FLP-18 functions through the G-protein coupled receptors NPR-1 and NPR-4 to modulate reversal length in Caenorhabditis elegans

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Running title: FLP-18 regulates reversal length in *C. elegans*

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Abstract:
Animal behavior is critically dependent on the activity of neuropeptides. Reversals, one of the most conspicuous behaviors in *Caenorhabditis elegans* plays an important role in determining the navigation strategy of the animal. Our experiments on hermaphrodite *C. elegans* show the involvement of a neuropeptide, FLP-18 in modulating reversal length in these hermaphrodites. We show that FLP-18 controls the reversal length by regulating the activity of AVA interneurons through the G-protein coupled neuropeptide receptors, NPR-4 and NPR-1. We go on to show that the site of action of these receptors is the AVA interneuron for NPR-4 and the ASE sensory neurons for NPR-1. We further show that mutants in the neuropeptide, *flp-18*, and its receptors show increased reversal lengths. Consistent with the behavioral data, calcium levels in the AVA neuron of freely reversing *C. elegans* were significantly higher and persisted for longer durations in *flp-18, npr-1, npr-4* and *npr-1 npr-4* genetic backgrounds in comparison to wild-type control animals. Finally, we show that increasing FLP-18 levels through genetic and physiological manipulations causes shorter reversal lengths. Taken together, our analysis suggests that the FLP-18/NPR-1/NPR-4 signaling is a pivotal point in the regulation of reversal length under varied genetic and environmental conditions.

Significance Statement:
In this study we elucidate the circuit and molecular machinery required for normal reversal behavior in hermaphrodite *C. elegans*. We delineate the circuit and the neuropeptide receptors required for maintaining reversal length in *C. elegans*. Our work sheds light on the importance of a single neuropeptide, FLP-18, and how change in levels in this one peptide could allow the animal to change the length of its reversal, thereby modulating how the *C. elegans* explores its environment. We also go on to show that FLP-18 functions to maintain reversal length through the neuropeptide receptors NPR-4 and NPR-1. Our study will allow for a better understanding of the complete repertoire of behaviors shown by freely moving animals as they explore their environment.
Introduction: Neuropeptides are used by neurons to communicate with each other and to modulate behavior. They modulate synaptic activity through synaptic and extra-synaptic neuropeptide receptors. Neurons generally signal through only one neurotransmitter, but they can secrete multiple neuropeptides. One difficulty in studying neuropeptide function is that a single neuropeptide can signal through multiple receptors. (Li and Kim, 2008).

Locomotion is the basis for most behaviors in *C. elegans*, including feeding, mating, sleeping, avoidance, and escape behaviors. The animal’s movement is mediated by multiple sets of motor neurons along the ventral nerve cord innervating the dorsal and ventral body-wall muscles (White et al., 1976). Muscle contractions are mediated by cholinergic A and B-type motor neurons that innervate these muscles (Alfonso et al., 1993; White et al., 1986), while muscle relaxations are mediated by the GABAergic D-type motor neurons (McIntire et al., 1993a; McIntire et al., 1993b; White et al., 1986). The multiple sets of motor neurons that innervate the body-wall muscles receive synaptic inputs from interneurons, which are in turn stimulated by sensory neurons (Goodman, 2006). Together, these neurons coordinate the activity of the ventral and dorsal muscles to generate the sinusoidal waves for forward locomotion, interspersed by short bouts of backward movement or reversals.

Reversals are critical determinants of the *C. elegans* navigation strategy as they increase the probability of change in direction. Reversal behavior can be parsed into two components, reversal initiation, which determines the frequency of reversals and reversal termination, which determines the reversal length. Shorter reversals allow for change in directions at angles less than 90 degrees while longer reversals are almost
always followed by omega turns that lead to an approximately 180 degrees change in direction of locomotion. Laser ablation of the AVA command interneuron completely eliminated longer reversals but the animal could still initiate reversals, suggesting that reversal initiation (frequency) and regulation of reversal length might have a different cellular basis (Gray et al., 2005).

Although a number of studies have highlighted the neurons, molecules and circuits that are required for the reversal behavior in *C. elegans* (Alkema et al., 2005; Brockie et al., 2001; Campbell et al., 2016; Chalfie et al., 1985; Cohen et al., 2009; Gray et al., 2005; Hart et al., 1995; Kaplan and Horvitz, 1993; Maricq et al., 1995; Piggott et al., 2011; Zhao et al., 2003; Zheng et al., 1999), most of these studies were focused on the reversal frequency. The role of neurons in regulating reversal length, has to date been elaborated in a single previous study involving laser ablation of neurons (Gray et al., 2005). A very recent study has shown that the AVA interneurons control reversal state by affecting the motor neurons and that the P/Q type Calcium channel, UNC-2 is required for maintaining the duration of reversals through its affect on motor neuron oscillations. The authors go on to show that enhancing UNC-2 activity shows an increase in reversal duration and velocity (Gao et al., 2018). However, although the neurons required for maintaining reversal length and the role of UNC-2 in this process have been studied, the molecular mechanisms that regulate the length of reversals remain largely unknown.

Studies have shown that the neuropeptide, FLP-18 is required for modulating spontaneous reversal frequency and swimming rates in *C. elegans* (Chang et al., 2015; Cohen et al., 2009; Kim and Li, 2004). However, the effects of FLP-18 on reversal length have not been studied. We show that FLP-18 controls reversal length by regulating the function of AVA interneurons and ASE sensory neurons through the
G-protein coupled neuropeptide receptors NPR-4 and NPR-1 respectively. Our experiments suggest a possible correlation between increased reversal length and increased activity of the AVA neuron in flp-18, npr-1 and npr-4 genetic backgrounds in freely moving animals. We further show that FLP-18 expression is regulated by the transcription factor CREB1/CRH-1 as well as by starvation and that both loss of creb1/crh-1 and starvation cause decrease in reversal length through the FLP-18 pathway. This indicates that FLP-18 could act as a behavioral switch from local search to global search behavior under different genetic conditions as well as during starvation. Our work identifies the FLP-18/NPR-1/4 signaling pathway as a pivotal point in defining an aspect of C. elegans locomotion under varied environmental and genetic conditions.

**Materials and Methods**

**Strains**

All the strains used in this work were grown at 20°C under standard conditions using NGM plates. The N2 strain was used as the reference wild-type (WT) strain. The list of strains used in this study is listed in Table 3. All experiments were performed with hermaphrodite C. elegans.

**Molecular Biology and Transgenic Lines**

The promoters used for this study include flp-18 (4.3kb), rig-3 (3kb), unc-30 (3kb), gcy-5 (3kb), gcy-7 (1.2kb), gpa-3 (5.8kb) and unc-4 (2kb) all of which have been previously described (Eastman et al., 1999; Feinberg et al., 2008; Lickteig et al., 2001; Rogers et al., 2003). These promoters were cloned into pPD95.75 or pPD49.26 along with the cDNA of the gene of interest expressed downstream of the promoter. The transgenic lines were prepared by microinjecting plasmids of interest as described.
previously (Lickteig et al., 2001; Mello and Fire, 1995; Mello et al., 1991).
A list of all the primers used to make constructs and genotype the *C. elegans* in this
work is listed in Table 1 and the plasmids used in this work are indicated in Table 2.

**Behavioral Assays**

Well-fed young adult animals were used to conduct all the behavioral studies, except
for the starvation experiments. The reversal assays were performed after transferring
the *C. elegans* to food-free NGM plates. Observation time started 1 min after transfer.
The *C. elegans* were transferred using halocarbon oil or eyelash picks to avoid any
injury to the animal. Spontaneous reversals were scored for 5 min as previously
described (Zhao et al., 2003). The reversal length is reported throughout this work as
body bends per reversal. We defined one body bend as equal to the one third of the
length of the *C. elegans*. For all reversal assays a 5 minute video was made and the
number of body bends per reversal calculated for every reversal made during the five
minutes that was recorded, the number of body bends was then averaged over every
reversal made during the recording and was plotted as a single dot in the scatter plot.
More than 20 such recordings were analyzed for each genotype. The results were
plotted as mean +/- SEM and evaluated using the Standard Student’s *t*-test.

For the starvation assays, animals were starved for 24h at the L4 stage (54h including
hatching time) on peptone free plates (to avoid contamination). After 24h, *C. elegans*
were assayed for reversal behavior. Again reversal length was determined for each
spontaneous reversal.

**Optogenetic Assays**

To stimulate a specific neuron, ChR2 was utilized as previously described (Husson et
al., 2013). The *sra-6* promoter was used to drive ASH neuron specific expression of
ChR2 as previously reported in the SRS85 strain (*sraIs49 V; lite-1 (ce314) X;*
Animals expressing ChR2 were grown on NGM agar plates seeded with OP50 containing 400 μM All-trans retinal. The *C. elegans* were grown in the dark till late L4/early adult stages. The assay was performed on freshly seeded NGM plates. During the assay, ChR2 was excited by blue light (460-490nm) sourced from an epi-fluorescence unit (OLYMPUS U-HGLGPS) attached to the Nikon SMZ2000 microscope. This experiment was performed under low illumination to avoid the pre-activation of ChR2. Blue light was illuminated for 3 seconds and reversal length was quantified as number of body bends in response to illumination. This assay was done with more than 20 animals per genotype and each dot in the scatter plot indicates the number of body bends in a single reversal brought about by stimulation of ASH. The results were plotted as mean +/- SEM and evaluated using the Standard Student’s t-test. The experimenter was blind to the genotypes of the strains while performing these experiments.

**Calcium Imaging**

The genetically encoded Ca2+ indicator GCaMP expressing strain *Prig-3*::GCaMP5 was used to visualize Ca2+ transients in the AVA command interneuron (Larsch *et al.*, 2013). Calcium transients were recorded in freely navigating *C. elegans* as previously described (Faumont *et al.*, 2011) using an Olympus IX73 inverted microscope fitted with the ASI based worm-tracker. Imaging was done at 40X objective with 0.6 NA. Images were acquired through QImaging camera using the ImageJ software. Videos were recorded at 10 frame per second with 100 ms exposure. The AVA neuron activity was analyzed according to the protocol described in (Kerr, 2006). The analysis was done using the FIJI ImageJ software. The Region of interest (ROI) was drawn as a 25X25 pixel circle over the AVA cell body expressing GCaMP.
The measured average pixel value from the ROI, $F_{\text{meas}}$, includes fluorescence from sample and background fluorescence ($F_{\text{bkg}}$). Then the fluorescence ($F$) from the given ROI was estimated by subtracting background fluorescence from the measured fluorescence value i.e. $F = F_{\text{meas}} - F_{\text{bkg}}$. The fluorescence value was estimated for each frame after 100 ms by manual repositioning of the ROI. Calcium transients were plotted as $\Delta F/F_o$, where $\Delta F$ is the change in the fluorescence value ($F$) from its baseline fluorescence ($F_o$). Calcium levels were estimated as $\Delta F/F_o$ max, which indicated the maximum $\Delta F/F_o$ value for each animal. Calcium raising duration in AVA was calculated from the frame when the animal initiated the reversal to the end of the reversal. The Standard Student’s $t$-test was used to compare the $\Delta F/F_o$, duration of AVA and the calcium raising duration ($\Delta t$) values between the WT and mutant strains.

**Microscopy**

For all fluorescence confocal microscopy based experiments, except the experiments involving starvation assays, late L4/early adult *C. elegans* were imaged. The animals were paralyzed using 2,3-ButaneDione Monoxime BDM (30mg/ml) for imaging $pflp-18::FLP-18::sl2::GFP$ in the neurons of the head region (Cohen et al., 2009). Images were acquired using a Leica TCS SP8 confocal microscope. Imaging of starvation experiments, were done 24h following the L4 stage. The fluorescence intensity from the region of interest (ROI) was quantified as the integrated intensity using ImageJ. The data are expressed as mean±/SEM. To perform neuron specific imaging, the neuron of interest was identified by expressing the mCherry marker under a neuron-specific promoter. The $Pnmr-1::mCherry$ and $Pttx-3::mcherry$ promoters were used as markers to identify AVA, RIM and AIY. GFP fluorescence quantification of $Pflp-$
ImageJ. Imaging results were evaluated using Standard Student’s $t$-test.

**Statistical analysis**

The Student’s $t$-test (unpaired $t$ test with Welch’s correction) was performed using Prism-6 (Graph Pad). All the statistical values for unpaired $t$ test were provided as ($p, t, df$). The level of significance was set as $p<0.05$

**Table 1 - List of Primers used in this study**

| Primer Code | Sequence | Comment       | Gene   |
|-------------|----------|---------------|--------|
| AB113       | AGGACGGAAATTACCTGTGC | Genotyping Forward External | flp-18 |
| AB114       | GCTTCGGGAAACGCTCATAT  | Genotyping Reverse Internal | flp-18 |
| AB115       | TTATTCTTTCTTGCCGGCC   | Genotyping Reverse External | flp-18 |
| AB116       | ACCTGTCACTTTACGCCGG   | Genotyping Reverse External | npr-1  |
| AB117       | TGATTTCGTTCCAGTTGAACG | Genotyping Reverse Internal | npr-1  |
| AB118       | GAACCTCATTCTCTCTGTG   | Genotyping Reverse External | npr-1  |
| AB119       | AGCTGTGGTCTCCTCCAGG   | Genotyping Reverse External | npr-4  |
| AB120       | CGATTTCGATGAGGAAACC   | Genotyping Reverse Internal | npr-4  |
| AB121       | CACAGCTTCTAATAGGAAAGGG | Genotyping Reverse External | npr-4  |
| AB122       | GCACGACGAACTGCAAATTT  | Genotyping Reverse External | npr-5  |
| AB123       | TCCTTGAGTTTTCTGGAATG  | Genotyping Reverse Internal | npr-5  |
| AB124       | AGGCATTTTTGGAACGGGCG | Genotyping Reverse External | npr-5  |
| AB64        | ACGCGTCGACAATGCTGTCCCAGGGAATT   | Cloning Forward Sall | Punc-4 |
| Accession | Sequence | Cloning Site | Restriction Site | Type |
|-----------|----------|--------------|-----------------|------|
| AB65      | CCCCCGGGAAAGAAGAACCCACTTCGGCT | Cloning Reverse | XmaI | Punc-4 |
| AB108     | ACGCGTCGAC TCTGTCACATACTGCTCGAA | Cloning Forward | SalI | Pflp-18 |
| AB109     | CCCCCGGGGTTGCTGTCTAACCCTGAAA | Cloning Reverse | XmaI | Pflp-18 |
| AB134     | CTAGCTAGCATGAATGGCTCCGATTGTCT | Cloning Reverse | NheI | npr-4a cDNA |
| AB136     | CGGGGTACCTTAAAGAAGCCTTCCTTTGG | Cloning Reverse | KpnI | npr-1 cDNA |
| AB130     | CTAGCTAGCATGGAATGGCTCCGATTGTCT | Cloning Reverse | NheI | npr-1 cDNA |
| AB131     | CGGGGTACCTTAAAGAAGCCTTCCTTTGG | Cloning Reverse | NheI | npr-1 cDNA |
| AB139     | GCGTCGACAAGTGAACCCACGCTTCACA | Cloning Reverse | SalI | Prig-3 |
| AB140     | CCCCCGGGGAGCTGTGAAATTTTATTAGCAGT | Cloning Reverse | XmaI | Prig-3 |
| AB192     | ACATTCATGCGGATTCTAAACATTGAAACTTG | Cloning Reverse | NcoI | crh-1 |
| AB193     | CCCCCGGGGTTAAGGCGGGTGCTGTC | Cloning Reverse | XmaI | Pflp-5 |
| AB209     | ACATTCATGCAAAATTTTTCTTTTTAAGTTGTGTATCGG | Cloning Reverse | SphI | Pgp3-3 |
| AB210     | CCCCCGGGAAAGCACAACCTCTAAAAGGCCA | Cloning Reverse | XmaI | Pgp3-3 |
| AB216     | ACATTCATGCGGATTCTTATTACTTACTTCTGG | Cloning Reverse | SphI | Pgc5-5 |
| AB217     | CCCCCGGGGATTAAAATCTCTAATTCTCTTCTGGGG | Cloning Reverse | XmaI | Pgc5-5 |
| AB219     | ACATTCATGCAATAAAAAAGCAAAACACGCTAAGCAGA | Cloning Reverse | SphI | Pgc5-7 |
| AB220     | CCCCCGGGGATTATTATTATTATGCTAAACTGGCAGA | Cloning Reverse | XmaI | Pgc5-7 |
| YD169     | ATTAGCTAGCATGCGGATCTGGTATTCTTCATGG | Cloning Reverse | XmaI | crh-1f cDNA |
| YD164     | ATTACCATGGTCACATTCCGTCTTTTCCTTCT | Cloning Reverse | NcoI | crh-1f cDNA |
| YD157     | TGGAAAGGAGGAGGATGGAAA | Genotyping Forward | crh-1 |

**Legend:**
- Cloning Site: 5' and 3' cloning sites.
- Restriction Site: Restriction enzyme sites.
- Type: Type of site (XmaI, SalI, NheI, KpnI, SphI, NcoI, etc.).
- **Accession numbers** are provided for reference.
Table 2 - List of Plasmids used in this study

| S. no. | Plasmid No. | Plasmid |
|--------|-------------|---------|
| 1      | pBAB501     | P/flip-18::NPR-4a |
| 2      | pBAB504     | Prig-3::NPR-4a |
| 3      | pBAB503     | Punc-25::NPR-1 |
| 4      | pBAB502     | Punc-4::NPR-1 |
| 5      | pBAB719     | Prab-3::CRH1f |
| 6      | pBAB505     | P/flip-18::CRH1f |
| 7      | pBAB509     | Punc-30::NPR-1::sl2::wrmScarlet |
| 8      | pBAB510     | P/flip-5::NPR-1::sl2::wrmScarlet |
| 9      | pBAB511     | Ppga-3::NPR-1::sl2::wrmScarlet |
| 10     | pBAB512     | Pgcy-5::NPR-1::sl2::wrmScarlet |
| 11     | pBAB513     | Pgcy-7::NPR-4::sl2::wrmScarlet |
| 12     | pBAB513     | Pgcy-7::NPR-4::sl2::wrmScarlet |

Table 3 - List of Strains used in this study

| Strain  | Genotype | Comment | Figure/s |
|---------|----------|---------|----------|
| BAB1541 | flip-18 (CGC strain VC2016) | From CGC (outcrossed 3X) | 1, 1-1 and 4 |
| BAB1542 | npr-1 (CGC strain RB1330) | From CGC (outcrossed 3X) | 1 and 1-1 |
| BAB1543 | npr-4 (Mittani strain tm1782) | From NBRP (outcrossed 3X) | 1 and 1-1 |
| BAB1544 | npr-5 (CGC strain CX14394) | From CGC (outcrossed 3X) | 1-1 |
| BAB1501 | npr-1 npr-4 | | 1, 1-1 and 4 |
| BAB1502 | flip-18 npr-1 npr-4 | | 1 and 1-1 |
| BAB765  | crh-1 (CGC strain YT17) | From CGC (outcrossed 3X) | 3 |
| BAB727  | crh-1; Prab-3::CRH-1f (indEx719) | | 3 |
| BAB1503 | flip-18; Pflip-18::FLP-18::sl2::GFP | | 3 |
| BAB1504 | npr-1 npr-4; Pflip-18::NPR-4a (indEx501) | | 1 |
| BAB1505 | npr-1 npr-4; Prig-3::NPR-4a (indEx504) | | 1 |
| BAB1506 | npr-1 npr-4; Punc-4::NPR-1 (indEx502) | | 1 |
| BAB1517 | npr-1 npr-4; Prra-6::CHOP-2(H134R)::mCherry + Psm-10::G-CaMP + Punc-122::mCherry; Prig-3::NPR4a (indEx504) | npr-4 rescue in AVA | 1 |
| AX1444  | Pflip-18::FLP-18::sl2::GFP | Reference (Cohen et al., 2009) | 3, 4 |
| BAB1509 | crh-1; Pflip-18::FLP-18::sl2::GFP | | 3 |
| BAB1510 | crh-1; Pflip-18::FLP-18::sl2::GFP; Prab-1 | | 3 |
| BAB1511 | crh-1; Pflp-18::FLP-18::sl2::GFP; Pflp-18::CRH-1f (indEx505) | 3 |
| BAB1512 | Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry | Reference (Guo et al., 2009) | 1, 3 |
| BAB1513 | fpl-18; Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry | 1 |
| BAB1514 | crh-1; Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry | 3 |
| BAB1515 | npr-1 npr-4; Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry | 1 |
| BAB1516 | fpl-18 npr-1 npr-4; Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry | 1 |
| BAB1519 | crh-1; Psra-6::CHOP-2(H134R)::mCherry + Posm-10::G-CaMP + Punc-122::mCherry; Pflp-18::CRH-1f (indEx505) | crh-1 rescue in FLP-18 expressing neurons | 3 |
| BAB1523 | fpl-18; Prig-3::GCaMP5 | 2 |
| BAB1525 | npr-4; Prig-3::GCaMP5 | 2 |
| BAB1526 | npr-1 npr-4; Prig-3::GCaMP5 | 2 |
| BAB1527 | crh-1; fpl-18 | 3 |
| BAB1528 | npr-1 npr-4; Punc-30::NPR1::sl2::wrmScarlet | 1 and 1-1 |
| BAB1529 | Ppca-3::NPR1::sl2::wrmScarlet; Punc122::GFP | 1 and 1-1 |
| BAB1530 | npr-1 npr-4; Ppca-3::NPR1::sl2::wrmScarlet; Punc122::GFP | 1 and 1-1 |
| BAB1531 | npr-1 npr-4; Ppca-3::NPR1::sl2::wrmScarlet; Punc122::GFP | 1 and 1-1 |
| BAB1532 | npr-1 npr-4; Ppca-3::NPR1::sl2::wrmScarlet; Punc122::GFP | 1 and 1-1 |
| BAB1533 | npr-1 npr-4; Ppca-3::NPR1::sl2::wrmScarlet; Ppca-3::NPR1::sl2::wrmScarlet; Punc122::GFP | 1 |
| BAB1534 | npr-1 npr-4; Prig-3::NPR4::sl2::wrmScarlet | 1 and 1-1 |
| BAB1535 | npr-1 npr-4; Prig-3::GCaMP5; Prig-3::NPR4::sl2::wrmScarlet | 2 |
| BAB1536 | npr-1 npr-4; Prig-3::GCaMP5; Ppca-3::NPR1::sl2::wrmScarlet; Ppca-3::NPR1::sl2::wrmScarlet | 2 |
| BAB1538 | crh-1; Pflp-18::FLP-18::sl2::GFP; Pflp-18::CRH-1f; Pnmr-1::mCherry; Pttx-3::mCherry | 2 |
| BAB1539 | Pflp-18::FLP-18::sl2::GFP; Pnmr-1::mCherry; Pttx-3::mCherry | 2 |
| BAB1540 | crh-1; Pflp-18::FLP-18::sl2::GFP; Pnmr-1::mCherry; Pttx-3::mCherry | 2 |
Results:

FLP-18 functions to control reversal length through the NPR-4 receptor in AVA interneurons and the NPR-1 receptor in ASE sensory neurons

A neuropeptide of the FMRF-amide like family, FLP-18 has been implicated in multiple behaviors in *C. elegans* (Chang *et al.*, 2015; Cohen *et al.*, 2009; Kim and Li, 2004). FLP-18 and its receptors, have been reported to be expressed in the neural circuitry involved in backward locomotion, and specifically in the AVA command interneuron (Kim and Li, 2004; Li and Kim, 2014; Rogers *et al.*, 2003).

We were interested in further understanding the role of FLP-18 in the modulation of the exploratory behavior of *C. elegans*. Similar to what was previously published by Cohen *et al.* we found that *flp-18* mutants showed decreased reversal frequency in comparison to wild-type (WT) animals (*p*=0.0052, *t*=2.981, *df*=34.8) ((Cohen *et al.*, 2009) and Figure 1-1A). We also observed longer reversals i.e. increased body bends per reversal in *flp-18* null mutants, in comparison to WT control animals (*p*<0.0001, *t*=17.36, *df*=26.45), Figure 1A) and

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XZxtXbquvzXR and

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QgO2JT5zHICGBa). This observation allowed us to postulate that FLP-18 could be modulating the reversal circuitry required to regulate the length of reversals. Further, expressing FLP-18::*sl2::*GFP under its own promoter partially rescued the increased reverse body bends seen in the mutants (*p*=0.0075, *t*=2.963, *df*=20.71), Figure 1A). Previous reports have indicated that the FLP-18::*sl2::*GFP line shows abnormal movement behaviors (Cohen *et al.*, 2009), this could account for the partial rescue of the *flp-18* mutant phenotype (*p*<0.0001, *t*=5.367, *df*=27.01). In order to affirm that *flp-
does not show obvious movement defects, we compared the frequency of forward body bends made by the \textit{flp-18} mutants to WT control animals and found this frequency to be similar to WT levels \((p=0.413, t=0.832, df=26.29)\), Figure 1-1B). We next wanted to delineate the receptors through which FLP-18 could be functioning to mediate reversal length. Previous work has shown that FLP-18 has high binding affinity for the receptors NPR-1, NPR-4, NPR-5, NPR-10 and NPR-11 (Li and Kim, 2014). AVA ablation eliminates longer reversals in both off and on food, therefore we reasoned that FLP-18 was likely modulating reversal length at the interneuron level (Gray \textit{et al.}, 2005). Sensory neurons give cues to activate the command interneuron AVA, which causes activation of reversals (Gordus \textit{et al.}, 2015; Piggott \textit{et al.}, 2011; Pirri and Alkema, 2012). We hypothesized that FLP-18 could be functioning through the command interneuron AVA or the upstream sensory neurons to regulate reversal length. The expression pattern of NPR-10 has not been well documented, however NPR-1 is expressed in sensory and motor neurons and NPR-4 is largely expressed in the AVA command interneurons while NPR-5 and NPR-11 are expressed mainly in sensory neurons (Chalasani \textit{et al.}, 2010; Cohen \textit{et al.}, 2009; de Bono and Bargmann, 1998; Wang and Wadsworth, 2002). Furthermore, FLP-18 has been shown to function through NPR-1 or NPR-4 receptors for multiple behavioral processes (Choi \textit{et al.}, 2013; Cohen \textit{et al.}, 2009; Frooninckx \textit{et al.}, 2012; Luo \textit{et al.}, 2015; Nagy \textit{et al.}, 2014; Rogers \textit{et al.}, 2003; Stawicki \textit{et al.}, 2013). We wanted to test if FLP-18 controls reversal length through the NPR-1 and NPR-4 receptors. We started with observing the reversal length in the \textit{npr-1} and \textit{npr-4} mutants. Both the mutants showed significant increase in body bends per reversal when compared to WT animals (WT and \textit{npr-1} \(p=0.0004, t=4.257, df=19.44\), WT and \textit{npr-4} \((p=0.0001, t=4.766, df=19.32)\), Figure 1A). We found that there was a
significant increase in the number of body bends per reversal in the npr-1 npr-4 double mutants when compared to either of the single mutants (npr-1 and npr-1 npr-4 ($p=0.0001$, $t=4.283$, $df=34.32$), npr-4 and npr-1 npr-4 ($p=0.0005$, $t=3.867$, $df=34.48$)), further the double mutants showed a phenotype that was very similar to the flp-18 mutants ($p=0.0982$, $t=1.706$, $df=30.41$), Figure 1A and https://www.amazon.com/clouddrive/share/CbcUUwOTQhF3btDNGG7112V8POJeGyWpRRDALaZXIn2). We also tested the body bends per reversal in npr-5 mutant animals to understand the role of other FLP-18 receptors in this process. We did not see an obvious difference in reversal lengths in these mutants in comparison to WT animals ($p=0.417$, $t=0.829$, $df=19.98$), Figure 1-1C).

We next wanted to identify the site of action of NPR-1 and NPR-4 receptors that would allow them to transduce the signal from FLP-18 to control reversal length. The command interneurons AVA, AVE and AVD are required to promote reversal behavior in C. elegans (Chalfie et al., 1985; de Bono and Maricq, 2005; Gray et al., 2005; Piggott et al., 2011). Of these command interneurons, FLP-18 is expressed in AVA (Kim and Li, 2004; Rogers et al., 2003), which also expresses the receptor NPR-4 (Cohen et al., 2009). We thought that it was possible that NPR-4 could function in the AVA interneuron to transduce the signal through FLP-18. To test this, we performed transgenic rescue experiments for NPR-4 under the flp-18 promoter in the npr-1 npr-4 double mutants. We found that this rescued the npr-4 phenotype in the npr-1 npr-4 double mutants ($p<0.0001$, $t=8.398$, $df=27.49$), Figure 1A). We further found that the rescued animals showed a reversal length very similar to that of WT animals ($p=0.5856$, $t=0.5513$, $df=19.98$). Since FLP-18 is expressed in multiple neurons including AVA, RIM, AIY, RIG, the pharyngeal neurons M2 and M3 and the ventral cord neurons (Kim and Li, 2004; McKay et al., 2003; Rogers et al., 2003), it
is possible that rescue of NPR-4 with this promoter completely rescues all aspects of
the *npr-1* *npr-4* double mutant phenotype. In order to delineate a more neuron specific
role of NPR-4, we went on to do a neuron specific rescue of NPR-4 in the AVA
neuron. We expressed NPR-4 under the *rig-3* promoter using *Prig-3::NPR-4:sl2::wrmScarlet*, which is expressed in the AVA command interneuron ((Schwarz
*et al.*, 2009) and Figure 1-1D), and found that this promoter could again rescue the
*npr-4* mutant phenotype in the *npr-1* *npr-4* double mutants (*p*<0.0001, *t*=5.723,
*df*=30.61), Figure 1A). The rescue of NPR-4 in the *npr-1* *npr-4* double mutants
showed a phenotype that was indistinguishable from the *npr-1* mutant animals
(*p*=0.2542, *t*=1.162, *df*=30.60), which is what would be expected if only NPR-4 is
rescued in the *npr-1* *npr-4* double mutant animals. These results indicate that FLP-18
could be partly functioning to maintain normal reversal body bends through the NPR-4 receptor acting in the AVA interneuron, which is one of the neurons that express
FLP-18.

We also performed rescue experiments to find out where NPR-1 functions to
modulate reversal length. The NPR-1 receptor has a strong expression in head sensory
neurons as well as in the GABAergic D-type motor neurons, and shows a weak
eexpression in the cholinergic A-type motor neurons (de Bono and Bargmann, 1998;
Wang and Wadsworth, 2002). We first decided to rescue NPR-1 in D-type motor
neurons using the *unc-30* promoter (McIntire et al., 1993a), and in the A-type motor
neurons using the *unc-4* promoter (Miller and Niemeyer, 1995). We found that the
expression of NPR-1 in both D-type (*p*=0.7909 *t*=0.2675, *df*=30.03) and A-type
(*p*=0.4462 *t*=0.7722, *df*=29.04) motor neurons could not rescue the increased reversal
length phenotype seen in the *npr-1* *npr-4* double mutants. We next tested NPR-1
functions in sensory neurons using *gpa-3* and *flp-5* promoters. Again we tested
expression of NPR-1 under these promoters with sl2::wrmScarlet (Figure 1-1D). The 
gpa-3 promoter is expressed in many sensory neurons including NPR-1 expressing 
neurons ASE, ASG, ASH, PHA and PHB while the flp-5 promoter is largely 
expressed in the ray sensory neurons and the NPR-1 expressing neurons ASE and 
RMG. Expressing NPR-1 under both gpa-3 and flp-5 promoters rescued the increased 
reversal length phenotype seen in the npr-1 npr-4 double mutants ((npr-1 npr-4 and 
npr-1 npr-4), Pgpa-3::NPR-1 (p<0.0001, t=9.447, df=22.73), npr-1 npr-4 and npr-1 
npr-4; Pfpl-5::NPR-1 (p<0.0001, t=5.944, df=35.80), Fig 1B). This rescue confirmed 
that NPR-1 could function in sensory neurons and further indicated that NPR-1 could 
be modulating reversal length through the ASE sensory neurons, which are the only 
neurons where NPR-1 is expressed under both gpa-3 and flp-5 promoters. The ASE 
neurons have been shown to be involved in CO2 sensing, chemotaxis to water-soluble 
compounds and avoidance behaviors in C. elegans (Bargmann and Horvitz, 1991; 
Bretscher et al., 2011; Pierce-Shimomura et al., 2001; Sambongi et al., 1999; Suzuki 
et al., 2008). To delineate a more neuron specific role for NPR-1, we expressed NPR-
1 in ASEL using the gcy-7 promoter, ASER using the gcy-5 promoter and in both 
ASEL/R using both promoters (Yu et al., 1997). Expressing NPR-1 in either ASEL or 
ASER and in both ASEL/R significantly rescued the increased reversal length of npr-
1 npr-4 double mutant ((npr-1 npr-4 and npr-1 npr-4; Pgcy-7::NPR-1 (p<0.0001, 
t=4.868, df=29.22), npr-1 npr-4 and npr-1 npr-4; Pgcy-5::NPR-1 (p<0.0001, t=5.853, 
df=33.96), npr-1 npr-4 and npr-1 npr-4; Pgcy-7::NPR-1; Pgcy-5::NPR-1 (p<0.0001, 
t=6.631, df=28.74), Figure 1B and 1-1D). The rescued npr-1 npr-4 double mutants 
showed reversal lengths similar to that seen in the npr-4 single mutant animals when 
NPR-1 was expressed only in ASEL. The reversal length was similar to WT animals 
when NPR-1 was expressed only in ASER or in ASER/L ((WT and npr-1 npr-4;
Pgcy-5::NPR-1 (p=0.0538, t=2.051, df=19.71), WT and npr-1 npr-4; Pgcy-7::NPR-1; Pgcy-5::NPR-1 (p=0.017, t=2.557, df=24.85), Figure 1B). Taken together, these results suggest that NPR-4 and NPR-1 act as receptors for FLP-18 in the AVA command interneuron and the ASE sensory neurons respectively to maintain reversal length.

Since FLP-18 appears to be functioning through NPR-1 and NPR-4, we went on to make a triple mutant of flp-18, npr-1 and npr-4. These mutants also showed longer reversals than WT animals (p=0.027, t=2.405, df=18.23), however the reversals were not as long as those seen in flp-18 single mutants or npr-1 npr-4 double mutants (Figure 1-1E and https://www.amazon.com/clouddrive/share/aPkQBaOufimeJUp3Acf3KkTVctaJnM3qj ST4O00WPDLr). On analyzing these mutants further we realized that their mobility was compromised, probably due to the presence of three mutations, which are involved in multiple processes other than maintaining reversal lengths (Choi et al., 2013; Cohen et al., 2009; Luo et al., 2015; Nagy et al., 2014; Rogers et al., 2003; Stawicki et al., 2013).

In order to get a cleaner readout of the reverse body bends in the triple mutants, we decided to combine optogenetics with behavioral studies. We used a transgenic line expressing Channelrhodopsin-2 (ChR2) specifically in the ASH sensory neurons using the sra-6 promoter. We induced reversals optogenetically by stimulating the ASH neuron, which forms functional connections with the AVA interneuron (Guo et al., 2009; Lindsay et al., 2011). We noted that ASH stimulation caused WT animals to undergo reversals (Illustrated in Figure 1C), even though the optogenetically activated neural circuit is different from the neural circuitry that activates reversals during exploratory behavior in C. elegans (Gray et al., 2005; Pirri and Alkema, 2012).
ChR2 mediated stimulation of the ASH neurons in flp-18 mutants resulted in a significant increase in the number of body bends per reversal when compared to WT control animals (\(p < 0.0001, t = 4.739, df = 32.95\), Figure 1D). A similar number of increased reversal body bends upon ChR2 activation were seen in npr-1 npr-4 double mutants (\(p = 0.0003, t = 4.222, df = 25.48\)), which were significantly rescued by expressing NPR-4 in the AVA command interneuron (\(p = 0.0003, t = 4.132, df = 27.82\)). Interestingly, expressing NPR-4 in just the AVA command interneurons could completely rescue the double mutant phenotype, indicating that overexpressing NPR-4 in the double mutant was enough to reduce reversal length to WT levels (\(p = 0.629, t = 0.49, df = 25.21\)) in optogenetically induced reversals. Further, we found that the flp-18 npr-1 npr-4 triple mutants showed a number of body bends per reversal that was similar to that seen in flp-18 mutants (\(p = 0.078, t = 1.824, df = 30.31\)) or in npr-1 npr-4 double mutants (\(p = 0.166, t = 1.432, df = 23.26\), Figure 1D). These data together indicated that FLP-18 functions through the G-protein coupled receptors NPR-1 and NPR-4 to control reversal length.

NPR-1 and the Brugia malayi homolog of NPR-4 have been shown to have inhibitory functions through FLP-18 (Anderson et al., 2014; Frooninckx et al., 2012). Hence, it is likely that FLP-18 functions through the NPR-4 and NPR-1 receptors and allows for modulation of the AVA and ASE neurons. Taken together, these results indicate that FLP-18 could modulate the activity of AVA and ASE sensory neuron through the NPR-4 and NPR-1 receptors and this modulation allows the animal to control reversal lengths. Although our experiments point to the fact that NPR-4 is functioning in the AVA neuron and NPR-1 functions in the ASE neurons, the fact that expressing NPR-1 in other sensory neurons and/or RMG neurons (with gpa-3 and flp-5 promoters) also allows for rescue of the increased reversal phenotype seen in the npr-1 mutants.
indicates the possibility of NPR-1 functioning in other neurons to maintain reversal length. Further analysis at the level of single neurons could allow one to understand if NPR-1 has a much more complex role in the process of regulating reversal length.

**Mutants in flp-18 show increased AVA activity**

Previous studies have shown an increase in calcium levels in the AVA command interneuron during spontaneous reversals (Ben Arous et al., 2010; Piggott et al., 2011; Zheng et al., 2012). Further, Gray and colleagues have shown that *C. elegans* with laser ablated AVA neurons do not show longer reversals during local search behavior (Gray et al., 2005). This information along with our previously described results where NPR-4 expression in AVA rescues the *npr-4* mutant phenotype, indicates a possible role of AVA in controlling the length of backward movement. How activity of AVA is related to length of reversals is not clear. We reasoned that calcium raising duration and/or calcium levels of AVA during reversals might have some relation with the length of reversals. To delineate the relation of AVA activity with length of reversals we used *C. elegans* with GCaMP5.0 expressed in the AVA interneuron using the *rig-3* promoter (Larsch et al., 2013) and measured calcium levels in freely navigating animals during spontaneous reversals. Consistent with our previous results, *flp-18* mutants showed increased reversal length when compared to WT animals (Figure 2). Furthermore, very strikingly *flp-18* mutants showed an increase in calcium levels ($p<0.0001$, $t=8.336$, $df=16.13$) and in the calcium rising duration for AVA during reversals ($p<0.0001$, $t=5.464$, $df=17.22$), Figure 2A-C and https://www.amazon.com/clouddrive/share/oESEQ4UJTziXidtYTldE2AKcggpXtZgp5luQd7RJYWD and https://www.amazon.com/clouddrive/share/2xZpGLfjhSBcfbe4GAFZVdQM6TRdo1CrZ4NvbUeoMp).
Our results show that FLP-18 functions through NPR-4 in AVA and NPR-1 in ASE to control reversal length (Figure 1A and B). Both npr-4 and npr-1 mutants showed increase in calcium levels (WT and npr-1 \( p<0.0001, t=4.656, df=20.78 \), WT and npr-4 \( p=0.0015, t=3.712, df=19.11 \)) and calcium raising duration of AVA during reversal (WT and npr-1 \( p=0.0054, t=3.085, df=22.06 \), WT and npr-4 \( p=0.0036, t=3.198, df=25.8 \)), when compared to WT control animals (Figure 2). Increased AVA activity in npr-4 mutants suggests that FLP-18 released from AVA and other neurons controls the activity of AVA through the NPR-4 receptor. The function of FLP-18 through NPR-4 in AVA provides a novel example of self-regulation of neuronal activity by neuropeptide signaling. NPR-4 has been reported to be involved in various functions in C. elegans, but the signaling mechanism underlying NPR-4 function is not completely clear. Increased activity of AVA in npr-4 mutants during reversals suggests an inhibitory function of the NPR-4 receptor. Our previous results show that NPR-1 functions in ASE to control reversal length (Figure 1A and C). NPR-1 has been shown to function through inhibitory signaling (Chang et al., 2006; Cheung et al., 2005; Rogers et al., 2006). Hence, npr-1 mutants might have increased activity in ASE sensory neurons. Increased activity of AVA during reversals in npr-1 mutants could be due to ASE mediated change in the activity of AIB and AWC neurons, which are known to play important roles in modulating reversal behavior (Chalasani et al., 2007; Gray et al., 2005; Piggott et al., 2011). Changes in the activity of AIB and AWC could in turn affect the AVA interneuron behavior.

Independently, both npr-1 and npr-4 mutants have increased calcium levels and calcium raising duration of AVA compared to WT animals but show significantly lower levels compared to flp-18 mutants (Calcium levels flp-18 and npr-1 \( p=0.0001, t=4.764, df=21.43 \) flp-18 and npr-4 \( p=0.0001, t=4.858, df=23.26 \), Figure 2A-C). To
test if increased calcium levels and calcium raising duration of AVA in flp-18 mutants
is due to both NPR-4 and NPR-1, we measured the calcium levels in npr-1 npr-4
double mutants. Similar to flp-18 mutants, npr-1 npr-4 double mutants showed longer
reversals with increased calcium levels (WT and npr-1 npr-4 (p<0.0001, t=6.772,
df=14.37), npr-1 and npr-1 npr-4 (p=0.0003, t=4.467, df=17.02), npr-4 and npr-1 npr-
4 (p=0.0002, t=4.582, df=18.25)) and calcium raising time of AVA (WT and npr-1
npr-4 (p=0.0003, t=4.533, df=17.43), npr-1 and npr-1 npr-4 (p=0.0476, t=2.096,
df=22.48), npr-4 and npr-1 npr-4 (p=0.0157, t=2.664, df=18.22)) when compared to
WT, npr-4 or npr-1 animals (Figure 2A-C and
https://www.amazon.com/clouddrive/share/STtTku2n4pkvrKDnHghDGUD0d3nEoPt
w5YxNaxazT1). This increase could be due to the synchronized effect of
independently increased activity of AVA due to loss of npr-4 and ASE due to loss of
npr-1. Finally, we were able to rescue the npr-1 phenotype seen in the npr-1 npr-4
double mutants by expressing NPR-1 in the ASE neurons, as can be seen from the
traces, the rescued line shows a calcium level (p=0.696, t=0.396, df=21.71) and
calcium raising time of AVA (p=0.956, t=0.0556, df=18.51) similar to that seen in
npr-4 mutants (Figure 2A-C). We were also able to rescue the npr-4 mutant
phenotype seen in the double mutants by expressing NPR-4 specifically in the AVA
neuron. The rescued line shows similar calcium levels (p=0.364, t=0.932 df=17.84)
and calcium raising time of AVA (p=0.0343, t=2.257 df=21.94) as seen in the npr-1
mutants (Figure 2A-C). These results show that NPR-1 expression in the ASE sensory
neurons and NPR-4 expression in the AVA command interneuron are able to rescue
the increased activity of the AVA neuron, seen in the npr-1 npr-4 mutant C. elegans.
CREB1/CRH-1 regulates FLP-18 levels
Having found FLP-18 to be required for controlling reverse body bends, we reasoned that changes in the cellular levels of FLP-18 could change the length of reversals, and hence food search strategy in response to different environmental conditions. We next asked how FLP-18 expression could be regulated in *C. elegans*. On analyzing the promoter sequence of FLP-18 we found multiple cAMP response element (CRE) sites (5'-'TGACGTCA-3') i.e. CREB1/CRH-1 binding sites (Craig *et al.*, 2001). Two additional pieces of evidence prompted us to see if CREB1/CRH-1 regulated the levels of FLP-18. First, *creb1/crh-1* mutants have been shown to effect reversal length, the mutants show short reversals when compared to WT animals in tap response assays (Timbers and Rankin, 2011). And second, there have been two recent reports implicating CREB1/CRH-1 in maintaining the levels of other FMRFamide like peptides, FLP-6 (Chen *et al.*, 2016) and FLP-19 (Rojo Romanos *et al.*, 2017). In order to see if CREB1/CRH-1 regulates FLP-18 expression, we analyzed the expression of FLP-18 using the *Pflp-18::FLP-18::sl2::GFP* reporter in WT and *creb1/crh-1* mutant animals. We found a significant increase in the FLP-18 expression in *creb1/crh-1* mutant animals when compared to WT control animals (*p=0.0049, t=2.982, df=38.53*), where neurons expressing FLP-18 showed increased expression in *creb1/crh-1* mutants (Figure 3A). Since CREB1/CRH-1 seems to act as a regulator of FLP-18 expression, we reasoned that CRH-1f (which has the DNA binding bZIP domain but lacks the N-terminal activating Kinase Inducible Domain (KID) (Wormbase gene: WBGene00000793)) could be inhibiting FLP-18 expression. In order to test this hypothesis we expressed CRH-1f under the pan-neuronal *rab-3* promoter in the *creb1/crh-1* mutant line. We found that pan-neuronally expressing CRH-1f could completely rescue the increased FLP-18 levels seen in *creb1/crh-1* mutant animals (*p=0.0015, t=3.411, df=38.32, Figure 3A). We next asked if
expressing CREB1/CRH-1 in only FLP-18 positive neurons could rescue the increased FLP-18 expression levels. Targeted expression of CRH-1f cDNA under the flp-18 promoter sequence restored the increased FLP-18 levels seen in the creb1/crh-1 mutant animals to WT levels (\(p=0.0103, t=2.687, df=42.04\), Figure 3A). We next went on to get a better understanding of the changes in FLP-18 levels in specific neurons. To do this experiment we used co-localization markers that would allow us to identify the AVA, AIY and RIM neurons (methods section). We quantitated the GFP expression from these neurons in WT and creb1/crh-1 mutant backgrounds. Our data indicated that in creb1/crh-1 mutants there was a significant increase in fluorescence in the AVA (\(p=0.0013, t=3.613, df=25.2\)) and AIY (\(p=0.029, t=3.613, df=25.2\)) neurons, while no changes were seen in the RIM neuron (\(p=0.955, t=0.0565, df=24.57\)) and (Figure 3B). We went on to express CREB1/CRH-1 under the flp-18 promoter in these mutant lines. In all the three neurons we saw a very significant reduction in Pf\textit{f}flp-18::FLP-18::\textit{sl2}::GFP expression upon expressing CREB1/CRH-1 (\(p<0.0001, t=6.297, df=21.41\)) AIY (\(p=0.0016, t=3.925, df=13.72\)) RIM (\(p<0.0001, t=5.692, df=18.71\)) and (Figure 3B). These data indicate that CREB1/CRH-1 could be acting as a repressor of FLP-18 in the neurons that are involved in the reversal behavior in \textit{C. elegans}.

If CREB1/CRH-1 is indeed required to inhibit FLP-18 levels, we hypothesized that creb1/crh-1 mutants may show differences in reversal lengths when compared to WT \textit{C. elegans}. Upon testing the mutants we found that the mutants show significant decrease in reverse body bends as would be expected if CREB1/CRH-1 inhibits FLP-18 expression (\(p=0.0013, t=3.613, df=25.2\), Figure 3C and https://www.amazon.com/clouddrive/share/FoxEFLU0U3uGnXdGsbo07rorAhQ6ro7dcPcj89zE87B). Again we could rescue this phenotype by expressing CRH-1f pan-
neuronally ($p=0.536, t=0.628, df=24.63$) as well as more specifically in FLP-18 expressing neurons ($p=0.881, t=0.141, df=25.25$) and (Figure 3C). Further, double mutants of creb1/crh-1 and flp-18 showed a phenotype that was indistinguishable from the flp-18 single mutants ($p=0.4966, t=0.6920, df=20.82$), Figure 3C), again indicating that CREB1/CRH-1 is acting upstream of FLP-18.

We next went on to explore the reversal body bends in creb1/crh-1 mutants upon activating the ASH neuron (Guo et al., 2009). We found a significant decrease in reverse body bends in these mutants ($p=0.0002, t=4.136, df=35.03$) and this phenotype could be rescued by expressing CRH-1f under the flp-18 promoter $(p=0.1037, t=1.664, df=41.1)$, Figure 3D).

Taken together, these results show that CREB1/CRH-1 inhibits FLP-18 expression in the C. elegans nervous system.

Starvation affects reverse body bends through increase in FLP-18 levels

Change in locomotion is required for effective exploration of the environment under different physiological conditions. Modulating reversal length is important to allow C. elegans to switch their navigational strategy during prolonged starvation. Our results thus far indicate that reversal length is affected by levels of FLP-18, this prompted us to investigate the expression of FLP-18 during starvation. In order to study the effect of starvation on FLP-18 levels, we starved the C. elegans for 24 hours and extracted RNA from fed and starved animals. We estimated the levels of flp-18 transcripts through qPCR under both conditions and found that there was a 20 fold increase in FLP-18 levels upon starvation (data not shown). We also validated this data by quantitating the levels of Pflp-18::FLP-18::sl2::GFP in well fed or 24 hours starved C. elegans. Again, we found an increase in Pflp-18::FLP-18::sl2::GFP levels upon
starvation ($p=0.0006$, $t=3.691$, $df=44.91$), Figure 4A). These two pieces of data indicate that starvation causes an increase in FLP-18 levels.

We next went on to ask if the increase in FLP-18 levels could cause changes in reversal body bends. Upon analyzing the reversal lengths in fed *C. elegans* and animals starved for 24 hours we saw a significant decrease in the number of body bends in starved animals during each reversal ($p<0.0001$, $t=4.619$, $df=30.71$), Figure 4B and

https://www.amazon.com/clouddrive/share/jErueH1BjmPVhpLuixNKGpG0XTYMQfULeVn0x6hY0r and

https://www.amazon.com/clouddrive/share/4nKmmo0ps2EK7JIMMYCaH63unAACD5tmNlasb9rJDf3t). If the decreased reversal body bends is indeed due to increased FLP-18 levels, then loss of FLP-18 should abolish the decrease in body bends that we see in starved WT animals. We went on to analyze the reverse body bends in *flp-18* mutants that were starved for 24 hours along with control animals that were not starved. We found that the decrease in body bends per reversal that is seen in WT animals upon starvation is completely abolished in *flp-18* mutant animals ($p=0.888$, $t=0.142$, $df=30.22$), Figure 4B and

https://www.amazon.com/clouddrive/share/RKqSKIVBWwrVFLsqiAmL2cgwxcI92mV2JfrGRsQfp6Sd and

https://www.amazon.com/clouddrive/share/ry5teKQOs5vNsYdp6WTnATS5S9c7kqh zg4Wkdl5kB7X). These data indicate that FLP-18 levels are regulated in *C. elegans* during starvation. Starved animals show increased FLP-18 expression and consistent with the fact that increased FLP-18 levels could cause shorter reversals, analysis of these *C. elegans* showed that they indeed made shorter reversals.
We next asked if FLP-18 is functioning through NPR-1 and NPR-4 during starvation. In order to do this we went on to look at the reversal length in the \textit{npr-1 npr-4} double mutants in animals that were either fed or starved for 24 hours. Again we found that the \textit{npr-1 npr-4} mutants behaved like the \textit{flp-18} mutants and had lost the ability to reduce reversal length as was seen in WT animals ($p=0.4001$, $t=0.856$, $df=24.13$), Figure 4B). Taken together, these experiments show that the FLP-18 pathway functioning through NPR-1 and NPR-4 is required for reducing the reversal length during starvation in \textit{C. elegans}.

One discrepancy that we found in these experiments was that there was a significant reduction in reversal length in \textit{flp-18} mutants when compared to our previous reversal data, further the \textit{npr-1 npr-4} double mutants appear to have a lower reversal length then \textit{flp-18} mutants at 24 hours fed and starved conditions (Figure 1A vs Figure 4B for \textit{flp-18} and \textit{npr-1 npr-4}). One possible reason could be the age of the animals that differs in both sets of experiments. At 78 hours of development, which is when the later experiments were performed all the animals have multiple eggs, this is not the case for the initial data which was done at 54 hours post egg-laying (including hatching) where the animals had no/few eggs (Chiba and Rankin, 1990). Although, the starvation data implicates \textit{flp-18}, and \textit{npr-1 npr-4} to be involved in modulating reversal length during starvation, more experiments would be required to pinpoint the exact mechanism of how these molecules function during satiety and starvation.

\textbf{Discussion}

Neuromodulators modify behaviors by shaping the properties of neural circuits. Here, we reveal the role of the neuropeptide FLP-18 in regulating reversal length in \textit{C. elegans}. Our initial observations showed that \textit{flp-18} null mutant animals make longer
reversals in comparison to WT control animals. Reversal length has been shown to be positively correlated with the probability of change in direction of *C. elegans* movement post reversal (Gray et al., 2005). Hence, reversal length is especially important for behaviors like chemotaxis and pathogen avoidance that require the animals to change locomotory strategies in order to move towards the source of food or away from a pathogen (Luo et al., 2013). Similarly, the switch that *C. elegans* make from local search to global search after prolonged starvation also depends upon the regulation of reversal length. Our study identifies FLP-18 as a key molecule in the regulation of reversal length under different genetic and environmental conditions. Null mutants of *creb1/crh-1* show shorter reversal length (Figure 3A) and low chemotaxis indices (Yogesh Dahiya; unpublished data) consistent with defects in the ability of the mutants to change direction in response to chemical gradients. We show that FLP-18 expression is negatively regulated by the transcription factor CREB1/CRH-1. Further, *flp-18; crh-1* double mutants show behaviors similar to *flp-18* null mutants indicating that FLP-18 is likely to be functioning downstream of CREB1/CRH-1. We go on to show that during starvation, WT *C. elegans* tend to make shorter reversals which in turn may result in a change in the animal’s exploratory strategy from local search to global search behaviors ((Gray et al., 2005) and this work). Our experiments suggest that this decrease in the reversal length could be due to increased levels of FLP-18, this is further corroborated by the fact that *flp-18* null mutants are unable to regulate reversal length under our assay conditions (Figure 4).

Reversal circuit analysis revealed that NPR-1, NPR-4 and NPR-5 could be the potential candidates affecting reversal behavior through FLP-18 (Li and Kim, 2014). Mutants in *npr-4* show significant increase in reversal length in comparison to wild-
type animals but significantly less than that seen in \textit{flp-18} mutants, which suggests that NPR-4 is not the only receptor through which FLP-18 is modulating the reversal circuit. Reversal behavior analysis of \textit{npr-1} mutants also showed a similar behavior to \textit{npr-4} mutants, while \textit{npr-1 npr-4} double mutants showed a significant increase in reversal length comparable to that seen in \textit{flp-18} mutants. Behavioral analysis showed that NPR-5, which is expressed in sensory neurons and the first layer of interneurons is not involved in this process. To further elaborate the site of action of FLP-18, we performed neuron specific rescue experiments. Our rescue experiments suggest that NPR-4 functions in AVA while NPR-1 could functions in the ASE sensory neurons. A possible circuit could involve NPR-1 functioning in the ASE sensory neuron to mediate changes in the AVA command interneuron through the AIB interneurons and/or the AWC sensory neurons, at the same time NPR-4 could affect the AVA neuron directly which is also one of the neurons that expresses FLP-18, thereby allowing for a possible mechanism for the AVA neuron to modulate it’s own activity during reversals (illustrated in Figure 5). However, it is plausible that NPR-1 could function in other sensory neurons or interneurons to allow for maintaining reversal length. Our data suggests that FLP-18 modulates the activity of reversal circuits at command interneuron and sensory neuron levels simultaneously. Finally, our data also suggests that shorter reversals during starvation could be mediated by the FLP-18/NPR-1/4 signaling pathway (Figure 4B and illustrated in Figure 5).

We also studied the reversal circuit through calcium imaging during exploratory behavior. Calcium imaging of AVA in \textit{flp-18} mutants showed similar patterns as observed in \textit{npr-1 npr-4} double mutants. Surprisingly, calcium levels of AVA during reversals in \textit{npr-1} and \textit{npr-4} mutants were comparable and significantly lower then \textit{flp-18} and \textit{npr-1 npr-4} mutants (Fig 2). These results suggest two important points.
First, FLP-18 functions through NPR-1 and NPR-4 supporting our behavioral experiments. Second, the FLP-18/NPR-1/4 system appears to modulate the reversal circuit through inhibitory signaling. Taken together, these observations point to a possible role of FLP-18 in modulating reversal length by regulating the duration of activity and calcium levels of AVA through NPR-4 and NPR-1. Our work provides molecular insights into the modulation of the locomotory circuit by showing that the FLP-18/NPR-1/NPR-4 signaling pathway plays a critical role in modulating locomotion based behaviors under various genetic and environmental conditions.
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Figure Legends:

Figure 1: Mutants in *flp-18* show increased body bends per reversal

A: Graph of body bends per reversal in wild-type (WT), *flp-18*, *npr-1*, *npr-4*, *npr-1 npr-4* and NPR-4 rescue lines (n=20). B: Graph of body bends per reversal in WT, *npr-4*, *npr-1 npr-4* and NPR-1 rescue lines (n=17-20). C: Illustration of ASH activation using ChR2. C: Graph of body bends per reversal upon ChR2 based activation of ASH interneurons in WT, *flp-18 npr-1 npr-4*, NPR-4 rescue line and the *flp-18 npr-1 npr-4* triple mutants (n=20). The error bars indicate standard errors in all figures. See also Figure 1-1.

Figure 2: Mutants in *flp-18* and its receptors *npr-1* and *npr-4* show increased AVA activity

A: Comparison of the mean values for calcium transients in AVA during a single reversal event WT *C. elegans*, *flp-18* mutants, *npr-1 npr-4* double mutants and NPR-1 rescue (left) or NRP-4 rescue lines (right). The dark solid line and light color shading represents mean+/−SEM (n=12-14 animals for each genotype). The Arrow indicates the reversal initiation point. B: indicates a graph of the maximum calcium transients in the AVA neuron. The Calcium peaks were compared for WT animals, *flp-18* mutants, *npr-4* mutants, *npr-1* mutants, *npr-1 npr-4* double mutants, NPR-1 rescue in ASE neurons and NPR-4 rescue in the AVA neuron (n=12-14). C: Comparison of calcium raising duration in AVA during reversal in WT, *flp-18* mutants, *npr-1* mutants, *npr-4* mutants, *npr-1 npr-4* double mutants, NPR-1 rescue in ASE neurons and NPR-4 rescue in the AVA neuron (n=12-14).

Figure 3: CREB1/CRH-1 regulates FLP-18 expression

A: Images and quantitation of P*flp-18::FLP-18::sl2::GFP* expression in WT, creb1/crh-1 and CREB1/CRH-1f rescue lines (n=20, scale bar; 10μm). In the graph to the right *crh-1*; *Prab-3::CRH-1f* and *creb1/crh-1* P*flp-18::CRH-1f* are compared to WT control animals (“ns” and “∗∗∗” just above each plot) and to *crh-1* mutants (indicated by lines drawn above the graph with respect to the *crh-1* plot). B: P*flp-18::FLP-18::sl2::GFP* fluorescence from the specific neurons AVA, AIY and RIM from WT, *crh-1* mutants and *crh-1* mutants expressing CRH-1f under the *flp-18* promoter (n=15). C: Graph of body bends per reversal in WT, creb1/crh-1, *flp-18*, creb1/crh-1; *flp-18* double mutants and CREB1/CRH-1f rescue lines (n=20). D: Graph of body bends per reversal upon ChR2 based activation of ASH interneurons in WT, creb1/crh-1 and rescue of the creb1/crh-1 mutant phenotype by expressing CRH-1f under the *flp-18* promoter (n=20).

Figure 4: Starvation causes increased FLP-18 levels in *C. elegans*

A: Images and graph of P*flp-18::FLP-18::sl2::GFP* expression in WT animals that are fed or starved for 24h (n=20, scale bar; 10μm). B: Graph of body bends per reversal in WT (fed and starved for 24h), *flp-18* (fed and starved for 24h) and *npr-1 npr-4* (fed and starved for 24h) (n=20).

Figure 5: Model

A: schematic model for the neuropeptide, FLP-18, based modulation of the reversal circuit at the level of sensory neurons and interneurons. In the absence of FLP-18 the reversals are longer and during starvation there is more FLP-18 and shorter reversals.
Please note that AVA and AIY are not the only neuron that expresses FLP-18, other neurons like RIM and RIG also express FLP-18 although they are not shown in the illustration.

**Extended Data**

Figure 1-1: (A) Graph of number of reversals per five minutes in WT animals in comparison to flp-18 mutant *C. elegans* (n=20), (B) Graph of forward body bends per 30s in WT and flp-18 mutants (n=20), (C) Graph of number of body bends per reversal in WT and npr-5 mutant animals (n=16-20), (D) Indicates images of NPR-4 and NPR-1 under specific promoters. The promoter is upstream of *npr-4* or *npr-1* cDNA and the sl2 sequence tagged to wrmScarlet (scale bar; 20µm). (E) Graph of body bends per reversal in wild-type (WT), flp-18, npr-1 npr-4 double mutants and flp-18 npr-1 npr-4 triple mutants (n=20). The error bars indicate standard errors in all figures.
Figure 1

A

Number of body bends/reversal

WT, flip-18, flip-18 rescue, npr-4, npr-1, npr-1 npr-4, Pflip:16; npr-4+, Pnp-1 npr-4+, Pnp-2 npr-4+, Pnp-3 npr-4+

ns

***

AVA/AIV/RIM/RIG/VNC, AVA

B

Number of body bends/reversal

WT, npr-1 npr-4, npr-4, Pnp-4-;npr-1, Pnp-30,npr-4, npr-1 npr-4, Pnp-2; npr-4, Pnp-5; npr-4, Pnp-7; npr-4, Pnp-1 npr-4, npr-1 npr-4, npr-1 npr-4, Pnp-1 npr-4, Pnp-2 npr-4, Pnp-3 npr-4

ns

Sensory, ASER/L, ASEL, ASER/L

C

ASH Chr2

AVA

Measuring number of body bends/reversal

D

Number of body bends/reversal

WT, flip-18, npr-1 npr-4, Pnp-2; npr-4, Pnp-7; npr-4, Pnp-1 npr-4

ns

***

AVA ns

***

AVA ns
Figure 5

- ASE
- AWC
- AIB
- AIY
- AVA

**WT**

- NPR-1
- NPR-4
- FLP-18

**Chemical Synapse**

- flp-18 (longer reversals)
- Starvation or loss of crh-1 (shorter reversals)