Hierarchical regulation

of functionally antagonistic neuropeptides

expressed in a single neuron pair

Ichiro Aoki,¹,²,* Luca Golinelli,³ Eva Dunkel,¹,² Shripriya Bhat,¹ Erschad Bassam,¹ Isabel Beets,³ and Alexander Gottschalk¹,²,*‡

¹Buchmann Institute for Molecular Life Sciences, Goethe University, Max-von-Laue-Strasse 15, D-60438 Frankfurt, Germany
²Institute of Biophysical Chemistry, Department of Biochemistry, Chemistry and Pharmacy, Goethe University, Frankfurt, Germany
³Department of Biology, KU Leuven, Leuven, Belgium

*Correspondence
‡Lead contact

Ichiro Aoki: Aoki@bio.uni-frankfurt.de

Alexander Gottschalk: a.gottschalk@em.uni-frankfurt.de

Short title
Hierarchical regulation of functionally antagonistic neuropeptide co-transmitters

Highlights (up to 4, each highlight cannot exceed 85 characters)

• A pair of neurons elicits opposing behaviors via two distinct neuropeptides

• The neuropeptide NLP-10 accelerates locomotion by activating premotor interneurons

• Release of the neuropeptide FLP-1 reduces NLP-10 release via autocrine feedback

• NLP-10 is crucial for escaping aversive mechanical stimuli and noxious blue light

eTOC blurb (should be 50 words or less)
Neuropeptidergic communication is complex due to the hundreds of proteinaceous transmitters and receptors involved. Aoki and colleagues demonstrate a further layer of regulation, where one neuropeptide
negatively influences release of an antagonistic neuropeptide by autocrine feedback, in a single neuron pair. This may represent a general principle in neuropeptidergic signaling.

Keywords
Neuropeptides; co-transmission; autocrine signaling; Caenorhabditis elegans; locomotion; aversive response

current word count: 7213 (including summary)

Summary
Neuronal communication involves small-molecule transmitters, gap junctions, and neuropeptides. While neurons often express multiple neuropeptides, our understanding of the coordination of their action and their mutual interactions remains limited. Here, we demonstrate that two neuropeptides, NLP-10 and FLP-1, released from the same interneuron pair, AVKL/R, exert antagonistic effects on locomotion speed in Caenorhabditis elegans. NLP-10 accelerates locomotion by activating the G protein-coupled receptor NPR-35 on premotor interneurons that promote forward movement. Notably, we establish that NLP-10 is crucial for the aversive response to mechanical and noxious light stimuli. Conversely, AVK-derived FLP-1 slows down locomotion by suppressing the secretion of NLP-10 from AVK, through autocrine feedback via activation of its receptor DMSR-7 in AVK neurons. Our findings suggest that peptidergic autocrine motifs, exemplified by the interaction between NLP-10 and FLP-1, might represent a widespread mechanism in nervous systems across species. These mutual functional interactions among peptidergic co-transmitters could fine-tune brain activity.
Introduction

Neuropeptides can establish orthogonal signaling networks with important effects on behavior, operating either in conjugation with, or independently of, classical small-molecule or monoamine transmitters and gap junctions. While neuropeptides can signal locally to postsynaptic or neighboring neurons, their capability extends to the global relay of information, received by numerous cells throughout the brain and body, as exemplified by peptide hormones like oxytocin, vasopressin, insulin, and pituitary-derived peptides. The versatility of neuropeptides is evident in their involvement in various disorders: For instance, individuals lacking pro-opiomelanocortin (POMC) suffer from severe obesity, dysfunction of the orexin system can lead to narcolepsy, and alterations in the level of the neuroprotective neuropeptide Y (NPY) are observed in neurodegenerative disorders.

Frequently, multiple (neuro)peptides, occasionally with diverse or opposing functions, are expressed in the same cell. However, it remains controversial whether distinct peptides are co-packaged into the same dense core vesicles (DCVs) and whether the general excitation of these neurons leads to the universal release of all peptides. Some cases of differential packaging or regulation of multiple neuropeptides expressed in the same cell have been reported: For example, murine chromaffin cells have two populations of DCVs. In pituitary neurons, POMC may be secreted from the constitutive pathway, while its derivative, adrenocorticotropic hormone (ACTH), is released from the regulated pathway. Vasopressin and galanin are also packaged into different DCV populations. Bag cells and the atrial gland of Aplysia californica contain DCVs with distinct sets of neuropeptides, and even multiple peptides from the same propeptide are distributed across different vesicles. To facilitate such segregation, at least the initial cleavage of propeptides has to occur in the Golgi apparatus, before their loading into secretory vesicles, as suggested in the processing of POMC. In C. elegans, the release of multiple neuropeptides from ASI sensory neurons could be differentially regulated by monoamines, and oxidative stress increases the secretion of FLP-1 but not FLP-18 neuropeptides from AIY interneurons. Co-transmission with multiple transmitters generally provides flexibility to neuronal microcircuits. This can happen either by convergently regulating the same target neuron conferring antagonistic, additive, or synergistic effects, or by divergently regulating multiple...
target neurons. Another strategy for flexibility involves packaging different transmitters into distinct vesicles and regulating their release specifically, providing an additional layer of adaptability\textsuperscript{10}. Despite these observations, little is known about whether and how different neuropeptides in the same neuron might be differentially regulated.

To address this issue, we studied the AVK interneurons in \textit{C. elegans}. This neuron pair stands out for its expression of a diverse array of neuropeptides and neuropeptide receptors, making it a central hub in the neuropeptide network of \textit{C. elegans}\textsuperscript{1}. Despite the absence of known small-molecule transmitters\textsuperscript{27,28}, AVK neurons signal to numerous cells in the nervous system, exerting influence over locomotion, roaming strategies, egg-laying\textsuperscript{29–31}, and pathogen avoidance\textsuperscript{32}. They integrate various sensory inputs, including information about food presence, oxygen levels, and more, through a range of chemical and electrical synapses, including dopamine signaling\textsuperscript{33–35}. Some of the neuropeptides expressed in AVK exhibit antagonistic effects on multiple aspects of locomotion. The most abundantly expressed neuropeptide in AVK, at the mRNA level, is FLP-1\textsuperscript{36}. Since \textit{flp-1} mutants are hyperactive, FLP-1 is supposed to suppress locomotion speed\textsuperscript{37,38}. In addition, \textit{flp-1} mutants show increased body bending\textsuperscript{29–31,37,38}. Bending angles correlate with locomotion strategy, thus altering bending angles can affect the directionality of locomotion, enabling the optimization of tactic behaviors\textsuperscript{29,30}. When overexpressed, NLP-49, the second-most abundant neuropeptide in AVK, enhances the increase of locomotion speed in response to mechanical stimuli\textsuperscript{39}. This suggests that FLP-1 and NLP-49 may exert antagonistic effects on locomotion speed, although the underlying mechanisms remain unclear.

Here, we demonstrate the antagonistic relationship between FLP-1 and NLP-10, two neuropeptides expressed in AVK. While FLP-1 released from AVK reduced locomotion speed, optogenetic depolarization of AVK unexpectedly accelerated locomotion. Through an AVK-specific RNAi-screen for neuropeptides, we found that NLP-10 mediates the increase of locomotion speed evoked by AVK-photoactivation. Furthermore, NLP-10 was required for the physiological escape response to mechanical stimuli and noxious blue light. Consistent with the functional antagonism, AVK-derived FLP-1 suppressed NLP-10 release from AVK through autocrine feedback. NLP-10 affected AVB and AIY interneurons, known to activate forward locomotion, via
the excitatory G protein-coupled receptor (GPCR) NPR-35. Our findings uncover a “wireless” microcircuit
governing locomotion speed and body posture, unpredicted by the anatomical connectome. Furthermore,
we demonstrate hierarchical regulation between functionally antagonistic neuropeptides secreted from the
same neuron.
Results

FLP-1 released from AVK neurons suppresses excitability in a modality-specific manner

*flp*-1 mutant animals are hyperactive. Although the FLP-1 neuropeptide is mainly produced in AVK, it appears to be synthesized and functional also in other neurons. Thus, the source of FLP-1 affecting locomotion speed remained unclear. To address this, we employed the Multi Worm Tracker (MWT, Figure 1A), a device allowing to monitor locomotion of *C. elegans*. In the presence of food bacteria, consistent with previous reports, *flp*-1 mutants displayed elevated basal locomotion speed compared to wild type animals, despite considerable variation during the accommodation phase (Figures 1B, S1A and S1B i, ii). In response to mechanical tapping stimuli on the culture dish, wild type animals exhibited a speed increase followed by gradual return to basal speed over the next 5 minutes. In contrast, *flp*-1 mutants showed an enhanced speed increase compared to wild type (Figures 1B and S1B i, iii). The body posture was also affected by the *flp*-1 mutation, as evidenced by increased body bending in *flp*-1 mutants (Figures S1Ai, iii and B iv, v). Upon tapping, wild type animals decreased body bending angles, while *flp*-1 mutants increased them (Figure 1B and S1B iv, vi). To assess locomotion directionality, we calculated ‘straightness’ (Figure S1A v and see STAR methods). After tapping, straightness was lower in *flp*-1 mutants, reflecting their “loopier” locomotion (Figure S1A vi-viii), which correlates with their increased bending angle, and can be recognized in the animal trajectories (Figure 1A). Most defects observed in *flp*-1 mutants were rescued by selectively expressing FLP-1 in AVK, using either the *twk-47* promoter or the truncated *flp*-1 promoter (Figures 1B and S1B). This suggests that FLP-1 released from AVK slows down basal locomotion, suppresses the speed increase upon tapping, decreases body bending and straightens locomotion direction. In a complementary experiment, we induced an AVK-specific knockdown of *flp*-1 by feeding dsRNA-producing bacteria (Figure 1C). To achieve AVK-specific RNA interference (RNAi), we employed animals expressing the RNA endonuclease RDE-1 and the RNA transmembrane transporter SID-1 in AVK of RNAi-deficient *rde*-1 mutants. Consistently, AVK-specific *flp*-1 knockdown enhanced the speed increase upon tapping. These results suggest that AVK-derived FLP-1 is required and sufficient to suppress (excessive) excitability, thereby mitigating hyperactivity.
To assess the general impact of FLP-1 on locomotion speed, we subjected animals to another type of acute sensory stimulation. Exposure to noxious blue light accelerates locomotion, a process primarily reliant on the light sensor protein LITE-1. Unlike the response to tapping, the flp-1 mutation did not enhance the speed increase upon blue light illumination (Figure S1C). This implies that FLP-1 modulates locomotion in a modality-dependent manner, rather than exerting a general slowing effect, which would be unfavorable in the case of escape behavior evoked by noxious stimuli.

Previously, the neuropeptide NLP-49 was implicated in the regulation of locomotion speed, since its overexpression in AVK enhanced the speed increase in response to mechanical stimulation. However, mutants carrying the nlp-49(gk546875) nonsense allele did not exhibit any obvious alteration in locomotion speed, prompting further consideration of whether NLP-49 genuinely regulates locomotion speed. We, therefore, created additional deletion alleles of nlp-49 and examined their effects on locomotion as well as responses to tapping and blue light. However, NLP-49 appeared not to exert an impact on locomotion speed during the tested scenarios, despite its abundant expression in AVK (Figure S2). Thus, we did not further investigate NLP-49.
Figure 1. AVK-derived FLP-1 suppresses excessive excitability

A. Multi-Worm Tracker (MWT) setup with blue and red illumination and tapping device. Representative trajectories obtained for wild type and flp-1 mutant animals are shown. Color code represents time as shown in B.

B. Locomotion of animals with the indicated genotype was tracked using MWT. NGM plates were tapped three times at 1 Hz, 900 seconds after recording began. ‘Body bending’ and ‘straightness’ were defined as described in Figure S1A. Basal speed (ii), body bending (v) and straightness (viii) indicate mean values from t = 840 to 899. ‘Speed increase’ (iii) and ‘Change in body bending’ (vi) indicate mean values from t = 911 to 960 subtracted by basal values. The mean straightness after tapping (t = 911~1020) was plotted in ix. Tukey test was performed. Note that tracks are temporally lost upon tapping, causing large variations and losses of data points.
C. Wild type N2 strain and animals for AVK-specific feeding RNAi strain (zxSi9[ptwk-47::rde-1:SL2:sid-1; rde-1(ne300)]) were fed with HT115 bacteria carrying a control vector or a plasmid encoding RNAi for flp-1. Animals were then subjected to behavioral analysis as described in A. Tukey test was performed. N indicates the numbers of independent experiments from different populations.

**AVK photoactivation accelerates directional locomotion, coordinated by AVK-derived FLP-1**

Previously, we demonstrated that acute manipulation of AVK activity off food, using Halorhodopsin (NpHR) or Channelrhodopsin-2 (ChR2), increased or decreased body bending, respectively, in line with the role of tonic FLP-1 release from AVK in reducing body bending angles. To probe the influence of AVK on locomotion speed without inducing photophobic behavior caused by blue light used for ChR2 activation, we selectively expressed the red-light activated ChR Chrimson in AVK using the twk-47 promoter (Figure S3A). In the absence of the ChR chromophore all-trans retinal (ATR), red light had no impact on locomotion (Figure S3B).

However, with ATR, despite AVK-derived FLP-1 slowing down locomotion (Figure 1), and despite far higher expression of flp-1 mRNA compared to other neuropeptide mRNAs in AVK, AVK-photoactivation slightly accelerated locomotion (Figure 2A i-iii), consistent with AVK activity correlating with locomotion speed. In flp-1(ok2811) mutants, the speed increase upon AVK-photoactivation was enhanced (Figure 2A i-iii). AVK photoactivation decreased the body bending angle in wild type but conversely increased it in flp-1 mutants, thus anti-correlating the straightness of locomotion (Figure 2A iv-vi and S3C). This is in accordance with the established role of FLP-1 in reducing body bending. Thus, AVK (photo)activation causes faster and straighter locomotion, that could facilitate efficient dispersal or escape behavior, while without FLP-1, signaling from AVK causes even faster but much less directional locomotion. The flp-1 mutation also enhanced speed increase of animals expressing ChR2(C128S), a ChR2 mutant activatable at lower light intensities, in AVK by the truncated flp-1(trc) promoter, affirming that FLP-1 suppresses the speed increase induced by AVK photoactivation (Figure S3D). Moreover, AVK-specific feeding RNAi for flp-1 enhanced the speed increase and reversed the change in body bending from decrement to increment (Figure 2B). In sum, these results indicate that a signal originating from AVK, distinct from FLP-1 and induced by cell depolarization, accelerates
Additionally, they highlight the role of FLP-1 released from AVK in decelerating and straightening locomotion. 

**Figure 2.** AVK photoactivation accelerates locomotion, which is suppressed by AVK-derived FLP-1

A. Wild type and *flop-1(ok2811)* mutant animals expressing Chrimson specifically in AVK were cultured on NGM plates supplemented with ATR and subjected to behavioral analysis with continuous red light illuminated from $t = 900$ to $1020$ to activate Chrimson. Basal values are the mean from $t = 780$ to $899$. ‘Speed increase’ (iii) and ‘Change in body bending’ (vi) indicate mean values from $t = 900$ to $1020$ subtracted by basal values. Welch test was performed.

B. Wild type and *flop-1* mutant RNAi strains expressing Chrimson in AVK were cultivated with HT115 bacteria carrying a control vector or a plasmid producing dsRNA for *flop-1* with ATR for 3 days and subjected to behavioral analysis. Dunnett test was performed against wild type animals fed with control HT115 bacteria.

**NLP-10 mediates the AVK-derived speed increase**

We asked whether chemical or electrical transmission from AVK may be responsible for the observed enhanced speed increase in response to photoactivation. Initially, we probed electrical transmission. AVK expresses gap junction (GJ) subunits such as UNC-7, INX-7 and INX-19, among others. However, RNAi knockdown of these GJ subunits, along with others, did not suppress the speed increase of *flop-1* mutants.
following AVK photoactivation (Figure S4A). Similarly, a dominant-negative variant of UNC-1, a stomatin protein required for function of GJs containing UNC-9\textsuperscript{52,53}, did not impede the speed increase (Figure S4B). Thus, GJs do not appear to be involved in the speed increase following AVK photoactivation.

Next, we examined the general role of chemical transmission. Tetanus toxin light chain (TeTx) blocks exocytosis both from synaptic vesicles and DCVs through cleavage of synaptobrevins\textsuperscript{54,55}. Expression of TeTx partially suppressed the increases in speed and body bending upon AVK photoactivation in \textit{flp}-1 mutants (Figures 3A and S5A), suggesting that exocytosis is at least partially responsible for the effect of AVK photoactivation. Although no small-molecule transmitter is known to be released by AVK\textsuperscript{27,28}, and most of the genes involved in biogenesis and transmission of acetylcholine (ACh), glutamate, GABA and monoamines are not expressed, trace amounts of \textit{eat}-4 and \textit{cat}-1, which encode vesicular transporters for glutamate and monoamines, respectively, might be expressed in AVK\textsuperscript{36}. However, RNAi for these did not suppress the speed increase (Figure S4C).

Hence, we investigated if a neuropeptide is responsible for the observed speed increase. To this end, 19 neuropeptide genes expressed in AVK besides FLP-1\textsuperscript{36} were knocked down by AVK-specific feeding RNAi. Among these, only the knockdown of \textit{nlp}-10 significantly attenuated the speed increase in \textit{flp}-1 mutant animals (Figures S4D and 3B). To further validate the role of NLP-10, we generated two \textit{nlp}-10 alleles, \textit{zx28} and \textit{zx29}, using CRISPR/Cas9-mediated genome editing. Consistent with the RNAi result, these alleles suppressed the speed increase upon AVK photoactivation in \textit{flp}-1(\textit{ok2811}) mutants (Figure 3C), solidifying the involvement of NLP-10. The extent of suppression was consistent with the nature of the mutations (Figure 3C): The \textit{zx28} allele might still produce one of four NLP-10 peptides (NLP-10-4), while the \textit{zx29} allele is a putative null (Figure S4E). The reduced straightness in \textit{flp}-1 mutants was suppressed by the \textit{nlp}-10 mutation (Figure S5F), suggesting that the directionality of the locomotion, which is accelerated by NLP-10, depends on FLP-1. The \textit{nlp}-10(\textit{tm6232}) allele, which deletes only the second exon, did not suppress the speed increase, likely due to the residual coding regions and intact splice sites allowing the generation of all four mature peptides (Figure S4E, F). In summary, our findings suggest that NLP-10 neuropeptides underlie the AVK-induced speed increase.
NLP-10 overexpression in AVK of wild type animals enhanced speed increase upon AVK photoactivation and reversed the effect of AVK photoactivation on body bending from negative to positive (Figures 3D iii and S5D iii). The expression and secretion of NLP-10 was confirmed by visualizing mScarlet fused to the NLP-10 prepropeptide in scavenger cells (coelomocytes) that endocytose and filter the body fluid (Figure S4G i)\(^{56}\). The flp-1 mutation increased body bending of animals expressing NLP-10 both before and during AVK photoactivation (Figure S5D i, iv), suggesting that FLP-1 has additional functions independent of NLP-10. Overexpression of NLP-10 in other neurons that natively express nlp-10, using the flp-21 and glr-1 promoters, did not induce an increase of locomotion speed or body bending upon AVK photoactivation (Figures 3E, S5E and S6A; we confirmed expression and secretion of NLP-10, see Figure S4G ii and iii). This suggests that systemically enriched NLP-10 is not sufficient to induce the speed increase upon AVK-photoactivation, emphasizing the necessity of spatially and/or temporally specific release of NLP-10 from AVK.
Figure 3.

A. Wild type and flp-1(ok2811) mutant animals, along with flp-1 mutants expressing either wild type or enzymatically inactive (E234Q) TeTx in AVK, all expressing Chrimson in AVK, were cultivated with ATR overnight and subjected to behavioral analysis with MWT under red light illumination. Dunnett test was performed against flp-1 mutants.

B. Wild type and flp-1(ok2811) mutant RNAi strains expressing Chrimson in AVK were cultivated with HT115 bacteria carrying a control vector or a plasmid producing dsRNA for nlp-10 in the presence of ATR for 3 days and subjected to behavioral analysis. Tukey test was performed.

C. Animals expressing Chrimson in AVK with indicated genotypes were analyzed. Tukey test was performed.

D. Wild type and flp-1(ok2811) mutant animals expressing Chrimson with or without AVK-specific NLP-10 overexpression were analyzed. Tukey test was performed.

E. Wild type and nlp-10(zx29) mutant animals, along with nlp-10 mutants expressing NLP-10 in indicated neurons, all expressing Chrimson in AVK, were analyzed. Dunnett test was performed against nlp-10 mutants.
AVK-derived FLP-1 reduces NLP-10 secretion

We investigated the mechanistic basis of how FLP-1 may suppress the NLP-10-mediated speed increase. As a premise, we confirmed that AVK excitability upon photoactivation was not increased in flp-1 mutants (Figure S7A). FLP-1 could suppress the speed increase either 1) through AVK-intrinsic effects on the expression or release of NLP-10; 2) through effects on downstream circuitry, e.g. having antagonistic effects on the same target neuron, or influencing different neurons with similar or antagonistic functions. To address whether FLP-1 affects secretion of NLP-10 from AVK, we quantified fluorescence of mScarlet co-secreted with NLP-10 and taken up by coelomocytes. The relative fluorescence in the coelomocytes over that in the AVK soma was largely decreased in unc-31(n1304) mutants, lacking the CAPS protein responsible for exocytosis from DCVs, as compared to wild type (Figure S7B). Thus, this method can also detect abnormalities in release of NLP-10.

In flp-1 mutants, the relative fluorescence in the coelomocytes over that in the AVK soma was increased compared to wild type (Figure 4A i). As the fluorescence in the AVK cell body was not significantly increased in flp-1 mutants, the rise in relative fluorescence is likely attributed not to an effect on NLP-10 protein level but to an increase of NLP-10 secretion from AVK (Figure 4A i). AVK-specific RNAi knockdown of flp-1 also increased NLP-10 release from AVK (Figure 4A ii). Taken together, although the possibility of FLP-1 effects on downstream circuits is not excluded, our results align with the idea that AVK-derived FLP-1 can suppress NLP-10 secretion from AVK.
Figure 4. AVK-derived FLP-1 suppresses NLP-10 secretion from AVK through autocrine feedback

A. (i) Fluorescence of mScarlet in L4 larvae of wild type and flp-1(ok2811) mutant animals expressing NLP-10::mScarlet in AVK was measured in AVK and in coelomocytes (CCs). The fluorescence intensity and ratio were plotted.

(ii) AVK-specific feeding RNAi strain expressing NLP-10::mScarlet in AVK was cultivated with HT115 bacteria carrying a control vector or a plasmid producing dsRNA for flp-1 for 3 days, and L4 larvae were analyzed as described in (A).
(iii) Wild type and dmsr-7(sy1539) mutant animals expressing NLP-10::mScarlet in AVK were analyzed.

B and C. Animals of indicated genotypes expressing Chrimson in AVK were cultivated with ATR and subjected to behavioral analysis. Tukey test was performed.

**The FLP-1 receptor DMSR-7 acts in AVK to mediate autocrine feedback**

How might FLP-1, released from AVK, suppress NLP-10 secretion from AVK? We addressed two hypotheses:

1) FLP-1 and NLP-10 compete for intracellular machinery during their biosynthesis or packaging into DCVs within AVK, or 2) FLP-1 secreted from AVK affects AVK itself by autocrine or circuitry feedback, thereby suppressing the release of NLP-10. Expression of truncated FLP-1 propeptides, comprising three or fewer of the N-terminal mature peptides, failed to rescue defects of the flp-1 mutants, although their secretion was confirmed by coelomocyte uptake of co-released mScarlet (Figure S7C). Thus, functional rescue seems to require more C-terminal FLP-1 peptides. Since the NLP-10 function was not suppressed even when excessive amounts of N-terminal FLP-1 species were expressed and released, it seems unlikely that FLP-1 suppresses NLP-10 release by competing for intracellular machinery.

We next addressed the possibility that AVK-derived FLP-1 affects AVK through autocrine feedback. To this end, there should be a receptor of FLP-1 that is expressed in AVK and relevant in this context. Various receptors for FLP-1 have been described *in vivo* and *in vitro*, such as NPR-6, FRPR-729, NPR-426,58, NPR-559, NPR-1160, NPR-22,61,62, DMSR-1, DMSR-5, DMSR-6 and DMSR-7. Among these, dmsr-6 and dmsr-7 are expressed in AVK neurons (Figure S6B)36. dmsr-7(sy1539) loss-of-function mutant animals exhibited an enhanced speed increase and increased body bending upon AVK photoactivation, similar to flp-1 mutants (Figure 4B). However, unlike flp-1 mutants, dmsr-7 mutants did not show an increase in basal speed or body bending, nor did they exhibit a prolonged speed increase after photostimulation, indicating that these effects of FLP-1 are possibly mediated by other receptor(s). flp-1; dmsr-7 double mutants did not display an additive defect compared to flp-1 single mutants. In addition, while the defects of flp-1 mutants were mostly rescued by FLP-1 expression in AVK, this rescue effect was largely compromised by loss of dmsr-7 (Figure 4B iii). The defects caused by the dmsr-7 mutation were rescued by expressing DMSR-7 specifically in AVK (Figure 4B iii).
The enhanced AVK-evoked speed increase of dmsr-7 mutants was suppressed by the nlp-10 mutation (Figure 4C), as was the case for flp-1 mutants (Figure 3C), suggesting that NLP-10 acts downstream of DMSR-7. The secretion of NLP-10 from AVK was increased in dmsr-7 mutants, similar to flp-1 mutants (Figure 4A iii), consistent with reported inhibitory effects of DMSR-7 through $G_{o/o}$-coupling$^{2,32,63}$. In sum, these results suggest that AVK-derived FLP-1 suppresses the AVK-evoked speed increase and promotes locomotion straightness by reducing body bending. This process involves suppressing NLP-10 secretion through autocrine feedback via DMSR-7 in AVK neurons.
Figure 5

A

i

Speed (mm/sec)

N = 3

ii

Basal speed

iii

Speed increase

npl-10
npr-35
flip-f

npl-10, flip-f

npl-10, npr-35, flip-f

wild type (zzix153)

zzix171

paw-47, flip-f

zzix171 [AVK, Cherry]

D

wild type (zzix153, zzix171)

npr-35

+ zziEx[paw-47; npr-35/]

+ zziEx[paw-47; npr-35/]

+ zziEx[paw-47; npr-35/]

E

i

Speed (mm/sec)

N = 3

ii

Basal speed

iii

Speed increase

npr-35

expression

AVB

AIV

ABG

BHG

AVD

AVB

etc

EVE

AVB

etc

AVC

AVB

etc

RM

etc

NPR-35

F

i

NPR-35

ND96

peptide

ND96

ii

2 µA

20 s

NLP-10-1

NLP-10-1 + BAPTA/AM

Glutamate

BAPTA-AM

G

AVK

FLP-1

DMSR-7

NLP-10

NLP-10

NLP-10

NLP-10

NPR-35

AVB

AVB

AVB

ABG

BHG

AVB

AVB

etc

etc

etc

etc

etc

Loopy locomotion

Locomotion speed

Body bending
Figure 5. NPR-35 mediates speed increase downstream of NLP-10

A-D. Animals of indicated genotypes expressing Chrimson in AVK were cultivated with ATR and subjected to behavioral analysis. Tukey test (A and B) or Dunnett test against npr-35 flp-1 double mutants (C) or npr-35 mutants (D) was performed.

E. flp-1(ok2811) animals expressing Chrimson in AVK and GCaMP6s in AIY were cultivated in the presence or absence of ATR and subjected to imaging analysis. Images were acquired at 1 frame per second (fps) while orange light (590 nm) was illuminated as indicated. Raw images were subjected to background subtraction and fluorescence intensity in AIY varicosities were quantified (i). (ii) Normalization between 0 to 1 was done by subtracting the minimum values followed by division by maximum values (after subtraction). (iii) Ratio of the average of the normalized intensity before (t = 26~30) and during (t = 31~45) illumination was plotted. p values were indicated (One-sample t-test).

F. (i) Two-electrode voltage-clamp (TEVC) recordings from untreated (black) and BAPTA-AM treated (red) Xenopus laevis oocytes expressing NPR-35 and perfused with 100 nM NLP-10-1 in ND96 bathing solution.

(ii) Oocytes expressing either NPR-35 or mGluR1a were or were not treated with BAPTA-AM and perfused with 100 nM NLP-10 peptides or 100 nM glutamate, respectively, as indicated. Activation current was calculated by subtracting baseline from the maximum current and plotted. Welch test was performed.

G. AVK-derived FLP-1 slows down locomotion speed and reduces body bending by suppressing NLP-10 release from AVK through autocrine feedback via the DMSR-7 receptor in AVK. NLP-10 accelerates locomotion speed by activating AVB and AIY, promoting forward locomotion. The directionality of the locomotion is dependent on the concurrent presence of FLP-1.

NPR-35 functions downstream of NLP-10 as its receptor

We previously identified NPR-35 as a receptor of NLP-10 in vitro. NPR-35 belongs to the neuropeptide FF (NPFF) and SIFamide receptor family, a conserved family across bilaterian animals, including vertebrate NPFF receptors and protostomian SIFamide receptors (Figure S8). Mammalian NPFF is involved in various behaviors such as nociception. Therefore, we examined the requirement of NPR-35 for the AVK-induced speed increase, aiming to support its role as a receptor for NLP-10 in vivo. Indeed, the speed increase was comparably suppressed by both the npr-35(ok3258) deletion and the nlp-10 mutation (Figure 5A i, iii).
Moreover, \textit{nlp-10; npr-35} double mutants did not exhibit an exacerbated phenotype, suggesting that NLP-10 and NPR-35 function in the same pathway as a ligand-receptor pair. The increased body bending in \textit{flp-1} mutants following AVK photoactivation was similarly suppressed by \textit{nlp-10} and \textit{npr-35} single mutations, as well as by double mutation of \textit{nlp-10} and \textit{npr-35} (Figure S9A i, iii). In addition, the loss of \textit{npr-35} completely suppressed the enhanced speed increase and the increased body bending caused by expressing NLP-10 in AVK (Figures 5B and S9B). The expression of NPR-35 from its own promoter rescued the compromised speed increase in \textit{npr-35; flp-1} double mutants compared to \textit{flp-1} mutants (Figure 5C). In conclusion, our findings strongly suggest that NPR-35 is indeed responsible for the increase of locomotion speed and body bending, serving as the sole receptor for NLP-10, at least in this specific context.

\textbf{NPR-35 excites interneurons that promote forward locomotion}

Which neurons may be the focus of action of NLP-10 through its receptor NPR-35? \textit{npr-35} is expressed in several neurons, including premotor interneurons that regulate forward or backward locomotion (Figure S6C)\textsuperscript{36}. Expression of NPR-35 in either AVB or AIY neurons, using the \textit{ttx-3}\textsuperscript{67} or \textit{lgc-55B}\textsuperscript{68} promoters, respectively, rescued the compromised speed increase in \textit{npr-35; flp-1} double mutants (Figure 5C). Furthermore, it rescued the abolished speed increase in \textit{npr-35} mutants overexpressing NLP-10 in AVK (Figure 5D). Considering that body bending decreased upon AVK photoactivation in \textit{npr-35} mutants expressing NPR-35 in AIY (Figure S9D, E), and that \textit{flp-1} mutants increase body bending upon AVK photoactivation, it suggests that the primary target of FLP-1’s inhibitory action might be the NLP-10-mediated transmission from AVK to AVB. Taken together, these findings suggest that NPR-35 functions in AVB and AIY to accelerate locomotion.

Given that AIY\textsuperscript{69} and AVB\textsuperscript{41,70} are known to be involved in the promotion of forward locomotion, it appears likely that NPR-35 activates these neurons in response to NLP-10. We thus recorded Ca\textsuperscript{2+} transients in AIY before and during AVK-photoactivation (Figure 5E). Though AIY was highly active before the stimulus, possibly as the immobilization startles sensory neurons innervating AIY\textsuperscript{71–77}, AVK photoactivation still significantly activated AIY in animals cultivated with ATR, but not without ATR. To further assess the
interaction between NPR-35 and NLP-10 and determine the G protein coupling specificity of NPR-35, we expressed NPR-35 in *Xenopus laevis* oocytes and activated it by administering NLP-10. If NPR-35 couples to G\(\alpha_q\), ligand binding should activate phospholipase C and the IP\(_3\) receptor, leading to Ca\(^{2+}\) mobilization from the ER. In oocytes, this evokes inward currents through an intrinsic Ca\(^{2+}\)-activated Cl\(^-\) channel\(^{78-80}\). Membrane currents were measured by means of two-electrode voltage-clamp (TEVC) recordings. NPR-35 strongly responded to each of the three synthetic mature NLP-10 peptides (NLP-10-1, 2 and 3) examined (Figure 5F).

Treatment with the cell-permeable Ca\(^{2+}\) chelator BAPTA-AM suppressed the currents evoked by NLP-10-1 in oocytes expressing NPR-35, as well as in oocytes expressing the known G\(\alpha_q\)-coupled mGluR1 receptor (Figure 5F), suggesting that NPR-35 is also G\(\alpha_q\)-coupled. In summary, NLP-10 released from AVK likely activates AIY and AVB through activation of the G\(\alpha_q\)-coupled GPCR NPR-35, promoting an increase in locomotion speed (Figure 5G).

**The NLP-10/NPR-35 signaling axis facilitates tap response and escape from noxious blue light exposure**

We discovered the role of NLP-10 and NPR-35 as locomotion accelerators during AVK photoactivation. To determine whether they are required for natural locomotion control, we initially addressed the tapping response, investigating whether NLP-10 antagonizes FLP-1 in a physiological context. The speed increase in response to tapping was reduced in *nlp-10* compared to wild type (Figure 6A). Similarly, *npr-35* mutants showed reduced responses, and *nlp-10; npr-35* double mutants did not show any additive effect compared to respective single mutants. These mutations also mitigated the enhanced response of *flp-1* mutants. Our findings suggest that during natural speed increase, NLP-10/NPR-35 signaling is required and antagonizes FLP-1 effects, mirroring the observations during AVK photoactivation.

The compromised speed increase observed in *nlp-10* mutants was rescued by expressing NLP-10 either in AVK using the *twk-47* promoter or in non-AVK neurons, some of which natively express NLP-10, using the *flip-21* promoter (Figure 6B). This outcome indicates that NLP-10 derived from multiple neurons contributes to the tap response, in contrast to our findings for AVK photoactivation (Figure 3E), posing the
question if FLP-1 derived from AVK can also suppress the release of NLP-10 from these other neurons. The impaired speed increase in npr-35 mutants was rescued by expressing NPR-35 from its own promoter, in AVB or in AIY (Figure 6C), suggesting that NLP-10 activates these interneurons to accelerate locomotion, as was the case for AVK photoactivation (Figure 5C, D).

**Figure 6**

A. Locomotion of animals of indicated genotypes was tracked with MWT while NGM plates were tapped. Tukey test was performed.
B. *nlp-10(*z29) mutants expressing NLP-10 in indicated neurons, along with wild type and *nlp-10* mutant animals, were analyzed as described in A. Dunnett test was performed against *nlp-10* mutants.

C. *npr-35(ok3258)* animals expressing NPR-35 in indicated neurons, along with wild type and *npr-35* mutant animals, were analyzed as described in A. Dunnett test was performed against *npr-35* mutants.

We next examined the involvement of NLP-10/NPR-35 signaling in escaping from noxious blue light. Wild type animals exposed to repeated satiating stimuli every 5 min robustly accelerated locomotion (Figure 7A). However, *nlp-10* mutants exhibited a slightly lower speed increase upon the first illumination (Figure 7A), with the difference between *nlp-10* mutants and wild type becoming progressively more pronounced with repeated illumination. Notably, the difference between the 4th and the 1st responses was significantly larger in *nlp-10* mutants. This is reminiscent of recent findings highlighting the role of neuropeptide signaling, involving FLP-14 and its receptor FRPR-19, in maintaining responsiveness to repeated nociceptive stimuli such as heat and harsh touch, or to optogenetic activation of the nociceptive neuron FLP. While the habituation to repeated blue light stimuli at 0.05 Hz or 0.4 Hz (i.e., every 20 sec or every 2.5 sec, respectively) was less pronounced compared to that following repeated mechanical stimuli or photoactivation of tap-responsive neurons at the same frequency (Figure S10), *nlp-10* mutants exhibited reduced responsiveness rather than enhanced habituation. Our results suggest that NLP-10 is required for a physiological response to blue light and the preservation of responsiveness when strong stimuli are repeated at low frequency. Of note, the blue light avoidance was unaffected by the *flp-1* mutation (Figures 6B and S1C), indicating that the FLP-1/NLP-10 antagonism may be inactive during nociceptive responses.

Since NLP-10 affected blue light avoidance behavior, we examined whether NPR-35 mediates this effect within the yet-to-be-defined signaling pathway governing the behavioral response to UV and blue light, initiated by the photoreceptor LITE-1. In accordance with our earlier results on AVK-evoked speed increase and tap response, *npr-35* mutants and *nlp-10; npr-35* double mutants exhibited reduced responses to blue light similar to *nlp-10* mutants (Figure 6B). The compromised speed increase in *nlp-10* mutants was rescued by expressing NLP-10, either in AVK using the twk-47 promoter or in non-AVK neurons, some of which natively
express NLP-10, using the *flp-21* or *glr-1* promoter (*Figure 7C*). This result suggests that NLP-10 derived from multiple neurons contributes to the response to blue light, as observed for the tap response. Cells other than AVK, potentially releasing NLP-10 in this context, are neurons expressing both LITE-1 and NLP-10. Two cells satisfying these criteria are PVT and AVG\(^{36}\), which might release NLP-10 in response to intracellular signaling following blue light-evoked excitation through LITE-1. Indeed, defective blue light responses in *lite-1* mutants were rescued by LITE-1 expression specifically in AVG, and furthermore, AVG photoactivation by Chrimson increased locomotion speed (*Figure 7D, E*). Finally, we probed where NPR-35 may act in this context. The defect in *npr-35* mutants was rescued by expressing NPR-35 from its own promoter, as well as in AVB or in AIY (*Figure 7F*). This suggests that NLP-10 activates these interneurons to accelerate locomotion, as observed for speed increase evoked by AVK photoactivation or by tapping (*Figures 5C, D and 6C*). In sum, these results suggest that during the physiological escape from noxious blue light, NLP-10 accelerates locomotion by activating AVB and AIY through NPR-35 (*Figure 7G*).
Figure 7. NLP-10/NPR-35 signaling facilitates escape from noxious blue light stimuli.
A. Wild type and nlp-10(zx29) animals were exposed to blue light (470 nm, 1.3 mW/mm2) for 30 seconds four times, as indicated, while their locomotion was monitored by MWT. (i) Locomotion speed was plotted over time (seconds). (ii) Speed during 1st (t = 301:330) to 4th (t = 1201:1230) illumination (v₁ to v₄) and (iii) the difference between the v₁ and v₄ were plotted. Despite expressing Chrimson in AVK, experiments were conducted in the absence of ATR, representing the response derived from innate mechanisms within the animals. Data points overlap with Figure 6B, 7A and 7C. In (ii), *** indicates p < 0.001 (Welch test), ## and ### indicate p < 0.01 and p < 0.001 (Dunnett test against v₁ of wild type) and $$$ indicates p < 0.001 (Dunnett test against v₁ of nlp-10). In (iii), * indicates p < 0.05 (Welch test), and ### indicates p < 0.001 (One-sample t-test).

B. Animals with indicated genotypes were analyzed as in A. Speed during v₄ was plotted. Tukey test was performed.

C. nlp-10(zx29) mutants expressing NLP-10 in indicated neurons, along with wild type and nlp-10 mutant animals, were analyzed as described in A. v₄ was plotted. Dunnett test was performed against nlp-10 mutants.

D. lite-1(ce314) mutants expressing LITE-1 under its own promoter or specifically in AVG, along with wild type and lite-1 mutant animals, all carrying juSi164, were exposed to continuous blue light for 30 seconds, as indicated.

E. Animals expressing Chrimson in AVG were cultivated with ATR, and exposed to red light, as indicated.

F. npr-35(ok3258) animals expressing NPR-35 in indicated neurons, along with wild type and npr-35 mutant animals, were analyzed as described in A. Dunnett test was performed against npr-35 mutants.

G. NLP-10 derived from AVK and other neurons contributes to tap- and blue light-evoked speed increase through NPR-35 on AVB and AIY. AVG, activated by blue light via LITE-1, might stimulate AVB through the co-transmission of NLP-10 and ACh in parallel to the AVK pathway. As some touch receptor neurons (TRNs) also express nlp-10, AVK-derived FLP-1 may selectively suppress speed increase upon gentle mechanical stimulation by reducing NLP-10 release from these specific neurons.
Discussion
Neuropeptides, serving as carriers of “wireless” communication, are released extrasynaptically, and enable signaling between cells that lack physical connections by synapses\(^1\). This study unveiled antagonistic effects of two neuropeptides, NLP-10 and FLP-1, originating from AVK interneurons, on locomotion speed and body posture. NLP-10 accelerated locomotion through the \(\text{G}_{\alpha_{q}}\)-coupled GPCR NPR-35 in AVB and AIY interneurons, promoting forward locomotion, with an additional impact on body bending, correlating with the straightness of locomotion direction. Despite the absence of anatomically defined chemical or electrical synapses between AVK and AVB/AIY\(^{33-35}\), our findings suggest direct communication mediated by NLP-10 and its receptor NPR-35. In addition, this pathway contributes to \textit{C. elegans} responses to mechanical and optical stimuli (elaborated below). Furthermore, AVK-derived FLP-1 decelerated but straightened locomotion by suppressing secretion of NLP-10 from AVK, through autocrine feedback mediated by the FLP-1 receptor DMSR-7 within AVK itself. This exemplifies an additional layer of complexity in neuropeptide signaling, wherein cascades of multiple neuropeptides expressed in the same neuron can reciprocally influence each other. These two neuropeptides, released from AVK and subject to hierarchical regulation of their antagonistic functions, coordinate both speed and directionality of locomotion, thereby enabling more efficient responses to environmental stimuli.

We presented evidence that AVK-derived FLP-1 suppresses NLP-10 secretion from the same neuron, proposing it to be the most probable mechanism. Nevertheless, there could be additional contributing mechanisms. First, FLP-1 might compete for the NPR-35 receptor with NLP-10. Yet, the evolutionary relationships of these peptide/GPCR systems suggest that they likely act in a cascade\(^2\). Second, since AVB also expresses DMSR-7, FLP-1 might directly affect AVB, alongside its primary effect via DMSR-7/NLP-10/NPR-35 signaling.

We suggest that FLP-1 could operate in a spatially specific manner, since the enhanced speed increase of \textit{flp-1} mutants was mitigated by FLP-1 expression in AVK but exacerbated by expression in AIY (Figure S6C). This spatial specificity could be due to FLP-1 signaling through autocrine feedback in AVK in this
context. In contrast to FLP-1, NLP-10 might act over longer distance, akin to some mammalian (neuro)peptides such as leptin, insulin and oxytocin\(^6,^{11}\). This inference is supported by the impaired response of \textit{nlp-10} mutants to tapping and blue light being rescued by NLP-10 expression either in AVK or in non-AVK neurons (\textbf{Figure 6B} and \textbf{7C}). Notably, the AVK-evoked speed increase was enhanced only by NLP-10 expression in AVK but not in non-AVK neurons (\textbf{Figure 3E}). Therefore, it appears that the precise temporal secretion of adequate amounts of NLP-10, rather than spatial specificity of the release site, triggers the observed speed increase. Considering that AVK-derived FLP-1 suppresses the response to tapping but not to blue light, and recognizing that NLP-10, derived from multiple neurons including AVK, could facilitate both responses, it is plausible that AVK-derived FLP-1 selectively inhibits the release of NLP-10 from neurons involved in the tapping response in addition to AVK.

The postulated difference in operational ranges of FLP-1 and NLP-10 aligns with FLP-1’s ability to activate a variety of receptors, contrasting with NLP-10’s specific activation of NPR-35\(^2\). Site-specific action is implied for several mammalian neuropeptides such as neuropeptide Y, dynorphin and somatostatin, which are expressed in various functionally distinct brain regions\(^6\). Consequently, these peptides are presumed to engage in local signaling, influencing target cells to yield context-dependent outcomes. Future work will explore whether FLP-1 is released from different regions of AVK neurons, whether the release sites of FLP-1 and NLP-10 are different, how far these neuropeptides can travel inside the animals’ body to reach different sets of receptors on diverse target cells, and whether these release sites and operation ranges vary in different contexts.

\textbf{NLP-10/NPR-35 signaling contributes to the nociceptive blue light avoidance}

\textit{C. elegans} exhibits aversion to blue light, primarily relying on LITE-1. Our results demonstrated the contribution of NLP-10/NPR-35 signaling to this process. \textit{lite}-1 is expressed in a small number of neurons and was shown to act in at least one head sensory neuron, ASK\(^{32}\). However, since posterior blue light illumination accelerates locomotion just like whole-body illumination\(^{46}\), and ASK lacks a posterior neurite, neurons with soma or neurite in the posterior body must contribute to the response. Among \textit{lite}-1 positive neurons, AVG
and PVT have neurites in the posterior body, and also express nlp-10, suggesting that they may relay blue light sensation to motor circuits and sustain responsiveness by releasing NLP-10. We found that LITE-1 expression in AVG rescued defects observed in lite-1 mutants, and that AVG photoactivation accelerated locomotion (Figure 7D, E). This collectively suggests that AVG is activated by blue light exposure, initiating signaling that leads to the avoidance behavior. AVG may directly activate AVB and the circuit for forward locomotion through co-transmission of ACh and NLP-10, in parallel to signals via AVK. Given the dense connectivity between PVT and AVK through gap junctions, PVT might activate AVK in response to blue light, in addition to releasing NLP-10. Future experiments will clarify the precise mechanisms by which blue light accelerates locomotion and how NLP-10 modifies this response.

Autocrine feedback enables functional interactions between neuropeptide co-transmitters

Autocrine signaling is extensively documented in various biological processes, playing crucial roles in cancer pathogenesis\(^{83}\), insulin secretion regulation\(^{84}\), etc., and is important in the nervous system as well, often as a positive feedback loop. Neurotrophins such as Brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF)\(^{85}\) provide essential survival cues to neurons. Autocrine positive feedback involving BDNF and its receptor TrkB is documented in both pre\(^{86}\) and post\(^{87}\) synaptic cells during synaptic plasticity and axon development\(^{88}\). In specific examples, Aplysia demonstrates an autocrine positive feedback loop involving a BDNF analog (ApNT), the neuropeptide sensorin\(^{89}\) and Transforming Growth Factor–β (TGF-β)\(^{90}\) in the presynaptic sensory neuron during synaptic plasticity consolidation. In Drosophila, an autocrine feedback loop by short neuropeptide F (sNPF) emerges in odorant receptor neurons (ORNs), particularly under starvation conditions, induced by the upregulation of its receptor\(^{91}\). Further, Oxytocin and vasopressin released from the soma and dendrites of magnocellular neurons in the hypothalamus engage in autocrine signaling through autoreceptors. This elicits retrograde feedback through endocannabinoids, inhibiting presynaptic GABAergic transmission to these neurons\(^{92,93}\).

In C. elegans, several cases of autocrine positive feedback by neuropeptides have been suggested, which may reinforce specific cellular states. For instance, the neuropeptide Pigment Dispersing Factor-2 (PDF-
2), originating from RIM interneurons, appears to form an autocrine feedback loop relevant to multisensory decision-making\(^4\). Similar autocrine excitatory feedback by PDF has also been documented in circadian clock neurons in \textit{Drosophila}\(^5\). Another example involves FLP-2 neuropeptides, derived from ASI sensory neurons, and the FRPR-18 receptor in ASI, suggesting the formation of an autocrine feedback loop that promotes locomotion during lethargus\(^6\). Last, FLP-14 neuropeptides released from \textit{glr-1}\(-\)expressing interneurons, known for promoting reversals, might form an autocrine loop onto those neurons through the FRPR-19 receptor, particularly after photoactivation of the nociceptive neuron FLP\(^81\).

In this study we uncovered, for the first time to our knowledge, a negative autocrine feedback loop mediated by neuropeptides. This mechanism potentially enables temporal integration of one signal before affecting another. Neuropeptide network maps predict many putative autocrine feedback motifs, consisting of neurons expressing both neuropeptides and their cognate receptors, not exclusive to \textit{C. elegans}\(^1\) but also in the rodent\(^9\) nervous system. Therefore, autocrine peptidergic signaling may facilitate mutual regulation between neuropeptide co-transmitters, optimizing brain function across species. Future work will define the mechanistic aspects of this distinct and specific signaling involving multiple neuropeptides.

\section*{Data Availability}
All strains and plasmids generated in this study are available upon request. Two supplementary figures, lists of plasmids and strains are provided.

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Author contributions
I.A. designed and performed experiments, wrote, and edited the manuscript, and supervised E.D., S.B. and E.B. L.G. designed and performed experiments and wrote the manuscript. E.D., S.P. and E.B. performed experiments. I.B. designed experiments, edited the manuscript, supervised, and secured funding. A.G. designed experiments, edited the manuscript, supervised, and secured funding.

Declaration of interest
The authors declare no competing interests.
Supporting information

S1 Fig (related to Figure 1)

A

i. N = 8

ii. Body bending (deg) from t = 30 to 600

iii. Body bending (t = 30–600)

iv. Straightness = \sqrt{\text{speed}^2 - \text{crab}^2}/\text{speed}

B

i. N = 0, 0, 4

ii. Basal speed

iii. Speed increase

iv. Change in body bending

C

i. N = 9

ii. Basal speed

iii. Speed increase

iv. Change in body bending

A. (i-iii) Representation of the whole recording duration (from 1 to 1200 sec, ii and iv) of data shown in Figure 1B. Mean body bending angles from t = 30 to 600 were plotted in iii. Tukey test was performed.
(iv) The MWT software saves an eleven-point spine along the center of every tracked animal and extracts ‘Body bending’.

(v) ‘Straightness’ is defined as the ratio of the vectorial speed component parallel to the orientation of the main body axis, relative to the overall speed (see STAR methods).

B. 60 animals of indicated genotype were transferred to a NGM plate with food one day before the experiment. Animals’ locomotion was tracked with MWT. NGM plates were tapped three times at 1 Hz, 900 seconds after recording was started. Basal speed (ii) and body bending (v) indicate mean values of each from t = 840 to 899. ‘Speed increase’ (iii) and ‘Change in body bending’ (vi) indicate mean values from t = 911 to 960 subtracted by basal values. Dunnett test was performed against flp-1 mutants.

C. Wild type or flp-1(ok2811) animals on NGM plates with bacterial food were subjected to behavioral analysis with MWT, while blue light was illuminated for 2 seconds at 900 seconds at 1.3 mW/mm². Basal speed (ii) and body bending (v) indicate mean values of each from t = 840 to 899. ‘Speed increase’ (iii) and ‘Change in body bending’ (vi) indicate mean values from t = 901 to 960 subtracted by basal values. Welch test was performed.
As the stop codon generated in the \textit{nlp-49(gk546875)} allele could potentially be read through\textsuperscript{98–100}, we generated distinct \textit{nlp-49} deletion alleles and characterized their locomotion (Figure S2A). Both \textit{nlp-49} deletion mutants and the \textit{gk546875} allele showed no remarkable defects in locomotion on food and exhibited normal speed increase upon tapping or in response to blue light illumination (Figure S2B and C). However, \textit{nlp-49} mutants displayed decreased body bending during locomotion off food, consistent with a previous report for \textit{nlp-49(gk546875)} mutants\textsuperscript{39} (Figure S2D).

These results confirm that \textit{nlp-49(gk546875)} is indeed a nonsense allele.

\begin{itemize}
  \item \textbf{A.} \textit{nlp-49} genomic locus. Positions of mutations were indicated.
  \item \textbf{B.} Animals of indicated genotypes on NGM plates with bacterial food were subjected to behavioral analysis with MWT, while mechanical stimuli were given by tapping plates three times at 1 Hz, 300 seconds after recording started, as
\end{itemize}
indicated by an arrow. (i) Locomotion speed along time, (ii) average basal locomotion speed before tapping \((t = 180 \sim 299, v_0)\) and (iii) speed increase after tapping \((\text{average speed } (t = 300 \sim 420) - v_0)\) were plotted.

C. Animals of indicated genotypes on NGM plates with bacterial food were subjected to behavioral analysis with MWT, while blue light was illuminated for 30 seconds at 300, 600, 900 and 1200 seconds. (i) Locomotion speed along time and (ii) average locomotion speed during the first blue light illumination \((t = 300 \sim 330, v_1)\) were plotted. Dunnett test was performed against wild type.

D. Animals of indicated genotypes were washed and transferred to NGM plates without bacterial food, and their locomotion was analyzed with MWT. (i) Locomotion speed and body bending angle along time, and average locomotion speed (ii) and body bending (iii) \((t = 1201 \sim 1800)\) were plotted. Dunnett test was performed against wild type.
S3 Fig (related to Figure 2)

A

1. Zi3153; zv/zs153; [mk8-47-Chimera]:P2A-GFP, pmec-4::NLS-GFP, pS64

B

i. Wildtype (zi3153) - ATR
ii. Wildtype (zi3153) + ATR
iii. Tp1 - ATR
iv. Tp1 + ATR

C

i. zi3153; [AVK-Chimera]
ii. Change in body bending (degree)
iii. Speed increase (mm/sec)
iv. Basal straightness
v. Basal body bending
vi. Basal speed

D

i. Wildtype (zi3153) - ATR
ii. Wildtype (zi3153) + ATR
iii. Tp1 - ATR
iv. Tp1 + ATR

E

i. Zi3296; flp-1; ok12811
ii. Zi354; [mk8-47-Chimera]; P2A::mCherry, pmec-4::NLS-GFP

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S3 Fig. (related to Figure 2)

A. (i) Green fluorescence of zxIs153[ptwk-47::Chrimson::P2A::GFP; pmec-4::NLS::GFP] strain was imaged.

(ii) Green and red fluorescence of flp-1(ok2811); zxIs164[ptwk-47::Chrimson::P2A::mCherry; pmec-4::NLS::GFP] strain was imaged.

B. Wild type and flp-1(ok2811) mutant animals expressing Chrimson specifically in AVK were cultivated on NGM plates with or without supplementation of ATR for 3 days and subjected to behavioral analysis with MWT with continuous red light illumination.

C. Close-up from 840 to 1140 sec of the data shown in Figure 2A (i and iv) for wild type (blue) and flp-1 mutants (orange), of locomotion speed (i), body bending (ii) and the straightness of locomotion direction (iii). Representative trajectories until 1020 sec are shown in iv, with color code representing time, as shown in i. Basal straightness (v) and the change in straightness (vi) were plotted, and a Welch test was performed.

D. Wild type and flp-1(ok2811) mutant animals expressing ChR2 in AVK by the flp-1(trc) promoter were cultivated on NGM plates with or without supplementation of ATR for 3 days and subjected to behavioral analysis with MWT with pulsed blue light illumination (0.08 mW, 250 ms, 1 Hz, 300 times).
Figure S4. (related to Figure 3)

A

N = 2

B

N = 1-3

C

N = 1

D

Relative Speed Increase

E

2000

nlp-10

tm6232

zx29 (#21, frame-shift, putatively null)

zx29 (#39, in-frame, NLP-10-4 could be intact)

NLP-10-1

NLP-10-2

NLP-10-3

NLP-10-4

sgRNA #1

sgRNA #6

zxlsf153[AVK-Chromoson]
xzlsf153 [nlp-10(TM6232)]
xzlsf153 [nlp-10(nlp10)]
xzlsf153 [nlp-10(nlp10) plp-1]
S4 Fig. NLP-10 is responsible for speed increase following AVK-photoactivation (related to Figure 3)

A. *flp-1(ok2811)* mutant animals expressing Chrimson alone or in combination with either wild type or dominant negative form (N494C) of UNC-1 specifically in AVK were cultivated with ATR and subjected to behavioral analysis with MWT.

B. Wild type and *flp-1(ok2811)* mutant of RNAi strain (zxSi9[ptwk-47::rde-1:SL2:sid-1]) expressing Chrimson in AVK were fed with HT115 bacteria expressing indicated dsRNA. Animals were then subjected to behavioral analysis with MWT.

C. *flp-1(ok2811)* mutant of RNAi strain (zxSi9[ptwk-47::rde-1:SL2:sid-1]; rde-1(ne300)) expressing Chrimson in AVK were fed with HT115 bacteria expressing indicated dsRNA.

D. *flp-1(ok2811)* mutant of RNAi strain (zxSi9[ptwk-47::rde-1:SL2:sid-1]; rde-1(ne300)) expressing Chrimson in AVK were fed with HT115 bacteria expressing dsRNA for indicated neuropeptides expressed in AVK. Animals were subjected to behavioral analysis with red illumination. Relative speed increase was calculated by subtracting speed increase of control animals analyzed on the same day from that of each species. two-tailed t-test was performed.

E. *nlp-10* genomic locus. Deletion sites in the respective mutant allele were indicated.

F-G. Animals expressing Chrimson in AVK with indicated genotypes were analyzed. Tukey test was performed (G).

H. Red fluorescence of indicated strains was imaged.
Figure S5. NLP-10 is responsible for acceleration, body bending (related to Figure 3)
A. Wild type and flp-1(ok2811) mutant animals, along with flp-1 mutants expressing either wild type or enzymatically inactive (E234Q) TeTx in AVK, all expressing Chrimson in AVK, were cultivated with ATR overnight and subjected to behavioral analysis with MWT under red light illumination. Dunnett test was performed against flp-1 mutants.

B. Wild type and flp-1(ok2811) mutant RNAi strains expressing Chrimson in AVK were cultivated with HT115 bacteria carrying a control vector or a plasmid producing dsRNA for nlp-10 in the presence of ATR for 3 days and subjected to behavioral analysis. Tukey test was performed.

C. Animals expressing Chrimson in AVK with indicated genotypes were analyzed. Tukey test was performed.

D. Wild type and flp-1(ok2811) mutant animals expressing Chrimson with or without AVK-specific NLP-10 overexpression were analyzed. Tukey test was performed.

E. Wild type and nlp-10(zx29) mutant animals, along with nlp-10 mutants expressing NLP-10 in indicated neurons, all expressing Chrimson in AVK, were analyzed. Dunnett test was performed against nlp-10 mutants.

F. To analyze straightness of animals of the indicated genotypes, datasets were extracted from experiments shown in Figures 3 to 5. Tukey test was performed.
S6 Fig. Expression pattern (related to Figure 3, 4 and 5)

Heatmaps illustrating gene expression patterns were generated using CenGENApp\(^{36}\) (https://cengen.shinyapps.io/CengenApp/). The heatmaps represent:

(A) Genes associated with \(nlp-10\) rescue experiments.

(B) FLP-1 receptors.

(C) Genes whose promoters were utilized in \(npr-35\) rescue experiments.
**Figure S7. FLP-1 suppresses NLP-10 release from AVK (related to Figure 4)**

A. Wild type and flp-1(ok2811) animals expressing GCaMP6s and Chrimson in AVK were cultivated with or without ATR and subjected to imaging at 1 frame per second (fps) with orange light (590 nm) illumination, as indicated. Row images were subjected to background subtraction, and fluorescence intensity in AVK soma were quantified. (i) Normalized
intensity was calculated by dividing raw intensity by the minimum value of each. (ii) Ratio of average of the normalized intensity before and during illumination was plotted. * indicates p < 0.05 (Tukey test).

B. Fluorescence of mScarlet in L4 larvae of wild type and *unc-31(n1304)* mutant animals expressing NLP-10::mScarlet (i) or FLP-1::mScarlet (ii) in AVK were measured in AVK and in anterior coelomocytes. The fluorescence intensity was plotted together with the ratio.

C. (i) The FLP-1 propeptide comprises multiple mature peptide species as numbered. A series of plasmids encoding truncated FLP-1 propeptide were generated for the rescue experiments in (ii).

(ii) Wild type and *flp-1(ok2811)* mutant animals, along with *flp-1* mutants expressing either full length or truncated FLP-1 propeptide, all expressing Chrimson in AVK, were cultivated with ATR and subjected to behavioral analysis with MWT. (ii) Representative image of an animal expressing truncated FLP-1 propeptide containing FLP-1-8, 2 and 3 was shown. The arrowhead indicates AVK, and arrows indicate coelomocytes.
Amino acid sequences of neuropeptide and monoamine receptors in *C. elegans*, along with homologs of NPR-35 in other species, were aligned using Clustal Omega. The resulting phylogenetic tree was generated and visualized with iTOL. NPR-35 and its homologs are highlighted in magenta and green, respectively.
Figure S9 (related to Figure 5)

A

- wild type (zzl153)
- nlp-10
- npr-35
- nlp-10 npr-35
- nlp-10 nlp-10
- nlp-10 nlp-10 nlp-10

B

- wild type (zzl153)
- zxl171
- nlp-10
- npr-35
- nlp-10 npr-35
- zxl171

C

- wild type (zzl153)
- nlp-10
- npr-35
- zxl171
- zxl153

D

- wild type (zzl153)
- nlp-10
- npr-35
- zxl171
- zxl153

E

- wild type (zzl153)
- nlp-10
- npr-35
- zxl171
- zxl153

S9 Fig. NPR-35 rescue (body bending related to Figure 5)

Animals of indicated genotypes expressing Chrimson in AVK were cultivated with ATR and subjected to behavioral analysis. Tukey test (A and B) or Dunnett test against npr-35 flip-1 double mutants (C) or npr-35 mutants (D and E) was performed.

Figure S10

Fig. 7

S10 Fig. Response to repetitive stimuli (related to Figure 7)
A. (i) Wild type N2 strain was exposed to blue light (470 nm, 1.3 mW/mm²) for 1 sec each, animals expressing Chrimson in tap-responsive neurons using mec-4 promoter were exposed to red light for 200 ms each, or wild type N2 strain was tap-stimulated, 30 times at 0.05 Hz as indicated while their locomotion was monitored by MWT. Locomotion speed was plotted.

(ii) Mean speed between stimuli (top) and the mean speed between stimuli normalized between 0 and 1 (bottom) for individual recordings were dot-plotted with different colors. Black open circles indicate mean values among different recordings.

B. CZ20310 strain carrying juSi164[mex-5p::HIs-72::miniSOG + Cbr-unc-119(+)] unc-119(ed3) was exposed to blue light (470 nm, 1.3 mW/mm²) for 500 ms each, or animals expressing Chrimson in tap-responsive neurons were exposed to red light for 200 ms each, 240 times at 0.4 Hz as indicated while their locomotion was monitored by MWT. Locomotion speed was plotted.

C and D. CZ20310 strain as wild type and nlp-10 mutants, both carrying juSi164, were illuminated as in A or B. The trace of wild type in D is the replot of the one in B.

S1 Table. Strain list.

S2 Table. Plasmid list.
STAR Methods

Experimental model and subject details

*C. elegans* strains were cultivated on nematode growth medium (NGM) plates seeded with *E. Coli* OP50-1 strain (Caenorhabditis Genetics Center (CGC), Twin Cities, MN, USA) as described\(^{101}\), unless otherwise described. N2 (Bristol) was used as the wild type strain unless otherwise indicated. Transgenic lines were generated by injecting plasmid DNA directly into hermaphrodite gonad as described\(^{102}\). Strains used in this study were listed in S1 Table.

Transgenes were integrated into genomes by optically activating Histone-miniSOG to mutagenize genomes as described previously\(^{103}\). Blue light was illuminated from Power-LED-Module MinoStar (2.37 W, 36 lm, 30 °C, Signal Construct GmbH, Niefern-Oeschelbronn, Germany) powered by LCM-40 constant current LED driver (Mean Well, New Taipei, Taiwan) and regulated at 3 Hz by Arduino Duemilanove (Arduino, Turin, Italy) and CMX100D10 solid state relay (Sensata technologies, Attleboro, MA, USA). Light intensity was 1.6 mW/mm\(^2\) during continuous illumination.

\(nlp-49(zx25), nlp-49(zx26), nlp-49(zx27), nlp-10(zx28), nlp-10(zx29)\) deletion alleles were generated by CRISPR/Cas9. sgRNA sequences were selected as described previously\(^{104,105}\) and using CHOPCHOP\(^{106}\). Expression of Cas9 was optimized as described\(^{107}\).

For AVK-specific feeding RNAi, \(zxSi9[Cbr unc-119(+); ptwk-47::rde-1:SL2:sid-1]; rde-1(ne300)\) strain, which expresses \(sid-1\) and \(rde-1\) in AVK of RNAi deficient \(rde-1(ne300)\) mutants\(^{108}\), was generated by Mos1-mediated Single Copy Insertion (MosSCI)\(^{109}\). Expression of Mos transposase was optimized as described\(^{107}\).

Defolliculated *Xenopus laevis* oocytes were purchased from EcoCyte Bioscience and maintained in ND96 solution (in mM: 96 NaCl, 1 MgCl\(_2\), 5 HEPES, 1.8 CaCl\(_2\), 2 KCl) at 4°C until RNA injection.

RNAi

Some HT115 bacteria clones expressing double strand RNA for target mRNA were recovered from the Ahringer library\(^{110}\), and the sequences were confirmed. For those genes not covered in the original version of the Ahringer library, coding regions of the genomic sequence were cloned into PstI-HindIII site of L4440.
vector, and the resulting plasmids were used for transformation of HCT15 cells. Used RNAi species were listed in Table S3.

HT115 bacteria were cultured and seeded on NGM plate containing 50 mg/ml ampicillin. For AVK-specific feeding RNAi, ZX3159 and ZX3154 strains were cultured on the seeded NGM plates for 3 days and subjected to behavioral or imaging analysis.

**Plasmids**

DN UNC-1 was a gift from Cori Bargmann.

pCFJ2474 pEXP[Psmu-2 | cas9(PATCs) | gpd-2 tagRFP-T(myr, patcs) smu-1 UTR] and pCFJ2475 pEXP[Psmu-2 | mosase(PATCs) | gpd-2 tagRFP-T(myr, patcs) smu-1 UTR] were gifts from Christian Froekjaer-Jensen (Addgene plasmid #159816 and #159813).

To express NPR-35 in Xenopus Laevis oocytes, the cDNA sequence of npr-35 was amplified by PCR using Q5 High-Fidelity DNA Polymerase (New England Biolabs (NEB), Ipswich, MA, USA) from cDNA of mixed-stage populations of wild-type C. elegans and inserted into the KSM vector, which contains Xenopus β-globin UTR regions and a T3 promoter, using HiFi assembly (NEB). Plasmid for mouse mGluR1 was prepared from mouse cDNA.

Other plasmids and primers used in this study are listed in S2 and S3 Table. Details regarding the plasmid constructs including sequences will be found in SnapGene files uploaded in FigShare.

**Behavioral assays**

L4 or adult animals were allowed to self-fertilize on NGM plates for 4 or 3 days at 25°C and subjected to behavioral analysis unless otherwise described. Animal locomotion was measured by Multi-Worm Tracker (MWT) equipped with a solenoid tapper and Dalsa Falcon 4M30 camera (Teledyne DALSA, Waterloo, Canada) with Optical Cast Plastic IR Longpass Filter (Edmund Optics, Barrington, NJ, USA) on the lens. An infrared back light was custom made with WEPIR3-S1 IR Power LED Star infrared (850nm) 3W (Winger Electronics GmbH & Co. KG, Dessau, Germany) powered by LCM-40 constant current LED driver and regulated
with a potentiometer (Vishay Intertechnology, Inc., Malvern, PA, USA). For optogenetics and stimuli, blue and red light was illuminated from LED modules ALUSTAR (3 W, 30°, 470 or 623 nm, 30 or 86.5 lm, respectively, Ledxon GmbH, Geisenhausen, Germany), powered by LCM-40 and regulated by Arduino Uno compatible board (Joy-IT, Neukirchen-Vluyn, Germany) with custom scripts.

The acquired data underwent analysis through the Choreography software, which is integrated with the MWT. This software yielded output values for ‘Speed’ and ‘body bending’. To compute ‘Straightness’, the following formula was applied, using a metric derived from the ‘crab’ values (representing speed perpendicular to body orientation) obtained from Choreography:

\[
\text{straightness} = \sqrt{\frac{\text{speed}^2 - \text{crab}^2}{\text{speed}}}
\]

The data was subsequently visualized using custom scripts in MATLAB (Mathworks, Natick, MA, USA). In timeseries plots, error bars represent the standard error of the mean (SEM).

Imaging analyses

Zeiss Observer Z1 (Carl Zeiss, Oberkochen, Germany) equipped with Kinetix22 (Teledyne Photometrics, Tucson, AZ, USA) and Prior Lumen LEDs (Prior Scientific, Cambridge, UK) was regulated with µManager. Illumination was synchronized for 2-color timelapse imaging with Z-stack by the Arduino Uno compatible board with AOTFcontroller script.

Coelomocyte (CC) uptake assay

Release of neuropeptides were analyzed by quantifying fluorescence of mScarlet fused to propeptide, co-released and taken up by coelomocytes basically as described before\(^5\). Briefly, L4 larvae of animals expressing mScarlet fused to FLP-1 or NLP-10 propeptides were mounted on slide glasses with 50 mg/ml tetramisole (Sigma-Aldrich, Burlington, MA, USA) and Polybead® Microspheres 1.00 µm (Polysciences, Warrington, PA, USA). Images were taken with Zeiss Observer Z1 with 10x objective with excitation light at
590 nm and were subjected to background subtraction and particle analysis with Fiji, an image processing package.

**Calcium imaging**

Animals expressing GCaMP6s were mounted on slide glasses with tetramizole and Polybead® Microspheres 1.00 µm. Images were taken with Zeiss Observer Z1 with 40x objective with excitation light at 460 nm with illumination of 590 nm light to activate Chrimson. Illumination pattern was generated by the Arduino Uno compatible board with a custom script. Images were subjected to background subtraction and quantification with Fiji.

**Two-electrode voltage clamp (TEVC) recording**

5′-capped cRNA was synthesized *in vitro* using the T3 mMessage mMachine transcription kit (Thermo Fischer Scientific, Waltham, MA, USA) using KSM plasmids linearised with NotI as templates. cRNA was finally purified with the GeneJET RNA purification kit (Thermo Fischer Scientific). *Xenopus* oocytes were placed individually into V-bottom 96 well plates and injected with RNA using the Roboinject system (Multi Channel Systems GmbH, Reutlingen, Germany). Each oocyte was injected with a total of 10 ng of the cRNA and incubated for 2 days in ND96 at 16°C until recording.

TEVC recordings were performed using the Robocyte2 recording system (Multi Channel Systems). Recording electrodes (Multichannel systems) normally had a resistance of 0.7–2 MΩ. The pipettes were filled with electrode solution prior to their use (1.5 M KCl and 1 M acetic acid). Some oocytes were incubated at 16°C for 3h in a solution of BAPTA-AM (Sigma-Aldrich) at 10 μM in ND96. Oocytes were then clamped at −80 mV, and current was continuously recorded at 500 Hz while flushing ND96 for 20s, the peptide solutions at 100 nM in ND96 for 30s and again ND96 to rinse.
Statistical Analysis

In the boxplots, the bottom and top of boxes represent the first and third quartiles, and the band inside the box represents the median. The ends of the lower and upper whiskers represent the lowest datum still within the 1.5 interquartile range (IQR), which is equal to the difference between the third and first quartiles, of the lower quartile, and the highest datum still within the 1.5 IQR of the upper quartile, respectively. Welch test was used for statistical test for two groups unless otherwise indicated. For multiple-comparison tests, one-way analyses of variance (ANOVAs) were performed, followed by Tukey-Kramer test or Dunnett test as indicated in each figure legend. Statistical tests were done by MATLAB. *, ** and *** indicate p < 0.05, p < 0.01 and p < 0.001, respectively.
Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 in order to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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