genetically downstream of AEX-2 and AEX-5. A) A gain of function mutation in the stimulatory Gα subunit GSA-1 in *C. elegans* fully suppresses aldicarb phenotypes in *aex-2* and *aex-5* mutants. B) The GABAergic expression of an activated *acy-1(js127)* transgene under the GABA synthase gene *unc-25* promoter sufficiently restores aldicarb sensitivity in both *aex-2* and *aex-5* animals.

Figure 5. Gsα pathway acts genetically downstream of AEX-2 and AEX-5.

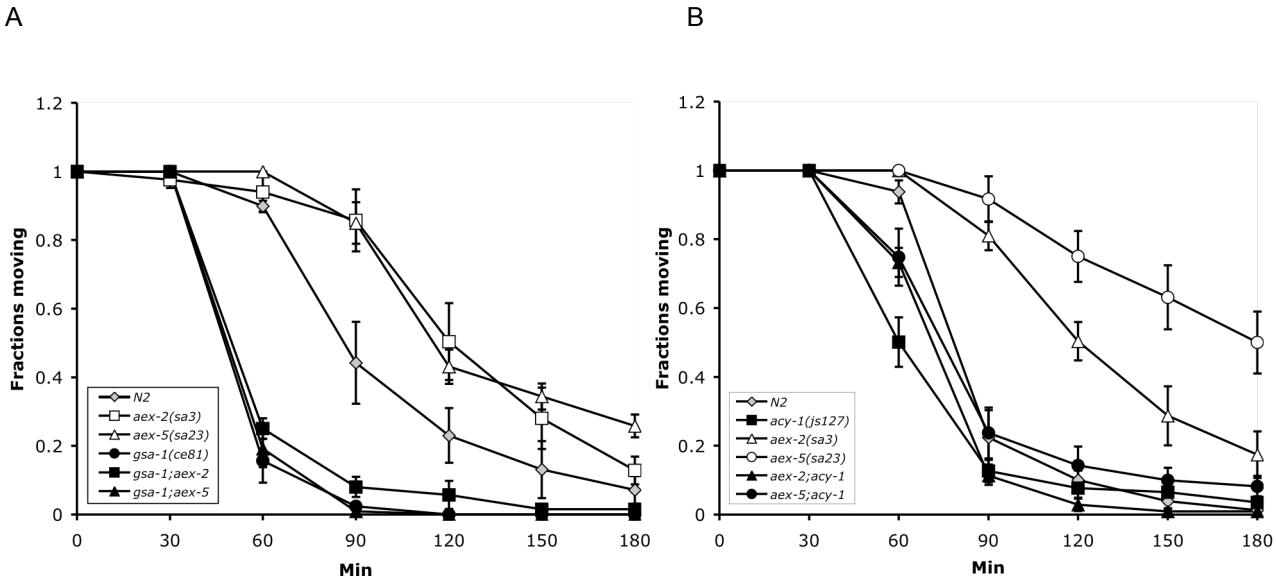

Figure 6. AEX-2 acts at least in part through the neuropeptide signaling. A) Disruption of either *aex-2* or *aex-5* gene functions in the *egl-3* mutant background does not enhance the aldicarb resistance. B) Neuronal expression of EGL-3::VENUS rescues aldicarb phenotypes in *egl-3* mutants, while muscular or intestinal expressions do not. C) Disrupting peptide processing enzymes EGL-3 or EGL-21 in GABAergic::*acy-1(js127, gf)* transgenic animals significantly blocks activated ACY-1 induced aldicarb hypersensitivity, leading to aldicarb resistance in double animals.

Figure 6. AEX-2 acts at least in part through the neuropeptide signaling.

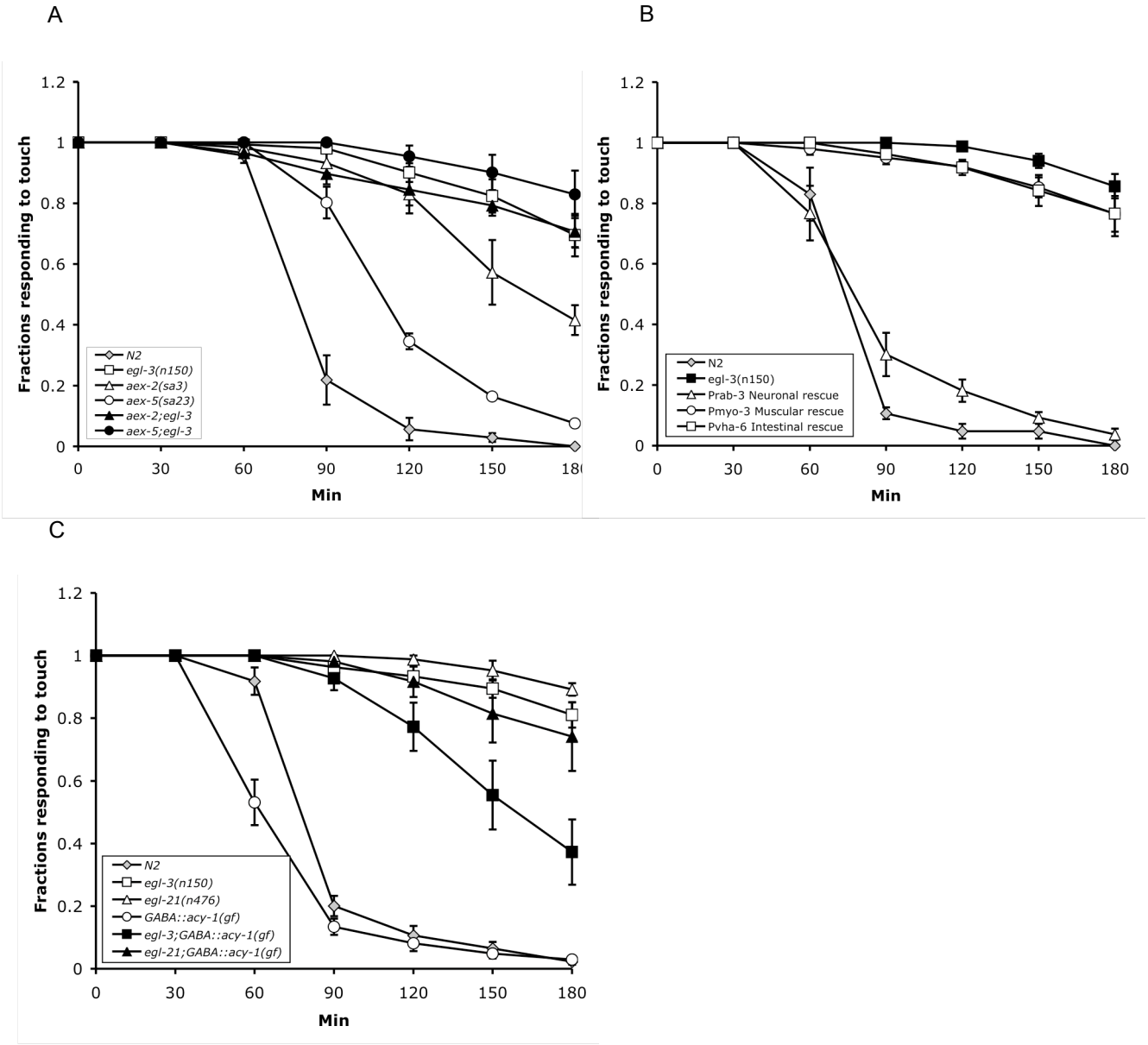

Figure 7. Food deprivation decreases intestinal activity and induces aldicarb resistance. A) A 10h starvation of young adult animals results in significant decrease in defecation frequency. Average number of defecation cycles in 5 minutes reduces from 4.7±0.8 to 1.5±1.1 for N2 animals, and from 4.7±0.9 to 1.1±1.1 for GABA::*acy-1(js127, gf)* animals. B) The 10h food deprivation does not affect defecation motor program, with most of N2 and GABA::*acy-1(js127, gf)* animals having functional enteric muscle contractions (EMC). Thus they are not defecation defective. C) Starved wild type animals have enhanced resistance to 1mM aldicarb treatment, while GABAergic expression of activated ACY-1 completely blocks the starvation-induced aldicarb resistance.

Figure 7. Food deprivation decreases intestinal activity and induces aldicarb resistance.

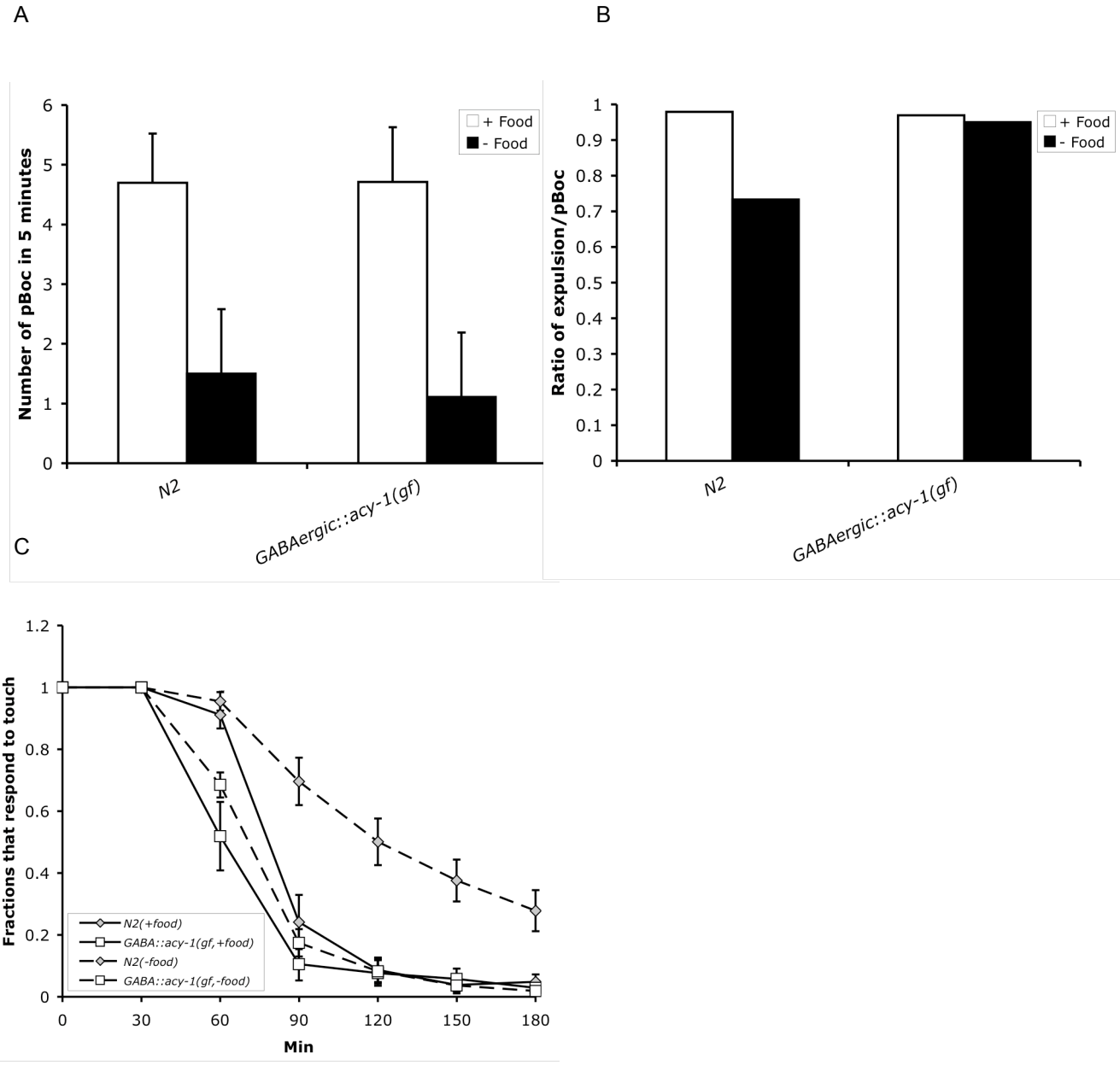

Figure 8. Model. The *C. elegans* intestine functions as an endocrine organ to coordinate the defecation and synaptic transmission regulation. The intestinal prohormone convertase (not shown) processes peptides (blue circles) that are secreted from the intestine (1). These intestine-derived signals act on GABAergic neurons (purple ellipse), which triggers the release of both GABA (small blue circles) and neuropeptides (green circles) via the Gsα-adenylate cyclase pathway (not shown) (2). Subsequently, GABA triggers enteric muscle (EM) contractions and defecation (3), while neuropeptides facilitate cholinergic transmission (4). The GABAergic GPCR AEX-2 likely functions as a receptor for intestinal peptide signals (not shown). A) An active intestine. This results in increased (1) and (2), which increases defecation activity (3) and cholinergic transmission (4). B) An inactive intestine. The reduced (1) and (2) leads to decreased defecation activity (3) and cholinergic function (4).

Figure 8. Model. The *C. elegans* intestine functions as an endocrine organ to coordinate the defecation and synaptic transmission regulation.

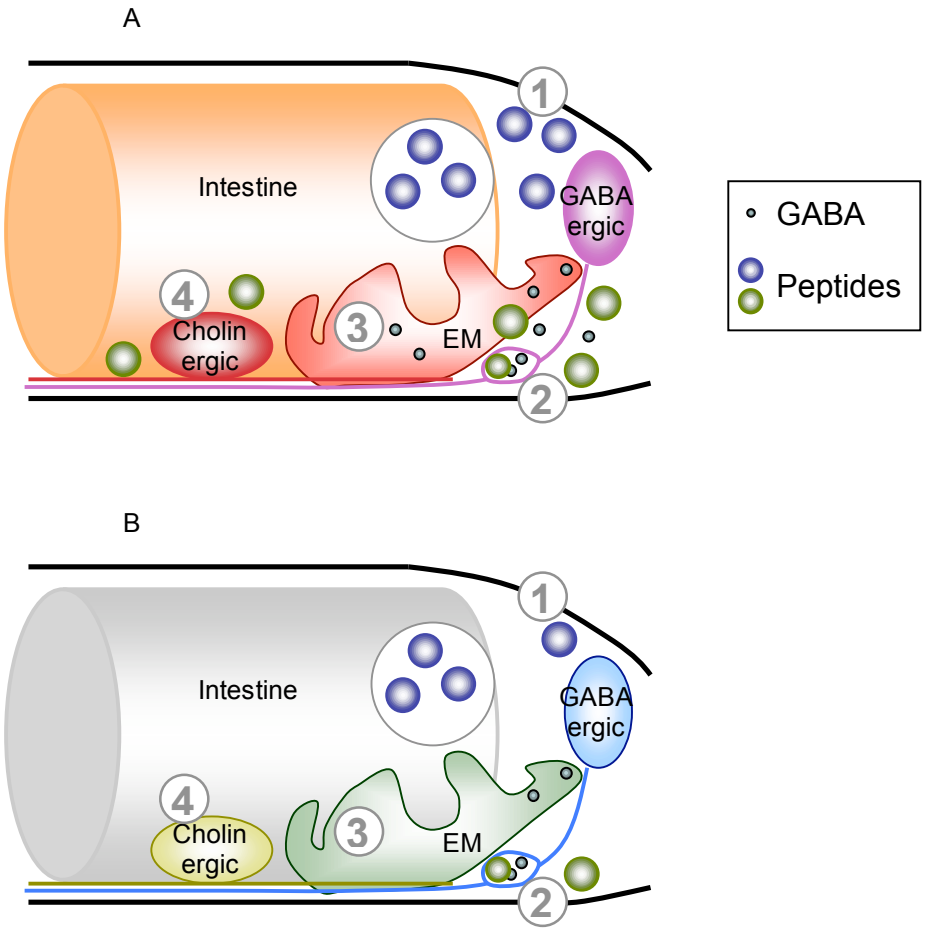

Table 1. Expressing EGL-3 in GABAergic neurons, cholinergic neurons or command interneurons partially rescues aldicarb phenotypes in *egl-3* mutants. The experiment was repeated at least three times and the fraction of worms that remained responsive to touch at 180min of the aldicarb assay was calculated and expressed as mean ± SEM.

Strains	Percentage of worms that respond to touch at 180min of the 1mM aldicarb treatment
N2	3.7% ±0.1%
egl-3(n150)	83.7% ±6.8%
Punc-17::egl-3::Venus (n=4)	32.9% ±12.9% - 71.6% ±6.2%
Pglr-1::egl-3::Venus (n=3)	38.9% ±5.6% - 63.5% ±1.9%
Punc-25::egl-3::Venus (n=4)	50.0% ±9.3% - 76.9% ±7.7%
Punc-47::egl-3::Venus (n=10)	27.8% ±1.9% - 72.5% ±0.5%

Chapter 4

A Homolog of the LIM Domain Focal Adhesion Protein ZYX-1 Regulates Synaptic Development in *C. elegans*

A homolog of the LIM domain focal adhesion protein ZYX-1 regulates synaptic development in *C. elegans*

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Abstract

The proper formation and maintenance of synapses is important for the correct wiring of the nervous system. Using a genetic screen looking for mutants that disrupt synaptic protein localization at mechanosensory PLM synapses, we identified *sam-6* as a gene that regulates synapse development in the nematode *C. elegans*. *sam-6* encodes a cytoskeletal LIM domain protein ZYX-1, a homologue of vertebrate Zyxin that is found in focal adhesion complexes and shuttles between the cytoplasm and nucleus. The *C. elegans zyx-1* locus encodes two ZYX-1 isoforms, and both are widely expressed in the nervous system, muscles, and spermatheca. Interestingly, the specific disruption of the splicing site in ZYX-1 LIM domain-only short isoform results in PLM synaptic defects, suggesting that LIM domains are involved in regulating synapse development. Using time-course imaging analysis of synaptic protein localizations, we revealed that *zyx-1* PLM synapses are still be able to form with apparently normal accumulations of presynaptic active zone proteins, synaptic vesicle proteins, and mitochondria. In contrast, PLM synapses fail to maintain in adult mutants, suggesting a defect in synapse

maintenance versus synaptogenesis. Tissue specific rescues reveal that ZYX-1 functions in mechanosensory neurons to regulate synapse maintenance. In an attempt to identify signal pathways downstream of ZYX-1, we examined several focal adhesion complex mutants and MAP kinase mutants, while we did not detect any defects in PLM synapses. Together, our results have provided evidence that the synapse maintenance in *C. elegans* PLM mechanosensory neurons involves a nuclear-cytoskeletal shuttling protein ZYX-1.

Introduction

The nervous system is a network made up of a large number of neurons that are electrically connected at synapses. During development, after a synapse is formed and stabilized, it needs to be maintained to carry out functions of both pre- and postsynaptic cells. In past few decades, several classes of molecules have been identified as regulators of synaptogenesis and synapse maintenance. Among them, the molecules that regulate neuronal cell contact and adhesion are implicated in organizing and maintaining synaptic structures. For example, at Drosophila giant fiber (GF) circuits, the L1-type cell adhesion molecules (CAMs) are required for maintaining normal presynaptic structures (Godenschwege et al., 2006). Disruption of L1-CAMs not only destabilizes presynaptic organizations, but also leads to decreased synaptic functions (Godenschwege et al., 2006). Moreover, the formation and stabilization of synapses also appear to involve cadherin molecules, as in the absence of functional cadherins such as cadherin-11, cadherin-13 or atypical cadherin flamingo, the neurons develop with either fewer synapses or destabilized synapses and axons (Bao et al., 2007; Paradis et al., 2007). These observations strongly suggest that cell adhesion molecules play important roles in organizing and maintaining synaptic structures. However, how these molecules act on synapses and what are their downstream signaling mechanisms remain not well understood.

The six-cell mechanosensory system of the nematode *C. elegans* provides a good model to study synapse development. Noticeably, two posteriorly localized mechanosensory neurons, PLML and PLMR, send out neuronal processes anteriorly

along the body, and two synapses are formed on the ventral nerve cord with the extended branches from PLM processes. The PLM synapses are large in size and separated from synapse rich regions, which make them an excellent system to study synapse formation and maintenance during development.

Here, we report the identification of a cytoskeletal protein ZYX-1 that likely acts in adhesion complexes to regulate PLM synapse development in *C. elegans*. ZYX-1 is a LIM domain protein that is conserved in worms and mammals. The nematode *zyx-1* gene encodes at least two ZYX-1 isoforms, and the LIM domain-containing short form appears to be required for the synaptic development. We found that ZYX-1 likely regulates both synapse formation and maintenance, as *zyx-1* young larva form fewer PLM synapses, which are apparently normal. In contrast, adult *zyx-1* mutants fail to maintain many of the synapses. ZYX-1 is broadly expressed in neurons and other non-neuronal tissues. We showed that ZYX-1 functions autonomously in mechanosensory neurons to regulate synapse maintenance. Interestingly, expression of ZYX-1 in yeast induces yeast cell to aggregate, suggesting a role of ZYX-1 in organizing adhesion complexes. We then intended to determine what cell adhesion molecules might be involved in ZYX-1 functions. So far, we did not observe any PLM defects in the absence of functional integrins or SYG-1/2. We are currently testing if cadherins are involved in maintaining PLM synapses. Together, our results support a role of a cytoskeletal protein ZYX-1 in regulating mechanosensory synapse development.

Results

zyx-1 mutants lack synaptic protein accumulation at PLM synapses

To identify genes that regulate synapse development in *C. elegans*, we used a transgenic line that specifically expresses GFP fused synaptobrevin in mechanosensory neurons. We performed ethylmethane sulfonate (EMS) mutagenesis using this transgenic line, and we recovered *sam-6* (which is *zyx-1*) as a mutant that lacks SNB-1::GFP at the PLM synapses. The SNB-1::GFP nicely labels PLM synapses in wild type animals. However, as it is relatively dim and not easy to score under dissection scopes, we then used a bright GFP::RAB-3 line (expressed in mechanosensory neurons) to analyze all the *zyx-1* mutant phenotypes. We first characterized the GFP::RAB-3 localization in wild type and mutant mechanosensory neurons. As shown in Fig 1A, in wild type animals, GFP::RAB-3 labels processes of PLMs and PVM neurons as well as two synaptic patches formed by PLM neurons on the ventral cord (Fig 1A, 1A'). Noticeably, in *zyx-1* mutants, no PLM synaptic structures are observed, while all the neuronal processes are normally labeled by GFP::RAB-3 (Fig 1B). We found that GFP::RAB-3 signals somehow accumulate on distal ends of PLM processes, forming a beads-on-a-thread structure (Fig 1B, 1B'). Moreover, the branches sending out by PLM processes are often missing in the mutant, while occasionally a branch and a single synaptic patch are observed on the ventral cord (Fig 1B'). Therefore, *zyx-1* mutants likely have disrupted PLM synapses. It remains unclear if the missing PLM synapse is caused by abnormalities in synapse formation, synapse maintenance, or both.

***zyx-1* mutants are defective in PLM synapse formation and maintenance**

As the lack of synaptic GFP::RAB-3 accumulation in *zyx-1* mutants could be caused by disrupted protein trafficking to synaptic sites, we first examined if a synaptic structure is still present in *zyx-1* mutants by using a transgenic line that expresses cytoplasmic RFP in mechanosensory neurons. As shown in Fig 2A, diffused RFP labels both PLM processes and synaptic patches on the ventral cord in wild type animals (Fig 2A). In contrast, no RFP is seen at the positions of PLM synapses in *zyx-1* animals, indicating the lack of synaptic structures in the mutant (Fig 2A). Therefore, the absence of GFP::RAB-3 accumulation at *zyx-1* PLM synapses is unlikely resulted from defective protein trafficking.

We then performed time-course analysis of GFP::RAB-3 localization at both wild type and mutant PLM synapses to see if ZYX-1 regulates synapse formation or maintenance. As shown in Fig 2B, both wild type and mutant PLM neurons have extended PLM processes in newly hatched L1 animals (0h). At 22.5°C, wild type PLM processes start extending branches towards the ventral cord at around 2 hours (2h). The appearance of GFP::RAB-3 at synapses slightly lags behind the branch extension, and at 4 hours significant fractions (38.6 ± 3.6%) of PLM processes have GFP::RAB-3 labeled synapses on the ventral nerve cord (4h) (Fig 2B, 2C). By the time of 8 hours, about 95% (95 ± 0%) of wild type PLM neurons have formed bright GFP::RAB-3 patches at synaptic sites (8h), which keep growing in size in developing larva until animals get to adulthood (Fig 2B, 2C, 8h-48h). Surprisingly, *zyx-1* mutants are still able to form PLM synapses as revealed by GFP::RAB-3 accumulations, while they have significantly

slowed synapse formation compared with wild type animals. As shown in Fig 2B, the first PLM synapse is not seen on the mutant ventral cord until 4 hours after hatch (Fig 2B). After that, the fractions of PLM processes with formed synapses increase slowly, and by 24 hours only 46.2 ± 3.8% of mutant PLM neurons have formed synapses on the ventral cord (Fig 2C). Intriguingly, after this developmental time point (24h), *zyx-1* mutants seem to start losing PLM synapses. This is revealed by the decrease in the GFP::RAB-3 labeled synaptic puncta, and by the time of young adults (48h) only 25.0 ± 2.5% of mutant PLM processes still maintain synapses on the ventral cord (Fig 2C). Accompanied with the decrease in synaptic numbers, there is an increase in the GFP::RAB-3 accumulation on mutant PLM processes, which form beads like structures (Fig 2A). Therefore, our results indicate that while ZYX-1 regulates synapse formation, it is also required to maintain synapses in the developing mechanosensory system.

The PLM synapses formed in *zyx-1* mutants are smaller and less structurally complicated compared with wild type PLM synapses. To examine if the mutant PLM synapse have proper localization of synaptic proteins, we crossed the mutant into transgenic lines that express either a mitochondria localized GFP or an active zone protein RIM-1::GFP in mechanosensory neurons. As shown in Fig 2B' and 2B", both mitochondria GFP and RIM-1::GFP are localized to PLM synapses in *zyx-1* animals (Fig 2B', 2B"). Interestingly, the GFP intensities of these synaptic markers are generally lower than those in wild type animals, indicating a decrease in the synapse size, complexity, or both, in *zyx-1* animals. We concluded that PLM synapses in mutants accumulate proper synaptic proteins at lower intensities.

***zyx-1* encodes a focal adhesion LIM domain protein that is widely expressed in the nervous system**

We mapped the original *sam-6* mutant to chromosome II and we demonstrated that it is *zyx-1*. In *C. elegans*, *zyx-1* encodes a focal adhesion LIM domain protein that shares homology with vertebrate zyxin, which has been found in cytoplasmic part of the focal adhesion complex (Wang and Gilmore, 2003) (Fig 3A). The *zyx-1* locus produces at least five transcripts, and they encode in total two ZYX-1 isoforms, a short form and a long form, in the nematode (Fig 3A). ZYX-1 contains three highly conserved C-terminal LIM domains (Fig 3A). Interestingly, a splice site mutation in the transcript of ZYX-1 short form that contains only C-terminal LIM domains disrupts PLM synapse development, suggesting that LIM domains are specifically required for PLM synapse formation and maintenance.

We then analyzed ZYX-1 expressions using rescuing ZYX-1 genomic constructs with either mCherry (a RFP variant) or GFP fused to the N- and C-terminus of ZYX-1, respectively. In the animals co-injected with the two constructs, the N-terminal mCherry labels only the long form of ZYX-1 protein, while C-terminal GFP labels all ZYX-1 isoforms, which we thought would help identify isoform specific ZYX-1 expression patterns. As shown in Fig 3B, ZYX-1::GFP is widely detected in neurons, muscles, and spermatheca (a structure where oocytes get fertilized by stored sperms). In the nervous system, the ZYX-1::GFP strongly labels cell bodies and axons of many neurons in the head ganglia, the ventral cord, and the tail ganglia, and we were unable to distinguish

between a nuclear and cytoplasmic localization of ZYX-1::GFP under such over-expression conditions (Fig 3B). In comparison, the expression of mCherry::ZYX-1 is pretty similar to that of ZYX-1::GFP, while it is much weaker in the intensity (Fig 3B). This suggests that the long form ZYX-1 is expressed at relatively low levels in *C. elegans*. Interestingly, despite its broad expressions, *zyx-1* animals show no noticeable defects in locomotion and other behaviors such as egg laying and mating. This suggests that either a long form ZYX-1 is required for these cellular functions, or there are redundant proteins that compensate the reduction-of-function of ZYX-1 in *zyx-1* mutants. We concluded that ZYX-1 is a broadly expressed protein with its long form maintained at relatively low levels in *C. elegans*.

ZYX-1 functions autonomously in mechanosensory neurons to regulate PLM synapse development

To assess where ZYX-1 functions to regulate PLM synapse development, we directed the expression of ZYX-1::GFP fusion proteins to mechanosensory neurons (driven by P*mec-7*), subset of command interneurons (driven by P*glr-1*), or muscles (driven by P*myo-3*) and examined which expression rescues PLM synapse defects. At PLM synapses, the presynaptic mechanosensory PLM neurons mainly synapse onto command interneurons AVA, AVD, AVE, and PVC, all of which express GLR-1 (Hart et al., 1995; Maricq et al., 1995). Therefore, by driving ZYX-1::GFP expression in mechanosensory neurons or command interneurons, we expected to distinguish a presynaptic versus a postsynaptic involvement of ZYX-1 in PLM synapse development.

As shown in Fig 4A, expression of ZYX-1::GFP in mechanosensory neurons significantly restores GFP::RAB-3 accumulation at PLM synapses in *zyx-1* mutants (Fig 4A). In contrast, glutamatergic or muscular expressions of ZYX-1::GFP do no rescue mutant PLM phenotypes, suggesting ZYX-1 acts presynaptically to regulate synapse development (Fig 4A). We then assessed if a short form of ZYX-1 is sufficient to restore PLM synapse development. For this purpose, we expressed either N- or C-terminal half of full-length ZYX-1 in mechanosensory neurons. As shown in Fig 4c, the expression of ZYX-1 C-terminal tandem LIM domains is sufficient to restore PLM synapses in *zyx-1* mutants, while the N-terminal ZYX-1 fails to rescue (Fig 4B). This indicates that the LIM domains of ZYX-1 are important for ZYX-1 functions. Together, our results suggest a cell autonomous role of ZYX-1 in regulating mechanosensory synapse development.

Intention to identify the interactions between ZYX-1 and other cell adhesion and signaling molecules

In vertebrates, the ZYX-1 homologue protein zyxin is found in focal adhesion complexes. It interacts with various integrins, actin binding proteins, and signaling molecules, and it is implicated in regulating cell adhesion, motility and gene transcription (Wang and Gilmore, 2003). Therefore, to assess if the *C. elegans* ZYX-1 functions in any of the adhesion complexes or signal pathways, we examined a series of mutants that are defective in integrins, focal adhesion proteins, IgG superfamily molecules, or signaling kinases to see if they have disrupted synapse development. The results are

summarized in Table 1. As shown in the table, we failed to detect significant defects in PLM synapse development in any of the mutants. Also we noticed that although mutations in the p38 MAP kinase pathway (*dlk-1*, *mkk-4*, *pmk-3*) suppress PLM synaptic defects in *rpm-1* mutants (Nakata et al., 2005 and data not shown), they do not suppress the synaptic defects in *zyx-1* animals (Table 1). Therefore, these molecules are unlikely to be involved in ZYX-1's functions.

We next performed a yeast two-hybrid screen to look for interacting proteins for the ZYX-1 LIM domains. As the LIM domains are suggested in PLM synapse development, we thought their interacting proteins might be involved in ZYX-1 functions. We recovered around 20 candidate molecules from the screen. Some of the candidates encode mitosis/meiosis related molecules, while the others are unannotated. We were able to get mutant alleles for five of the candidates, i.e. *C44B9.2(tm3522)*, *F29G6.3(tm3495)*, *zfp-1(ok554)*, *div-1(or148)*, and *atn-1(ok84)*, and we examined their PLM synapses. We failed to detect any defects in the PLM synapse development in the mutants examined (data not shown). We will need to wait for more available mutants to determine if the remaining candidate molecules are involved in regulating PLM synapse development.

Discussion

Previous studies have established a role for cell adhesion molecules in regulating synapse development, while how they transmit the signals inside the cell remains not well understood. Here we provide evidence that a *C. elegans* focal adhesion protein ZYX-1 may act in adhesive complexes to regulate synapse formation and maintenance in mechanosensory neurons. ZYX-1 is widely expressed in neurons, and it functions cell-autonomously in mechanosensory neurons to regulate PLM synapse development. Interestingly, the C-terminal LIM domains are important for ZYX-1 functions, suggesting that ZYX-1 may regulate synapse development via LIM domain mediated protein interactions. Our results provide a possible link between the synaptic regulatory cell adhesion molecules and their cytoplasmic signaling.

ZYX-1 is a conserved focal adhesion protein that contains a putative N-terminal actin binding domain and three C-terminal LIM domains. In vertebrates, the vertebrate homologue of ZYX-1 has been implicated in actin dynamics, and it is actively involved in the regulation of cell adhesion, motility, and mechanotransduction (Hirata et al., 2008; Wang and Gilmore, 2003). Interestingly, several lines of evidence suggest that the ZYX-1 homologue is able to shuttle between focal adhesion sites and nucleus in vertebrates (Nix and Beckerle, 1997; Nix et al., 2001). This suggests that the *C. elegans* ZYX-1 may regulates PLM synapse development by a similar mechanism, where it modulates gene transcriptions during early PLM neuron development. Unfortunately, we were unable to detect a nuclear accumulation of ZYX-1 in mechanosensory neurons using either a multi-copy *mCherry::zyx-1::gfp* transgenic line or a single-copy integrated line (Fig 3 and data

not shown). Thus, it is likely that ZYX-1 mainly localizes to the cytoplasm. As in vertebrates, the mutation in the putative nuclear export signal (NES) in zyxin induces nuclear localization of the protein, it will be interesting to determine if there is a similar NES in the *C. elegans* ZYX-1, and if there is, whether deleting the sequence leads to ZYX-1 nuclear accumulation.

We have tried several ways to identify proteins that may act together with ZYX-1 in regulating synapse development. In summary, we have examined three types of molecules, while so far we did not detect any functions of them in regulating PLM synapse development: 1) Cell adhesion complex molecules. These include integrins, focal adhesion proteins UNC-97 and UNC-98, and IgG superfamily proteins SYG-1 and SYG-2 that have been shown to regulate HSN synapse development (Shen et al., 2004); 2) Signaling molecules. These include p38 MAP kinase pathway components DLK-1, MKK-4, PMK-3, which have been shown to regulate PLM and GABAerigc synapse development (Nakata et al., 2005); 3) ZYX-1 LIM domain interacting proteins. These include an actin binding protein, a zinc finger protein, a cell division protein, and two unannotated proteins (Table 2). As a result, we think ZYX-1 may depend on other classes of molecules to carry out its functions. One of the sets of molecules we are currently looking at are cadherins/catenins, as evidence suggests that the vertebrate zyxin and N-cadherin/catenins are present in a complex (Lee et al., 2004). This leads us to examine PLM synapse development in available cadherin/catenin mutants. If there are any defects detected, we will test the interactions of cadherins/catenins with the ZYX-1 pathway using genetic and biochemical approaches. Also we are trying to characterize

more mutants of LIM domain interacting proteins, as some of them might be available in the near future. Together, the characterization of ZYX-1 pathway and the involved proteins will eventually lead to a better understanding on the molecular mechanisms that underlie synapse development.

Materials and methods

Strain maintenance

All *C. elegans* strains were maintained on *E. coli* OP50 spotted NGM plates at RT using standard procedures unless otherwise stated (Wood, 1988).

Strains

N2 (wild type) REF (Brenner, 1974), *sam-6(js417)*, *zyx-1(gk190)*, *syg-1(ky652)*, *syg-2(ky671)*, *ina-1(gm144)*, *ina-1(gm39)*, *pat-3(st564);mwEx31[pat-3(Y804F),sur-5::gfp]*, *pat-4(st551);zpEx225[gfp::pat-4(S334A);rol-6]*, *unc-97(su10)*, *unc-98(su130)*, *mkk-4(ok1545)*, *pmk-3(ok169)*, *C44B9.2(tm3522)*, *F29G6.3(tm3495)*, *zfp-1(ok554)*, *div-1(or148)*, *atn-1(ok84)*, *jsIs821[*P*mec-7::gfp::rab-3,*Cbr*unc-119]*, *jsIs973[*P*mec-7::mrfp,*Cbr*unc-119]*, *jsIs608[*P*mec7::mtgfp;* pJM23*(lin-15)]*, *jsEx448[*P*mec-7::rim-1::gfp;*pJM23*(lin-15)];lin-15(n765)*

jsIs821;jsIs973, *jsIs608;jsIs973*, *jsEx448;jsIs973*, *sam-6(js417);jsIs821;jsIs973*, *sam-6(js417);jsIs608;jsIs973*, *sam-6(js417);jsEx448;jsIs973*, *zyx-1(gk190);jsIs821;jsIs973*, *zyx-1(gk190);jsIs608;jsIs973*, *zyx-1(gk190);jsEx448;jsIs973*, *syg-1(ky652);jsIs821*, *syg-2(ky671);jsIs821*, *ina-1(gm144);jsIs821*, *ina-1(gm39);jsIs821*, *pat-3(st564);jsIs973;mwEx31*, *pat-4(st551);jsIs821;zpEx225*, *unc-97(su110);jsIs821*, *jsIs973;unc-98(su130)*, *mkk-4(ok1545);jsIs821*, *zyx-1(gk190);mkk-4(ok1545);jsIs821*, *pmk-3(ok169);jsIs821*, *zyx-1(gk190);pmk-3(ok169);jsIs821*, *C44B9.2(tm3522);jsIs821*,

F29G6.3(tm3495);jsIs821, *zfp-1(ok554);jsIs821*, *div-1(or148);jsIs821*, *atn-1(ok84);jsIs821*

jsEx1013[NM1860(*mcherry::zyx-1*), NM1874(*zyx-1::gfp*)], *sam-6(js417);jsEx1015*[NM1934(P*mec-7::zyx-1(fl)::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *sam-6(js417);jsEx1017*[NM1934(P*mec-7::zyx-1(fl)::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *sam-6(js417);jsEx1020*[NM1938(P*glr-1::zyx-1(fl)::gfp*) + pPD118.33(P*myo-2::gfp*) line1], *sam-6(js417);jsEx1021*[NM1938(P*glr-1::zyx-1(fl)::gfp*) + pPD118.33(P*myo-2::gfp*) line2], *sam-6(js417);jsEx1024*[NM1941(P*myo-3::zyx-1(fl)::yfp*) + pPD118.33(P*myo-2::gfp*) line1], *sam-6(js417);jsEx1025*[NM1941(P*myo-3::zyx-1(fl)::yfp*) + pPD118.33(P*myo-2::gfp*) line2], *sam-6(js417);jsEx1043*[NM2004(P*mec-7::gfp::zyx-1(aa 1-293)*) + pPD118.33(P*myo-2::gfp*) line1], *sam-6(js417);jsEx1044*[NM2004(P*mec-7::gfp::zyx-1(aa 1-293)*) + pPD118.33(P*myo-2::gfp*) line2], *sam-6(js417);jsEx1047*[NM2006(P*mec-7::gfp::zyx-1(aa 294-603)*) + pPD118.33(P*myo-2::gfp*) line1], *sam-6(js417);jsEx1048*[NM2006(P*mec-7::gfp::zyx-1(aa 294-603)*) + pPD118.33(P*myo-2::gfp*) line2]

Constructs

NM1860-*zyx-1* genomic construct with *mCherry* fused at the N-terminus. A recombineering pipeline protocol has been adapted to make the *mCherry::zyx-1* fusion plasmid (Sarov et al., 2006). Briefly, a 1.8kb KanRmch fragment was amplified from the plasmid NM1849 pR6KKanRmch using oligos 5’ -

CGTGCTCGCTCCTTCAGCACTATTCCAGACTCGGCATCCGCTACTGATCTGAA TTCTGAAGTTCCTATTCTCT - 3' and 5'-CTTACGGATGGGAGTAGAGGGGGTGGTGGAGGCGGAGGCGGGGGTCCCATC TTGTACAGCTCGTCCATGC - 3'. The PCR product was digested with DpnI to remove the template plasmid, followed by electroporation into the cells containing *zyx-1* fosmids. After recombination, the KanR was removed by anhydrotetracycline treatment, and the *mCherry::zyx-1* fragment was gap repaired into an Amp vector backbone using oligos 5'-AAATAAAAAAATAGAAAAATACTTGAAAAATATTGAAAAAGATTTTAAAAA TACAATTCGTTATGCATTATGGGTAC - 3' and 5' - TACAAATTCGGCAAATCGACAACTTGCCGGTTTGCCGGAAACTATCAATTTAC CAATCTAAGTCTGTGCTCC - 3'. This gave the plasmid *mCherry::zyx-1*.

NM1874- *zyx-1* genomic construct with *gfp* fused at the C-terminus. A recombineering pipeline protocol has been adapted to make the *zyx-1::gfp* fusion plasmid. Briefly, a 1.8kb GFPKanR fragment was amplified from the plasmid NM1835 pR6KGFP using oligos 5' - TGCTCTGCAAGACCTGTAATGGAAACCGGCTCCGCGTGGTCAGCTCCACGAG CTCAGGAGGTAGCGGCA - 3' and 5'-GAAGAAAAACGGATGGGGGGAATGGAAATTGTTGACTGATGGCTCGCTTAAC CGGCAGATCGTCAGTCAG - 3'. The PCR product was digested with DpnI to remove the template plasmid, followed by electroporation into the cells containing *zyx-1* fosmids.

After recombination, the KanR was removed by anhydrotetracycline treatment, and the *zyx-1::gfp* fragment was gap repaired into an Amp vector backbone using oligos 5'-AAATAAAAAAATAGAAAAATACTTGAAAAATATTGAAAAAGATTTTAAAAA TACAATTCGTTATGCATTATGGGTAC - 3' and 5' - TACAAATTCGGCAAATCGACAACTTGCCGGTTTGCCGGAAACTATCAATTTAC CAATCTAAGTCTGTGCTCC - 3'. This gave the plasmid *zyx-1::gfp*.

NM1934-*zyx-1* full-length cDNA with *gfp* fused at the C-terminus expressed under the mechanosensory specific *mec-7* promoter. The full-length *zyx-1* cDNA was amplified from the cDNA clone yk1054c6 using oligos 5'- TGAC ACGT GGA TCC ATGGGACCCCCGCCTCCG - 3' and 5'- TGAC ACGT CGG TAC CAA CGTGGAGCTGACCACGCGG - 3'. Both the PCR product and the plasmid NM776 pPD117.01 (P*mec-7::gfp*) were digested with BamHI and KpnI, and the digestion products were gel purified, ligated, and transformed into DH5α competent cells. This gave the plasmid P*mec-7::zyx-1(fl)::gfp*.

NM1938-*zyx-1* full-length cDNA with *gfp* fused at the C-terminus expressed under the *glr-1* promoter. The full-length *zyx-1* cDNA was amplified from the cDNA clone yk1054c6 using oligos 5'- TGAC ACGT GGA TCC ATGGGACCCCCGCCTCCG - 3' and 5'- TGAC ACGT ACT AGT CGTGGAGCTGACCACGCGG - 3'. Both the PCR product and the plasmid NM1707 P*glr-1::aex-2::gfp* were digested with BamHI and

SpeI, and the digestion products were gel purified, ligated, and transformed into DH5α competent cells. This gave the plasmid P*glr-1::zyx-1(fl)::gfp*.

NM1941-*zyx-1* full-length cDNA with *yfp* fused at the C-terminus expressed under the muscle specific *myo-3* promoter. The *zyx-1* full-length cDNA was amplified from the cDNA clone yk1054c6 using oligos 5'- TGAC ACGT GCT AGC ATGGGACCCCCGCCTCCG - 3' and 5'- TGAC ACGT AGG CCT CGTGGAGCTGACCACGCGG - 3'. Both the PCR product and the plasmid NM2102 P*myo-3::aex-2::yfp* were digested with NheI and StuI, and the digestion products were gel purified, ligated, and transformed into DH5α competent cells. This gave the plasmid P*myo-3::zyx-1(fl)::yfp*.

NM2004-*zyx-1* N-terminal (aa 1-293) cDNA fragment fused with *gfp* at the N-terminus under the mechanosensory specific *mec-7* promoter. The *gfp::zyx-1(aa 1-293)* fragment was amplified with overlap PCR using oligos 5'- TGAC ACGT GGT ACC ATGAGTAAAGGAGAAGAACTTTTC - 3', 5'- CGGAGGCGGGGGTCCCATCTTGTATGGCCGGCTAGCGA - 3' (for *gfp* amplification, from the plasmid NM776 pPD117.01), and 5'- TCGCTAGCCGGCCATACAAGATGGGACCCCCGCCTCCG - 3', 5'- TGAC ACGT GAT ATC TATCGTTGATAAAGATCTGGTGGT - 3' (for *zyx-1(aa 1-293)* amplification from the *zyx-1* cDNA clone yk1054c6). Both the PCR product and the plasmid NM445 pPD96.41 were digested with KpnI and EcoRV, and the digestion

products were gel purified, ligated, and transformed into DH5α competent cells. This gave the plasmid P*mec-7::gfp::zyx-1(aa 1-293)*.

NM2006-*zyx-1* C-terminal (aa 294-603) cDNA fragment fused with *gfp* at the N-terminus under the mechanosensory specific *mec-7* promoter. The *gfp::zyx-1(aa 294-603)* fragment was amplified with overlap PCR using oligos 5'- TGAC ACGT GGT ACC ATGAGTAAAGGAGAAGAACTTTTC - 3', 5'-GGAAAGTTCTTGCTTGAGTCATCTTGTATGGCCGGCTAGCGA - 3' (for *gfp* amplification, from the plasmid NM776 pPD117.01), and 5'-TCGCTAGCCGGCCATACAAGATGACTCAAGCAAGAACTTTCC - 3', 5'- TGAC ACGT GAT ATC TTACGTGGAGCTGACCACGC - 3' (for *zyx-1(aa 294-603)* amplification from the *zyx-1* cDNA clone yk1054c6). Both the PCR product and the plasmid NM445 pPD96.41 were digested with KpnI and EcoRV, and the digestion products were gel purified, ligated, and transformed into DH5α competent cells. This gave the plasmid P*mec-7::gfp::zyx-1(aa 294-603)*.

Cloning of *zyx-1*

We mapped *sam-6* to a 190kb region on chromosome II using single nucleotide polymorphism (SNP) analysis. Subsequent germ line transformation experiments revealed that fosmid 17Bb03 fully restores GFP::RAB-3 localization at PLM synapses in *sam-6* mutants. Using a candidate gene approach, we verified that *sam-6* is *zyx-1* based on following observations: 1) *zyx-1* mutants do not complement *sam-6* mutants; 2)

sequencing of *sam-6* allele reveals mutation in *zyx-1* locus; 3) a *zyx-1* mutant allele *gk190* exhibits defective PLM synapse phenotypes that resemble *sam-6* animals, and 4) A *zyx-1* plasmid is able to rescue both *sam-6* and *zyx-1* mutant phenotypes. Thus, we concluded that *sam-6* encodes ZYX-1.

Fluorescence imaging and time-course analysis

The images of fluorescence labeled mechanosensory neuronal processes were captured by a Retiga 2000R 12-bit RGB camera (Q-imaging) under an Olympus BX60 epifluorescence microscope using X60 objective and X1 optivar, which were then processed in Adobe Photoshop. For the time-course imaging of mechanosensory GFP::RAB-3, RFP, MITO::GFP and RIM::GFP, the eggs of appropriate fluorescent lines were collected and allowed to hatch at RT, and about 20 L1 larvae were picked at various time points after the hatch to analyze the fluorescence protein expression and localization at PLM synapses. The quantification was calculated by dividing the numbers of branching or synapse-bearing PLM processes with the total numbers of PLM processes scored. These experiments were repeated twice, and mean values and stand error of mean (SEM) were reported.

Germ line transformation

The transgenic lines were constructed using standard germ line transformation procedures (Mello et al., 1991). All the ZYX-1 rescue plasmids were injected at the final concentration of 30ng/μl, while the co-injection marker P*myo-2::gfp* and the plasmid

vector pcDNA3 were injected at 5ng/µl and 100ng/µl, respectively. At least two independent transgenic lines of each injection were analyzed for the rescue of PLM synapse development phenotypes.

Acknowledgements

We would like to thank Dr. A Francis Stewart for providing reagents for recombineering cloning of the *mCherry/gfp zyx-1* genomic fusion constructs. Some strains used in this work were obtained from the *Caenorhabditis elegans* Genetics Center, which is supported by the National Institutes of Health National Center for Research Resources. This work was supported by the grant NS040094 from the National Institute of Health to M.L.N. This work was partly supported by NIH Neuroscience Blueprint Core Grant NS057105 to Washington University and the Bakewell Family Foundation.

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Figures and figure legends

Figure 1. PLM synapses are missing in *zyx-1* mutants. A) The GFP::RAB-3 expressed by the *jsIs821* transgene labels mechanosensory neurons PLM and PVM in an L4 animal. Two PLM synaptic patches are localized on the ventral nerve cord. A') GFP::RAB-3 labeled PLM processes and synapses in a young adult wild type animal. B) PLM synapses are absent in young adult *zyx-1* mutants. Instead, the RAB-3 signal accumulates on PLM processes, forming beads like structures (arrow heads). B') Another example of *zyx-1* mutant animal bearing one PLM synapse (asterisk) on the ventral cord. Scale bar, 20μm.

Figure 1. PLM synapses are missing in *zyx-1* mutants.

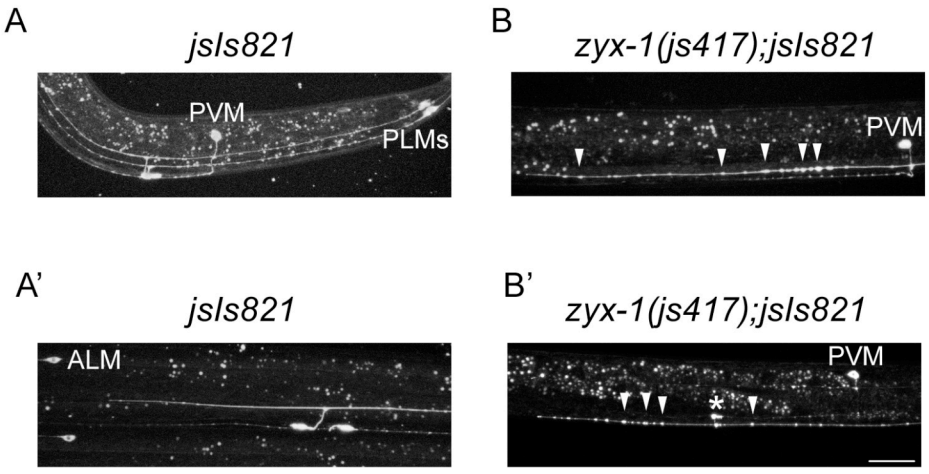
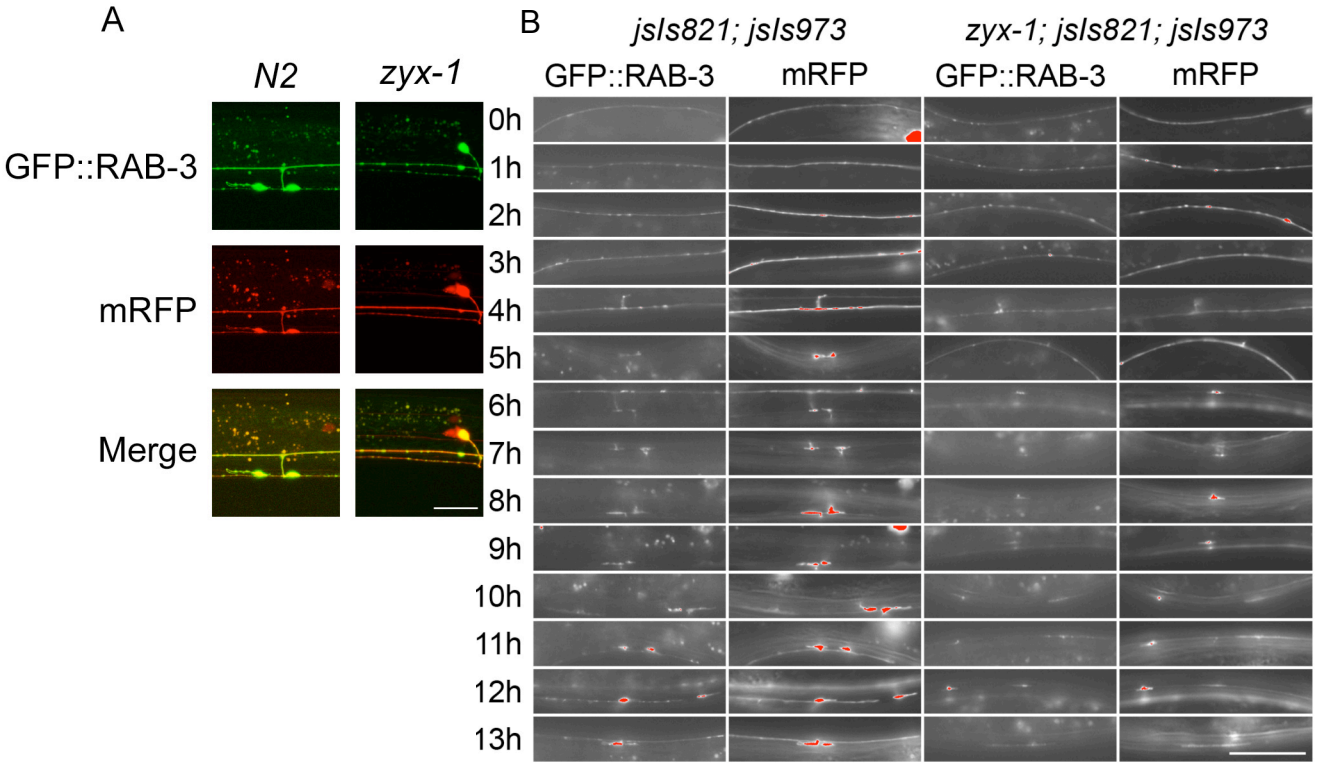


Figure 2. *zyx-1* mutants have defects in synapse formation and maintenance. A) A *zyx-1* mutant with mechanosensory neurons double labeled by GFP::RAB-3 and cytoplasmic mRFP. No mRFP signals are detected at PLM synapses in the mutant, indicating a lack of synaptic structures. B-B") Time-course imaging of PLM synapse development in wild type and *zyx-1* animals. The mechanosensory processes and synapses are double labeled by either GFP::RAB-3 (B), Mito::GFP (B'), or RIM-1::GFP (B") and cytoplasmic mRFP. Red color denotes intensity saturations. The fractions of PLM processes that have sent out branches or formed synapses at various time points after hatch are quantified in C). Scale bar, 20μm.

Figure 2. *zyx-1* mutants have defects in synapse formation and maintenance.



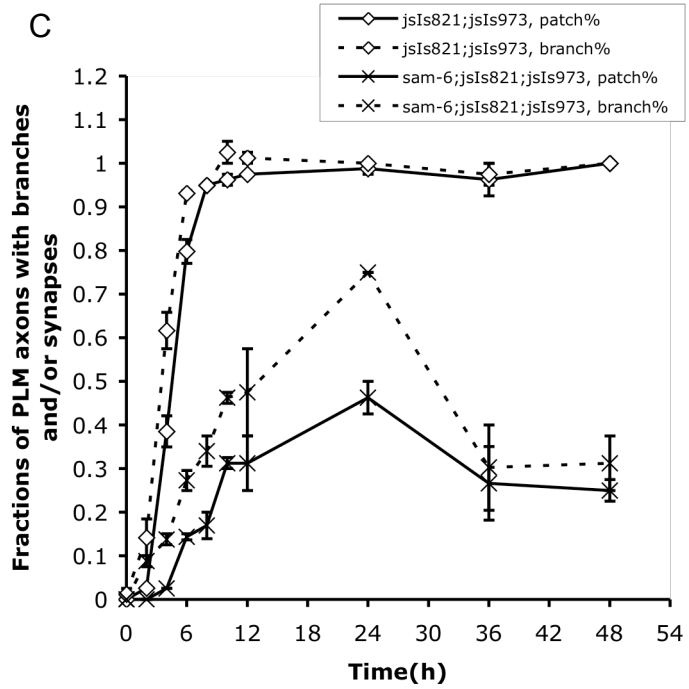


Figure 2. *zyx-1* mutants have defects in synapse formation and maintenance.

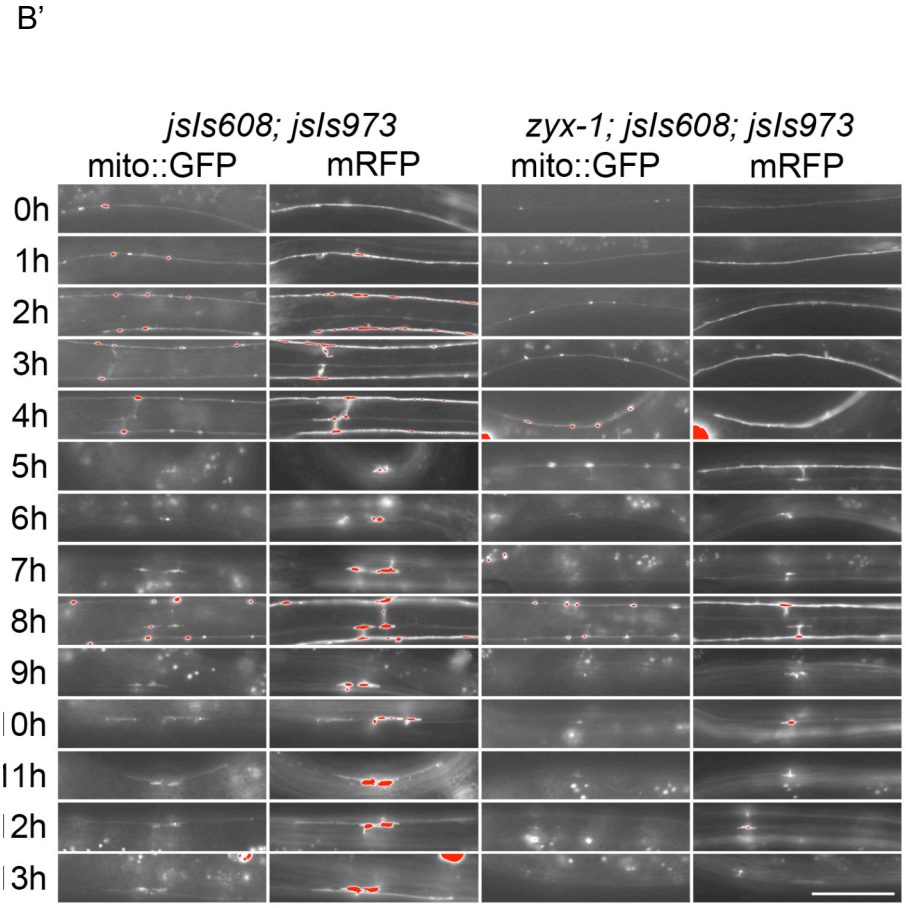


Figure 2. *zyx-1* mutants have defects in synapse formation and maintenance.

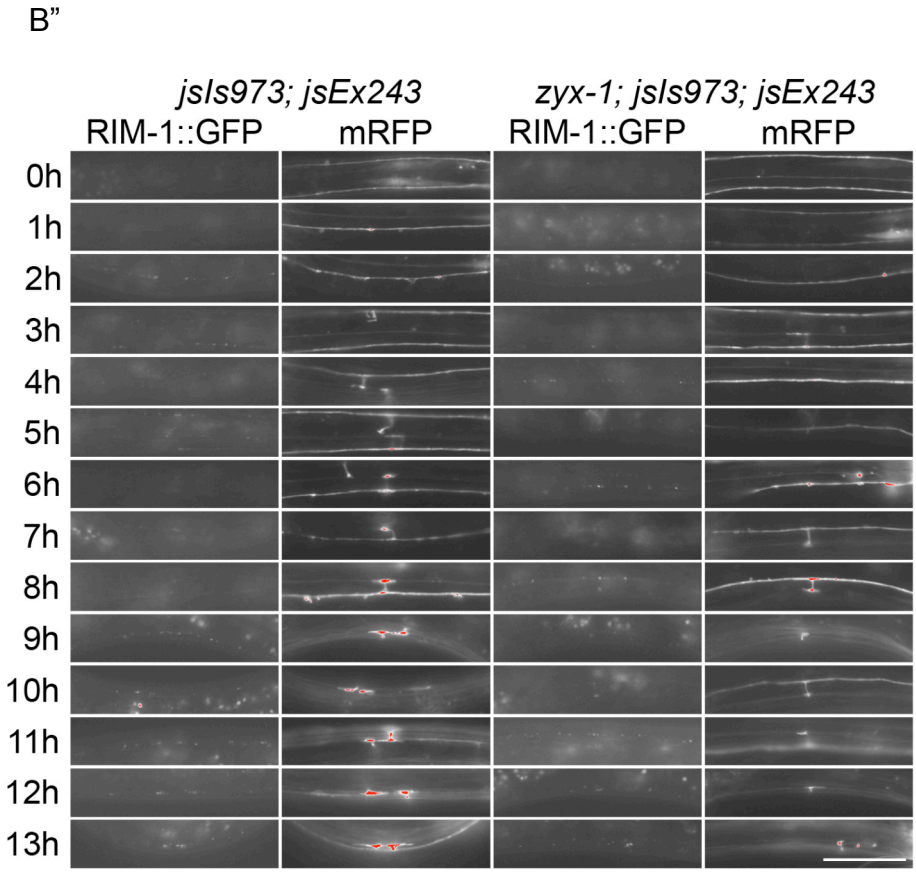
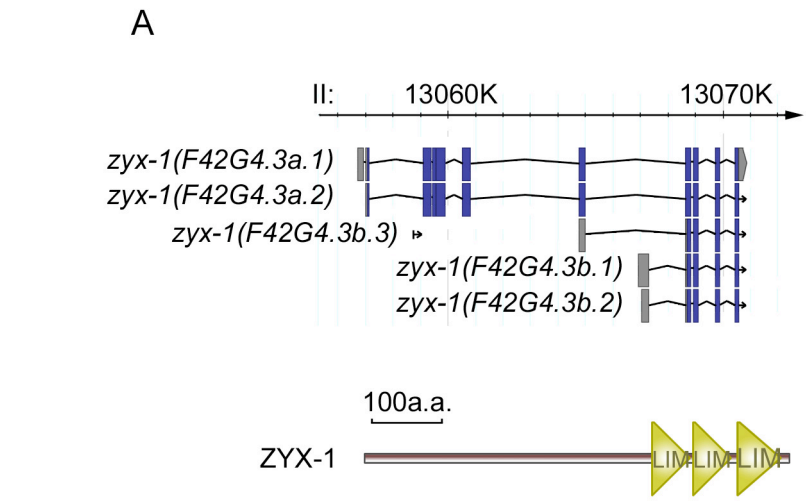


Figure 3. *zyx-1* encodes a focal adhesion LIM domain protein and is widely expressed in the nervous system and muscles. A) Gene structure of *zyx-1*. The *zyx-1* locus is located on the *C. elegans* chromosome II and encodes at least five different transcripts. Two isoforms of ZYX-1 protein, a long form and a short form, are predicted. Blue boxes, coding exons; gray boxes, non-coding exons; black lines, introns. Similar to its vertebrate homologue, the *C. elegans* ZYX-1 is predicted to have three LIM domains at the C-terminus. B) The MCHERRY::ZYX-1 and ZYX-1::GFP genomic fusion proteins are widely expressed in the nervous system and muscles. M, muscle; NR, nerve ring; VNC, ventral nerve cord; SP, spermatheca. ZYX-1 signals label muscle nuclei (asterisks). Due to high expression levels, the subcellular localization of ZYX-1 in neurons is not clearly distinguished. Scale bar, 40μm.

Figure 3. *zyx-1* encodes a focal adhesion LIM domain protein and is widely expressed in the nervous system and muscles.



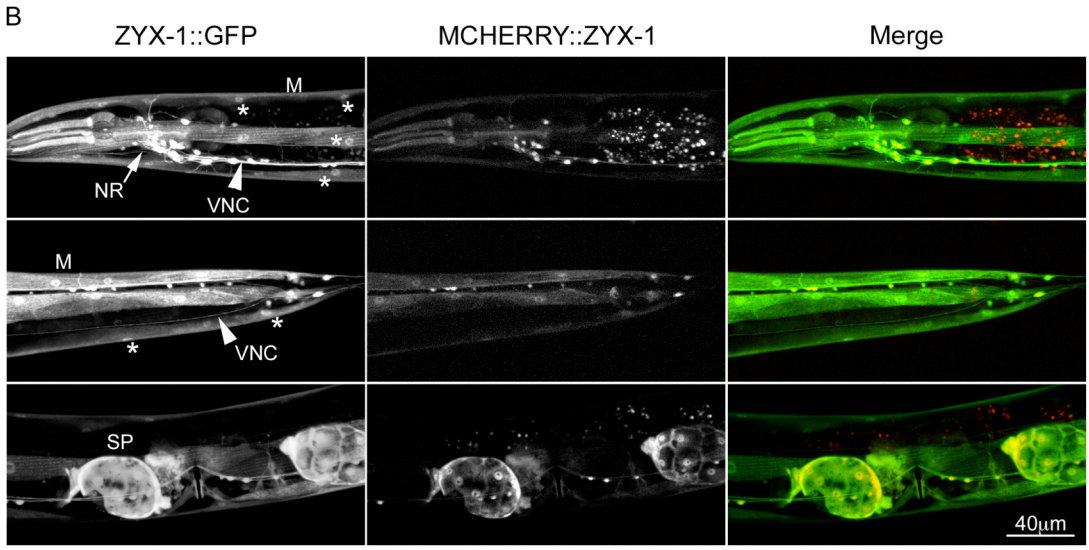


Figure 4. ZYX-1 functions autonomously in mechanosensory neurons to regulate PLM synapse development. The percentage of rescued animals with 2 PLM synapses, 1 PLM synapse, and no PLM synapses are quantified. A) Expression of full length ZYX-1 cDNA in presynaptic mechanosensory neurons significantly rescues *zyx-1* mutant phenotype, while muscular or interneuronal expressions do not. B) Expression of ZYX-1 LIM domains in mechanosensory neurons significantly rescues *zyx-1* mutant phenotypes. The error bars represent standard deviation (SD).

Figure 4. ZYX-1 functions autonomously in mechanosensory neurons to regulate PLM synapse development.

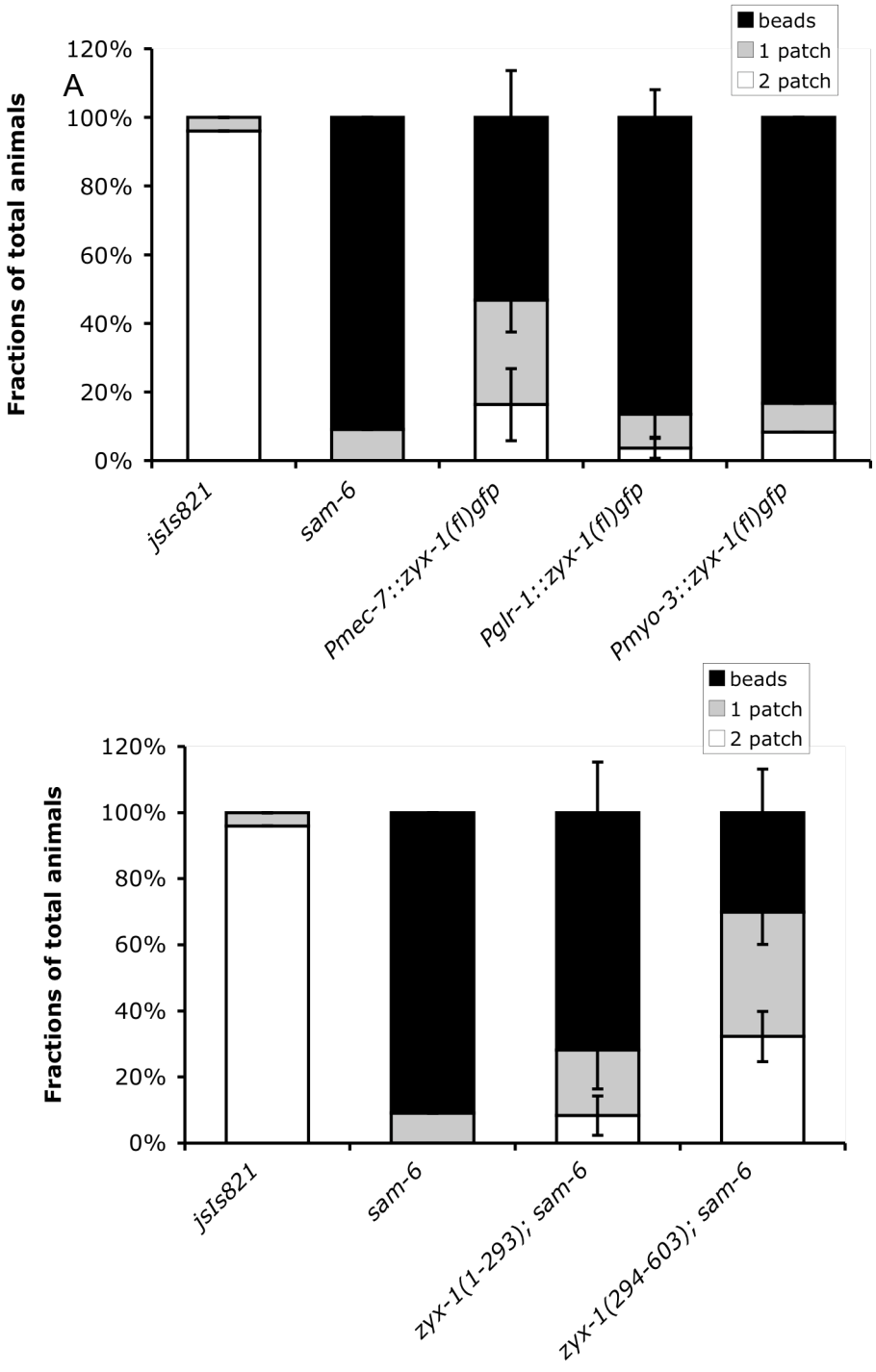


Table 1. Test of candidate molecules that may regulate PLM synapse development. None of the genes examined have significant roles in PLM synapse formation or maintenance.

Genotype	N	Two patches	One patch	No patches
Wild type	29	97%	3%	0%
zyx-1(js417)	50	0%	8%	92%
zyx-1;mkk-4	128	15.6%	22.7%	61.7%
zyx-1;pmk-3	128	7.0%	16.4%	76.6%
zyx-1;dlk-1	55	14.5%	9.1%	76.4%
unc-97(su110)				
unc-98(su130)	12	66.7%	33.3%	0%
ina-1(gm39)	25	100%	0%	0%
ina-1(gm144)	50	96%	2%	2%
pat-3;[pat-3(Y804F)]	79	74.7%	17.7%	7.6%
pat-4;[pat-4(S334A)::gfp]	90	87.8%	7.8%	4.4%
syg-1(ky652)	48	96%	4%	0%
syg-2(ky671)	34	97%	3%	0%
syd-1(ju2); syd-2(ju487)	41	88%	12%	0%

Chapter 5

Concluding Remarks

When Sydney Brenner first settled on *Caenorhabditis elegans* as a genetic model about half a century ago, his goal was to ultimately use this organism to unravel the mysteries of the nervous system. Realizing that the animal only has a limited number of neurons, he proposed the complete circuitry of the nervous system could be determined by serial-section electron microscopy, and based on this it is possible to dissect the roles of every single gene involved in neural development and function (Riddle et al., 1997). With White and his colleagues' efforts, the first part of Brenner's proposal had already come true 20 years before. Now with many new technologies not available at old days (RNAi, for example), people are making rapid progresses towards realizing the second part of the proposal.

My dissertation presented here has added another example of the power of *C. elegans* as a model organism to study nervous system function. In this dissertation, I have described the identification of intestinal peptide-related signals, and they likely function as modulators of multiple aspects of neural functions, such as defecation and synaptic transmission. Several lines of evidence in mammals have demonstrated that small peptides and hormones can modulate nervous system functions (Moult and Harvey, 2008). Interestingly, as a small organism as *C. elegans*, its genome contains over 100 identified peptide genes that encode over 250 peptides (Li, 2005). Evidence suggests that the peptides, together with their processing enzymes (proprotein convertases, PC), are present in many neuronal classes across the nematode nervous system, and they could be potentially co-released with conventional neurotransmitters (Li, 2005; Li et al., 1999). On the other hand, the *C. elegans* intestine is likely the largest endocrine organ in the

animal. With both the PC (i.e. AEX-5) and peptides present in this organ, the intestine likely plays an important role in modulating other neuronal functions. In the future, it will be interesting to adopt efficient genetic approach (such as RNAi) to disrupt intestinal peptide expressions to see what other neuronal phenotypes could occur. If there is any, by scoring one easily observable phenotype (such as defecation), one may be able to identify molecules, such as peptides, peptide-processing enzymes, and peptide-release related exocytic factors that are involved in the nervous system function. The *C. elegans* intestine will serve as a powerful start point to demystify genes involved in neural function, and this, together with other studies, will ultimately help people move towards realizing what Brenner had proposed when he first adopted this small organism.

References

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