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Neuropeptide Modulators of the C. elegans locomotor circuit

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Neuropeptide Modulators of the *C. elegans* locomotor circuit

A Thesis submitted in partial satisfaction of the requirements for the degree

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in

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by

Kingston Zhou

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The Thesis of Kingston Zhou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California San Diego

2018

DEDICATION

This thesis is dedicated to my mother, of whom I cannot ask for any more because she has already given me everything.

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I would like to acknowledge members of the Jin & Chisholm lab for their continuous support throughout my program; I would be utterly lost without their willingness to help.

Data involving the fluorescent reporter of *ins-29* and convulsion frequency of *frpr-14(0); acr-2(gf)* animals were provided with permission by McCulloch, Katherine. The thesis author was the primary author of this material.

ABSTRACT OF THE THESIS

Neuropeptide modulators of the *C. elegans* locomotor circuit

by

Kingston Zhou

Master of Science in Biology

University of California San Diego, 2018

Professor Yishi Jin, Chair

Neuropeptides are small peptides secreted from neurons that modulate the activity of the nervous system. Aberrant neuropeptide signaling, then, can be detrimental to the behaviors they modulate.

Studying neuropeptides can characterize how neural circuits are regulated to maintain optimal activity. I utilized a genetic model of epilepsy in *C. elegans* to identify neuropeptide modulators of the locomotor circuit. Based on a list of genes that were previously found to be up-regulated in this model, I generated compound mutants. Using compound and confocal microscopy, I found that one up-regulated neuropeptide was ectopically expressed due to circuit hyperactivity. Following genetic and pharmacological analyses, I identified four neuropeptides that promote hyperactivity in the locomotor circuit.

Introduction

The balance of excitatory and inhibitory inputs is required for optimal neural circuit function. During human development, genes and experience drive the pruning of synapses, achieving an optimal excitatory/inhibitory (E/I) balance (Tierney, 2009). Aberrant E/I balance in neural circuits has been observed in many neurological diseases, such as epilepsy (Mann et al., 2008). Mutations in activity-dependent genes are implicated in many neurological diseases, such as MeCP2 in Rett syndrome (Ebert, 2013). Thus, activity-dependent changes in gene expression may modulate neural circuit function under aberrant contexts to restore homeostasis.

Neuropeptide-encoding genes are commonly upregulated in response to changes in circuit activity. Neuropeptides are small signaling peptides that are stored and released from dense core vesicles within neurons and target G-protein coupled receptors (Suudhof 2008, Jekely, 2018). Neuropeptides have been revealed to play a variety of modulatory roles in neural circuits; hence, studying neuropeptides offers key insights into how circuits respond to E/I imbalance (Kow et al., 1988). For instance, in mice, the peptide galanin was found to play a regulatory role for

epileptic activity (Lerner et al., 2008). *Caenorhabditis elegans,* an organism whose neural circuit has been completely mapped, express neuropeptides that are structurally similar to human neuropeptides. Genetic crosses and transgenic lines are relatively easily to generate in *C. elegans,* which present an advantageous model organism in which neuropeptides may be studied.

In all animals including humans, *Drosophila*, and *C. elegans,* neuropeptide genes code for similar precursor molecules that undergo a variety of processing (Figure 1). These precursor molecules, starting as pre-proneuropeptides, first undergo signal peptide cleavage by a signal peptidase at the rough endoplasmic reticulum, producing the proneuropeptide (Jekely, 2018). Afterwards, the proneuropeptide is trafficked to the Golgi apparatus and packaged into secretory vesicles for proteolytic processing. Here, the endopeptidases cathepsin L and proprotein convertases cleave dibasic residues of the proneuropeptide (Funkelstein et al., 2010). Basic residues remaining at the amino- or carboxy- terminus following endopeptidase cleavage are then removed by the exopeptidases aminopeptidase and carboxypeptidase E, respectively, generating the active neuropeptide (Hook et al., 2008). Lastly, neuropeptides undergo varying posttranslational modification, such as C-terminal amidation and disulfide bond formation, which alters their

biological activity (Brange et al., 1993; Eipper et al., 1992). Figure 1 outlines the general pathway for processing of a proneuropeptide into an active neuropeptide. While diverse, neuropeptides are commonly observed to serve redundant roles to affect the same behavior, which is a feature of convergent signaling. For instance, in the sea slug *Aplysia*, the neuropeptides FCAP and CP2 released from the same neuron both work to prime a downstream neuron to fire quickly (Koh, 2003).

Neuropeptide-encoding genes in *C. elegans* are organized into 3 classes: FMRFamide-like peptides (*flp*), insulin-like genes (*ins*), and neuropeptide-like proteins (*nlp*) (Li et al, 2008). Members of the *ins* class encode for peptides with a B and A domain after processing with varied disulfide linkages, and some *ins* gene products have been shown to signal through the DAF-2 insulin-like receptor to regulate reproductive growth (Pierce, 2000). Members of the *flp* class share a common C-terminus RFamide group (Li, 2014). All other neuropeptides that lack the features of *ins* and *flp* gene structures are classified as *nlp* genes (Li, 2009). Many *C. elegans* neuropeptide functions and signaling pathways remain to be discovered.

I have used the motor circuit of *C. elegans* to study the regulation of E/I balance. Previous studies have found that neuropeptides play

modulatory roles in regulating locomotion (Li et al., 2005). *C. elegans* locomotion results from coordinated cholinergic excitation and GABAergic inhibition of muscles, producing a regular sinusoidal pattern of movement (Richmond et al., 1999). Cholinergic transmission from nerve to the muscle involves acetylcholine release and subsequent binding to muscle nicotinic acetylcholine receptors (nAchRs), which are evolutionarily conserved pentameric ion channels (Albquerque et al., 2009). The *C. elegans* gene *acr-2* encodes for one of the five subunits of a neuronal acetylcholine receptor (Jospin et al., 2009). The ACR-2 receptor regulates cholinergic neurotransmission pre-synaptically by controlling acetylcholine release within cholinergic ventral cord motor neurons. Null mutations in *acr-2* result in reduced acetylcholine neurotransmission and slower movement (Jospin et al., 2009). In contrast, a valine to methionine substitution in *acr-2,* [*acr-2(gf)*], results in a gain-of-function mutation that causes increased cholinergic excitation and a cell non-autonomous reduction in GABAergic inhibition in the motor circuit, and whole-body spontaneous shrinking or convulsions (Jospin et al., 2009). A similar valine to methionine substitution in a human nAchR gene, *CHRNB2,* has been associated with autosomal dominant nocturnal frontal lobe epilepsy (Phillips et al., 2001).

Several lines of evidence have shown interactions between *acr-2(gf)* and neuropeptide signaling. Previously in *C. elegans*, loss-of-function mutations in *unc-31* [*unc-31(lf)*], which is required for neuropeptide release, were found to reduce the convulsions of *acr-2(gf)* animals, suggesting the collective effect of neuropeptide action on locomotion was promoting circuit hyperactivity (Stawicki et al., 2013). In contrast, the neuropeptide *flp-18* was shown to work synergistically with *flp-1* to reduce circuit hyperactivity, highlighting two specific neuropeptides that are inhibitory in *acr-2(gf)* animals. In addition, they found *flp-18* expression to be increased in *acr-2(gf)* motor neurons, suggesting transcriptional changes in neurons in response to hyperactivity. Therefore, although the totality of neuropeptide function in *C. elegans* is to promote hyperactivity in *acr-2(gf)*, the specific neuropeptides involved remained to be discovered.

In efforts to investigate changes in the expression of activity-dependent genes in an imbalanced locomotor circuit, previous RNA-seq analyses have described changes in the neuronal transcriptome in an *acr-2(gf)* background relative to wild-type, (McCulloch, K., unpublished data). Over 200 genes were mis-expressed in *acr-2(gf)* mutants. Interestingly, RNA-seq analyses revealed upregulated transcription of members of all classes of neuropeptide-encoding genes in *acr-2(gf)* mutants (McCulloch, K., unpublished data). These genes include the neuropeptide-like proteins

and FMRFamide-like peptides *nlp-1, flp-24, flp-12,* and *flp-18,* and insulin-like genes *ins-29* and *ins-25.* Notably, the identification by RNA-seq of *flp-18* up-regulation validated prior findings of increased *flp-18* expression in the *acr-2(gf)* animals (Stawicki et. al, 2013). From these enriched genes, I aimed to identify and elucidate the function of additional neuropeptide-encoding genes closely relevant to the *C. elegans* motor circuit.

To identify neuropeptide modulators of *C. elegans* locomotion, used a combination of genetics and pharmacological approaches to identify and characterize neuropeptides involved in motor behavior. I firstly present my finding that *flp-12(0)* combined with either a null mutation in *ins-29 ins-25* [*ins-29 ins-25(0)*] or *flp-24(0)* reduced the convulsion frequency in the *acr-2(gf)* background. This observation led me to pursue how *flp-12* functions to affect locomotion. Next, I present experiments I performed to elucidate the function of *flp-12*. Lastly, I discuss other enriched genes in the *acr-2(gf)* background.

Materials and Methods

Maintenance and Construction of Strains

All strains were maintained on nematode growth media (NGM) plates as described in Brenner 1974. Strains containing *acr-2(n2420)* and a single mutation in a gene on another chromosome were generated by crossing males carrying the gene of interest into MT6241 [*acr-2(n2420)*] hermaphrodites, followed by isolation of double mutant self-progeny through genotyping and the convulsion phenotype. Different crossing schemes were used to generate *acr-2(gf)* animals with mutations in *nlp-1* and *flp-12; acr-2, nlp-1, and flp-12* are all linked on the X chromosome. CZ26047 [*nlp-1(ok1469) acr-2(n2420)*] was generated by crossing males heterozygous for *nlp-1(ok1469)* into MT6241 [*acr-2(n2420)*] hermaphrodites. Self-progeny from doubly heterozygous F1 hermaphrodites were selected for *acr-2(n2420)* by the convulsion phenotype. *acr-2(n2420)* homozygous progeny heterozygous for *nlp-1(ok1469)* via a recombination event were selected for by genotyping by PCR (with primers KM163 and KM164, see Table 1). Double homozygous animals were identified in the next generation by PCR genotyping. CZ26269 [*flp-12(ok2409) acr-2(n2420)*] was generated in the same fashion, except the marker *lon-2(e678)* in the strain with genotype *lon-2(e678) acr-2(n2420)* was used to select for recombinant heterozygotes for *flp-12(ok2409)*, in which the disappearance of the long phenotype indicated chromosomal recombination in *acr-2(n2420)* homozygotes with

flp-12(ok2409). Compound mutants containing at least two mutations combined with *acr-2(n2420)* were generated by crossing males carrying the gene of interest into previously generated strains containing *acr-2(n2420)* and another gene of interest and monitored by genotyping.

Convulsion Assays

Convulsion assays were performed on NGM plates seeded with OP50. On the day prior to scoring convulsions, about 10 L4 worms of each genotype were separately picked onto seeded NGM plates that were randomized to prevent bias. 10 L4 worms of strain MT6241 [*acr-2(n2420)*] were picked onto the same set of randomized plates the day prior to assays. 24 hours later, 1-day old adult worms were monitored for a period of 1 minute and 30 seconds, in which convulsions were counted.

Aldicarb sensitivity assays

Aldicarb sensitivity assays were performed on OP50-seeded NGM plates containing the acetylcholinesterase inhibitor aldicarb. On the day prior to scoring, 10 L4 worms of each strain to be scored were picked together onto seeded, randomized NGM plates. The following day, 10 1-

day old adult worms of each strain were transferred to seeded plates containing 500 μM aldicarb. The number of paralyzed worms for each strain was counted every 30 minutes for 3 hours.

Levamisole sensitivity assays

Levamisole sensitivity assays were performed on seeded NGM plates containing the acetylcholine receptor agonist levamisole. On the day prior to scoring, 10 L4 worms of each strain to be scored were picked together onto seeded, randomized NGM plates. The following day, 10 1-day old adult worms of each strain were transferred to seeded plates containing 1 mM levamisole. The number of paralyzed worms for each strain was counted every 15 minutes for 1 hour.

Molecular Cloning

Plasmids used for rescue of *flp-12* and as fluorescent reporters of neuropeptide genes were all constructed in a similar fashion. Inserts were amplified from genomic DNA by PCR, which were then ligated to a vector linearized by restriction enzyme digest or PCR using a Gibson Assembly mix from New England Biolabs, Inc.

The *nlp-1* fluorescent reporter [*Pnlp-1::gfp*] was constructed using the endogenous 2 kb sequence upstream of the start codon of *nlp-1* amplified by PCR and the vector pPD95.75. pPD95.75 was linearized by XbaI at the multi-cloning site, and the amplified *Pnlp-1* sequence was ligated to the vector by Gibson Assembly.

A plasmid was constructed for overexpression of *flp-12* by heat-shock [*Phsp-16.2:;flp-12::SL2::mKate2*]. The expression of *flp-12* and the red fluorescent reporter *mKate2* is controlled by the heat-shock promoter *Phsp-16.2*. *mKate2* is trans-spliced using the *SL2* sequence. To construct this plasmid, the endogenous DNA sequence of *flp-12* was amplified by PCR, which was inserted downstream of *Phsp-16.2* in the vector pPD49.78 linearized by NcoI and EcoRV. *SL2::mKate2* amplified by PCR was then inserted downstream of *flp-12* at the SacI restriction site.

The plasmid used as a transcriptional reporter and rescue of *flp-12* was generated using the genomic sequence of *flp-12* and its endogenous 2 kb sequence promoter upstream of its start codon. This sequence was amplified by PCR and ligated to the *Phsp-16.2::flp-12::SL2:mKate2* plasmid, which was linearized by PCR to exclude the *Phsp-16.2:;flp-12* sequence. See Table 2 for primers used in cloning reactions.

Confocal microscopy

On the day prior to imaging, L4 animals were transferred to NGM plates seeded with OP50. The following day, one-day old adult worms were prepared for confocal microscopy as described previously using a Zeiss LSM 710 microscope (Shaham, 2006). Animals not rolled were paralyzed with levamisole when picked onto slides with a 1% agar pad under a cover slip. Animals that were rolled were picked onto M9 solution on slides prepared a 10% agar pad, then rotated by a cover slip. Images were taken at 63x magnification.

DiI staining and imaging

On the day of imaging, animals were transferred into an Eppendorf tube with 1 ml M9 and spun down at 3000 rev/min. The supernatant was decanted by vacuum, and the worm pellet was resuspended with 1 ml M9 and 5 µl DiI stock dye solution (Molecular Probes, 2 mg/ml concentration) was added. Animals were then incubated on a slow shaker for approximately 3 hours and transferred to an OP50-seeded NGM plate afterwards. Stained animals were picked onto levamisole on slides prepared with 1% agar pads under a cover slip for visualization by fluorescence microscopy. Images were taken with the Zeiss Axiocam microscope camera at 40x magnification.

Results

Ectopic expression of neuropeptide genes in *acr-2(gf)* animals.

nlp-1 was found to be the most up-regulated *nlp* in *acr-2(gf)*. In wild type animals, *nlp-1* is expressed in sensory amphid neurons in the head (AWC and ASI), as well as the BDU interneuron and PHB neuron in the tail (Nathoo et al., 2001). Amphid neurons serve chemosensory functions and have ciliated nerve endings exposed to the exterior environment. I constructed a green fluorescent transcriptional reporter of *nlp-1* [*Pnlp-1::gfp*] to document expression in *acr-2(gf)* animals (Figure 2A). This transgene was microinjected into wild type animals. I then crossed the transgene into *acr-2(gf)* animals and characterized its expression across wild type and *acr-2(gf)* animals. Using confocal microscopy, images of the heads of animals rolled onto the ventral plane were taken of one day old adult wild type and *acr-2(gf)* animals (Figure 2B). Compared to wild type, *acr-2(gf)* animals were found to have two additional cell bodies in the head fluorescing GFP. These observations suggest that the increased expression of *nlp-1* detected by RNA-seq is due, at least in part, to expression in additional cells compared to wild type. A DiI staining protocol was performed to identify cell bodies ectopically expressing *nlp-1.*

The DiI dye stains ADL, ASH, ASI, ASJ, ASK, and AWB amphid neurons red-orange. The dendrites of these sensory neurons are exposed to the external environment, allowing uptake of the dye. DiI staining revealed that neither ectopic cell is an amphid neuron. Additionally, colocalization of red and green signal marked the ASI cell body in both wild type and *acr-2(gf)* animals, indicating that within *acr-2(gf)*, *nlp-1* is still transcribed in cells in which it is normally expressed (Figure 2C).

A fluorescent transcription reporter of *ins-29* was also constructed [*Pins-29::gfp*] (McCulloch, K., unpublished data). *ins-29* is the second-most up-regulated neuropeptide gene in the *acr-2(gf)* background. In wild type animals, weak GFP expression was sometimes observed in two head neurons. However, *acr-2(gf)* animals show strong GFP expression in these cells, indicating elevated expression of *ins-29* in *acr-2(gf)* animals (Figure 2D). DiI staining showed that these cells are not amphid neurons (Figure 2E).

Previous studies had found *flp-18* to be expressed in the motor neurons to regulate locomotor activity, and its expression was elevated in *acr-2(gf)* animals in the same cells (Stawicki et. al, 2013). Analysis of *ins-29* and *nlp-1* transcriptional reporters, in contrast, suggest that these peptides function in neurons in the head, perhaps non-amphid command

interneurons, in response to motor hyperactivity. My studies also show that, in addition to inducing upregulation of neuropeptides in the same cells as wild type, *acr-2(gf)* can also result in ectopic expression of neuropeptides, suggesting complex transcriptional effects due to hyperactivity.

flp-12(0) combined with either *ins-29 ins-25(0)* or *flp-24(0)* reduces the convulsion rate of *acr-2(gf)* animals.

To determine neuropeptide modulators of locomotor circuit function, I generated *acr-2(gf)* animals that contained mutant alleles for several neuropeptide genes that were found to be up-regulated in *acr-2(gf).* I measured the convulsion frequency between these compound mutants and animals with *acr-2(gf)* alone. Mutant alleles introduced were large deletions that include the large portions of the short coding regions (less than 2 kb total) of neuropeptide genes; therefore, they were likely null *(0)* mutations (Figure 3A). None of these mutants alone had a significant effect on convulsion frequency. Interestingly, when *flp-12(0)* was combined with either *flp-24(0)* or *ins-29 ins-25(0)* in *acr-2(gf)*, the rate of convulsions of each strain was markedly reduced (Figure 3B). These mutations' combined effect on convulsions suggests that their respective

neuropeptides work to affect locomotor behavior through convergent pathways. Given that these mutant alleles suppressed convulsive activity, it is suggested that these neuropeptide genes normally promote chronic hyperactivity of the locomotor circuit. This finding is consistent with previous evidence that showed reduced convulsions in *unc-31(lf); acr-2(gf)* animals, in which *unc-31(lf)* prevented neuropeptide release globally (Stawicki et al., 2013). My results highlight *flp-12, ins-29, ins-25,* and *flp-24* as potential contributors to the *acr-2(gf)* convulsion phenotype. Hence, I sought to investigate the effects of *flp-24*, *ins-29 ins-25*, and *flp-12* on neurotransmission.

flp-12(0), ins-29 ins-25(0), and *flp-24(0)* do not alter presynaptic cholinergic transmission.

As *flp-12(0), ins-29 ins-25(0),* and *flp-24(0)* suppressed convulsive activity of *acr-2(gf)* animals, I examined how neurotransmission is altered in these animals to result in this phenotype. To this end, I performed pharmacological assays. One such assay involves the acetylcholinesterase inhibitor aldicarb, which, over time, leads to paralysis in *C. elegans* (Mahoney et al., 2006). I employed this assay to find differences in presynaptic cholinergic release between strains. I found that

mutants for *flp-12* in the *acr-2(gf)* background showed no significant differences in their sensitivity to aldicarb relative to *acr-2(gf)* alone (Figure 4A and B). My findings from aldicarb sensitivity assays indicate that changes in presynaptic components of neurotransmission by mutant alleles of *flp-12, ins-29, ins-25,* and *flp-24* are not likely to be the primary cause of suppressed locomotor circuit activity. This led me to employ another pharmacological assay to test postsynaptic differences in neurotransmission between mutants.

flp-12(0) with *ins-29 ins-25(0)* reduces postsynaptic excitation of the muscle.

The drug levamisole, a muscle acetylcholine receptor agonist, also causes paralysis in *C. elegans* over time and provides a test for differences in postsynaptic excitation of the muscle between mutants (Lewis et al., 1980). I found that with *acr-2(gf)*, *ins-29 ins-25(0)* alone or with *flp-12(0)* decreased animals' sensitivity to levamisole compared to *acr-2(gf)* alone (Figure 5A and B). This suggests that rather than mediating presynaptic acetylcholine release, *flp-12* and *ins-29 ins-25* may be altering postsynaptic neurotransmission to reduce overexcitation of muscle. Interestingly, no significant differences in sensitivity were found

between *flp-24(0); flp-12(0) acr-2(gf)* mutants and *acr-2(gf)* animals alone. The mechanism by which *flp-24* mediates locomotor circuit activity remains to be elucidated. I next tested mutants outside of the *acr-2(gf)* background in order to examine their effects on neurotransmission without *acr-2(gf)*.

Increased resistance to levamisole by *flp-12(0)* and *ins-29 ins-25(0)* is specific to the *acr-2(gf)* background.

I crossed mutant alleles for *ins-29 ins-25, flp-24,* and *flp-12* out of the *acr-2(gf)* background to determine whether the previously observed sensitivities to aldicarb and levamisole would be maintained in the absence of chronic circuit hyperactivity. I performed aldicarb and levamisole sensitivity assays on mutants without *acr-2(gf)*, including the *flp-24(0); flp-12(0)* and *ins-29 ins-25(0); flp-12(0)* combinations (Figure 6). Notably, no significant differences in the percentage of animals left non-paralyzed by either drug between wild type and any mutant strains were found, indicating that *ins-29 ins-25(0); flp-12(0)* conferred decreased sensitivity to levamisole solely in the context of chronic circuit hyperactivity provided by *acr-2(gf)*.

Other enriched genes in *acr-2(gf)*

Mutant alleles for non-neuropeptide genes that were also enriched in *acr-2(gf)* were crossed into the *acr-2(gf)* background to identify regulators of the locomotor circuit. I crossed alleles for enriched genes involved in fatty acid metabolism (*hacd-1* and *acox-1*) and synaptic transmission (*shw-3, lgc-10,* and *frpr-14*). *hacd-1* encodes for hydroxyl-CoA dehydrogenase, which participates in fatty acid β-oxidation in mitochondria. *acox-1* is the *C. elegans* ortholog to human peroxisomal acyl-CoA oxidase 1 and participates in a peroxisomal oxidation pathway. As both *hacd-1* and *acox-1* were up-regulated in *acr-2(gf)* animals and are involved in separate oxidation pathways, I hypothesized that combining mutations for these genes within *acr-2(gf)* may affect locomotion. However, no significant differences were found in the convulsion frequencies in *acr-2(gf)* animals with mutated genes involved in fatty acid metabolism and *acr-2(gf)* alone (Figure 7A).

shw-3 is the *C. elegans* ortholog to several human potassium voltage-gated channels. Its expression in mechanosensory neurons, as well as its predicted ion channel function, suggests *shw-3* to have a potential role in the locomotor circuit (Spencer et al., 2011). Similar to *shw-3, lgc-10* is an ortholog of a human ion channel family, specifically the

human zinc-activated ion channel. In contrast, *frpr-14* putatively encodes a putative FMRFamide neurotransmitter receptor. I proposed that mutant combinations of these genes associated with synaptic transmission with *acr-2(gf)* would affect convulsive activity. However, no significant differences were found between *acr-2(gf)* double mutants and *acr-2(gf)* alone (Figure 7B). Still, triple mutants have yet to be generated and assayed for changes in locomotion.

Data involving the fluorescent reporter of *ins-29* and convulsion frequency of *frpr-14(0); acr-2(gf)* animals were provided with permission by McCulloch, Katherine. The Thesis author was the primary investigator and author of this paper.

Discussion

<u>Ectopic expression of neuropeptide genes</u>

In *acr-2(gf)* animals, *nlp-1* is ectopically expressed in two additional cells in the head, while *ins-29* showed increased expression in the same cells as wild type (Figure 2). These cells are yet to be identified, but this expression profile suggests that other neuropeptides, such as *flp-12,* may also be ectopically expressed under the genetic background of *acr-2(gf)*,

yielding different phenotypes. Interestingly, unlike *flp-18,* which regulated excitation with *acr-2* in motor neurons, *nlp-1, ins-29,* and *ins-25* are not expressed in motor neurons. Still, *flp-12(0)* with *ins-29 ins-25(0)* altered postsynaptic excitation of the muscle in *acr-2(gf)* animals. Neuropeptides are secreted molecules; therefore, it is likely they function from a distance, secreted from head neurons to regulate locomotion. As previously discussed, control of *flp-12* expression by LIM-4 altered the neuronal subtype of the SMB neuron, indicating that other neuronal subtypes may be significantly changed due to ectopic expression of other neuropeptides in *acr-2(gf)* (Kim et al., 2015).

flp-12, ins-29, ins-25, and *flp-24* are neuropeptide modulators of the locomotor circuit.

In the *acr-2(gf)* background, *flp-12(0)* combined with *ins-29 ins-25(0)* or *flp-24(0)* resulted in significantly lower convulsion rates compared to *acr-2(gf)* alone (Figure 3). Hence, they function to promote chronic circuit hyperactivity in *acr-2(gf)* mutants. This is in contrast to the findings of *flp-1* and *flp-18,* which reduce the hyperactivity of *acr-2(gf)* (Stawicki et al., 2013). As elimination of global neuropeptide function by *unc-31(lf)* resulted in a lower convulsion rate in *acr-2(gf)* animals, the loss of function

of *flp-12, ins-29, ins-25,* and *flp-24* then are significant contributors to the observed reduction in convulsions in *unc-31(lf); acr-2(gf)* animals (Stawicki et al., 2013). However, other neuropeptide regulators of the circuit remain to be discovered, because more mutant combinations for up-regulated neuropeptide genes are yet to be generated and tested for locomotion.

flp-12, ins-29, and *ins-25* promote hyperactivity postsynaptically in the muscle.

Pharmacology experiments indicated that *flp-12* and insulin-like peptides regulate motor function post-synaptically in the muscle, rather than regulating cholinergic neuron activity. Additionally, changes were only observed in the *acr-2(gf)* genetic background (Figure 6). *flp-12, ins-29,* and *ins-25,* then, may only be mediating excitation of the muscle in the context of chronic circuit hyperactivity. Prior to this research, *ins-29* and *ins-25* have had no reported function; *flp-12* mutants are reported to have repetitive turning behavior in males, indicating that *flp-12* is involved with locomotion (Liu et al., 2007). This work is the first to report a function for *flp-12* in hermaphrodite locomotion.

To date, most of the neuropeptides that regulate *acr-2(gf)* behavior, aside from *flp-18*, are not expressed in the motor neurons, but in the head.

The neurons expressing *ins-29* and *ins-5* have not yet been identified. In wild type *C. elegans*, *flp-12* has been shown to be expressed in the AVH/AVJ, BAG, PDA, PVR, SAA, SDQ, and SMB neurons (Chang et al., 2015). The SMB neuron is a cholinergic pre-motor interneuron involved in local search behavior. *flp-12* expression is regulated by the transcription factor LIM-4 and determines the neuronal subtype of the SMB neuron (Kim et al., 2015). I have constructed a plasmid in which *flp-12* and the red fluorescent protein *mKate2* are transcribed under the control of endogenous *flp-12* promoter [*Pflp-12::flp-12::SL2::mKate2*], which should serve to not only characterize the expression of *flp-12*, but also to rescue *flp-12* function. I expect that wild type *flp-12* expression would rescue the convulsion rate and levamisole sensitivity of *ins-29 ins-25(0); flp-12(0) acr-2(gf)* mutants back to *acr-2(gf)* levels.

The receptor targets through which *flp-12* signals to affect locomotor circuit function would further elucidate the action of these neuropeptides. However, determining the primary GPCRs that neuropeptides bind to remains a challenge, as neuropeptides and their receptors are both commonly found to be promiscuous, meaning that they may have more than one binding partner (Janssen et al., 2010). Still, efforts to de-orphanize GPCRs in *C. elegans* may provide candidate receptors for neuropeptides. Such efforts have suggested that *frpr-8,*

which encodes a GPCR, may be a candidate receptor for *flp-12* (I. Beets, personal communication). Genetic knock-out of *frpr-8* and further locomotive and pharmacologic analysis of *ins-29 ins-25(0); frpr-8(0) acr-2(gf)* mutants would determine whether *flp-12* mediates locomotion primarily through *frpr-8* signaling if these mutants are observed to have convulsive activity and sensitivity to levamisole similar to *ins-29 ins-25(0); flp-12(0) acr-2(gf)* animals as reported here.

Non-neuropeptide genes enriched in *acr-2(gf)*

While locomotive analyses determined no significant changes in convulsions between *acr-2(gf)* animals and animals with mutant alleles of enriched non-neuropeptide genes combined with *acr-2(gf)*, other phenotypes remain to be characterized in these mutants (Figure 7). Research presented here primarily examined locomotion, as *acr-2(gf)* animals' most prominent and characterized phenotype is their convulsive behavior. Up-regulation of non-neuropeptide genes by the chronic circuit hyperactivity of *acr-2(gf)* may affect other behaviors, including, but not limited to: egg-laying, stress-induced sleep, feeding, olfaction, and mating. Phenotypes that are primarily controlled by cholinergic signaling and neuropeptides, such as egg-laying, are of special interest in future studies.

Analysis of these behaviors within genetic combinations of mutant alleles for enriched non-neuropeptide genes in the *acr-2(gf)* background may shed light on the functional importance of these genes in additional behaviors.

Table 1. Genotyping primers

Gene(allele)	Mutation	Analysis method	Primer name	Sequence (5' to 3')
nlp-1(ok1469)	641 bp deletion	Gel	YJ12276	GATCAGCCGAGCAGAATGA
			YJ12277	ATTCAGAAGCGGAAAGAGCA
flp-12(ok2409)	564 bp deletion	Gel	KM163	TGCATTTTAGGAACTCGTCT
			KM164	TCTCGTTCTCGTTTCTGATT
ins-29 ins-25(ju1580)	2.2 kb deletion	Gel	KM175	CGCCCACTTTTGACCTATTC
			KM176	CTTTGAAGTTCGCCCACAGT
			KM207	GCAAGATTTGAAGGACAGCAC
flp-24(gk3109)	1180 bp deletion	Gel	KM151	CGATGTTCCGCTCTGAGCTTC
			KM152	TGGTCACAGTGCATTGCTCTC
flp-18(tm2179)	1286 bp deletion	Gel	YJ5881	ATGATGATGCCACCTGGAACAC
			YJ5882	TTAGGAGGAATTTGGACGCTTAG
nlp-8(ok1799)	695 bp deletion	Gel	KZ1000	CCCACCCACACTATCTTCTT
			KZ1001	CAAGACGTCGAGAGTCACTG
hacd-1(ok2776)	555 bp deletion	Gel	KM169	CTTGACCGCGAAAATAGAAA
			KM170	TCTGCAGAACATCAAGTCCA
acox-1(ok2257)	1064 bp deletion	Gel	KM159	TGGATTCCTAGGCTTCAACAA
			KM160	TGTCGCTTTTGTTACAATGTCTT
shw-3(ok1884)	1112 bp deletion	Gel	YJ7245	CTCTGGCCCTTCATTCTCTG
			YJ7243	TCTGCCAGGCACTGAGACTA
frpr-8	CRISPR-Cas9 mediated deleiton	Gel	KZ1040	GACCCCTACGTCACTGCTTC
			KZ1041	TTGGGACTGTTGAGTGAGGA
			KZ1042	CCCTAGTGTGGCAAGGTTTT
frpr-14(gk751210)	C -> T point mutation	Loss of Cac8I cut site in mutant: gel	KZ1002	GGCGGTAGCTTAAGGTTTTT
			KZ1004	CAGGCCTCCAACACAAATAG

Table 2. Primers used in cloning reactions

Amplicon	Purpose/notes	Primer name	Sequence (5' to 3')
flp-12	Insertion into pPD49.78 for *Phsp16.2::flp-12::SL2::mKate2*	KZ1007	TAGCGTCGACGGTACATGAATGTTCAAGTCATCATC
		KZ1008	GATGCGGAGCTCAGATCTACTTTCGTCCAAATCG
SL2::mKate2	Linearizing backbone for *Pflp-12::flp-12::SL2::mKate2*	KM221	GCTTGCATGCCTGCAGGT
		KM224	GCTGTCTCATCCTACTTTC
Pflp-12::flp-12	Insertion into *SL2::mKate2* vector for *Pflp-12::flp-12::SL2::mKate2*	KZ1034	TGCATGCCTGCAGGTCGGGAATTAGATTGACATAG
		KZ1031	AGTAGGATGAGACAGCCTACTTTCGTCCAAATCG
Pnlp-1	Insertion into pPD95.75 vector for *Pnlp-1::gfp*	KM161	TGCATGCCTGCAGGTCGACTTTGTTTTATCCAACATTATTCAC
		KM162	TGGCCAATCCCGGGGATCCTCGTTGCCTCAAGTTGATG

Table 3. List of strains

Strain number	Genotype	Notes
CZ26047	*nlp-1(ok1469) acr-2(n2420) X*	
CZ26121	*hacd-1(ok2776) V; acr-2(n2420) X*	
CZ26266	*shw-3(ok1884) V; acr-2(n2420) X*	
CZ26267	*nlp-8(ok1799) I; acr-2(n2420) X*	
CZ26268	*lgc-10(gk5709) IV; acr-2(n2420) X*	
CZ26269	*flp-12(ok2409) acr-2(n2420) X*	
CZ26270	*ech-7(et6) I*	*Paqr-2(tm3410)* crossed out of strain QC119 [*ech-7(et6);paqr-2(tm3410)*]
CZ26475	*Pnlp-1::gfp (juEx7879)*	*Pnlp-1::gfp* & *Pmyo-2::rfp* transgenic line (1/2)
CZ26476	*Pnlp-1::gfp (juEx7880)*	*Pnlp-1::gfp* & *Pmyo-2::rfp* transgenic line (2/2)
CZ26477	*acr-2(n2420) X; Pnlp-1::gfp (juEx7879)*	*juEx7879* crossed into *acr-2(n2420)*
CZ26478	*acr-2(n2420) X; Pnlp-1::gfp (juEx7880)*	*juEx7880* crossed into *acr-2(n2420)*
CZ26817	*flp-24(gk3109) III; acr-2(n2420) X*	
CZ26818	*flp-24(gk3109) III; nlp-1(ok1469) acr-2(n2420) X*	
CZ26819	*nlp-8(ok1799) I; flp-18(tm2179) acr-2(n2420) X*	
CZ26820	*acox-1(ok2257) I; hacd-1(ok2776) V; acr-2(n2420)*	

(Table 3 continued)

CZ26821	*ins-25 ins-29 (ju1580) I; flp-12(ok2409) acr-2(n2420) X*	
CZ26822	*ins-25 ins-29 (ju1580) I; flp-12(ok2409) X*	*acr-2(n2420)* crossed out of CZ26821.
CZ26823	*flp-24(gk3109) III; flp-12(ok2409) X*	*acr-2(n2420)* crossed out of CZ26824
CZ26824	*flp-24(gk3109) III; flp-12(ok2409)* *acr-2(n2420) X*	
VC1971	*flp-24(gk3109) III*	
RB1863	*flp-12(ok2409) X*	
VC1309	*nlp-8(ok1799) I*	
RB1340	*nlp-1(ok1469) X*	
CZ25972	*ins-29 ins-25(ju1580) I*	
FX02179	*flp-18(tm2179) X*	
VC1785	*acox-1(ok2257) I*	
RB2102	*hacd-1(ok2776) V*	
RB1556	*shw-3(ok1884) V*	
VC3751	*lgc-10(gk5709) IV*	
VC40662	*frpr-14(gk751210) II*	
QC119	*ech-7(et6) I; paqr-2(tm3410) III*	
MT6448	*lon-2(e678) acr-2(n2420) X*	

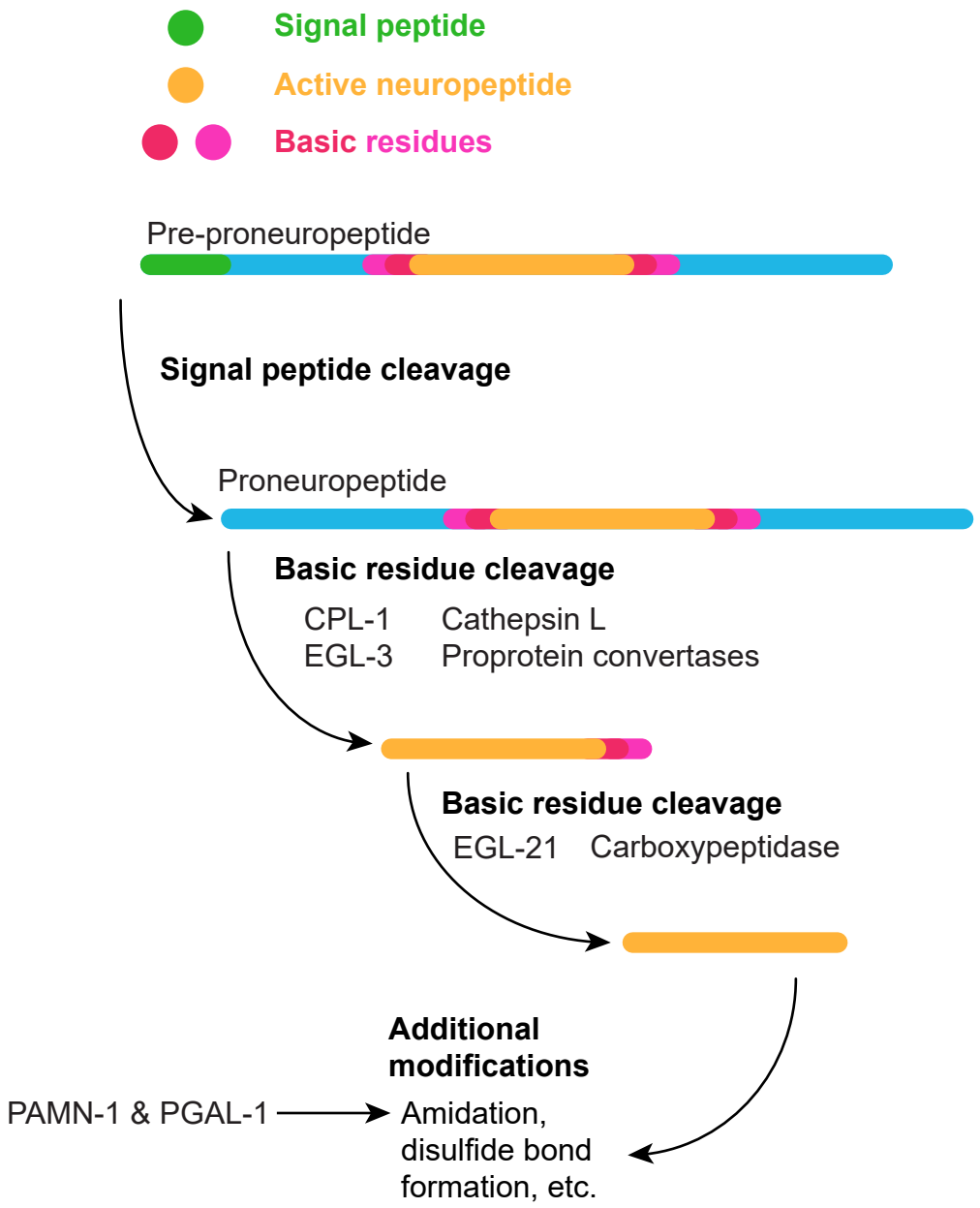

Figure 1. Neuropeptide processing. Neuropeptides are first translated as pre-proneuropeptides with a signal peptide that is cleaved by a signal peptidase. Dibasic residues are then cleaved by endopeptidases. Remaining basic residues are cleaved by exopeptidases, and the active neuropeptide undergoes additional modifications. Relevant *C. elegans* proteins are written next to their function.

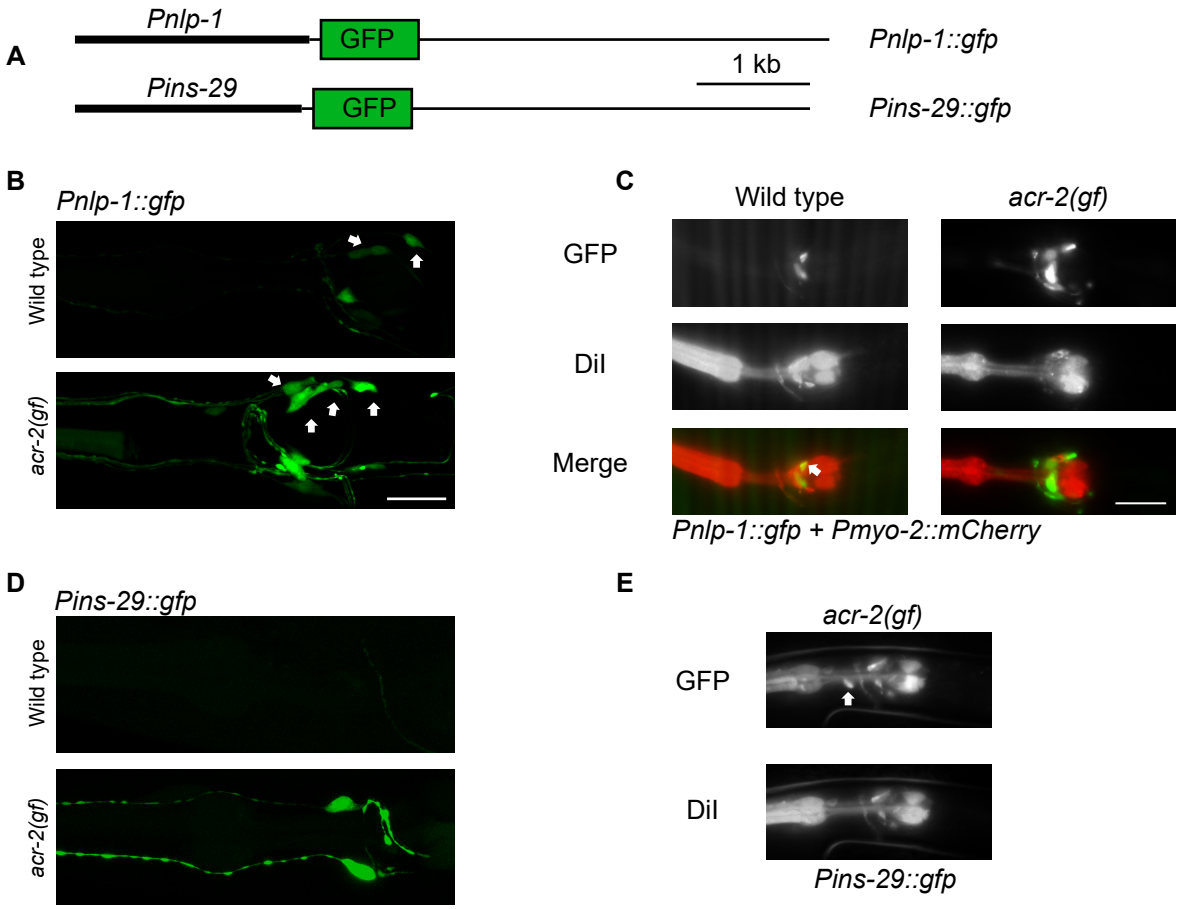

Figure 2. *nlp-1* is ectopically expressed in *acr-2(gf)* animals. A Plasmid constructs for fluorescent reporters of *nlp-1* and *ins-29.* GFP expression was driven by the endogenous promoters of *nlp-1* and *ins-29.* **B** Images of the head of one day old adult animals expressing the *nlp-1* fluorescent reporter transgene taken by confocal microscopy. Animals were rolled onto the ventral plane before images were taken. Arrowheads designate cell bodies of one side (left) of the animal. In wild type, two GFP-positive AWC and ASI cell bodies are visualized. In *acr-2(gf)* animals, four ectopic cell bodies express GFP. Scale bar = 20 μm. **C** DiI staining of amphid neurons of wild type and *acr-2(gf)* animals expressing the same transgene. Colocalization of red and green signal (yellow) in wild type animals mark the ASI cell body, indicated by the arrowhead. Staining indicated that cells ectopically expressing *nlp-1* in *acr-2(gf)* animals are not amphid cells. Note that the red signal included a red fluorescent pharyngeal head marker that was co-expressed with the *nlp-1* reporter transgene; this head marker was distinguishable from the DiI stain on the microscope. Scale bar = 2 μm. **D** Confocal microscopy of the heads of one day old adult animals expressing an *ins-29* fluorescent reporter transgene. Wild type animals show weak GFP expression in two head cells, while *acr-2(gf)* animals show increased GFP expression in the same cells. **E** DiI staining of amphid neurons of *acr-2(gf)* animals expressing the *ins-29* reporter. Staining showed that the GFP-positive head cell, indicated by the arrowhead, is not an amphid neuron. Images in panels D and E were provided with permission by McCulloch, Katherine.

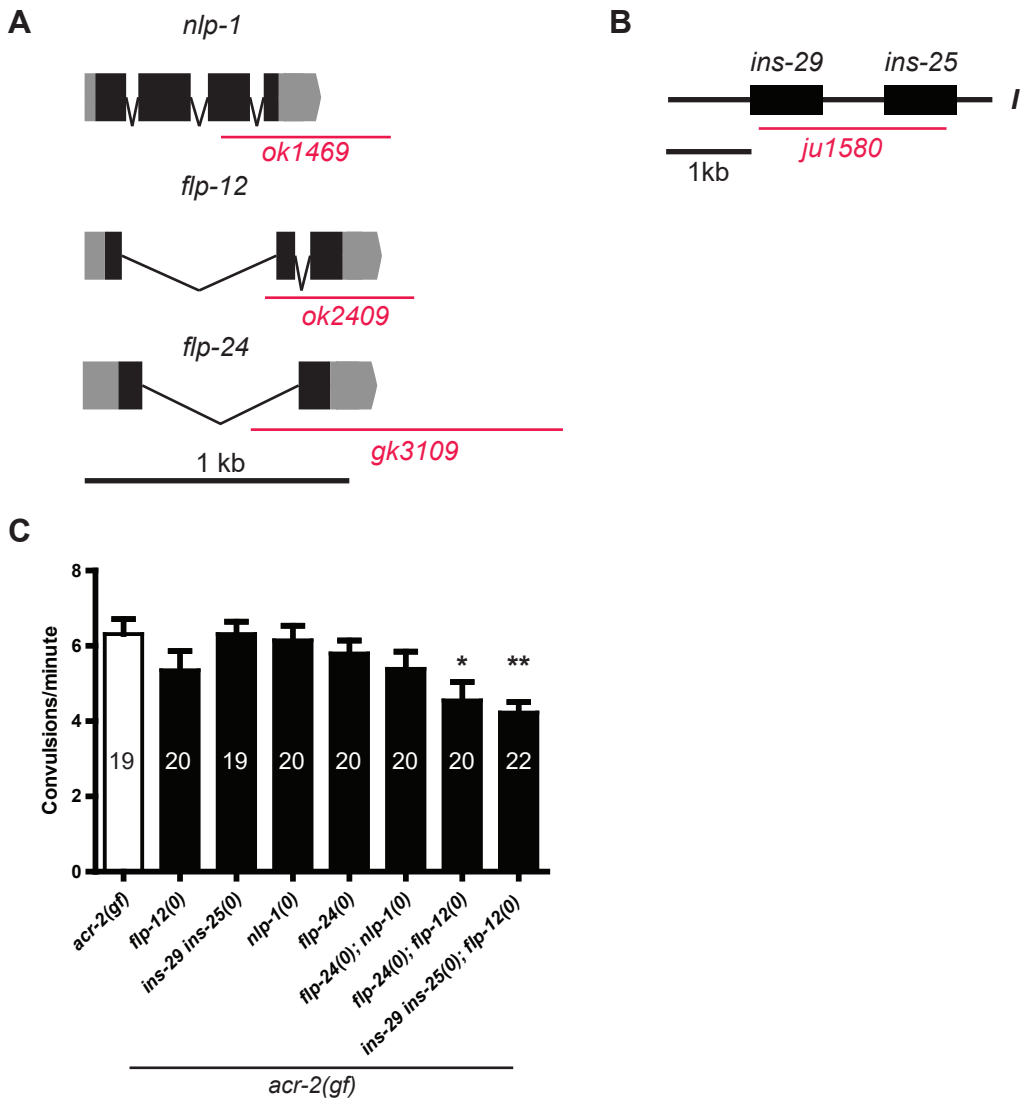

Figure 3. *flp-12(0)* combined with either *ins-29 ins-25(0)* or *flp-24(0)* reduces the convulsion rate of *acr-2(gf)* animals. A Genetic structure of *flp-12, flp-24,* and *nlp-1.* Grey boxes denote untranslated regions, while solid black lines denote introns. Exons are represented by dark boxes. Deletion alleles are marked by red lines. *ok1469, ok2409,* and *gk3109* are 641, 564, and 1180 bp deletions on the 3' end of *nlp-1, flp-12,* and *flp-24*, respectively. *ok1469* deletes the fourth and most of the third exon of *nlp-1*, resulting in a frameshift causing a premature stop codon. *ok2409* deletes the second and third exons of *flp-12* and results in a premature stop codon. *gk3109* deletes the second exon of *flp-24* and results in a premature stop codon. **C** *ins-29* and *ins-25* are clustered in the same region on chromosome I, and *ju1580* is a CRISPR-Cas9 mediated deletion of both genes. **C** Convulsion rates of indicated strains shown. Statistical one-way ANOVA analysis indicates a significant difference in convulsion rates between *acr-2(gf)* alone and *flp-24(0); flp-12(0) acr-2(gf)* ($p < 0.05$) and *ins-29 ins-25(0); flp-12(0)* ($p < 0.01$) animals. N values for each group are noted in each bar.

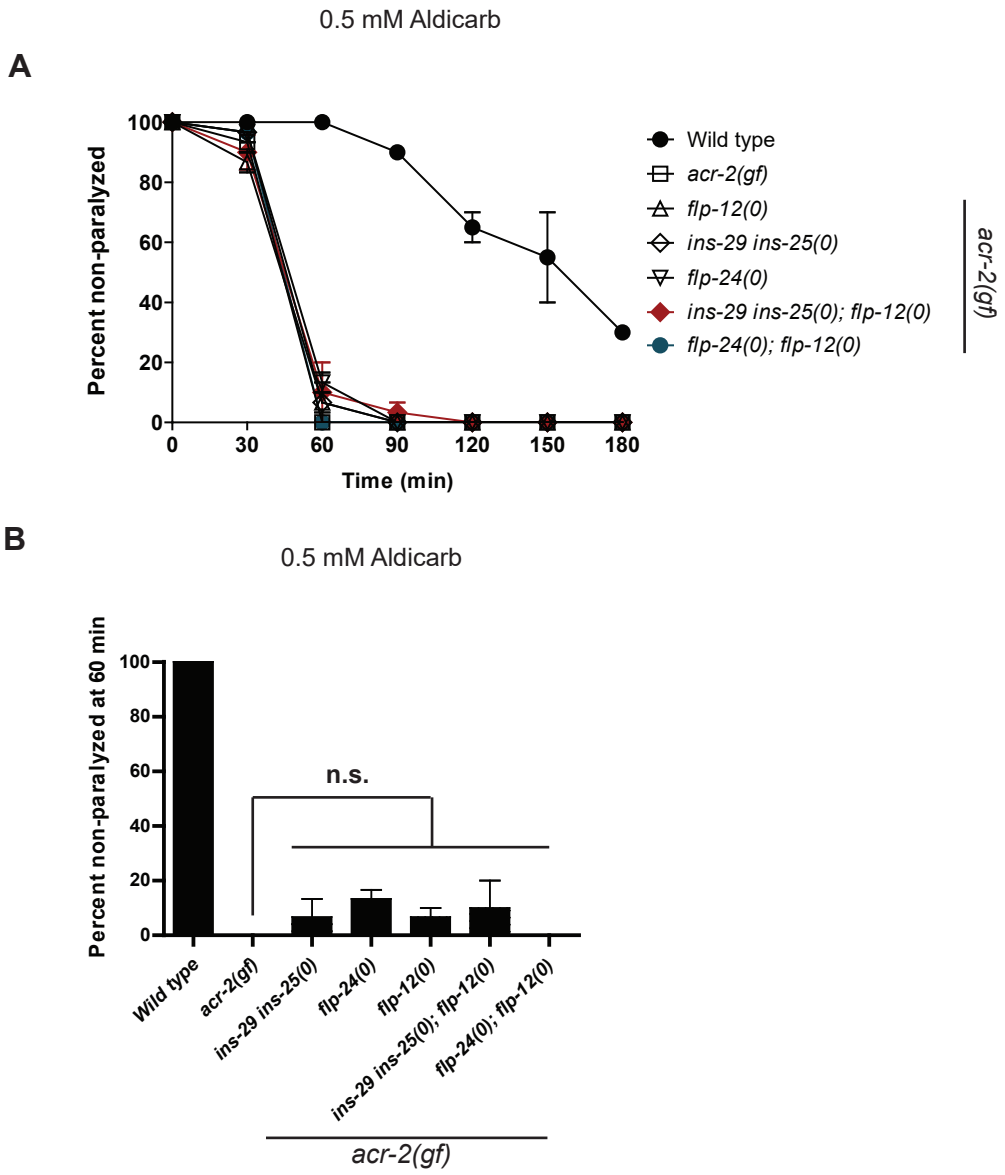

Figure 4. *flp-12(0), ins-29 ins-25(0),* and *flp-24(0)* do not alter presynaptic cholinergic transmission. An aldicarb sensitivity assay was performed on indicated strains. **A** At any 30-minute time interval, two-way ANOVA analysis indicated no significant differences in the number of animals paralyzed by aldicarb over a time course of 3 hours between *acr-2(gf)* animals and other strains with *acr-2(gf)*. **B** At 60 minutes into the assay, no significant differences in the number of paralyzed animals were found between *acr-2(gf)* animals and other mutant strains. Aldicarb concentration = 500 μM; n = 3; 10 animals per replicate strain.

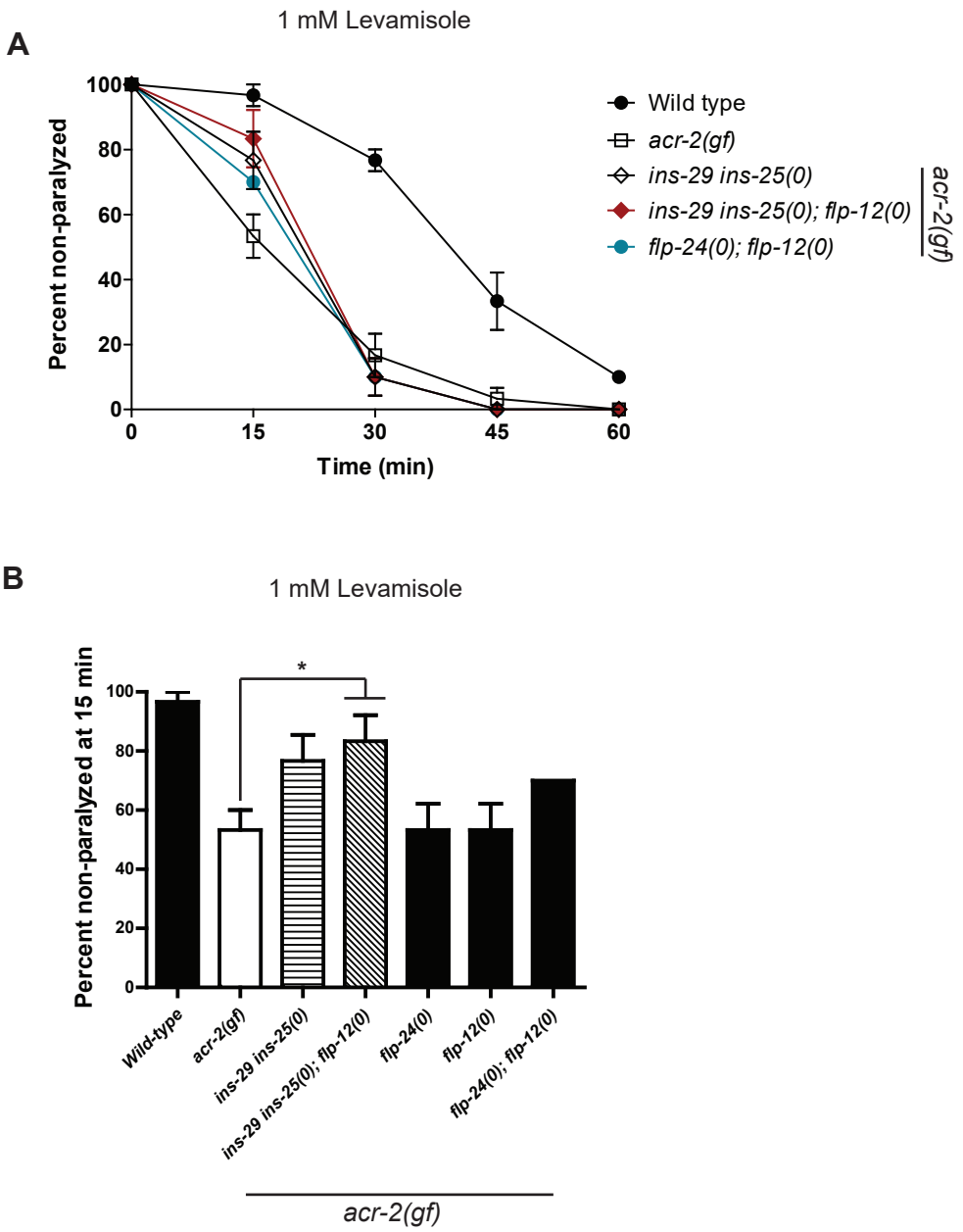

Figure 5. i*ns-29 ins-25(0)* alone or with *flp-12(0)* reduces postsynaptic excitation of the muscle. A levamisole sensitivity assay was performed on indicated strains. **A** Over a time course of one hour, two-way ANOVA analysis found a significant difference in the number of animals paralyzed by levamisole in *ins-29 ins-25(0); acr-2(gf)* and *ins-29 ins-25(0); flp-12(0) acr-2(gf)* animals compared to *acr-2(gf)* alone. **B** At 15 minutes into the assay, one-way ANOVA analysis indicated a significant difference in the number of paralyzed animals between i*ns-29 ins-25(0); flp-12(0) acr-2(gf)* animals and *acr-2(gf)* alone. Levamisole concentration = 1 mM; n = 3; 10 animals per replicate strain. * $p < 0.05$.

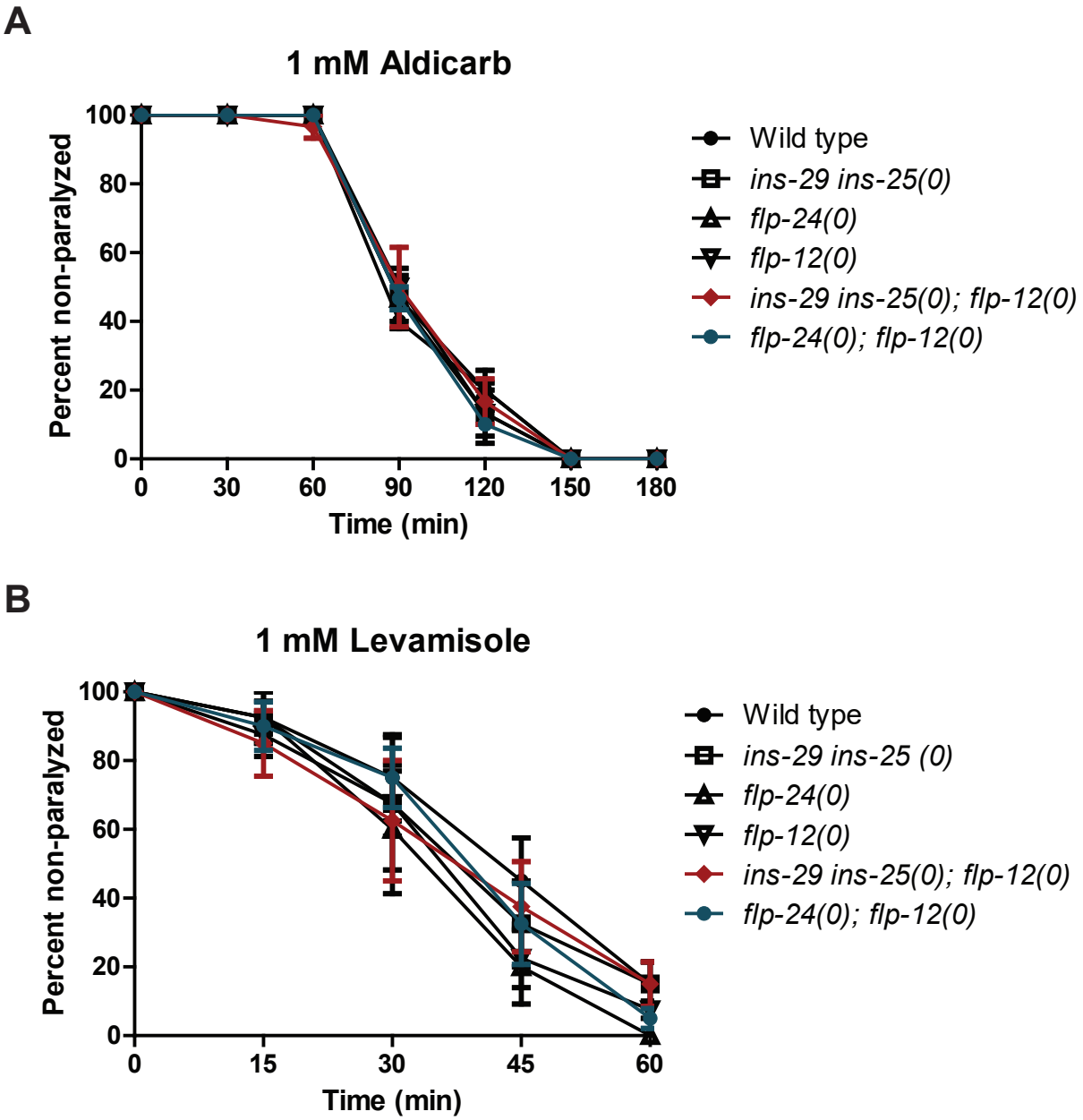

Figure 6. Increased resistance to levamisole by *flp-12(0)* and *ins-29 ins-25(0)* is specific to the *acr-2(gf)* background. An aldicarb and levamisole sensitivity assay was performed on indicated strains. **AB** At any time interval, two-way ANOVA analyses indicated no significant differences in the number of animals non-paralyzed by either drug between wild type and mutant strains. **A** Aldicarb concentration = 1 mM; n = 3; 10 animals per replicate strain. **B** Levamisole concentration = 1 mM; n = 4; 10 animals per replicate strain.

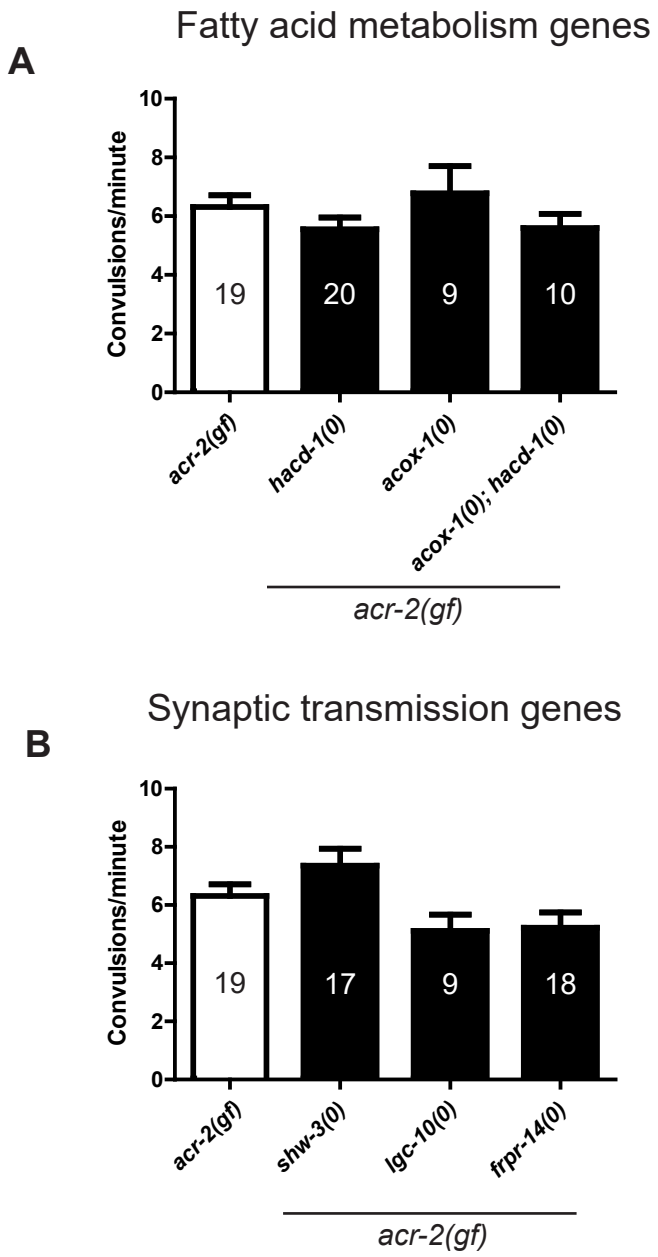

Figure 7. Convulsion rates of other mutants. A Convulsion rates of *acr-2(gf)* animals with mutant alleles for enriched genes in *acr-2(gf)* involved in fatty acid metabolism pathways. **B** Convulsion rates of *acr-2(gf)* animals with mutant alleles for enriched genes involved in synaptic transmission. Statistical one-way ANOVA analyses indicate no significant difference in convulsion frequencies between strains listed and *acr-2(gf)* animals. Data for *frpr-14(0); acr-2(gf)* mutants were provided with permission by McCulloch, Katherine.

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