Neuropeptides Function in a Homeostatic Manner to Modulate Excitation-Inhibition Imbalance in C. elegans

Tamara M. Stawicki¹,²*, Seika Takayanagi-Kiya¹,²*, Keming Zhou¹, Yishi Jin¹,²*

¹ Division of Biological Sciences, Section of Neurobiology, University of California San Diego, La Jolla, California, United States of America, ² Howard Hughes Medical Institute, University of California San Diego, La Jolla, California, United States of America

Abstract

Neuropeptides play crucial roles in modulating neuronal networks, including changing intrinsic properties of neurons and synaptic efficacy. We previously reported a Caenorhabditis elegans mutant, acr-2(gf), that displays spontaneous convulsions as the result of a gain-of-function mutation in a neuronal nicotinic acetylcholine receptor subunit. The ACR-2 channel is expressed in the cholinergic motor neurons, and acr-2(gf) causes cholinergic overexcitation accompanied by reduced GABAergic inhibition in the locomotor circuit. Here we show that neuropeptides play a homeostatic role that compensates for this excitation-inhibition imbalance in the locomotor circuit. Loss of function in genes required for neuropeptide processing or release of dense core vesicles specifically modulate the convulsion frequency of acr-2(gf). The proprotein convertase EGL-3 is required in the cholinergic motor neurons to restrain convulsions. Electrophysiological recordings of neuromuscular junctions show that loss of egl-3 in acr-2(gf) causes a further reduction of GABAergic inhibition. We identify two neuropeptide encoding genes, flp-1 and flp-18, that together counteract the excitation-inhibition imbalance in acr-2(gf) mutants. We further find that acr-2(gf) causes an increased expression of flp-18 in the ventral cord cholinergic motor neurons and that overexpression of flp-18 reduces the convulsion of acr-2(gf) mutants. The effects of these peptides are in part mediated by two G-protein coupled receptors, NPR-1 and NPR-5. Our data suggest that the chronic overexcitation of the cholinergic motor neurons imposed by acr-2(gf) leads to an increased production of FMRFamide neuropeptides, which act to decrease the activity level of the locomotor circuit, thereby homeostatically modulating the excitation and inhibition imbalance.

Introduction

Neuropeptides are widespread and diverse modulators of neuronal circuit function, and have long been known to play regulatory roles in complex behaviors, such as learning, feeding, temperature regulation, and pain sensation [1,2]. Additionally, neuropeptide modulation is implicated in a number of neurological diseases including epilepsy and autism [3–8]. In recent years great strides have been made in the recognition of the diverse regulatory roles in complex behaviors, such as learning, feeding, neuronal circuit function, and have long been known to play important insights on the precise mechanisms underlying endogenous neuropeptide function in animal behaviors [13,17–19].

The C. elegans genome contains over 100 peptide-encoding genes, which are generally classified as ffp for FMRFamide-like peptides, ins for insulin-like genes, and nlp for neuropeptide-like proteins [20]. Recent proteomic studies have detected expression of over 150 distinct mature peptides [20–23]. As in higher vertebrates and other organisms, neuropeptide precursors are packaged into large dense core vesicles, and are further processed into functionally mature neuropeptides through a series of conserved enzymatic reactions [24,25]. The release of dense core vesicles occurs in response to Ca²⁺ influx, and relies on several unique proteins in addition to those that are also involved in fast neurotransmitter release [26].

The two best characterized enzymes for neuropeptide processing in C. elegans are the proprotein convertase (PC2), EGL-3, and the carboxypeptidase E (CPE), EGL-21 [20,27,28]. EGL-3/PC2 cleaves the propeptide after the basic amino acid residues located in the C-terminus of the individual peptides [20]. EGL-21/CPE then removes the basic amino acids of the newly cleaved peptides [20]. Both genes are expressed primarily in the nervous system [20,27,28]. An early report using an antibody that recognizes fully processed FMRFamide-related peptides showed loss of most staining in egl-21 mutants, and a great reduction of staining in egl-3 mutants [27]. Recent peptidomic analyses fail to detect any processed neuropeptides in egl-3 null mutants [21]. While egl-21 mutants show incomplete processing of the majority of FLP and NLP peptides, they also express a number of fully processed peptides [22]. Thus, these two enzymes are important for the
Imbalanced neuronal circuit activity is considered a major underlying cause in many neurological disorders, such as epilepsy and autism. Neuropeptides are small polypeptides that are released from neurons. They are widely known to provide neuromodulatory functions and have diverse roles in the nervous system. By investigating a C. elegans mutant that exhibits convulsions as the result of an imbalanced excitation and inhibition in the locomotor circuit, we have identified a homeostatic mechanism involving two distinct neuropeptide genes. We find that the expression of the neuropeptides is up-regulated in response to over-excitation and that, in turn, they act to increase inhibitory transmission. While current treatment strategies for epilepsy have focused on targeting fast synaptic transmission, this work supports the general notion that manipulating slow neuropeptide neurotransmission can strongly influence neural excitation and inhibition imbalance.

Neuropeptide release in C. elegans is well known to influence neural circuit activity and behavior [15,29,30]. The UNC-31 CAPS (Calcium-dependent Activator Protein for Secretion) protein is essential for peptide-containing dense core vesicle release, and unc-31 mutants exhibit many sensory deficits and impaired locomotion [31–35]. Examples of specific neuropeptides regulating the locomotor circuit activity include the neuropeptide NLP-12, which is released by the stretch sensitive neuron DVA and can influence cholinergic motor neuron neurotransmitter release [14]. The levels of the FLP-1 FMRFamide peptides can also alter locomotor behavior such that flp-1(lf) mutants are hyperactive while overexpression of flp-1 causes reduced mobility [36].

C. elegans sinusoidal locomotion is the result of coordinated muscle contraction due to innervation by the excitatory cholinergic motor neurons and inhibitory GABAergic motor neurons in the ventral cord [37]. Neuropeptide signaling has been implicated in modulating the activity of both types of motor neurons as well as the muscles [12,20]. We have previously reported that the ACR-2 nicotinic acetylcholine receptor is expressed in the cholinergic motor neurons and plays a key role in balancing excitatory and inhibitory neurotransmission in the locomotor circuit [38]. Specifically, a gain of function mutation (Val309Met), designated as acr-2(gf), in the pore-lining transmembrane domain of the ACR-2 subunit causes an increase in cholinergic excitation, accompanied with a decrease in GABAergic inhibition. This imbalance in excitation and inhibition results in stochastic convulsive behavior due to spontaneous contractions of body muscles. Thus, the frequency of convulsions of the acr-2(gf) mutant can be used as an indicator for the imbalanced activity of the locomotor circuit.

In this study we examined the roles of neuropeptides in modulating excitation and inhibition imbalance in the locomotor circuit. We show that neuropeptides processed by EGL-3 and released from the cholinergic motor neurons inhibit the convulsions caused by acr-2(gf). We find that two neuropeptide-encoding genes, flp-1 and flp-18, act together to reduce excitation and inhibition imbalance in the locomotor circuit. acr-2(gf) causes a specific up-regulation of flp-18 expression in the cholinergic motor neurons. Electrophysiological recordings of the neuromuscular junctions indicate that egl-3 and flp genes primarily influence GABAergic synaptic transmission. We also identify two neuropeptide receptors, NPR-1 and NPR-5 that are likely involved in the regulation of convulsions by the FLP-18 neuropeptides. These data suggest that neuropeptide production is regulated by activity, and that in turn neuropeptides function in a homeostatic manner to modulate output of the locomotor circuit. Our findings have implications for our understanding of excitation-inhibition imbalance in disease conditions, and support a general notion that neuropeptide modulation can provide effective strategies in disease management.

Results

Loss of function in the proprotein convertase EGL-3 increases the convulsion frequency of acr-2(gf)

To specifically test the roles of neuropeptides on acr-2(gf) induced convulsions, we first examined a set of mutants that are known to disrupt peptide processing. We found that multiple alleles of egl-3 caused a significant increase in the convulsion frequency of acr-2(gf) (Figure 1A). A null mutation in sbt-1, a molecular chaperone necessary for EGL-3 function [23], showed a similar enhancement. egl-3(lf); sbt-1(lf); acr-2(gf) triple mutants showed a similar level of increased convulsions as egl-3(lf); acr-2(gf) and sbt-1(lf); acr-2(gf) double mutants, consistent with SBT-1 and EGL-3 acting in the same pathway. The overall locomotion pattern and speed of sbt-1; acr-2(gf) was indistinguishable from that of acr-2(gf) [Videos S1, S2], supporting the specific effects of SBT-1 and EGL-3 on convulsion frequency.

The carboxypeptidase E EGL-21 generally functions together with EGL-3 in producing mature neuropeptides [20]. However, we tested three mutations in egl-21, including a large deletion tm5578, which removes most of the exons 2 and 3 and causes premature stop after 85 amino acids (Table S1), and did not observe any effects on acr-2(gf) convulsions (Figure 1A). A null mutation in epl-2, another carboxypeptidase, also showed no effects. Moreover, egl-21(lf); egl-3(lf); acr-2(gf) triple mutants behaved similarly to egl-3(lf); acr-2(gf). These observations suggest that epl-2 may not be required, or has a partial role, for processing the specific neuropeptides involved in acr-2(gf) convulsive behavior. As addressed later, we found the latter interpretation to be true. Overall, these observations indicate that EGL-3-dependent neuropeptides modulate the convulsive behavior of acr-2(gf) animals.

The function of neuropeptides is dependent on dense core vesicle release that requires the CAPS protein UNC-31 [26]. To test further the role of neuropeptides in modulating acr-2(gf) convulsions, we introduced a null mutation of unc-31 into the acr-2(gf) background. In contrast to egl-3(lf); acr-2(gf), unc-31(lf); acr-2(gf) double mutants showed a significant reduction in the convulsion frequency as compared to the acr-2(gf) mutants alone (Figure 1A, Videos S1, S3). Importantly, unc-31(lf) blocked the enhancement of egl-3(lf); acr-2(gf) convulsions, as egl-3(lf); unc-31(lf); acr-2(gf) triple mutants convulsed to the same degree as unc-31(lf); acr-2(gf) (Figure 1A). Dense core vesicles contain complex components that include neuropeptides, whose processing most likely depends on EGL-3, as well as INS-like peptides, whose processing generally does not depend on EGL-3. Upon release, peptides can act in a combinatorial manner to modulate specific pathways. The fact that the enhanced convulsion in egl-3(lf); acr-2(gf) is dependent on unc-31 led us to propose that the effective mature neuropeptides...
processed by EGL-3 are a specific subset of dense core vesicle components released via UNC-31.

We further addressed in which cells neuropeptide processing by EGL-3 is required to modulate \textit{acr-2(gf)}. We found that expression of \textit{egl-3} in the cholinergic motor neurons using the \textit{unc-17} or the \textit{acr-2} promoter [32], fully rescued the enhanced convulsions in \textit{egl-3(lf)}; \textit{acr-2(gf)}, whereas expression of \textit{egl-3} in pre-motor command neurons, driven by the \textit{glr-1} promoter [40], did not show any effect (Figure 1B, Tables S1, S2). Together, these data reveal that neuropeptides processed in the cholinergic motor neurons modulate the convulsive behavior of \textit{acr-2(gf)}, and suggest that the neuropeptide products act to restore the balance of excitation and inhibition in the locomotor circuit.

FMRFamide-like peptides encoded by \textit{flp-1} and \textit{flp-18} act synergistically to decrease locomotor circuit activity in \textit{acr-2(gf)} mutants

We next sought to determine the specific neuropeptides responsible for the inhibition of \textit{acr-2(gf)} convulsions. We tested a set of candidate neuropeptide genes that had either been shown to be expressed in the locomotor circuit, or were known to affect locomotion [20]. Of 23 neuropeptide mutants tested, none showed significant enhancement of the \textit{acr-2(gf)} conviction phenotype (Figure 2A, Table S1). We reasoned that the observed inhibitory effects of \textit{egl-3} could be due to a group of neuropeptides produced by more than one gene. To test this idea, we made selected double mutants among \textit{flp} and \textit{nlp} genes chosen based on similarity in expression patterns or phenotypes. In doing so we found that eliminating both \textit{flp-1} and \textit{flp-18} resulted in a significant enhancement of \textit{acr-2(gf)} convulsions (Figure 2D, Figure 3A). Two independent \textit{flp-18(lf)} mutants, \textit{flp-18(tm2179)} and \textit{flp-18(db99)}, gave similar effects (Figure 3A). None of the other seven neuropeptide gene double mutants affected \textit{acr-2(gf)} conviction frequency (Figure 2D). We note that while \textit{flp-1}; \textit{flp-18} \textit{acr-2(gf)} mutants display a significant enhancement of the conviction frequency, the extent of the conviction is often less obvious than that seen in \textit{egl-3(lf)}; \textit{acr-2(gf)} animals (Videos S4, S5), suggesting that other as yet unidentified neuropeptides may also be influencing \textit{acr-2(gf)}.

In the recent peptidomic studies of \textit{egl-21(lf)} animals, fully processed FLP-1 peptides are reported to be largely undetectable; however, four of the six fully processed mature peptides from FLP-18 are produced [22]. The presence of functional FLP-18-derived peptides would explain why \textit{egl-21(lf)} single mutants did not show detectable effects on \textit{acr-2(gf)} (Figure 1A). To test this idea, we constructed \textit{egl-21(lf)}; \textit{flp-18(lf)} \textit{acr-2(gf)} triple mutants, and observed that the convolution frequency in these animals was comparable to that of \textit{flp-1(lf)}; \textit{flp-18(lf)} \textit{acr-2(gf)} (Figure 3A). Thus, these observations support a role of EGL-21 in the processing of FLP-1 neuropeptides, and imply other unidentified carboxypeptidases in the processing of FLP-18 neuropeptides.

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**Figure 1. Neuropeptide processing and release pathway regulate \textit{acr-2(gf)} convulsions.** All mutations are loss of function alleles, except for \textit{acr-2(gf)}, which designates \textit{acr-2(n2420)}. Mean convolution frequencies are shown. Error bars indicate SEM. Numbers in the graph indicate sample sizes. Statistics: **: \(p<0.001\), ***: \(p<0.01\), *: \(p<0.05\) by ANOVA and Bonferroni post hoc test. (A) Loss of function in \textit{egl-3} and \textit{sbt-1} significantly enhances \textit{acr-2(gf)} convulsions; and the increased convolution caused by \textit{egl-3(lf)} is dependent on \textit{unc-31}. (B) \textit{egl-3} functions in the cholinergic motor neurons to suppress \textit{acr-2(gf)} convulsions. The number of independent transgenic lines tested are the following: \textit{Prgef-1::egl-3}; 4 lines, \textit{Punc-17::egl-3}; 3 lines, \textit{Pglr-1::egl-3}; 3 lines, \textit{Pacr-2}; 2 lines. Quantification data is shown for one representative line.

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As an independent assay for the effects of *flp-1* and *flp-18* neuropeptides on the locomotor circuit activity associated with *acr-2(gf)* convulsions, we tested the sensitivity of animals to the acetylcholinesterase inhibitor aldicarb [41]. *acr-2(gf)* animals show hypersensitivity to aldicarb, consistent with increased cholinergic transmission and decreased GABAergic transmission [38] (Figure 3B, 3C). *flp-1(lf)* mutants showed mild resistance to aldicarb (Figure S1A), consistent with a previous report [29]. *flp-18(lf)* showed sensitivity to aldicarb similar to wild type and suppressed the resistance of *flp-1(lf)* (Figure S1A). The hypersensitivity of *acr-2(gf)* to aldicarb was slightly, but not significantly enhanced by loss of function mutations in either *flp-1* or *flp-18* alone (Figure 3B). Notably, triple mutants of *flp-1(lf)*; *flp-18(lf)* *acr-2(gf)* showed significantly increased sensitivity to aldicarb, compared to *acr-2(gf)* alone (Figure 3B). Both the increased convulsion frequency and the increased aldicarb sensitivity of the *flp-1(lf)*; *flp-18(lf)* *acr-2(gf)* triple mutants were rescued by transgenic expression of *flp-18*, as well as pan-neuronal expression of *flp-1(+) (Figure 3A, 3C), indicating FLP genes act in the nervous system to modulate the excitation-inhibition imbalance caused by *acr-2(gf)*.

**EGL-3 and FLP neuropeptides primarily regulate GABAergic inhibition**

To address more precisely how neuropeptides influence locomotor circuit activity in the *acr-2(gf)* background, we performed electrophysiological recordings at the neuromuscular junction. As reported previously [38, 42], when recordings were performed with 2 mM Ca^{2+} in the bath solution, *acr-2(gf)* showed slightly increased frequencies of endogenous acetylcholine release (EPSC), but a striking reduction of endogenous GABAergic activity (IPSC) (Figure 4A). Loss of *egl-3* function in *acr-2(gf)* caused a further reduction in endogenous IPSC frequency (Figure 4A). We observed a similar, but milder, effect on IPSC rate in *flp-1(lf)*; *flp-18(lf)* *acr-2(gf)* triple mutants, consistent with the milder...
enhancement in convulsions in these animals (Videos S4, S5). 

\[ \text{egl-3}^{(f)}; \text{acr-2}^{(gf)} \text{ and } \text{flp-1}^{(yn4)}; \text{acr-2}^{(gf)} \text{ both showed slightly reduced endogenous EPSC rates compared to } \text{acr-2}^{(gf)} \text{ single mutant, although the average rate did not significantly differ among the strains (Figure 4A). The amplitudes of endogenous EPSCs and IPSCs were similar in all four genotypes tested (Figure 4B), suggesting that the muscle ACh and GABA receptors are largely unaltered. Thus, the electrophysiology analysis indicates that neuropeptides processed by EGL-3 compensate for the excitation-inhibition imbalance caused by } \text{acr-2}^{(gf)} \text{ primarily by influencing GABAAergic transmission, and that FLP-1 and FLF-18 peptides account for most, but not all, of the neuromodulatory effects of EGL-3.} 

The \text{acr-2}^{(gf)} mutation increases FLP-18 expression in the cholinergic motor neurons

The specific effect of \text{flp-1} and \text{flp-18} on \text{acr-2}^{(gf)} could be caused by either increased expression or release of these neuropeptides in this mutant background. To address the possibility of increased neuropeptide release, we examined two fluorescent reporters for dense core vesicle release from the cholinergic motor neurons: \text{Punc-129::NLP-21::venus} and \text{Punc-129::INS-22::venus} [29,43]. Neither reporter showed significant changes in fluorescence intensity or pattern (Figure S2), suggesting that the general release machinery is largely normal in \text{acr-2}^{(gf)}. 

We next tested for increased expression of neuropeptides using a bicistronic \text{flp-18} reporter that contains the entire genomic locus of \text{flp-18}, including the 3.6 kb upstream promoter, followed by a trans-spliced SL2::GFP (designated as \text{Pflp-18::flp-18::SL2::gfp}) [44]. In the wild type background this reporter was strongly expressed in several head neurons and was detectable at low levels in the ventral nerve cord. In the \text{acr-2}^{(gf)} background, we found that \text{Pflp-18::flp-18::SL2::gfp} expression in the ventral cord neurons was strongly enhanced (Figure 5A–5C), while its expression in the head neurons was not changed (Figure S3). GFP expression pattern in the cholinergic motor neurons under the \text{acr-2}^{(gf)} promoter was also not affected by \text{acr-2}^{(gf)} mutation (Figure S4). We quantified the number of ventral cord neuron cell bodies that showed expression of \text{Pflp-18::flp-18::SL2::gfp}, and found that more cell bodies were observed with elevated expression in \text{acr-2}^{(gf)} animals than in wild type (Figure 5D). We were not able to examine \text{flp-1} expression due to variable expression patterns of different transgenic \text{flp-1} reporter lines (our unpublished data). 

The cells that showed up-regulation of \text{Pflp-18::flp-18::SL2::gfp} in \text{acr-2}^{(gf)} were evenly spaced along the ventral nerve cord (Figure 5B). To determine in which class of motor neurons \text{Pflp-18::flp-18::SL2::gfp} expression was affected, we crossed \text{acr-2}^{(gf)}; \text{Pflp-18::flp-18::SL2::gfp} with a set of mCherry reporter lines driven by specific motor neuron promoters. We observed consistent co-expression of GFP and mCherry in B-type cholinergic motor neurons, labeled by \text{Pttr-3}, and occasional expression in A-type cholinergic motor neurons, labeled by \text{Punc-4}, but no overlapping expression in GABAAergic D-type motor neurons, labeled by \text{Pttr-5} (Figure 5E–5H). These data indicate that \text{acr-2}^{(gf)} primarily up-regulates \text{flp-18} expression in the cholinergic B-type motor neurons.

Elevated \text{flp-18} expression correlates with the onset of convulsions and is likely induced by neuronal activity

To further correlate the \text{acr-2}^{(gf)}-dependent up-regulation of \text{flp-18} expression, we examined the developmental onset of \text{flp-18}...
Neuropeptide Modulation of Convulsions

Neuropeptide generally act through G-protein coupled receptors (GPCRs). Next we sought to identify which GPCRs are involved in the regulation of convulsions by the flp neuropeptides. The CKR-2 receptor can be activated by FLP-1 at high concentration, and is also shown to act as a high-affinity receptor for NLP-12 [14,45]. We found that ckr-2(lf) or ckr-2(lf); flp-18(lf) had no effects on acr-2(gf) (Figure S6), consistent with the observation that nlp-12(lf) did not affect acr-2(gf) either alone or in combination with flp-18 (Figure 2D). Three receptors NPR-1, NPR-4 and NPR-5 can be activated by all six FLP-18 neuropeptides when expressed in Xenopus oocytes [44,46–48]. NPR-1 is expressed in the ventral cord GABAergic motor neurons and in multiple head sensory neurons [46]. NPR-4 is expressed in the AVA, RIV, BDU and PQR neurons as well as in coelomocytes and the intestine [44]. NPR-5 expression is found in the amphid and phasmid neurons, interneurons AIA and AUA, as well as in the muscles [44]. We found that loss of function mutations in individual npr genes neither suppressed nor enhanced acr-2(gf) (Figure 7A). We then made selected double mutant combinations among npr-1, npr-4, and npr-5, in the presence or absence of flp-18(lf)(Figure 2D). Eliminating both npr-1 and npr-5 in acr-2(gf) resulted in increased convulsions, while npr-4(lf) showed detectable effects only when both npr-3 and flp-1 were eliminated (Figure 7A, 7B). To further test the roles of these npr genes, we examined the suppression effects of acr-2(gf) by the overexpression of flp-18. We found that the suppression of convulsion by overexpression of flp-18 were reduced by either npr-1(lf); npr-5(lf) or npr-4(lf); npr-5(lf) double mutations, but not by npr-1(lf) or npr-5(lf) single mutation (Figure 6B). Based on these observations, we conclude that NPR-1 and NPR-5 likely play a major role in mediating the modulatory action of FLP-18 in acr-2(gf), while NPR-4 has a minor role. Similar npr receptor combinations had no effects on the suppression of convulsion by overexpression of flp-1 driven by a pan-neuronal promoter (Figure 6B).

npr-1 and npr-5 appear to be the major receptors mediating the suppression of convulsions by FLP-1 and FLP-18

As FLP-18 functions together with FLP-1 to reduce acr-2(gf) convulsions (Figure 2D, Figure 3A), we hypothesized that the induced expression of flp-18 could be a homeostatic response to the elevated cholinergic neuronal activity in acr-2(gf). If so, overexpression of flp-18(+)(+) or flp-1(+)(+) should ameliorate the extent of convulsions. Indeed, overexpressing flp-18 under the control of its endogenous promoter caused a significant suppression of convulsions (Figure 6B). Overexpression of flp-1, driven by a pan-neuronal promoter, also resulted in a similar suppression of convulsions (Figure 6B). Together, these observations support the conclusion that in the acr-2(gf) background where excitation and inhibition balance is impaired, increased expression of flp-18, and possibly of flp-1, acts as a homeostatic response to dampen imbalanced circuit activity.

acr-2(gf) in mutants occurs in mid-larval stage [38]. We found that in acr-2(gf) mutants the expression of the flp-18 reporter also increased sharply in mid-larval stages (Figure 6A). The close temporal correlation between the onset of acr-2(gf) convulsions and that of flp-18 up-regulation in cholinergic motor neurons is consistent with flp-18 up-regulation being caused by increased cholinergic activity. Supporting this idea, we observed increased expression of flp-18::flp-18::SL2::gfp in wild type animals acutely treated with aldicarb (Figure S5, Protocol S1). In contrast, the expression of flp-18::flp-18::SL2::gfp in acr-2(gf) animals was decreased when the animals were grown on plates with the acetyleholine receptor antagonist mecamylamine (Figure S5), which suppresses the convulsion behavior as previously reported [38].

To further test the roles of these acr-2(gf) in the presence or absence of flp-18(lf) (Figure 2D). Eliminating both npr-1 and npr-5 in acr-2(gf) resulted in increased convulsions, while npr-4(lf) showed detectable effects only when both npr-3 and flp-1 were eliminated (Figure 7A, 7B). To further test the roles of these npr genes, we examined the suppression effects of acr-2(gf) by the overexpression of flp-18. We found that the suppression of convulsion by overexpression of flp-18 were reduced by either npr-1(lf); npr-5(lf) or npr-4(lf); npr-5(lf) double mutations, but not by npr-1(lf) or npr-5(lf) single mutation (Figure 6B). Based on these observations, we conclude that NPR-1 and NPR-5 likely play a major role in mediating the modulatory action of FLP-18 in acr-2(gf), while NPR-4 has a minor role. Similar npr receptor combinations had no effects on the suppression of convulsion by overexpression of flp-1 driven by a pan-neuronal promoter (Figure 6B). It is possible that this reflects
non-physiological effects caused by overexpression of flp-1.

Alternatively, FLP-1 and FLP-18 may act through distinct signaling pathways. Supporting the latter idea, we observed a slight but significant difference in aldcarb sensitivity between npr-5(fj); npr-1(fj) acr-2(gf) and flp-1(fj); npr-5(fj); npr-1(fj) acr-2(gf) (Figure 5C). Nonetheless, this difference did not result in significant changes in the convulsion frequency of flp-1(fj); npr-5(fj); npr-1(fj) acr-2(gf) from that of npr-5(fj); npr-1(fj) acr-2(gf) (Figure 7A, 7B), which could reflect the limitation of our visual detection methodology.

We next addressed the cell types in which npr-1 or npr-5 may act. We found that expression of npr-5 in muscles by the Pmyo-3 promoter reduced the convulsion frequency of npr-5(fj); npr-1(fj) acr-2(gf) animals to a similar degree as did npr-5 expression under its endogenous promoter (Figure 7C). We also expressed npr-1 in the GABAergic motor neurons by the Punc-25 promoter, and did not detect any significant effect on the convulsion frequency of npr-5(fj); npr-1(fj) acr-2(gf). Overall, our analysis supports a conclusion that npr-5 acts in the muscle, while Npr-1 expressed in other neurons such as sensory head neurons may be contributing to the locomotor circuit activity in an indirect manner.

Discussion

In this study we have identified two neuropeptide-encoding genes, flp-1 and flp-18 that act in a homeostatic manner to dampen the effects of the excitation-inhibition balance of the locomotor circuit caused by the acr-2(gf) mutation. The role of flp-1 and flp-18 in suppressing overexcitation of the locomotor circuit is dependent on neuropeptide processing by egl-3 in the cholinergic motor neurons. We provide electrophysiological evidence that this neuropeptide modulation primarily acts on the GABAergic neural transmission at the neuromuscular junctions. Our previous studies have shown that acr-2(gf) elevates the activity of the cholinergic motor neurons [38]. Here, we find that acr-2(gf) causes up-regulation of flp-18 expression in the cholinergic motor neurons, and that over-expression of flp-18 or flp-1 is able to suppress acr-2(gf). Our analyses of known flp neuropeptide receptors suggest that npr-1 and npr-5 play a major role in mediating the peptide’s function. We show that npr-5 primarily acts in the muscles. Yet, the combinatorial effects of these receptors on the excitation-inhibition imbalance caused by acr-2(gf) likely involve multiple cell types.

The production of mature neuropeptides generally requires sequential enzymatic reactions starting with proprotein convertases, followed by carboxypeptidases [20]. Peptide mass spectrometry studies indicate that the majority of mature neuropeptides in C. elegans require EGL-3/PC2, its chaperone SBT-1, and EGL-21/CPE [20]. Previous studies have shown that egl-3 and egl-21 generally exhibit similar behavioral defects, although they differ in severity [27,28]. Here, we find that loss of function in egl-3 and elt-1, but not in egl-21, enhances the convulsion frequency of acr-2(gf) animals. Proteomic analyses show that several mature peptides of FLP-18 are present in egl-21 mutants [22]. We find that egl-21(fj); flp-18(fj) acr-2(gf) triple mutants show an increased convulsion frequency similar to flp-1(fj); flp-18(fj) acr-2(gf). These data provide an explanation for the lack of effect on the acr-2(gf) convulsion frequency by the egl-21 mutations, and also imply the involvement of other carboxypeptidases besides EGL-21 in mature neuropeptide production. Once mature peptides are processed in the dense core vesicles, the release of peptides requires UNC-31/CAPS. Intriguingly, we observed a suppression of acr-2(gf) convulsions by unc-31(fj), an opposite effect from that of egl-3(fj). egl-3 is necessary for processing primarily NLP and FLP neuropeptides [21,27], while unc-31 is required for release of all neuropeptides including INS-like peptides and may also affect fast neurotransmitter release. We find that loss of both flp-1 and flp-18 largely mimicked the effects of egl-3(fj). Importantly, the effects of egl-3(fj) are dependent on unc-31(fj). These data support a conclusion that flp-1 and flp-18 peptides, processed by EGL-3, are released via dense core vesicles and in turn act to modulate the locomotion circuit in an inhibitory manner. We infer that the suppression of convulsions of acr-2(gf) by unc-31(fj) is likely due to the involvement of other pathways or unidentified neuropeptides that play excitatory roles in locomotion.

Previous studies on flp-18 have focused on the functions of FLP-18 released from the head interneurons [44]. flp-18(fj) mutants show defects in fat accumulation and foraging behavior, and the defects can be rescued by flp-18 expression in the AY or RIG neurons [44]. The role of FLP-18 neuropeptides in the locomotor circuit is unknown. In wild type animals, flp-18 expression, visualized using a reporter that expresses FLP-18 and GFP under its endogenous promoter [21], extends with carboxypeptidases besides EGL-21 in mature neuropeptide production. Once mature peptides are processed in the dense core vesicles, the release of peptides requires UNC-31/CAPS. Intriguingly, we observed a suppression of acr-2(gf) convulsions by unc-31(fj), an opposite effect from that of egl-3(fj). egl-3 is necessary for processing primarily NLP and FLP neuropeptides [21,27], while unc-31 is required for release of all neuropeptides including INS-like peptides and may also affect fast neurotransmitter release. We find that loss of both flp-1 and flp-18 largely mimicked the effects of egl-3(fj). Importantly, the effects of egl-3(fj) are dependent on unc-31(fj). These data support a conclusion that flp-1 and flp-18 peptides, processed by EGL-3, are released via dense core vesicles and in turn act to modulate the locomotion circuit in an inhibitory manner. We infer that the suppression of convulsions of acr-2(gf) by unc-31(fj) is likely due to the involvement of other pathways or unidentified neuropeptides that play excitatory roles in locomotion.
combinatorial manner to modulate the convulsion in acr-2(gf) animals, with NPR-1 and NPR-5 having a major, and NPR-4 a minor role. These receptors are expressed in multiple cell types. Our data show that muscle-specific expression of NPR-5 can rescue the increased convulsions, suggesting that the FLP-18 neuropeptides can act directly on muscles to inhibit contraction, or to promote relaxation. NPR-1 and NPR-5 may also be activated by neuropeptides other than FLP-18, since npr-5(f0); npr-1(f0) acr-2(gf) triple mutants show a more severe phenotype than fip-18(f0) acr-2(gf) double mutants. The effect of fip-1 and fip-18 double loss of function is milder than that of egl-3(f0) (Figure 4), implying the involvement of other neuropeptides.

We previously showed that GABAergic transmission at the neuromuscular junctions is reduced in acr-2(gf) animals [38]. Our neuromuscular physiology analysis here shows that the neuropeptide modulation by egl-3 and fip-1 and fip-18 primarily acts on GABAergic transmission. npr-1 is expressed in the GABAergic motor neurons [44,48]. However, our data suggests that this

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**Figure 6. Induced expression of FLP-18 in acr-2(gf) correlates with the onset of convulsions, and high levels of FLP-18 or FLP-1 suppress convulsions.** (A) Quantification of the number of cell bodies in the ventral cord that showed Pflp-18::flp-18::SL2::gfp expression in larval and adult stages. Each dot indicates quantification from one animal. Means are indicated by lines. Error bars indicate SEM. Two independent lines juEx4062 and juEx4073 were tested. Result from juEx4073 is shown. (B) Convulsion of acr-2(gf) was suppressed by expression of Pflp-18::flp-18::SL2::gfp or pan-neuronal expression of fip-1. The suppression by fip-18 overexpression was blocked by loss of both npr-1 and npr-5, or npr-4 and npr-5. The same set of npr mutations did not affect the suppression effect of fip-1 overexpression. Mean convolution frequencies are shown. Error bars indicate SEM. Statistics: ****, p<0.001, * p<0.05 by ANOVA and Dunnett’s post-hoc test. (+/-) indicates strains with no mutations in any of the neuropeptide receptor genes.
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expression is unlikely to be directly responsible the effect of neuropeptides on GABA neurons. flp-1 is reported to be expressed primarily in the head neurons including AIA, AII, AVA, AVE, AVK, RIG, RMG, M5 [36]. The effects of flp-1 on convulsions appear to be independent of CKR-2, presently the only known receptor for FLP-1 (Figure S6). npr-1(nf); npr-5(nf) double mutants cause enhanced convulsions of acr-2(gf), similar to flp-1(nf); flp-18(nf) double mutants. Yet, double loss of function in npr-1 and npr-5 does not significantly affect the suppression of convulsions by flp-1(+) overexpression under a pan-neuronal promoter. It is possible that flp-1(+) overexpression activates other inhibitory pathways that do not require npr-1 and npr-5. We observed an enhanced aldicarb sensitivity of the flp-1(nf); npr-5(nf); npr-1(nf) acr-2(gf) animals comparing to npr-5(nf); npr-1(nf) acr-2(gf) (Figure S7). However, this difference did not result in detectable differences in convulsion, which may likely be due to the limitation in our methodology of visual observation of convulsion. The modest effects of these receptors make it difficult to determine the precise contribution of their signaling in the context of convulsive behavior of acr-2(gf).

Identification of additional GPCRs that respond to FLP-1 will be necessary for fully understanding the peptidergic transmission pathway that modulates acr-2(gf) convulsions. Overall, our results are consistent with a model in which these neuropeptides act on multiple cell types, one of which is body wall muscle, to coordinate the activity state of the locomotion circuit.

The molecular nature and the physiological basis of C. elegans acr-2(gf) mutants share similarities with mutations causing epileptic seizures including an imbalance between excitation and inhibition of the nervous system. Examples of neuropeptides acting to inhibit altered neuronal circuit activity, such as in seizures, have also been observed in vertebrates. For example, the neuropeptide galanin has been shown to play a key role in epilepsy [5, 52]. Galanin agonists inhibit seizures [5], and expression of galanin is increased in the mouse brain upon the induction of seizures [53]. A model for the role of galanin in epilepsy has been proposed in that increased excitation increases galanin levels in an attempt to normalize the excitation and inhibition balance by reducing glutamatergic transmission [6]. Likewise, our studies have revealed that activity-dependent expression of neuropeptides provides a homeostatic mechanism to modulate neuronal network balance. Together, these findings provide support for manipulations of slow neuropeptide signaling in controlling neuronal circuit activity disruption underlying neurological disorders.

Materials and Methods

Genetics and alleles

All C. elegans strains were grown on NGM plates at room temperature (20–22°C) following standard methods. Deletion mutant strains were backcrossed two times against N2 before being used for strain construction. All double mutants were constructed using standard procedures, and genotypes were confirmed by PCR verification of the deletions. Table S1 lists the information on the alleles and strains. Specific alleles used in the figures are: acr-2(gf) indicates acr-2(n2420), unc-31(e926), egl-3(n389), egl-5(n979), egl-5(m2090), elt-1(ok901), egl-21(n611), egl-21(n476), egl-21(n3578), ospd-2(ok3147), flp-1(nf4), flp-9(ok2730), flp-11(tm2706), flp-13(tm2427), flp-18(tm2179), flp-20(ok2964), flp-21(ok889), nlp-3(tm3023), nlp-7(tm2984), nlp-9(tm3572), nlp-12(ok335), nlp-14(tm1809), nlp-15(ok1512), ins-3(ok2488), ins-4(ok3534), ins-11(tm1053), ins-18(ok3444), ins-22(ok3616), ins-27(ok2474), ins-28(ok2722), ins-30(ok2343), ins-35(ok3297), ins-36(ok3278), ins-37(ok3276), ins-38(ok3275).
solution containing (in mM): 120 CH₃O₃SCs, 4 CsCl, 15 CsF, 4

scored as paralyzed when no body movements were observed, and young adults were observed using a Zeiss Axioplan 2 fluorescence microscope the following day. The number of cell bodies with GFP and mCherry fluorescence was counted, L4 animals were picked the day before an experiment, and at least two trials were performed per genotype. Videos were scored by an observer blind to genotype. A “convulsion” was defined as a visible shortening in the animal’s body length.

Pharmacology analysis

L4 animals were picked the day before an experiment. The day of the experiment ten young adults per genotype were placed on plates containing 150 μM aldicarb, and the effects on animal movement were observed at 30 minute intervals. Animals were scored as paralyzed when no body movements were observed, even in response to touch.

FLP-18 imaging

Confocal images were taken on a Zeiss LSM 510 with 1 μm per section, and processed using ImageJ. Maximum projection images were created from confocal stacks and the average intensity was measured of the ventral cord posterior to the vulva. For cell body counting, L4 animals were picked the day before an experiment, and young adults were observed using a Zeiss Axioplan 2 fluorescence microscope the following day. The number of cell bodies of the ventral nerve cord with visible GFP fluorescence was counted. For identification of the cells expressing Pflp-18::flp-18::SL2::gfp cell bodies with GFP and mCherry fluorescence were observed and counted. For the observation in different stages of animals, animals were synchronized at L1 stage and observed under Zeiss Axioplan 2 Fluorescence microscope at each developmental stage.

Electric physiology

NMJ dissection methods were adapted from previous studies [42]. In brief, adult worms were immobilized on Sylgard-coated cover slips with cyanacrylate glue. A dorsolateral incision was made with a sharp glass pipette and the cuticle flap was folded back and glued down to expose the ventral medial body wall muscles. The preparation was then treated by collagenase type IV (Sigma-Aldrich) for ~30 s at a concentration of 0.4 mg/ml. The bath solution contained (in mM): 127 NaCl, 5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 4 MgCl₂, 10 glucose, and sucrose to 340 mOsm, bubbled with 5% CO₂, 95% O₂ at 20°C. The pipette solution contained (in mM): 120 CH₃O₃SCs, 4 CsCl, 15 CsF, 4 MgCl₂, 5 EGTA, 0.25 CaCl₂, 10 HEPES and 4 Na₂ATP, adjusted to pH 7.2 with CsOH. Conventional whole-cell recordings from muscle cells were performed at 20°C with 2–3 MΩ pipettes. An EPC-10 patch-clamp amplifier was used together with the Patchmaster software package (HEKA Electronics, Lambrecht, Germany). Endogenous acetylcholine postsynaptic currents were recorded at −60 mV and GABA postsynaptic currents were recorded at 0 mV. The current traces were imported to IGOR Pro (WaveMetrics, Lake Oswego, OR) for further analysis.

Ethics statement

This work does not use human subjects or animals. The research was performed following the ethical conduct rules of University of California San Diego.

Supporting Information

**Figure S1** Aldicarb sensitivity of flp-1 and flp-18 mutants. (A) in wild type and (B) in acr-2(fly) background. Animals were placed on an NGM plate with 1 mM (A) or 500 μM (B) aldicarb and non-paralyzed worms were counted every 30 minutes. (A) flp-1(fly) show aldicarb resistance. (B) Loss of flp-1 and flp-18 does not significantly affect the aldicarb sensitivity in wild type or acr-2(fly) background. Statistics show the comparison of flp-1(fly) vs flp-1(fly); flp-18(fly) and acr-2(fly); flp-18(fly) acr-2(fly). ***: p<0.001, **: p<0.01, *: p<0.05 by two-way ANOVA and Bonferroni post-hoc test.

**Figure S2** Expression of nlp-21 and ins-22 is not affected by acr-2(fly). L4 stage animals of Punc-129::NLP-21::venus and Punc-129::INS-22::venus were subjected to confocal imaging. (Top) Images from Punc-129::NLP-21::venus and Punc-129::INS-22::venus animals. Dorsal nerve cord near the bend of the gonad was imaged. NLP-21::venus was observed in coelomocyte and in the dorsal nerve cord (DNC). INS-22::venus was observed mainly in the DNC. (Middle, bottom) The fluorescence intensity was measured using ImageJ from three fluorescent patches in the coelomocyte, and the average was used for statistics. Fluorescence intensity of DNC was also examined using ImageJ. The NLP-21::venus expression pattern or fluorescence intensity is not affected by acr-2(fly). Dashed circle indicate the position of coelomocytes. Dashed rectangles indicate the DNC region used for the measurement. Student’s t-test was performed to compare fluorescence intensities. Error bars indicate SEM. Numbers in the graph indicate sample sizes.

**Figure S3** Head neuron expression of Pflp-18::flp-18::SL2::gfp is not different between wild type and in acr-2(fly). (A) Representative confocal images of the head neurons (top) and the ventral nerve cord (bottom) in L4 animals. (B) Fluorescence intensity in a head neuron RIG is not different between wild type and acr-2(fly) animals. Intensity was quantified using ImageJ. Average of fluorescence intensity of the two cell bodies of RIG neuron was taken from each animal. Dashed circle in images indicates the region with two cell bodies of RIG. Numbers in the graph indicate sample sizes. Two transgenic lines (yuEx4062 and yuEx4073) were examined and no difference was observed between the two. Results from yuEx4073 are shown.

**Figure S4** Expression pattern of Punc-17β::zgfp. Images of Punc-17β::zgfp expression in the wild type (left) and acr-2(fly) (right) genetic background. Expression is only seen in the A and B type motor neurons.

**Figure S5** Expression of flp-18 by aldicarb and mecamylamine treatment. (A) Pflp-18::flp-18::SL2::zgfp expression in the ventral
nerve cord is increased by aldicarb and decreased by mecamylamine treatment. (top) Representative images of the ventral nerve cord with and without the drug treatment. White boxes indicate the region of cell body and the enlarged images of the region are shown on right of each image. (bottom) Quantification of GF fluorescence intensity in cell body. Two transgenic lines (muEx4062 and muEx4073) were examined. Results from muEx4073 are shown. (B) Expression level of Punc-17ß-actin was not affected by drug treatments. GF fluorescence intensity in cell body was quantified using ImageJ.

Figure S6  acr-2 does not affect acr-2(gf) convulsions. Loss of acr-2 or acr-2 in combination with flp-18 does not affect the convulsion frequency of acr-2(gf). Quantification method is the same as in others.

Figure S7  Loss of flp-1 causes increased aldicarb sensitivity in npr-5: npr-1 acr-2(gf) background. Animals were placed on an NGM plate with 150 µM aldicarb and non-paralyzed worms were counted every 30 minutes. Loss of flp-1 enhances aldicarb sensitivity of npr-5: npr-1 acr-2(gf). Statistics show the comparison of flp-1;flp-1 vs flp-1;flp-1 vs npr-5; npr-5; npr-1 flp-1;flp-1 acr-2(gf). * p<0.05 by two-way ANOVA and Bonferroni post-hoc test.

Protocol S1  Supplementary procedure for Figure S5.

Table S1  Strains and Genotypes.

Table S2  DNA Constructs.

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Video S1  Locomotion of adult acr-2(gf) animals. (WMV)

Video S2  Locomotion of adult sbt-1(gf):acr-2(gf) animals. (WMV)

Video S3  Locomotion of adult unc-31(gf); acr-2(gf) animals. (WMV)

Video S4  Locomotion of adult eg-3(gf); acr-2(gf) animals. (WMV)

Video S5  Locomotion of adult flp-1(gf); flp-18(gf) acr-2(gf) animals. (WMV)

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Author Contributions
Conceived and designed the experiments: TMS ST-K KZ YJ. Performed the experiments: TMS ST-K KZ YJ. Analyzed the data: TMS ST-K KZ YJ. Contributed reagents/materials/analysis tools: TSM ST-K KZ YJ. Wrote the paper: TSM ST-K KZ YJ.
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