



## Original article

Neuropeptide signaling regulates the susceptibility of developing *C. elegans* to anoxia

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## ABSTRACT

Inadequate delivery of oxygen to organisms during development can lead to cell dysfunction/death and life-long disabilities. Although the susceptibility of developing cells to low oxygen conditions changes with maturation, the cellular and molecular pathways that govern responses to low oxygen are incompletely understood. Here we show that developing *Caenorhabditis elegans* are substantially more sensitive to anoxia than adult animals and that this sensitivity is controlled by nervous system generated hormones (e.g., neuropeptides). A screen of neuropeptide genes identified and validated *nlp-40* and its receptor *aex-2* as a key regulator of anoxic survival in developing worms. The survival-promoting action of impaired neuropeptide signaling does not rely on five known stress resistance pathways and is specific to anoxic insult. Together, these data highlight a novel cell non-autonomous pathway that regulates the susceptibility of developing organisms to anoxia.

## 1. Introduction

Molecular oxygen (dioxxygen or O<sub>2</sub>), hereafter referred to as oxygen, is essential for the function and survival of multicellular organisms. During mitochondrial oxidative phosphorylation, oxygen serves as the final electron-acceptor of the electron transport chain and thus is critical for producing the vast majority of ATP to fuel cellular operations [1]. While even short periods of oxygen deprivation can cause lasting cell damage and death, there is variation in the sensitivity of specific cell types to oxygen deprivation. The origin of variable sensitivity to hypoxia is likely to be multifactorial including differences in tissue vascularization and intrinsic differences in metabolic rate. In addition, sensitivity of tissues to oxygen deprivation evolves during organismal development, and several different molecular pathways are engaged upon hypoxic insult, which ultimately dictate stress responses and cell survival or death. Identification of the specific molecular pathway(s) engaged by a specific clinical setting is essential for devising optimal therapeutic approaches. We are particularly interested in hypoxic insults incurred during development because perinatal hypoxic-ischemic insults in humans can have devastating effects on newborn infants [2] leading to multi-tissue dysfunction with lifelong disabilities [3].

*C. elegans* has proven to be a superb tool for studying the genetic and

molecular response to oxygen deprivation across its lifespan [4–7]. As soil dwellers, *C. elegans* generally prefer environmental oxygen in the 5–12% range [8]. Interestingly, they have distinct responses to moderate and severe oxygen deprivation [6]. Worms can survive, develop and reproduce under low oxygen, hypoxic conditions (0.5–1% oxygen) by decreasing their oxygen consumption and locomotion [9]. However, under extreme oxygen deprivation or anoxia (< 0.1% oxygen), worms become hypometabolic and suspend development, feeding and reproduction [5,6,10]. Depending on growth conditions, they can survive in this ‘suspended animation’ state for a few days, but eventually die if oxygen is not reintroduced. Upon reoxygenation, they can resume normal activity including reproduction. While the hypoxic response in *C. elegans* is extensively studied and depends on the canonical hypoxia induced factor (*hif-1*) pathway, the anoxic response is *hif-1*-independent and is less well understood [6].

Distinct cell types in the worm sense upshifts and downshifts in oxygen, and their activity controls behavioral responses of the animals to variation in environmental oxygen conditions [11]. Flibotte et al. showed that elimination of a pair of neurons specifically responsible for sensing downshifts in oxygen (e.g., BAG neurons) protected developing worms from an anoxic insult [12]. This highlights a potentially novel cell non-autonomous mechanism controlling organismal survival under

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anoxic conditions. It is possible this effect is mediated by humeral factors since Flibotte et al., also found that developing worms deficient in several neuropeptide processing and secretion genes, *egl-3* and *unc-31* respectively, were protected from anoxia [12]. These observations raise several important questions about the relationship between neuropeptide signaling and developing worm responses to anoxic stress. Does neuropeptide signaling in developing worms control responses to many stressors or specifically anoxia? Does neuropeptide signaling in developing worms operate through known or novel stress-resistance pathways? If the pathways are novel and specific to anoxia it would be critical to determine whether sensitivity to anoxia during development is mediated by the collective action of many neuropeptides or few (perhaps only one). Together the answers to these questions could identify new pathways for modulating the vulnerability of developing organisms to anoxia.

## 2. Materials and methods

### 2.1. Worm husbandry

*C. elegans* were cultivated at 20 °C on nematode growth medium (NGM) agar surface, unless otherwise stated, using standard techniques [13]. NGM plates were seeded with a 40 µL lawn of the *E. coli* strain OP50 as the food source, except in RNAi experiments, generally 1–2 days prior to worms being placed on them.

### 2.2. Worm strains

Strains used in this study are listed in [Supplementary Material \(Table 1\)](#). Candidates from the neuropeptide screen were explicitly backcrossed to rule out false positives due to genetic background. Double mutants were made using standard genetic techniques. Mutations were confirmed by PCR genotyping, and the primers used are also listed in [Table 1](#). For neuropeptide fosmid rescue experiments, the appropriate neuropeptide mutant worm was injected with 160 ng/µL of the corresponding fosmid, along with 2.5 ng/µL of *P<sub>myo-2</sub>::mCherry* as a coinjection marker.

### 2.3. Anoxia assay

Worms were synchronized by hypochlorite treatment and eggs were placed on fresh plates. For each experiment, 30–50 synchronized L4 animals from each genotype were placed on three fresh plates. Each plate was placed in a separate anaerobic Bio-Bag (Becton Dickinson, catalog # 261216) along with appropriate co-bagged controls (generally N2 and *unc-31/egl-3* mutants). The bags were sealed and an anoxic environment (< 0.1% O<sub>2</sub>) was generated by palladium catalyst mediated consumption of ambient oxygen in the bag (per manufacturer instructions). A resazurin indicator was included in each bag to confirm oxygen deprivation. Unless otherwise stated, worms were placed under anoxia for 48 h at 20 °C, after which the bags were opened, and the resazurin indicator confirmed reintroduction of ambient oxygen in the bag. Anoxia exposure was also confirmed by ‘stunned’ phenotype of worms once the bags were opened [6,12]. Any bags that failed to achieve anoxia according to the indicator and/or due to the absence of ‘stunning’ in one or all plates were excluded from the experiment. Animals were allowed to recover for 24 h at 20 °C after anoxia, after which dead and alive animals were manually scored. Animals were considered alive if they were moving, pumping their pharynx and/or responded to light touch. Each experiment thus had three technical replicates (3 bags), and experiments were generally repeated 3–6 independent times. The mean survival fraction across independent experiments was reported.

### 2.4. Heat shock assay

Synchronized L4 worms (50 per genotype group) were picked onto fresh plates, sealed with parafilm and placed in a plastic bag submerged in a 34 °C water bath. Individual plates were removed after 5 h and 12 h to manually score living/dead animals and promptly returned to the water bath. Animals were scored alive if they were moving, pumping their pharynx and/or responded to light touch.

### 2.5. ER stress assay

NGM plates were supplemented with 5 mg/mL tunicamycin or equivalent DMSO vehicle (0.05%) and seeded with OP50 bacteria. Synchronized L4 worms raised on NGM OP50 plates were placed on vehicle or tunicamycin plates and lifespan of worms on either plate was determined by scoring live/dead animals daily.

### 2.6. UV stress assay

To determine ‘germ cell’ survival, young adult animals were irradiated with 0, 80 and 120 J/m<sup>2</sup> UV-B and allowed to recover for 24 h. Survival of the progeny (eggs laid for ~3 h) of the irradiated adults was then determined by counting dead and alive eggs. Average results across two independent experiments are presented. In each experiment, survival on four replicate plates was determined (the offspring of 16–25 irradiated animals). The *xpa-1 (ok698)* mutant, deficient in nucleotide excision repair, was included as a positive control to demonstrate UV toxicity.

Larval survival was determined by irradiating L1 larvae and scoring the percentage of animals that arrested/died or survived (developed into L4/adult) 48 h later. Average results across two independent experiments are presented. In each experiment, survival of three replicate plates, each containing between 40 and 160 L1 larvae, was determined. The *xpa-1 (ok698)* mutant was included as a positive control [14].

### 2.7. Hypercarbia assay

Carbon dioxide was introduced into a sealed chamber fitted with a CO<sub>2</sub> controller (ProCO<sub>2</sub> from BioSpherix, Inc.) until its concentration was 10% of room air, as measured by a CO<sub>2</sub> sensor in the chamber. A separate sensor measured O<sub>2</sub> concentration, which was 18% of room air. Synchronized L4 worms were placed in this chamber for 48 h, after which they were removed and assessed for a ‘stunned’ phenotype and survival.

### 2.8. RNAi experiments

NGM plates were seeded with *E. coli* expressing empty vector (L4440) or *egl-3* RNAi-encoding (C51E3.7) plasmids in order to perform feeding RNAi experiments. We tested the worm strain TU3311 [15], enhanced for neuronal import of dsRNA from the bacterial source and RNAi-dependent silencing of the target gene, to achieve knockdown of *egl-3* specifically in neurons. L4-staged worms were placed on either empty vector or *egl-3* RNAi plates for 3–4 generations to ensure successful knockdown of *egl-3*. Subsequently, worms were placed in anaerobic Bio-Bags for 54 h and allowed to recover for 24 h before scoring survival.

### 2.9. Neuropeptide screen

We assembled a list of all *nlp*'s and *flp*'s [16] and interrogated Wormbase ([www.wormbase.org](http://www.wormbase.org)) to identify mutants that were putative null. This list of neuropeptide mutants was obtained from either the Caenorhabditis Genetics Center (Minneapolis, MN) or the National BioResource Project (Tokyo, Japan). We posited that deletion mutants would have the most severe loss of function phenotype. Any *flp* or *nlp*

that did not have a deletion mutant available was excluded from the screen. Using this criterion, we were able to test 29 of 31 total *flp*'s (all but *flp-23* and *flp-32*) and 36 of 42 total *nlp*'s (all but *nlp-4*, *nlp-6*, *nlp-13*, *nlp-16*, *nlp-33* and *nlp-39*). Similar to a chemical mutagenesis forward genetic screen, the *flp* and *nlp* mutant strains were not backcrossed to N2 worms prior to the initial screen. We began the screen with each strain was tested two independent times, each time with three plates (one in each Bio-Bag) and with co-bagged N2 and *unc-31* worms. The mean survival of each strain across these 6 plates was used to narrow candidates for the secondary screen. Any strain with > 50% mean survival was selected for secondary screening. In the secondary screen, strains were (i) tested in at least 3 additional independent experiments, (ii) backcrossed 2x to N2 and retested, and (iii) subjected to rescue experiments using available fosmid DNA for each mutant (Source Bioscience, Nottingham UK). Fosmids were injected at 160 ng/μL along with 2.5 ng/μL *P<sub>myo-2</sub>::mCherry* as a coinjection marker. For *nlp-40* rescue experiments, the genomic rescue line used (OJ949) was generously donated by Dr. Derek Sieburth [17].

### 2.10. Statistical analysis

Data were analyzed using Prism (GraphPad Software, La Jolla, CA). Significant differences between two groups were determined using paired Student's *t*-test (two-tailed). Significant differences within groups greater than two were determined using one-way ANOVA followed by Tukey's test for multiple comparisons. Survival curves were analyzed using the log-rank (Mantel-Cox) test for significance. For all tests, the significance threshold was set to  $p < 0.05$ .

## 3. Results

### 3.1. Loss of neuropeptide signaling protects against anoxic stress in developing *C. elegans*

Flibotte et al. found that mutations in *egl-3* and *unc-31* protected developing worms from anoxic insult [12]. These conserved genes are essential for neuropeptide signaling; *egl-3* encodes a proprotein convertase necessary for processing neuropeptides into their mature forms, and *unc-31* encodes a calcium-dependent protein necessary for secretion of neuropeptides from dense core vesicles. However, whether these observations were probing a novel pathway regulating oxygen deprivation was unclear. To gain insight into this issue we began by exploring the boundaries of these findings.

For purposes of clarity, the low oxygen system we use achieves an ambient oxygen level of < 0.001 kPa (< 0.1%) and we define this as anoxia. We began by reproducing the original observations: 30–50% wild-type (N2) animals in the L4 stage (4th larval stage) of development survive 48 h of anoxia [5,6,12] while 70–80% of *egl-3(ok979)* and *unc-31(e928)* L4 animals survive 48 h of anoxia (group effect:  $F_{(2,9)} = 8.329$ ,  $p = 0.009$ ) (Fig. 1A). We would like to note that while this was the survival range for most experiments, we see a high degree of variability in this assay that we were unable to attribute to factors such as position of plates in the bag. Despite this, these consistently significant results imply that neuropeptide processing and secretion regulate the vulnerability of L4 worms to anoxia. To consolidate these observations we looked at the effects of another gene involved in neuropeptide processing, *egl-21(n476)*. EGL-21 is a carboxypeptidase that removes the C-terminal basic residue(s) from small peptides generated by EGL-3. We find that loss of *egl-21(n476)* also protects L4 worms against 48 h of anoxia compared to N2 ( $t_{(5)} = 3.841$ ,  $p = 0.0121$ , paired *t*-test) (Fig. 1B). That loss of function of multiple genes required for neuropeptide synthesis and secretion protects L4 worms from anoxic stress provides unambiguous evidence that neuropeptides regulate developing organism sensitivity to anoxic insult. Interestingly, these genes are conserved in humans both in sequence and function [18–20].

We find that while 30–50% of L4 worms survive 48 h of anoxia, 90–100% of young adult N2 worms survived the same insult ( $t_{(1)} = 25.89$ ,  $p = 0.0246$ , paired *t*-test) (Fig. 1C). Since the L4 stage of worm development can be as long as 8 h, during which worms undergo a variety of physiological changes, we wondered if early L4 worms would differ in their sensitivity to anoxia in comparison with late L4 worms. We used a reporter line *qls56 [(lag-2::GFP) + unc-119(+)]* for distal tip cell maturation [21] to precisely distinguish early and late L4 worms. In early L4, the distal tip cells are towards the ventral surface of the body, while in late L4, they have formed a U-shaped gonad toward the dorsal surface. There was no significant difference in anoxic survival between the younger and older L4 animals ( $t_{(4)} = 0.5139$ ,  $p = 0.6344$ , paired *t*-test) (Fig. 1D). Thus developmental processes occurring during the L4 larval stage do not strongly influence susceptibility to anoxia, but once the animals transition into adulthood, they acquire resistance to a 48 h anoxic insult. We conducted all subsequent experiments at the L4 stage.

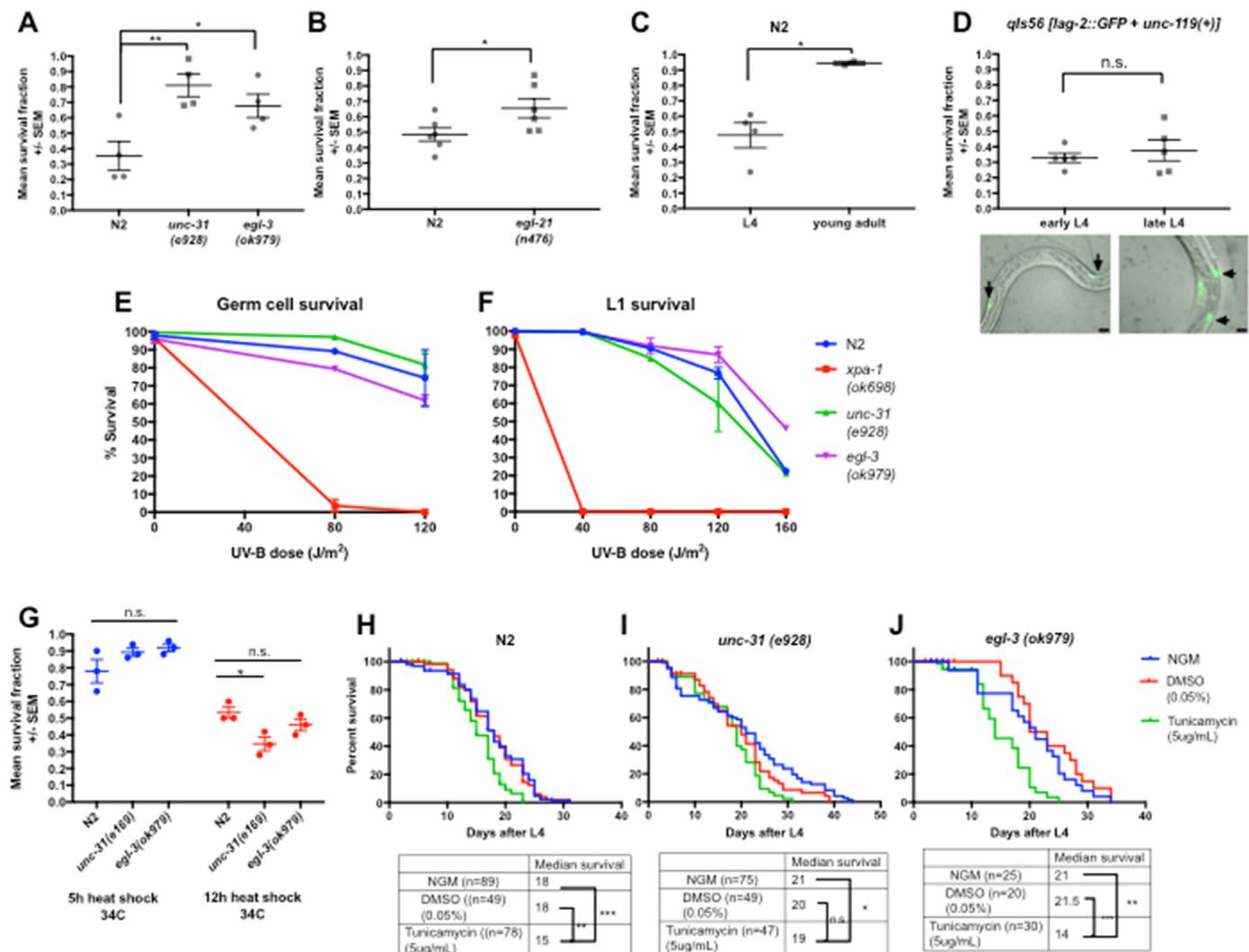
In addition to O<sub>2</sub> depletion, 8–12% CO<sub>2</sub> is generated in the Bio-Bags used for these studies (per manufacturer). Work by others suggests that worms reduce pharyngeal pumping in response to high CO<sub>2</sub> levels, and this is in part mediated by *egl-21* and *unc-31* [22]. This raises the possibility that we are studying the effects of hypercarbia on worms instead of anoxia. To examine this issue, we placed L4-staged N2, *egl-3(ok979)* and *unc-31(e928)* worms in a chamber with 20% O<sub>2</sub> and 10% CO<sub>2</sub>. After 48 h in this chamber, none of the worms had developmentally arrested or died, as they do in the Bio-Bags. Rather, they transitioned into adulthood and laid viable eggs (data not shown). GCY-9 is known to be necessary for sensing CO<sub>2</sub> and regulates worm avoidance to the gas [23]. We found that *gcy-9(n4470)* null animals were as sensitive to 48 h of anoxia as N2 animals (data not shown). We conclude that the survival phenotype we observed is most likely to reflect a response to decreased ambient O<sub>2</sub> levels rather than increased CO<sub>2</sub> levels.

Next, we explored insult-specificity. Mutants for the insulin receptor *daf-2* are known to resist a large number of toxic insults [24]. Do mutations in neuropeptide processing and secretion similarly confer broad protection against imposed stressors? To test general stress resistance, we examined the behavior of neuropeptide mutants to UV stress, heat stress and ER stress. Neuropeptide signaling mutants *egl-3(ok979)* and *unc-31(e928)* subjected to UV stress at the L1 stage for developmental maturation or adult stage for germ cell maturation (Fig. 1E, F) or heat stress at the L4 stage (Fig. 1G) had similar or worse survival compared to N2 worms. In the heat shock experiment, we see a duration-dependent decrease in survival across all groups (10–20% worms died at 5 h, while 50–65% worms died at 12 h), and find that *unc-31* is significantly more sensitive to 12 h of heat shock compared to N2. Additionally, *egl-3(ok979)* and *unc-31(e928)* worms at the L4 stage were as sensitive to tunicamycin-induced ER stress as N2 worms (Fig. 1H, I, J). Since loss of neuropeptide signaling does not protect developing animals from a disparate set of stressors, our observations suggest a specific linkage of loss of neuropeptide signaling to anoxic stress.

### 3.2. Known stress-resistance/longevity pathways do not regulate neuropeptide-mediated survival under anoxia

Previous work has identified a variety of cellular pathways that impact survival after anoxia. Since many aspects of our system are unique (i.e., study of L4 animals, solid NGM culture and Bio-Bags to induce anoxia), we wondered if genes and pathways previously shown to regulate stress responses also underlie the neuropeptide-mediated anoxic susceptibility phenotype.

We began by interrogating loss of function mutations in a variety of such genes in the *unc-31(e928)* and/or *egl-3(ok979)* background. We tested the following genes due to their described role in anoxia, hypoxia or general stress resistance/longevity: (i) the worm homolog for the FOXO transcription factor which is involved in the insulin signaling pathway, *daf-16(mgDf50)* [4,25], (ii) the worm homolog for hypoxia



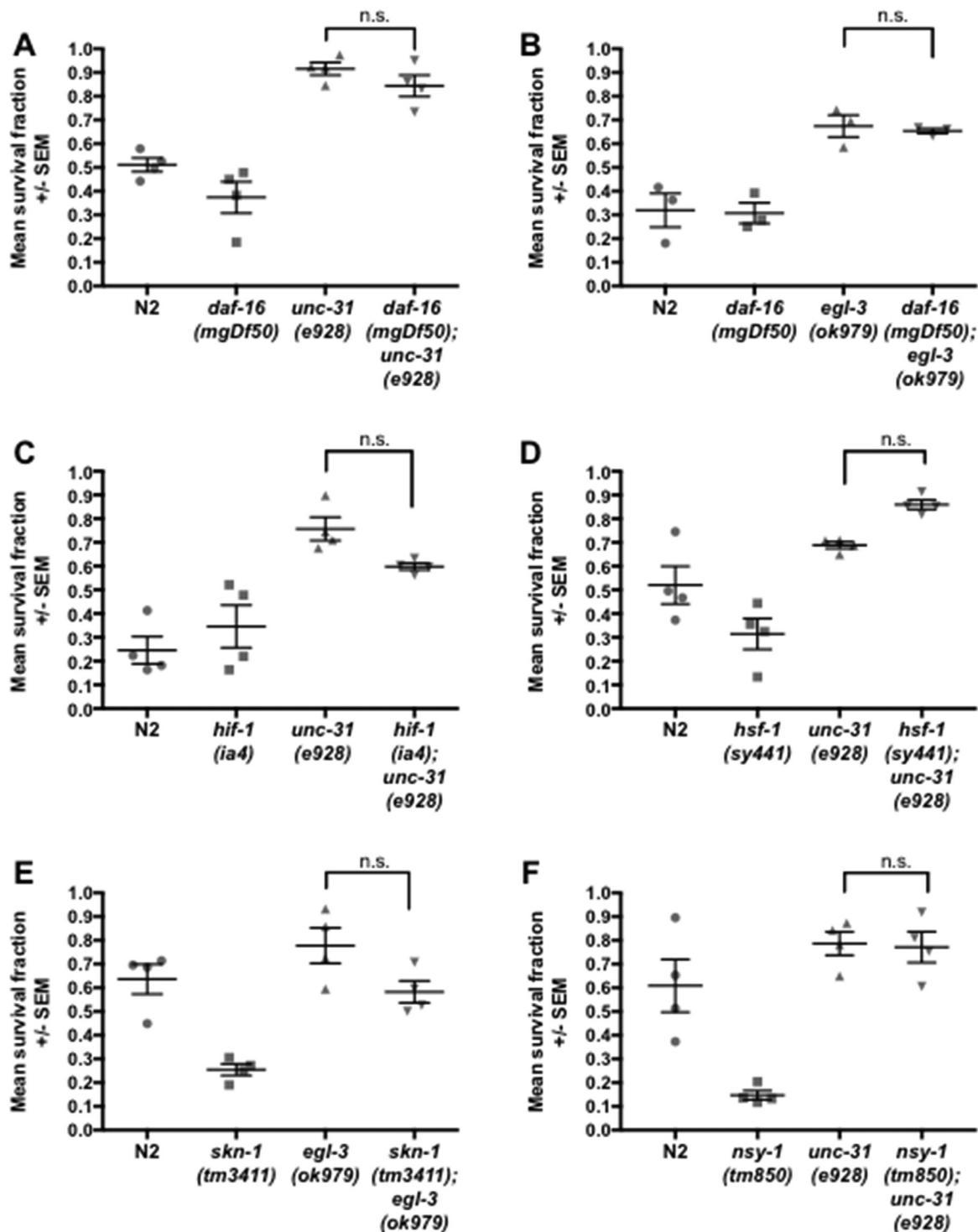
**Fig. 1.** Loss of neuropeptide signaling protects L4 *C. elegans* from anoxia. A) Survival of L4 *unc-31* and *egl-3* mutants after 48 h anoxia compared to N2 (n = 4 independent experiments). B) Survival of L4 *egl-21* mutant after 48 h anoxia compared to N2 (n = 6 independent experiments). C) Survival of L4 and young adult N2 worms after 48 h anoxia (n = 4 independent experiments). D) Survival of early and late L4 worms after 48 h anoxia using reporter line *qls56* (n = 5 independent experiments). E) Survival of *unc-31* and *egl-3* mutant embryos compared to N2 embryos after adult parents (n = 16–25 worms each) are irradiated with increasing doses of UV-B (*xpa-1* is a positive control for UV sensitivity). F) Survival of L1 *unc-31* and *egl-3* mutants compared to N2 (n = 40–160 worms each) irradiated with increasing doses of UV-B. G) Survival of L4 *unc-31* and *egl-3* mutants compared to N2 (50 worms each) after 5 h and 12 h of 34 °C heat shock (n = 3 independent experiments). H–J) Survival curve of L4 N2, *unc-31* and *egl-3* mutants placed on NGM compared to NGM containing DMSO (vehicle) or tunicamycin (5 mg/mL). Data are analyzed using paired t-test (comparing 2 groups), one-way ANOVA with Tukey's test for multiple comparisons (comparing 3 or more groups) or Mantel-Cox test for survival curve comparison. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

induced transcription factor, *hif-1*(*ia4*) [26], (iii) the worm homolog for the heat shock transcription factor, *hsf-1*(*sy441*) [27], (iv) the worm homolog for Nrf transcription factor, *skn-1*(*tm3411*) [28], and (v) the worm homolog for the MAP kinase kinase kinase, *nsy-1*(*tm850*) [29]. Briefly, we made double mutants between the mutant gene of interest (i–v above) and either *unc-31*(*e928*) or *egl-3*(*ok979*) mutants, and subjected them to anoxia at the L4 stage. Our reasoning was that if increased anoxic survival of *unc-31*(*e928*) or *egl-3*(*ok979*) mutants was due to the activity of any one of the genes i–v, then loss of that gene in the *unc-31*(*e928*) or *egl-3*(*ok979*) mutant background would blunt the survival benefit.

We found no statistically significant difference in survival between *daf-16*(*mgDf50*);*unc-31*(*e928*) double mutants compared to *unc-31*(*e928*) mutants after correcting for multiple comparisons (group effect:  $F_{(4,15)} = 26.38$ ,  $p < 0.0001$ ), or between *daf-16*(*mgDf50*);*egl-3*(*ok979*) double mutants compared to *egl-3*(*ok979*) alone (group effect:  $F_{(4,10)} = 15.76$ ,  $p = 0.0003$ ) (Fig. 2A, B). Similarly, we found no statistically significant differences in survival between *hif-1*(*ia4*);*unc-*

*31*(*e928*) and *unc-31*(*e928*) animals (group effect:  $F_{(3,12)} = 15.62$ ,  $p = 0.0002$ ) (Fig. 2C), between *hsf-1*(*sy441*);*unc-31*(*e928*) and *unc-31*(*e928*) animals (group effect:  $F_{(3,12)} = 19.4$ ,  $p < 0.0001$ ) (Fig. 2D), between *skn-1*(*tm3411*);*egl-3*(*ok979*) and *egl-3*(*ok979*) animals (group effect:  $F_{(2,9)} = 25.19$ ,  $p = 0.0002$ ) (Fig. 2E) or between *nsy-1*(*tm850*);*unc-31*(*e928*) and *unc-31*(*e928*) animals (group effect:  $F_{(4,15)} = 16.77$ ,  $p < 0.0001$ ) (Fig. 2F). Some pathways, such as *skn-1* (Fig. 2E), show small, trending effects that are not statistically significant across 4–6 experiments. It is worth noting that the main group comparisons here are between *egl-3*/*unc-31* and the double mutant. Thus, we conclude that none of these genes were able to significantly reverse the survival benefit conferred by loss of *unc-31*(*e928*) or *egl-3*(*ok979*). These data suggest that these 5 genes either act upstream of neuropeptide signaling or in pathways unrelated to the L4 worm response to anoxia.

Together, these data provide evidence for a novel pathway that controls the specific vulnerability of developing worms to anoxic conditions. The key features of this pathway are: a) L4-staged worms are



**Fig. 2.** Known stress-resistance and longevity promoting genes do not regulate neuropeptide-mediated anoxia sensitivity. All experiments were done with L4 animals under 48 h anoxia. Survival of the following double mutants was compared with *egl-3* or *unc-31* alone. A) *daf-16*; *unc-31* B) *daf-16*; *egl-3* C) *hif-1*; *unc-31* D) *hsf-1*; *unc-31* E) *skn-1*; *egl-3* F) *nsy-1*; *unc-31*. (n = 3 independent experiments for b), n = 4 for rest). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons.

uniquely sensitive to anoxia but not hypercarbia, b) neuropeptide processing and secretion fundamentally control this vulnerability c) neuropeptide control of this anoxic vulnerability operates independent of the major known pathway stress resistance pathways. Based on these results we set the goal of identifying the potential humoral factor(s) that drive this process.

### 3.3. Biogenic amines do not underlie *unc-31*-mediated anoxic survival

While *egl-3* and *egl-21* have established roles in processing neuropeptides, *unc-31* is involved in mediating fusion of dense-core vesicles containing biogenic amines as well as neuropeptides. Berendzen et al. found that protein aggregation in neurons led to metabolic changes in non-neuronal tissues, and this cell non-autonomous regulation was due to dense-core vesicle mediated release of the biogenic amine serotonin

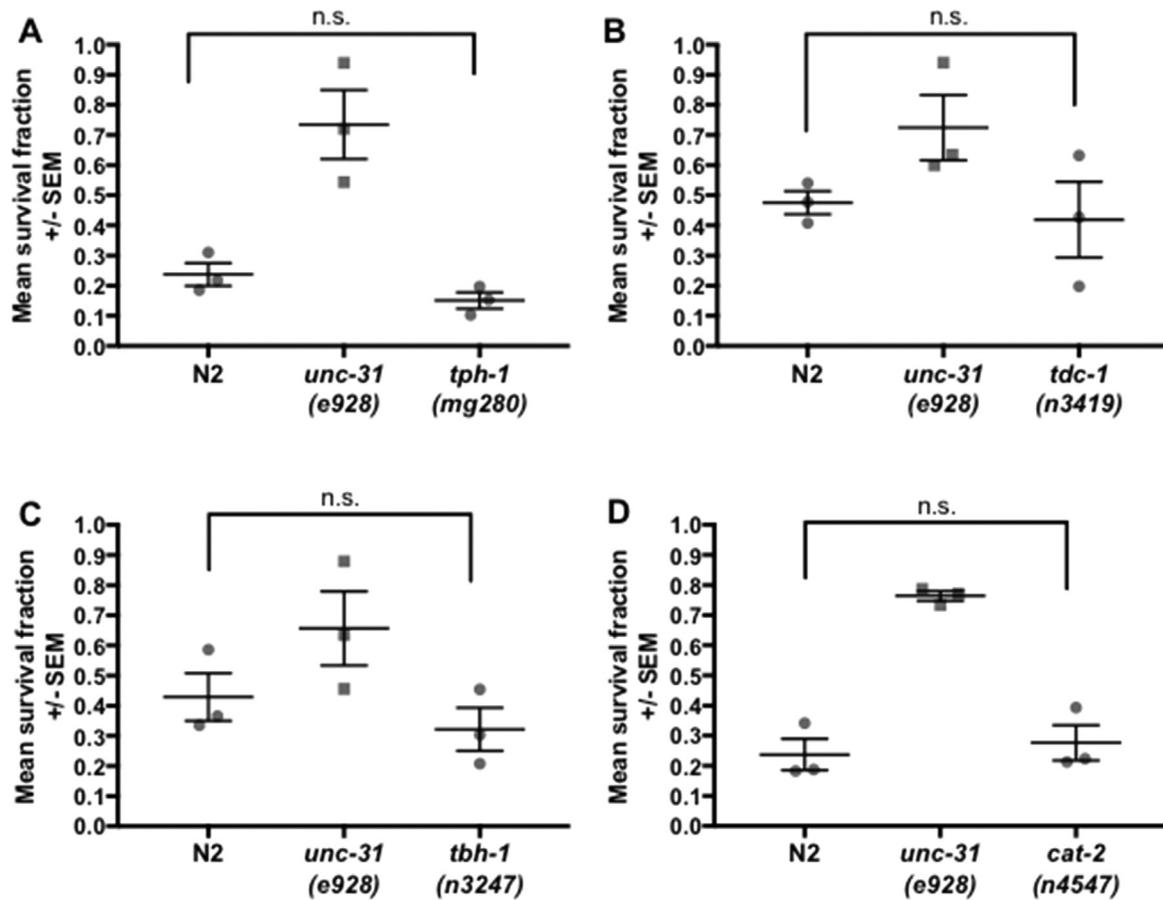


Fig. 3. Survival of biogenic amine synthesis mutants at L4 after 48 h of anoxia. N2 and *unc-31* survival was compared against survival of A) tryptophan hydroxylase (*tph-1*), B) tyrosine decarboxylase (*tdc-1*), C) tyramine  $\beta$ -hydroxylase (*tbh-1*) and D) tyrosine hydroxylase (*cat-2*). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons ( $n = 3$  independent experiments for all).

from neurons [30]. We wondered if loss of dense-core vesicle signaling in *unc-31* mutants might be similarly regulating whole animal survival under anoxia by biogenic amine signaling. There are four biogenic amines in *C. elegans*: serotonin, dopamine, tyramine and octopamine. These are synthesized by tryptophan hydroxylase (*tph-1*), tyrosine hydroxylase (*cat-2*), tyrosine decarboxylase (*tdc-1*) and tyramine  $\beta$ -hydroxylase (*tbh-1*) respectively. If increased survival of *unc-31* mutants under anoxia is due to deficient signaling by one of these amines, their loss should phenocopy *unc-31* mutants. Thus, we assessed the survival of worms with loss of function mutations in each of these enzymes. We found that none of these mutants had a significant survival benefit compared to N2 (*tph-1* group effect:  $F_{(3,8)} = 10.52$ ,  $p = 0.0038$ ; *tdc-1* group effect:  $F_{(2,6)} = 2.75$ ,  $p = 0.01420$ ; *tbh-1* group effect:  $F_{(2,6)} = 3.315$ ,  $p = 0.1072$ ; *cat-2* group effect:  $F_{(2,6)} = 40.25$ ,  $p = 0.0003$ ) (Fig. 3A, B, C, D), suggesting that loss of *unc-31* confers a survival benefit independent of biogenic amine biogenesis.

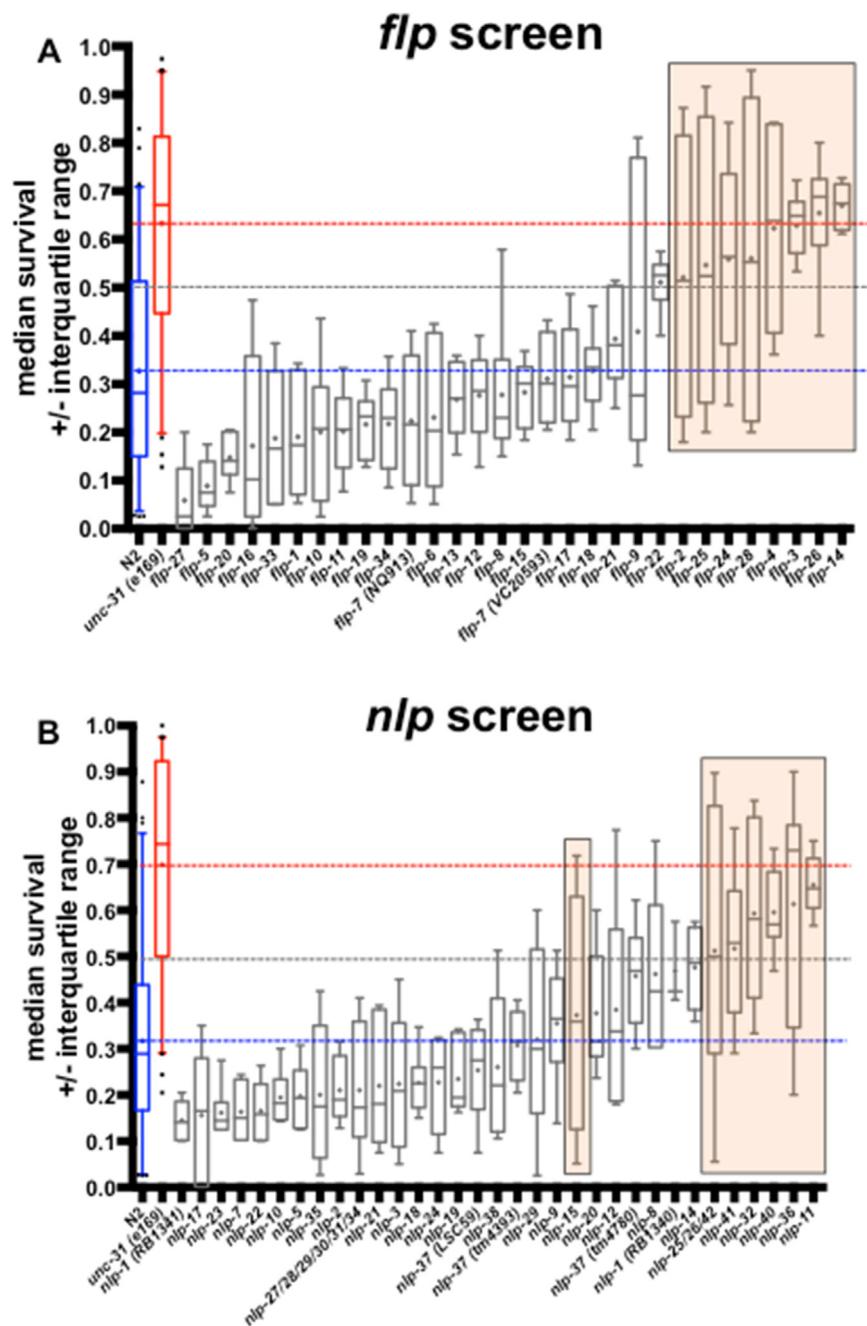
### 3.4. The neuropeptide *nlp-40* partly regulates *C. elegans* response to anoxia

Loss of *egl-3*, *egl-21* and *unc-31* likely leads to broad inhibition of neuropeptide signaling in the worm. To understand neuropeptide-mediated regulation of anoxia, we asked which neuropeptide(s) are necessary for survival under this stress. Having ruled out biogenic amines, we considered two competing hypotheses: a) loss of multiple neuropeptides together influence anoxic sensitivity, or b) the loss of a single (or a few) neuropeptides is/are responsible for the survival benefit in animals with *egl-3*, *egl-21* or *unc-31* mutations. While the second hypothesis might seem unlikely *a priori*, we note the rich body of literature implicating single neuropeptides in regulating complex

behaviors and pathways in worms, such as sleep induction [31,32], thermosensation [33], mitochondrial unfolded protein response induction [34] and feeding [35]. In an attempt to distinguish between these hypotheses, we undertook a screen to find a putative neuropeptide that may control the sensitivity of L4 worms to anoxia.

There are 113 genes encoding over 250 distinct neuropeptides in *C. elegans*, most of which do not have clearly defined functions [16,36]. These neuropeptide genes are broadly divided into three groups based on their peptide structure and sequence: insulin-like peptides (*ins*), FMRF-like peptides (*flp*) and non-insulin/non-FMRF like peptides (*nlp*).

We first considered the insulin class of neuropeptides for two primary reasons. a) EGL-3 is known to process INS pro-peptides to generate their mature, active forms [37]. b) Previous work shows that loss of function in the insulin receptor gene *daf-2* is also protective against anoxia in different developing stages and different anoxia assays, and this protection is entirely dependent on its downstream transcription factor encoded by *daf-16* [4,12,38]. Our finding that *daf-16(mgDf50);unc-31(e928)* and *daf-16(mgDf50);egl-3(ok979)* double mutants have similar survival compared to *unc-31(e928)* and *egl-3(ok979)* mutants alone (Fig. 2A, B) suggested that the *unc31* and *egl-3* effect on anoxia is likely to be independent of the *daf-2/daf-16* pathway. This served to focus our search to *flp*'s and *nlp*'s. There are 31 *flp*'s and 42 *nlp*'s, each of which has multiple mutant alleles. In most cases, it has not been determined whether these are loss of function alleles. We curated these mutants *in silico* and compiled a list of available deletion alleles for each *flp* and *nlp* that would most likely be null alleles. This yielded 29 *flp* mutants and 36 *nlp* mutants which we subsequently obtained from the CGC (Minneapolis, MN) and/or the NBRP (Tokyo, Japan).

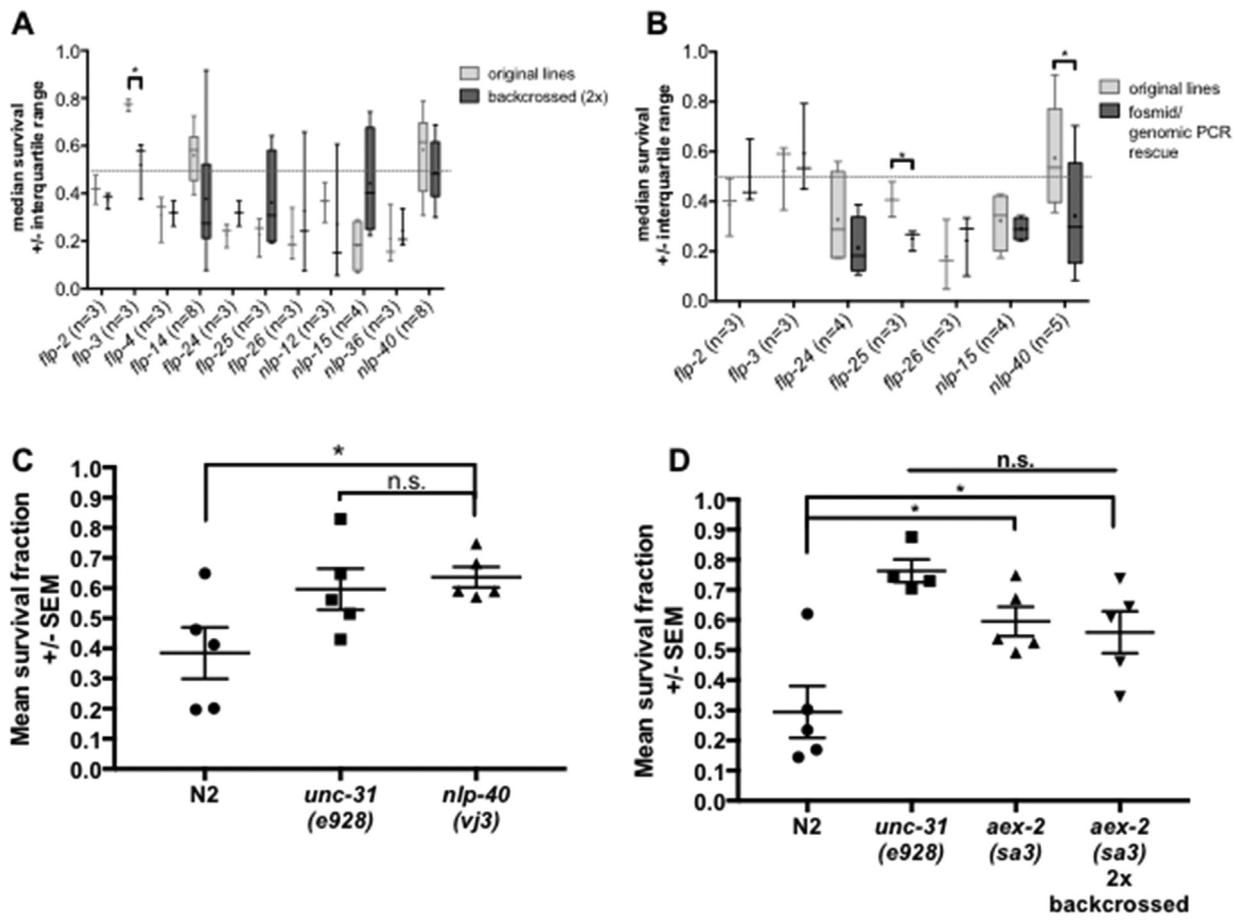


**Fig. 4.** Screen for *flp* and *nlp* classes of neuropeptides regulating survival against anoxia. All experiments were done with L4 animals under 48 h anoxia. A) Survival of 31 strains containing deletions of individual *flp*'s. B) Survival of 33 strains comprising deletions of 36 individual *nlp*'s.  $n = 2$  independent experiments, with 3 bags per experiment, for each *flp* and *nlp* strain. Data show distribution of survival of each strain across 6 bags. Strains highlighted in the orange box in (A) and (B) were selected as candidates for further screening.

Our goal was to identify individual *flp* or *nlp* mutants that mimicked the survival benefit seen in *unc-31* loss of function worms. In addition, we wanted to exhaustively test mutants from this list and repeat the experiment multiple times. We had to reconcile the number of independent experiments we could carry out with the need to balance two competing issues –screening a large number of available mutants and thoroughly testing them to overcome the substantial biological variability that is a consistent feature of this anoxia assay. Thus, we decided to measure survival of each strain after 48 h anoxia in two independent experiments, with co-bagged N2 and *unc-31(e928)* controls, and with three technical replicates per experiment. Thus, we had 6 total data points for each mutant across two experiments (Fig. 4A, B). As expected, we did encounter a large amount of biologic variability in these

experiments, making candidate identification challenging. We decided to narrow down an initial list of mutants that had a mean survival greater than 50% across these 6 observations (orange boxes, Fig. 4A, B). The only exception was *nlp-15(ok1512)*, which was included in the candidate list because it had very high survival in one of the two experiments. This left us with 8 *flp*'s and 9 *nlp*'s for the secondary screen. One of the strains we tested had a very large deletion that removed many hundreds of genes (VC30122, which spanned *nlp-25*, *nlp-26* and *nlp-42*). Because of this, we had 15 strains encompassing the 17 total mutations after our initial screen.

To filter these candidates further, we undertook a secondary screen with the following tests: a) repeat the experiment for each candidate > 3–4 more times, b) backcross each line to N2 at least twice to



**Fig. 5.** Secondary screen of candidate *flp* and *nlp* mutants identifying *nlp-40* as a partial regulator of anoxia. For all experiments, worms at the L4 stage were subjected to 48 h anoxia. **A)** Survival of candidate *flp*'s and *nlp*'s before and after 2X backcrossing to N2 ( $n = 3$ –9 independent experiments). **B)** Survival of candidate *flp*'s and *nlp*'s before and after rescue with a fosmid or a PCR fragment containing the genomic locus ( $n = 3$ –5 independent experiments). Data were analyzed using a two-tailed paired *t*-test for each *flp/nlp*. Data appear as 'boxes' instead of 'lines' if the experiment was conducted more than 3 times. \* $p < 0.05$ . **C)** Survival of a second allele of *nlp-40*, *vj3* after 48 h anoxia ( $n = 5$  independent experiments). **D)** Survival of the *nlp-40* receptor *aex-2* compared to *unc-31* ( $n = 4$  independent experiments). For **C)** and **D)**, data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons. \* $p < 0.05$ .

remove mutations in the background genotype and c) use fosmids to rescue the deleted locus. We were able to repeat the experiment in 11 of 14 strains, twice backcross 11 of 14 strains and rescue 7 of 14 strains. For most lines, repeating the experiment many times dropped the average survival to well below 50%, suggesting a high false-positive rate in our initial screen (light gray points, Fig. 5A, B). Additionally, we noticed that backcrossing the lines led to high variability in the data (dark gray points, Fig. 5A). One line with high survival (*flp-3(ok3265)*), had to be excluded because backcrossing reduced its survival to N2 levels (Fig. 5A), and fosmid rescue did not reduce its high survival (Fig. 5B). This indicated that mutations at loci other than *flp-3* are likely to be conferring a benefit in our assay. Since we did not backcross all the lines before initially testing them, it is possible that by the same rationale, we may have missed potential hits due to mutations in non-neuropeptide genes that suppressed a putative benefit of the neuropeptide mutant we studied. Despite the limitations of this approach, one of the candidates, *nlp-40(tm4085)*, was able to pass all three tests of the secondary screen (backcross:  $t_{(7)} = 1.797$ ,  $p = 0.1155$ , paired *t*-test; rescue:  $t_{(4)} = 2.868$ ,  $p = 0.456$ , paired *t*-test) (Fig. 5A, B). We tested a second loss of function allele, *nlp-40(vj3)* to verify the survival benefit of the *nlp-40(tm4085)* allele, and found a similar survival benefit in *nlp-40(vj3)* mutants (group effect:  $F_{(2,12)} = 4.226$ ,  $p = 0.0408$ ) (Fig. 5C). Since *nlp-40* mutant survival hovers around 50–60% for both alleles despite backcrossing, we suggest that loss of *nlp-40* is only partially responsible for the anoxia survival phenotype of *unc-31(e928)*. It is likely that more than one neuropeptide regulates anoxic survival in *C.*

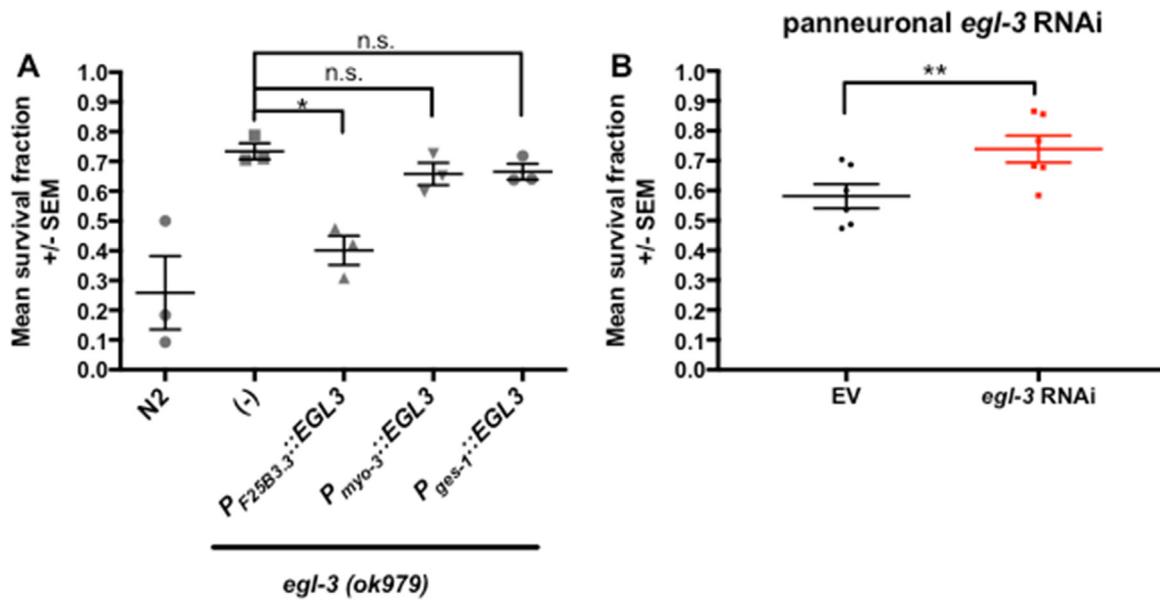
*elegans*.

To further explore the contribution of *nlp-40*, we tested the survival of its putative receptor gene *aex-2* [17]. If *nlp-40* requires *aex-2* for its survival benefit under anoxia, loss of *aex-2* should mimic loss of *nlp-40*. Indeed, we found that *aex-2(sa3)* mutant worms have increased survival 48 h of anoxia compared to N2 worms, and similar to *unc-31(e928)* mutant worms, and this effect persists after backcrossing (group effect:  $F_{(3,15)} = 8.346$ ,  $p = 0.0017$ ) (Fig. 5D).

Together, these data indicate that the neuropeptide *nlp-40* partially regulates survival under anoxia, and we predict that it does so likely through its receptor *aex-2*.

### 3.5. Neuropeptide synthesis in the nervous system is necessary for survival under anoxic stress

Reporter studies have shown that UNC-31 and EGL-21 are ubiquitously expressed in the nervous system and have little detectable expression in other tissues [19,20]. Similarly, EGL-3 is broadly expressed in the nervous system, but there is evidence that it is also expressed in non-neuronal cells such as the intestine [37]. We asked if EGL-3 function in specific tissue(s) was sufficient for its regulation of anoxic survival. To this end, we generated worms expressing genomic *egl-3* under either a pan-neuronal ( $P_{F25B3.3}$ ), intestinal ( $P_{ges-1}$ ) or muscle-specific ( $P_{myo-3}$ ) promoter in the *egl-3(ok979)* mutant background. These lines will be null for *egl-3* in the whole animal except neurons, muscles and intestine, respectively. We found that *egl-3(ok979)* worms had



**Fig. 6.** Neuronal expression of *egl-3* is necessary for regulating survival under anoxia. A) Survival of worm lines expressing genomic *egl-3* in neurons ( $P_{F25B3.3}$ ), body wall muscle ( $P_{myo-3}$ ) and intestine ( $P_{ges-1}$ ) in an *egl-3(ok979)* null background ( $n = 3$  independent experiments). Data were analyzed using one-way ANOVA with Tukey's test for multiple comparisons. B) Survival of the line TU3311 ( $P_{unc-119}::sid-1$ ) which is sensitized for neuronal knockdown of a target gene, fed empty vector (EV) or *egl-3* RNAi bacteria for at least 2 generations. ( $n = 6$  independent experiments), Data were analyzed using paired *t*-test between EV and *egl-3* for each line. \* $p < 0.05$ , \*\* $p < 0.01$ .

significantly greater survival compared to N2 after 48 h anoxia, and survival of *egl-3(ok979);P<sub>myo-3</sub>::EGL-3* and *egl-3(ok979);P<sub>ges-1</sub>::EGL-3* was not significantly different from *egl-3(ok979)*. However, *egl-3(ok979);P<sub>F25B3.3</sub>::EGL-3* worms lost this survival benefit and had similar survival as N2 worms (group effect:  $F_{(4,10)} = 10.1$ ,  $p = 0.0015$ ). Since restoration of *egl-3* to neurons suppressed the benefits of whole organism ablation of *egl-3* (Fig. 6A), the nervous system is likely to generate the neuropeptide that controls susceptibility to anoxia during development.

To test neuronal contribution of *egl-3* in a different way, we turned to RNA interference (RNAi). We employed the worm strain TU3311(*uls60[unc-119p::YFP+unc-119p::sid-1]*), which expresses the dsRNA channel *sid-1* under the *unc-119* promoter to enhance dsRNA uptake specifically in the nervous system [15]. We then fed these worms either empty vector (EV) bacteria as a control or *egl-3* RNAi bacteria to induce pan-neuronal knockdown of *egl-3*. Compared to worms raised on EV bacteria, those raised on bacteria expressing pan-neuronal RNAi against *egl-3* survived anoxia significantly better group effect: ( $t_{(5)} = 6.808$ ,  $p = 0.0010$ , paired *t*-test) (Fig. 6B). Together, these observations indicate that loss of *egl-3* in neurons is necessary to confer survival under anoxia. Since *unc-31* and *egl-21* are thought to be exclusively expressed in neurons, these observations make a strong case that neuropeptides synthesized in the nervous system make worms susceptible to anoxic stress.

### 3.6. *nlp-40* function in cholinergic or ASK sensory neurons does not account for its regulation of anoxic survival

Recently, Cao et al. undertook a comprehensive single cell RNA sequencing analysis of *C. elegans* [39]. We interrogated their data to determine the expression pattern of *nlp-40* at the transcript level and found that it is most highly expressed in neurons (Fig. 7A). Among neurons, *nlp-40* expression was highest in the pair of ASK sensory neurons located in the head, followed by its expression in cholinergic neurons (Fig. 7B). We decided to see if *nlp-40* expression in these cell-types accounted for its regulation of worm survival under anoxia. Indeed, neuronal knockdown of *nlp-40* using the TU3311 strain led to increased survival of L4 worms under 48 h anoxia compared to those

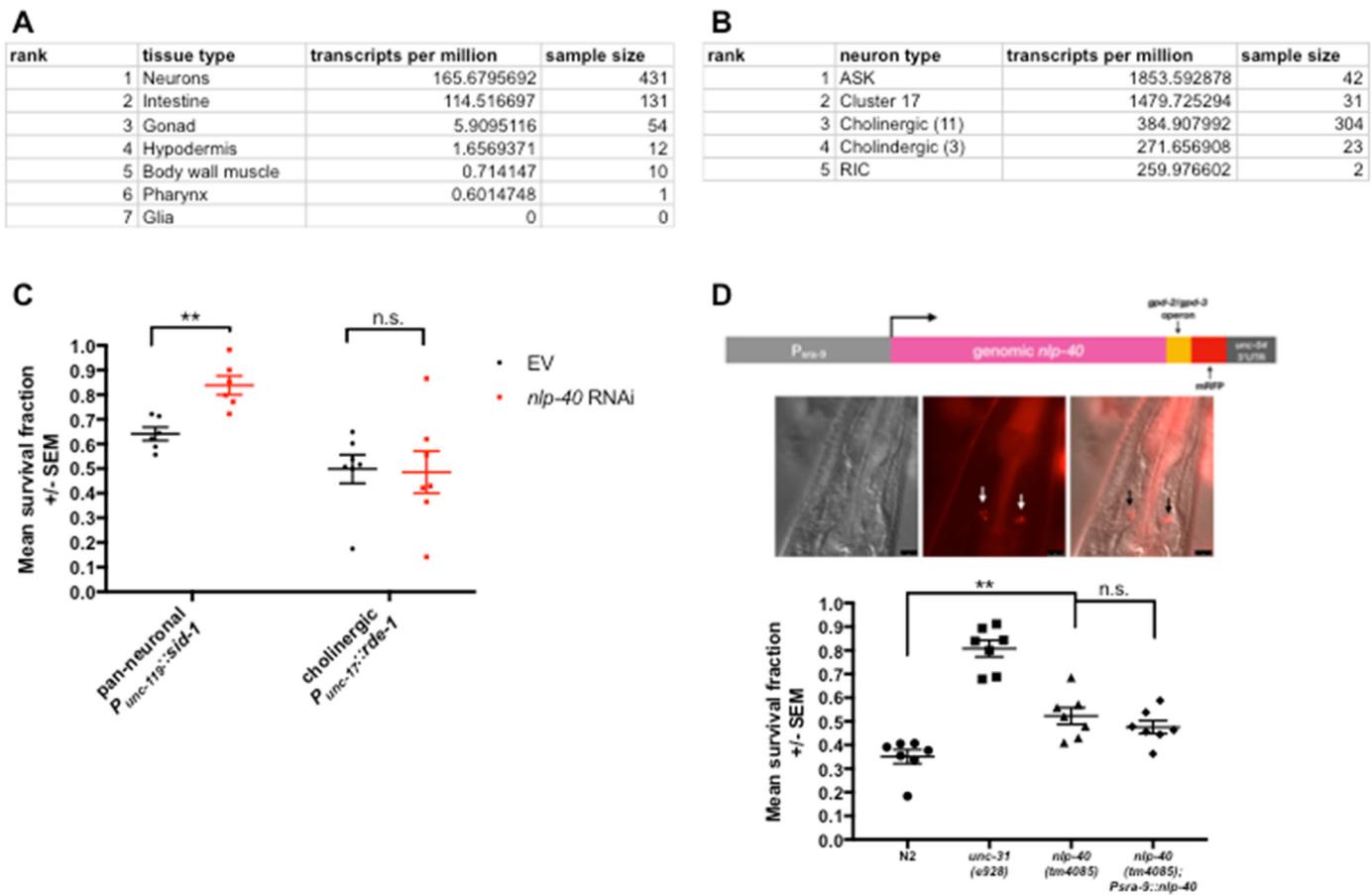
fed empty vector bacteria ( $t_{(5)} = 6.148$ ,  $p = 0.0017$ , paired *t*-test) (Fig. 7C). Additionally, we performed RNAi mediated knockdown of *nlp-40* specifically in cholinergic neurons using the strain XE1581 (*wpSi10[unc-17p::rde-1::SL2::sid-1 + Cbr-unc-119(+)]*; *eri-1(mg366)*; *rde-1(ne219)*; *lin15(n744)*) [40]. Unexpectedly, we found that *nlp-40* knockdown in cholinergic neurons did not lead to a survival benefit in worms when compared to those fed empty vector bacteria ( $t_{(4)} = 0.02699$ ,  $p = 0.9798$ , paired *t*-test) (Fig. 7C). To test whether *nlp-40* expression in the ASK neuron is necessary for its regulation of survival in anoxia, we generated worms expressing *nlp-40* genomic DNA under the *sra-9* promoter ( $P_{sra-9}$ ) in the *nlp-40(tm4085)* mutant background. These worms are deficient in *nlp-40* expression in all cells except the ASK neurons (Fig. 7D). If *nlp-40* functions in the ASK neuron to regulate developing worm anoxic survival, we would expect that restoring *nlp-40* expression in the ASK neuron would suppress the survival benefit of *nlp-40* mutation. We find ASK-specific rescue of *nlp-40* modestly suppress the survival benefit of *nlp-40* mutation however this did not achieve significance after correcting for multiple comparisons (group effect:  $F_{(3,24)} = 36.41$ ,  $p < 0.0001$ ) (Fig. 7D). Together these observations indicate that *nlp-40* elaboration by neurons is a critical determinant of developing worm survival after anoxic insult. The participation of neither cholinergic nor ASK neurons in isolation adequately account for this phenomenon.

## 4. Discussion

Here we investigated a novel role of neuropeptides in regulating the susceptibility of developing *C. elegans* to the specific stress of anoxia. We find that loss of neuropeptide biogenesis and secretion by neurons enhances survival of animals and this effect operates independent of five well-defined stress resistance pathways. This effect is mediated in part by neuronally derived by *nlp-40* and its receptor *aex-2*. To our knowledge this is the first identification of a secreted factor that renders animals susceptible to anoxic insult.

### 4.1. Developmental stage and anoxia tolerance

Intuitively, it would be logical for worms stressed by anoxia to make



**Fig. 7.** *nlp-40* does not function in cholinergic or ASK sensory neurons for its contribution to worm survival under anoxia. Expression pattern of *nlp-40* RNA in different A) tissue types and B) neuronal subtypes obtained from single cell transcriptomics database [39]. C) Survival of L4-staged worms from the strain XE1581 (*Punc-17::rde-1*) fed empty vector (EV) or *nlp-40* RNAi bacteria, to achieve cholinergic-specific knockdown of *nlp-40*, after 54 h anoxia. D) Generation of the ASK-specific *nlp-40* rescue line [*nlp-40(tm4085);Psra-9::nlp-40::SL2::mRFP*] and its survival after 48 h anoxia. Arrows show mRFP-expressing ASK neurons. Scalebar = 10  $\mu$ m. Data were analyzed using paired *t*-test between EV and *nlp-40* in (C) and using one-way ANOVA with Tukey's test for multiple comparisons in (D). \*\**p* < 0.01.

and secrete factors that promote resistance to insult. And yet, we find that neuropeptide signaling, in fact, can make developing worms sensitive to anoxia. Why might this be? Neuropeptides are essential for guiding behavioral adaptations in response to environmental cues. For example, heat and UV stress lead to a neuropeptide mediated sleep-like response [41,42]; food quality and availability leads to neuropeptide-mediated changes in dwelling and roaming behaviors [43]. Neuropeptide signaling between developmental stages in part regulates the establishment of non-genetic, stereotyped behaviors across these stages in individual worms [44]. In response to adverse environmental cues, worms enter the stress-resistant 'dauer' state, and this is enabled by the upregulation of neuropeptide signaling during the L2d stage of development [45]. To reconcile these beneficial actions of neuropeptides on behavior with what we find, we hypothesize a trade-off between competing imperatives. According to this view, neuropeptides can promote advantageous behavioral adaptations to certain environmental conditions at the expense of disadvantageous susceptibility to non-ordinary conditions such as anoxia. Further study of developmental regulation of neuropeptide signaling, including the specific neuropeptides and tissues involved, will be necessary to understand how this balance of biological effects is optimized.

#### 4.2. Genetic pathways regulating anoxia

There are several canonical pathways regulating *C. elegans* response to various stressors, including oxidative stress and oxygen deprivation.

The insulin signaling DAF-2/DAF-16 pathway is known to regulate stress-resistance and longevity in worms, flies and mammals [46–48]. Loss of function in *daf-2* leads to increased lifespan and increased tolerance to many stressors including heat, UV radiation, oxidative stress, hypoxia and anoxia, and this requires its downstream transcription factor *daf-16* [4,38,49–51]. The *hif-1* transcription factor is required to survive hypoxia, and its stabilization extends lifespan [9,52]. The *hsf-1* transcription factor is essential for lifespan extension and the response to heat and oxidative stress [27]. The *skn-1* transcription factor is similarly necessary for resistance to oxidative stress and lifespan extension [53], and is necessary for mitochondrial fusion and recovery of worms after anoxia [28]. The MAPK pathway is also critical for the response to oxidative stress, and worms mutant for *nsy-1*, the worm homolog of ASK family proteins, were shown to be resistant to extended anoxia [29]. Interestingly, *skn-1* and *nsy-1* mutants were hypersensitive to anoxia, and merit further investigation for their independent roles in regulating anoxia. Some, like the *skn-1;egl-3* double mutants, showed a trend of decreased survival compared to *egl-3* alone (Fig. 2E). While this result was not statistically significant over 4 independent experiments, it indicates that *skn-1* may play a small role in the neuropeptide regulation of anoxia, and could benefit from additional focused investigation to fully understand this phenotype. However, in light of the connections between these well-established pathways to survival under low oxygen conditions, it is remarkable that none genetically interacted with the neuropeptide regulation of developing worms vulnerability to anoxia. This leads to the exciting prospect that additional stress

resistance pathways remain to be found.

#### 4.3. Cell non-autonomous actions of neuropeptides

A pair of sensory neurons in the head, the BAG neurons, are responsible for detecting a decrease in environmental oxygen in worms [11]. Genetic ablation of BAG neurons protects worms from anoxia [12]. One potential explanation for BAG-mediated regulation of whole animal survival could be that the BAG neuron directly secretes a peptide that coordinates the response of different tissues to anoxia and regulates animal survival. This is unlikely because surviving anoxic stress during development does not require neuropeptide signaling from BAG neurons [12]. Worms null for *egl-3* with BAG-specific rescue of *egl-3* continue to display high survival under anoxia. Additionally, the neuropeptide *flp-17* is primarily expressed in BAG neurons [54]. Here, we find that loss of *flp-17* does not protect L4 worms from anoxia (Fig. 4A), further suggesting that neuropeptide-mediated regulation of survival under anoxic stress acts in non-BAG neurons.

Another explanation for the Flibotte et al. finding is that BAG coordinates a network of neurons, which in turn secrete factor(s) that influence organismal vulnerability to anoxia. If BAG neurons leads to neuropeptide release from different downstream neuron(s), this would argue for a cell non-autonomous regulation of the response to anoxia. There are many examples of neuropeptide-mediated cell non-autonomous effects in worms. For example, the neuropeptide *flp-2* is used by neurons when they experience mitochondrial stress, leading to a systemic mitochondrial unfolded protein response in distal tissues [34]. The neuropeptide *nlp-20* is released downstream of pathogen-sensing ASH and ASI neurons to regulate the immune response in worms [55]. Neuropeptides, in part, non cell-autonomously regulate lifespan extension in worms downstream of ATG-18 mediated neuroendocrine signaling during dietary restriction or insulin signaling [56].

Genes encoding neuropeptides are expressed widely throughout the nervous system but are also found in non-neuronal tissue such as intestine, the vulval hypodermis and the gonads. They act both locally at synapses and over long distances as hormones to coordinate these behaviors within and between tissues [57]. Here, we find that for inducing sensitivity to anoxia, neuropeptide synthesis is necessary in neurons. We identify *nlp-40* as a regulator of the worm response to anoxia during development. In support of this, feeding RNAi mediated neuronal knockdown of *nlp-40* enhances developing worms survival after anoxia. This was an unexpected finding because prior work showed that *nlp-40* is expressed by the intestine [17]; its neuronal expression pattern is not well described. However, Cao et al. published a single cell transcriptomic profile of *C. elegans* in the L2 stage of development [39]. Our probing their database indicates that *nlp-40* is highly expressed in cholinergic and ASK neurons. Cell-type specific knockdown and rescue experiments do not, however, precisely define which neuron(s) is/are responsible for elaborating NLP-40 in our paradigm. In addition, we do not know which cells respond to NLP-40, and likely other neuropeptides, to control anoxic sensitivity in developing worms. We anticipate this information will enable identification of new molecular pathways that confer anoxia resistance.

#### 5. Conclusions

Together, this body of work introduces neuropeptide signaling as a novel regulator of anoxic stress in developing worms. It begins to describe the mechanisms by which neuropeptides regulate survival of the whole organism in cell autonomous and non-autonomous ways, and how they might differ in development and adulthood.

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SD and RGK designed the experiments and wrote the article. H.L. proofread the article. SD, EP, JL, UB and MS conducted the experiments.

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#### Conflict of interest

The authors declare no competing financial interests.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.freeradbiomed.2018.12.006.

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