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EGL-3 and EGL-21 are required to trigger nocifensive response of *Caenorhabditis elegans* to noxious heat

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ABSTRACT

Caenorhabditis elegans (*C. elegans*) is a widely used model organism to examine nocifensive response to noxious stimuli, including heat avoidance. Recently, comprehensive analysis of the genome sequence revealed several pro-neuropeptide genes, encoding a series of bioactive neuropeptides. *C. elegans* neuropeptides are involved in the modulation of essentially all behaviors including locomotion, mechanosensation, thermosensation and chemosensation. The maturation of pro-neuropeptide to neuropeptide is performed by ortholog pro-protein convertases and carboxypeptidase E (e.g. EGL-3 and EGL-21). We hypothesized that *C. elegans egl-3* or *egl-21* mutants will have a significant decrease in mature neuropeptides and they will display an impaired heat avoidance behavior. Our data has shown that thermal avoidance behavior of *egl-3* and *egl-21* mutants was significantly hampered compared to WT(N2) *C. elegans*. Moreover, *flp-18*, *flp-21 and npr-1* mutant *C. elegans* displayed a similar phenotype. EGL-3 pro-protein convertase and EGL-21 carboxypeptidase E are essential enzymes for the maturation of pro-neuropeptides to active neuropeptides in *C. elegans*. Quantitative mass spectrometry analyses with *egl-3* and *egl-21* mutant *C. elegans* homogenates demonstrated that proteolysis of ProFLP-18 and ProFLP-21 are severely impeded, leading to a lack of mature bioactive neuropeptides. Not only FLP-21 but also FLP-18 related mature neuropeptides, both are ligands of NPR-1 and are needed to trigger nocifensive response of *C. elegans* to noxious heat.

1. Introduction

Caenorhabditis elegans (*C. elegans*) is a remarkable animal model system for functional genomics pertinent to mammalian and human biology and diseases (Komuniecki et al., 2012; Avila et al., 2012). *C. elegans* genome sequencing was finalized in 1998 and it is now comprehensively annotated and publicly available (The *C. elegans* Sequencing Consortium, 1998; the *C. elegans* genome sequencing project, 1999). This is an important benefit for proteomic investigations particularly in the context of comprehensive biochemical and signaling network studies (Tullet, 2014; Boucher and Jenna, 2013). Interestingly, adult *C. elegans* consists of 959 cells including 302 neurons, which make this model attractive to study neuronal communication at the physiological and molecular levels (Wittenburg and Baumeister, 1999). *C. elegans* is particularly useful for the study of nociception as it exhibits a well-defined and reproducible nocifensive behavior, involving a reversal and change in direction away from the noxious stimulus (Wittenburg and Baumeister, 1999; Carr and Zachariou, 2014). Following the genome sequencing of *C. elegans*, it was determined that specific genes encode transient receptor potential (TRP) ion channel proteins with significant sequence similarities to mammalian TRP channels including the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Kahn-Kirby and Bargmann, 2006). Specifically, five TRP subfamilies including TRPV analogs (e.g. OSM-9 and OCR-1-4) were characterized (Xiao and Xu, 2011). Furthermore, it has been recently established that *C. elegans* TRP channels are associated with behavioral and physiological processes, including sensory transduction of thermal and chemical information (Glauser et al., 2011; Venkatachalam et al., 2014). Many *C. elegans* TRP channels share similar activation and regulatory mechanisms with their mammal counterparts. Interestingly, it was revealed that the thermal avoidance response of *C. elegans* is amplified when animals are exposed to capsaicin a well-known agonist of the TRPV1 (Wittenburg and Baumeister, 1999; Tobin et al., 2002). Furthermore, the TRPV1 can be activated by other physical and chemical stimuli including noxious heat, low pH, divalent cations and animal toxins (Yang and Zheng, 2017). The activation of the TRPV1 triggers the release of several neuropeptides, including substance P and calcitonin gene-related peptide central to synaptic and

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nociceptive transmission in mammals (Jara-Oseguera et al., 2008; Gazzieri et al., 2007).

Neuronal molecular communication in *C. elegans* was recently investigated and exploratory immunochemical analyses exposed the presence of numerous neuropeptides in *C. elegans* (Mills et al., 2012). Additionally, a comprehensive analysis of the genome sequence revealed several pro-neuropeptide genes, encoding a series of bioactive neuropeptides and specific neuropeptide receptors playing a central role in synaptic transmission (Li, 2005; Hu et al., 2011; Choi et al., 2013). Neuropeptides are involved in the modulation of essentially all behaviors including locomotion, mechanosensation, thermosensation and chemosensation (Komuniecki et al., 2012; Biron et al., 2008). Neuropeptides act as neuromodulators and as fast neurotransmitters. The broad existence of these neuropeptides in nematodes suggests a fundamental role of neuropeptidergic signaling in *C. elegans* but the molecular pathways and networks are poorly understood. Moreover, *C. elegans* uses classical neurotransmitter systems like acetylcholine (Ach), glutamate, γ-amunobutyric acid (GABA), serotonin, dopamine and octopamine found in mammals (Barclay et al., 2012). Several neuropeptide receptors were identified and were shown to play an important role in signal transduction. It has been recently demonstrated that neuropeptide receptor 1 (NPR-1) play a central role in locomotion and thermal avoidance. NPR-1 and its ligands FLP-18 and FLP-21 play an important role in the regulation of locomotion (Choi et al., 2013). Additionally, data suggested that TRPV channels (i.e. OSM-9 and OCR-2) and the FLP-21/ NPR-1 neuropeptide signaling pathway are essential for the nocifensive response associated to heat avoidance in *C. elegans* (Glauser et al., 2011). Therefore, the key proteases (e.g. ELG-3 and EGL-21) involved in the processing of pro-neuropeptides into neuropeptides can potentially regulate nociceptive behavior in *C. elegans*.

In mammals, several neuropeptides are synthesized by the action of pro-protein convertases (PCs) and endopeptidases during the axonal transport (Hook et al., 2008; Saidi et al., 2016; Ruiz and Beaudry, 2016; Saidi and Beaudry, 2017; Ben Salem et al., 2018). Recently, it was revealed that *C.elegans egl-3* gene encodes a protein (i.e. EGL-3) with 57% sequence homology compared to mammalian pro-protein convertase type 2 (PC2) (Kass et al., 2001). EGL-3 is a serine endoprotease which cleaves pro-proteins at paired basic amino acids as shown in Fig. 1 and is an ortholog of the human PC2. EGL-3 is an essential enzyme involved in the maturation of pro-neuropeptides to active neuropeptides in *C. elegans* (Hook, 2008; Li and Kim, 2008). Analogously to human, *C. elegans egl-21* gene encodes a protein (i.e. EGL-21) that is an ortholog of the human carboxypeptidase E and this enzyme is broadly expressed in several neurons. EGL-21 is essential for removing basic residues from the C-terminal following EGL-3 pro-neuropeptide processing (Jacob and Kaplan, 2003). Thus, we believe that *egl-3* and *egl-21* mutant animals will lack mature neuropeptides and consequently synaptic chemical communication will be impaired. Changes in nocifensive behavior are orchestrated by specific altered activity during synaptic communication in *C. elegans*.

The objectives of the study were to characterize nocifensive responses of wild type (N2 strain), *egl-3* and *elg-21* mutants as well as specific neuropeptide mutant nematodes (*flp-21* and *flp-18*) and neuropeptide receptor *npr-1* following exposition to noxious heat (i.e. 33–35 °C). Chemotaxis results are shown in supplementary figures. Relative quantification of FLP-21 and FLP-18 related neuropeptides were performed using high performance liquid chromatography coupled to a hybrid Orbitrap high-resolution mass spectrometer.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were obtained from Fisher Scientific (Fair Lawn, NJ, USA) or MilliporeSigma (St-Louis, MO, USA). For mass spectrometry analysis, formic acid, water (HPLC-MS Optima grade), acetonitrile (HPLC-MS Optima grade), trifluoroacetic acid (TFA), were purchased from Fisher Scientific.

2.2. C. elegans strains

The N2 (Bristol) isolate of *C. elegans* was used as a reference strain. Mutant strains used in this work included: *egl-21* (KP2018); *egl-21* (MT1206); *egl-3* (MT1541); *egl-3* (VC461); *flp-18* (AX1410); *flp-21* (RB982); *npr-1* (CX4148). N2 (Bistrol) and other strains were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota (Minneapolis, MN, USA). Strains were maintained and manipulated under standard conditions as described (Brenner, 1974; Margie et al., 2013). Worms were grown and kept on Nematode Growth Medium (NGM) agar at 22 °C in a Thermo Scientific Heratherm refrigerated incubator. Analyses were performed at temperature ranging from 22 to 25 °C unless otherwise noted.

2.3. Thermal avoidance and chemotaxis assays

The principle behind evaluating the *C. elegans* response to a stimulus (i.e thermal or chemical) is to observe and quantify the movement evoked in response to a specific stimulus. The methods proposed in this manuscript for the evaluation of thermal avoidance or chemotaxis were adapted from the two and four quadrants strategies previously described (Margie et al., 2013; Porta-De-La-Riva et al., 2012). We decided to use the same observation strategy for all the test performed to enable better comparison. The experimental schematics are illustrated in Fig. 2. Chemotaxis assay results are available as supplemental figures. Experiments were performed on 92 × 16 mm petri dishes divided into four quadrants. A middle circle delimited (i.e. 1 cm diameter) an area where animals were not counted. This prevents immobile worms from distorting the behavior data. The quadrants create an alternating configuration of thermal (or chemical) stimuli areas and control areas to prevent any bias that may appear resulting from the initial location of the nematodes. Petri dishes were divided into quadrants; two stimulus areas (A and D) and two control areas (B and C). Sodium azide (i.e. 0.5 M) was used in all quadrants to paralyze the nematodes. Nociceptive heat was generated using an electronically heated metal tip (0.8-mm diameter) producing a constant radial temperature gradient (e.g. 32–35 °C on the NGM agar at 2 mm from the tip measured with an infrared thermometer). Nematodes were isolated, prepared and washed according to protocol outline by Margie et al. (2013). From this point, they were off food pending and throughout all experimentations. The nematodes (i.e. 50 to 200 young adult worms) were deposited at the center of a marked petri dish and after 30 min, they were counted per quadrant. Nematodes that did not cross the inner circle were not counted. The derived Thermal avoidance Index (TI) formula is shown in Fig. 2. Both TI and the animal avoidance percentage were used to phenotype each tested *C. elegans* genotype. The selection of quadrant temperature was based on previous experiments (Wittenburg and Baumeister, 1999).

2.4. Analysis of neuropeptides

2.4.1. C. elegans homogenization and peptides extraction

The N2 (Bristol) isolate of *C. elegans* was used as a reference strain. Mutant strains used for the neuropeptide analyses work included: *egl-21* (KP2018); *egl-21* (MT1206); *egl-3* (MT1541); *egl-3* (VC461). Strains were cultured in liquid media standard as described (Brenner, 1974; Margie et al., 2013). The liquid media was centrifuged at 1000 *g* for 10 min and nematodes were collected and aliquoted to re-enforced 1.5 mL homogenizer tubes containing 500 µm glass beads. A solution of 0.1% TFA in water was added at a ratio of 1:5 (w:v) and a Disruptor Genie was used at 2800 rpm for 15 min. Nematodes were homogenized in a TFA solution to inhibit the enzymatic degradation of neuropeptides. The homogenates were centrifuged at 12,000 *g* for 10 min. The

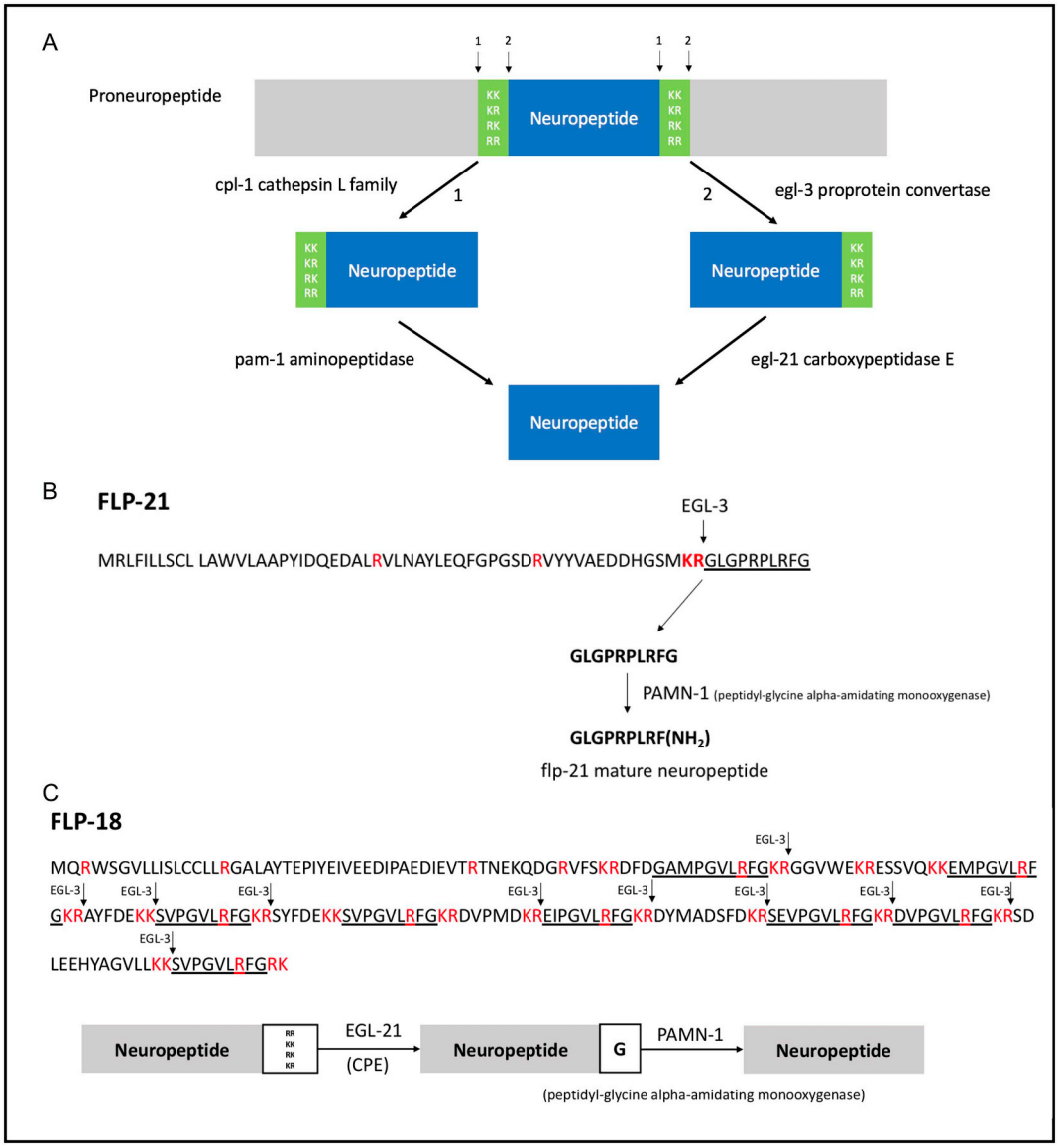

Fig. 1. Neuropeptide processing by pro-protein convertases (EGL-3) and carboxypeptidase E (EGL-21). Most pro-neuropeptides have several paired basic amino acid cleavage sites and based on the sequence, various processing intermediates can be derived leading to mature neuropeptide. A) Generalyse protease pathways for pro-neuropeptide processing in C.elegans (adapted from Hook et al., 2008). B) FLP-21 processing by pro-protein convertases EGL-3 leading to active FLP-21 neuropeptide. C) FLP-18 processing by pro-protein convertases EGL-3 and carboxypeptidase E EGL-21 leading to several actives neuropeptides with common C-terminal sequence of GVLRFG.

protein concentration for each homogenate was determined using a Bradford assay and all samples were normalized to avoid any bias. One hundred microliters of sample were taken and mixed with 100 µL of methanol containing 0.1% TFA to precipitate large and medium size proteins. Samples were centrifuge at 12,000 *g* for 10 min. The supernatant was dried using an Eppendorf vacuum concentrator, re-suspended in 50 µL of 5% acetonitrile in water containing 0.1% formic acid and transferred into low volume HPLC vials.

2.4.2. Chromatographic conditions

The HPLC system was a Thermo Scientific Vanquish FLEX UHPLC system (San Jose, CA, USA). The chromatography was performed using a gradient mobile phase along with a microbore column Thermo Biobasic C18 100 × 1 mm, with a particle size of 5 µm. The initial mobile phase condition consisted of acetonitrile and water (both fortified with 0.1% of formic acid) at a ratio of 5:95. From 0 to 2 min, the ratio was maintained at 5:95. From 2 to 22 min, a linear gradient was applied up to a ratio of 50:50 and maintained for 3 min. The mobile phase composition ratio was reverted at the initial conditions and the column was allowed to re-equilibrate for 15 min for a total run time of 40 min. The flow rate was fixed at 75 µL/min and 2 µL of sample were injected.

2.4.3. Mass spectrometry conditions

A Thermo Scientific Q-Exactive Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced with a Thermo Scientific Vanquish FLEX UHPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode and operating in scan mode at high-resolution and accurate-mass (HRAM). Nitrogen was used for sheath and auxiliary gases and they were set at 10 and 5 arbitrary units. Auxiliary gas was heated to 200 °C. The heated ESI probe was set to 4000 V and the ion transfer tube temperature was set to 300 °C. The scan range was set to *m/z* 400–2000. Data was acquired at a resolving power of 140,000 (FWHM using automatic gain control target of 3.0×10^6 and maximum ion injection time of 200 msec. Product ion spectra (MS^2) were acquired at a resolving power of 17,500

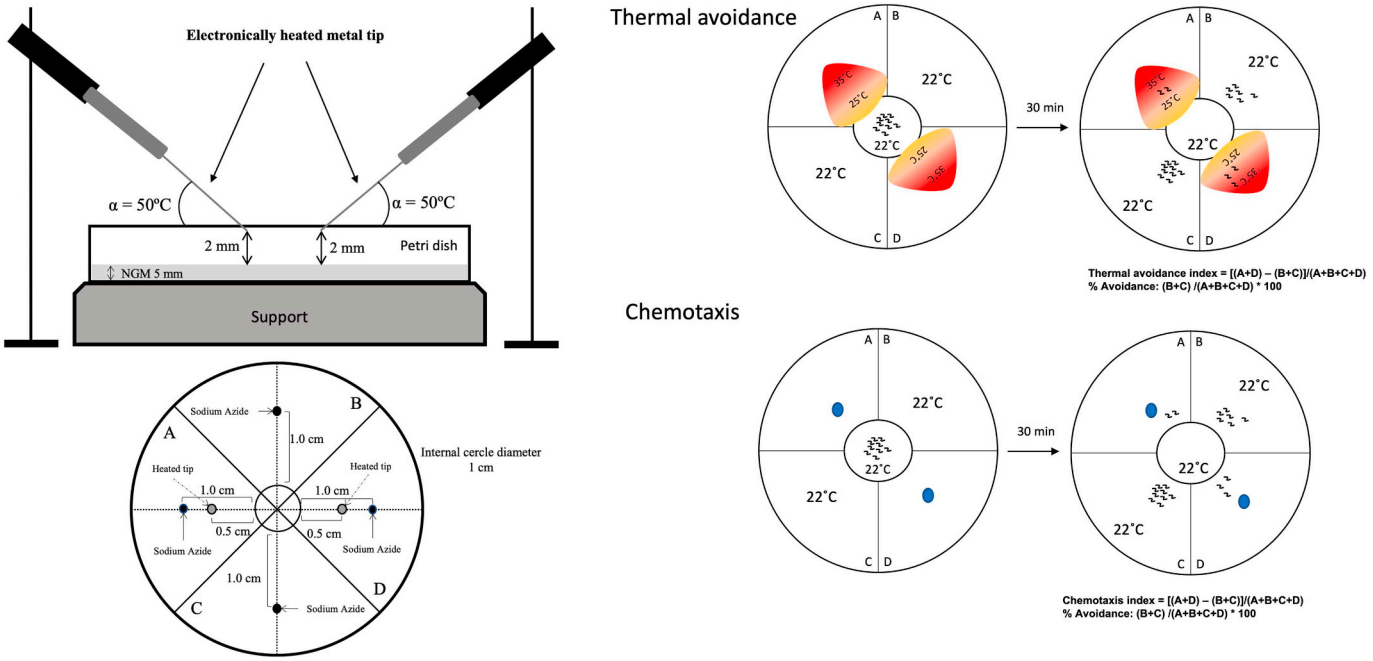

Fig. 2. Technical schematics of the four quadrants assay adapted from Margie et al. (2013). For thermal avoidance assay and chemotaxis, plates were divided into quadrants two test (A and D) and two controls (B and C). Sodium azide was added to all four quadrants to paralyze nematodes. C.elegans were added at the center of the plate (n = 50 to 200) and after 30 min, animals were counted on each quadrant. Only animals outside the inner circle were scored. The calculation of the thermal avoidance index and chemotaxis index were performed has outlined. Quadrants temperature was continuously monitored using an infrared thermometer.

(FWHM), using automatic gain control target of 2.0×10^5 and maximum ion injection time of 50 msec. The normalized collision energy was set to 28 (HCD) and the isolation window was set to 2 Da. Targeted peptide quantifications were performed at MS^1 level using specific precursor masses based on the monoisotopic masses. Peptide quantification was performed by extracting specific precursor ions using a 5 ppm mass window and a label free approach. The data were normalized based on the peak areas observed of the mean WT (N2) strain for relative quantification. Analyses were performed in triplicate. Instrument calibration was performed prior to all analyses and mass accuracy was notably below 1 ppm using Thermo Pierce calibration solution and automated instrument protocol. Peptide sequences were verified base on typical b and y fragments observed at the MS^2 level.

2.5. Statistical analysis

All behavior data were analyzed using a one-way ANOVA followed by Dunnett multiple comparison test (e.g. WT(N2) was the control group used). Significance was set a priori to $p < .05$. The statistical analyses were performed using PRISM (version 7.0d). Neuropeptide data were analyzed with a two-tailed Student-*t*-test with significance set a priori to $p < .01$.

3. Results and discussion

3.1. C. elegans thermal avoidance behavior

Thermal avoidance assays are widely used as a model to study how sensory information is integrated to alter nematode behavior. Noxious temperatures (> 30 °C) provoke a temperature avoidance response in *C. elegans* that can be quantified using a standard thermal avoidance assay. Comprehensive studies have suggested that AFD neurons are the main thermosensors in *C. elegans* (Mori and Ohshima, 1995; Koutarou et al., 2004). Also, FLP neurons located in the head and PHC neurons in the tail act as thermonociceptive neurons and both express heat- and capsaicin-sensitive TRPV channels, OCR-2 and OSM-9 (Liu et al., 2012; Glauser et al., 2011). Interestingly, a recent study has shown that heat avoidance is not related to AFD sensory neurons but does rely on functional NPR-1 receptors located in the RMG interneuron (Glauser et al., 2011). Moreover, NPR-1 controls heat avoidance coactively with OSM-9 and OCR-2. NPR-1 function as a receptor for *flp-18*- and *flp-21*-encoded neuropeptides. It was extensively demonstrated that FLP-21/NPR-1 are key players for nocifensive response triggered by noxious heat. The ASH neuron is considered an important nociceptor involved in avoidance responses from noxious stimuli, but its role in heat avoidance is not clear despite ASH neurons expressing OCR-2 and OSM-9 channels (Sassa et al., 2013). However, it is well established that signaling from ASH neurons induce avoidance behavior through gap junctions with RMG interneurons and that communication is regulated by the NPR-1 receptor (Metaxakis et al., 2018; Glauser et al., 2011). EGL-3 is an essential enzyme involved in the maturation of pro-neuropeptides to active neuropeptides and EGL-21 removes basic residues from the C-terminal following EGL-3 pro-neuropeptide processing leading to active neuropeptides, including FLP-21- and FLP-18- related neuropeptides.

Thermal avoidance assays described in Fig. 2 were performed to assess if *egl-3* and *egl-21* mutant *C. elegans* will display an impeded nocifensive response to noxious heat. The initial experiment involved an assessment of the mobility and bias for WT (N2) and mutants *egl-3*, *elg-21*, *flp-18*, *flp-21* and *npr-1* nematodes. Animals were placed in the center of plates divided into quadrants conserved at constant temperature (i.e. 22 °C) and no stimulus was applied (negative control). As shown in Fig. 3, there was no quadrant selection bias observed for all *C. elegans* genotypes tested. The nematodes were not preferably selecting any quadrant and were uniformly distributed after 30 min following the initial placement at the center of the marked petri dish.

The thermal behavior of *C. elegans* was studied on petri dishes in which two opposite quadrants had a surface temperature of 33 °C to 35 °C and the other two were at room temperature. The results depicted in Fig. 4 suggest that *egl-3* and *egl-21* thermal avoidance is defective for these mutants. These results are coherent with our initial hypothesis since both of these enzymes are important for the maturation of pro-

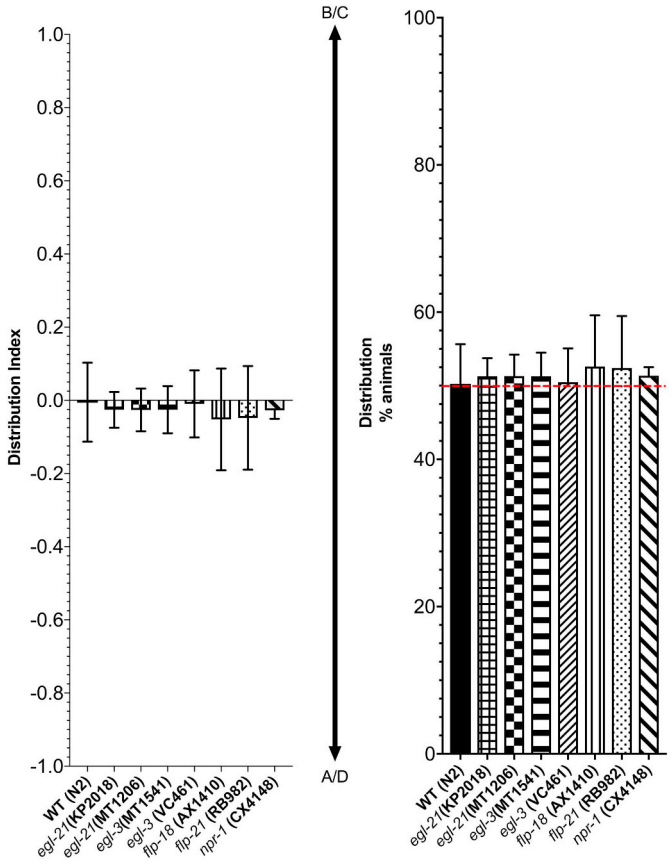

Fig. 3. Comparison of the mobility and bias for WT (N2) and mutants *egl-3, elg-21, flp-18, flp-21 and npr-1* animals in plates divided into quadrants conserve at constant temperature and no stimulus was applied (negative control). No quadrant selection bias was observed for all C. elegans genotype tested.

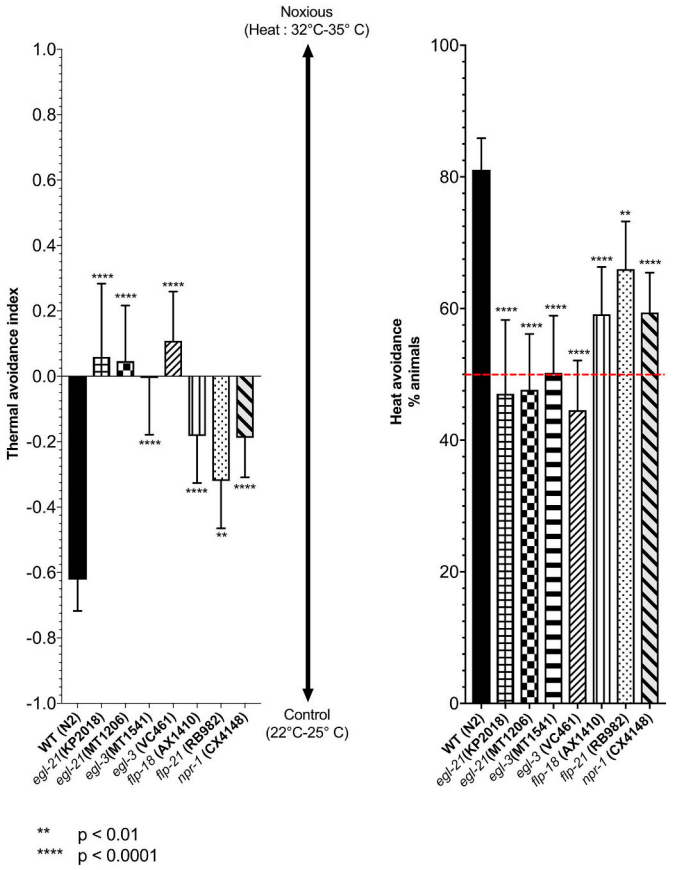

Fig. 4. Thermal avoidance index and avoidance were evaluated for WT (N2) and mutants *egl-3, elg-21, flp-18, flp-21 and npr-1* animals. Display values (mean ± SD) were calculated from at least 3 independent experiments (n > 150 nematodes) for each genotype. Heat avoidance is severely impaired in *egl-3 and egl-21* mutant animals.

neuropeptides to neuropeptides. Additionally, *flp-18*, *flp-21* and *npr-1* mutants also displayed an impeded thermal avoidance behavior. However, it was significantly less pronounced compared to *egl-3* and *egl-21* mutants. Both *egl-3* and *egl-21* mutant animals lack a wide variety of mature neuropeptides. Thus, synaptic chemical communication is impaired, probably with RMG and other interneurons (Glauser et al., 2011; Peymen et al., 2014). As it was previously established, heat avoidance relies on functional NPR-1 receptors located in the RMG interneuron and both, FLP-18 and FLP-21 mature neuropeptides are ligands of NPR-1. Changes in nocifensive behavior are coordinated by specific molecular modulation of the neurotransmitters involved in synaptic communications in *C. elegans*. Chemotaxis experiments performed (e.g. chemical repellent, acid and osmotic pressure) are presented in supplementary figures (i.e. Fig. S1, Fig. S2 and Fig. S3). The results do not show any significant difference between WT (N2) and all tested mutants. Classical small molecule neurotransmitters modulate behavior in response to various stimuli in *C. elegans* and subsequently, neuropeptides may not be essential for *C. elegans* to respond to chemical repellents (Li and Kim, 2008; Hilliard et al., 2002).

3.2. High-resolution mass spectrometry analysis of specific neuropeptides

The analysis of *C. elegans* homogenates was performed using a hybrid Quadrupole-Orbitrap mass spectrometer operating in full scan MS mode at a resolution of 140,000 (FWHM) and in full scan MS^2 mode at a resolution of 17,500 (FWHM). Based on specific cleavage points identified in Fig. 1 and in silico C- and N-ladder fragmentation, we generated an in silico mass list for FLP-18 (UniProtKB – Q9N4V0) and FLP-21 (UniProtKB – Q18234) related pro-neuropeptides and neuropeptides to extract specific *m/z* (± 5 ppm) from the total ion current (TIC) chromatogram. Moreover, MS^2 spectra were collected using an inclusion list to trigger MS^2 analyses based upon accurate mass of targeted precursor ions. As illustrated in Table 1, all targeted pro-neuropeptides and neuropeptides were detected with mass accuracy ranging from − 2.47 ppm to 2.53 ppm (< 3 ppm). The mass accuracy observed was within the instrument specifications. Moreover, all the acquired MS^2 spectra confirmed the targeted peptide sequences based on typical a,b,c and x,y,z positive ion fragments (Roepstorff and Fohlman, 1984). An example of TIC, extracted ion chromatogram (XIC) and MS^2 spectra is presented in supplemental Fig. 4S.

The relative peptide quantification was based on a label free approach well described in the proteomic literature (Blein-Nicolas and Zivy, 2016; Neilson et al., 2011). More specifically, peak areas based on MS^1 XIC's were compared between samples and relative peptide concentrations were determined based on the ratio of the mean area of the WT (N2) group. Technical triplicate precision assessments were performed, and all groups provided %CV < 10%. As shown in Fig. 5, there was no significant concentration difference for FLP-21G and FLP-21 neuropeptides between WT(N2) and *egl-21* mutants. This was expected since EGL-21 is not involved in the biosynthesis of mature FLP-21 neuropeptide as shown in Fig. 1. However, significant FLP-21G pro-neuropeptide and FLP-21 concentration differences were observed for *egl-3* mutants. Importantly, this was an expected result even if mature FLP-21 neuropeptide was detected. As shown in Fig. 1A, other pathways can lead to the biosynthesis of FLP-21G and FLP-21 neuropeptides. Moreover, redundant proteolytic reactions from other proteases can explain these results (Hook et al., 2008; Husson et al., 2007). The

Table 1
FLP-18 and FLP-21 related neuropeptides observed in *C. elegans*.

Peptide	Sequence	Charge state z	Theoretical mass	Observed mass	Relative error (ppm)
FLP21-G	GLGPRPLRFG	+2	535.3169	535.3158	−2.05
FLP21(NH_2)	GLGPRPLRF-NH_2	+2	506.3142	506.3145	0.59
FLP18-1GKR	GAMPGVLRFGKR	+2	644.8690	644.8682	−1.24
FLP18-1	GAMPGVLRF-NH_2	+2	473.7682	473.7694	2.53
FLP18-2GKR	EMPGVLRFGKR	+2	645.3610	645.3614	0.62
FLP18-2	EMPGVLRF-NH_2	+2	474.2602	474.2611	1.90
FLP18-3GKR	SVPGVLRFGKR SVPGVLRFGRK	+2	608.3697	608.3682	−2.47
FLP18-3	SVPGVLRF-NH_2	+2	437.2689	437.2692	0.69
FLP18-4GKR	EIPGVLRFGKR	+2	636.3828	636.3824	−0.63
FLP18-4	EIPGVLRF-NH_2	+2	465.2820	465.2812	−1.72
FLP18-5GKR	SEVPGVLRFGKR	+2	672.8910	672.8899	−1.63
FLP18-5	SEVPGVLRF-NH_2	+2	501.7902	501.7909	1.40
FLP18-6GKR	DVPGVLRFGKR	+2	622.3671	622.3664	−1.12
FLP18-6	DVPGVLRF-NH_2	+2	451.2663	451.2668	1.11

relative quantification results for FLP-18-related neuropeptides are presented in Fig. 6. Interestingly, all FLP-18 pro-neuropeptides incorporating at the C-terminal GKR sequence appear to accumulate in *egl-21* mutants. These mutants have a deficit in the expression of the EGL-21 carboxypeptidase E and, therefore, the catalytic reaction to remove C-terminal R or K residues from peptides is significantly hampered. Similarly, *egl-3* mutants display a significant deficit in both FLP-18 pro-neuropeptides including C-terminal GKR sequence, and mature FLP-18 neuropeptides. These results are coherent with prior published qualitative results (Husson et al., 2006). EGL-3 is an ortholog of the mammalian prohormone convertase 2 (PC2), an important enzyme involved in the processing of neuropeptide precursors at paired basic residues (i.e. RR, KR, RK and KK). As presented in Fig. 1A, the CPL-1 and PAM-1 pathway does not appear to compensate for the deficit in expression of the EGL-3 pro-protein convertase and EGL-21 carboxypeptidase E in *egl-3* and *egl-21 C. elegans* mutants. These results are coherent with other studies we have performed on mammalian CNS tissue fractions (Saidi et al., 2015; Orduna and Beaudry, 2016; Saidi and Beaudry, 2017; Ben Salem et al., 2018). This is a very interesting finding for further translational studies using *C. elegans* as a model organism for nociceptive pharmacology studies. Although, the neuropeptide analyses were performed using whole-animal lysate, the results demonstrated that *egl-3* and *egl-21* mutant *C. elegans* have a noteworthy deficit in mature neuropeptides which results in impaired synaptic chemical communication, possibly with the interneuron RMG (Glauser et al., 2011; Peymen et al., 2014). As discovered previously, NPR-1 ligands/NPR-1 neuropeptide signaling pathway is necessary to trigger nocifensive response of *C. elegans* to noxious heat (Glauser et al., 2011). In *C. elegans*, modulation in nocifensive behavior is intimately associated to specific altered activity during synaptic communication.

4. Conclusion

The thermal avoidance behavior of *egl-3* and *egl-21* mutant *C. elegans* was significantly hampered compared to WT(N2) *C. elegans*. Moreover, *flp-18*, *flp-21* and *npr-1* mutant *C. elegans* displayed a similar phenotype. EGL-3 pro-protein convertase and EGL-21 carboxypeptidase E are essential enzymes for the maturation of pro-neuropeptides to active neuropeptides in *C. elegans*. The ability to avoid noxious heat is strongly associated with the neuropeptide receptor gene *npr-1*. FLP-18/FLP-21/NPR-1 neuropeptide signaling pathways appear to be important to determine the threshold for heat avoidance in *C. elegans* but other neuropeptides and neuropeptide receptors are most likely playing an important role. Experiments using *egl-3* and *egl-21* mutant *C. elegans* homogenates demonstrate that proteolysis of ProFLP-21 and ProFLP-18 are severely hindered leading to a lack of mature bioactive neuropeptides. Pharmacological manipulations of pro-protein convertases could be an interesting strategy to alter sensitivity threshold in higher species.

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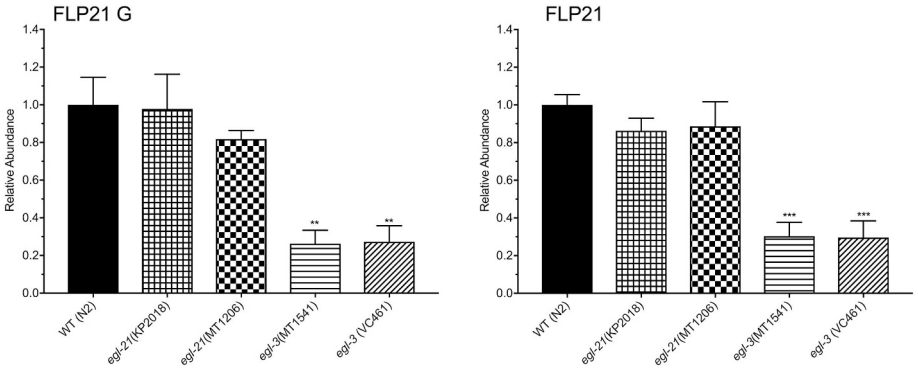

Fig. 5. FLP-21G and FLP-21(NH2) neuropeptide relative concentrations. Peak areas were compared based on MS1 XIC's. The concentration of mature FLP-21 is hampered in *egl-3* mutants.
** $p < 0.01$; *** $p < 0.001$

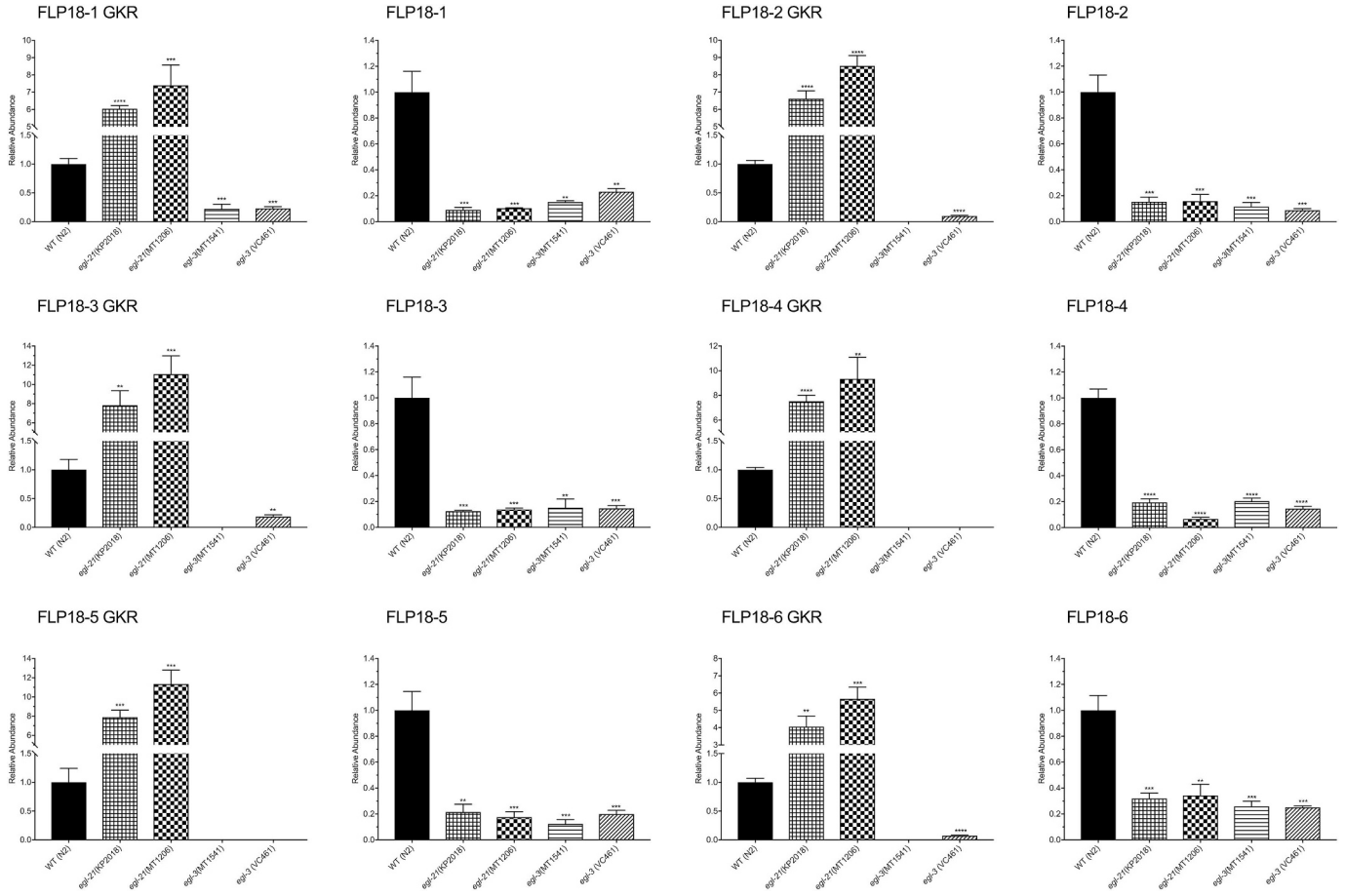

Fig. 6. Relative concentration of FLP-18 related neuropeptides. Peak areas were compared based on MS1 XIC's. The concentration of mature FLP-18 is severally impaired in both, *egl-21 and egl-3* mutants. Moreover, pro-neuropeptides including C-terminal GKR sequence accumulated in *egl-21* mutants as suspected based on proteolytic mechanism outline in Fig. 1. Consequently, *egl-21 and egl-3* have a deficit of mature FLP-18 and FLP-21 neuropeptides essential for neurotransmission. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.npep.2018.11.002.

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