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Orcokinin neuropeptides regulate sleep in *Caenorhabditis elegans*

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Abstract

Orcokinin neuropeptides are conserved among ecdysozoans, but their functions are incompletely understood. Here, we report a role for orcokinin neuropeptides in the regulation of sleep in the nematode *Caenorhabditis elegans*. The *C. elegans* orcokinin peptides, which are encoded by the *nlp-14* and *nlp-15* genes, are necessary and sufficient for quiescent behaviors during developmentally-timed sleep (DTS) as well as during stress-induced sleep (SIS). The five orcokinin neuropeptides encoded by *nlp-14* have distinct but overlapping functions in the regulation of movement and defecation quiescence during SIS. We suggest that orcokinins may regulate behavioral components of sleep-like states in nematodes and other ecdysozoans.

Introduction

Ecdysozoa is comprised of the most diverse group of animals on earth. This clade includes arthropods and nematodes, as well as other smaller phyla, which are united by having a molting cycle (Aguinaldo, Turbeville et al. 1997). Molts occur periodically during growth and are accompanied by elaborate and specific molting behaviors. In the nematode *C. elegans*, the molting cycle is similar to the circadian cycle in other animals (Hendriks, Gaidatzis et al. 2014). Molt timing is regulated by LIN-42, a worm homolog of the circadian protein PERIOD, and behavior during the molt resembles sleep controlled by circadian timing in other animals (Raizen, Zimmerman et al. 2008).

Orcokinin neuropeptides are strikingly conserved across Ecdysozoa. They have been described in nematodes (Nathoo, Moeller et al. 2001), in several arthropods including cockroaches (Hofer, Dircksen et al. 2005, Hofer and Homberg 2006), kissing bugs (Wulff, Sierra et al. 2017), fruit flies (Chen, Choi et al. 2015), crayfish (Yasuda-Kamatani and Yasuda 2000), lobsters (Dickinson, Stemmler et al. 2009), and in tardigrades (Koziol 2018). Orocokinins are related to pedal peptides (Kim, Go et al. 2018), identified in mollusks (Lloyd and Connolly 1989) and to smooth muscle relaxant peptides (SMPs), identified in echinoderms (Rowe and Elphick 2012, Kim, Kim et al. 2016), suggesting that an ancestor to ecdysozoan orcokinins was present in early bilaterians (Jekely 2013, Semmens and Elphick 2017).

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Orcokinins regulate insect ecdysis and circadian activity. In kissing bugs, disruption of orcokinin signaling causes molting defects (Wulff, Sierra et al. 2017), perhaps partially due to a role for these peptides in the biosynthesis of the ecdysteroids (Zitnan, Ross et al. 1999, Yamanaka, Roller et al. 2011). In cockroaches, orcokinins peptides injected into the brain induce a phase shift in circadian sleep/wake behavior (Hofer and Homberg 2006). A role in the regulation of both molting and circadian rhythms suggests the intriguing hypothesis that an ancestral role for orcokinin is in the regulation of behavioral rhythms, specifically sleep/wake behavior. We pursued this hypothesis in *C. elegans*, given the similarity of its molting cycle to circadian rhythms in other organisms.

C. elegans orcokinins are encoded by the genes *nlp-14* and *nlp-15* (Nathoo, Moeller et al. 2001). NLP-14 peptides modulate cholinergic signaling during male mating (Sherlekar, Janssen et al. 2013) and mediate decision-making during nociceptive behaviors (Hapiak, Summers et al. 2013). *nlp-14* transcripts are upregulated during the 2-hour period prior to ecdysis (George-Raizen, Shockley et al. 2014), when the animals display sleep-like quiescent behavior (Raizen, Zimmerman et al. 2008). Single-cell transcriptomic data suggests that *nlp-14* is expressed in the sleep-promoting ALA neuron (Nath, Chow et al. 2016, Taylor, Santpere et al. 2019), while its paralog *nlp-15* is expressed in both the ALA neuron and in another sleep promoting neuron called RIS (Taylor, Santpere et al. 2019). Thus, we sought to test the hypothesis that orcokinin neuropeptides regulate sleep.

C. elegans sleep is regulated by neuropeptides. Developmentally timed sleep (DTS) occurs during larval transitions, coincident with the molt (Singh and Sulston 1978, Raizen, Zimmerman et al. 2008). Movement quiescence during DTS is controlled primarily by the RIS neuron which releases FLP-11 neuropeptides (Turek, Lewandrowski et al. 2013, Turek, Besseling et al. 2016) with a minor role for NLP-22 peptides released from the RIA neurons (Nelson, Trojanowski et al. 2013). Arousal during DTS is mediated by pigment dispersing factor neuropeptides (PDF), which also mediate arousal in insects (Renn, Park et al. 1999, Choi, Chatzigeorgiou et al. 2013). SIS is controlled by both the ALA and RIS interneurons, via the release of a collection of neuropeptides (Nelson, Lee et al. 2014, Lenz, Xiong et al. 2015, Turek, Besseling et al. 2016). Based on their spatial and temporal expression patterns, and on their roles in regulating behavioral rhythms in other ecdysozoans, we hypothesized that NLP-14 and NLP-15 play a role in sleep regulation in *C. elegans.*

We combined the analysis of loss-of-function with over-expression to characterize the function of the *C. elegans* orcokinins and find that NLP-14 and NLP-15 are required for movement and defecation quiescence that occur during sleep; NLP-14 peptides play a larger role. This work expands our knowledge of the function of orcokinins and suggests a previously unappreciated role in sleep regulation.

Methods

Worm maintenance and strains

Animals were maintained at 20°Celsius on agar plates containing nematode growth medium and fed the OP50 derivative bacterial strain DA837 (Davis, Somerville et al. 1995). The following strains were used in this study:

- N2 (Bristol - *wild type*)
- KG532 = *kin-2*(*ce179*) X
- VC1063 = *nlp-15*(*ok1512*) I
- PS5009= *pha-1*(*e2132ts*); *syEx723*[*hsp-16.2p*::*lin-3C*;*myo-2p*:*gfp*; *pha-1*(*+*)]
- SJU6 = *stjEx3*[*hsp-16.2p*::*nlp-14*; *myo-2p*::*mCherry*]
- SJU27 = *stjIs2*[*hsp-16.2p*::*nlp-14*; *myo-2p*::*mCherry*]
- SJU44 = *stjEx32*[*hsp-16.2p*::*nlp-14*(1-3); *myo-2p*::*gfp*]
- SJU47 = *stjEx36*[*hsp-16.2p*::*nlp-14*(1-2); *myo-2p*::*gfp*]
- SJU95 = *stjEx76*[*ida-1p*::*mCherry*; *nlp-14p*::*gfp*]
- SJU96 = *stjEx77*[*ida-1p*::*mCherry*; *nlp-14p*::*gfp*]
- SJU102 = *kin-2*(*ce179*) X; *stjIs2*
- SJU109 = *sjtEx123*[*hsp-16.2p*::*nlp-14*(1); *myo-2p*::*mCherry*]
- SJU110 = *sjtEx123*[*hsp-16.2p*::*nlp-14*(1); *myo-2p*::*mCherry*]
- SJU121 = *stjEx91*[*hsp-16.2p*::*nlp-15*; *myo-3p*::*mCherry*]
- SJU122 = *stjEx92*[*hsp-16.2p*::*nlp-15*; *myo-3*::*mCherry*]
- SJU154 = *nlp-14*(*tm1880*) X
- SJU178 = *nlp-14*(*stj10*) X
- SJU207= *nlp-14*(*tm1880*) X; *stjEx146*[*ida-1p*::*nlp-14*; *myo-3p*::*mCherry*] (Line#1)
- SJU208= *nlp-14*(*tm1880*) X; *stjEx147*[*ida-1p*::*nlp-14*; *myo-3p*::*mCherry*] (Line#2)
- SJU209= *nlp-14*(*tm1880*) X; *stjEx148*[*ida-1p*::*nlp-14*;*myo-3p*::*mCherry*] (Line#3)
- SJU232= *stjEx163*[*hsp-16.2p*::*nlp-14*(3); *myo-2p*::*mCherry*]
- SJU233= *nlp-14*(*stj18*) X
- SJU241= *stjIs160*[*hsp-16.2p*::*nlp-14*; *myo-2p*::*mCherry*]
- SJU244= *stjEx167*[*hsp-16.2p*::*nlp-14*(3); *myo-2p*::*mCherry*]
- SJU245= *stjEx168*[*hsp-16.2p*::*nlp-14*(3); *myo-2p*::*mCherry*]
- SJU246= *stjEx169*[*hsp-16.2p*::*nlp-14*(3); *myo-2p*::*mCherry*]
- SJU247= *stjEx170*[*hsp-16.2p*::*nlp-14*(3); *myo-2p*::*mCherry*]
- SJU254= *stjEx171*[*hsp-16.2p*::*nlp-14*(1-4); *myo-2p*::*mCherry*]
- SJU255= *stjEx172*[*hsp-16.2p*::*nlp-14*(1-4); *myo-2p*::*mCherry*]
- SJU256= *stjEx173*[*hsp-16.2p*::*nlp-14*(1-4); *myo-2p*::*mCherry*]

- SJU257= *stjEx174*[*hsp-16.2p*::*nlp-14*(1-4); *myo-2p*::*mCherry*]
- SJU258= *stjEx175*[*hsp-16.2p*::*nlp-14*(1-4); *myo-2p*::*mCherry*]
- SJU260= *nlp-14*(*stj19*) X
- SJU262= *nlp-15*(*ok1512*) I*; nlp-14*(*stj18*) X
- SJU272= *nlp-14*(*stj18*) X; *pha-1*(*e2132ts*) III; *syEx723*[*hsp16.2p*::*lin-3C*; *myo-2p*::*gfp*; *pha-1*(+)]
- SJU273= *nlp-14*(*tm1880*) X; *pha-1*(*e2132ts*) III; *syEx723*[*hsp16.2p*::*lin-3C*; *myo-2p*::*gfp*; *pha-1*(+)]
- SJU281= *nlp-14(stj13)* X*; nlp-15(stj25)* I
- SJU282= *stjEx184*[*nlp-15p*::*gfp*;*ida-1p*::*mCherry*; *myo-3p*::*mCherry*]
- SJU312= *nlp-15*(*stj25*) I

Molecular biology and transgenesis

DNA for transgenesis was constructed using overlap extension-polymerase chain reaction (OE-PCR) (Nelson and Fitch 2011). The promoter of the gene *hsp-16.2* and the coding sequences of *nlp-14* and *nlp-15* were amplified from genomic DNA by PCR. The amplicons were fused together by OE-PCR. To over-express subsets of NLP-14 peptides, the *hsp-16.2* promoter and a portion of the *nlp-14* gene coding for the N-terminal signal peptide and NLP-14(1), (1-2), (1-3) or (1-4) peptide(s), followed by a stop codon, were amplified from genomic DNA. Next, the operon sequence from the genes *gpd-2* and *gpd-3* and the coding sequence for the red fluorescent protein RFP were amplified from the plasmid pLR304. The three amplicons were fused by OE-PCR. To over-express the NLP-14(3) peptide, the plasmid pSJU8 was commercially engineered to contain sequence for the *hsp-16.2* promoter, the coding sequence for the N-terminal signal peptide and NLP-14(3) peptide of the *nlp-14* gene and the 3'untranslated region of the gene *unc-54* (GeneScript ©). The *nlp-14*, *nlp-15* and *ida-1* fluorescent reporters were constructed by amplifying 5' regulatory DNA for each gene from genomic DNA and the green fluorescent protein (*gfp*) or mCherry coding sequence from the plasmids pPD95.75 or pCFJ90 (Addgene). The promoter and *gfp* amplicons were fused for each gene using OE-PCR. To express *nlp-14* in the ALA neuron, 5' regulatory DNA of the gene *ida-1* was fused by OE-PCR to the *nlp-14* coding sequence. Transgenesis was performed by microinjection, as described (Stinchcomb, Shaw et al. 1985). The strains SJU27, SJU141 and SJU142 were integrated using UV irradiation, as described (Mello and Fire 1995).

Reverse transcription-PCR (RT-PCR) of *nlp-14*(*tm1880*) was accomplished by isolating total RNA using an RNeasy mini kit (Qiagen ©), followed by cDNA synthesis using SuperScript™ One-Step RT-PCR System (ThermoFisher ©). Oligonucleotides used are in Table S1. Extrachromosomal arrays and DNA concentrations are listed in Table S2.

Construction of mutants

SJU178, SJU233, SJU262, SJU260, SJU262 and SJU281 were constructed by CRISPR/Cas9 gene editing. Using a published protocol (Arribere, Bell et al. 2014), insertions were made in the *nlp-14* or *nlp-15* gene at defined sites. Simultaneously, an edit of the *dpy-10* gene was made which resulted in an easily identifiable dumpy (Dpy) or roller (Rol) phenotype, to allow for screening. Specifically, a mixture of guide RNA (gRNA) duplexed with Alt-R® CRISPR-Cas9 tracrRNA (IDT ©), Alt-R ® S.p. Cas9 Nuclease V3 (IDT) and oligonucleotide repair templates were injected into day-1 adult wild-type or SJU233 animals. Dpy or Rol progeny of the injected animals were transferred to individual plates and maintained to the next generation. The genomic DNA of 10-15 progeny was used as templates for PCR to amplify a portion of the *nlp-14* or *nlp-15* gene. The amplicon was treated with NheI restriction enzyme and analyzed by agarose gel electrophoresis. 15-20 non-Dpy non-Rol animals from plates with the desired edit were transferred individually to fresh plates and grown to the next generation. These worms were again screened by PCR combined with restriction digest, and the alleles were confirmed by sequencing (Genewiz ©). The sequences of reagents are listed in Table S3.

The strain SJU154 was generated by crossing the strain FX18180(*tm1880)* X, which was obtained from the National BioResource Project (PI, Shohei Mitani), to male N2 animals, and then crossing resultant males back to tm1880. This procedure was repeated 3 times to reduce the number of unlinked mutations on the five autosomal chromosomes.

WorMotel behavioral assays

Movement quiescence was quantified during both developmentally timed sleep (DTS) and stress induced sleep (SIS), using the WorMotel, as previously described (Churgin, Jung et al. 2017). For DTS, we monitored active L4 animals (pre-lethargus) of each genotype for 12-hours. Because of day-to-day and chip-to-chip variability in sleep, we statistically compared strains housed in different wells of the same WorMotel. A combination of 24 wild-type, mutant, and/or transgenic active L4 animals were picked onto the agar surfaces of individual wells of the WorMotel polydimethylsiloxane (PDMS) chip. Images were captured every 10-seconds for 12 hours, and quiescence was quantified and DTS was manually measured based on a definable peak of quiescence, as previously described (Raizen, Zimmerman et al. 2008). For SIS, a combination of 24 wild-type, mutant, and/or transgenic day-1 adults were picked individually onto the agar surfaces of a welled PDMS microchip. The chip was placed into a UV-cross linker (Ultraviolet, 254 UVP) and exposed to 1500 J/m2 of UV light to induce SIS (DeBardeleben, Lopes et al. 2017). For over-expression experiments, day-1 adults were heat-shocked on standard growth plates, by submerging them, wrapped in para-film, in a 33°C water bath for 30 minutes. The heat-shocked animals were individually transferred to the agar surfaces of a welled PDMS microchip. For SIS and over-expression, images were captured every 10-seconds for 8 or 4 hours, respectively, and total minutes of quiescence was determined.

Body bending analysis following over-expression

Day-1 adults were heat-shocked by submerging standard growth plates, wrapped in para-film, in a 33°C water bath for 30 minutes. Body bends were counted manually using a stereomicroscope for 60-seconds, 2 hours after heat exposure. A body bend was defined as the movement of the body just posterior to the pharynx to the opposite position from the previous maximal bend.

Defecation analysis during SIS and following overexpression

The defecation cycle was measured manually for 5-6 minutes by visual inspection using a stereomicroscope, using described criteria (Thomas 1990). For over-expression, day-1 adults, on standard growth plates, were submerged, wrapped in para-film, in a 33°C water bath for 30 minutes and analyzed for 5 minutes between 2 and 2.5 hours after heat exposure. For SIS, day-1 adults were exposed to 1500 J/m2 of UV light in a UV-cross linker (Ultraviolet, 254 UVP) on growth plates and the defecation cycle was measured for 5-6 minutes, 85-95 minutes post-UV. For temporal SIS analyses, a single animal was examined for 5-minutes every 30 minutes for 4-hours, post-UV.

Microscopy

Fluorescence microscopy was conducted using an Olympus BX63 wide-field fluorescence microscope equipped with a Hamamatsu FLASH 4.0V3 digital camera and CellSens Dimension Version 2 software. Day-1 adult transgenic animals were immobilized on glass slides containing a 5% agar pad supplemented with 25mM sodium azide.

Alignments

Peptide sequences were obtained from the National Center for Biotechnology Information (NCBI) and aligned using online T-coffee software (Notredame, Higgins et al. 2000). Peptide alignments were annotated using Boxshade (https://embnet.vital-it.ch/software/BOX_doc.html).

Results

NLP-14 and NLP-15 are orcokinin homologs expressed in the ALA and RIS neurons

Neuropeptides coded by the genes *nlp-14* and *nlp-15* are classified as orcokinins based on sequence similarity to insect and crustacean peptides (Nathoo, Moeller et al. 2001). All five NLP-14 peptides are conserved at the C-terminus, while the NLP-15 peptides show greater conservation at the N-terminus (Figure 1A). *nlp-14* expression has been demonstrated in the ventral cord and some sensory and interneurons (Nathoo, Moeller et al. 2001), as well as in male-specific neurons (Sherlekar, Janssen et al. 2013). Single-cell gene expression studies revealed enrichment of both *nlp-14* and *nlp-15* transcripts in the ALA neuron and enrichment of *nlp-15* (but not *nlp-14*) in the RIS neuron (Nath, Chow et al. 2016, Taylor, Santpere et al. 2019). ALA and RIS are central sleep-promoting neurons (Turek, Lewandrowski et al. 2013, Hill, Mansfield et al. 2014, Konietzka, Fritz et al. 2020). In support of the single-cell transcriptomic data, we found that a GFP transcriptional reporter for *nlp-14* colocalizes with an mCherry transcriptional reporter for *ida-1*, which is strongly expressed in ALA (Zahn, Macmorris et al. 2001) (Figure 1B). Similarly, a GFP transcriptional reporter for *nlp-15* showed expression in both ALA and RIS neurons (Figure 1C). The combination of expression during the molt, a *C. elegans* sleep state, with expression in sleep-regulating neurons led us to hypothesize that *nlp-14* and *nlp-15* regulate sleep.

To test this hypothesis, we obtained mutant strains for *nlp-14* and *nlp-15*, which carry the deletion alleles, *tm1880* and *ok1512*, respectively. *tm1880* is predicted to cause an in-frame deletion that preserved the signal sequence as well as peptides 2, 4, and 5 of *nlp-14*. We confirmed this *in silico* prediction using RT-PCR (Figure 1D). We refer to *tm1880* from hereon as *nlp-14*(2, 4, 5). *ok1512* too is predicted to cause an in-frame deletion, which preserves the signal sequence as well as peptides 3-5 of *nlp-15* (Figure 1E). We refer to *ok1512* as *nlp-15*(3-5).

To construct complete loss-of-function mutants as well as other mutants, we used CRISPR/Cas9 gene editing technology (Paix, Folkmann et al. 2017). The *nlp-14*(*stj18*) strain contains a stop codon in the first exon, after the signal sequence but prior to the sequence encoding all NLP-14 peptides (Figure 1D). Similarly, the *nlp-15*(*stj25*) strain contains an insertion of a stop codon 5’ of all five NLP-15 peptides (Figure 1E). Hence, *stj18* and *stj25* are predicted to make null alleles of *nlp-14* and *nlp-15*, respectively, so we will refer to them as *nlp-14*(*null*) and *nlp-15*(*null*). The *nlp-14*(*stj10*) strain carries an insertion of a stop codon at the 3’-end of the sequence encoding peptide 3 so we will refer to it as *nlp-14*(*1-3*). The *nlp-14*(*stj19*) strain contains an in-frame deletion that removes only peptide 3, so we will refer to it from hereon as *nlp-14*(*1, 2, 4, 5*).

The *C. elegans* orcokinins are required for DTS

DTS occurs prior to ecdysis in *C. elegans* (Singh and Sulston 1978, Raizen, Zimmerman et al. 2008). Orcokinins regulate ecdysis and ecdysteroid biosynthesis in insects (Yamanaka, Roller et al. 2011, Wulff, Sierra et al. 2017). We hypothesized that DTS and/or the molt would be disrupted in *nlp-14* and/or *nlp-15* mutants. Using the WorMotel (Churgin, Jung et al. 2017), we found that DTS was unaltered in *nlp-14*(*null*), *nlp-15*(*3-5*) or *nlp-15*(*null*) single mutant animals (Figure 2A–C). Because we suspected there might be functional redundancy between the two genes, we tested animals that were mutant for both *nlp-14*(*null*) and *nlp-15*(*3-5*) and *nlp-14*(*null*) and *nlp-15*(*null*). DTS was modestly reduced in both double mutants (Figure 2D,E). We found no molting defects by carefully scanning double mutant strains via stereomicroscopy or by inspecting select animals at 1000X using wide-field differential interference contrast (DIC) microscopy. Based on these results, we conclude that NLP-14 and NLP-15 are required for movement quiescence during DTS but play a minor role in this behavior perhaps due to compensatory action of other neuropeptides (Nelson, Trojanowski et al. 2013, Turek, Besseling et al. 2016). They are not required for the successful completion of the molt.

NLP-14 peptides are required for movement quiescence during SIS

Since *nlp-14* and *nlp-15* are expressed in the RIS and/or ALA neurons, which are central regulators of SIS (Hill, Mansfield et al. 2014, Konietzka, Fritz et al. 2020), we tested their necessity for movement quiescence. SIS was induced by UV irradiation (DeBardeleben, Lopes et al. 2017) and animals were monitored on a WorMotel (Churgin, Jung et al. 2017). The *nlp-14*(*null*), *nlp-14*(*2, 4, 5*) and *nlp-14*(*1-3*) animals all displayed reductions in movement quiescence (Figure 3A–C,H,I; Figure S1), while *nlp-14*(*1,2,4,5*), *nlp-15*(*3-5*) and *nlp-15*(*null*) animals did not (Figure 3D,E; Figure S1). *nlp-14*(*null*); *nlp-15*(*3-5*) and *nlp-14*(*null*); *nlp-15*(*null*) double mutants displayed reduced total movement quiescence similar to that observed in *nlp-14*(null) single mutants; i.e. *nlp-15* mutations did not enhance the phenotype caused by *nlp-14* mutations (Figure 3F,G,J; Figure S1). However, the *nlp-15* mutations increased the variance of the *nlp-14*(*null*) phenotype (p=0.03, *nlp-14*(*null*);*nlp-15*(*null*); p=0.003, *nlp-14*(*null*);*nlp-15*(*3-5*); Levene’s test). Based on these results, we conclude that one or more of the NLP-14 peptides (but not peptide 3) are required for movement quiescence during SIS and that NLP-15 peptides are likely dispensable for SIS total quiescence, but modulate the *nlp-14* phenotype in some way.

The timing of movement quiescence is regulated by NLP-14 and NLP-15 peptides

We noted that the *nlp-14*(*null*), *nlp-14*(*2,4,5*) and *nlp-14*(*1-3*) mutants all displayed movement quiescence earlier than wild-type controls (Figure 3H,I). In the first hour post UV-stress, each mutant displayed significantly more quiescence than wild-type animals (Figure S2). *nlp-14*(2, 4, 5) mutants had the most severe defect. We expected that *nlp-15* mutants may enhance this phenotype but, to our surprise, the early quiescence phenotype of *nlp-14* mutants was suppressed rather than enhanced by *nlp-15* mutations. *nlp-14*(*null*); *nlp-15*(*3-5*) and *nlp-14*(*null*); *nlp-15*(*null*) double mutants did not show these timing defects (Figure 3J; Figure S2). These data suggest that removal of *nlp-14* alters the timing of SIS through *nlp-15*-dependent mechanisms.

NLP-14 peptides are required for defecation quiescence during SIS

Insect and crustacean orcokinins regulate rhythmic intestinal muscle contractions (Stangier, Hilbich et al. 1992, Hofer and Homberg 2006, Chen, Choi et al. 2015). In *C. elegans*, the rhythmic defecation cycle is inhibited during SIS. Defecation is precisely timed by NLP-40 neuropeptides released from the posterior intestines (Wang, Girskis et al. 2013), and during sleep it is partially inhibited by peptides released from the ALA (Nath, Chow et al. 2016). Defecation consists of three behaviors, which occur every 50-60 seconds in the following order: a posterior body contraction (pBoc), an anterior body contraction (aBoc) and an expulsion (Exp) (Thomas 1990). The defecation rates of unstressed wild-type and *nlp-14*(*2, 4, 5*) animals were similar (Figure 4A), however, in the 10-minute period beginning 85 minutes after UV exposure, the number of expulsions was significantly increased in the *nlp-14*(*2, 4, 5*) and *nlp-14*(*null*) animals (Figure 4B,D). We also observed that *nlp-14*(*2, 4, 5*) animals performed more pBoc and aBoc events without an Exp (Figure 4C). In contrast, *nlp-14*(*1-3*) mutant animals did not display defects in defecation quiescence (Figure 4E), suggesting that NLP-14 peptides 4 and 5 are not needed for this behavior. Based on defects observed in the various mutants, we conclude that the NLP-14 peptides 1 and/or peptide 3 are required for the quiescence of defecation during SIS.

We performed a temporal analysis of defecation quiescence by counting expulsions for five minutes every 30 minutes after UV irradiation (Table S4). As expected, wild-type animals slowed their defecation rate throughout the first 4 hours of UV-induced SIS. Also as expected, *nlp-14*(*2, 4, 5*) and *nlp-14*(*null*) animals displayed more frequent events, but there was high variation between animals (Figure 4F,G, Table S4). Both the *nlp-14*(*1-3*) and *nlp-14*(*1,2,4,5*) animals showed defecation temporal profiles similar to wild type (Figure 4H; Table S4). However, the *nlp-14*(*1-3*) mutant animals showed reduced expulsions throughout SIS, which is significantly lower than wild-type animals at later time points. The *nlp-15*(*3-5*) single mutants showed no defect in defecation quiescence (Table S4); surprisingly, both *nlp-14*(*null*); *nlp-15*(*3-5*) and *nlp-14*(*null*); *nlp-15*(*null*) double mutants also were similar to wild-type animals during these 4 hours (Table S4). Taken together, these data suggest that NLP-14 peptides 1 and 3 are required for defecation quiescence. However, similar to what was observed with the timing defects of *nlp-14*(*null*) and *nlp-14*(*2, 4, 5*) animals, the defecation defects may be dependent upon the presence of *nlp-15.*

NLP-14 peptides are secreted from the ALA during SIS

Since *nlp-14* is required for quiescence during SIS and is expressed in the ALA neuron (Nath, Chow et al. 2016, Taylor, Santpere et al. 2019), we predicted that quiescence induced by strong activation of this neuron would be blunted in *nlp-14* mutants. Epidermal growth factor (EGF) induces sleep in mammals (Kushikata, Fang et al. 1998, Kramer, Yang et al. 2001), *Drosophila* (Foltenyi, Greenspan et al. 2007) and *C. elegans* (Van Buskirk and Sternberg 2007), and acts during SIS by stimulating neuropeptide release from both the ALA and RIS neurons (Nelson, Lee et al. 2014, Nath, Chow et al. 2016, Konietzka, Fritz et al. 2020). Overexpression of *lin-3*, coding for EGF, induces movement quiescence (i.e. EGF-induced sleep) (Van Buskirk and Sternberg 2007). Using the WorMotel, we found that *lin-3* overexpression caused prolonged movement quiescence in otherwise wild-type animals (Figure 5A,B), but this quiescence was significantly attenuated in *nlp-14*(*null*)*, nlp-14*(*2, 4, 5*) and *nlp-14*(*1-3*) mutant animals (Figure 5A,B). These results suggest that one or more NLP-14 peptides are required for EGF-induced sleep.

In addition to quiescence of body and feeding movements, *lin-3* over-expression also induced quiescence of defecation in wild-type animals, where not a single animal performed a pBoc, aBoc, or Exp (Figure 5C–E). EGF-induced defecation quiescence was variably attenuated (i.e. defecation events were observed) in *nlp-14*(*null*) and in *nlp-14*(*2, 4, 5*) mutants (Figure 5C–E). We conclude that NLP-14 peptides are functioning downstream of EGF signaling to promote quiescence of both movement and defecation.

To test whether activity of NLP-14 peptides in ALA is sufficient to restore quiescent behavior, we made transgenic animals in which *nlp-14* expression was controlled by the *ida-1* promoter; *ida-1* is expressed strongly in ALA, but is also expressed in a few other neurosecretory neurons (Zahn, Macmorris et al. 2001). In three independent transgenic lines, movement quiescence during SIS was significantly increased relative to *nlp-14*(*2,4,5*) mutants (Figure 5F). Also, the timing defects we observed in *nlp-14*(*2, 4, 5*) animals were corrected by expressing *nlp-14* from the *ida-1* promoter (Figure S2). Additionally, defecation quiescence during SIS was much more prevalent in these *ida-1p*::*nlp-14* transgenic animals (Figure 4B) and, in fact, it was reduced below that of wild-type control levels (Table S4). Based on expression pattern, the requirement of *nlp-14* for EGF-induced sleep and rescue from the *ida-1* promoter, our data suggest that the NLP-14 peptides are released from the ALA neuron to regulate both movement and defecation quiescence during SIS. However, our data does not rule out the possibility that NLP-14 peptides are released from other cells as well.

Overexpression of *nlp-14* induces movement and defecation quiescence

We predicted that overexpression of *nlp-14* would induce quiescence of movement and defecation in active animals, like that observed for other somnogenic neuropeptides such as *flp-11, flp-13, flp-24, nlp-8,* and *nlp-22* (Nelson, Trojanowski et al. 2013, Nelson, Lee et al. 2014, Nath, Chow et al. 2016, Turek, Besseling et al. 2016). We constructed multiple transgenic lines in which *nlp-14* expression is controlled by a heat-inducible promoter. To induce strong pan-somatic expression of the gene, we subjected *hsp-16p*::*nlp-14* animals to a 30-minute heat pulse and then waited 2 hours before analysis of behavior. At this 2-hour time point, any direct effect of heat on behavior, which is minor at temperatures less than 35 degrees, had fully dissipated. Wild-type control animals were exposed to the same conditions.

Overexpression of *nlp-14* strongly suppressed body movement, which we measured by counting body bends (Figure 6A; Table S5) and by using machine vision, the WorMotel (Figure 6B,C). Overexpression of *nlp-14* also caused a significant reduction in defecation events (Figure 6D; Table S6). Thus NLP-14 peptides are both required for and capable of inducing quiescence of movement and defecation. In addition to the movement and defecation phenotypes we were focused on, we incidentally noted that many *hsp-16.2p*::*nlp-14* transgenic animals, even before induced over-expression, displayed a kinked body posture phenotype, where their body resembled a question mark (Video S1).

Many neuropeptides signal through GPCRs, which increase or decrease signaling of second messenger pathways. Movement quiescence is antagonized by signaling through the cyclic adenosine monophosphate/Protein Kinase A pathway (Cianciulli, Yoslov et al. 2019). In *C. elegans*, PKA activity can be experimentally increased by genetic impairment in the gene *kin-2*, which encodes a regulatory subunit of PKA (Charlie, Thomure et al. 2006). We found that the increased PKA activity of *kin-2*(*ce179*) mutants (Charlie, Thomure et al. 2006), stimulated movement but not defecation following *nlp-14* overexpression (Figure 6E,F). These data suggest that NLP-14 peptides inhibit cAMP/PKA in cells regulating movement quiescence.

Based on our analysis of different *nlp-14* loss-of-function alleles, suggesting that removal of subsets of NLP-14 peptides affected behavioral quiescence in unique ways, we over expressed different combinations of the peptides. Overexpression of NLP-14-1 or NLP-14-3 induced quiescence of movement but not defecation, while overexpression of NLP-14-1 and NLP-14-2 strongly induced quiescence of movement and weakly of defecation. Overexpression of NLP-14-(1-3) or NLP-14-(1-4) strongly induced quiescence of both movement and defecation (Figure 6G,H). These data, together with the loss-of-function analyses, suggest that all five NLP-14 peptides regulate movement quiescence; defecation quiescence, however, is prominently regulated by peptides 1 and 3, while the other peptides may play more subtle modulating roles.

Orcokinin receptors are unknown for all ecdysozoans

Orcokinin receptors have not been identified in any animal, despite screening attempts using heterologous expression systems (Yamanaka, Hua et al. 2010, Yamanaka, Roller et al. 2011). In *C. elegans*, the receptor NPR-10 has been proposed as an NLP-14 receptor, based on genetic interactions and anatomical connectivity (Hapiak, Summers et al. 2013). We did not detect changes in movement quiescence during SIS in the presumptive *npr-10*(*ok1442*) null mutants. *ok1442* mutants have a 788bp deletion that is predicted to result in a frameshift and premature stop in exon 5 and therefore a truncated protein composed of only five transmembrane domains (Figure S1). Our data suggest that NPR-10 is not the receptor for NLP-14 during sleep regulation or that other receptors function redundantly together with NPR-10. To date, no orcokinin receptor for any animals has been convincingly identified.

Discussion

Orcokinin neuropeptides are conserved in Ecdysozoa, which consists of organisms that undergo molting (Aguinaldo, Turbeville et al. 1997). Millions of years of evolution separate these animals, yet orcokinin peptide sequences are highly similar (Yasuda-Kamatani and Yasuda 2000, Nathoo, Moeller et al. 2001, Hofer, Dircksen et al. 2005, Hofer and Homberg 2006, Dickinson, Stemmler et al. 2009, Chen, Choi et al. 2015, Wulff, Sierra et al. 2017, Koziol 2018). Functional studies have demonstrated that they regulate insect circadian rhythms and molting (Hofer and Homberg 2006, Wulff, Sierra et al. 2017), rhythmic smooth muscle contractions of insects and crustaceans (Stangier, Hilbich et al. 1992, Li, Pulver et al. 2002, Skiebe, Dreger et al. 2002) and decision making behaviors and male mating in *C. elegans* (Hapiak, Summers et al. 2013, Sherlekar, Janssen et al. 2013). Here we described a novel function for the orcokinin encoded by *nlp-14* and *nlp-15* during the regulation of sleep.

Using a combination of loss-of-function and overexpression studies we find that NLP-14 and NLP-15 peptides regulate two sleep states, developmentally timed sleep (DTS), which resembles sleep in animals that are strongly circadian (Trojanowski and Raizen 2016), and stress-induced sleep (SIS), a behavior required for recovery following exposure to damaging stress (Hill, Mansfield et al. 2014). The five NLP-14 peptides play a larger role in the regulation of SIS than DTS. They promote movement and defecation quiescence, the latter of which is largely regulated by NLP-14 peptides 1 and 3. We propose that these sleep-regulatory roles are more conserved in Ecdysozoa.

Developmentally timed sleep

Both *nlp-14* and *nlp-15* are expressed in the sleep regulating neurons ALA and RIS (Van Buskirk and Sternberg 2007, Turek, Lewandrowski et al. 2013). Individually, they are dispensable for DTS but removal of both genes reduces movement quiescence without causing molting defects. This is in contrast to studies done with the kissing bug *Rhodnius prolixus* where disruption of orcokinin by RNA interference (RNAi) caused molting defects (Wulff, Sierra et al. 2017). We also did not observe molting difficulties when *nlp-14* was over-expressed, suggesting that either the role of these peptides is strictly behavioral or that there is degeneracy in the control of molting (Choi, Chatzigeorgiou et al. 2013, Nelson, Trojanowski et al. 2013, Turek, Besseling et al. 2016).

Stress induced sleep

In contrast to its relatively minor roles in DTS, NLP-14 is more important during stress-induced sleep (SIS), where the removal of all or subsets of peptides causes strong defects in movement and defecation quiescence. Numerous neuropeptides regulate movement quiescence, including FLP-11, secreted from the RIS (Konietzka, Fritz et al. 2020) and

FLP-13, FLP-24, and NLP-8 (Nelson, Janssen et al. 2015, Nath, Chow et al. 2016), released from ALA. These molecules signal through many GPCRs (Nelson, Janssen et al. 2015, Iannacone, Beets et al. 2017), reducing cAMP/PKA signaling in different cells (Cianciulli, Yoslov et al. 2019). The orcokinins can be added to this expanding list of somnogenic neuropeptides. This observation in *C. elegans* that multiple peptides can induce quiescence when over-expressed is consistent with studies in fish, which have identified several somnogenic neuropeptides using an over-expression approach (Chiu, Rihel et al. 2016, Lee, Andreev et al. 2017). Therefore, this complexity to sleep regulation appears to be phylogenetically conserved and demonstrates the importance of sleep to all animals.

Our data, however, suggest that the orcokinins in *C. elegans* are not acting strictly as somnogens. Removal of *nlp-14* shifts the timing of SIS, such that it occurs earlier. We propose that NLP-14 peptides may be functioning to promote aversive behaviors associated with nocioception, a previously described role (Hapiak, Summers et al. 2013), and act as a somnogen only at later stages, to facilitate recovery from the stressful exposure.

Surprisingly, this early increased quiescence in *nlp-14* mutants is dependent on the presence of *nlp-15*. An interpretation of this could be that NLP-15 and NLP-14 peptides are antagonizing one another during the injurious response to UV, promoting both quiescence and arousal, respectively. At early time points after UV exposure, NLP-14 peptides may promote behavioral arousal, perhaps to allow for an escape response, whereas NLP-15 may promote quiescence at all time points.

At later time points, both promote sleep. After exposures to injurious conditions such as UV light or high heat, animals must balance the benefits of aversion and escape with those of recovery, which are linked to sleep. More work needs to be done to test this idea.

The defecation motor program is both stimulated (Wang, Girskis et al. 2013) and inhibited by neuropeptides (Nath, Chow et al. 2016). When awake, NLP-40 peptides are released from the posterior intestines following rhythmic calcium fluxes, bind their receptor AEX-2, stimulating cAMP/PKA and calcium signaling in the AVL and DVB neurons. This stimulates GABA release, which excites the enteric muscles and initiates an expulsion (Wang, Girskis et al. 2013). During SIS, the ALA neuron releases NLP-8 peptides to inhibit defecation (Nath, Chow et al. 2016). We find that ALA and/or RIS also release NLP-14 peptides. Our data indicates that NLP-14's effects on defecation are cAMP/PKA-independent. PKA functions in the AVL and DVB motor neurons during defecation to increase their activity leading to enteric muscle contractions that drive the expulsion events (Wang and Sieburth 2013). Based on this, our data suggest that NLP-14 peptides are functioning either directly on the enteric muscles or downstream of PKA in AVL and DVB. The notion that orcokinins act directly on GI motility would be consistent with observations of crustacean orcokinins, which directly regulate smooth muscle of the gut (Li, Pulver et al. 2002) and deuterostome starfish myorelaxant peptides (SMPs), which promote the relaxation of stomach muscle (Lin, Egertova et al. 2018). There is a sequence similarity between the SMPs and NLP-14 peptides (Kim, Kim et al. 2016). Therefore the role of orcokinin/SMPs in smooth muscle regulation may be conserved.

A conserved sleep-regulating role for orcokinins neuropeptides

Are these sleep functions of NLP-14 and NLP-15 more broadly conserved in Ecdysozoa? Though prior studies have not reported the requirement of orcokinins for sleep, some observations point towards a sleep-regulating role in insects too. Elegant work by Hofer and Homber showed that orcokinin injections result in a circadian phase shift, measured by wheel-running activity in cockroaches. Interestingly, while the authors do not emphasize this point, their actographic data indicate strong inhibition of activity 24-48 hours after the orcokinin injection (Hofer and Homberg 2006). Hence, they observed both a change in sleep timing and in sleep/activity in response to orcokinins injections, much as we observe a change in timing and in sleep following *nlp-14* overexpression in *C. elegans.*

DTS in *C. elegans* occurs coincident with the molt (Raizen, Zimmerman et al. 2008). Insect larvae can sleep between molts (Szuperak, Churgin et al. 2018) and also become quiescent during the molt, a behavior called molt-sleep (Reinecke, Buckner et al. 1980). Gene-expression analysis suggests that molt-sleep is regulated by neuropeptide signaling (MacWilliam, Arensburger et al. 2015). Removal of orcokinins in the kissing bug causes molting failure and death (Wulff, Sierra et al. 2017). It is possible that inhibition of either the behavioral or physiological aspects of molt-sleep is the cause of this lethality. To test for a conserved sleep-regulating role during the molt, it will be important to measure sleep following orcokinin manipulation during inter-molt and molt-sleep in insects and sleep in crustaceans.

In contrast to effects during DTS, the NLP-14 peptides play a more important role during SIS regulation. Heat-induced recovery sleep occurs in *Drosophila melanogaster* and is regulated by the same family of neuropeptides controlling SIS in *C. elegans* (Lenz, Xiong et al. 2015). In an effort to test the generalizability of our findings, we propose that an initial approach would be to test the necessity of orcokinins in *Drosophila.* It would be particularly interesting to test for an orcokinin role in crayfish, which display slow-wave brain activity, similar to mammals (Ramon, Hernandez-Falcon et al. 2004, Ramon, Mendoza-Angeles et al. 2012). Considering that sickness and injury increase sleep in mammals (Imeri and Opp 2009), SIS may exist in crustaceans as well.

What about tardigrades? These amazingly hardy animals can survive some of the harshest conditions, like desiccation and extreme heat and osmotic pressure. They do so by entering a state of extended quiescence referred to as cryptobiosis (Crowe 1975). This can promote survival for years, but is reversible, at which point their bodies can be remarkably repaired (Wright, Westh et al. 1992). Cryptobiosis may represent an extreme version of SIS. Is this protective behavioral state regulated by orcokinins? If so, orcokinins may be an evolutionarily ancient mechanism controlling protective behavioral quiescence in Ecdysozoa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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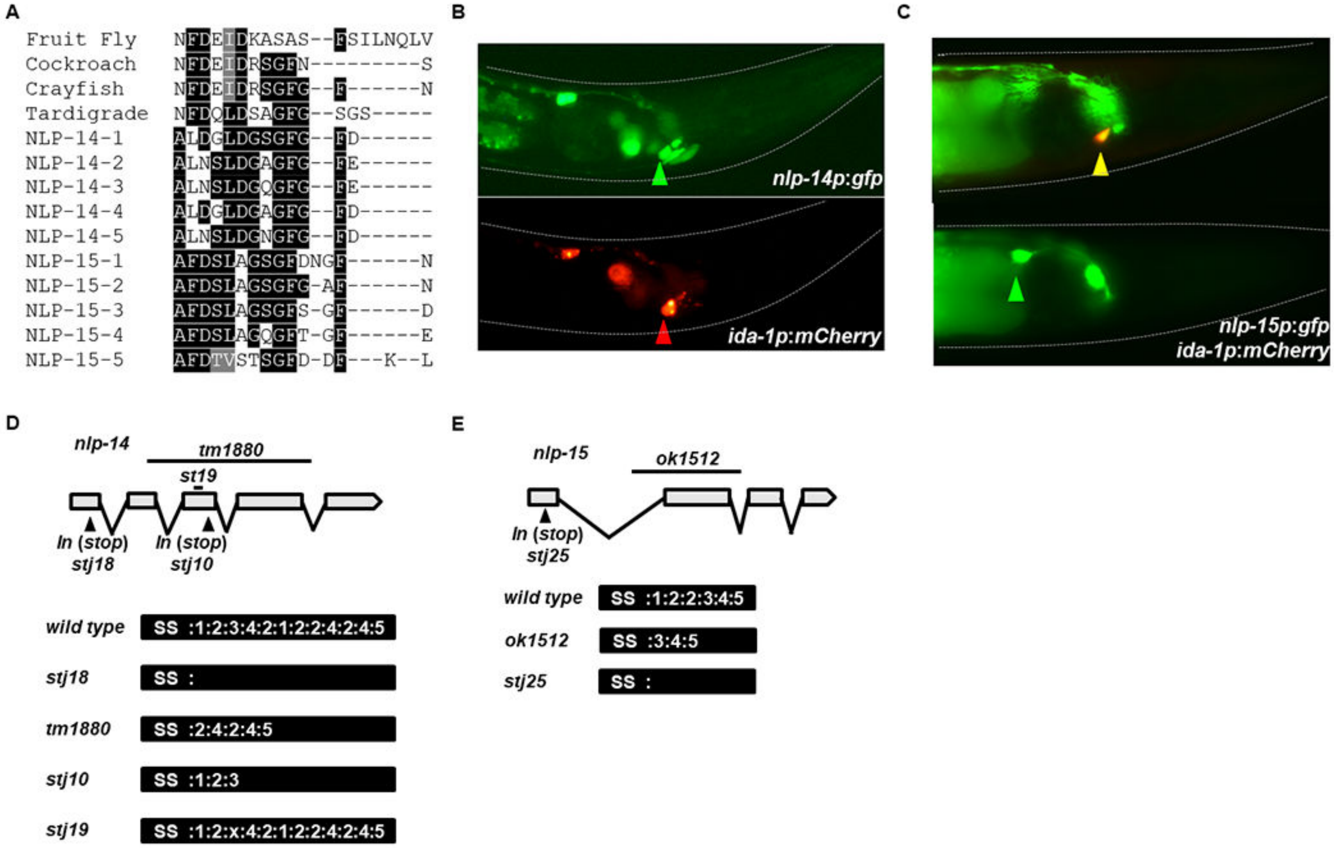

Figure 1: NLP-14 and NLP-15 neuropeptides are related to arthropod orcokinins and are expressed in sleep-promoting cells.

A) Peptide alignment of orcokinins from: *Drosophila melanogaster* (fruit fly), *Blatella germanica* (cockroach), *Orconectes limosus* (crayfish), *Hypsibius dujardini* (Tardigrade) and *C. elegans* (Nematode). **B)** Representative images of an animal expressing *gfp* from the promoter of *nlp-14* (top image) and mCherry from the promoter of *ida-1* (bottom image). ALA expression is denoted by a green and red arrowhead. Anterior – Right; Dorsal – Bottom, Ventral – Top. **C)** Representative images of an animal expressing *gfp* from the promoter of *nlp-15* and the same image superimposed with mCherry from the promoter of *ida-1*. Expression in the RIS (*gfp* only) and ALA (*gfp* and *mCherry*) are denoted with green and yellow arrowheads, respectively. Anterior – Right; Dorsal – Bottom, Ventral – Top. **D)** Gene and protein structure for NLP-14, highlighting the location of deletion and insertion alleles. **E)** Gene and protein diagram for NLP-15, highlighting the location of a deletion and insertion allele. SS denotes signal sequence.

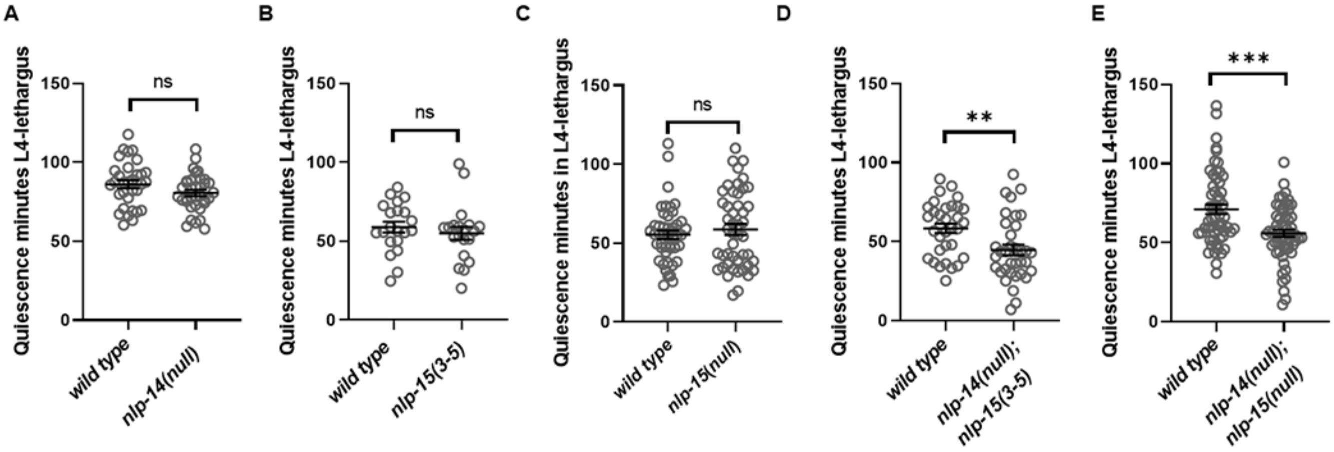

Figure 2: The *C. elegans* orcokinins play a small role during DTS.

A) Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*) animals (N ≥ 32). **B)** Movement quiescence during L4 lethargus in wild-type and *nlp-15*(*ok1512*) animals (N ≥ 20). **C)** Movement quiescence during L4 lethargus in wild-type and *nlp-15*(*stj25*) animals (N ≥ 46). **D)** Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*); *nlp-15*(*ok1512*) animals (N ≥ 33, $**p < 0.01$). **E)** Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals (N ≥ 58, $***p < 0.001$). Statistical significance was calculated using Student’s t-test. All error bars represent mean +/− SEM.

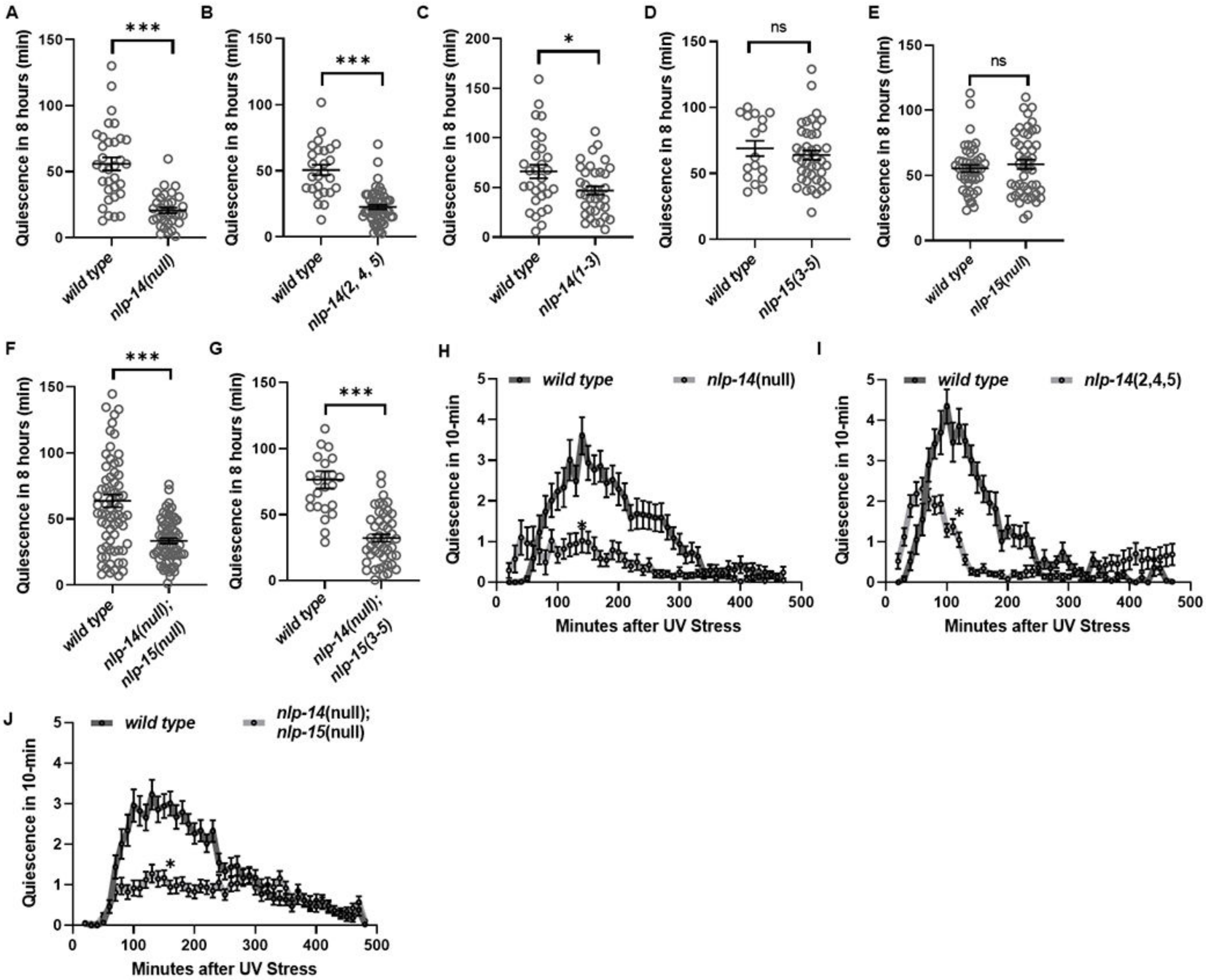

Figure 3: NLP-14 neuropeptides are required for movement quiescence during SIS.

A) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*) animals (N ≥ 33, ***p < 0.001). **B)** Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*tm1880*) animals (N ≥ 26, ***p < 0.001). **C)** Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj10*) animals (N ≥ 30, *p<0.05). **D)** Movement quiescence during UV-induced SIS in wild-type and *nlp-15*(*ok1512*) animals (N ≥ 17). **E)** Movement quiescence during UV-induced SIS in wild-type and *nlp-15*(*stj25*) animals (N ≥ 33). **F)** Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals (N ≥ 68, ***p < 0.001). **G)** Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*); *nlp-15*(*ok1512*) animals (N ≥ 24, ***p < 0.001). **A-G)** Statistical significance was calculated using Student's t-test. **H)** Average quiescence in 10-minute windows over 8-hours during UV-induced SIS of wild-type and *nlp-14*(*stj18*) animals (N ≥ 33, * P<0.01 at 70, 90-270 minutes). **I)** Average quiescence in 10-minute windows over 8-hours during UV-induced SIS of wild-type and *nlp-14*(*tm1880*) animals (N ≥ 26, * P<0.01 at 40,50 80-190 minutes). **J)** Average quiescence in 10-minute windows over

8-hours during UV-induced SIS of wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals (N ≥ 68, * P<0.01 at 80-230 minutes). **H-J)** Statistical significance was calculated using two-way ANOVA followed by Sidak's multiple comparisons test. All error bars represent mean +/− SEM.

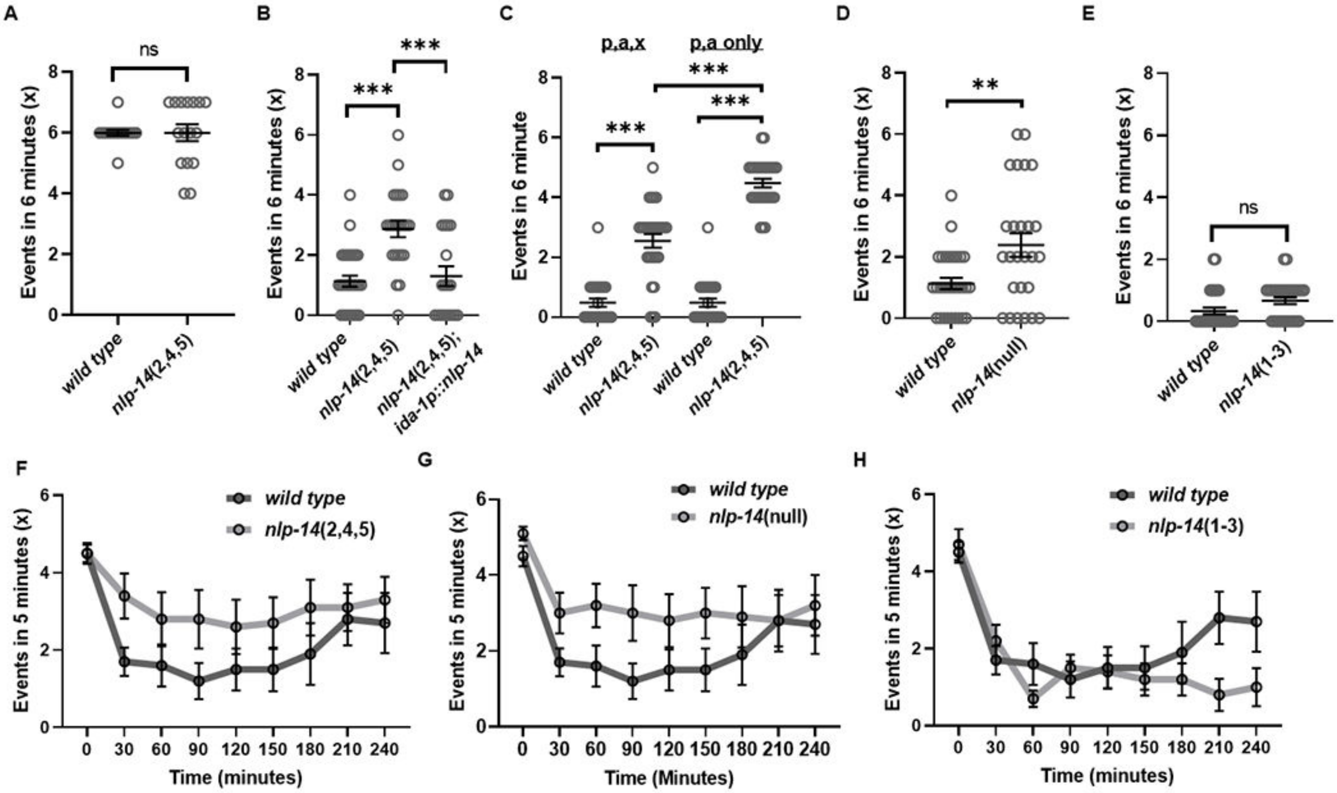

Figure 4: NLP-14 neuropeptides are indispensable for defecation quiescence during SIS.

A) Expulsions (x) performed in 6-minutes, of wild-type and *nlp-14*(*tm1880*) animals, who had not been exposed to any external stress. **B)** Wild-type, *nlp-14*(*tm1880*) and *nlp-14*(*tm1880*); *ida-1p*::*nlp-14* animals 85-95 minutes following UV-stress (N ≥ 20, ***P<0.001). **C)** p- (Posterior body contraction or pBoc) and a- (Anterior body contraction or aBoc) events or p-, a- and x-events in 6-minutes of wild-type and *nlp-14*(*tm1880*) animals, 85-95 minutes post-UV stress (N ≥ 26, ***p<0.001). **D)** x-events performed in 6-minutes, of wild-type and *nlp-14*(*stj18*) animals (N ≥ 26, **p<0.01) and **E)** wild-type and *nlp-14*(*stj10*) animals (N=30). **F)** Defecation quiescence profile of *nlp-14*(*tm1880*), **G)** *nlp-14*(*stj18*), and **H)** *nlp-14*(*stj10*) animals (N=10, *p<0.05 210 minutes). **A,D,E)** Statistical significance was calculated using Student's t-test. **B-C)** Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparisons test. **H)** Statistical significance was calculated using two-way ANOVA followed by Sidak's multiple comparisons test. All error bars represent mean +/− SEM.

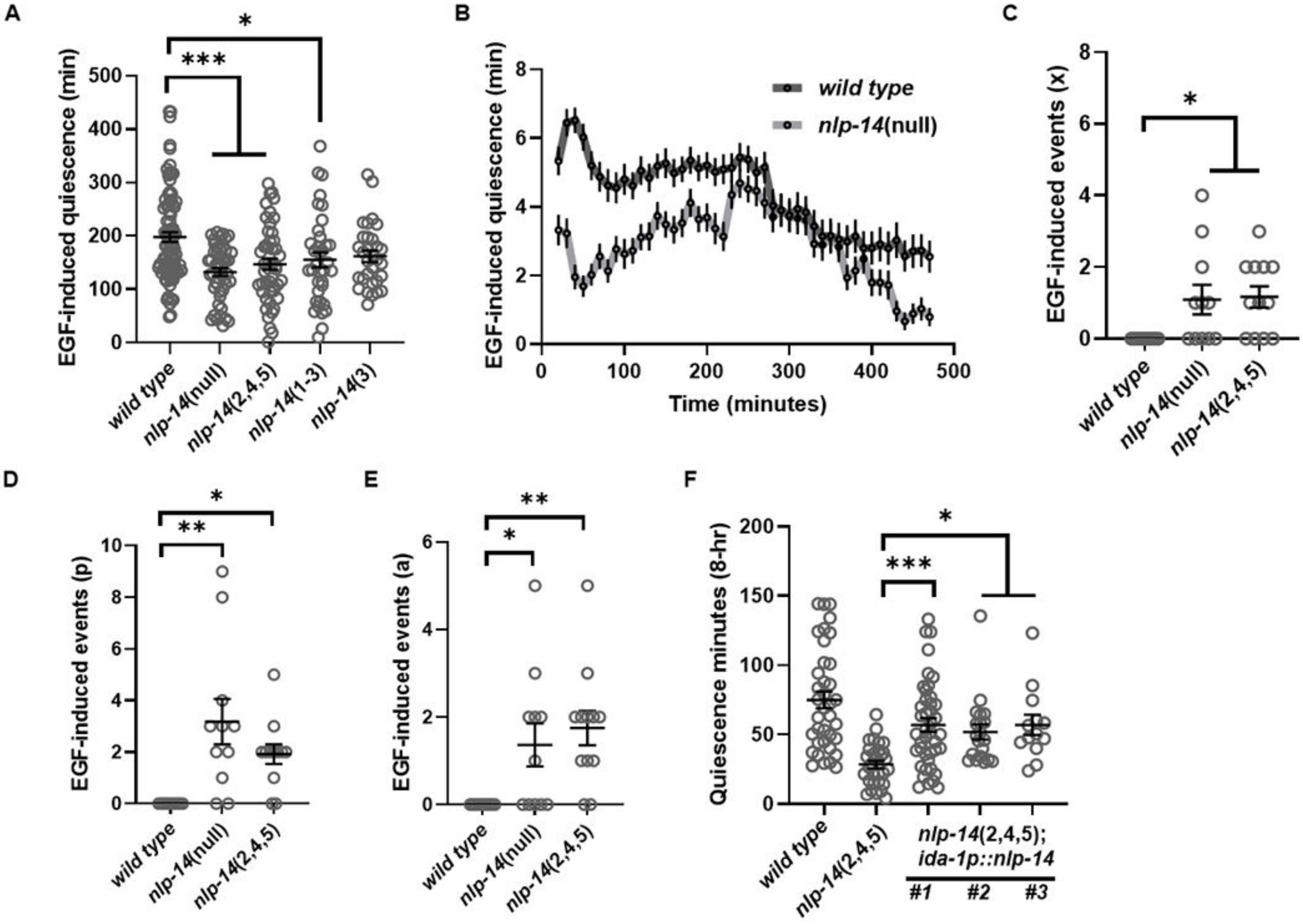

Figure 5: NLP-14 peptides function downstream of EGF from the ALA neuron.

A) Total minutes of quiescence in 4-hours, following overexpression of *lin-3*, in the following genetic backgrounds: Wild type, *nlp-14*(*stj18*), *nlp-14*(*tm1880*), *nlp-14*(*stj10*), and *nlp-14*(*stj19*) (N ≥ 31, *p<0.05, ***p<0.001). **B)** Average quiescence in 10-minute windows over 4-hours following *lin-3* over-expression in wild-type and *nlp-14*(*stj18*) animals (N =48, p<0.05 100-120 minutes, p<0.001 20-80 minutes). Statistical significance was calculated using two-way ANOVA followed by Sidak's multiple comparisons test. **C)** x-, **D)** p- and **E)** a-events performed in 5 minutes, 2-hours after the overexpression of *lin-3* in wild-type, *nlp-14*(*stj18*) and *nlp-14*(*tm1880*) animals (N ≥ 10, *p<0.05, **p<0.01). **F)** Movement quiescence during UV-induced SIS in wild-type, *nlp-14*(*tm1880*) and *nlp-14*(*tm1880*); *ida-1p*::*nlp-14* animals (N ≥ 11, *p<0.05, ***p<0.001). Numbers below *nlp-14*(*tm1880*); *ida-1p*::*nlp-14* indicate distinct transgenic lines. **A,C-F)** Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparisons test. All error bars represent mean +/− SEM.

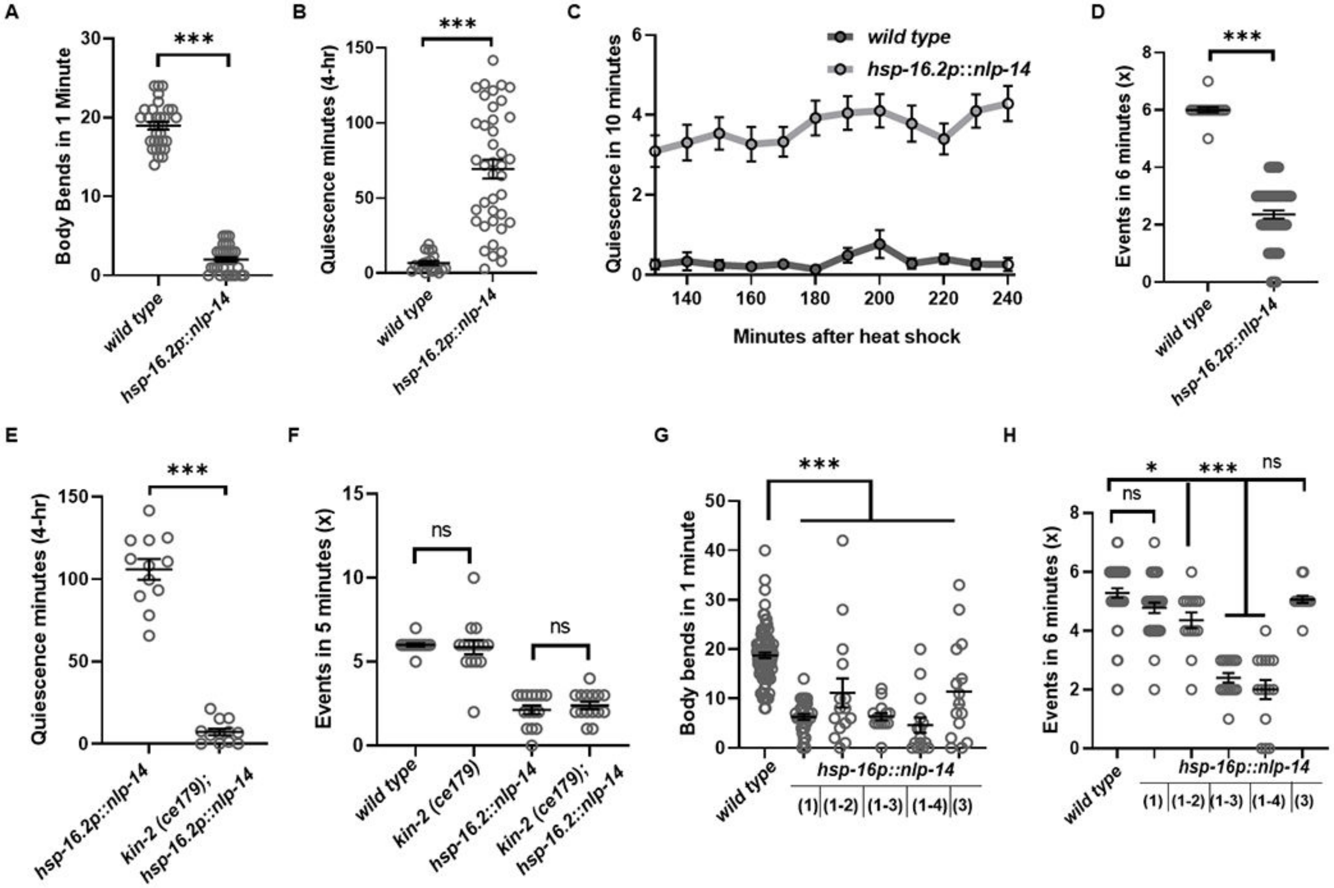

Figure 6: NLP-14 peptides are capable of inducing sleep-like behaviors.

A) Body bends performed in 1 minute, 2-2.5-hours after a 30-minute, 33 degree Celsius heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals (N = 32, ***p<0.001). **B)** Movement quiescence during 4-hours following a 30-minute, 33 degree Celsius heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals (N ≥ 19, ***p<0.001). **C)** Average quiescence in 10-minute windows over 4-hours following a 30-minute, 33 degree Celsius heat shock, wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals (N ≥ 19, ***P<0.001 at all time points displayed). **D)** x-events performed in 6-minutes, 2-2.5-hours after a 30-minute, 33 degree Celsius heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals (N = 16, ***p<0.001). **E)** Movement quiescence during 4-hours following a 30-minute, 33 degree Celsius heat shock, *hsp-16.2p*::*nlp-14* (strain SJU27) and *kin-2*(*ce179*); *hsp-16.2p*::*nlp-14* animals (N =12, *p<0.001). **F)** x-events performed in 5-minutes by wild-type and *kin-2*(*ce179*) animals without exposure to heat shock and by *hsp-16.2p*::*nlp-14* (strain SJU27) and *kin-2*(*ce179*); *hsp-16.2p*::*nlp-14* animals 2-2.5-hours after a 30-minute, 33 degree Celsius heat shock (N = 15). **G)** Body bends performed in 1-minute, 2-2.5-hours after a 30-minute, 33 degree Celsius heat shock, by wild type and animals over-expressing *nlp-14*(1), (1-2), (1-3), (1-4) or (3) (N ≥15, ***p<0.001). **H)** x-events performed in 5-minutes, 2-2.5-hours after a 30-minute, 33 degree Celsius heat shock, by wild type and animals overexpressing *nlp-14*(1), (1-2), (1-3), (1-4) or (3) (N ≥ 15, *p<0.05, ***p<0.001). **A,B,D,E)** Statistical significance was calculated using Student's t-test. **C)** Statistical significance was calculated using two-way ANOVA followed by Sidak’s multiple comparisons test. **F-H)** Statistical significance was calculated using one-way ANOVA followed by Tukey’s multiple comparisons test. All error bars represent mean +/− SEM.