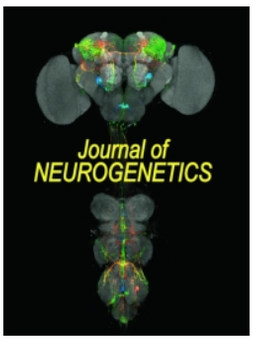

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ORIGINAL RESEARCH ARTICLE

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.

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KEYWORDS
C. elegans; sleep; *nlp-14*; *nlp-15*; orcokinin

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 *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, Aeschimann, & Großhans, 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 *et al.*, 2008).

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

Orcokinins regulate insect ecdysis and circadian activity. In kissing bugs, disruption of orcokinin signaling causes molting defects (Wulff *et al.*, 2017), perhaps partially due to a role for these peptides in the biosynthesis of the ecdysteroids (Yamanaka *et al.*, 2011; Zitnan *et al.*, 1999). In cockroaches, orcokinin peptides injected into the brain induce a phase shift in circadian sleep/wake behavior (Hofer & Homberg 2006). A role in the regulation of both molting and circadian rhythms suggests the intriguing hypothesis that an ancestral role for orcokinins 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 *et al.*, 2001). NLP-14 peptides modulate cholinergic signaling during male mating (Sherlekar *et al.*, 2013) and mediate decision-making during nociceptive behaviors (Hapiak *et al.*, 2013). *nlp-14* transcripts are upregulated during the 2-h period prior to ecdysis (George-Raizen, Shockley, Trojanowski, Lamb, & Raizen, 2014), when the animals display sleep-like quiescent behavior (Raizen *et al.*, 2008). Single-cell transcriptomic data suggests that *nlp-14* is expressed in the sleep-promoting ALA neuron (Nath, Chow, Wang, Schwarz, & Sternberg, 2016; Taylor *et al.*, 2019), while its paralog *nlp-15* is expressed in both the ALA neuron and in another sleep promoting neuron called RIS (Taylor *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 (Raizen *et al.*, 2008; Singh & Sulston, 1978). Movement quiescence during DTS is controlled primarily by the RIS neuron which releases FLP-11 neuropeptides (Turek, Besseling, Spies, Konig, & Bringmann, 2016; Turek, Lewandrowski, & Bringmann, 2013) with a minor role for NLP-22 peptides released from

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Supplemental data for this article can be accessed here.

the RIA neurons (Nelson *et al.*, 2013). Arousal during DTS is mediated by pigment dispersing factor (PDF) neuropeptides, which also mediate arousal in insects (Choi, Chatzigeorgiou, Taylor, Schafer, & Kaplan, 2013; Renn, Park, Rosbash, Hall, & Taghert, 1999). SIS is controlled by both the ALA and RIS interneurons, via the release of a collection of neuropeptides (Lenz, Xiong, Nelson, Raizen, & Williams, 2015; Nelson *et al.*, 2014; Turek *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 overexpression 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 °C on agar plates containing nematode growth medium and fed the OP50 derivative bacterial strain DA837 (Davis *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 & 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, Watertown, MA). 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, Carr, & Hirsh, 1985). The strains SJU27, SJU241 and SJU242 were integrated using UV irradiation, as described (Mello & Fire, 1995).

Reverse transcription-PCR (RT-PCR) of *nlp-14*(*tm1880*) was accomplished by isolating total RNA using an RNeasy mini kit (Qiagen ©, Hilden, Germany), followed by cDNA synthesis using SuperScript™ One-Step RT-PCR System

(Thermo Fisher ©, Waltham, MA). 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 *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 was 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. Fifteen to twenty 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 nlp-14 (*tm1880*) strain 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 three times to reduce the number of unlinked mutations on the five autosomal chromosomes.

WorMotel behavioral assays

Movement quiescence was quantified during both DTS and stress-induced sleep (SIS), using the WorMotel, as previously described (Churgin *et al.*, 2017). For DTS, we monitored active L4 animals (pre-lethargus) of each genotype for 12-h. Due to 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-s for 12 h, and quiescence was quantified and DTS was manually measured based on a definable peak of quiescence, as previously described (Raizen *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/m^2 of UV light to induce SIS (DeBardeleben, Lopes, Nessel, & Raizen, 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 min. 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-s for 8 or 4 h, 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 min. Body bends were counted manually using a stereomicroscope for 60-s, 2 h 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 min 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 min and analyzed for 5 min between 2 and 2.5 h after heat exposure. For SIS, day-1 adults were exposed to 1500 J/m^2 of UV light in a UV-cross linker (Ultraviolet, 254 UVP) on growth plates and the defecation cycle was measured for 5–6 min, 85–95 min post-UV. For temporal SIS analyses, a single animal was examined for 5-min every 30 min for 4-h, 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 25 mM sodium azide.

Alignments

Peptide sequences were obtained from the National Center for Biotechnology Information (NCBI) and aligned using online T-coffee software (Notredame, Higgins, & Heringa, 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 *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 1(A)). *nlp-14* expression has been

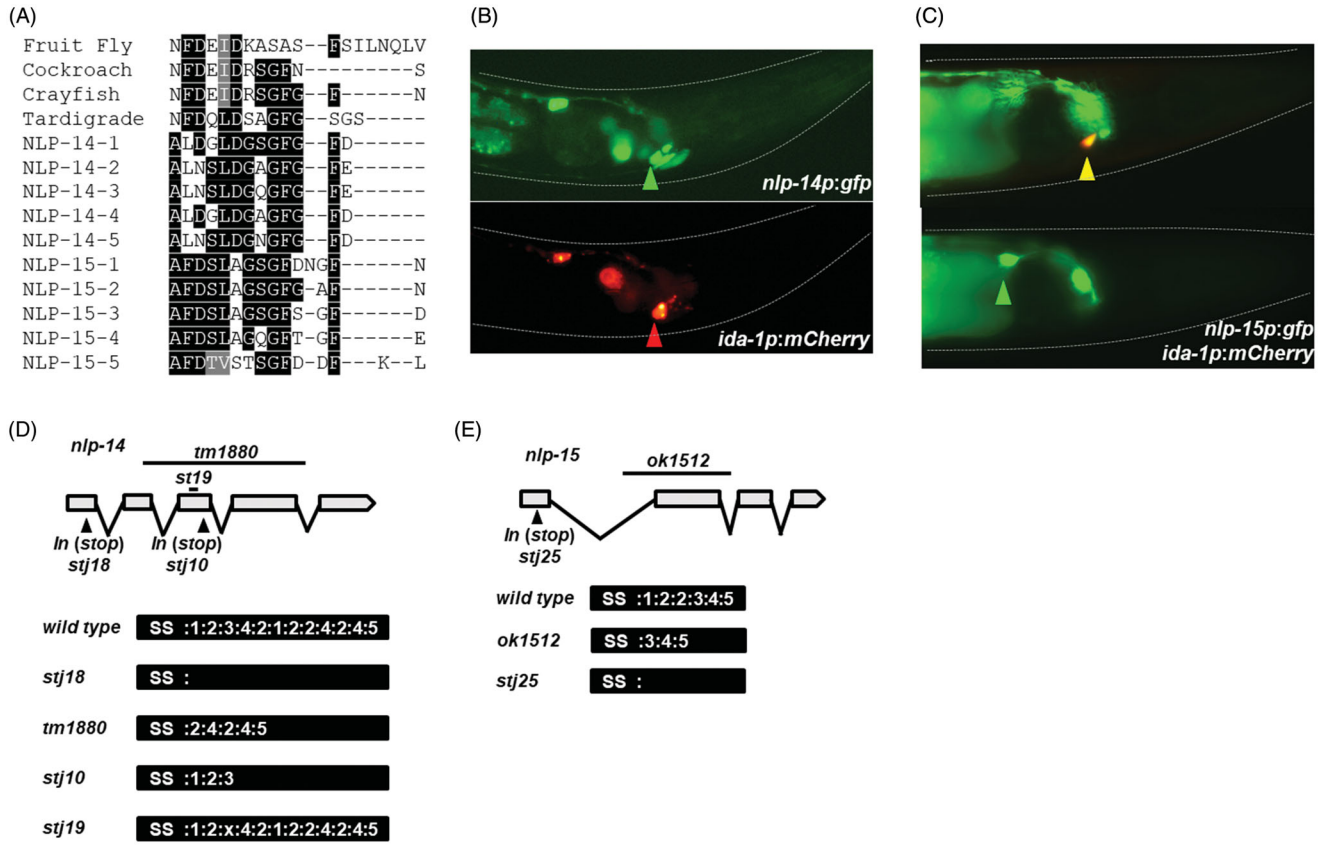

Figure 1. NLP-14 and NLP-15 neuropeptides are related to arthropod orcokinins and are expressed in sleep-promoting cells. (A) Peptide alignment of orcokinins from: *Drosophila melanogaster* (fruit fly), *Blattella germanica* (cockroach), *Orconectes limosus* (crayfish), *Hypsibius dujardini* (Tardigrade) 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 arrowheads. 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 ALA (top - gfp and mCherry) and RIS (bottom - gfp only) are denoted by arrowheads. 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.

demonstrated in the ventral cord and some sensory and interneurons (Nathoo *et al.*, 2001), as well as in male-specific neurons (Sherlekar *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 *et al.*, 2016; Taylor *et al.*, 2019). ALA and RIS are central sleep-promoting neurons (Hill, Mansfield, Lopez, Raizen, & Van Buskirk, 2014; Konietzka *et al.*, 2020; Turek *et al.*, 2013). 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, Dong, Day, & Hutton, 2001) (Figure 1(B)). Similarly, a GFP transcriptional reporter for *nlp-15* showed expression in both ALA and RIS neurons (Figure 1(C)). 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 1(D)). 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 1(E)). 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, & Seydoux, 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 1(D)). Similarly, the *nlp-15*(*stj25*) strain contains an insertion of a stop codon 5′ of all five NLP-15 peptides (Figure 1(E)). 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* (Raizen *et al.*, 2008; Singh & Sulston, 1978). Orcokinins regulate ecdysis

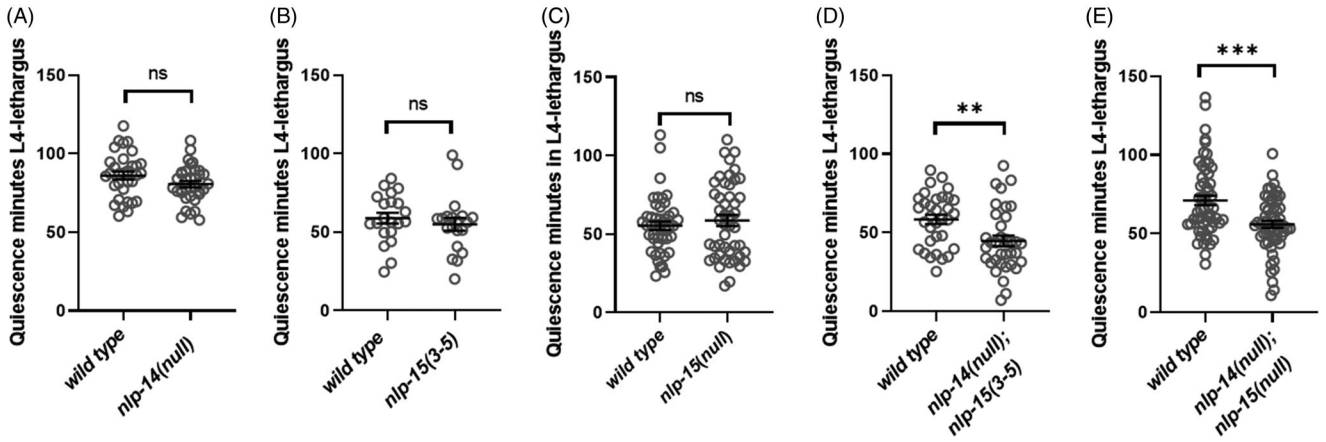

Figure 2. The *C. elegans* orcokinins play a small role during DTS. (A) Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*) animals ($N \geq 32$). (B) Movement quiescence during L4 lethargus in wild-type and *nlp-15*(*ok1512*) animals ($N \geq 20$). (C) Movement quiescence during L4 lethargus in wild-type and *nlp-15*(*stj25*) animals ($N \geq 46$). (D) Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*); *nlp-15*(*ok1512*) animals ($N \geq 33$, $^{**}p<.01$). (E) Movement quiescence during L4 lethargus in wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals ($N \geq 58$, $^{***}p<.001$). Statistical significance was calculated using Student's *t*-test. All error bars represent mean ± SEM.

and ecdysteroid biosynthesis in insects (Wulff *et al.*, 2017; Yamanaka *et al.*, 2011). We hypothesized that DTS and/or the molt would be disrupted in *nlp-14* and/or *nlp-15* mutants. Using the WorMotel (Churgin *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 2(A–C)). As 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 2(D,E)). We found no molting defects by carefully scanning double mutant strains via stereomicroscopy or by inspecting select animals at 1000× 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 *et al.*, 2013; Turek *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 *et al.*, 2014; Konietzka *et al.*, 2020), we tested their necessity for movement quiescence. SIS was induced by UV irradiation (DeBardeleben *et al.*, 2017) and animals were monitored on a WorMotel (Churgin *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 3(A–C,H,I); Figure S1), while *nlp-14*(*1,2,4,5*), *nlp-15*(*3-5*) and *nlp-15*(*null*) animals did not (Figure 3(D,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 3(F,G,J); Figure S1). However, the *nlp-15* mutations increased the variance of the *nlp-14*(*null*) phenotype ($p=.03$, *nlp-14*(*null*);*nlp-15*(*null*); $p=.003$, *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 3(H,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 3(J); 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 (Chen *et al.*, 2015; Hofer & Homberg 2006; Stangier, Hilbich, Burdzik, & Keller, 1992). 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 *et al.*, 2013), and during sleep it is partially inhibited by peptides released from the ALA (Nath *et al.*, 2016). Defecation consists of three behaviors, which occur every 50–60 s 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*)

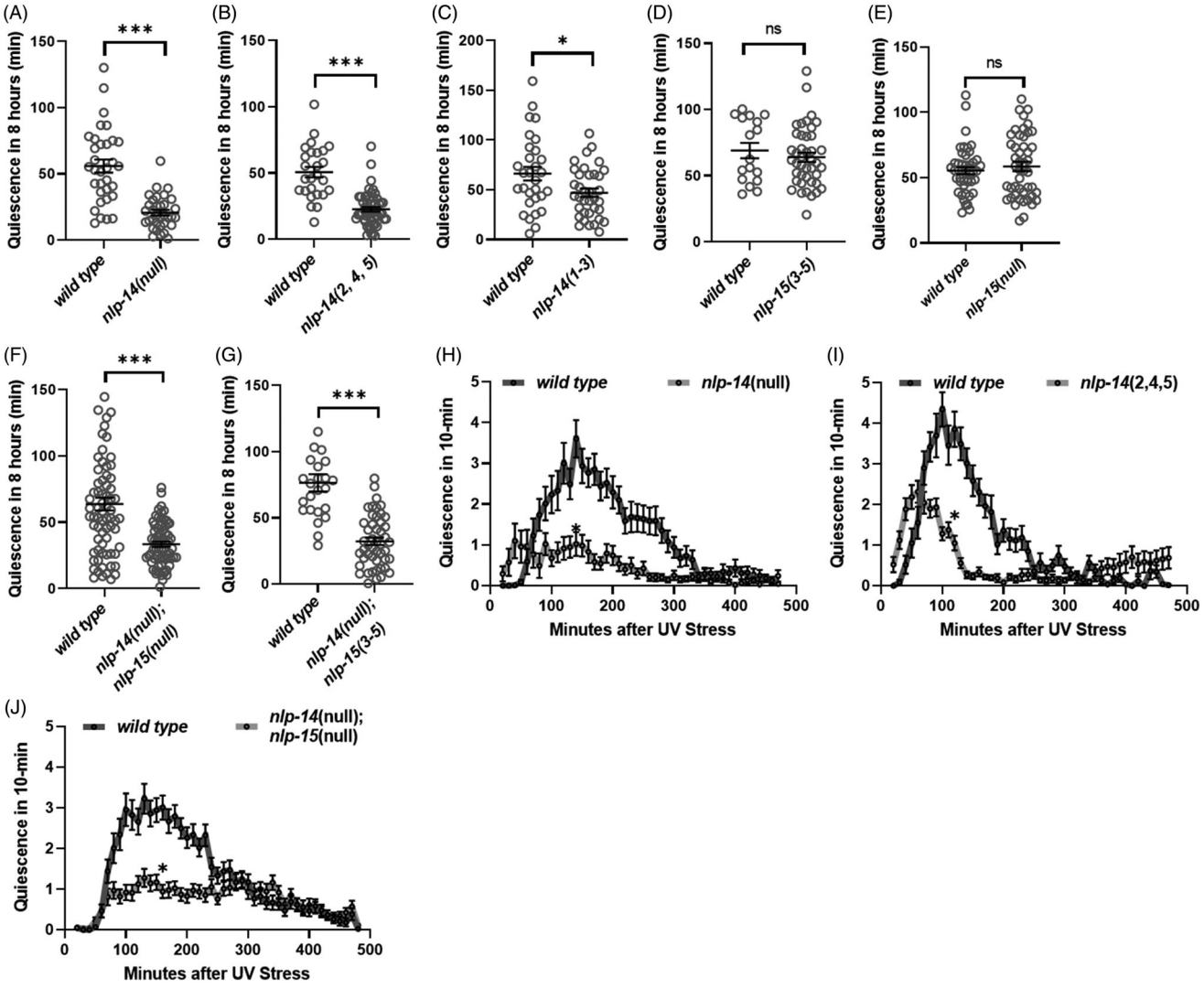

Figure 3. NLP-14 neuropeptides are required for movement quiescence during SIS. (A) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*) animals ($N \geq 33$, ***$p<.001$). (B) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*tm1880*) animals ($N \geq 26$, ***$p<.001$). (C) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj10*) animals ($N \geq 30$, *$p<.05$). (D) Movement quiescence during UV-induced SIS in wild-type and *nlp-15*(*ok1512*) animals ($N \geq 17$). (E) Movement quiescence during UV-induced SIS in wild-type and *nlp-15*(*stj25*) animals ($N \geq 33$). (F) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals ($N \geq 68$, ***$p<.001$). (G) Movement quiescence during UV-induced SIS in wild-type and *nlp-14*(*stj18*); *nlp-15*(*ok1512*) animals ($N \geq 24$, ***$p<.001$). (A–G) Statistical significance was calculated using Student's *t*-test. (H) Average quiescence in 10-min windows over 8-h during UV-induced SIS of wild-type and *nlp-14*(*stj18*) animals ($N \geq 33$, *$p<.01$ at 70, 90–270 min). (I) Average quiescence in 10-min windows over 8-h during UV-induced SIS of wild-type and *nlp-14*(*tm1880*) animals ($N \geq 26$, *$p<.01$ at 40, 50, 80–190 min). (J) Average quiescence in 10-min windows over 8-h during UV-induced SIS of wild-type and *nlp-14*(*stj18*); *nlp-15*(*stj25*) animals ($N \geq 68$, *$p<.01$ at 80–230 min). (H–J) Statistical significance was calculated using two-way ANOVA followed by Sidak's multiple comparisons test. All error bars represent mean ± SEM.

animals were similar (Figure 4(A)); however, in the 10-min period beginning 85 min after UV exposure, the number of expulsions was significantly increased in the *nlp-14*(*2,4,5*) and *nlp-14*(*null*) animals (Figure 4(B,D)). We also observed that *nlp-14*(*2,4,5*) animals performed more pBoc and aBoc events without an Exp (Figure 4(C)). In contrast, *nlp-14*(*1-3*) mutant animals did not display defects in defecation quiescence (Figure 4(E)), 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 5-min every 30 min after UV irradiation (Table S4). As expected, wild-type animals slowed their defecation rate throughout the first 4 h 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 4(F,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 4(H); 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 h (Table S4). Taken together, these data suggest that NLP-14 peptides 1 and 3 are required for defecation quiescence. However,

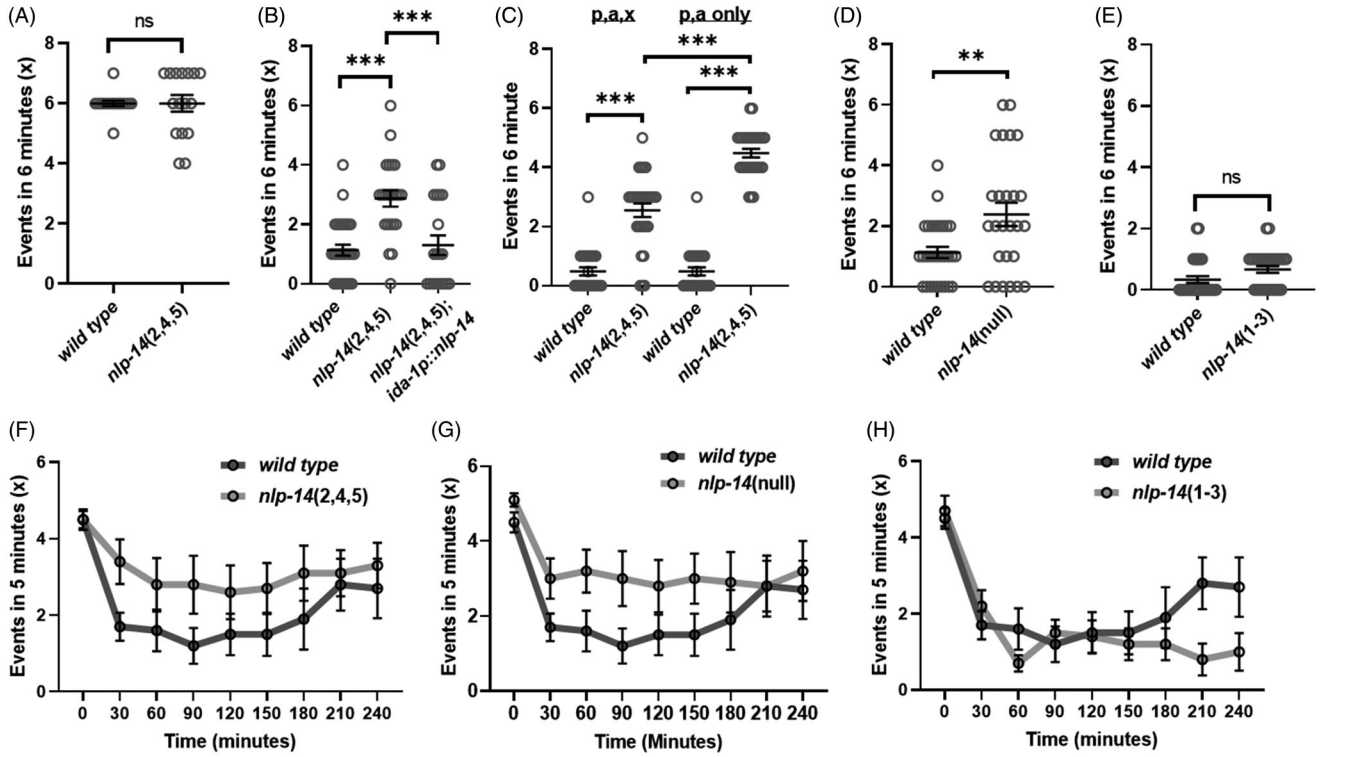

Figure 4. NLP-14 neuropeptides are indispensable for defecation quiescence during SIS. (A) Expulsions (x) performed in 6-min, of wild-type and *nlp-14*(*tm1880*) animals, who had not been exposed to any external stress. (B) Wild-type, *nlp-14*(*tm1880*) and *nlp-14*(*tm1880*); *ida-1p*::*nlp-14* animals 85–95 min following UV-stress ($N \geq 20$, $^{***}p < .001$). (C) p- (posterior body contraction or pBoc) and a- (anterior body contraction or aBoc) events or p-, a- and x-events in 6-min of wild-type and *nlp-14*(*tm1880*) animals, 85–95 min post-UV stress ($N \geq 26$, $^{***}p < .001$). (D) x-events performed in 6-min, of wild-type and *nlp-14*(*stj18*) animals ($N \geq 26$, $^{**}p < .01$) 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 ($N = 10$, $^{*}p < .05$, 210 min). (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.

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 *et al.*, 2016; Taylor *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 (Kramer *et al.*, 2001; Kushikata, Fang, Chen, Wang, & Krueger, 1998), *Drosophila* (Foltenyi, Greenspan, & Newport, 2007) and *C. elegans* (Van Buskirk & Sternberg, 2007), and acts during SIS by stimulating neuropeptide release from both the ALA and RIS neurons (Konietzka *et al.*, 2020; Nath *et al.*, 2016; Nelson *et al.*, 2014). Overexpression of *lin-3*, coding for EGF, induces movement quiescence (i.e. EGF-induced sleep) (Van Buskirk & Sternberg, 2007). Using the WorMotel, we found that *lin-3* overexpression caused prolonged movement quiescence in otherwise wild-type animals (Figure 5(A,B)), but this quiescence was significantly attenuated in *nlp-14*(*null*), *nlp-14*(*2,4,5*) and *nlp-14*(*1-3*) mutant animals (Figure 5(A,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 5(C–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 5(C–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 *et al.*, 2001). In three independent transgenic lines, movement quiescence during SIS was significantly increased relative to *nlp-14*(*2,4,5*) mutants (Figure 5(F)). 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 4(B)) 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

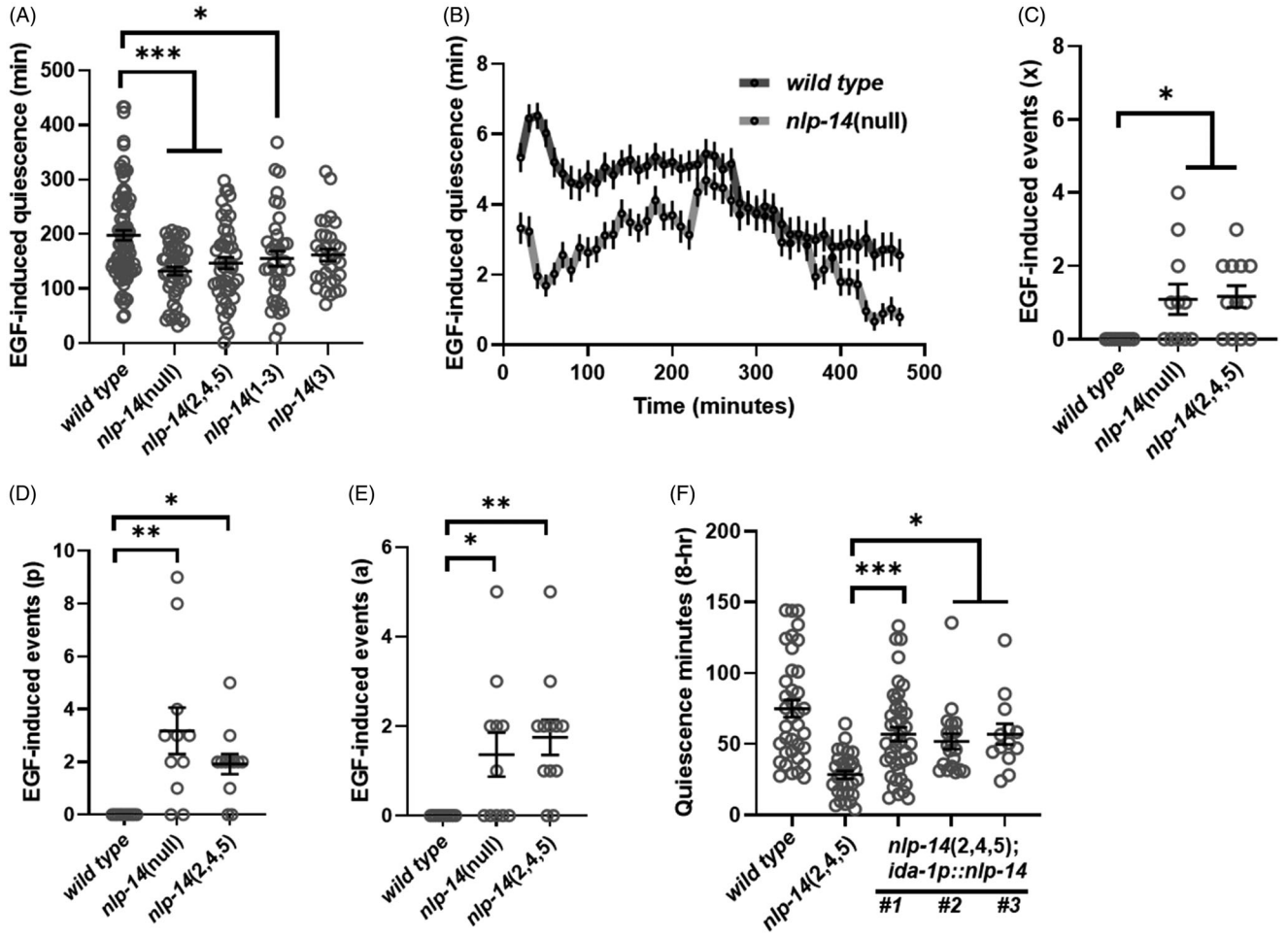

Figure 5. NLP-14 peptides function downstream of EGF from the ALA neuron. (A) Total minutes of quiescence in 4-h, following overexpression of *lin-3*, in the following genetic backgrounds: wild type, *nlp-14*(*stj18*), *nlp-14*(*tm1880*), *nlp-14*(*stj10*), and *nlp-14*(*stj19*) ($N \geq 31$, $^{*}p<.05$, $^{***}p<.001$). (B) Average quiescence in 10-min windows over 4-h following *lin-3* over-expression in wild-type and *nlp-14*(*stj18*) animals ($N = 48$, $p<.05$, 100–120 min, $p<.001$, 20–80 min). 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 min, 2-h after the overexpression of *lin-3* in wild-type, *nlp-14*(*stj18*) and *nlp-14*(*tm1880*) animals ($N \geq 10$, $^{*}p<.05$, $^{**}p<.01$). (F) Movement quiescence during UV-induced SIS in wild-type, *nlp-14*(*tm1880*) and *nlp-14*(*tm1880*); *ida-1p*::*nlp-14* animals ($N \geq 11$, $^{*}p<.05$, $^{***}p<.001$). 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.

movement and defecation quiescence during SIS. However, our data do 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* (Nath *et al.*, 2016; Nelson *et al.*, 2013, 2014; Turek *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-min heat pulse and then waited 2 h before analysis of behavior. At this 2-h time point, any direct effect of heat on behavior, which is minor at temperatures less than 35°, 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 6(A); Table S5) and by using machine vision, the WorMotel (Figure 6(B,C)). Overexpression of *nlp-14* also caused a significant reduction in defecation events (Figure 6(D); 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 overexpression, 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 *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, Schade, & Miller, 2006). We found that the increased PKA activity of *kin-2*(*ce179*) mutants (Charlie *et al.*, 2006), stimulated movement but not

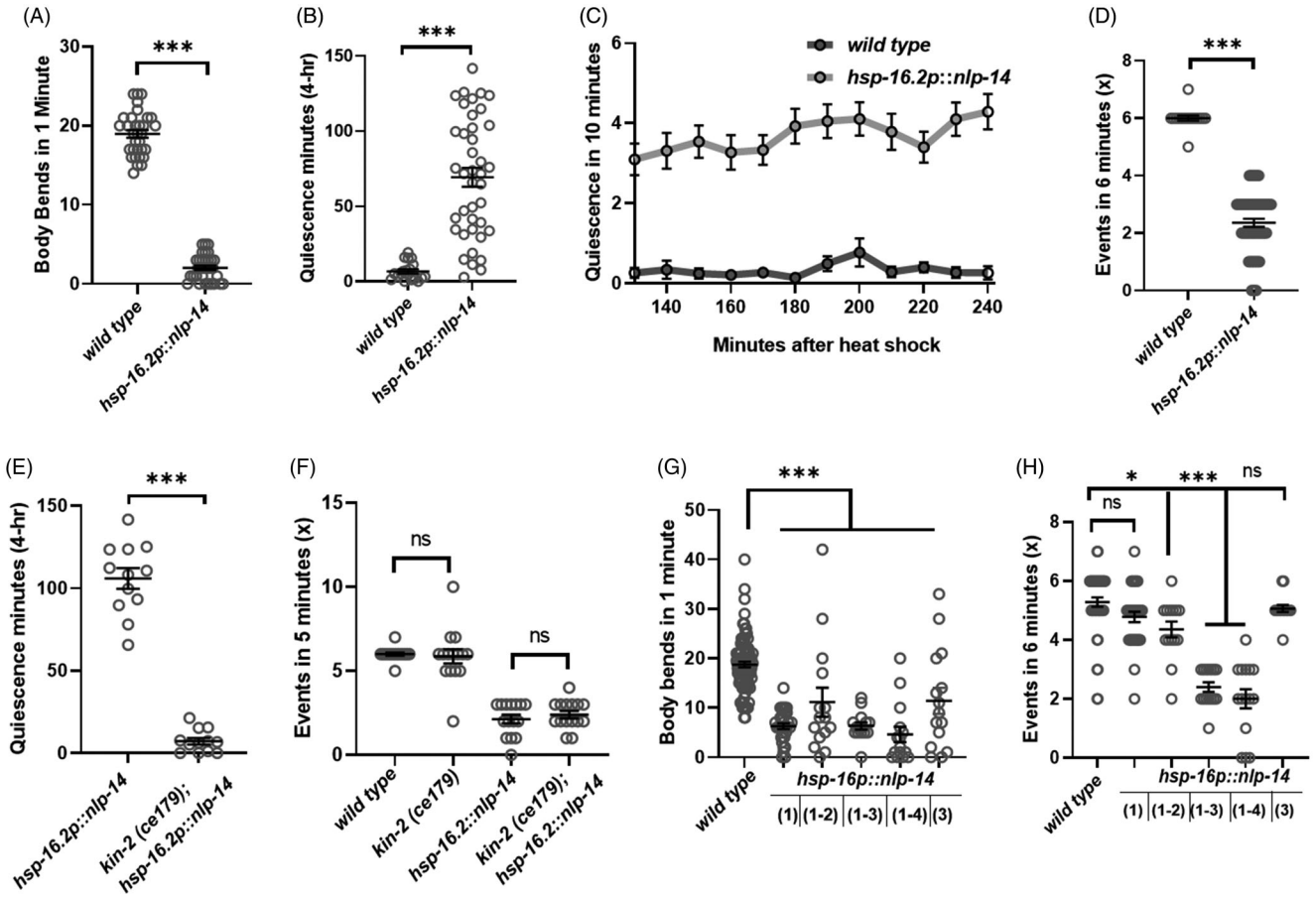

Figure 6. NLP-14 peptides are capable of inducing sleep-like behaviors. (A) Body bends performed in 1 min, 2–2.5-h after a 30-min, 33 °C heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals ($N = 32$, ***$p < .001$). (B) Movement quiescence during 4-h following a 30-min, 33 °C heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals ($N \geq 19$, ***$p < .001$). (C) Average quiescence in 10-min windows over 4-h following a 30-min, 33 °C heat shock, wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals ($N \geq 19$, ***$p < .001$ at all time points displayed). (D) x-events performed in 6-min, 2–2.5-h after a 30-min, 33 °C heat shock, by wild-type and *hsp-16.2p*::*nlp-14* (strain SJU27) animals ($N = 16$, ***$p < .001$). (E) Movement quiescence during 4-h following a 30-min, 33 °C heat shock, *hsp-16.2p*::*nlp-14* (strain SJU27) and *kin-2*(*ce179*); *hsp-16.2p*::*nlp-14* animals ($N = 12$, *$p < .001$). (F) x-events performed in 5-min 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-h after a 30-min, 33 °C heat shock ($N = 15$). (G) Body bends performed in 1-min, 2–2.5-h after a 30-min, 33 °C heat shock, by wild type and animals over-expressing *nlp-14*(1), (1–2), (1–3), (1–4) or (3) ($N \geq 15$, ***$p < .001$). (H) x-events performed in 5-min, 2–2.5-h after a 30-min, 33 °C heat shock, by wild type and animals overexpressing *nlp-14*(1), (1–2), (1–3), (1–4) or (3) ($N \geq 15$, *$p < .05$, ***$p < .001$). (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.

defecation following *nlp-14* overexpression (Figure 6(E,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 6(G,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 *et al.*, 2010, 2011). In *C. elegans*, the receptor NPR-10 has been proposed as an NLP-14 receptor, based on genetic interactions and anatomical connectivity (Hapiak *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 788 bp 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 animal has been convincingly identified.

Discussion

Orcokinin neuropeptides are conserved in Ecdysozoa, which consists of organisms that undergo molting (Aguinaldo *et al.*, 1997). Millions of years of evolution separate these animals, yet orcokinin peptide sequences are highly similar (Chen *et al.*, 2015; Dickinson *et al.*, 2009; Hofer *et al.*, 2005; Hofer & Homberg 2006; Koziol, 2018; Nathoo *et al.*, 2001; Wulff *et al.*, 2017; Yasuda-Kamatani & Yasuda, 2000). Functional studies have demonstrated that they regulate insect circadian rhythms and molting (Hofer & Homberg 2006; Wulff *et al.*, 2017), rhythmic smooth muscle contractions of insects and crustaceans (Li *et al.*, 2002; Skiebe, Dreger, Meseke, Evers, & Hucho, 2002; Stangier *et al.*, 1992) and decision making behaviors and male mating in *C. elegans* (Hapiak *et al.*, 2013; Sherlekar *et al.*, 2013). Here, we described a novel function for the orcokinins 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, DTS, which resembles sleep in animals that are strongly circadian (Trojanowski & Raizen, 2016), and SIS, a behavior required for recovery following exposure to damaging stress (Hill *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 (Turek *et al.*, 2013; Van Buskirk & Sternberg, 2007). 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 orcokinins by RNA interference (RNAi) caused molting defects (Wulff *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 *et al.*, 2013; Nelson *et al.*, 2013; Turek *et al.*, 2016).

Stress-induced sleep

In contrast to its relatively minor roles in DTS, NLP-14 is more important during 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 *et al.*, 2020) and FLP-13, FLP-24, and NLP-8 (Nath *et al.*, 2016; Nelson *et al.*, 2015), released from ALA. These molecules signal through many GPCRs (Iannacone *et al.*, 2017; Nelson *et al.*, 2015), reducing cAMP/PKA signaling in different cells (Cianciulli *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 *et al.*, 2016; Lee *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 nociception, a previously described role (Hapiak *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 *et al.*, 2013) and inhibited by neuropeptides (Nath *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 *et al.*, 2013). During SIS, the ALA neuron releases NLP-8 peptides to inhibit defecation (Nath *et al.*, 2016). We find that ALA also releases NLP-14 peptides. Our data indicate 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 & 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 *et al.*, 2002) and deuterostome starfish myorelaxant peptides (SMPs), which promote the relaxation of stomach muscle (Lin, Egertova, Zampronio, Jones, & Elphick, 2018). There is a sequence similarity between the SMPs and NLP-14 peptides (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 h after the orcokinin injection (Hofer & Homberg 2006). Hence, they observed both a change in sleep timing and in sleep/activity in response to orcokinin 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 *et al.*, 2008). Insect larvae can sleep between molts (Szuperak *et al.*, 2018) and also become quiescent during the molt, a behavior called molt-sleep (Reinecke, Buckner, & Grugel, 1980). Gene-expression analysis suggests that molt-sleep is regulated by neuropeptide signaling (MacWilliam, Arensburger, Higa, Cui, & Adams, 2015). Removal of orcokinins in the kissing bug causes molting failure and death (Wulff *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 *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, Nguyen, & Bullock, 2004; Ramon, Mendoza-Angeles, & Hernandez-Falcon, 2012). Considering that sickness and injury increase sleep in mammals (Imeri & 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, & Ramlov, 2010). 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.

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Disclosure statement

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References

Aguinaldo, A.M., Turbeville, J.M., Linford, L.S., Rivera, M.C., Garey, J.R., Raff, R.A., & Lake, J.A. (1997). Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature*, *387*(6632), 489–493. doi:10.1038/387489a0

Arribere, J.A., Bell, R.T., Fu, B.X., Artiles, K.L., Hartman, P.S., & Fire, A.Z. (2014). Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics*, *198*(3), 837–846. doi:10.1534/genetics.114.169730

Charlie, N.K., Thomure, A.M., Schade, M.A., & Miller, K.G. (2006). The Dunce cAMP phosphodiesterase PDE-4 negatively regulates G alpha(s)-dependent and G alpha(s)-independent cAMP pools in the *Caenorhabditis elegans* synaptic signaling network. *Genetics*, *173*(1), 111–130. doi:10.1534/genetics.105.054007

Chen, J., Choi, M.S., Mizoguchi, A., Veenstra, J.A., Kang, K., Kim, Y.J., & Kwon, J.Y. (2015). Isoform-specific expression of the neuropeptide orcokinin in *Drosophila melanogaster*. *Peptides*, *68*, 50–57. doi:10.1016/j.peptides.2015.01.002

Chiu, C.N., Rihel, J., Lee, D.A., Singh, C., Mosser, E.A., Chen, S., … Prober, D.A. (2016). A zebrafish genetic screen identifies neuromedin U as a regulator of sleep/wake states. *Neuron*, *89*(4), 842–856. doi:10.1016/j.neuron.2016.01.007

Choi, S., Chatzigeorgiou, M., Taylor, K.P., Schafer, W.R., & Kaplan, J.M. (2013). Analysis of NPR-1 reveals a circuit mechanism for behavioral quiescence in *C. elegans*. *Neuron*, *78*(5), 869–880. doi:10.1016/j.neuron.2013.04.002

Churgin, M.A., Jung, S.K., Yu, C.C., Chen, X., Raizen, D.M., & Fang-Yen, C. (2017). Longitudinal imaging of *Caenorhabditis elegans* in a microfabricated device reveals variation in behavioral decline during aging. *eLife*, *6*, e26652. doi:10.7554/eLife.26652

Cianciulli, A., Yoslov, L., Buscemi, K., Sullivan, N., Vance, R.T., Janton, F., … Nelson, M.D. (2019). Interneurons regulate locomotion quiescence via cyclic adenosine monophosphate signaling during stress-induced sleep in *Caenorhabditis elegans*. *Genetics*, *213*(1), 267–279. doi:10.1534/genetics.119.302293

Crowe, J. (1975). The physiology of cryptobiosis in tardigrades. *Memorie dell'Istituto italiano di idrobiologia 32*, 37–59.

Davis, M.W., Somerville, D., Lee, R.Y., Lockery, S., Avery, L., & Fambrough, D.M. (1995). Mutations in the *Caenorhabditis elegans* Na,K-ATPase alpha-subunit gene, eat-6, disrupt excitable cell function. *The Journal of Neuroscience*, *15*(12), 8408–8418. doi:10.1523/JNEUROSCI.15-12-08408.1995

DeBardeleben, H.K., Lopes, L.E., Nessel, M.P., & Raizen, D.M. (2017). Stress-induced sleep after exposure to ultraviolet light is promoted

by p53 in *Caenorhabditis elegans*. *Genetics*, *207*(2), 571–582. doi:10.1534/genetics.117.300070

Dickinson, P.S., Stemmler, E.A., Barton, E.E., Cashman, C.R., Gardner, N.P., Rus, S., … Christie, A.E. (2009). Molecular, mass spectral, and physiological analyses of orcokinins and orcokinin precursor-related peptides in the lobster Homarus americanus and the crayfish *Procambarus clarkii*. *Peptides*, *30*(2), 297–317. doi:10.1016/j.peptides.2008.10.009

Foltenyi, K., Greenspan, R.J., & Newport, J.W. (2007). Activation of EGFR and ERK by rhomboid signaling regulates the consolidation and maintenance of sleep in *Drosophila*. *Nature Neuroscience*, *10*(9), 1160–1167. doi:10.1038/nn1957

George-Raizen, J.B., Shockley, K.R., Trojanowski, N.F., Lamb, A.L., & Raizen, D.M. (2014). Dynamically-expressed prion-like proteins form a cuticle in the pharynx of *Caenorhabditis elegans*. *Biology Open*, *3*(11), 1139–1149. doi:10.1242/bio.20147500

Hapiak, V., Summers, P., Ortega, A., Law, W.J., Stein, A., & Komuniecki, R. (2013). Neuropeptides amplify and focus the monoaminergic inhibition of nociception in *Caenorhabditis elegans*. *The Journal of Neuroscience*, *33*(35), 14107–14116. doi:10.1523/JNEUROSCI.1324-13.2013

Hendriks, G.-J., Gaidatzis, D., Aeschimann, F., & Großhans, H. (2014). Extensive oscillatory gene expression during *C. elegans* larval development. *Molecular Cell*, *53*(3), 380–392. doi:10.1016/j.molcel.2013.12.013

Hill, A.J., Mansfield, R., Lopez, J.M., Raizen, D.M., & Van Buskirk, C. (2014). Cellular stress induces a protective sleep-like state in *C. elegans*. *Current Biology: CB*, *24*(20), 2399–2405. doi:10.1016/j.cub.2014.08.040

Hofer, S., Dircksen, H., Tollback, P., & Homberg, U. (2005). Novel insect orcokinins: Characterization and neuronal distribution in the brains of selected dicondylian insects. *The Journal of Comparative Neurology*, *490*(1), 57–71. doi:10.1002/cne.20650

Hofer, S., & Homberg, U. (2006). Evidence for a role of orcokinin-related peptides in the circadian clock controlling locomotor activity of the cockroach *Leucophaea maderae*. *The Journal of Experimental Biology*, *209*(Pt 14), 2794–2803. doi:10.1242/jeb.02307

Hofer, S., & Homberg, U. (2006). Orcokinin immunoreactivity in the accessory medulla of the cockroach *Leucophaea maderae*. *Cell and Tissue Research*, *325*(3), 589–600. doi:10.1007/s00441-006-0155-y

Iannacone, M.J., Beets, I., Lopes, L.E., Churgin, M.A., Fang-Yen, C., Nelson, M.D., … Raizen, D.M. (2017). The RFamide receptor DMSR-1 regulates stress-induced sleep in *C. elegans*. *eLife*, *6*, e19837. doi:10.7554/eLife.19837

Imeri, L., & Opp, M.R. (2009). How (and why) the immune system makes us sleep. *Nature Reviews. Neuroscience*, *10*(3), 199–210. doi:10.1038/nrn2576

Jekely, G. (2013). Global view of the evolution and diversity of metazoan neuropeptide signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(21), 8702–8707. doi:10.1073/pnas.1221833110

Kim, C.H., Go, H.J., Oh, H.Y., Elphick, M.R., & Park, N.G. (2018). Identification of evolutionarily conserved residues required for the bioactivity of a pedal peptide/orcokinin-type neuropeptide. *Peptides*, *103*, 10–18. doi:10.1016/j.peptides.2018.03.007

Kim, C.H., Kim, E.J., Go, H.J., Oh, H.Y., Lin, M., Elphick, M.R., & Park, N.G. (2016). Identification of a novel starfish neuropeptide that acts as a muscle relaxant. *Journal of Neurochemistry*, *137*(1), 33–45. doi:10.1111/jnc.13543

Konietzka, J., Fritz, M., Spiri, S., McWhirter, R., Leha, A., Palumbos, S., … Bringmann, H. (2020). Epidermal growth factor signaling promotes sleep through a combined series and parallel neural circuit. *Current Biology: CB*, *30*(1), 1–16e13. doi:10.1016/j.cub.2019.10.048

Koziol, U. (2018). Precursors of neuropeptides and peptide hormones in the genomes of tardigrades. *General and Comparative Endocrinology*, *267*, 116–127. doi:10.1016/j.ygcen.2018.06.012

Kramer, A., Yang, F.C., Snodgrass, P., Li, X., Scammell, T.E., Davis, F.C., & Weitz, C.J. (2001). Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science (New York, N.Y.)*, *294*(5551), 2511–2515. doi:10.1126/science.1067716

Kushikata, T., Fang, J., Chen, Z., Wang, Y., & Krueger, J.M. (1998). Epidermal growth factor enhances spontaneous sleep in rabbits. *The American Journal of Physiology*, *275*(2), R509–R514. doi:10.1152/ajpregu.1998.275.2.R509

Lee, D.A., Andreev, A., Truong, T.V., Chen, A., Hill, A.J., Oikonomou, G., … Prober, D.A. (2017). Genetic and neuronal regulation of sleep by neuropeptide VF. *eLife*, *6*, e25727. doi:10.7554/eLife.25727

Lenz, O., Xiong, J., Nelson, M.D., Raizen, D.M., & Williams, J.A. (2015). FMRFamide signaling promotes stress-induced sleep in *Drosophila*. *Brain, Behavior, and Immunity*, *47*, 141–148. doi:10.1016/j.bbi.2014.12.028

Li, L., Pulver, S.R., Kelley, W.P., Thirumalai, V., Sweedler, J.V., & Marder, E. (2002). Orcokinin peptides in developing and adult crustacean stomatogastric nervous systems and pericardial organs. *The Journal of Comparative Neurology*, *444*(3), 227–244. doi:10.1002/cne.10139

Lin, M., Egertova, M., Zampronio, C.G., Jones, A.M., & Elphick, M.R. (2018). Functional characterization of a second pedal peptide/orcokinin-type neuropeptide signaling system in the starfish *Asterias rubens*. *The Journal of Comparative Neurology*, *526*(5), 858–876. doi:10.1002/cne.24371

Lloyd, P.E., & Connolly, C.M. (1989). Sequence of pedal peptide: A novel neuropeptide from the central nervous system of Aplysia. *The Journal of Neuroscience*, *9*(1), 312–317. doi:10.1523/JNEUROSCI.09-01-00312.1989

MacWilliam, D., Arensburger, P., Higa, J., Cui, X., & Adams, M.E. (2015). Behavioral and genomic characterization of molt-sleep in the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology*, *62*, 154–167. doi:10.1016/j.ibmb.2015.01.012

Mello, C., & Fire, A. (1995). DNA transformation. *Methods in Cell Biology*, *48*, 451–482. doi:10.1016/S0091-679X(08)61399-0

Nath, R.D., Chow, E.S., Wang, H., Schwarz, E.M., & Sternberg, P.W. (2016). *C. elegans* stress-induced sleep emerges from the collective action of multiple neuropeptides. *Current Biology: CB*, *26*(18), 2446–2455. doi:10.1016/j.cub.2016.07.048

Nathoo, A.N., Moeller, R.A., Westlund, B.A., & Hart, A.C. (2001). Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 14000–14005. doi:10.1073/pnas.241231298

Nelson, M.D., & Fitch, D.H. (2011). Overlap extension PCR: An efficient method for transgene construction. *Methods in Molecular Biology (Clifton, N.J.)*, *772*, 459–470. doi:10.1007/978-1-61779-228-1_27

Nelson, M.D., Janssen, T., York, N., Lee, K.H., Schoofs, L., & Raizen, D.M. (2015). FRPR-4 is a G-protein coupled neuropeptide receptor that regulates behavioral quiescence and posture in *Caenorhabditis elegans*. *PLoS One*, *10*(11), e0142938. doi:10.1371/journal.pone.0142938

Nelson, M.D., Lee, K.H., Churgin, M.A., Hill, A.J., Van Buskirk, C., Fang-Yen, C., & Raizen, D.M. (2014). FMRFamide-like FLP-13 neuropeptides promote quiescence following heat stress in *Caenorhabditis elegans*. *Current Biology*, *24*(20), 2406–2410. doi:10.1016/j.cub.2014.08.037

Nelson, M.D., Trojanowski, N.F., George-Raizen, J.B., Smith, C.J., Yu, C.C., Fang-Yen, C., & Raizen, D.M. (2013). The neuropeptide NLP-22 regulates a sleep-like state in *Caenorhabditis elegans*. *Nature Communications*, *4*, 2846. doi:10.1038/ncomms3846

Notredame, C., Higgins, D.G., & Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology*, *302*(1), 205–217. doi:10.1006/jmbi.2000.4042

Paix, A., Folkmann, A., & Seydoux, G. (2017). Precision genome editing using CRISPR-Cas9 and linear repair templates in *C. elegans*. *Methods (San Diego, California)*, *121–122*, 86–93. doi:10.1016/j.ymeth.2017.03.023

Raizen, D.M., Zimmerman, J.E., Maycock, M.H., Ta, U.D., You, Y.J., Sundaram, M.V., & Pack, A.I. (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature*, *451*(7178), 569–572. doi:10.1038/nature06535

Ramon, F., Hernandez-Falcon, J., Nguyen, B., & Bullock, T.H. (2004). Slow wave sleep in crayfish. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(32), 11857–11861. doi:10.1073/pnas.0402015101

Ramon, F., Mendoza-Angeles, K., & Hernandez-Falcon, J. (2012). Sleep in invertebrates: Crayfish. *Frontiers in Bioscience (Scholar Edition)*, *4*, 1190–1200. doi:10.2741/s325

Reinecke, J., Buckner, J.S., & Grugel, S.R. (1980). Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. *The Biological Bulletin*, *158*(1), 129–140. doi:10.2307/1540764

Renn, S.C., Park, J.H., Rosbash, M., Hall, J.C., & Taghert, P.H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*, *99*(7), 791–802. doi:10.1016/S0092-8674(00)81676-1

Rowe, M.L., & Elphick, M.R. (2012). The neuropeptide transcriptome of a model echinoderm, the sea urchin *Strongylocentrotus purpuratus*. *General and Comparative Endocrinology*, *179*(3), 331–344. doi:10.1016/j.ygcen.2012.09.009

Semmens, D.C., & Elphick, M.R. (2017). The evolution of neuropeptide signalling: Insights from echinoderms. *Briefings in Functional Genomics*, *16*(5), 288–298. doi:10.1093/bfgp/elx005

Sherlekar, A.L., Janssen, A., Siehr, M.S., Koo, P.K., Caflisch, L., Boggess, M., & Lints, R. (2013). The *C. elegans* male exercises directional control during mating through cholinergic regulation of sex-shared command interneurons. *PLoS One*, *8*(4), e60597. doi:10.1371/journal.pone.0060597

Singh, R.N., & Sulston, J.E. (1978). Some observations on moulting in *Caenorhabditis elegans*. *Nematologica*, *24*(1), 63–71. doi:10.1163/187529278X00074

Skiebe, P., Dreger, M., Meseke, M., Evers, J.F., & Hucho, F. (2002). Identification of orcokinins in single neurons in the stomatogastric nervous system of the crayfish, Cherax destructor. *The Journal of Comparative Neurology*, *444*(3), 245–259. doi:10.1002/cne.10145

Stangier, J., Hilbich, C., Burdzik, S., & Keller, R. (1992). Orcokinin: A novel myotropic peptide from the nervous system of the crayfish, *Orconectes limosus*. *Peptides*, *13*(5), 859–864. doi:10.1016/0196-9781(92)90041-Z

Stinchcomb, D.T., Shaw, J.E., Carr, S.H., & Hirsh, D. (1985). Extrachromosomal DNA transformation of *Caenorhabditis elegans*. *Molecular and Cellular Biology*, *5*(12), 3484–3496. doi:10.1128/mcb.5.12.3484

Szuperak, M., Churgin, M.A., Borja, A.J., Raizen, D.M., Fang-Yen, C., & Kayser, M.S. (2018). A sleep state in *Drosophila* larvae required for neural stem cell proliferation. *eLife*, *7*, e33220. doi:10.7554/eLife.33220

Taylor, S.R., Santpere, G., Reilly, M., Glenwinkel, L., Poff, A., McWhirter, R., … Miller, D.M. (2019). Expression profiling of the mature *C. elegans* nervous system by single-cell RNA-sequencing. bioRxiv: 737577. doi:10.1101/737577

Thomas, J.H. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. *Genetics*, *124*(4), 855–872.

Trojanowski, N.F., & Raizen, D.M. (2016). Call it worm sleep. *Trends in Neurosciences*, *39*(2), 54–62. doi:10.1016/j.tins.2015.12.005

Turek, M., Besseling, J., Spies, J.P., Konig, S., & Bringmann, H. (2016). Sleep-active neuron specification and sleep induction require FLP-11 neuropeptides to systemically induce sleep. *eLife*, *5*, e12499. doi:10.7554/eLife.12499

Turek, M., Lewandrowski, I., & Bringmann, H. (2013). An AP2 transcription factor is required for a sleep-active neuron to induce sleep-like quiescence in *C. elegans*. *Current Biology: CB*, *23*(22), 2215–2223. doi:10.1016/j.cub.2013.09.028

Van Buskirk, C., & Sternberg, P.W. (2007). Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nature Neuroscience*, *10*(10), 1300–1307. doi:10.1038/nn1981

Wang, H., Girskis, K., Janssen, T., Chan, J.P., Dasgupta, K., Knowles, J.A., … Sieburth, D. (2013). Neuropeptide secreted from a pacemaker activates neurons to control a rhythmic behavior. *Current Biology: CB*, *23*(9), 746–754. doi:10.1016/j.cub.2013.03.049

Wang, H., & Sieburth, D. (2013). PKA controls calcium influx into motor neurons during a rhythmic behavior. *PLoS Genetics*, *9*(9), e1003831. doi:10.1371/journal.pgen.1003831

Wright, J.C., Westh, P., & Ramlov, H. (2010). Cryptobiosis in tardigrada. *Biological Reviews*, *67* (1), 1–29. doi:10.1111/j.1469-185X.1992.tb01657.x

Wulff, J.P., Sierra, I., Sterkel, M., Holtof, M., Van Wielendaele, P., Francini, F., … Ons, S. (2017). Orcokinin neuropeptides regulate ecdysis in the hemimetabolous insect *Rhodnius prolixus*. *Insect Biochemistry and Molecular Biology*, *81*, 91–102. doi:10.1016/j.ibmb.2017.01.003

Yamanaka, N., Hua, Y.J., Roller, L., Spalovska-Valachova, I., Mizoguchi, A., Kataoka, H., & Tanaka, Y. (2010). Bombyx prothoracicostatic peptides activate the sex peptide receptor to regulate ecdysteroid biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(5), 2060–2065. doi:10.1073/pnas.0907471107

Yamanaka, N., Roller, L., Zitňan, D., Satake, H., Mizoguchi, A., Kataoka, H, Tanaka, Y. (2011). Bombyx orcokinins are brain-gut peptides involved in the neuronal regulation of ecdysteroidogenesis. *The Journal of Comparative Neurology*, *519*(2), 238–246. doi:10.1002/cne.22517

Yasuda-Kamatani, Y., & Yasuda, A. (2000). Identification of orcokinin gene-related peptides in the brain of the crayfish *Procambarus clarkii* by the combination of MALDI-TOF and on-line capillary HPLC/Q-Tof mass spectrometries and molecular cloning. *General and Comparative Endocrinology*, *118*(1), 161–172. doi:10.1006/gcen.1999.7453

Zahn, T.R., Macmorris, M.A., Dong, W., Day, R., & Hutton, J.C. (2001). IDA-1, a *Caenorhabditis elegans* homolog of the diabetic autoantigens IA-2 and phogrin, is expressed in peptidergic neurons in the worm. *The Journal of Comparative Neurology*, *429*(1), 127–143. doi:10.1002/1096-9861(20000101)429:1<127::AID-CNE10>3.0.CO;2-H

Zitnan, D., Ross, L.S., Zitnanova, I., Hermesman, J.L., Gill, S. S., & Adams, M. E., (1999). Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron*, *23*(3), 523–535. doi:10.1016/S0896-6273(00)80805-3