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Neuropeptide Secreted from a Pacemaker Activates Neurons to Control a Rhythmic Behavior

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Summary

Background—Rhythmic behaviors are driven by endogenous biological clocks in pacemakers, which must reliably transmit timing information to target tissues that execute rhythmic outputs. During the defecation motor program in *C. elegans*, calcium oscillations in the pacemaker (intestine), which occur about every 50 seconds, trigger rhythmic enteric muscle contractions through downstream GABAergic neurons that innervate enteric muscles. However, the identity of the timing signal released by the pacemaker and the mechanism underlying the delivery of timing information to the GABAergic neurons are unknown.

Results—Here we show that a neuropeptide-like protein (NLP-40) released by the pacemaker triggers a single rapid calcium transient in the GABAergic neurons during each defecation cycle. We find that mutants lacking *nlp-40* have normal pacemaker function, but lack enteric muscle contractions. NLP-40 undergoes calcium-dependent release that is mediated by the calcium sensor, SNT-2/synaptotagmin. We identify AEX-2, the G protein-coupled receptor on the GABAergic neurons, as the receptor of NLP-40. Functional calcium imaging reveals that NLP-40 and AEX-2/GPCR are both necessary for rhythmic activation of these neurons. Furthermore, acute application of synthetic NLP-40-derived peptide depolarizes the GABAergic neurons in vivo.

Conclusions—Our results show that NLP-40 carries the timing information from the pacemaker via calcium-dependent release and delivers it to the GABAergic neurons by instructing their activation. Thus, we propose that rhythmic release of neuropeptides can deliver temporal information from pacemakers to downstream neurons to execute rhythmic behaviors.

Keywords

neuropeptide secretion; rhythmic behavior; synaptotagmin; G protein-coupled receptor (GPCR); GABAergic neurons; calcium imaging; defecation motor program; *C. elegans*

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Introduction

Rhythmic behaviors are widely observed in multicellular organisms. The periods of these rhythms are determined by endogenous biological clocks in pacemakers and range from seconds to even years [1, 2]. Genetic, biochemical and electrophysiological studies have shed light on how pacemakers generate biological clocks with different periods [3-5]. However, how pacemakers impact the physiology of target tissues to generate rhythmic behaviors is largely unknown. Perturbations in the communication between pacemakers and downstream targets can disrupt the orchestrated rhythmic behavioral outputs and can lead to disorders such as insomnia and arrhythmia [6, 7].

The *C. elegans* defecation motor program is a very simple rhythmic behavior with a period of about 50 seconds [8]. It is composed of three stereotypical, sequential muscle contractions: first, the posterior body wall muscles contract (pBoc); three seconds later, the anterior body wall muscles contract (aBoc); next, the enteric muscles contract which leads to the expulsion (Exp) of digested food from the intestine (Figure 1A). Previous studies have shown that the intestine functions as the pacemaker, and the period is set by calcium oscillations in the intestine that peak every 50 seconds [9-12]. It has been proposed that the intestine may secrete different signals which act on different circuits to coordinate these three muscle contractions [13].

Among the candidate signals are neuropeptides. Neuropeptides are derived from larger neuropeptide precursors, which are packaged into dense core vesicles (DCVs) where they are cleaved and processed to produce small bioactive peptides [14]. Neuropeptides are released when DCVs undergo calcium-dependent exocytosis upon stimulus, which is mediated by the synaptotagmin family of calcium sensors [15]. After secretion, neuropeptides activate G protein-coupled receptors (GPCRs) on target cells to regulate diverse biological processes [16]. While it has been well known that neuropeptides in pacemakers are critical for rhythmic behavioral output [17-19], it is still unclear how neuropeptide signaling establishes rhythmicity in target tissues to generate rhythmic behaviors [20].

The Exp step in the defecation motor program is controlled by a pair of GABAergic neurons, AVL and DVB [21]. These two neurons release the neurotransmitter γ-aminobutyric acid (GABA), which activates the excitatory GABA receptor, EXP-1, on enteric muscles to cause muscle contraction [22]. It has been suggested that a secreted signal from the intestine may act through AEX-2, a GPCR, on the GABAergic neurons to control the Exp step [23]. However, the identity of the signal and how it conveys the temporal information from the intestine to the downstream GABAergic neurons are unknown.

Here, we report that a conserved neuropeptide-like protein (NLP-40) is required for the Exp step in *C. elegans*. We show that the calcium oscillations in the intestine drive the release of NLP-40, which is mediated by the calcium sensor, SNT-2/synaptotagmin. In vivo calcium imaging shows that NLP-40 is the instructive cue for rhythmic calcium influx in the GABAergic neurons by activating its receptor AEX-2/GPCR. We propose a model whereby rhythmic release of neuropeptides encodes temporal information that couples pacemakers to downstream neurons to coordinate rhythmic behaviors.

Results

nlp-40 is required for the Exp step

The gene *nlp-40* (neuropeptide-like protein 40) was identified in a forward genetic screen for genes that regulate synaptic transmission (see Experimental Procedures). Two

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independently isolated *nlp-40* mutants, *vj3* and *tm4085*, both of which delete significant portions of the *nlp-40* coding region (Figure 1D and Supplemental Information), displayed distended intestinal lumens, nearly complete elimination of Exp and reductions in aBoc (Figure 1B, 1C and S1B). However, both pBoc frequency and calcium oscillations in the intestine were normal (Figure S1A and data not shown). The constipated phenotype and Exp defects of *nlp-40* mutants could be fully rescued by a transgene containing *nlp-40* genomic DNA (Figure 1B and 1C). In addition, knockdown of *nlp-40* expression by RNA interference (RNAi) also produced similar Exp defects (Figure 1C). Thus, *nlp-40* is necessary for the execution of the Exp step. *nlp-40* is predicted to encode a 123-amino-acid neuropeptide precursor protein, which is highly conserved in nematodes. The NLP-40 precursor contains a predicted signal sequence and three dibasic consensus cleavage sites predicted to serve as processing sites to generate four small peptides (P1 to P4) (Figure 1E and S2).

NLP-40 is secreted from the intestine

Examination of the expression pattern of *nlp-40* using a functional, endogenous *nlp-40* promoter to drive the expression of GFP with a nuclear localization sequence (NLS) revealed that *nlp-40* was exclusively expressed in intestinal cells (Figure 2A). Expression of *nlp-40* cDNA under the control of a heterologous intestine-specific promoter completely rescued the Exp defects of *nlp-40* mutants (Figure 2B).

To determine whether NLP-40 is secreted from the intestine, we expressed a functional NLP-40::YFP fusion protein in the intestine. This fusion protein adopted a highly punctate pattern of fluorescence on the basolateral surface of the intestinal cells (Figure 2C and 2D). YFP fluorescence was also detected in six coelomocytes (Figure 2C and 2E). They are scavenger cells in *C. elegans* whose function is to take up proteins, which are secreted from other tissues, by bulk endocytosis [24]. Thus, our results are consistent with the notion that NLP-40 is packaged in dense core vesicles (DCVs) and is secreted from the intestine.

snt-2/synaptotagmin functions in the intestine to regulate the Exp step

If calcium oscillations in the intestine instruct the execution of rhythmic Exp by triggering NLP-40 secretion, we reasoned that NLP-40 release would be calcium dependent. To test this idea, we determined whether synaptotagmin, which is the principal calcium sensor for synchronous exocytosis of synaptic vesicles and DCVs [15], mediates the Exp step and NLP-40 release from intestine. In *C. elegans*, there are seven genes encoding synaptotagmin family members, *snt-1* to *snt-7* (www.wormbase.org). Only RNAi-mediated knockdown of *snt-2* produced detectable Exp defects, reducing the Exp frequency to approximately 40% (Figure 3A and data not shown). *snt-2 (tm1711)* mutants, which contain a 862bp deletion that is predicted to introduce an early stop codon in *snt-2* before the C2B domain (Supplemental Information), displayed similar Exp defects (Figure 3B).

A transcriptional reporter of *snt-2*, in which GFP was driven by the endogenous *snt-2* promoter, was expressed throughout the intestine, as well as in several neurons in the head and tail (Figure 3C). However, GFP fluorescence was not detected in the two GABAergic neurons, AVL and DVB, which control the Exp step (Figure S3A and S3B). Expression of *snt-2* cDNA specifically in the intestine rescued the Exp defects of *snt-2* mutants (Figure 3B). Thus, we conclude that *snt-2/*synaptotagmin functions in the intestine to regulate the Exp step.

snt-2 is the calcium sensor for the release of NLP-40 from the intestine

Functional SNT-2::CFP fusion proteins adopted a punctate pattern of fluorescence on basolateral surfaces of intestinal cells that co-localized with NLP-40::YFP puncta (90.4%±

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1.9% co-localization, n=13 worms) (Figure 3D). To directly test whether *snt-2* regulates the secretion of NLP-40 from the intestine, we examined the accumulation of NLP-40::YFP in coelomocytes. The intensity of YFP-tagged neuropeptide fluorescence in coelomocytes is a measure of the efficacy of neuropeptide secretion in *C. elegans* [25, 26]. NLP-40::YFP fluorescence in coelomocytes was reduced by approximately 50% in *snt-2* mutants, compared to wild type controls (Figure 2E, 2F and 2G). Thus, SNT-2 is associated with the DCVs containing NLP-40 and *snt-2* is required for normal secretion of NLP-40 from the intestine.

SNT-2 contains two calcium binding domains, C2A and C2B. The C2B domain contains all five conserved aspartic acid residues (Figure S3C), which are the key residues that coordinate calcium [27]. To determine whether calcium binding is critical for SNT-2 function, we mutated two aspartic acid residues in the C2B domain of SNT-2 corresponding to the residues in *Drosophila* synaptotagmin 1 that are required for calcium-dependent synchronous transmitter release [28]. Expression of this calcium-binding defective SNT-2 (referred to as *snt-2* [D247, 253N], Figure S3C) in the intestine failed to rescue the Exp defects of *snt-2* mutants in three independent transgenic lines (Figure 3B). Thus, SNT-2 is likely to be an important calcium sensor for NLP-40 release from the intestine. The residual NLP-40 secretion observed in *snt-2* mutants may be mediated by other calcium sensors or by calcium-independent NLP-40 release.

NLP-40 regulates rhythmic calcium influx in the GABAergic neurons

Once released from the intestine, NLP-40 could control Exp by either activating the GABAergic neurons or by directly activating the enteric muscles. To distinguish between these possibilities, we tested whether optogenetic activation of the GABAergic neurons could bypass the requirement of *nlp-40*. It was previously shown that activation of channelrhodopsin-2 (ChR-2) in the GABAergic neurons with blue light in the presence of all *trans*-retinal but not in its absence could restore Exp to mutants lacking *aex-2*, the GPCR on GABAergic neurons [23]. We found that activation of ChR-2 in the GABAergic neurons rescued the Exp defects of *nlp-40* mutants to a similar extent as *aex-2* mutants, but failed to rescue the Exp defects of mutants lacking *exp-1*, the excitatory GABA receptor on enteric muscles (Figure 4A). Therefore, NLP-40 does not function to activate the muscles directly, but instead is likely to participate in the activation of the GABAergic neurons during the Exp step.

To determine whether *nlp-40* activates the GABAergic neurons, we performed calcium imaging of GABAergic neurons during the defecation cycle using GCaMP3.0, a genetically-encoded calcium indicator [29]. Expression of GCaMP3.0 in the DVB neuron allows the visualization of the DVB cell body and the synaptic region where the DVB axon enters the ventral cord and innervates the enteric muscles (Figure 4B) [30]. In wild type worms, we observed a single robust calcium spike in the synaptic region of the DVB neuron (and also often in the cell body) that began approximately three seconds following each pBoc step, peaked immediately before each Exp step, and returned to baseline about two to three seconds following Exp. Calcium spikes were not observed at any other time in the cycle (51 out of 51 cycles in 14 worms, Figure 4B, 4C, Movie S1 and data not shown). These results show that DVB neurons undergo rhythmic activation that correlates with the Exp step. *nlp-40* mutants displayed no calcium spikes in DVB neurons (0 out 46 cycles in 14 worms, Figure 4D and Movie S2), except for two calcium spikes that were associated with two passive Exp steps (see Supplemental Information). Expression of *nlp-40* cDNA in the intestine completely restored rhythmic calcium transients to *nlp-40* mutants (35 out of 35 cycles in 12 worms, Figure 4E and Movie S3). Thus, the GABAergic neurons are rhythmically activated and this rhythmic activation is dependent on NLP-40 from the intestine.

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AEX-2/GPCR is the receptor for NLP-40

We hypothesized that AEX-2/GPCR might be the receptor on the GABAergic neurons for NLP-40, since *aex-2* mutants phenocopied *nlp-40* mutants for defects in the Exp and aBoc steps (Figure 4A, S1 and [23]) and calcium influx in DVB neurons (0 out of 47 cycles in 12 worms, Figure 4F and Movie S4).

To directly test this hypothesis, we first determined which of the four predicted mature peptides derived from NLP-40 (P1 to P4, Figure 1E) mediates the Exp step. To do this, we generated four NLP-40 variants (M1 to M4, Figure 5A), each of which contains missense mutations in one of the four predicted mature peptides. Only the NLP-40 variant carrying mutations in the P3 peptide (termed M3), was not able to rescue the Exp defects of *nlp-40* mutants (Figure 5A). Next, we generated chimeras between NLP-40 and NLP-21 (C1 to C4, Figure 5A). *nlp-21* encodes a neuropeptide precursor that is expressed in the intestine [31] but does not regulate the Exp step (data not shown). Only chimeras (C2 and C3) that included the P3 peptide region could rescue *nlp-40* mutants (Figure 5A), whereas chimeras that did not contain P3 were not functional (C1 and C4). Thus, the region including the P3 peptide is both necessary and sufficient for NLP-40 function. P3 encodes a seven-amino-acid peptide, MVAWQPM (referred to as P3-1). This peptide and a possible partial degradation product of it, VAWQPM (referred to as P3-2), have previously been biochemically identified by mass spectrometry of *C. elegans* peptide enriched extracts [32].

Finally, we tested whether the P3 peptides could activate AEX-2/GPCR in a heterologous CHO cellular assay system. In this system, activation of the GPCR is coupled to release of intracellular calcium, which leads to an increase of aequorin luminescence [33]. We found that both P3-1 and P3-2 peptides were able to activate AEX-2/GPCR. The NLP-40 P3-1 peptide (EC_{50}=3.39 nM±1.17 nM) was a more potent ligand for AEX-2/GPCR, compared to the NLP-40 P3-2 peptide (EC_{50}=26.73 nM±1.18 nM) (Figure 5B). In contrast, NLP-40 P1 (APSAPAGLEEKL), which was not required for *nlp-40* function (Figure 5A), failed to activate AEX-2/GPCR at all concentrations tested (Figure 5B). Also, CHO cells transfected with the empty vector that did not include *aex-2* cDNA did not generate responses to any of the three peptides tested (Figure S5). Taken together, we conclude that AEX-2/GPCR is the receptor for the NLP-40 P3 peptides.

NLP-40 is an instructive cue for the depolarization of the GABAergic neurons

If NLP-40 is the timing signal that delivers the temporal information encoded by the calcium oscillations in the intestine to the downstream GABAergic neurons, we predict that NLP-40 would be an instructive cue for the excitation of GABAergic neurons. To test this prediction, we examined whether acute delivery of the NLP-40 P3-1 peptide to the GABAergic neurons in vivo would be sufficient to activate them. To mimic the endogenous release of NLP-40 into the pseudocoelom, we injected the synthetic NLP-40 P3-1 peptide into the pseudocoelom of immobilized *nlp-40* mutants, and recorded calcium response in GCaMP3.0-expressing DVB neurons. We found that the NLP-40 P3-1 peptide consistently induced a single calcium spike in DVB neurons 3.5±0.60 (mean± s.d.) seconds following injection (10 out of 10 injections, n=10 worms) (Figure 6A, 6B and Movie S5). The onset delay and duration of the NLP-40 P3-1 induced calcium spikes in DVB were similar to those induced by endogenously released NLP-40 (Figure 4C), suggesting the injected NLP-40 P3-1 recapitulates endogenous NLP-40 function. In contrast, injecting the NLP-40 P3-1 peptide into *nlp-40; aex-2* double mutants failed to elicit any calcium responses (0 out of 8 injections, n=8 worms) (Figure 6D and Movie S7). In addition, injection of the NLP-40 P1 peptide, which did not activate AEX-2 in vitro, also failed to elicit any calcium responses in DVB neurons (0 out of 7 injections, n=7 worms) (Figure 6C and Movie S6). Thus, we conclude that NLP-40 is instructive for the excitation of the GABAergic neurons.

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Discussion

In this study, we demonstrate that NLP-40 is the timing signal that couples the calcium oscillations in the intestine to the rhythmic activation of the downstream GABAergic neurons during the defecation motor program in *C. elegans*. Based on our work and previous studies [10-13, 23], we propose a model for the control the Exp step (Figure 6E). During the defecation motor program, calcium oscillations in the intestine drive calcium-dependent rhythmic secretion of NLP-40, which is mediated by the calcium sensor SNT-2/synaptotagmin. Once released, NLP-40 binds to its receptor, AEX-2/GPCR, which activates Gαs and adenylate cyclase to produce cAMP and thus triggers calcium influx to depolarize the downstream GABAergic neurons. These neurons then release GABA, which activates its receptor EXP-1 on the enteric muscles to drive the Exp step. Therefore, rhythmic release of neuropeptides may be a mechanism by which temporal information is encoded to mediate the communication between pacemakers and the downstream neurons to control rhythmic behaviors.

How does NLP-40 carry the timing information from the pacemaker?

Our results support the model that NLP-40 carries timing information from the intestine via calcium-dependent rhythmic release (Figure 6E). First, we find that the calcium sensor SNT-2/synaptotagmin resides on NLP-40-containing DCVs and mediates the release of NLP-40 from the intestine, and that calcium binding of SNT-2/synaptotagmin is required for the Exp step (Figure 2G, 3B and 3D). Second, DVB neurons undergo NLP-40 dependent rhythmic activation and NLP-40 can instruct the activation of DVB neurons (Figure 4C, 4D, 6B and Movie S1, S5). The short period (about 50 seconds) of the defecation cycle makes regulation of NLP-40 release by transcriptional or translational control unlikely. Therefore, we speculate that during the defecation cycle, calcium oscillations in the intestine may drive rhythmic release of NLP-40 through SNT-2/synaptotagmin.

Interestingly, capacitance measurements in rat gonadotropes have shown that calcium oscillations can induce rhythmic exocytosis [34]. In addition, immunohistological evidence suggests that cyclic release of the neuropeptide pigment-dispersing factor (PDF) from the pacemaker may be important for circadian locomotor activity in *Drosophila* [35]. Thus, rhythmic release of neuropeptides may represent a general mechanism by which timing information is transmitted from pacemakers.

Calcium-dependent NLP-40 release

SNT-2 is most similar to the mammalian synaptotagmin 1, 2, and 9 family members, which mediate fast, synchronous secretory vesicle secretion in the brain [36]. It is interesting to note that the relatively mild Exp defects observed in *snt-2* mutants differ from the phenotype of *nlp-40* mutants. In the absence of *snt-2*, NLP-40 release from the intestine may be coupled less efficiently or be uncoupled from the calcium oscillations. Nonetheless, the residual NLP-40 released in *snt-2* mutants may drive the Exp step if it reached a certain threshold to activate AEX-2/GPCR in some cycles.

In principle, SNT-2 could function redundantly with one or more of the six other *C. elegans* synaptotagmin family members. Alternatively, other types of calcium binding proteins may mediate the residual NLP-40 release in *snt-2* mutants. One interesting candidate is AEX-1, a Munc13-4 like protein, which is a C2 domain containing protein that functions in the intestine to regulate the Exp step [37]. However, it has not been tested whether calcium binding of the C2 domain in AEX-1 is required for its function.

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How does NLP-40 deliver the temporal information to the GABAergic neurons?

Many studies have established a classic neuromodulatory role of neuropeptides in which neuropeptides fine-tune the excitability of neurons and circuits [38-41]. However, our results suggest that NLP-40 can act more like a classic neurotransmitter by depolarizing the GABAergic neurons through its receptor AEX-2/GPCR. First, the rhythmic calcium spikes in DVB neurons are abolished in *nlp-40* and *aex-2* mutants (Figure 4C, 4D and 4F). Second, injection of the NLP-40-derived P3-1 peptide into the pseudocoelom is sufficient to reliably elicit a calcium transient in the DVB neuron within a few seconds, and this response is dependent on AEX-2/GPCR (Figure 6B, 6D). Third, it is unlikely that classic neurotransmitters are involved in activating DVB neurons during the defecation cycle, since mutants defective in the biosynthesis, transport or release of classical neurotransmitters (such as *unc-13/Munc13, eat-4* for glutamate, *cat-2* for dopamine, *tph-1* for serotonin, *tdc-1* for tyramine and octopamine, *unc-17* for acetylcholine) have grossly normal Exp frequency (Figure S4 and [42]). Therefore, NLP-40 delivers timing information by serving as an instructive cue for the excitation of the GABAergic neurons via its receptor AEX-2/GPCR.

NLP-40 P3-1 injection caused consistent calcium responses in DVB but failed to induce robust enteric muscle contractions (Exp) (data not shown). This is likely due to the additional unknown permissive signal proposed by Mahoney et al., which restricts the Exp step from occurring outside of a small window within a few seconds of the pBoc step [23]. Our results show that this permissive signal most likely controls the refractory property of enteric muscles, since peptide injection (presumably at any time in the cycle) always elicited a calcium response in DVB. Thus the refractory period may be a mechanism by which the robust rhythmicity of Exp, instructed by NLP-40/AEX-2 signaling in the GABAergic neurons, is ensured.

That neuropeptides are involved in controlling the Exp step has been implied by the identification of a well-conserved set of proteins involved in the processing and secretion of neuropeptides, including AEX-5/pro-protein convertase, AEX-4/SNAP25, AEX-1/Munc13-like protein, and SYN-1/syntaxin, which are necessary for the Exp step [23, 37, 43]. Our results extend these studies by identifying the peptide and providing mechanisms by which the peptide is released from the pacemaker and activates its downstream targets. The identification of additional downstream components in this GPCR signaling pathway will promote further understanding of how neuropeptides can instruct rhythmic behaviors.

Experimental procedures

Strains, plasmids and transgenes

All *C. elegans* strains were maintained as described at 20°C on NG M plates seeded with OP50 *E. coli* [44]. The *vj3* allele was originally isolated from an EMS screen for aldicarb resistant mutants. The construction of plasmids was performed with standard protocols. Transgenic worms were generated by microinjection [45]. A detailed list of all the strains, plasmids and oligos is described in the Supplemental Information.

Behavioral assays

The defecation motor program was scored as previously described [8, 46]. Each animal was scored for 10 consecutive defecation cycles. 8-10 worms were examined for each genotype. Unpaired two-tail Student's t test with unequal variance was used to determine statistical significance between two samples. The assay with channelrhdopsin-2(ChR2) in GABAergic neurons was performed as previously described [23]. See Supplemental Information for details.

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Fluorescence Imaging and analysis

Fluorescence imaging of NLP-40::YFP and SNT-2::CFP in the intestine was performed in *glo-1(zu391)* mutant background, which has normal Exp steps (Figure S4), to reduce intestinal auto-fluorescence [47]. The coelomocyte assay was performed and quantified as previously described [25]. See Supplemental Information for details.

In vivo calcium imaging

The transgenic animals vjIs58[pmyo-2::NLS::mCherry, Punc-47mini::GCaMP3.0]) was used for calcium imaging in DVB neurons. The strains, used in Figure 4B to 4F, contained the *unc-13(s69)* mutation to immobilize animals, because *unc-13(s69)* mutants are almost completely paralyzed [48]. Both *vjIs58* and *unc-13(s69)* had normal Exp steps (Figure S4). Live imaging of young adults was performed on NGM-agarose plates with food topped with a coverslip, using a Nikon eclipse 90i microscope equipped with a Plan Apo 40× oil objective (N.A.=1.0) and a standard GFP filter set. The GCaMP3.0 fluorescence in the synaptic region of the DVB neuron was quantified. For imaging and quantification details, see Supplemental Information.

In vitro cellular assay for AEX-2/GPCR activation by NLP-40-derived peptides

Chinese hamster ovary cells (CHO-K1), stably expressing the mitochondrially targeted apo-aequorin (mtAEQ) and the human $G_{\alpha 16}$ subunit, were used for the Ca^{2+} measurements to detect the activation of GPCR [33]. The cells were transiently transfected with the *aex-2* cDNA construct (pHW149) or the empty vector (pHW148), and then treated with one of the synthetic NLP-40-derived peptides (NLP-40 P1, P3-1 or P3-2). See Supplemental Information for details.

Injection of NLP-40 derived peptides and calcium imaging

Injections were performed using the Eppendorf Femtojet express under an inverted Olympus IX70 microscope equipped with an Olympus UPlan FLN 40× oil objective (N.A.=1.30) and a Photometrics cascade 512B camera. Adult worms were glued on sylgard-coated cover slides using histoacryl (B. Braun, Germany), as previously described [48]. Worms were superfused with external solution (150mM NaCl, 5mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 15 mM HEPES, pH=7.3, 330 mOsm). Real time calcium imaging was performed using Metamorph 6.0 (Universal Imaging), at 4 frames per second for 1 minute (15-100ms exposure time, 2×2 binning, with injection at around the fifteenth second). The injection solution was prepared as following: 10 M of synthetic peptide (NLP-40 P1 or P3-1), 0.5mg/ml of Dextran, rhodamine B (10,000 MW, Molecular Probes) in the external solution. Successful injections were verified with the red co-injection marker in pseudocoelom after injection. Quantification of GCaMP3.0 signal in synaptic region or cell body of the DVB neuron was measured as described in the “in vivo calcium imaging” section of the Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The neuropeptide-like protein NLP-40 is critical for a rhythmic behavior.
- NLP-40 undergoes SNT-2/synaptotagmin-dependent release from the pacemaker.
- NLP-40 activates AEX-2/GPCR on GABAergic neurons.
- NLP-40 is instructive for the rhythmic activation of GABAergic neurons.

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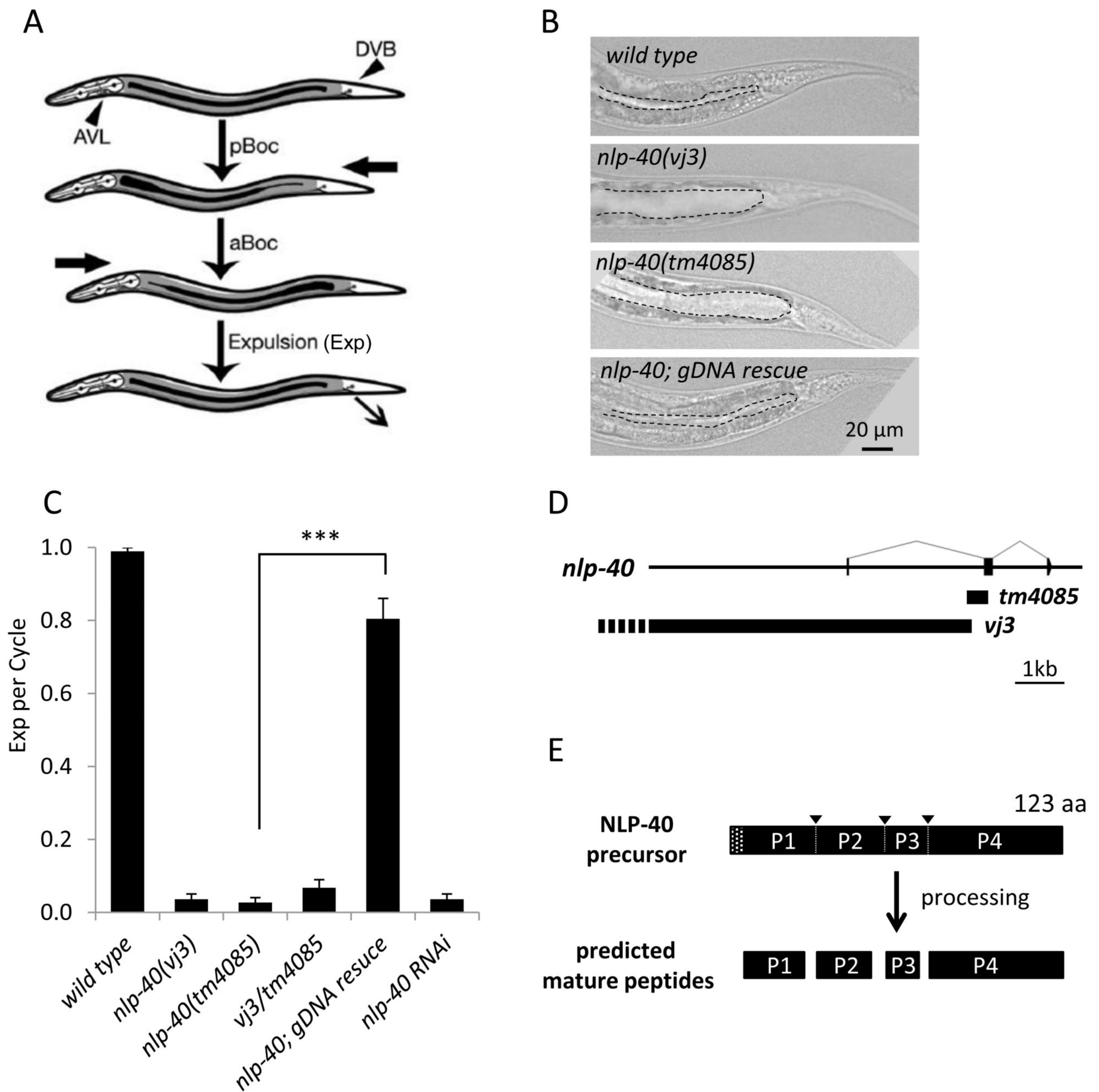

Figure 1. *nlp-40* mutants lack the Exp step

(A) Diagram of the defecation cycle in *C. elegans*, which repeats every 50 seconds (adapted from [23]). Each cycle is initiated with posterior body wall muscle contraction (pBoc), and 3 seconds later, anterior body wall muscles contract (aBoc), which is immediately followed by enteric muscle contraction (the expulsion (Exp) step). The two GABAergic neurons, AVL and DVB, which control the Exp step, are indicated by arrowheads. (B) Representative DIC images of the posterior intestines of young adult worms with indicated genotypes. The enlarged space within the intestinal lumen (black lines) indicates that the lumen is distended in *nlp-40* mutants. (C) Quantification of the Exp frequency in young adult worms of the indicated genotypes. *nlp-40* gDNA rescue denotes *nlp-40(tm4085)* mutant animals expressing genomic *nlp-40* transgenes. “Exp per cycle” is defined as the ratio of Exp over

pBoc. (D) The gene structure of *nlp-40* and the positions of the *vj3* and *tm4085* deletions are indicated. The region labeled *nlp-40* represents the genomic DNA fragment (Supplemental Information) used for rescue. (E) Schematic of the NLP-40 protein, which is a neuropeptide precursor with 123 amino acids. The signal sequence is shown and arrowheads indicate the three consensus cleavage sites by pro-protein convertases. NLP-40 is predicted to yield four small peptides (P1 to P4). The mean and standard errors are shown. Asterisks (***) indicate significant difference: $p < 0.0005$ in Student's t test. (See also Figure S1 and S2)

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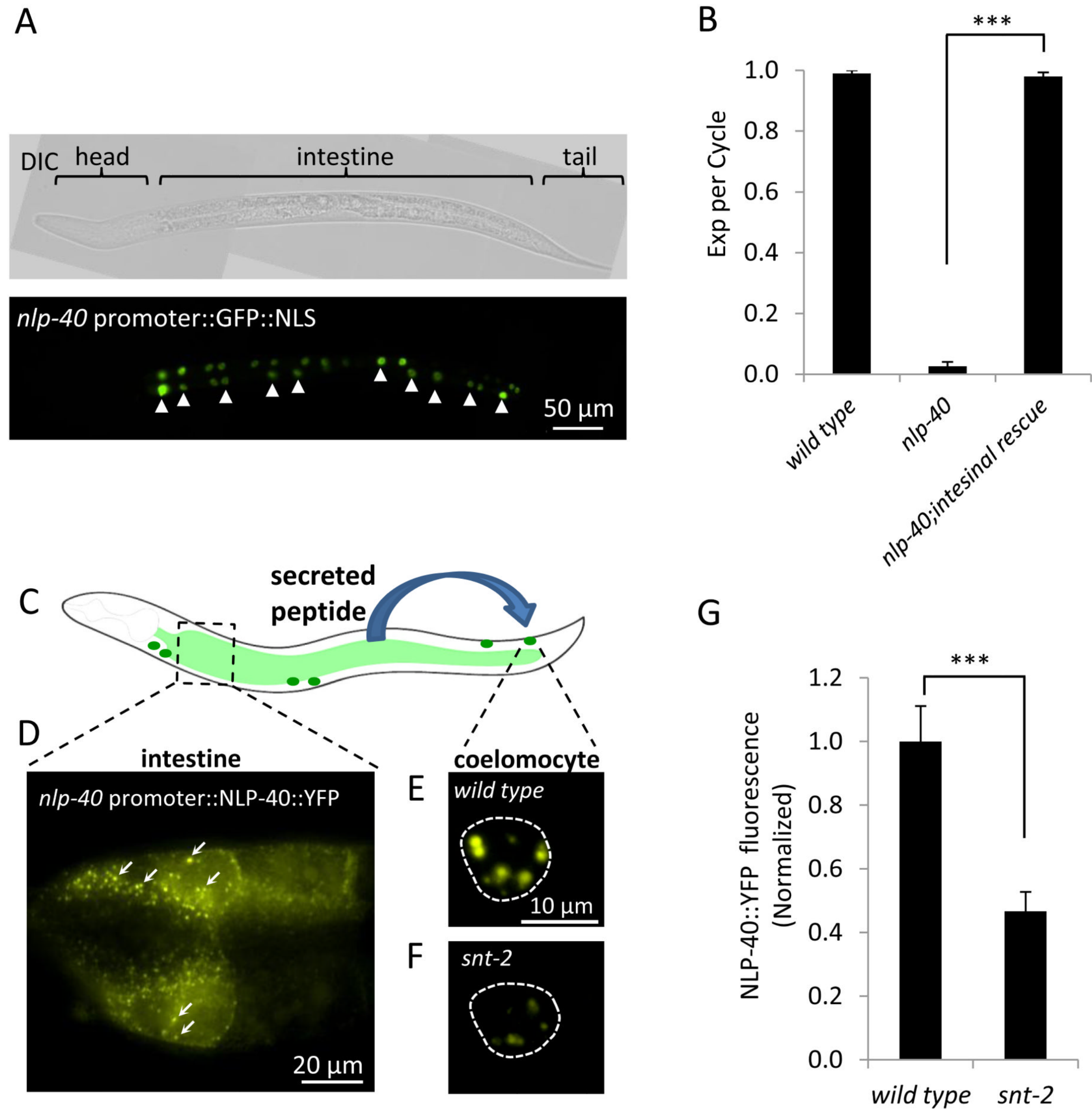

Figure 2. NLP-40 undergoes SNT-2/synaptotagmin dependent release from the intestine

(A) The expression pattern of *nlp-40*. Representative DIC and fluorescent images of an L3 stage worm expressing a transcriptional reporter of *nlp-40*, in which GFP::NLS is driven by the *nlp-40* endogenous promoter fragment (3511bp upstream of ATG). White arrowheads indicate the nuclei of intestinal cells. (B) Quantification of the Exp frequency in young adults with the indicated genotypes. The intestinal rescue denotes transgenic *nlp-40(tm4085)* worms expressing *nlp-40* cDNA under the intestine-specific *ges-1* promoter. (C) Diagram showing peptide expressed the intestine can be secreted and taken up by coelomocytes (green ovals) through bulk endocytosis. (D) Representative image of NLP-40::YFP localization on the basolateral surface of the anterior intestine (some puncta are indicated by arrows). (E) and (F) Representative image of the posterior coelomocytes (outlined by a dashed line) in wild type (E) and *snt-2* mutants (F). Fluorescent patches within the cell reflects secreted NLP-40::YFP taken up by the coelomocyte. (G) Quantification of the average NLP-40::YFP fluorescence in coelomocytes of L4 worms of wild type (n=25), and

snt-2(tm1711) mutants (n=24). The mean and standard error are shown. Asterisks (***) indicate significant difference of $p < 0.0005$ in Student's t test. (See also Figure S4)

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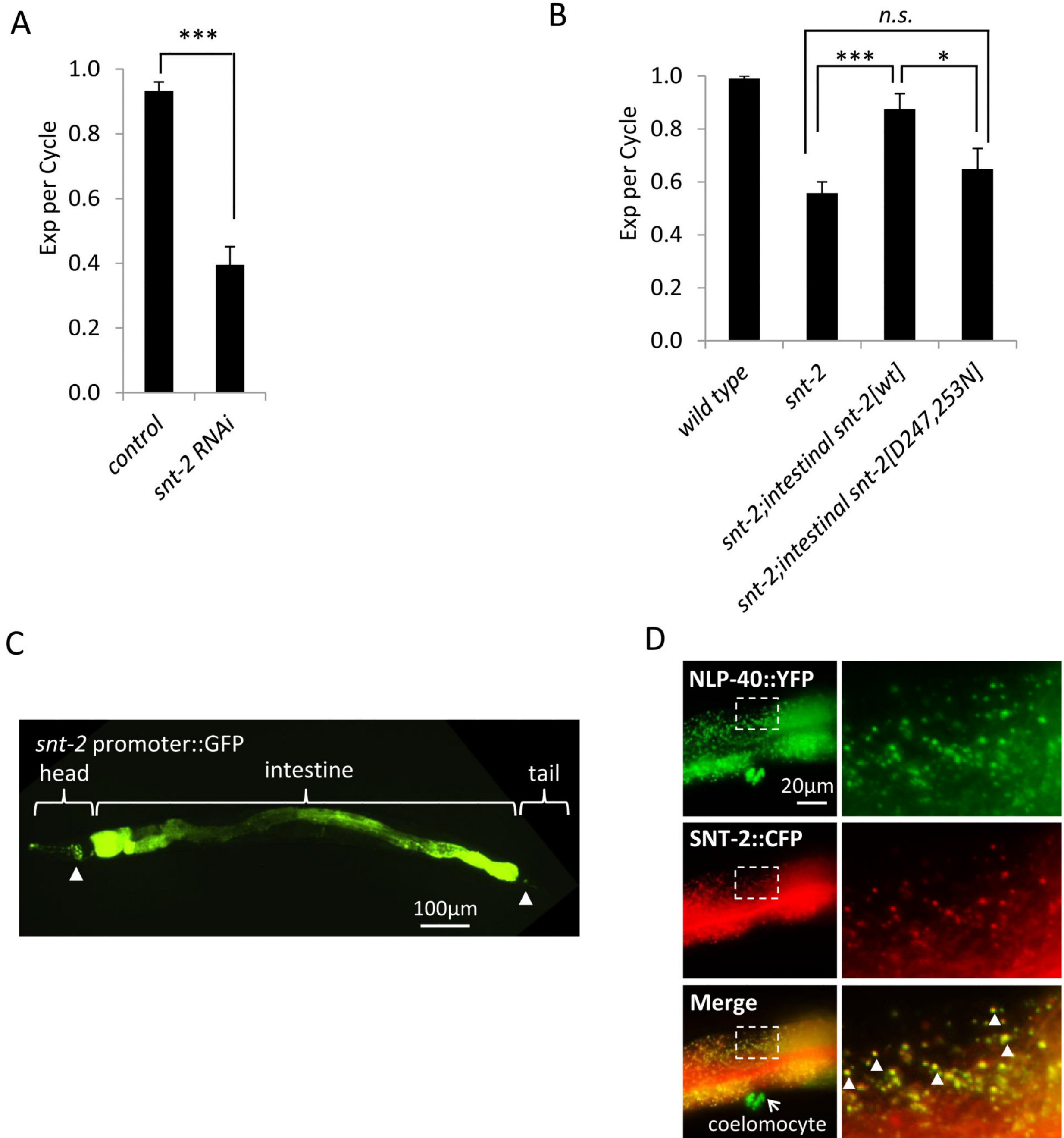

Figure 3. ***snt-2*/synaptotagmin functions in the intestine to regulate the Exp step**

(A) and (B) Quantification of the Exp frequency of RNAi-treated young adults (A) and in young adults with indicated genotypes (B). (C) The expression pattern of *snt-2*. Representative fluorescent image of an adult expressing a *snt-2* transcriptional reporter, in which GFP is driven by the *snt-2* promoter fragment (4177 bp, from 792bp upstream of ATG to 33 bp of the second exon of *snt-2* gene). Arrowheads indicate fluorescence in head and tail. (D) Co-localization of NLP-40::YFP (false colored in green) and SNT-2::CFP (false colored in red) in the intestine (using the *nlp-40* promoter). The arrow indicates the coelomocyte, which only takes up NLP-40::YFP. Right panels: 5× magnification of the indicated regions (dashed rectangles). Some co-localized puncta are indicated by

arrowheads. Asterisks indicate significant differences: * $P < 0.05$, *** $P < 0.0005$ in Student's t tests. (See also Figure S1, S3 and S4)

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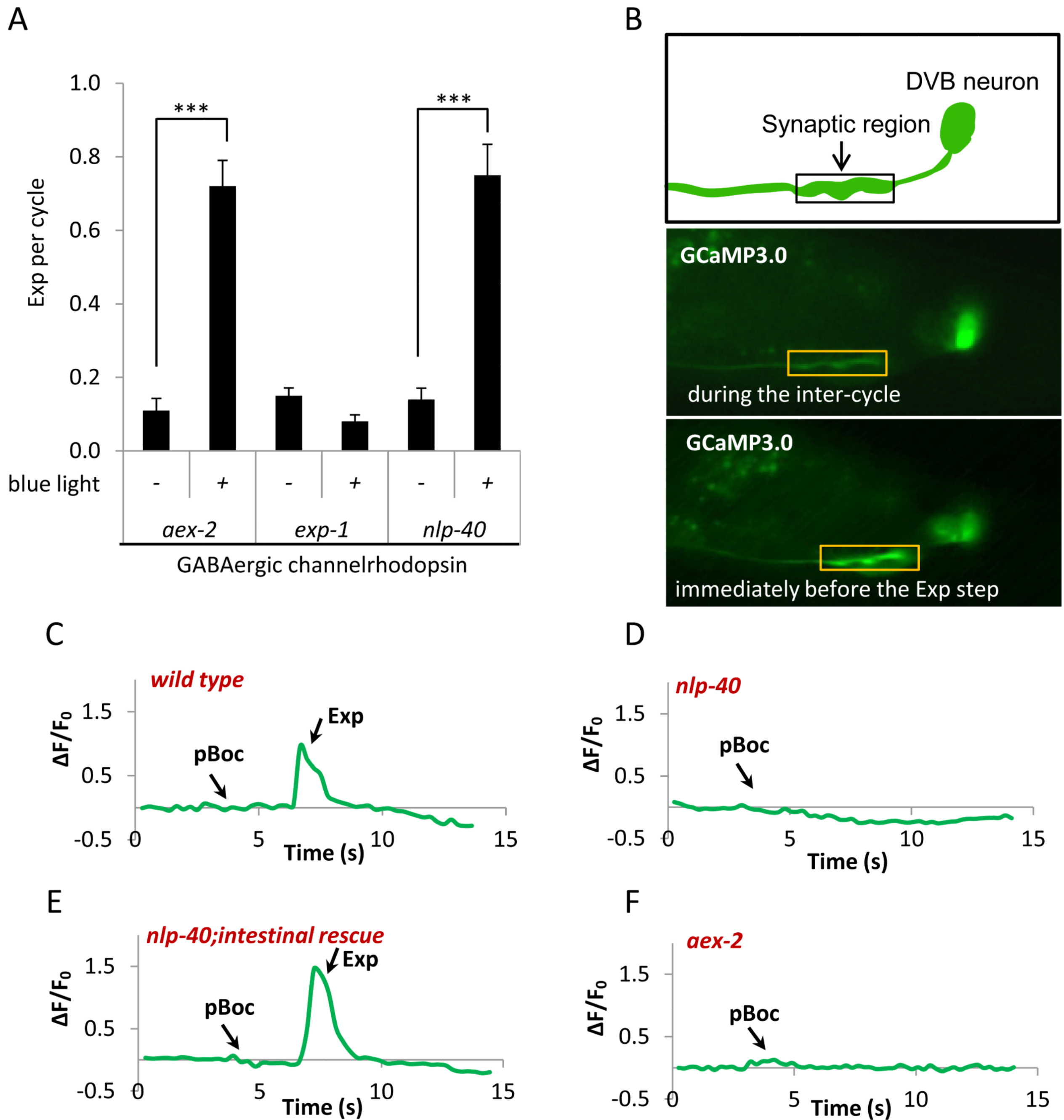

Figure 4. ***nlp-40*** **is required for rhythmic calcium influx in the GABAergic neurons**
(A) Quantification of the Exp frequency in young adults with indicated genotypes. In the presence of all-*trans* retinal, A 1-5 second blue light pulse after pBoc was used to activate the GABAergic neurons expressing ChR-2::GFP (using the *unc-47* full length promoter), as previously reported [23]. (B) Expression of the calcium indicator, GCaMP3.0 in the DVB neuron (*vjIs58*). Top: schematic showing the cell body, axon and synaptic region (boxed) of the DVB neuron in the tail. Middle and Bottom: representative snapshots from a real-time video showing increase in GCaMP3.0 fluorescence in the synaptic region right before the Exp step. Worms are oriented anterior toward the left and dorsal side on the top of the image. (C to F) Representative traces showing changes of GCaMP3.0 fluorescence in DVB

synapses during the defecation process in worms with indicated genotypes. All calcium imaging in (B) to (F) was performed in the *unc-13(s69)* mutant background (See Experimental Procedures). Intestinal rescue denotes transgenic *nlp-40(tm4085)* worms expressing *nlp-40* cDNA under the intestine-specific *ges-1* promoter. The mean and standard errors are shown. Asterisks (***) indicate significant differences: *** $P<0.0005$ in paired two-sample t test. (See also Figure S4 and Movie S1 to S4)

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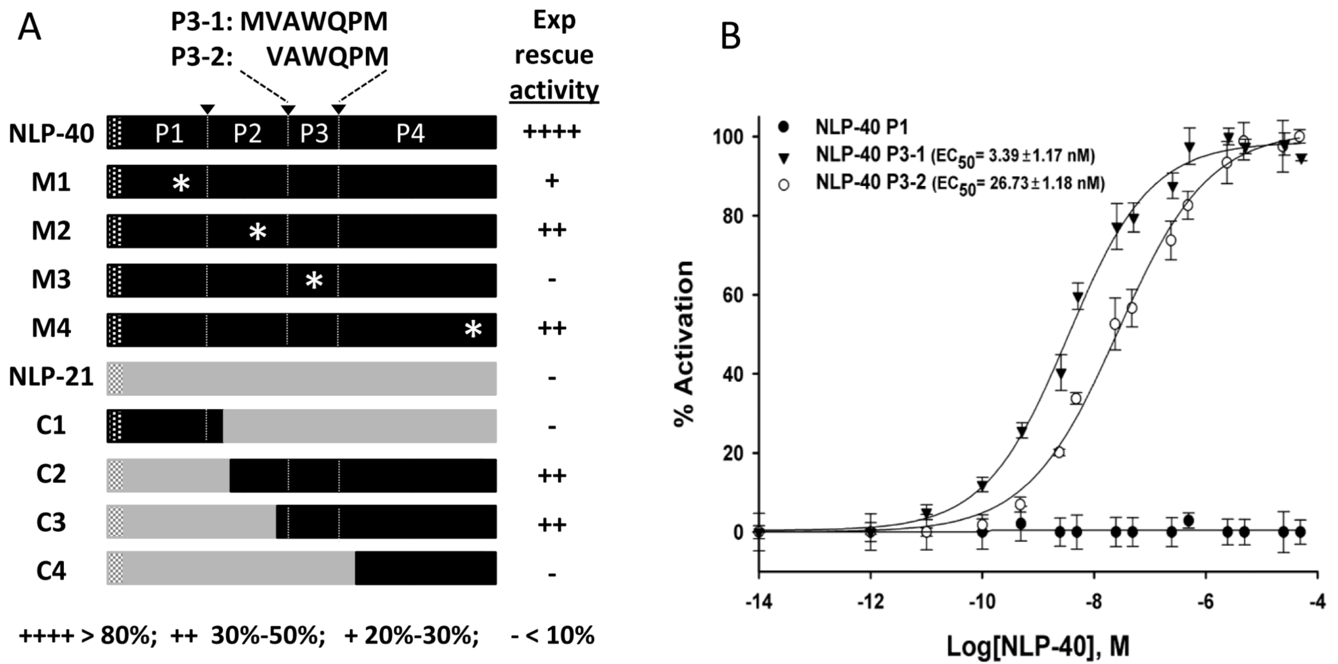

Figure 5. AEX-2 is the receptor for NLP-40

(A) Structure-function analysis of NLP-40 variants. P1-P4 denotes the four predicted peptides derived from NLP-40, based on the consensus dibasic cleavage sites (black arrowheads). Hatched regions represent signal sequences. P3 may give rise to two peptides as determined by mass spectrometry, P3-1 and P3-2. M1-M4 represents variants with three consecutive residues replaced with alanine (white asterisk). C1-C4 represents chimeras with NLP-21 at the indicated positions. Incomplete rescue of some variants may be due to inefficient processing of mutant proteins. (B) Dose-response curves of CHO/mtAEQ/$G_{\alpha 16}$ cells transfected with AEX-2/GPCR and treated with the indicated synthetic NLP-40 peptides: NLP-40 P1: APSAPAGLEEKL; NLP-40 P3-1: MVAWQPM; NLP-40 P3-2: VAWQPM. Percent activation is based on calcium dependent aequorin luminescence. The mean and standard errors are shown. (See also Figure S5)

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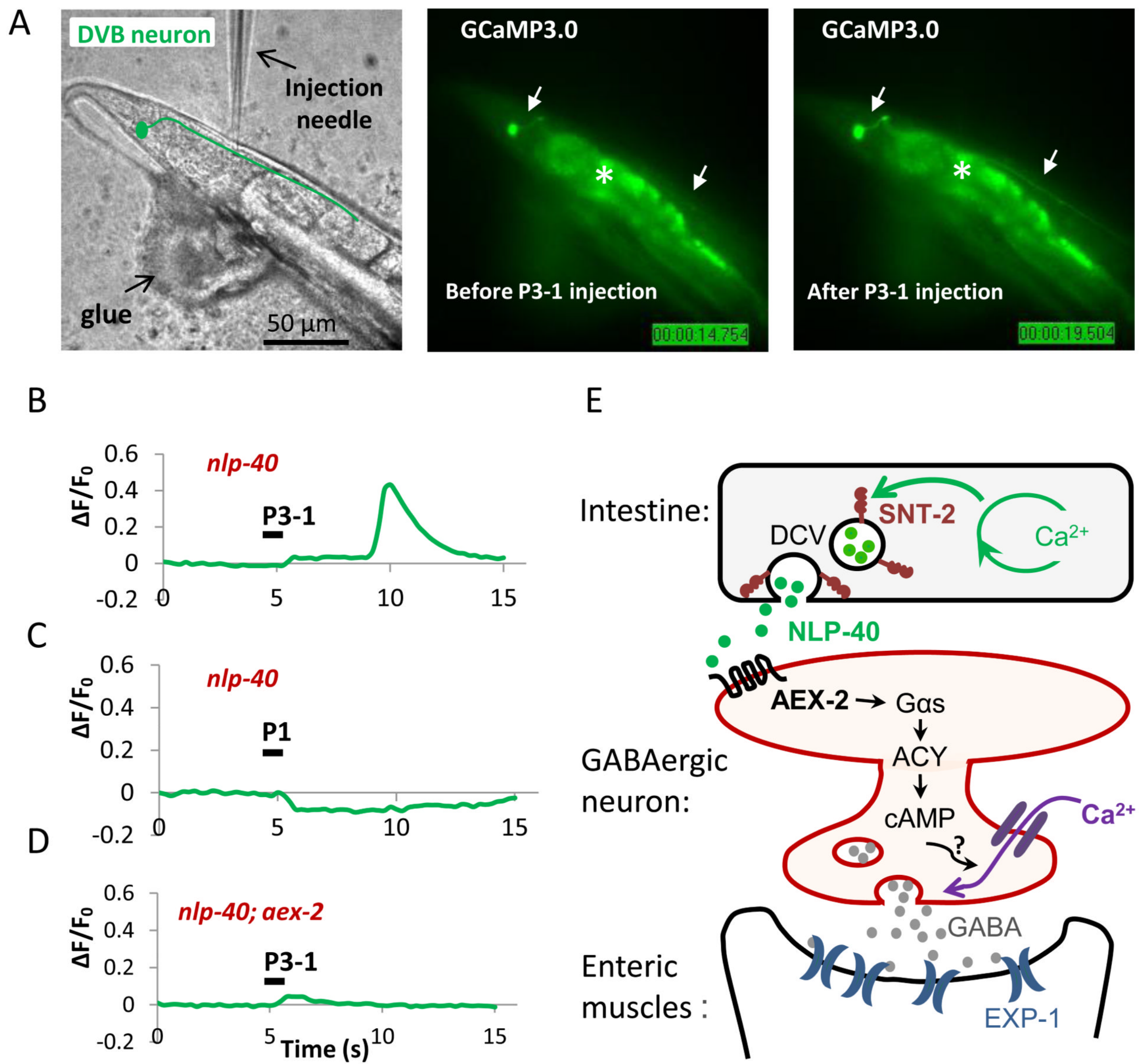

Figure 6. NLP-40 is instructive for the excitation of the GABAergic neurons

(A) Left: bright field image showing the injection needle inserted into the pseudocoelom of worms that have been immobilized by gluing. Middle and Right: representative snapshots from a real-time video showing increase in GCaMP3.0 fluorescence in the cell body and axon (white arrows) of the DVB neuron within 4 seconds after injection of NLP-40-derived bioactive peptide P3-1 in *nlp-40; vjIs58* animals (GCaMP3.0 in DVB neurons). White asterisks (*) represent auto-fluorescence from the intestine. (B) to (D) Representative traces showing changes of GCaMP3.0 fluorescence in the cell body of the DVB neuron in worms with indicated genotypes after injection with the indicated NLP-40-derived peptides. (E) Model of the circuit controlling the Exp step. Each defecation cycle is initiated with a calcium spike in the intestine. SNT-2/synaptotagmin on DCVs senses the increase in calcium and promotes the release of NLP-40 derived peptides from the intestine. Once secreted, NLP-40 activates its receptor AEX-2/GPCR, which is coupled to the activation of the Gαs adenylate cyclase (ACY) to produce cAMP. The activation of AEX-2/GPCR triggers calcium influx in AVL and DVB neurons. These two neurons become excited and

release GABA, which in turn binds to the excitatory GABA receptor, EXP-1, and thus leads to the enteric muscle contractions (Exp). The downstream effectors of cAMP are unknown. (See also Movie S5 to S7)

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