KU LEUVEN

FACULTY OF SCIENCE

The efferent neuropeptidergic circuit underlying arousal and sensitization in *C. elegans*

Lore DEVRIENDT

Thesis presented in
fulfillment of the requirements
for the degree of Master of Science
in Biochemistry and Biotechnology

Supervisor: Prof. I. Beets
Animal physiology and Neurobiology

Co-supervisor: Prof. W. Schafer
Animal physiology and Neurobiology

Mentor: K. Venkatesh
Animal physiology and Neurobiology

Academic year 2022-2023

Host lab information page

Master theses performed in the Beets/Schafer lab comply with following house rules:

1. *Maximal education and scientific self-empowerment of students*

   Students are encouraged to learn from their mistakes through dialogue, rather than by simply being corrected by their supervisors. All writing in this document is the result of this process.

   Students get two feedback moments with respect to their writing process. One from their daily supervisor, who will advise them on format and content (*e.g.* scientific language, proper representation of results and structure of their interpretations). For this, daily supervisors interact either in person with the student, or via comments added to the manuscript. A second feedback session includes comments from (co)supervisors and only focuses on content. This implicates additional input with regards to the student's findings and the final discussion of results. The student's interpretation of this feedback is integrated in the final version of this manuscript, as it is presented here.

2. *Authenticity in science*

   Potential plagiarism was verified using TurnItIn. This work overall scored 4% of potential plagiarism. Sections underlying this result were manually verified by the daily supervisor, as such supporting the authenticity of this work.

Acknowledgements

This past year has been amazing, being submerged in the academic world and experiencing personal progress has been extremely rewarding.

I want to express my sincere gratitude to Prof. Isabel Beets and Prof. William Schafer for the opportunity to work in their lab. Their passion and dedication for the topic is energizing. Many thanks for your expertise and constructive feedback sessions.

Special thanks to Keertana Venkatesh to trust me with part of her research. Her mentorship has been instrumental in improving the quality of this work. Thank you for your unwavering support and guidance. May your hard work be rewarded with many discoveries! Enjoy London!

I would be remiss if I did not include the entire lab. It is a wonderful team, which helped keeping my spirit and motivation high throughout this process.

Finally, I would like to thank my family, especially my parents, sister and boyfriend, for the encouragement and emotional support.

Table of contents

Acknowledgements i

Table of contents ii

Abbreviations iv

Summary v

Samenvatting vi

Introduction 1

1. Arousal and sensitization 1

2. Role of neuropeptides and monoamines in arousal and sensitization 3

3. *Caenorhabditis elegans*: a powerful model organism for delineating the neuropeptidergic circuitry underlying arousal 5

3.1. Wired circuitry for mechanical and chemosensory cues 6

3.1.1. Chemosensory circuit 7

3.1.2. Mechanosensory circuit 9

3.2. Neuropeptide signalling in *C. elegans* 10

3.2.1. Neuropeptide biosynthesis 11

3.2.2. Neuropeptide receptors 12

4. Neuropeptides involved in arousal and sensitization in *C. elegans* 14

Research objectives 17

Materials and Methods 18

1. *C. elegans* strains 18

2. General *C. elegans* maintenance 20

2.1. Preparing *C. elegans* culture plates 20

2.2. Transferring of *C. elegans* 20

2.3. Staging of young adults for assays 21

2.4. Production of males 21

2.5. Handling of contamination 21

2.6. Freezing of *C. elegans* 22

3. Generating new *C. elegans* strains 23

3.1. Genotyping using PCR and gel electrophoresis 23
3.2. Genotyping using PCR and sequencing 25
4. Behavioural assays 26
4.1. Tap assay 26
4.2. Sensitization assay 26
5. Analysis 28
5.1. Tap assay 28
5.2. Sensitization assay 28
Results 29
1. Standardizing the mechanosensory stimulus for arousal 29
2. Generating transgenic mutant strains 30
3. All candidate mutants show a normal locomotory arousal response 32
4. Sensitization assays 33
4.1. The *npr-2* mutant shows a partial sensitization defect 33
4.2. The *ckr-1* mutant shows a sensitization defect 34
4.3. The frpr-*16* mutant does not show a sensitization defect 35
Discussion 37
Conclusion 41
References 42
Appendix i
1. Risk assessment i
2. Buffers and solutions ii

Abbreviations

Abbreviation	Meaning
ASH	Aphid Single Cilium H
ATR	All-Trans-Retinal
C. elegans	*Caenorhabditis elegans*
CAPS	calcium-dependent activator protein for secretion
CCK	cholecystokinin
CGC	*Caenorhabditis* Genetics Centre
CGRP	calcitonin gene-related peptide
ChR2	channelrhodopsin2
D. melanogaster	*Drosophila melanogaster*
DCV	dense core vesicle
DMSO	dimethylsulfoxide
E. coli	*Escherichia coli*
FLP	FMRFamide-related peptides
GPCR	G protein-coupled receptors
INS	insulin-like peptide
MRN	mechanosensory neuron
NCBI	National Center for Biotechnology Information
NGM	nematode growth medium
NJM	neuromuscular junction
NLP	neuropeptide-like proteins
NMDA	N-methyl-D-aspartate
PC	protein convertase
PCR	polymerase chain reaction
PDF	pigment dispersing factor
PrRP	prolactin releasing hormone peptide
RID	Ring Interneuron D
S. littoralis	*Spodoptera littoralis*
SK	sulfakinins
TAE	Tris-acetate-EDTA
TRN	touch receptor neuron
UV	ultraviolet

Summary

Arousal is a behavioural state that correlates with a higher alertness and responsiveness to stimuli, which is critical for survival. Sensitization is an aspect of arousal in which previous, most often aversive, stimuli prime a subsequent reaction causing an exaggerated response. Researchers have studied arousal caused by external stimuli across species and discovered that neuromodulators, in particular neuropeptides, play a crucial role in modulating such behaviour. However, a detailed understanding of the underlying circuitry remains unknown.

In order to unravel the neuropeptides regulating arousal, we studied the molecular basis of arousal in the model organism *Caenorhabditis elegans (C. elegans). C. elegans* shows both locomotory arousal and sensitization when it is exposed to an aversive mechanosensory cue. As a result, subsequent aversive chemical cues also result in an enhanced response compared to worms that did not receive any arousal stimulus.

A previous candidate screen in the host labs identified the neuropeptide FLP-20 as a key player in this cross-modal paradigm. The aversive mechanosensory cue results in the release of FLP-20 peptides from the touch receptor neurons which then bind to its receptor, FRPR-3 in a neuroendocrine cell, RID. However, the downstream signalling from RID to the chemosensory circuit is not well understood. The neuroendocrine RID neuron primarily relies on gap junctions and neuromodulatory signals to communicate with other neurons. Moreover, knockdown of the calcium-dependent activator protein for secretion (CAPS) UNC-31, which is essential for neuropeptide release, results in a sensitization defect, suggesting that neuropeptides are involved in arousal-evoked sensitization. The aim of this project is to unravel the molecular players leading to this cross-modal sensitization.

In this work, we investigated the putative role of three neuropeptide receptors expressed by the primary nociceptive chemosensory neuron, ASH, namely NPR-2, CKR-1, and FRPR-16.

Worms lacking their respective genes were assessed in two behavioural assays: a tap assay, to discover defects in locomotor arousal, followed by the sensitization assay. None of our candidates showed a locomotory arousal defect. Neither does the *frpr-16* mutant show a defect in the sensitization assay. The *npr-2* mutant shows a variable, partial sensitization phenotype. In addition, our data suggests the involvement of the CKR-1 receptor in cross modal sensitization.

Although more trials are needed, our results identify two neuropeptide receptors that are potentially involved in the cross-modal sensitization of chemosensory circuits during arousal. Furthermore, a detailed analysis of the arousal speed curve could bring interesting new arousal characteristics to light. Additionally, it will be interesting to investigate the ligands for these receptors to delineate the circuitry involved.

Samenvatting

Arousal is een gedragsstaat die correleert met een hogere alertheid en responsiviteit op stimuli, wat cruciaal is voor overleving. Sensitisatie is een aspect van arousal waarbij eerdere, meestal aversieve, stimuli een daaropvolgende reactie voorbereiden om een sterkere reactie uit te lokken. Onderzoekers hebben arousal veroorzaakt door externe stimuli bestudeerd bij verschillende dieren en ontdekten dat neuromodulatoren, voornamelijk neuropeptiden, een cruciale rol spelen in de modulatie van dit gedrag. Echter, de moleculaire basis van arousal en de onderliggende circuits blijven nog grotendeels onbekend.

Om de neuropeptiderge signaalwegen die aan de basis liggen van arousal verder te ontrafelen, voerden we een studie uit in het modelorganisme *Caenorhabditis elegans (C. elegans).* Wanneer *C. elegans* wordt blootgesteld aan een mechanosensorische prikkel, fungeert het als een arousal stimulus en sensibiliseert het de worm. Een daaropvolgende aversieve chemische prikkel zal vervolgens ook een versterkte chemosensorische respons uitlokken, in vergelijking met wormen die geen arousal stimulus hebben ontvangen.

Eerder onderzoek identificeerde het FLP-20 neuropeptide als een cruciale speler in dit model. FLP-20 wordt tot expressie gebracht in de mechanosensorische neuronen die het peptide vrijstellen bij een mechanosensorische prikkel. Het neuropeptide bindt vervolgens zijn receptor, FRPR-3, in de neuroendocriene cel RID. De signaaloverdracht tussen RID en het chemosensorisch circuit is echter nog niet gekend. RID communiceert met de rest van het zenuwstelsel voornamelijk via gap junctions en neuropeptiden. Voorgaand onderzoek toonde aan dat knockdown van een cruciale factor (UNC-31) betrokken bij de vrijgave van neuropeptiden, leidt tot een defect in sensitisatie. Dit suggereert dat neuropeptide signalering betrokken is bij sensitisatie van het chemosensorisch circuit tijdens arousal. In dit project trachten we de moleculaire spelers te ontrafelen die leiden tot deze cross-modale sensitisatie.

In dit werk onderzochten we de rol van drie neuropeptidereceptoren die tot expressie worden gebracht in het primaire chemosensorische nociceptor neuron ASH, namelijk NPR-2, CKR-1 en FRPR-16. De gedragsstaat van wormen zonder hun respectievelijk gen werden getest met behulp van twee paradigma's: Een tap assay, om defecten in locomotorische arousal te ontdekken, gevolgd door de sensitisatie assay. Geen van onze kandidaten vertoonde een locomotorisch defect. Ook vertoont de *frpr-16* mutant geen defect in de sensitisatietest. De *npr-2* mutant vertoont een variabel, gedeeltelijk sensitisatiedefect. Daarnaast suggereren onze resultaten dat de CKR-1-receptor betrokken is bij de cross-modale sensitisatie van de chemosensorische ASH neuronen.

Hoewel meer herhalingen van deze gedragstesten nodig zijn om finale conclusies te trekken, wijzen onze resultaten twee neuropeptidereceptoren aan die mogelijk betrokken zijn bij de

cross-modale sensitisatie van chemosensorische circuits tijdens arousal. Bovendien kan een meer gedetailleerde analyse van het verloop van de locomotorische snelheid tijdens arousal interessante nieuwe kenmerken van deze gedragsstaat aan het licht brengen. Daarnaast is het interessant om het effect van de liganden van deze receptoren op arousal te onderzoeken, om zo het betrokken circuit verder te achterhalen.

Introduction

1. Arousal and sensitization

“The only constant in life is change” as Heraclitus describes (Ephesius 1979). Living in a constantly changing environment requires that we respond appropriately. To survive, it is important to be capable of discriminating between harmful and tolerable stimuli and subsequently fine tune later reactions. This behavioural state of higher alertness, causing enhanced reactions is described as arousal.

An arousal state is widely characterised by higher responsiveness to sensory inputs, enhanced motor activity and a heightened emotional reaction (Pfaff et al. 2008). Furthermore, it is possible to quantify these behavioural outputs (Pfaff 2015). There are multiple types of arousal states, the most prominent being fear, hunger, sexual drive and pain, altering a variety of behavioural decisions (Jing, Gillette, and Weiss 2009). We can classify arousal into two types: local arousal is limited to one form of behaviour that enhances specific effects relevant to this behaviour. Secondly, general arousal impacts multiple networks and provokes specific along with non-specific events (Jing, Gillette, and Weiss 2009). Additionally, this state can be caused by both internal or endogenous stimuli, like sleep, and external or exogenous stimuli from the environment, primarily danger signals (Chew et al. 2018). Arousal has been observed and explored in many species, like humans (Hubbard et al. 2011), rats (Lee and Dan 2012), fish (Woods et al. 2014; Yokogawa, Hannan, and Burgess 2012), flies (Asahina et al. 2014; Babcock, Landry, and Galko 2009) and worms (Chew et al. 2018).

Complications in arousal circuits are associated with multiple health disorders, including sleep problems, attention difficulties, depression, vegetative states, anaesthesia, cognitive declines, apathy, and mood changes (Pfaff et al. 2008). Dysfunctional circuits can lead to many cognitive problems as these changes are needed for anticipation of internal and external stimuli (Lee and Dan 2012).

The mollusc *Aplysia* is one of the first organisms where the mechanisms underlying arousal were extensively studied, as both behavioural readouts and the nervous system are relatively simple compared to vertebrate animals. The major focus were two types of arousal: feeding arousal and defensive arousal (Jing, Gillette, and Weiss 2009).

A fundamental feature of arousal is sensitization. This phenomenon is a form of behavioural plasticity leading to an elevated behavioural response after an initial stimulus (Rose and

Rankin 2001). Sensitization is needed for survival, to make adjustments in behaviour depending on the internal state as well as the environment. The pathway that becomes sensitized is not necessarily the same as the input, which is often referred to as cross-modal sensitization (Chew et al. 2018).

In the moth *Spodoptera littoralis* (*S. littoralis*), brief pre-exposure to sugar still has an effect on the behaviour after 24 hours, demonstrating long term sensitization after a short taste incentive. The short contact with the tastant does not only sensitize the corresponding circuit but also improves detection of pheromones, which is governed by other circuits, illustrating cross-modal sensitization (Minoli et al. 2012). The sensitivity to this sex pheromone is also altered by other inputs such as predator sounds (Anton et al. 2011). Similar long-term sensitization was also observed in *Aplysia* (Barbas et al. 2003).

Another example of cross-modal sensitization is nociceptive sensitization in *Drosophila melanogaster* (*D. melanogaster*). The exposure of *Drosophila* larvae to ultraviolet (UV) light alters the activation properties of sensory neurons, thereby increasing their sensitivity to pain. This lowering of the pain threshold is called thermal allodynia. Researchers additionally observed a second characteristic of sensitization, namely thermal hyperalgesia, referring to the exaggerated reaction caused by repeated UV exposure (Babcock, Landry, and Galko 2009).

General pain sensitization is also observed in humans. For example, Hubbard studied the startle response elicited by a nociceptive cue, namely an electric shock. It appeared that the startle reaction is more severe in an expected context compared to a period which was considered safe (Hubbard et al. 2011).

2. Role of neuropeptides and monoamines in arousal and sensitization

It is widely seen that arousal and sensitization are modulated by neuromodulators, including monoamines and neuropeptides (Lee and Dan 2012).

A thoroughly studied example of sensitization in humans is pain sensitization. General sensitization lowers the pain threshold by changing neural properties, allowing a more sensitive detection the next time. Additionally, the brain generates a potent pain response leading to avoid these harmful stimuli and prevent further injury (Latremoliere and Woolf 2009). Glutamate together with neuropeptide signalling via calcitonin gene-related peptide (CGRP) and substance P peptides result in the activation of N-methyl-D-aspartate (NMDA) receptors, leading to a calcium influx that establishes this lower pain threshold (Latremoliere and Woolf 2009). Besides its involvement in central sensitization, substance P also performs a function in the regulation of aggressive behaviour in mammals (Katsouni et al. 2009). This enhancement in aggression between males has also been demonstrated for the invertebrate homologue, tachykinin, in *D. melanogaster* (Asahina et al. 2014).

There are many more detailed studies concerning conserved neuropeptides involved in arousal. For example, impaired orexin signalling in mice results in sleep fragmentation, similar to narcolepsy in humans (Inutsuka and Yamanaka 2013). Studies in zebrafish also highlight the importance of neuropeptides in arousal. It was observed that elevated orexin/hypocretin levels result in a heightened response to dark flashes (Woods et al. 2014).

Another illustration of neuropeptide involvement in arousal is the prolactin releasing hormone peptide (PrRP). It was found to mediate stress arousal in addition to being involved in the regulation of energy metabolism and food intake (Pražienková et al. 2019).

The mollusc *Aplysia* is thoroughly studied in the context of sensitization. Sensitization is a form of learning from noxious cues. In *Aplysia*, an electric shock to the tail enhances the animal's overall defences (Mackey et al. 1987). The sensory neurons that detect tail shock stimulate downstream interneurons to release serotonin onto the presynaptic site of the syphon sensory neurons and, in doing so, increasing synaptic excitation evoking sensitization (Kandel 1995).

Dispensing psychostimulants like methamphetamine lead to changes in concentrations of multiple neurotransmitters such as dopamine, serotonin, and noradrenaline. They can lead to either blockage (cocaine) or promoted transmitter release (amphetamines). In both cases, this leads to adapted signalling of arousal states, like sleep, locomotion, attention and overall activity (Andretic, Van Swinderen, and Greenspan 2005).

However, the detailed circuits and the underlying molecular players regulating arousal and associated sensitization are still unclear. More so, it remains unclear how extrasynaptic signals such as neuropeptides modulate the wired circuitry for arousal behaviours. As behavioural research in humans is complex, we use organisms with conserved pathways and discrete, quantifiable behavioural outputs to gain further insight into these mechanisms. For this, we specifically use *Caenorhabditis elegans* as model system to dissect the neuropeptidergic circuitry underlying arousal and sensitization behaviours.

3. *Caenorhabditis elegans*: a powerful model organism for delineating the neuropeptidergic circuitry underlying arousal

Caenorhabditis elegans (*C. elegans*) is a free living, microscopic nematode that in nature is predominantly found on rotten fruits and plants (Félix 2010). This transparent worm has a relatively compact neuronal circuit, consisting of only 302 neurons, contrary to the approximate 86 billion neurons that compose the human brain (Allen et al. 2015). Moreover, all the synaptic connections between the neurons in the *C. elegans* nervous system are mapped and displayed in a network called the connectome (Cook et al. 2019).

Since the full genome is sequenced, we can predict possible neuropeptides and specifically mutate their genetic sequences. Over the years, a databank has been generated of *C. elegans* mutants with specific deletions or other genetic deviations (Corsi, Wightman, and Chalfie 2015). Together with the ability to both self-fertilize and mate with males, the nematode is highly amenable to genetic studies. In addition, *C. elegans* has a short generation time and produces many offspring. Consequently, crosses can be followed for generations while minimizing the experimental time (Corsi, Wightman, and Chalfie 2015). The life cycle of *C. elegans* starts with a fertilized egg. In the first three days the embryo hatches and goes through three more moults to become a L4 larva. After the final moult the nematode develops into an adult worm, ready to produce offspring. These adults can still live up to 3 weeks. Development can also follow the alternative route of the dauer stage, which is highly stress resistant. (Corsi, Wightman, and Chalfie 2015). Furthermore, *C. elegans* has a number of practical advantages as a model organism: they are easily cultivated and have a small size. Together with the variety of available genetic tools, they are a prime candidate for genetic studies.

Along with the known wired circuitry, *C. elegans* has an extensive, to a certain extent conserved, neuromodulatory system, which allows to understand how these systems interact to bring about behaviour. Neuromodulators, like neuropeptides and monoamines, have been seen to modulate a myriad of behaviours in *C. elegans* such as locomotion, learning and arousal states (Bhat et al. 2021; Flavell, Raizen, and You 2020). As behaviour is clearly defined and quantifiable, *C. elegans* is a powerful model organism for unravelling neural mechanism underlying behaviours (Hart 2006).

Sensitization to aversive external inputs has been observed in *C. elegans* as well. Prior work from the lab has shown that an aversive mechanosensory cue (tap) to the culture plate results in locomotory arousal wherein the worms speed up. When a second aversive cue of a chemical repellent is given, the worms show an enhanced response compared to a condition where no tap was applied. Thus, a prior stimulus results in cross-modal sensitization, thereby

eliciting an altered behavioural response. This behavioural state of arousal was seen to be dependent on afferent neuropeptide signals from the mechanosensory circuit. However, the efferent signals to the chemosensory circuit, regulating the sensitization response, are not well understood (Chew et al. 2018). We aim to further gain insight on this efferent neuropeptidergic circuit. The next section first introduces what we already know about the mechanosensory and chemosensory circuits with respect to their wired connections. Thereafter, the focus shifts to the extrasynaptic neuropeptidergic circuitry.

3.1. Wired circuitry for mechanical and chemosensory cues

The *C. elegans* connectome (figure 1) displays a highly structured arrangement consisting of sensory neurons that detect inputs, interneurons mediating the response, and motor neurons with synapses onto muscles for execution (Bargmann 2012). This outline was mapped by use of electron microscopy and provides insight into the gap junctions and chemical synapses between the 302 neurons in the worm's nervous system (White et al. 1986).

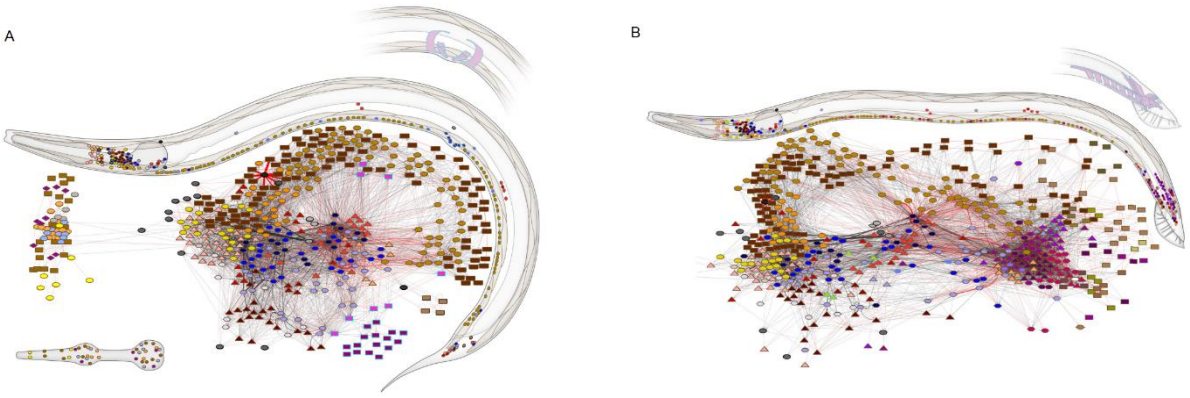

Figure 1. C. elegans *connectome of a hermaphrodite (A) and male (B) (Cook et al. 2019).*

In a hermaphrodite there is a total of around 6,000 gap junctions in *C. elegans*, compared to approximately 10,000 in males. These gap junctions consist of a varying combination of six subunits. In invertebrates the molecules encoding for these building blocks are called innexins, and *C. elegans* has 25 innexin types (Hall 2017). Gap junctions provide electrical coupling and can also serve as a gateway allowing diffusion of ions and small molecules.

Chemical synapses give neurons the opportunity to convey electrical signals via neurotransmission. The signalling molecules involved, called neurotransmitters, are released upon calcium entry into the cell. After diffusion over the synaptic cleft, they bind to receptors at the postsynaptic site of the adjacent neuron (Richmond 2007). Neurotransmitters are

synthesized from amino acids and other simple molecules through a multistep enzymatic process and stored in clear synaptic vesicles (Hobert 2013). In total the *C. elegans* connectome accounts for around 7,000 chemical synapsis, of which 2,000 occur at neuromuscular junctions (NJMs) (Richmond 2007).

C. elegans has multiple sensory circuits to detect and react to environmental cues. Apart from the chemosensory and mechanosensory circuits, which are the focus of this project and explained in detail below, there are others like thermosensory, electrosensory, pheromone sensing, and oxygen sensing circuits (Metaxakis, Petratou, and Tavernarakis 2018).

3.1.1. Chemosensory circuit

Due to the lack of auditory and visual senses, *C. elegans* relies heavily on chemosensation to mediate responses (Artyukhin, Schroeder, and Avery 2013). For instance, after hatching, larvae assess food levels as well as population density via the concentration of pheromones and use this information to decide whether to continue the normal life cycle or go into the resistant dauer stage upon unfavourable, environmental conditions (Sengupta 2007). Approximately 8.5% of *C. elegans* genes encode chemosensory G protein-coupled receptors (GPCRs) (Ferkey, Sengupta, and L'Etoile 2021). Through chemosensory neurons, the nematode is also able to detect a variety of chemicals that provoke reactions like escape or mating (Bargmann 2006).

A hermaphrodite has assumably 32 chemosensory neurons: 11 pairs in the head amphids (ASE, ASG, ASH, ASI, ASJ, ASK, ADF, ADL, AWA, AWB, AWC), a thermosensory pair (AFD), 4 neurons in the inner labial organs (URX(L/R), AQR, PDR) and 2 pairs of tail phasmids (PHA, PHB) (Ferkey, Sengupta, and L'Etoile 2021). The chemosensory cells are located close to the cuticle with their cilia directly or indirectly exposed to the environment. The neurons in the amphid (figure 2) and phasmid exist in symmetric pairs that in some cases, however, are functionally distinct. For example, the left and right ASE sensory neurons show a different distribution of GPCRs (Bargmann 2006). Bilateral pairs of neurons are often connected by gap junctions and send an integrated signal (Hall 2017).

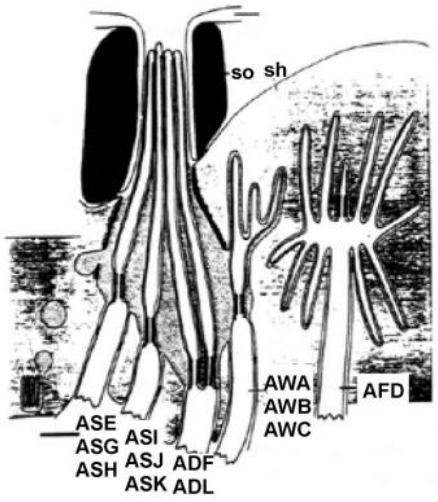

Figure 2. Detailed schematic of the different amphid neurons of C. elegans *(Bargmann 2006).*

The ASE neurons detect various gustatory cues, namely salts and other water-soluble attracts (Bargmann 2006). Considering that the main food source of *C. elegans* are bacteria and they produce countless volatile organic compounds through their metabolic pathway, these can also be interpreted as an attractive stimulus. They are detected by two pairs of sensory neurons, called AWC (responsible for detecting benzaldehyde, butanone, isoamyl alcohol, 2,4,5-trimethylthiazole and 2,3-pentanedione) and AWA (diacetyl, pyrazine, and 2,4,5-trimethylthiazole). AWB (2-nonanone) is a structurally similar neuron but the compounds detected by this neuron are considered repulsive (Bargmann 2006).

C. elegans determines its movements based on concentration changes in the chemical gradient. This method of movement is called chemotaxis and can also be found in bacteria. This pattern of activity is fashioned on the decision to continue in the current direction or randomly pick another course (Bargmann 2006). To mediate a rapid escape response, sensory neurons involved in sensing harmful chemicals, such as AWB, ASH and ADL, are directly connected with backward motor neurons. Sensory neurons that mediate a response for attractive cues on the other hand have multiple layers of interneurons integrating and assessing the inputs (Sengupta 2007).

Interestingly, the chemosensory ASH neuron is defined as a polymodal nociceptor neuron because it can respond to both chemical as well as mechanical noxious cues. Examples of repellents include high osmolarity, acidic environments, heavy metals, organic odours, and nose touch (Ezcurra et al. 2016). All stimulate the same behaviour: the nematode stops, moves backward and turns before moving away, making an omega-shaped turn (Goodman 2006).

3.1.2. Mechanosensory circuit

C. elegans is also equipped with an extensive mechanosensory system, counting 30 mechanosensory neurons (MRNs) in hermaphrodites and an additional 46 in males. They are dedicated to sensing external inputs, such as detecting a bacterial lawn, obstacles or other worms. In addition, self-generated stimuli, like stretch, are also recognized by these neurons (Goodman 2006).

There are several MRNs that are able to sense various mechanical stimuli. For light touch, there are six touch neurons: ALM(L/R), AVM, PVM and PLM(L/R) (figure 3). Using mutations and laser ablations, researchers discovered touch is involved in many behaviours such as feeding, egg laying, mating, defecation and locomotion (Goodman 2006). When the worm is gently touched, it will move in the opposite direction, away from the stimulus. Contact with the tail results in an accelerated forward movement, while contact with the head results in a reverse motion. These are regulated by the posterior neurons (PLML and PLMR) and the neurons in the anterior part of the body (ALML, ALMR, and AVM), respectively (Chalfie et al. 1985). Additionally, these five neurons are also involved in tap response. A tap is a vibrational cue, causing a backwards movement. This reverse motion is caused by the imbalanced activity, a stronger reverse than forward circuit. Because AVM only develops post-embryonic, tap in young larvae leads in half of the cases in a forward movement and the other half in a backward motion (Bozorgmehr et al. 2013; Bounoutas and Chalfie 2007). We will be using this tap response as a first aversive cue to study arousal behaviours.

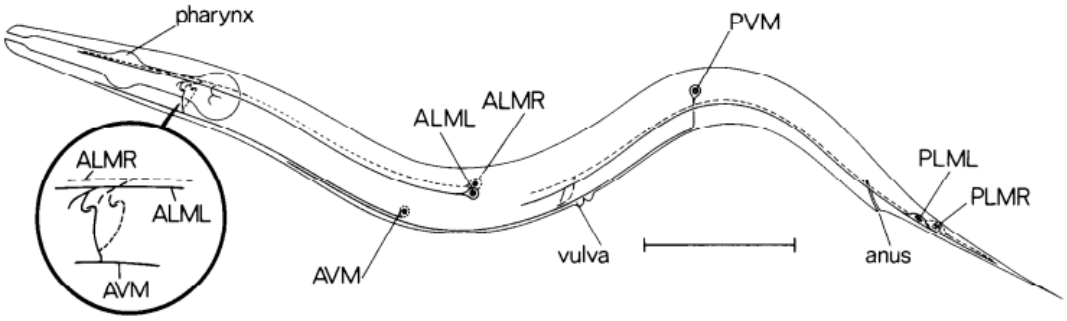

Figure 3. The six touch neurons in C. elegans *(M. Chalfie and Thomson 1979).*

The touch neurons hold essential sodium channels (DEG/ENaC) that enable touch sensitivity. Kung hypothesized that due to the relative change in position of the channel to the membrane, a force arises and forces the channel to open (Kung 2005). This sodium

current is followed by a calcium flux, starting signal transduction (Bounoutas and Chalfie 2007).

The sensory neurons form gap junctions with the interneurons needed for a locomotor response (Bounoutas and Chalfie 2007). They are determined using laser ablations: AVD and AVA are needed for backwards movement, while PVC and AVB are required for forward movement (Chalfie et al. 1985). To counter for possible simultaneous activation, the touch neurons also send inhibitory glutamatergic signals to the antagonistic interneurons (Bounoutas and Chalfie 2007).

Hard touch responses are regulated by other sensory neurons such as PVD and FLP in addition to ALM and PLM that also play a role in gentle touch (Bounoutas and Chalfie 2007; Hart 2006).

The wired circuit alone is not sufficient to account for all forms of behaviour. The anatomical diagram shows the potential interactions but is incomplete since neuropeptides and biogenic amines, which are able to work extrasynaptically in addition to synaptic, are not included (Bentley et al. 2016).

3.2. Neuropeptide signalling in *C. elegans*

In order to adapt behavioural reactions to the changing environment, alteration in synaptic communication is often necessary. In addition to the above discussed wired circuitry, extrasynaptic signalling via neuropeptides is known to modulate many if not all behaviours, such as locomotion, learning, feeding, mating, and sleep (Bhat et al. 2021). These modulators can change properties like excitability and synaptic efficiency, and thereby are able to regulate the information flow among all the possible connections in the connectome, whether or not wireless (Bargmann 2012).

Part of the neurons that release monoamines and neuropeptides are not synaptically connected to the cells expressing their corresponding receptors. This suggests that monoamines and neuropeptides work outside the wired circuitry (Bentley et al. 2016). A recent study from the host lab aimed to highlight key features of the neuropeptide connectome. They integrated data from a deorphanization screen of neuropeptide receptors combined with expression patterns of neuropeptides and their receptors, and anatomic neuronal information. The resulting neuropeptide signalling network of the *C. elegans* nervous system constitutes 91 peptide-GPCR couples and shows a high density of putative peptidergic connections as well as high degree interconnected hubs (Ripoll Sánchez et al.

2022). Extrasynaptic signalling lends the opportunity to execute local functions as well as modulating GPCRs at a distance (Bargmann 2012).

Over 350 neuropeptides have been identified in the *C. elegans* genome using a combination of computational and peptidomics techniques. These neuropeptides can be divided into three classes based on their sequence similarity: insulin-like peptides (ILP), FMRFamide-related neuropeptides (FLP) and the neuropeptide like proteins (NLP), which contains the non-insulin, non-FMRFamide-related peptides (Li and Kim 2008).

3.2.1. Neuropeptide biosynthesis

Neuropeptides are encoded in the genome as part of a larger peptide precursor. This pre-pro-protein contains one or multiple copies of one or numerous different neuropeptides and undergoes various post-translational steps (figure 4), yielding active neuropeptides (Husson et al. 2007). In some organisms the precursor can also be alternatively cleaved to yield even a bigger variety of peptides. In addition, mechanisms can be in place to sort the peptides to different locations (Li and 2008).

After the neuropeptide gene is transcribed in the nucleus, the contained signal peptide directs the polypeptide chain into the endoplasmic reticulum during translation. There the first step in neuropeptide processing is to cut off the signal-peptide from the preproprotein by a signal peptidase, producing the proprotein. This product is packaged in dense core vesicles (DCVs) together with processing enzymes. The proprotein is further chopped by endoproteolytic protein convertases (PCs) at a C-terminal position of two basic amino acids. The *C. elegans* genome encodes for four PCs, with EGL-3 being used most often (Husson et al. 2007). The basic residues are then removed by carboxypeptidase E. EGL-21 is broadly used as it is expressed in 60% of the neurons (Jacob and Kaplan 2003). To create a mature neuropeptide, some peptides require further modification such as amidation (van Bael et al. 2018). As most neuropeptides require EGL-3 or EGL-21 for maturation, deletion mutants of these genes can give an indication for involvement of neuropeptides in regulation of the studied behaviour (Bhat et al. 2021).

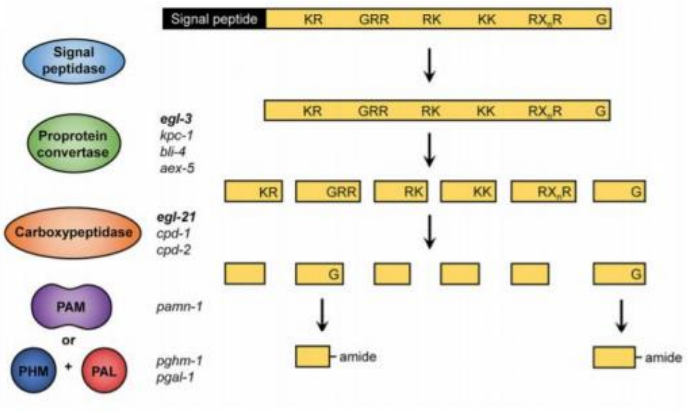

Figure 4. Scheme displaying the steps in neuropeptide processing from the preproprotein to mature neuropeptides in C. elegans *(van Bael et al. 2018).*

The DCVs containing the mature neuropeptides are scattered along the axon, as opposed to the clear neurotransmitter vesicles that are mainly present at the synapse. Neuropeptide release upon calcium stimulation is regulated by CAPS (calcium-dependent activator protein for secretion), named UNC-31 in *C. elegans* (Husson et al. 2007). Post secretion, neuropeptides are cleared by proteolytic enzymes. Therefore, for the next release all the peptides need to be synthesised *de novo*.

3.2.2. Neuropeptide receptors

To exert their function, neuropeptides mostly bind to GPCRs. These receptors typically span the membrane seven times. GPCRs are integral in many signalling processes and do not necessarily need a ligand. In total there are five classes; however, only two families, namely Rhodopsin and Secretin, are known to have neuropeptide ligands (Hobert 2013). Some studies also show that neuropeptides are able to activate channels and kinases (Lingueglia et al. 1995). Neuropeptides do not always show exclusive receptor binding. Contrary to non-RF-amide peptides, who have in most cases distinct receptors, are RF-amide peptides known for their ‘many to many’ and ‘one to many’ binding characteristics, causing functional overlap (Beets et al. 2022).

The signalling cascade of the GPCRs depend on the associated G protein. This is a trimeric protein containing three subunits: Gα, Gβ, and Gɣ. The *C. elegans* genome encodes for 21 Gα, 2 Gβ, and 2 Gɣ proteins, generating a variety of possibilities. In mammals the Gα proteins can be subdivided in four different classes, each with their specific signalling pathway: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ (figure 5) (Frooninckx et al. 2012). The same subtypes

are found in *C. elegans*, the remaining are thought to be unique for the GPCRs in the chemosensory neurons (Frooninckx et al. 2012). In short, when a ligand binds its respective GPCR a conformation change occurs and GDP, bound at the innermembrane, is replaced by GTP, causing the G protein to split in GαGTP and GβGɣ (Cabrera-vera et al. 2003). These effector molecules are then free to carry out their functions via different intracellular cascades. These receptors are tightly regulated. Phosphorylation of the receptor itself can result in reduced affinity. The receptor can be completely shut down by binding of arrestin. On the other hand, the Gα protein can also be activated independent of the receptor, or the GTP exchange can be made more difficult. In addition, recycling of subunits can be inhibited by phosphorylation (Hancock 1997).

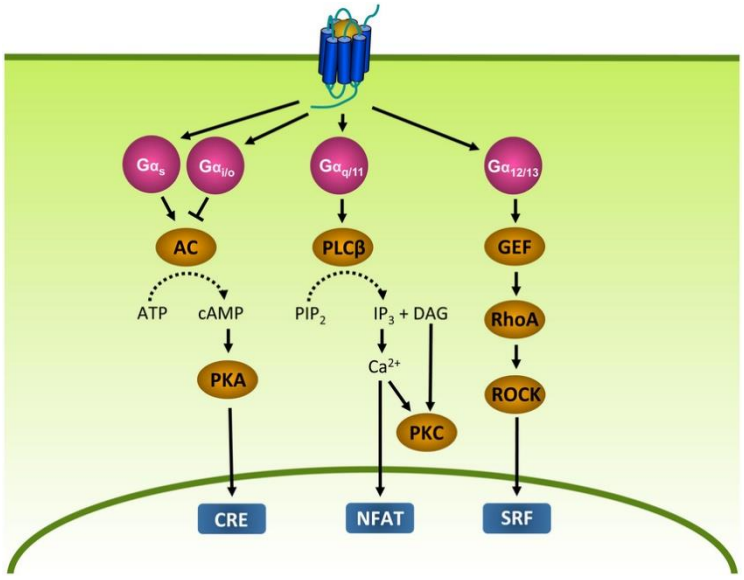

Figure 5. The four main different classes of the Gα subunit and their accompanying signalling cascade (de Munnik et al. 2015).

Most behavioural responses require both wired connections as well as a broad range of neuropeptidergic inputs (Bhat et al. 2021). For example, *C. elegans* egg-laying requires the HSN interneurons that receive synaptic inputs form other neurons, like PLM mechanosensory neurons, to initiate egg laying behaviour, while being regulated themselves by neuropeptides (Schafer 2006). Another example is male mating that apart from synaptic inputs also requires neuropeptides, in particular nematocin (Garrison et al. 2012). Other examples are social behaviour, learning, locomotion, sleep, etc. (Bhat et al. 2021). Our focus will be on the regulation of arousal and sensitization behaviour.

4. Neuropeptides involved in arousal and sensitization in *C. elegans*

The most often discussed example of endogenous inputs leading towards arousal is sleep. A sleep like pattern is also observed in *C. elegans*. After each larval state there is a period of quiescence, called lethargus, lasting 2 to 3 hours. Characteristic to this sleep-like state is that feeding and locomotion is supressed, resulting in lower sensory responses to arousal cues (Cho and Sternberg 2014). During this sleep-like state, *C. elegans* shows a reduced avoidance behaviour evoked by chemicals or mechanical cues (Cho and Sternberg 2014). Many neuropeptides are involved in the regulation of lethargus. Similar to the 24 hours circadian rhythm in mammals, regulated by the protein PERIOD, does *C. elegans* express *lin-42*. This transcription regulator works as a molecular clock with its expression at a maximum during larval moults. LIN-42 regulates expression of the neuropeptide gene *nlp-22* that promotes quiescence (Nelson et al. 2013). During lethargus, secretion of the pigment dispersing factor (PDF)-like neuropeptide PDF-1 is diminished, lowering touch sensitivity and consequently decreasing locomotion. Moreover, it is demonstrated that the neuropeptide receptor NPR-1 can inhibit the production of PDF-1 (Choi et al. 2013). The reduced responsiveness is regulated on multiple levels. Research demonstrated a decrease in calcium flux in the sensory neuron ASE as well as a change in excitability of interneurons (Cho and Sternberg 2014). Many more neuropeptides are involved in regulating sleep-like behaviour in *C. elegans*, such as FLP-11 (Bhat et al. 2021), FLP-13, FLP-24, and NLP-8 (Nath et al. 2016).

Locomotory arousal is most extensively studied and can originate from various aversive stimuli (figure 6) (Chew et al. 2018). A much discussed example is the locomotory arousal induced by the mechanical stimulus resulting from tapping the worms' culture plate (Rankin, 1990). Prior work from the lab has shown that after mechanosensory arousal, by giving taps to the petri dish, also behavioural responses to chemical stimuli were increased in *C. elegans*. An aversive chemical, like glycerol, sensed by the nociceptive ASH neurons evokes an enhanced escape response after tapping. Using genetically encoded calcium indicators to directly measure the calcium activity of ASH, it was found that ASH shows a higher stimulus-evoked calcium response after mechanosensory arousal (figure 7). This increase in activity of the ASH neuron is also observed after optogenetic activation of ASH in aroused animals. This shows that the chemosensory pathway can be sensitized by mechanosensory arousal (Chew et al. 2018).

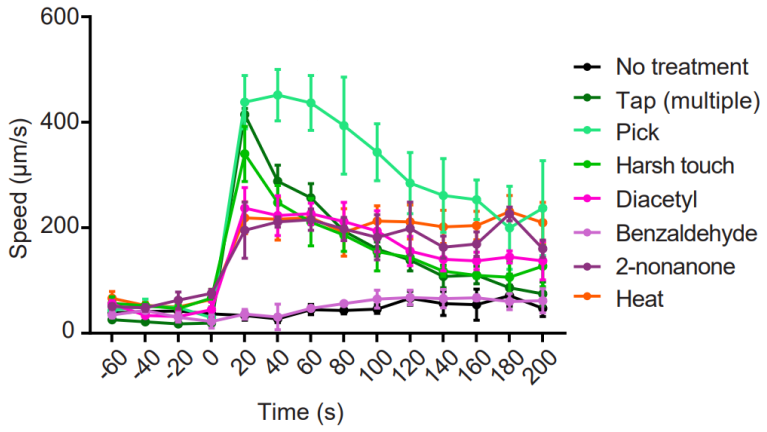

Figure 6. Locomotion assay of wild type C. elegans *displaying increased speed in response to different aversive stimuli and a gradual decline afterwards, indicating locomotor arousal (Chew et al. 2018).*

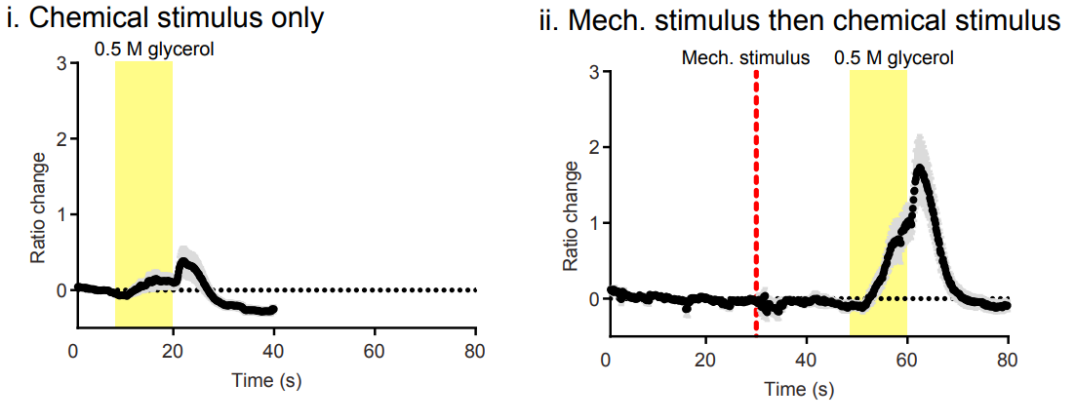

Figure 7. The calcium response measured in ASH as reaction to glycerol both (i) separately or (ii) following a mechanical cue, indicating cross-modal sensitization (Chew et al. 2018).

To investigate the involvement of neuropeptides establishing the arousal state, a candidate screen was performed which identified the neuropeptide FLP-20 as a crucial regulator of arousal. Mutants of *flp-20* are no longer capable to increase their speed or to exhibit an enhanced chemical sensitization after a mechanosensory arousing stimulus, although the worms are still able to sense mechanical cues (Chew et al. 2018). Using mKate2, a red fluorescent protein, researchers disclosed that *flp-20* is expressed in the touch receptor neurons (TRNs). They also identified the FLP-20 receptor, FRPR-3, by employing an aequorin bioluminescence assay. Mutants for both FRPR-3 and FLP-20 were as defective as single mutants, implying that they work in the same genetic pathway (Chew et al. 2018). It is interesting to note that FLP-20 is also involved in another form of non associative learning, namely habituation, more specifically in generating intermediate memory by mass training (Li

et al. 2013). Contrary to sensitization, habituation is a process where a decreased responsiveness is measured due to the exposure of a repeating familiar stimulus (Rose and Rankin 2001). The opposing effects of FLP-20 are regulated by different pathways. While habituation is presumable caused by creating a higher amount of presynaptic vesicles, sensitization is regulated by the FRPR-3 receptor (De Fruyt et al. 2020).

FRPR-3 receptors are expressed in multiple neurons. Using cell-specific RNAi, it was found that knockdown of the *frpr-3* gene specifically in the neuroendocrine RID cells disrupts ASH sensitization. The same study showed that optogenetic activation of RID was sufficient to mimic both locomotor arousal and ASH sensitization. RID is a neuroendocrine cell that produces a variety of neuropeptides. To test if these neuropeptides might be involved in the signalling cascade towards ASH, they knocked down the CAPS protein, UNC-31, an essential gene necessary for dense core vesicle release. These worms indeed showed a sensitization defect after arousal (Chew et al. 2018). Figure 8 shows an overview of the known players in this cross-modal sensitization paradigm.

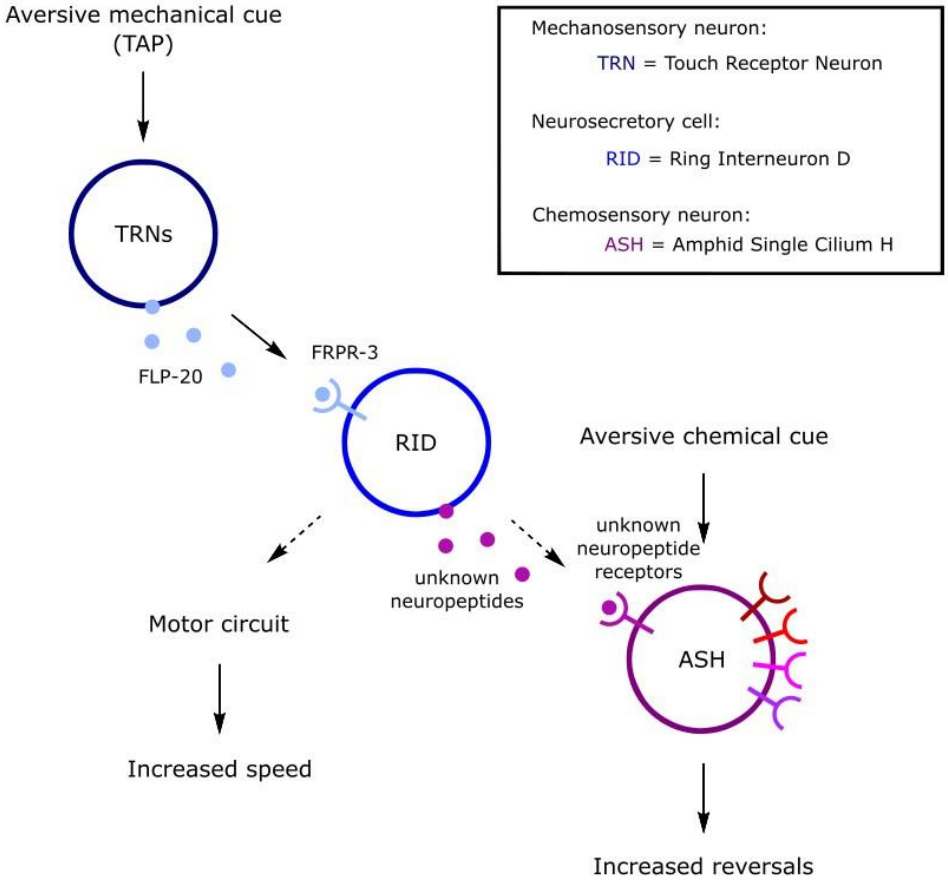

Figure 8. Schematic representation of the sensitization of the escape response mediated by ASH regulated by the neuropeptide FLP-20 and their receptor FRPR-3 expressed by RID.

Research objectives

To be able to make the proper behavioural adaptations it is needed to modulate the neuronal activity accordingly. The wired circuitry is not sufficient to coordinate these adaptations since also long-range signals like neuropeptides are needed.

In order to begin to understand the complex underlying circuitry governing arousal and sensitization we are using *Caenorhabditis elegans* as a model organism. Chew, Tanizawa, et al. (2018) discovered the role of *flp-20* in the sensitization response to an adverse chemical after mechanosensory arousal by tapping. In this project we aim to further unravel the molecular players of this pathway by which the neuroendocrine RID cell communicates with the chemosensory ASH neuron. ASH expresses a variety of neuropeptide GPCRs that could possibly be involved in this signalling cascade. The purpose of this project is to determine the impact of 3 of the 11 candidates with characterized ligands, namely the genes *npr-2*, *ckr-1* and *frpr-16*.

These genes are already studied in other contexts. The *npr-2* gene participates in the lowering of the responsiveness and raising the adaptation to repellents in off food conditions (Ezcurra et al. 2016). In addition, *npr-2* is responsible for regulating the avoidance behaviour of Methyl salicylate (MeSa) (Luo et al. 2014). The *ckr-1* gene is known to be activated by the cholecystokinin-like peptide NLP-12 and acts on the head neurons, increasing turning needed for local searching (Ramachandran et al. 2021). Further, the receptor CKR-1 is involved in the escape response by binding NLP-18, which promotes turning behaviour (Chen et al. 2022). The *frpr-16* gene is less studied and is thought to partially mediate male attraction to the pheromone ascr#8. Additionally, in males, *frpr-16* also supresses the avoidance behaviour to this pheromone observed in hermaphrodites (Reilly et al. 2021).

The first step to assess the role of these genes in sensitization is to generate loss-of-function mutants of these genes. For practical convenience, we will make use of optogenetic activation of the chemosensory ASH neurons to mimic the response to an encountered chemical. Therefore, the loss-of-function mutants should contain the required optogenetic transgene and the mutated gene (*npr-2*, *ckr-1* and *frpr-16)*. In a second step, behavioural responses of these mutants are measured in the established arousal and sensitization paradigm.

The functional characterization of these genes in the context of sensitization ought to provide the basis to further investigate the molecular mechanisms underling this arousal state, and more specific the sensitization process. Given the evolutionary conservation of many neuropeptidergic pathways, this may also lay a foundation to gain further insight into the peptidergic control of arousal in more complex nervous systems.

Materials and Methods

1. *C. elegans* strains

The strains used in the experiments are listed in table 1 and were all generated in the Bristol N2 background.

Table 1. List of C. elegans strains used in this research project

Strain	Genotype	Source	Description
N2	/	/	wild-type control
AQ2047	*lite-1(ce314) X*	Schafer lab Cambridge	*lite-1*: point mutation (substitution G/A)
AQ2235	*lite-1(ce314) ljIs114[gpa-13p::FLPase, sra-6p::FTF::ChR2::YFP] X*	(Chew et al. 2018)	*lite-1*: point mutation; *ljIs114*: Transgene expressing Channelrhodopsin2 (*ChR2*) in the ASH neurons (*ASH::ChR2*) under control of the *sra-6p* promoter. The transgene is present on the X chromosome.
AQ4133	*frpr-3(ok3302) V; lite-1(ce314) X; ljIs124[gpa-13p::FLPase,sra-6p::FTF::ChR2::YFP] not X*	(Chew et al. 2018)	*frpr-3*: partial deletion (591 bp); *lite-1*: point mutation; *ljIs124*: Transgene expressing *ChR2* in the ASH neurons (*ASH::ChR2*) under control of the *sra-6p* promoter. The transgene is on a non X chromosome.
AQ2056	*npr-2(ok419) IV*	Schafer lab Cambridge	*npr-2*: partial deletion (1374 bp)
LSC1247	*ckr-1(ok2502) I*	Schoofs lab	*ckr-1*: partial deletion (1289 bp)
PS8490	*frpr-16(sy1366) II*	*Caenorhabditis* Genetics Centre (CGC)	*frpr-16*: insertion of a STOP-IN cassette (43bp)
IBE534	*npr-2(ok419) IV; lite-1(ce314) ljIs114[gpa-13p::FLPase, sra-6p::FTF::ChR2::YFP] X*	This project	*npr-2*: partial deletion; *lite-1*: point mutation; *ljIs114*: Transgene expressing Channelrhodopsin2 (*ChR2*) in the ASH neurons (*ASH::ChR2*). The transgene is present on the X chromosome.

IBE549	*ckr-1(ok2502) I; lite-1(ce314) ljIs114[gpa-13p::FLPase, sra-6p::FTF::ChR2::YFP] X*	This project	*ckr-1*: partial deletion; *lite-1*: point mutation; *ljIs114*: Transgene expressing Channelrhodopsin2 (*ChR2*) in the ASH neurons (*ASH::ChR2*). The transgene is present on the X chromosome.
IBE563	*frpr-16(sy1366) II; lite-1(ce314) ljIs114[gpa-13p::FLPase, sra-6p::FTF::ChR2::YFP] X*	This project	*frpr-16*: insertion; *lite-1*: point mutation; *ljIs114*: Transgene expressing Channelrhodopsin2 (*ChR2*) in the ASH neurons (*ASH::ChR2*). The transgene is present on the X chromosome.

2. General *C. elegans* maintenance

2.1. Preparing *C. elegans* culture plates

The food source of *C. elegans* are bacteria, which are grown on agar plates. Subsequently, the worms are transferred to these plates to reproduce.

The agar plates on which the *C. elegans* strains are kept contain nematode growth medium (NGM). Most often we use NGM with a concentration of 2.5 g/L peptone. For 1L solution, we dissolve, apart from 2.5g Bacto™ Peptone (Gibco), 3g NaCl (Sigma-Aldrich) and 17g Difco™ Agar (BD BIOsciences) in 800mL AD water. Next, this bottle is autoclaved together with a bottle containing 200mL AD water. Afterwards the bottles are placed in a 60° oven. Before pouring, the liquids of the two bottles are combined to attain a final volume of 1L. Next, 1mL $MgSO_4$, 1mL $CaCl_2$, 1mL cholesterol and 25mL phosphate buffer is added (appendix 2). To avoid the formation of crystals, it is recommended to add these slowly and give them a few minutes to dissolve. For medium plates, which have a diameter of 55mm (Corning), 12mL media is poured into each plate using the stripettor™ ultra (Corning®) and a 50mL serological pipet (Greiner CELLSTAR®). After the plates have solidified, they are stored invertedly at room temperature.

The bacteria used as food source for *C. elegans* is *Escherichia coli* (*E. coli*) OP50; this mutated strain is an uracil auxotroph, meaning its growth is limited on NGM plates. One colony is picked from a streak plate and inoculated in a 50mL glass tube containing around 40mL 2x Tryptone Yeast Extract (2xTY) broth medium (appendix 2). Next, the sealed tube is placed in a shaking incubator with a temperature of 37°C and set at 180 rpm. The next morning (circa 16h later), the tube is ready for use. It can be stored in the fridge at 4°C for up to 2 weeks.

Thereafter the NGM plates are seeded: 200µL of the OP50 culture is dispensed on the solid NGM agar using a stepper pipette (Eppendorf Multipette® plus). Afterwards this droplet is distributed over the plate using a glass spreader. Plates are left open to dry in the laminar flow and are afterwards inverted and stored at room temperature. The bacteria are left to grow on the plate for at least one day before use.

2.2. Transferring of *C. elegans*

With *C. elegans* being a fast growing and multiplying organism, it is important to renew the plates every 3 to 4 days when kept at 20°C. This is preformed by transferring five hermaphrodites of different life stages to a new plate, which can form the next generation. For behavioural studies it is especially important that there is always plenty of food as

behaviour can drastically change in starved conditions. When the worms are grown at 16°C, transferring once per week is sufficient.

When only transferring a few worms, we can choose between picking (scooping) and sticking using a food patch. When there is need to transfer many worms at the same time, we can opt for chunking, meaning we cut a piece of the agar plate and put it upside-down on a new plate. Since *C. elegans* is a microscopic nematode, all proceedings happen under the microscope (Nikon SMZ 745).

2.3. Staging of young adults for assays

For our assays we need 1-day old adults. To assure that we have enough L4 worms when starting up the assay, they need to be staged. This is possible by transferring ten adult worms to a fresh plate, the morning 2 days prior of the assay, and let them lay eggs during 3 hours before burning them. Another option is to pick late L3s, 4 days prior.

2.4. Production of males

Gender in *C. elegans* is determined by the ratio of X chromosomes. Contrary to a hermaphrodite, which has two X chromosomes, males only have one X chromosome. This is caused by a meiotic nondisjunction, which occurs with a frequency of less than 0.2% (Corsi, Wightman, and Chalfie 2015). This percentage can be increased by heat-shocking the hermaphrodites at the L4 stage. To enhance the chance of generating males, hermaphrodite L4s are placed at 30°C for 6 hours and then overnight at 16°C. After obtaining at least one male, the animal can be crossed with a hermaphrodite and normal segregation will yield 50% males and 50% hermaphrodites in the next generation. Males are maintained by crossing twelve males with three hermaphrodites with the same background (4:1 ratio) and are also transferred every 3 to 4 days at 20°C.

2.5. Handling of contamination

The method to get rid of the contamination depends on the kind of microorganism. When it is fungal contamination, you can serially chunk the plate or transfer worms frequently to a new plate to slowly get rid of the fungal spores. A bacterial contamination is more difficult as these bacteria are a food source for the worm and hence are present in the gut. In this case we can bleach the worms, thereby breaking open the worms without damaging the eggs and have them hatch and start a new population. For this, a droplet of 10µL bleach solution (appendix 2) is added on the side of a seeded plate, together with six adult worms. The contaminants and hermaphrodites will be killed by the solution and the remaining fluid will be absorbed by

the plate before the embryos hatch. When the eggs hatch, the L1 larvae can crawl unharmed to the food.

2.6. Freezing of *C. elegans*

C. elegans can survive a freeze over a longer period, allowing to make backup stocks. Worms are ideally frozen as freshly starved L1s as this stage survives freezing the best. Freezing requires four plates that are going to starve around the same time; to accomplish this, each plate should contain the same amount of the same state worms. The plates are ready for freezing when there is no longer any food and most eggs have hatched into L1s (Stiernagle 2006). 4mL S Basal (1mL / plate) (appendix 2) is used to rinse the starved plates in a serial manner. After collection of the fluid with worms (~3ml), the same amount of freezing solution (appendix 2) is added. Pipet up and down before distributing the solution over six freezing tubes. The cryovials (Thermo Scientific) are placed in a Styrofoam box to ensure a gradient freeze when placing them in the -80°C freezer. Two weeks later one vile is thawed and poured on a seeded plate to assess if enough worms have survived the freeze. This plate is checked after a day and if enough worms are alive, the freeze was successful.

3. Generating new *C. elegans* strains

New strains are made by crossing males and hermaphrodites of different genotypes. To facilitate mating, we make use of crossing plates: NGM plates seeded with only a small droplet of 20µL OP50. Thus, forcing the worms to remain in closer proximity and enhancing the chance of mating. It is possible to transfer the parents the day after to a new plate to increase the percentage of cross progeny on the second plate.

Depending on the kind of mutation or transgene present, different methods are used to screen the genetic background of each worm. A deletion or insertion can be detected using polymerase chain reaction (PCR) followed with gel electrophoresis, while a point mutation requires sequencing. Fluorescent markers can be visualized using a fluorescence microscope (Leica M165 FC). Information on the kind of mutations used in the work can be found in table 2.

Table 2. Overview mutation characteristics and their verification approach

Gene or transgene	Mutation		Verification
npr-2	*ok419*	Deletion	PCR + gel
ckr-1	*ok2502*	deletion	PCR + gel
frpr-16	*sy1366*	insertion	PCR + gel
lite-1	*ce134*	point mutation	PCR + sequencing
ljIs114		Fusion YFP	Fluorescence microscopy

3.1. Genotyping using PCR and gel electrophoresis

First, the genomic DNA from the worms is extracted by lysis using proteinase K. Therefore, 5µL proteinase K (appendix 2) and 100µL PCR worm lysis buffer (appendix 2) is thawed and mixed. 5µL solution is transferred into each vial and, depending on the experiment, one or multiple worms are added. Next, the vials are laid on dry ice for 15 minutes to further break open the cuticula. Afterwards, the vials are placed in a thermocycler (Biometra) to set free the genetic material (table 3).

Table 3. Thermocycler program used for worm lysis

Phase	Temperature	Duration
Release of genomic DNA	60°C	60 min
Inactivation Proteinase K	95°C	15 min
Hold	4 – 10°C	pause

To amplify our gene of interest and check whether the incorporated deletion/insertion is either not present, hetero- or homozygous, we design gene specific primers using the Primer-BLAST tool by the National Center for Biotechnology Information (NCBI). Good primer sets comply with a length of around 20bp, a GC content between 40 and 60%, no formation of secondary structures in or between the two primers, and a similar annealing temperature with less than 5 degree difference. Ordered primers first need to be resuspended. The powder is centrifuged for 30 seconds and the appropriate amount of Milli Q is added to obtain a 100µM stock. To determine the amount of Milli Q (µL) needed, the oligo yield (nmol) needs to be multiplied by 10. Afterwards, the solution is briefly vortexed. Subsequently, a working stock is made containing 10µL primer with 90µL Milli Q, to attain a final concentration of 10µM that can be stored in the freezer. When using a new primer set the optimal annealing temperature needs to be determined with a gradient PCR. The primers we used in the experiments are depicted in table 4.

Table 4. List depicting the primer couples with their sequence, annealing temperature, and the length of their formed transcript in the presence and absence of the mutation.

	Type primer	Sequence	Annealing temp.	Length with mutation	Length without mutation
npr-2	Forward primer	5' - CGTGGTTCGTCCTCATAAAC -3'			
	Reverse primer	5' - CAGCCACCGTACATTAGCAC -3'	58.6°C	432 bp	1721 bp
	Poison primer	5' - GGGAAACTGGATGGAAATTG -3'	58.6°C	/	808 bp
ckr-1	Forward primer	5' - CTGTTATCAACATTCGGGTCAC -3'			
	Reverse primer	5' - CCCTGATGGTCGGTCTCTAT -3'	58.6°C	326 bp	1700 bp
	Poison primer	5' - CAAGGGTTTCCTTCTAACGG -3'	58.6°C	/	605 bp
frpr-16	Forward primer	5' - CTTCTTCTTCTCCTTTTTCCAC -3'			
	Reverse primer	5' - GAAGAGTGCCAACCTGAAAG -3'	62.0°C	373 bp	416 bp
	Poison primer	5' - CTTATCACTTAGTCACCTCTGCTC -3'	62.0°C	258	/

To perform the PCR for both primer sets, testing the forward primer with both the reverse and poison primer, the genomic DNA sample is split in two by transferring 2.5µL to a new vial. We opted to use REDTaq® PCR (Sigma-Aldrich). In each vial, 1µL of each primer, 12.5µL Readymix™ and 9.5µL MilliQ are added. The PCR program used is described in table 5.

Table 5. PCR program used for REDTaq PCR®

Phase	Temperature	Duration
Initial denaturation	60°C	2 min
Cycle x30		
- Denaturation	95°C	1 min
- Hybridisation	Annealing temp	2 min
- Extension	72°C	1 min/kilobase
Final extension	72°C	5 min
Hold	4 – 10°C	pause

Lastly, the gel electrophoresis is performed. A gel containing 1% agarose (Sigma-Aldrich) is used, made from 2g agarose mixed with 200mL Tris-acetate-EDTA (TAE) buffer (appendix 2). This solution is heated in the microwave for 3 minutes. Thereafter 10µL GelRed (10000x in Dimethylsulfoxide (DMSO), Biotium) is added, which allows us to visualize the DNA with UV light since it intercalates between the DNA strands. This hot solution is poured in a holder with combs and left to solidify. After removing the comb, the gel is placed in the electrophoresis chamber (PowerPac™ Basic, Biorad) filled with TAE. To load the gel 7µL of the samples are carefully dispensed in the wells, flanked on both sides with 5µL 1Kb Plus DNA ladder (Invitrogen). The container is connected and run for 40min at 120V to obtain a clear distribution. Afterwards the DNA is visualized using the ProXima 2500-T gel imager (Isogen Life Sciences).

3.2. Genotyping using PCR and sequencing

For confirmation or when screening for point mutations, the PCR product is sent for sequencing. First the PCR is completed, as explained in section 2.1. Before sending the PCR amplicon for sequencing, the sample needs to be purified. The Wizard SV Gel and PCR Clean-Up kit (Promega) is used, following the accompanied protocol that covers DNA binding, washing and elution steps. Next, the concentration is measured using the NanoPhotometer® (Implen) and the samples are diluted accordingly, for small constructs of 500 bp a final concentration of 10ng/µL is needed. 10µL of the DNA construct along with 4µL primer in a 1.5mL Safe-lock micro centrifuge tube (VWR) is sent to the company LGC genomics.

4. Behavioural assays

In this project we will perform two different behavioural assays: a tap assay and a sensitization assay, using 1-day adult worms. Both assays are similar and require multiple steps over a period of three days.

4.1. Tap assay

On the first day of preparing for a tap assay, the required low peptone plates (0.13g/L peptone), are seeded with a 50µL spot of OP50 (see section 1.1 Preparing *C. elegans* culture plates) and dried in the laminar flow for approximately 40 minutes. Once dried, they are kept inverted at room temperature. Low peptone plates are NGM maintenance plates with a concentration of only 0.13g/L peptone (other contents remain the same) and are used to obtain a thin layer of bacteria to clearly register the movement of the worms. To ensure that each assay is performed with young adults of the same age, the second day around 16u00, 10 L4s of the required genotypes are transferred to each seeded plate and placed at 20°C. To guarantee enough L4 worms, they can be priorly staged (see section 3.3 Staging of young adults for assays). On the third day the behaviour of the now young adult worms is tested.

For every recording, the plate is placed in a holder with an attached automatic tapper. The movement of the worms is recorded using a Pixelink camera (PL-D734MU-NIR-T) with a capture rate of five frames per second. The worms are given 2 minutes to acclimatise before the recording is started. After 30 seconds of the start of the video, five consecutive taps are given to the bottom of the plate in 3 seconds. The total duration of the recording is set to 5 minutes. The detailed time scheme can be consulted in figure 9.

The set up itself is made in house by Keertana Venkatesh, PhD student. The programs to automatically orchestrate the tap at the timepoint of 30 seconds is controlled and regulated by a Bonsai-RX (version 2.7.0) program, controlling the Arduino (version IDE 2.0.2) connected to the tapper.

4.2. Sensitization assay

In this assay, two stimuli are presented including a mechanosensory stimulus of tap and a chemosensory stimulus using optogenetics (channelrhodopsin expressed in the chemosensory neuron, ASH). The NGM plate preparation for the sensitization assay is similar. On the first day of a sensitization assay, low peptone plates (0.13g/L peptone) are seeded. For channelrhodopsin to function appropriately, the cofactor All-Trans-Retinal (ATR) needs to be added through feeding. For this, we make use of OP50 mixed with ATR (see

appendix), with a ratio of 1µL of the 100mM ATR stock for each 200µL OP50 (Rabinowitch, Treinin, and Bai 2016). As ATR is light sensitive, the plates must be wrapped in aluminium foil. For the control plates ethanol, the dilution solvent of ATR, is incorporated instead. Similar to the tap assay, the L4s are picked 1 day prior to the assay.

This assay is strictly performed in a dark room. The tap stimulus is similar to the procedure explained in the previous section (see section 4.1 Tap assay). The recordings follow the same pattern (see figure 9) but in case of a sensitization assay, there is an additional blue light input of 3 seconds, 50 seconds into the recording. The blue light lamp (CoolLED pE-100, Intensity 100%, wavelength 470nm) is pointed to the fixed plate and controlled by another Arduino panel. A Bonsai-RX program controls both the Arduino panels (for the tapper and the blue light) and the camera acquisition. These are in-house programs written by a PhD student in the lab, Keertana Venkatesh. The videos only have a length of 3 minutes due to time constraints, but this presents no problem since sufficient data can still be obtained.

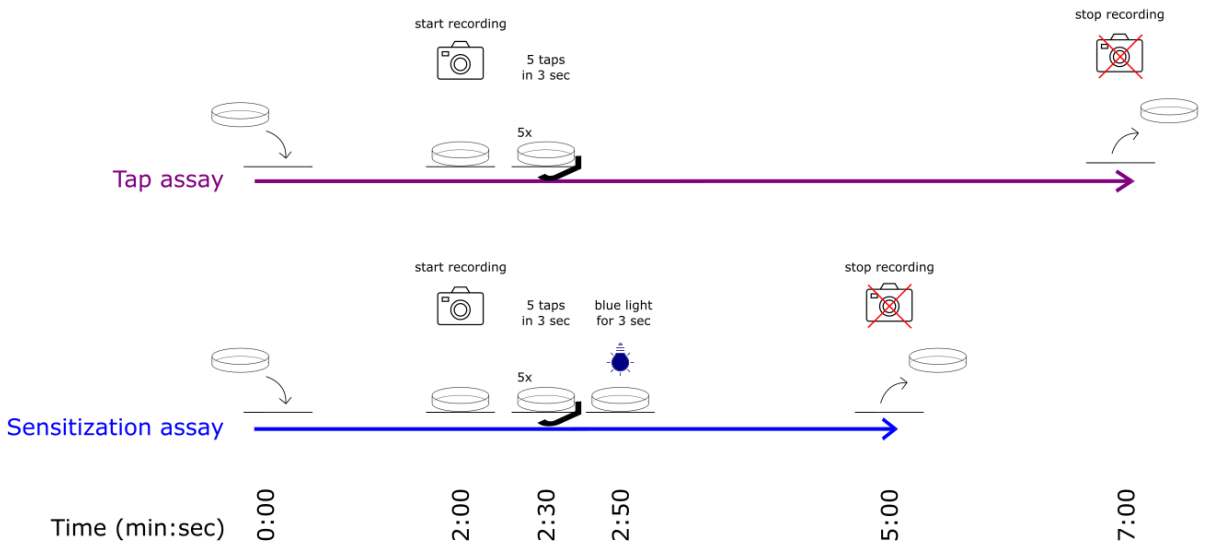

Figure 9. Overview of the course of the assays, both the tap and the sensitization assay are depicted.

5. Analysis

5.1. Tap assay

The recordings are analysed using Tierspy (version 1.5.3a+18aaba9), a multi worm tracker program (Javer et al. 2018; Javer, Ripoll-Sánchez, and Brown 2018). Afterwards the obtained data is run through an in-house python script, written by Keertana Venkatesh, generating the mean absolute speed (absolute speed of both forward and backward motion) for each plate. This absolute mean speed is then plotted for the speed trace graphs and is used further for comparing the response to wild type control using two parameters, the arousal integral and the arousal speed. The arousal integral, a measure of the distance travelled, is calculated by taking the area under the curve for the first 20 seconds post tap and the baseline is calculated from the average speed 30 seconds before tap for the respective genotype. This is achieved using GraphPad Prism (version 8.0.1). Further, arousal speed is compared by quantifying the ten second average speed before tap and after tap. This is extracted from the absolute mean speed trace using a custom Python script. Statistical tests are carried out using GraphPad Prism (version 8.0.1). One way ANOVA is used to compare the means of the arousal integrals between the different genotypes. Two-way ANOVA, Bonferroni post-test is used to study the arousal speed before and after tap and make a comparison between the genotypes.

5.2. Sensitization assay

The Tierpsy tracker is also used as multi worm tracker for the sensitization assay but an additional step is performed for these assays. Tierpsy tracks single worms by giving a worm index; however, if the worms collide or cluster during the tracking process, the identities of the worms are lost and new worm indices are assigned. As we wish to compare the maximum reversal speed of each individual worm when the blue light is switched on, we manually join the trajectories for single worms whenever there is a worm clustering (Github page Tierpsy/tierpsy-tracker). The obtained data is again processed with a python script written by Keertana Venkatesh, yielding information on the mean speed for each plate and the maximum backwards speed after exposure to the blue light for each individual worm. Contrary to the tap assay, we look at the mean speed and thus differentiate between backward and forward directed movements. First the speed trace is plotted. The reversal integral is calculated as the area under the curve for the interval of 45 to 90 seconds (these numbers included). The maximal reversal speed is plotted for each worm to determine the influence of a tap on the reversal speed. The data is plotted and undergoes a statistical analysis using GraphPad Prism (version 8.0.1). For all comparisons, Two-way ANOVA, Bonferroni post-test is used.

Results

1. Standardizing the mechanosensory stimulus for arousal

The candidate genes will be evaluated for their potential role in arousal and sensitization. Arousal assays have been previously established in the lab by giving an aversive mechanosensory stimulus in the form of five manual taps within a time period of three seconds. This results in an increase in speed that only gradually decreases back to baseline speed over the course of minutes, referred to as locomotory arousal (Chew et al. 2018).

To avoid variability when applying the taps manually, an automated taper was built. To test for the tapper's efficiency as an arousal stimulus, tap assays were preformed comparing manual taps to automated taps. For this comparison, two genotypes were tested, N2 and *lite-1(ce314)*. *lite-1(ce314*) is a point mutation in the *lite-1* gene. Worms inherently respond to blue light and this response is mediated by the lite-1 gene. It is essential to have this mutation in the background for our sensitization assay that uses optogenetics to activate the primary chemosensory neuron, ASH, using channelrhodopsin by shining blue light. Thus, in order to delineate the response to activation of ASH from the inherent blue light response, this *lite-1* mutation is essential.

For all conditions, we see a similar trajectory after administering the tap, a rapid increase in speed that afterwards gradually decreases. According to both the arousal characteristics, the arousal integral and the arousal speed, there are no significant differences between manual tapping and using the automated tapper for the two genotypes (Figure 10). This experiment suggests that it is possible to use the automated tapper as an arousal stimulus.

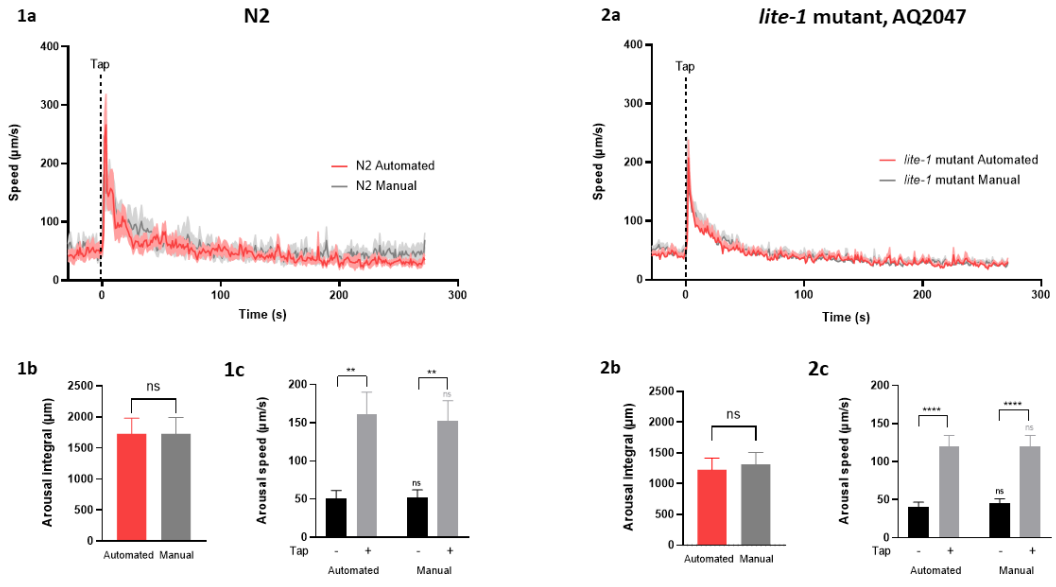

Figure 10. Tap arousal assay comparing manual tapping with the automated tapper for C. elegans strains: (1) N2 and (2) AQ2047, a lite-1(ce314) mutant. The graphs depict (a) the speed trace, (b) the arousal integral and (c) the average arousal speed before and after tap stimulation. For each trial around 5 to 10 animals were assayed and the number of trials are: N2 ≥ 7, AQ2047 ≥ 12. For all graphs, error bars depict mean ± SEM and statistical comparisons were done using (b) one-way ANOVA and (c) two-way ANOVA with Bonferroni correction. p-values < 0.05 were deemed significant.

2. Generating transgenic mutant strains

To evaluate if our candidate genes of interest, *npr-2, frpr-16* and *ckr-1,* are involved in the sensitization pathway, we first needed to generate the desired mutant strains. Since the sensitization assay involves optogenetic activation of ASH neurons, the worms should carry multiple transgenes or genomic mutations. Apart form a loss-of-function allele of the gene of interest, each mutant strain should also express channelrodopsin specifically in the ASH neuron, which is the primary nociceptive neuron in *C. elegans*. Additionally, the worms should have a defective *lite-1* gene to circumvent the innate reaction to blue light.

We started our crosses from a strain that already contains these two X chromosomal modifications. The strain AQ2235, expresses channelrhodopsin in the ASH neuron and contains a point mutation in the *lite-1* gene (*lite-1(ce314)*) which was shown to be a null mutation of *lite-1* (Edwards et al. 2008). We further crossed this strain with available mutant strains of the genes of interest. The *npr-2* and *ckr-1* mutants are partial deletion alleles from the *C. elegans* Gene Knockout consortium that have been further backcrossed to the wild-type N2 strain. The *frpr-16* allele has a STOP-IN cassette in the *frpr-16* gene causing loss of function. The insert, 43 nucleotide in length, causes a frameshift and additionally encodes a stop codon for all three reading frames. In addition, the cassette has a Cas9 target side that allows for further modification and can also be used to revered the strain back to wild-type. (Wang et al. 2018). Crosses were set up according to a scheme that minimizes screening steps and avoids sequencing for the *lite-1* point mutation located on the X chromosome (figure 11).

Afterwards the three generated strains (IBE534, IBE549, IBE563) were verified by checking: 1) the presence of the three receptor mutations by PCR and gel electrophoresis of the respective insertion/deletion alleles, 2) by sequencing of the *lite-1* locus, and 3) by validating the presence of the channelrhodpsin transgene (*ljIs114*) based on the presence of a fluorescent marker. Hence forth, these strains were used for behavioural assays and the strain carrying only the *lite-1* mutation and channelrhodopsin transgene (as used for the cross) was used as the wild-type control strain.

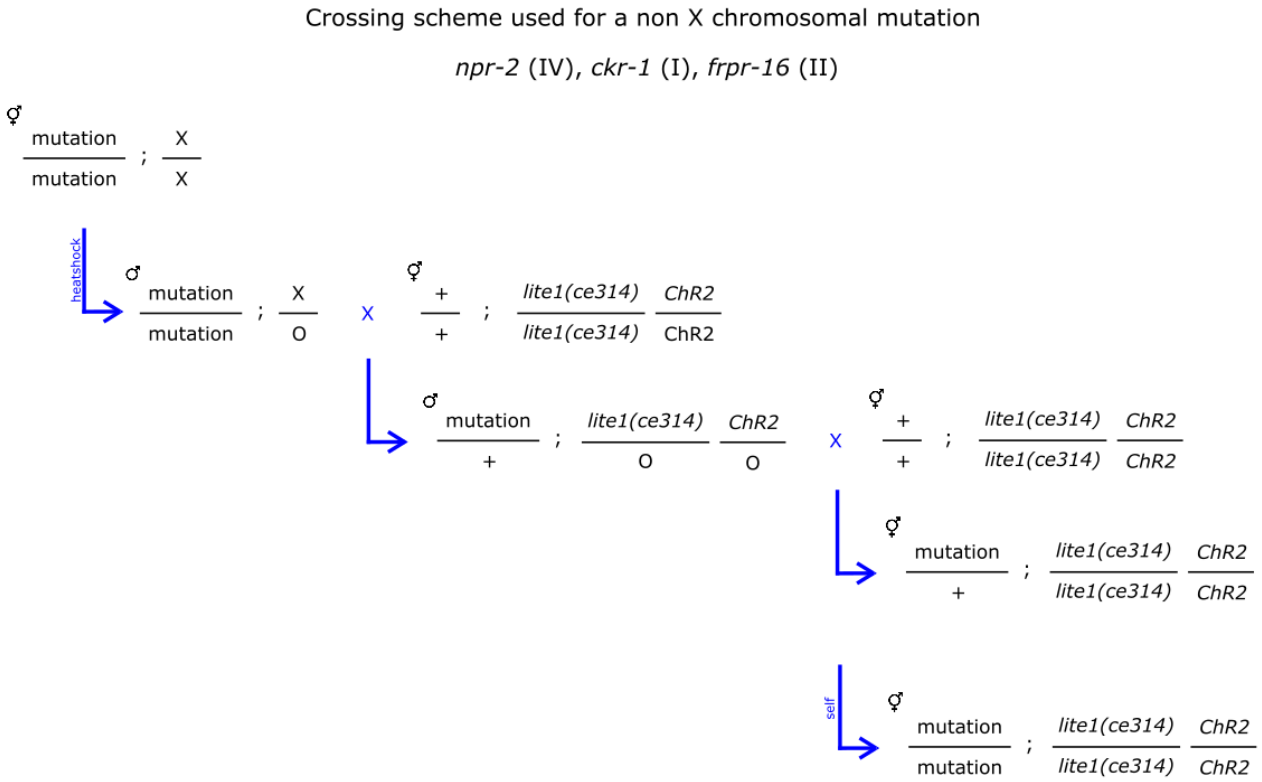

Figure 11. Crossing scheme used for generating mutant strains IBE534, IBE549, IBE563 with npr-2(ok419);lite-1(ce314) ljIs114, with ckr-1(ok2502);lite-1(ce314)ljIs114 and with frpr-16(sy1366);lite-1(ce314)ljIs114 genotypes, respectively.

3. All candidate mutants show a normal locomotory arousal response

To evaluate if the genes of interest (*npr-2*, *ckr-1* and *frpr-16*) are affecting the response to tap we first performed a tap assay. The worms' locomotory responses to the five consecutive taps, administered in a 3 second interval, were recorded and the speed trace, arousal integral and arousal speed parameters were extracted and analysed.

As a control strain, we used worms carrying the *lite-1* point mutation and channelrhodopsin transgene, as mentioned in section 2. This is the wild-type-like control and will be referred to as wild type henceforth. In addition, we also used a positive control strain that is defective in locomotory arousal after tap. For this, we used a *frpr-3* mutant carrying the *lite-1* point mutation and the same channelrhodopsin transgene. We opted for this control as the FRPR-3 receptor has been shown to be involved in mediating arousal and sensitization responses earlier in the lab and shows a locomotory arousal defect (Chew et al. 2018).

As visualized in the graphs (figure 12), all three neuropeptide receptor mutants show a normal locomotory arousal response similar to wild type. Yet, the arousal speed indicates a significantly lower and higher arousal speed for *npr-2* (*) and *frpr-16* (**), respectively. In all panels, the response pattern of the controls are as expected. While the speed of the wild-type control quickly rises before gradually decreasing, the *frpr-3* mutant control worms speed up but only to a lower extend after which they quickly fall back to their resting speed.

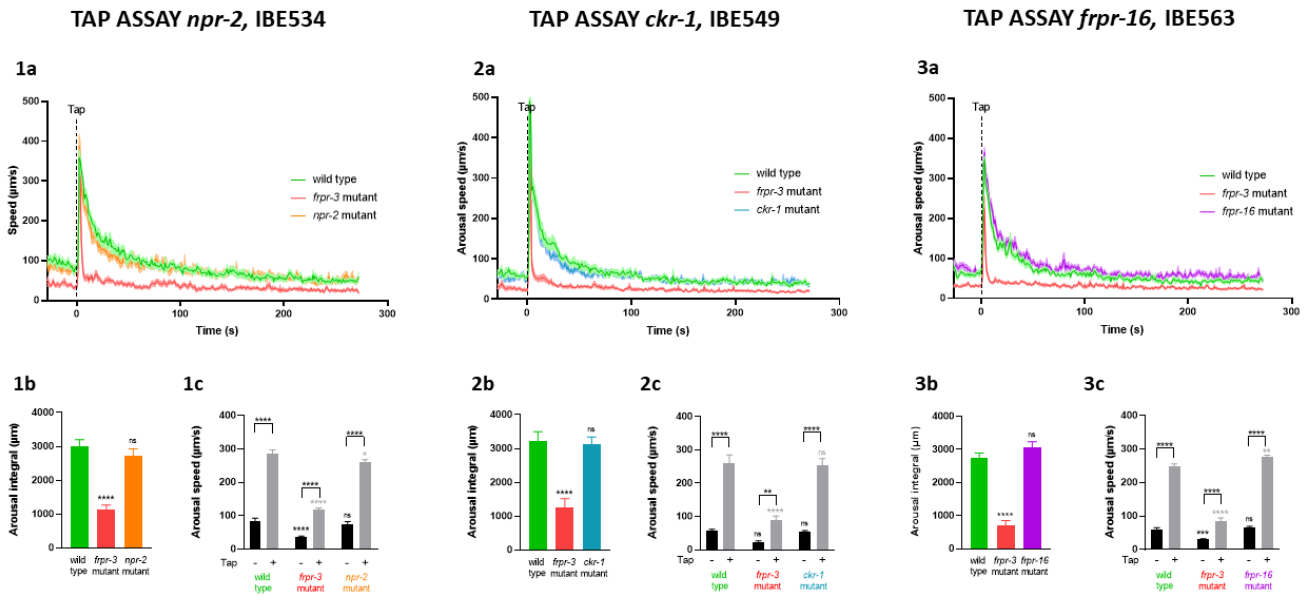

Figure 12. Tap arousal assay testing the mutant C. elegans strains: (1) Test for IBE534 = npr-2(ok419);lite-1(ce314) ljIs114, (2) Test for IBE549 = ckr-1(ok2502);lite-1(ce314)ljIs114 and (3)Test for IBE563 = frpr-16(sy1366);lite-1(ce314)ljIs114. The graphs depict (a) the speed trace, (b) the arousal integral and (c) the average arousal speed before and after tap stimulation. For each trial around 5 to 10 animals were assayed and the number of trials were: IBE534 ≥ 10, IBE549 ≥ 12 and IBE563 ≥ 14. For all graphs, error bars depict mean ± SEM and statistical comparisons were done using (b) one-way ANOVA and (c) two-way ANOVA with Bonferroni correction. p-values < 0.05 were deemed significant.

4. Sensitization assays

Besides locomotory arousal, we also assessed the effects of the candidate receptor genes on the sensitization of chemosensory responses during arousal using a sensitization assay. In essence, this assay compares the reaction to an aversive chemosensory cue, here mimicked via blue light mediated ASH activation, between worms exposed to a previous mechanosensory cue (tap) and worms that did not receive this arousing stimulus. In doing so, we can determine if the genes of interest are involved in this specific type of cross-modal sensitization.

After exposure to the blue light cue, wild-type worms rapidly reverse before moving forward with an elevated speed and gradually coming back to their normal pace (figure 13A). This backward response was found to be higher in aroused animals compared to worms that did not receive the prior tap stimulus (figure 13B). The *frpr-3* mutants reverse over a shorter distance at a lower speed before moving forward, but they do not show a prior acceleration (figure 13A). In addition, there is no difference between locomotory responses of *frpr-3* mutants exposed to only blue light and worms that receive the prior tap stimulus and subsequent blue light activation of ASH, thereby showing a sensitization defect (figure 13B). These two controls are used in each experiment to validate the credibility of the set up.

4.1. The *npr-2* mutant shows a partial sensitization defect

When deleting the *npr-2* gene we do not see a clear defect in sensitization (figure 13A). The reversal integral of the *npr-2* mutant candidate, when exposed to both tap and blue light, differs significantly (**) from the wild type (figure 13B). When looking closely at the analysis, we observed that from the three days with each three repeats, a significant difference between the mutant and wild-type control strain was seen on only one of these days. Moreover, the three repeats of that day show much variation. Despite this, the reversal integral of *npr-2* mutants still significantly differs (****) between the two conditions with and without tap (figure 13B). Contrary to the arousal integral, the maximum reversal speed does not differ from wild type (figure 13C). Thus, although the pooled data shows a partial sensitization defect in *npr-2* mutants, due to the variability seen across assay days, more repeats need to be carried out to confirm this phenotype. Both the wild type and the *frpr-3* mutant control reacted as expected.

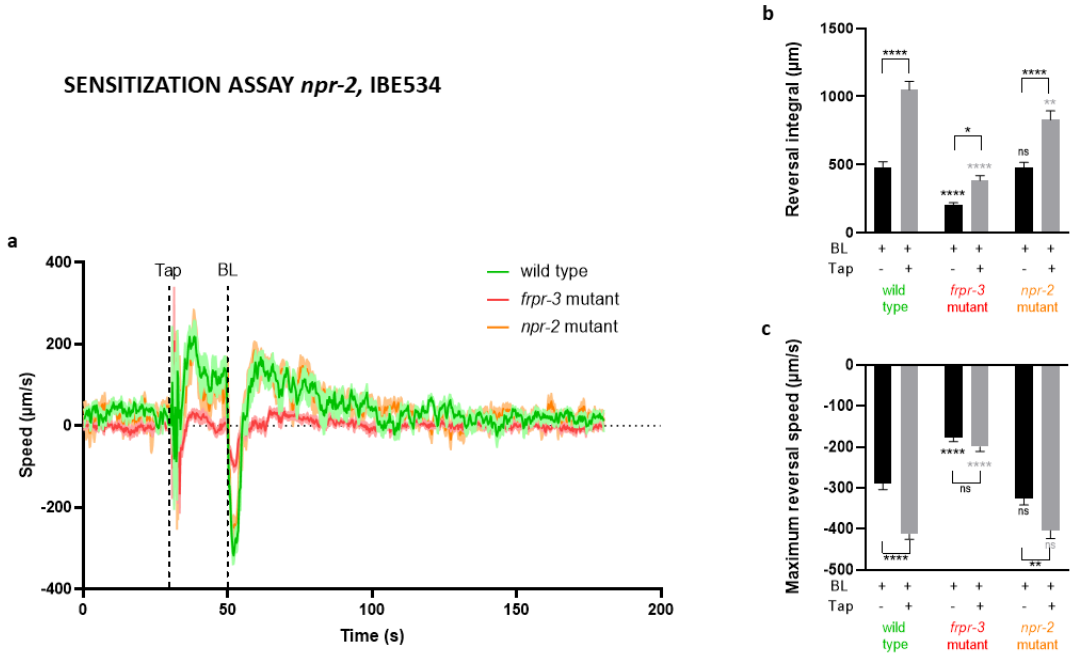

Figure 13. Sensitization assay testing the mutant C. elegans strain IBE534 = npr-2(ok419);lite-1(ce314) ljIs114. The graphs depict (a) the speed trace, (b) the reversal integral and (c) the maximum reversal speed for conditions with (grey bars) and without (black bars) tap stimulation. For each genotype 9 assays were performed; on average each trial had ≥ 5 worms. For all graphs, error bars depict mean ± SEM and statistical comparisons were made using two-way ANOVA with Bonferroni post-hoc test, with p-values < 0.05 deemed significant.

4.2. The *ckr-1* mutant shows a sensitization defect

The mutant strain carrying a deletion in the *ckr-1* gene did not react similarly to the blue light pulse as the wild-type control (figure 14A). The exposure to the blue light after tap causes an increased reversal reaction but not to the same extend as the wild-type control strain. The subsequent increase in forward speed is also reduced, after which it quickly falls back to its normal pace.

The reversal integral (figure 14B), a proxy for the length of the reversal, shows a significant reduction (****) for the *ckr-1* mutant compared to the wild-type control after tap and blue light. The condition with only blue light shows a slight but significant (*) increase in reversal integral. Most importantly, there is no longer a significant difference between the reversal response with or without tap in the *ckr-1* mutant strain, suggesting a sensitization defect. When we take a closer look at the data of each day separately, we see that for two out of the tree days the “tap with blue light” condition is diminished and therefore no longer shows a significant difference to the “only blue light” condition. However, for the data of one day there is still a significant (**) difference between the reversal response with and without tap. Nonetheless, we still see a significant (****) reduction for response to “tap with blue light” in

ckr-1 mutants compared to wild type for all the days. This suggests that *ckr-1* might be involved in mediating specifically cross-modal sensitization.

The maximum reversal speed (figure 14B) when previously exposed to tap does not differ significantly from wild type. Without tap stimulation the mutant candidate shows a significantly (*) higher speed compared to wild type. Taking the two arousal characteristics into account, when both tap and blue light is administered, our results suggest that *ckr-1* mutants reverse at the same maximum speed but for a shorter period. Again, the controls react as expected, validating the credibility of the obtained results.

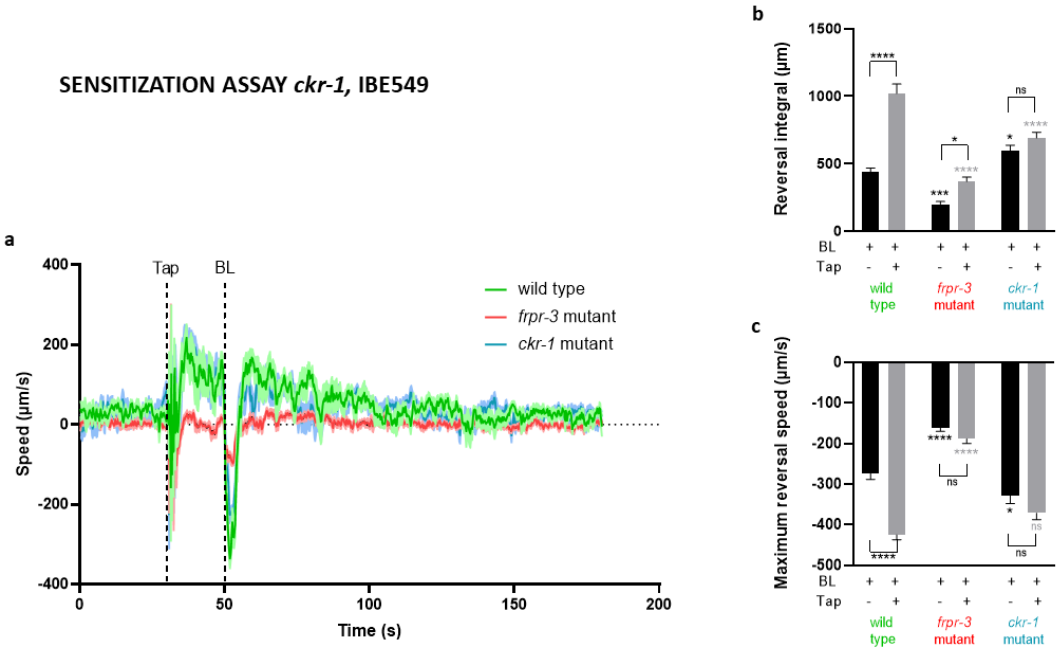

Figure 14. Sensitization assay testing the mutant C. elegans strain IBE549 = ckr-1(ok2502);lite-1(ce314) ljIs114. The graphs depict (a) the speed trace, (b) the reversal integral and (c) the maximum reversal speed for conditions with (grey bars) and without (black bars) tap stimulation. For each genotype ≥8 assays were performed; on average each trial had ≥ 5 worms. For all graphs, error bars depict mean ± SEM and statistical comparisons were made using two-way ANOVA with Bonferroni post-hoc test, with p-values < 0.05 deemed significant.

4.3. The *frpr-16* mutant does not show a sensitization defect

Our results suggest that the *frpr-16* gene does not play a role in sensitization. Compared to the wild-type control, *frpr-16* mutants show a very similar reversal dynamic (figure 15A). However, the speed trace does show a higher initial speed after reversal that decreases at a steady but lower pace. The two observed arousal characteristics (figure 15B and C) show no significant differences compared to wild type control. Moreover, there remains a significant difference (****) for response to “only blue light” versus “tap and blue light” in *frpr-16* mutant worms. The *frpr-3* mutant control is as expected, showing a defective sensitization response.

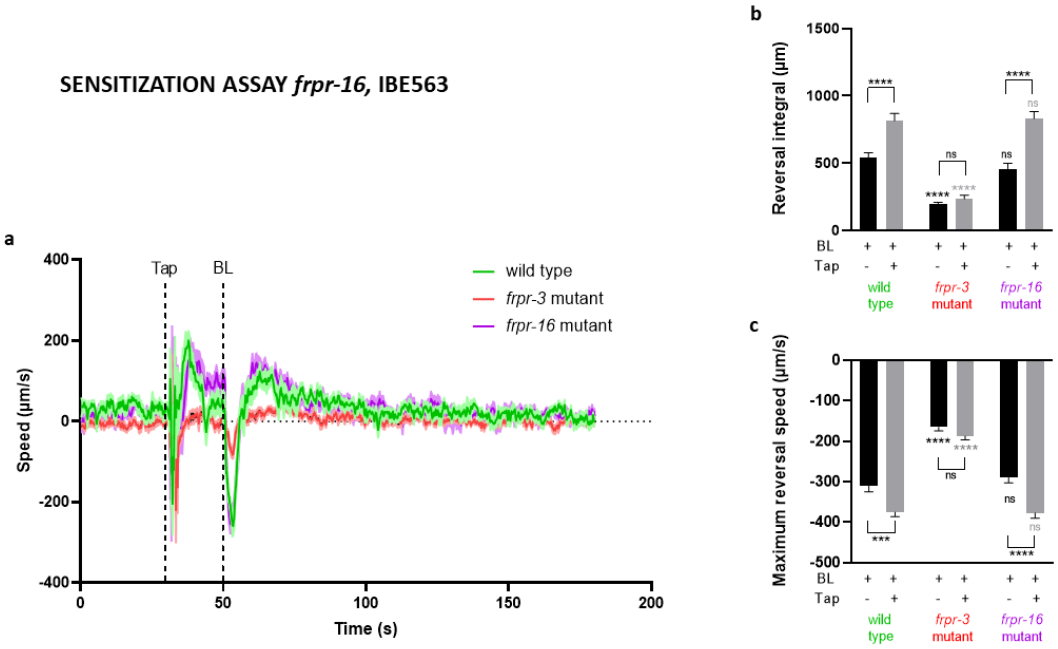

Figure 15. Sensitization assay testing the mutant C. elegans strain IBE563 = frpr-16(sy1366);lite-1(ce314) ljIs114. The graphs depict (a) the speed trace, (b) the reversal integral and (c) the maximum reversal speed for conditions with (grey bars) and without (black bars) tap stimulation. For each genotype 9 assays were performed; on average each trial had ≥ 6 worms. For all graphs, error bars depict mean ± SEM and statistical comparisons were made using two-way ANOVA with Bonferroni post-hoc test, with p-values < 0.05 deemed significant.

Discussion

This work investigates the efferent neuropeptidergic signals mediating cross-modal sensitization and arousal in *C. elegans*, in particular, the efferent neuropeptidergic signals mediating sensitization of the chemosensory circuit.

In 2018, Chew et al. described a new behavioural paradigm for arousal in *C. elegans*, where a mechanosensory cue results in both a direct increase in locomotor arousal as well as cross modal sensitization to chemical cues. The researchers started to elucidate the molecular players and discovered the involvement of FLP-20, released from the mechanosensory neurons. To orchestrate the cross-modal sensitization, the neuropeptide FLP-20 was shown to signal to the neuroendocrine cell RID by binding of the receptor FRPR-3. Next, RID redirects the signal to the chemosensory neuron ASH, but the mechanism is unknown (Chew et al. 2018).

The purpose of this research was to take a closer look at the signalling pathways between RID and ASH. We believe this efferent signal is neuropeptidergic in nature since a knockdown of *unc-31,* a gene necessary for regulating neuropeptide release, confirmed the involvement of neuropeptides. To identify the identity of these neuropeptide signals, we looked at neuropeptide receptor mutants for genes that are specifically present in the chemosensory circuit. We started our search by picking three GPCRs expressed in ASH, namely, NPR-2, CKR-1 and FRPR-16.

All these three mutants were tested for arousal and sensitization responses. In order to reduce variability in these behavioural assays, by administering the arousal stimulus through manual tapping, we standardised an automated tapper. When comparing results from manual tapping to results obtained with the installed automated tapper, we observed no significant differences in the arousal response. After analysis, the automated tapper was incorporated in the standard protocol and used in all upcoming assays. The use of the automated tapper eliminates human inaccuracies, as the taps are now administered at fixed time points with constant time intervals and consistent force.

Our behavioural assay entails quantifying responses to an aversive mechanical cue, tap, as arousal stimulus and further responses to an aversive chemosensory cue, thereby testing for sensitization. Firstly, all the three mutants underwent tap assays to uncover a possible defect to the mechanosensory stimulation. All assayed worms showed a normal arousal response to tap stimulation. This was in line with our expectations since these candidates were picked for there expression in the chemosensory neuron, ASH, and are not highly expressed in the mechanosensory circuit (Taylor et al. 2021).

Interestingly, the *frpr-16* mutant did show an overall higher speed. To discover the reason why, this aspect needs detailed analysis, by looking at just the tap response with, for

example, varying numbers of taps. If the locomotion phenotype can be confirmed, cell-specific rescue experiments can be performed to assess whether FRPR-16 indeed regulates locomotory tap responses. Single-cell RNA-sequencing data indicates that *frpr-16* does show expression in some mechanosensory neurons like PVM, PLM and ALM (Taylor et al. 2021). Thus, it is possible that *frpr-16* may be involved in the negative regulation or a feedback mechanism for inhibiting high speed in response to a tap stimulus, but this needs to be further investigated in detail.

A recent deorphanization screen discovered that FRPR-16 is one of the three receptors able to bind neuropeptides from different classes: FLP-3, NLP-23 (Beets et al. 2022). The pathway requiring FLP-3 regulates males attraction to ascr#8 (Reilly et al. 2021).

The sensitization characteristics of the *frpr-3* mutant are similar to wild type, suggesting that this gene is not involved in locomotory arousal or cross-modal sensitization. However, it should be noted that the forward speed after reversal (during rebound) in response to tap together with optogenetic activation of ASH in this mutant is increased. We have currently not quantified this rebound forward speed. However, it might be a unique feature of arousal that needs further investigation.

The *npr-2* gene shows a variable and partial sensitization phenotype. This receptor is activated by the RFamide neuropeptide FLP-21, which is also a ligand of NPR-1 (Beets et al. 2022). It is documented that both these receptors, NPR-1 and NPR-2, work together to orchestrate the avoidance of Methyl salicylate, a volatile molecule produced as stress signal by plants when they are damaged that serves as warning for infections and herbivores (Luo et al. 2014). The receptors also work together to coordinate adaptation of ASH to repellents in off food conditions (Ezcurra et al. 2016).

When studying the arousal characteristics of the *npr-2* mutants, the maximum reversal speed does not suggest that the gene is involved as it looks similar to wild type. However, the reversal integral is significantly (**) lower for the condition with both tap and optogenetic ASH activation compared to the wild-type control. This data was highly variable across days, hence more repeats would be needed to say anything conclusively for this candidate.

The *ckr-1* mutant strain, however, does show a strong sensitization defect. The *ckr-1* gene is homologous to the cholecystokinin (CCK) receptors in humans (Frooninckx et al. 2012). These molecules mediate multiple functions in the gastrointestinal tract, like pancreas enzyme secretion, gall blader contractions and food intake, additionally they work in brain circuits regulating aggression, sleep, anxiety, memory, nociception and sexual arousal. In invertebrates, this group of conserved peptides, called sulfakinines (SK), play roles in food ingestion, satiety and secretion (Nässel and Wu 2022). In *C. elegans*, the *ckr-1* gene is previously studied for its binding to NLP-12 and NLP-18 peptides, which both regulate

locomotion. Activation of CKR-1 by NLP-12 in head neurons increases turning (Ramachandran et al. 2021), while NLP-18 signalling through CKR-1 is involved in regulating omega-shaped turning in escape responses (Chen et al. 2022).

The results of the sensitization experiment with the *ckr-1* mutant show that there is no longer a significant difference in the locomotory response evoked by optogenetic ASH activation in the presence or absence of the arousing tap stimulus for both the reversal integral and the maximum reversal speed. This suggests that *ckr-1* is at least partially involved in the heightened reaction after the secondary chemical cue, here mimicked by the optogenetic blue light impulse. It also further suggests that there could be independent neuropeptidergic circuits regulating responses to locomotory arousal and sensitization.

However, it is counterintuitive that the mutant worms missing the *ckr-1* gene, exposed to blue light only, were significantly (*) more aroused compared to wild type. This is interesting and needs further investigation. It is possible that *ckr-1* is involved in controlling reversal responses upon exposure to aversive chemosensory cues, thereby having an inhibitory effect. These aspects can be investigated further by performing cell-specific rescue experiments in the chemosensory circuit and also calcium imaging in the ASH neuron. Nevertheless, there is no longer a significant difference in response to optogenetic ASH activation with and without the tap stimulus suggesting a strong sensitization defect.

Contrary to the reversal integral, we do not see a significant reduction in the maximum reversal speed. This would mean that the initial backward motion has the same speed but does not last as long compared to the wild-type control.

Additionally, the presence of the *ckr-1* mutation clearly affects the rebound speed after optogenetic activation of ASH, making it an interesting aspect to investigate in more detail. So far, we only looked at the reversal integral, which is a measure for the total reversal calculated as the negative area under the curve, and the maximal reversal speed, a measure for the amplitude of the peak. It could be interesting to study more aspects of the arousal curve, in particular the difference in the maximal reversal speed in the absence and presence of the arousing tap stimulus, defined as the fold change. Another interesting characteristic is the rebound integral, the increased forward speed after the worms reverse. Until now this has not been included in our analysis, while these aspects also clearly differ between the wild type and *frpr-3* control. This shift is also visible in the *ckr-1* mutant.

To verify that it is in fact the *ckr-1* gene that is responsible for these defects and not a background mutation, we can reintroduce the wild-type gene in the mutant. For this study we worked with a complete knockout where the expression of *ckr-1* in all neurons is disrupted. To further understand the mechanism of action and role of *ckr-1*, it is important to carry out cell-specific rescue experiments. This would give us information on the neuron through which

it is acting to regulate the sensitization responses. We hypothesize that it is functioning in the ASH neuron itself but this could also be via other neurons. The earlier studies on *ckr-1* show that *ckr-1* mediates escape responses of omega turns and local search behaviour via AIB in combination with SMD (Chen et al. 2022) and SMD (Ramachandran et al. 2021) neurons, respectively. It will be interesting to pinpoint if it functions via the same or different neurons for cross-modal sensitization.

Additionally, it will be interesting to further look at the previously identified ligands of CKR-1, NLP-12 and NLP-18, to further delineate upstream signals and to investigate the underlying neurons. Deletion experiments with these respective ligands can point out their potential involvement and additional cell specific rescue can give information about their origin. According to single cell RNA sequencing data, neither NLP-12 or NLP-18 are expressed by the neuroendocrine cell RID (Taylor et al. 2021). For this reason we hypothesize that it is coordinated by a multi step mechanism with more players involved. The prior lab research showed the involvement of RID, ASH and neuropeptides but did not yet demonstrated their relationship (Chew et al. 2018). Despite RID being a neuroendocrine cell, should the neuropeptide signal not necessarily begin in RID itself. Whether the neuropeptide originated in RID can be verified in a RID specific *unc-31* RNAi knockdown experiment.

Apart from *ckr-1, C. elegans* has a second cholecystokinin like receptor, namely *ckr-2*. It is seen that both the cholecystokinin receptors show affinity for the NLP-12 ligand (Beets et al. 2022). Moreover, *ckr-2* is also expressed in ASH but in a lesser extent (Taylor et al. 2021). In consequence, it might be interesting to investigate the role of *ckr-2* in this sensitization paradigm.

Besides the three genes studied here, ASH expresses many more receptors which can possibly be involved. Eleven of these candidate receptors have known ligands, therefore they should be prioritized. It would be interesting to test the candidate receptor pairs *pdfr-1* with the PDF-1 neuropeptide as ligand and *frpr-19* with the neuropeptide ligand FLP-14, as these ligands are directly expressed in RID (Taylor et al. 2021). These receptors can be screened in parallel while further uncovering the role played by *ckr-1* in this cross modal sensitization paradigm.

Conclusion

This research project helped to understand the potential involvement of three neuropeptide receptor genes expressed in the nociceptive ASH neurons, *npr-2*, *ckr-1* and *frpr-16,* in arousal and more specifically in the cross-modal sensitization of ASH responses. Our data suggest that the *npr-2* and *frpr-16* genes are not involved in this sensitization pathway. However, the results generated in this study indicate a putative role of the CKR-1 receptor in cross-modal sensitization. We further hypothesise that CKR-1 could be mediating this effect via its expression in the chemosensory circuit.

Overall, this study helped to get a better understanding of the molecular players involved in arousal and the efferent extrasynaptic signals regulating cross-modal sensitization to chemical cues.

References

Allen, E. N., J. Ren, Y. Zhang, and J. Alcedo. 2015. “Sensory Systems: Their Impact on *C. Elegans* Survival.” *Neuroscience* 296: 15–25. https://doi.org/10.1016/j.neuroscience.2014.06.054.

Andretic, Rozi, Bruno Van Swinderen, and Ralph J. Greenspan. 2005. “Dopaminergic Modulation of Arousal in *Drosophila*.” *Current Biology* 15 (13): 1165–75. https://doi.org/10.1016/j.cub.2005.05.025.

Anton, Sylvia, Katarina Evengaard, Romina B. Barrozo, Peter Anderson, and Niels Skals. 2011. “Brief Predator Sound Exposure Elicits Behavioral and Neuronal Long-Term Sensitization in the Olfactory System of an Insect.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (8): 3401–5. https://doi.org/10.1073/pnas.1008840108.

Artyukhin, Alexander B, Frank C Schroeder, and Leon Avery. 2013. “Density Dependence in *Caenorhabditis* Larval Starvation.” *Scientific Reports* 3 (1): 2777. https://doi.org/10.1038/srep02777.

Asahina, Kenta, Kiichi Watanabe, Brian J. Duistermars, Eric Hoopfer, Carlos Roberto González, Eyrún Arna Eyjólfsdóttir, Pietro Perona, and David J. Anderson. 2014. “Tachykinin-Expressing Neurons Control Male-Specific Aggressive Arousal in *Drosophila*.” *Cell* 156 (1–2): 221–35. https://doi.org/10.1016/j.cell.2013.11.045.

Babcock, Daniel T., Christian Landry, and Michael J. Galko. 2009. “Cytokine Signaling Mediates UV-Induced Nociceptive Sensitization in *Drosophila* Larvae.” *Current Biology* 19 (10): 799–806. https://doi.org/10.1016/j.cub.2009.03.062.

Bael, Sven van, Jan Watteyne, Kurt Boonen, Wouter de Haes, Gerben Menschaert, Niels Ringstad, H Robert Horvitz, Liliane Schoofs, Steven J Husson, and Liesbet Temmerman. 2018. “Mass Spectrometric Evidence for Neuropeptide-Amidating Enzymes in *Caenorhabditis Elegans*.” *Journal of Biological Chemistry* 293 (16): 6052–63. https://doi.org/https://doi.org/10.1074/jbc.RA117.000731.

Barbas, Demian, Luc DesGroseillers, Vincent F. Castellucci, Thomas J. Carew, and Stéphane Marinesco. 2003. “Multiple Serotonergic Mechanisms Contributing to Sensitization in *Aplysia*: Evidence of Diverse Serotonin Receptor Subtypes.” *Learning and Memory*. https://doi.org/10.1101/lm.66103.

Bargmann, Cornelia I. 2006. “Chemosensation in *C. Elegans*.” *WormBook : The Online Review of C. Elegans Biology*, 1–29. https://doi.org/10.1895/wormbook.1.123.1.

Bargmann, Cornelia I. 2012. “Beyond the Connectome: How Neuromodulators Shape Neural Circuits.” *BioEssays* 34 (6): 458–65. https://doi.org/10.1002/bies.201100185.

Beets, Isabel, Sven Zels, Elke Vandewyer, Jonas Demeulemeester, Jelle Caers, Esra Baytemur, William R Schafer, Petra E Vértes, Olivier Mirabeau, and Liliane Schoofs. 2022. “System-Wide Mapping of Neuropeptide-GPCR Interactions in *C. Elegans* Lead Contact.” https://doi.org/10.1101/2022.10.30.514428.

Bentley, Barry, Robyn Branicky, Christopher L. Barnes, Yee Lian Chew, Eviatar Yemini, Edward T. Bullmore, Petra E. Vértes, and William R. Schafer. 2016. *The Multilayer Connectome of Caenorhabditis Elegans*. *PLoS Computational Biology*. Vol. 12. https://doi.org/10.1371/journal.pcbi.1005283.

Bhat, Umer Saleem, Navneet Shahi, Siju Surendran, and Kavita Babu. 2021. “Neuropeptides and Behaviors: How Small Peptides Regulate Nervous System Function and Behavioral Outputs.” *Frontiers in Molecular Neuroscience* 14 (December): 1–16. https://doi.org/10.3389/fnmol.2021.786471.

Bounoutas, Alexander, and Martin Chalfie. 2007. “Touch Sensitivity in *Caenorhabditis Elegans*.” *Pflugers Archiv European Journal of Physiology* 454 (5): 691–702. https://doi.org/10.1007/s00424-006-0187-x.

Bozorgmehr, Tahereh, Evan L. Ardiel, Andrea H. McEwan, and Catharine H. Rankin. 2013. “Mechanisms of Plasticity in a *Caenorhabditis Elegans* Mechanosensory Circuit.” *Frontiers in Physiology* 4 AUG (August): 1–11. https://doi.org/10.3389/fphys.2013.00088.

Cabrera-vera, Theresa M, Jurgen Vanhauwe, Tarita O Thomas, Martina Medkova, Anita Preininger, Maria R Mazzoni, Heidi E Hamm, Molecular Pharmacology, and Biological Chemistry T M C. 2003. “Insights into G Protein Structure , Function , and Regulation” 24 (6): 765–81. https://doi.org/10.1210/er.2000-0026.

Chalfie, Martin, John E. Sulston, John G. White, Eileen Southgate, J. Nichol Thomson, and Sydney Brenner. 1985. “The Neural Circuit for Touch Sensitivity in *Caenorhabditis Elegans*.” *Journal of Neuroscience* 5 (4): 956–64. https://doi.org/10.1523/jneurosci.05-04-00956.1985.

Chen, Lili, Yuting Liu, Pan Su, Lili Chen, Yuting Liu, Pan Su, Wesley Hung, et al. 2022. “Escape Steering by Cholecystokinin Peptidergic Signaling.” *CellReports* 38 (6): 110330. https://doi.org/10.1016/j.celrep.2022.110330.

Chew, Yee Lian, Yoshinori Tanizawa, Yongmin Cho, Buyun Zhao, Alex J. Yu, Evan L. Ardiel, Ithai Rabinowitch, et al. 2018. “An Afferent Neuropeptide System Transmits

Mechanosensory Signals Triggering Sensitization and Arousal in *C. Elegans*." *Neuron* 99 (6): 1233-1246.e6. https://doi.org/10.1016/j.neuron.2018.08.003.

Cho, Julie Y., and Paul W. Sternberg. 2014. "Multilevel Modulation of a Sensory Motor Circuit during *C. Elegans* Sleep and Arousal." *Cell* 156 (1–2): 249–60. https://doi.org/10.1016/j.cell.2013.11.036.

Choi, Seungwon, Marios Chatzigeorgiou, Kelsey P. Taylor, William R. Schafer, and Joshua M. Kaplan. 2013. "Analysis of NPR-1 Reveals a Circuit Mechanism for Behavioral Quiescence in *C. Elegans*." *Neuron* 78 (5): 869–80. https://doi.org/10.1016/j.neuron.2013.04.002.

Cook, Steven J., Travis A. Jarrell, Christopher A. Brittin, Yi Wang, Adam E. Bloniarz, Maksim A. Yakovlev, Ken C.Q. Nguyen, et al. 2019. "Whole-Animal Connectomes of Both *Caenorhabditis Elegans* Sexes." *Nature* 571 (7763): 63–71. https://doi.org/10.1038/s41586-019-1352-7.

Corsi, Ann K, Bruce Wightman, and Martin Chalfie. 2015. "A Transparent Window into Biology : A Primer on *Caenorhabditis Elegans*," 1–31. https://doi.org/10.1895/wormbook.1.177.1.

Edwards, Stacey L., Nicole K. Charlie, Marie C. Milfort, Brandon S. Brown, Christen N. Gravlin, Jamie E. Knecht, and Kenneth G. Miller. 2008. "A Novel Molecular Solution for Ultraviolet Light Detection in *Caenorhabditis Elegans*." *PLoS Biology* 6 (8): 1715–29. https://doi.org/10.1371/journal.pbio.0060198.

Ephesius, Heraclitus. 1979. *Fragmenten* . *Fragmenten*. Baskerville serie. Amsterdam: Athenaeum-Polak en Van Gennep.

Ezcurra, Marina, Denise S. Walker, Isabel Beets, Peter Swoboda, and William R. Schafer. 2016. "Neuropeptidergic Signaling and Active Feeding State Inhibit Nociception in *Caenorhabditis Elegans*." *Journal of Neuroscience* 36 (11): 3157–69. https://doi.org/10.1523/JNEUROSCI.1128-15.2016.

Félix, Marie-anne. 2010. "The Natural History of *Caenorhabditis Elegans*." *Current Biology* 20 (22): R965–69. https://doi.org/10.1016/j.cub.2010.09.050.

Ferkey, Denise M., Piali Sengupta, and Noelle D. L'Etoile. 2021. "Chemosensory Signal Transduction in *Caenorhabditis Elegans*." *Genetics*. Genetics Society of America. https://doi.org/10.1093/GENETICS/IYAB004.

Flavell, Steven W., David M. Raizen, and Young Jai You. 2020. "Behavioral States." *Genetics*. Genetics Society of America. https://doi.org/10.1534/genetics.120.303539.

Frooninckx, Lotte, Liesbeth van Rompay, Liesbet Temmerman, Elien van Sinay, Isabel Beets, Tom Janssen, Steven J. Husson, and Liliane Schoofs. 2012. “Neuropeptide GPCRs in *C. Elegans*.” *Frontiers in Endocrinology* 3 (DEC): 1–18. https://doi.org/10.3389/fendo.2012.00167.

Fruyt, Nathan De, Alex J. Yu, Catharine H. Rankin, Isabel Beets, and Yee Lian Chew. 2020. “The Role of Neuropeptides in Learning: Insights from *C. Elegans*.” *International Journal of Biochemistry and Cell Biology*. Elsevier Ltd. https://doi.org/10.1016/j.biocel.2020.105801.

Garrison, Jennifer L., Evan Z. Macosko, Samantha Bernstein, Navin Pokala, Dirk R. Albrecht, and Cornelia I. Bargmann. 2012. “Oxytocin/Vasopressin-Related Peptides Have an Ancient Role in Reproductive Behavior.” *Science* 338 (6106): 540–43. https://doi.org/10.1126/science.1226201.

Goodman, Miriam B. 2006. “Mechanosensation.” *WormBook : The Online Review of C. Elegans Biology*, 1–14. https://doi.org/10.1895/wormbook.1.62.1.

Hall, David H. 2017. “Gap Junctions *in C. Elegans*: Their Roles in Behavior and Development.” *Developmental Neurobiology* 77 (5): 587–96. https://doi.org/10.1002/dneu.22408.

Hancock, John T. 1997. *Cell Signalling*. Book. Harlow: Longman.

Hart, Anne. 2006. “Behavior.” *WormBook*. https://doi.org/10.1895/wormbook.1.87.1.

Hobert, Oliver. 2013. “The Neuronal Genome of *Caenorhabditis Elegans*.” *WormBook : The Online Review of C. Elegans Biology*, August, 1–106. https://doi.org/10.1895/wormbook.1.161.1.

Hubbard, Catherine S., Edward Ornitz, John X. Gaspar, Suzanne Smith, Jenny Amin, Jennifer S. Labus, Lisa A. Kilpatrick, Jamie L. Rhudy, Emeran A. Mayer, and Bruce D. Naliboff. 2011. “Modulation of Nociceptive and Acoustic Startle Responses to an Unpredictable Threat in Men and Women.” *Pain* 152 (7): 1632–40. https://doi.org/10.1016/j.pain.2011.03.001.

Husson, Steven J, Inge Mertens, Tom Janssen, Marleen Lindemans, and Liliane Schoofs. 2007. “Neuropeptidergic Signaling in the Nematode *Caenorhabditis Elegans*.” *Progress in Neurobiology* 82 (1): 33–55. https://doi.org/https://doi.org/10.1016/j.pneurobio.2007.01.006.

Inutsuka, Ayumu, and Akihiro Yamanaka. 2013. “The Regulation of Sleep and Wakefulness by the Hypothalamic Neuropeptide Orexin/Hypocretin.” *Nagoya J. Med. Sci*. Vol. 75.

Jacob, Tija C, and Joshua M Kaplan. 2003. “The EGL-21 Carboxypeptidase E Facilitates Acetylcholine Release at *Caenorhabditis Elegans* Neuromuscular Junctions.” *The Journal of Neuroscience: The Official Journal of the Society for Neurosience* 23 (6): 2122–30. https://doi.org/10.1523/JNEUROSCI.23-06-02122.2003.

Javer, Avelino, Michael Currie, Chee Wai Lee, Jim Hokanson, Kezhi Li, Céline N Martineau, Eviatar Yemini, et al. 2018. “An Open Source Platform for Analyzing and Sharing Worm Behavior Data.” https://doi.org/10.5281/zenodo.1323782.

Javer, Avelino, Lidia Ripoll-Sánchez, and André E.X. Brown. 2018. “Powerful and Interpretable Behavioural Features for Quantitative Phenotyping *of Caenorhabditis Elegans*.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 373 (1758). https://doi.org/10.1098/rstb.2017.0375.

Jing, Jian, Rhanor Gillette, and Klaudiusz R. Weiss. 2009. “Evolving Concepts of Arousal: Insights from Simple Model Systems.” *Reviews in the Neurosciences* 20 (5–6): 405–27. https://doi.org/10.1515/REVNEURO.2009.20.5-6.405.

Kandel, Eric R. 1995. “The Molecular Biology of Memory Storage: A Dialogue Between Genes and Synapses.” *Proc. Natl. Acad. Sci. U.S.A*. Vol. 92. https://www.science.org.

Katsouni, Eleni, Pavlos Sakkas, Apostolos Zarros, Nikolina Skandali, and Charis Liapi. 2009. “The Involvement of Substance P in the Induction of Aggressive Behavior.” *Peptides*. https://doi.org/10.1016/j.peptides.2009.05.001.

Kung, Ching. 2005. “A Possible Unifying Principle for Mechanosensation.” *Nature* 436 (7051): 647–54. https://doi.org/10.1038/nature03896.

Latremoliere, Alban, and Clifford J. Woolf. 2009. “Central Sensitization: A Generator of Pain Hypersensitivity by Central Neural Plasticity.” *Journal of Pain*. https://doi.org/10.1016/j.jpain.2009.06.012.

Lee, Seung Hee, and Yang Dan. 2012. “Neuromodulation of Brain States.” *Neuron* 76 (1): 209–22. https://doi.org/10.1016/j.neuron.2012.09.012.

Li, Chris, and Kyuhyung Kim. 2008. “Neuropeptides.” *WormBook : The Online Review of C. Elegans Biology*, no. 212: 1–36. https://doi.org/10.1895/wormbook.1.142.1.

Li, Chris, Tiffany A. Timbers, Jacqueline K. Rose, Tahereh Bozorgmehr, Andrea McEwan, and Catharine H. Rankin. 2013. “The FMRFamide-Related Neuropeptide FLP-20 Is Required in the Mechanosensory Neurons during Memory for Massed Training in *C. Elegans*.” *Learning and Memory* 20 (2): 103–8. https://doi.org/10.1101/lm.028993.112.

Lingueglia, E, G Champigny, M Lazdunski, and P Barbry. 1995. “Cloning of the Amiloride-Sensitive FMRFamide Peptide-Gated Sodium Channel.” *Nature* 378 (6558): 730–33. https://doi.org/10.1038/378730a0.

Luo, Jintao, Zhaofa Xu, Zhiping Tan, Zhuohua Zhang, and Long Ma. 2014. “Neuropeptide Receptors Npr-1 and Npr-2 Regulate *Caenorhabditis Elegans* Avoidance Response to the Plant Stress Hormone Methyl Salicylate.” *Genetics* 199 (2): 523–31. https://doi.org/10.1534/genetics.114.172239.

Mackey, S L, D L Glanzman, S A Small, A M Dyke, E R Kandel, and R D Hawkins. 1987. “Tail Shock Produces Inhibition as Well as Sensitization of the Siphon-Withdrawal Reflex of *Aplysia*: Possible Behavioral Role for Presynaptic Inhibition Mediated by the Peptide Phe-Met-Arg-Phe-NH2 (Behavioral Inhibition/Synaptic Inhibition/Identified Neuron).” *Proc. Nati. Acad. Sci. USA*. Vol. 84. https://www.pnas.org.

Metaxakis, Athanasios, Dionysia Petratou, and Nektarios Tavernarakis. 2018. “Multimodal Sensory Processing in *Caenorhabditis Elegans*.” *Open Biology*. Royal Society Publishing. https://doi.org/10.1098/rsob.180049.

Minoli, Sebastian, Isabella Kauer, Violaine Colson, Virginie Party, Michel Renou, Peter Anderson, Christophe Gadenne, Frédéric Marion-Poll, and Sylvia Anton. 2012. “Brief Exposure to Sensory Cues Elicits Stimulus-Nonspecific General Sensitization in an Insect.” *PLoS ONE* 7 (3): 11–14. https://doi.org/10.1371/journal.pone.0034141.

Munnik, Sabrina M de, Martine J Smit, Rob Leurs, and Henry F Vischer. 2015. “Modulation of Cellular Signaling by Herpesvirus-Encoded G Protein-Coupled Receptors.” *Frontiers in Pharmacology* 6. https://www.frontiersin.org/articles/10.3389/fphar.2015.00040.

Nässel, Dick R., and Shun Fan Wu. 2022. “Cholecystokinin/Sulfakinin Peptide Signaling: Conserved Roles at the Intersection between Feeding, Mating and Aggression.” *Cellular and Molecular Life Sciences*. Springer Science and Business Media Deutschland GmbH. https://doi.org/10.1007/s00018-022-04214-4.

Nath, Ravi D., Elly S. Chow, Han Wang, Erich M. Schwarz, and Paul W. Sternberg. 2016. “*C. Elegans* Stress-Induced Sleep Emerges from the Collective Action of Multiple Neuropeptides.” *Current Biology* 26 (18): 2446–55. https://doi.org/10.1016/j.cub.2016.07.048.

Nelson, M D, N F Trojanowski, J B George-Raizen, C J Smith, C-C Yu, C Fang-Yen, and D M Raizen. 2013. “The Neuropeptide NLP-22 Regulates a Sleep-like State in *Caenorhabditis Elegans*.” *Nature Communications* 4: 2846. https://doi.org/10.1038/ncomms3846.

Pfaff, Donald, Ana Ribeiro, James Matthews, and Lee Ming Kow. 2008. “Concepts and Mechanisms of Generalized Central Nervous System Arousal.” *Annals of the New York Academy of Sciences* 1129: 11–25. https://doi.org/10.1196/annals.1417.019.

Pfaff, Donald W. 2015. “1. Toward a Universal Theory of Brain Arousal.” *Brain Arousal and Information Theory*, 1–25. https://doi.org/10.4159/9780674042100-001.

Pražienková, Veronika, Andrea Popelová, Jaroslav Kuneš, and Lenka Maletínská. 2019. “Prolactin-Releasing Peptide: Physiological and Pharmacological Properties.” *International Journal of Molecular Sciences*. MDPI AG. https://doi.org/10.3390/ijms20215297.

Rabinowitch, Ithai, Millet Treinin, and Jihong Bai. 2016. “Artificial Optogenetic TRN Stimulation of *C. Elegans*.” *Bio-protecol* 6 (20). https://doi.org/10.21769/bioprotoc.1966.

Ramachandran, Shankar, Navonil Banerjee, Raja Bhattacharya, Michele L. Lemons, Jeremy Florman, Christopher M. Lambert, Denis Touroutine, et al. 2021. “A Conserved Neuropeptide System Links Head and Body Motor Circuits to Enable Adaptive Behavior.” *ELife* 10: 1–28. https://doi.org/10.7554/eLife.71747.

Reilly, Douglas K, Emily J McGlame, Elke Vandewyer, Annalise N Robidoux, Caroline S Muirhead, Haylea T Northcott, William Joyce, et al. 2021. “Distinct Neuropeptide-Receptor Modules Regulate a Sex-Specific Behavioral Response to a Pheromone.” *Communications Biology* 4 (1): 1018. https://doi.org/10.1038/s42003-021-02547-7.

Richmond, Janet. 2007. “Synaptic Function * Table of Contents,” 1–15. https://doi.org/10.1895/wormbook.1.69.1.

Ripoll Sánchez, Lidia, Jan Watteyne, Haosheng Sun, Robert Fernandez, Seth Taylor, Alexis Weinreb, Marc Hammarlund, et al. 2022. *The Neuropeptidergic Connectome of C. Elegans*. https://doi.org/10.1101/2022.10.30.514396.

Rose, J. K., and C. H. Rankin. 2001. “Analyses of Habituation in *Caenorhabditis Elegans*.” *Learning and Memory* 8 (2): 63–69. https://doi.org/10.1101/lm.37801.

Schafer, William F. 2006. “Genetics of Egg-Laying in Worms.” *Annual Review of Genetics*. https://doi.org/10.1146/annurev.genet.40.110405.090527.

Sengupta, Piali. 2007. “Generation and Modulation of Chemosensory Behaviors in *C. Elegans*.” *Pflugers Archiv European Journal of Physiology* 454 (5): 721–34. https://doi.org/10.1007/s00424-006-0196-9.

Stiernagle, Theresa. 2006. “Maintenance of *C. Elegans*.” *WormBook : The Online Review of C. Elegans Biology*. https://doi.org/10.1895/wormbook.1.101.1.

Taylor, Seth R., Gabriel Santpere, Alexis Weinreb, Alec Barrett, Molly B. Reilly, Chuan Xu, Erdem Varol, et al. 2021. “Molecular Topography of an Entire Nervous System.” *Cell* 184 (16): 4329-4347.e23. https://doi.org/10.1016/j.cell.2021.06.023.

Wang, Han, Heenam Park, Jonathan Liu, and Paul W. Sternberg. 2018. “An Efficient Genome Editing Strategy to Generate Putative Null Mutants in *Caenorhabditis Elegans* Using CRISPR/Cas9.” *G3: Genes, Genomes, Genetics* 8 (11): 3607–16. https://doi.org/10.1534/g3.118.200662.

White, J G, E Southgate, J N Thomson, and S Brenner. 1986. “The Structure of the Nervous System of the Nematode *Caenorhabditis Elegans*.” *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 314 (1165): 1–340. https://doi.org/10.1098/rstb.1986.0056.

Woods, Ian G., David Schoppik, Veronica J. Shi, Steven Zimmerman, Haley A. Coleman, Joel Greenwood, Edward R. Soucy, and Alexander F. Schier. 2014. “Neuropeptidergic Signaling Partitions Arousal Behaviors in Zebrafish.” *Journal of Neuroscience* 34 (9): 3142–60. https://doi.org/10.1523/JNEUROSCI.3529-13.2014.

Yokogawa, Tohei, Markus C. Hannan, and Harold A. Burgess. 2012. “The Dorsal Raphe Modulates Sensory Responsiveness during Arousal in Zebrafish.” *Journal of Neuroscience* 32 (43): 15205–15. https://doi.org/10.1523/JNEUROSCI.1019-12.2012.

Appendix

1. Risk assessment

The entire lab is safety level 1 since we solely work with non-pathogenic organisms namely, *C. elegans* and *E. coli OP50*. It is required by lab protocol to wear gloves and a lab coat. For safety measures, lab coats are not allowed in places intended for eating and conversely, there should be no food or drinks in the lab area. Before leaving the lab hands are disinfected.

In the lab there are some general rules. All volatile materials are handled in the laminar flow cabinet. Everything entering the laminar flow cabinet should be disinfected with 70% ethanol. The autoclave can be used under supervision for sterilization of both liquids as solids. Furthermore, is it important to sort your waste depending on the type into the right containers.

When using following products, handle with extra care:

Acetic acid (CH3COOH) is a highly flammable substance and can cause skin burns and eye damage. Protective gear should be worm.

Calcium chloride (CaCl2) can cause severe eye irritation it is recommended to wear eye protection.

Disodium Ethylenediaminetetraacetic acid (EDTA) is harmful when inhaled, repeated exposure may cause organ damage. Restrict use to within the flow cabinet.

Ethanol (EtOH) is a highly flammable substance and can cause serious eye irritation.

GelRed The long-term dangers of GelRed are not yet clear, therefor is the use of GelRed only allowed in the designated area while wearing a different lab coat with a red collar. Everything that enters this room must remain there or be tossed.

Hydrochloric acid (HCl) can cause serious eye damage and skin burns. Additionally, it may also be corrosive.

Ultraviolet light (UV) can cause severe skin and eye damage, use the Proxima imager with care.

2. Buffers and solutions

2xTY Broth (200mL)

Weigh 3.2g Bactotryptone (Gibco), 2.0g yeast extract (Sigma-Aldrich) and 1.0g NaCl (Sigma-Aldrich) in a Duran bottle. Next, add 200mL AD water, homogenise by swirling and put in the autoclave.

50x TAE Buffer (1L)

Weigh 242g Trizma base (Sigma-Aldrich), 18.61g EDTA (Sigma-Aldrich), 700mL AD, use stirrer to dissolve, add 57.1mL acetic acid (Sigma-Aldrich), homogenize and add 1L AD water.

ATR

Dilute ATR (R2500 Sigma-Aldrich) in ethanol (VWR Prolabo Chemicals) to 100 mM in a 1.7 ml tube. Next, wrap the vial with aluminium foil to avoid light exposure and store at -20 °C.

Bleach solution (1mL)

Mix 500µL 1M NaOH with 500µL house hold bleach (5% sodium hypochlorite)

$CaCl_2$ 1M (200mL)

Weigh 29.4g $CaCl_2$ (Sigma-Aldrich) in a Duran bottle, add 200mL Milli Q, swirl to homogenise and autoclave.

Cholesterol 5mg/ml (200mL)

Weigh 1.0g cholesterol (Sigma-Aldrich), fill with 200mL 100% ethanol (VWR Prolabo Chemicals) and homogenise.

Freezing solution (100mL)

Weigh 0.585g NaCl (Sigma-Aldrich), 0.592g KH_2PO_4 (Sigma-Aldrich) and 0.112g K_2HPO_4 (VWR Prolabo Chemicals). After adding 30mL glycerol (Sigma-Aldrich), fill up with Milli Q until 100mL. Afterwards homogenise and autoclave.

$MgSO_4$ 1M (200mL)

Weigh 49.3g $MgSO_4$ (Supelco®) in a Duran bottle, add 200mL Milli Q water, homogenise and autoclave.

PCR worm lysis buffer (100mL)

Measure following ingredients 1mL 1M Tris/HCL ph 8.3, 5mL KCl, 0.25 mL MgCl2, 4.5mL 10% NP-40, 4.5mL 10% Tween 20 and 1mL 1% gelatin. Next, add Milli Q until a final volume of 100mL. To facilitate use, aliquot 100µL in PCR tubes and store at -20°C.

Phosphate buffer 1M (1L)

Weigh 108.87g KH_2PO_4 (Sigma-Aldrich) and 34.84g K_2HPO_4 (VWR Prolabo Chemicals), in Duran bottle and add 1L Milli Q, homogenise and autoclave.

Proteinkinase K (1mL)

Weigh 1mg of proteinkinase K form *Tritirachium album* and dissolve in 1mL AD. Next, aliquot 5µL in PCR tubes and store at -20°C.

S-basal (200mL)

Weigh 1.17g NaCl (Sigma-Aldrich), 1.2g KH_2PO_4 (Sigma-Aldrich) and 0.2g K_2HPO_4 (VWR Prolabo Chemicals), fill with 200mL MQ, homogenise and autoclave.

AFDELING
Straat nr bus 0000
3000 LEUVEN, BELGIË
tel. + 32 16 00 00 00
fax + 32 16 00 00 00
www.kuleuven.be