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University of Southampton

Faculty of Environmental and Life sciences

School of Biological Sciences

**Functional Investigation of Molecular Determinants of Nociception in
*Octopus vulgaris***

DOI: <https://doi.org/10.5258/SOTON/PG/T086>

by

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Thesis for the degree of Doctor of Philosophy

May 2026

University of Southampton

Abstract

Faculty of Environmental and Life Sciences

School of Biological Sciences

Doctor of Philosophy

Functional Investigation of Molecular Determinants of Nociception in *Octopus vulgaris*

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Eleonora Maria Pieroni

Cephalopods are characterised by an exceptional neuroanatomy, formed of a central brain mass and an extended arm sensory system which contributed to the evolution of complex behaviours that might underly brain states comparable to vertebrates, including pain. This possibility is a major reason behind their inclusion in the UK and EU legislations regulating animal welfare in research. Despite irrefutable evidence of nociception in cephalopods, the occurrence of pain in these animals requires further studies.

This thesis investigated putative genes involved in *Octopus vulgaris* nociception. To overcome the current limited direct genetic tractability in this species, I designed my work around a model hopping approach and used *in vivo* and *in vitro* approaches to characterise gene function.

In silico analysis identified *O. vulgaris* orthologues of well-characterised vertebrate and invertebrate genes involved in nociception based on the rationale that the strong adaptive value of nociception led to the evolution of conserved molecules across metazoans. I prioritised genes that showed orthologues in *Caenorhabditis elegans*. This allowed me to probe mutant strains for deficiency in avoidance behaviours triggered by low pH. This verified 19 putative nociceptive-related genes from octopus with a discernible nociception defect in *C. elegans*.

The validity of this approach was reinforced by complementation experiments using TRPV receptors, a family of highly conserved ion channels directly involved in sensory detection of noxious stimuli. Two TRPV sequences, *Ovtrpv1* and *Ovtrpv2*, were identified in *O. vulgaris* genome and exhibited a tissue distribution consistent with a selective role in nociception. Heterologous expression in *C. elegans* and successful rescue of loss of function mutants for the orthologous genes (*ocr-2* and *osm-9* respectively), as well as complementary work carried out in the *Xenopus laevis* oocyte expression system, confirmed a polymodal function of *Ovtrpv1* and *Ovtrpv2*.

Finally, I searched for potential candidates of an antinociceptive system in *O. vulgaris*. As the approach described above found no evidence for an opioid peptide system, I used an evolutionary strategy to define the closest protostome orthologue of the opioid system that evolved to modulate pain, namely allatostatin C. *In silico* and experimental evidence revealed the presence of two receptors, *OvAstCR1* and *OvAstCR2*. These were successfully de-orphanised by the identified putative endogenous peptide *OvAstC* when investigated for functional signalling in HEK293G5A recombinant system. The tissue distribution of the allatostatin C system implies broad functional outcomes in *O. vulgaris*.

Overall, I identified conserved genes that could concur to trigger and modulate *O. vulgaris* nociceptive responses, paving the way for functional studies aimed at investigating pain-like responses in cephalopods, ultimately increasing the level of welfare attention these molluscs deserve in scientific, cultural and economic contexts.

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List of Accompanying Materials

Supplementary Tables S1-S3 from “Chapter 2: Identification of molecular nociceptors in *Octopus vulgaris* through functional characterisation in *Caenorhabditis elegans*” are electronic files accessible from the journal: <https://doi.org/10.1242/bio.062268>

Supplementary Tables S1-S4 and Supplementary Files S1-S6 from “Chapter 3: Identification and functional investigation of *Octopus vulgaris* TRPV channels: molecular determinants of nociception in cephalopods?” are electronic files accessible from the following link:

[10.5281/zenodo.18377710](https://doi.org/10.5281/zenodo.18377710)

Supplementary Tables S1-S3 and Supplementary Files S1-S6 from “Chapter 4: Tracing the evolutionary history of allatostatin C signalling through GPCR deorphanisation in *Octopus vulgaris*” are electronic files accessible from the following link:

<https://zenodo.org/records/19974511>

Research Thesis: Declaration of Authorship

Print name: Eleonora Maria Pieroni

Title of thesis: Functional Investigation of Molecular Determinants of Nociception in *Octopus vulgaris*

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published as:
 - **Chapter 2: Pieroni, E. M., O'Connor, V., Holden-Dye, L., Imperadore, P., Fiorito, G. and Dillon, J.** Identification of molecular nociceptors in *Octopus vulgaris* through functional characterisation in *Caenorhabditis elegans*. *Biology Open*, 2026: bio.062268. DOI: [10.1242/bio.062268](https://doi.org/10.1242/bio.062268).
 - **Chapter 3: Pieroni E.M., Baylis H., O'Connor V., Holden-Dye L., Yañez-Guerra L.A., Imperadore P., Fiorito G., Dillon J.** Identification and functional investigation of *Octopus vulgaris* TRPV channels as potential nociceptors in cephalopods. *bioRxiv*, 2026 DOI: <https://doi.org/10.64898/2026.03.27.714695>
 - **Chapter 4: Pieroni E.M., Dillon J, O'Connor V., Holden-Dye L., Imperadore P., Fiorito G., Yanez-Guerra L.A.** *In silico* identification and deorphanisation of an allatostatin C GPCR system in the cephalopod *Octopus vulgaris* reveal two receptors with distinct potency. *bioRxiv*, 2026 DOI: <https://doi.org/10.64898/2026.02.18.706622>

- **Appendix A: Lamb J., Pieroni E.M., Al Khawaja F., Deinhardt K., O'Connor V., Dillon J.** Sensory and developmental phenotyping of *C. elegans* parses autism associated genes into behavioural classifications. *bioRxiv*, 2026 DOI: <https://doi.org/10.64898/2026.03.27.714775>
- **Appendix C: Guan L., Reynoldson T.E., Pieroni E.M., Dillon J., Hardege I., Yanez-Guerra L.A.** Development of pXeDNA3.1-LacZ, a Dual-System Plasmid for Membrane Protein Expression in *Xenopus* and Mammalian Cells. *microPublication Biology*, 2025. DOI: [10.17912/micropub.biology.001866](https://doi.org/10.17912/micropub.biology.001866)

Signature: Date:.....

Acknowledgements

I never thought the moment I would have handed my PhD thesis would have ever come. And yet here we are, as this felt as an endless but nonetheless fruitful and rewarding journey.

The first thank you goes obviously to my supervisory team, especially to Dr James Dillon, Professor Vincent O'Connor and Professor Lindy Holden-Dye for being not just my scientific mentors, but true wonderful human beings. You were there to support me throughout my best and, most importantly, my worst moments of this project. I am very grateful to have met you and I hope I can meet more researchers that value science but at the same time are fair, kind and generous as you are.

I would like to thank my present and past lab mates (most of them already doctors): Lucy, Henry, James, Liliana, Jo, Kirsten, Yogendra and Fernando. And I am grateful of having met some sincere souls like Kam and Zahra, energetic and determined ones like Beatriz, and light-hearted ones like Olimi.

A special thank you goes to Dr Luis Alfonso Yañez-Guerra, for being an extended mentor of mine. Passion and curiosity for science are something we have in common, and confidence something we don't, and I envy.

From my Italian side, thank you to my lab partners Federica, Marianna, Clara, Erica and Valerio. A warm thank you to my friend Marco. We started as passionate master students and are now good friends. Don't give up, keep writing your thesis, you are almost there too!

Finally, my family. To Papà Massimo, Mamma Grazia, Marco and Federica. There are no words available to describe the love and gratitude I feel and I hope this work whom you have contributed to with all your sacrifices and endless support, would be partial evidence of it. I would also like to dedicate this work to my sweet nephew Massimo, whose smiles and cuteness have made these last few months more bearable. And to my grandparents and my lovely cats Lanù and Tigro, always missed and loved.

Finally, I would like to thank my Happiness, Bobolino, for always being there for me through my highs and lows, failures and successes. Love you.

Definitions and Abbreviations

2-AG	2-Arachidonoylglycerol
ACE	Angiotensin-converting enzyme
AEA	Anandamide
AchR	Acetylcholine Receptor
AI	Artificial Intelligence
ANO	Anoctamin
ANOVA	Analysis of variance
ASC	Amiloride Sensitive ion Channel
ASD	Autism Spectrum Disorder
ASG	Anterior Salivary Gland
ASIC	Acid Sensing Ion Channel
ASPA	Animal (Scientific Procedures) Act
AstA	Allatostatin A
AstAR	Allatostatin A Receptor
AstC	Allatostatin C
AstCR	Allatostatin C Receptor
ATP	Adenosine triphosphate
BGH	Bovine Growth Hormone
BLAST	Basic Local Alignment Search Tool
BLOSUM	BLOcks SUBstitution Matrix
bp	base pair
BrH	Branchial Heart
BSA	Bovine serum albumin
BUSCO	Benchmarking Universal Single-Copy Orthologs
°C	Degrees Celsius
C-terminal	Carboxy terminal
CACNA	Calcium voltage-gated ion channel
cAMP	cyclic Adenosine Monophosphate
CB	Cannabinoid
CBR	Cannabinoid Receptor
cDNA	complementary DNA
CGC	Caenorhabditis Genetics Centre
cGMP	cyclic Guanosine Monophosphate

CGRP	Calcitonin Gene-Related Peptide
CHO	Chinese Hamster Ovary
CLANS.....	CLuster ANalysis of Sequences
CNG	Cyclic Nucleotide Gated ion channel
CNV	Copy Number Variant
CPE	Carboxypeptidase
CTR	Chemotactile Receptor
CRB.....	Chemotactile Receptor Bitter substances
CRISPR.....	Clustered Regularly Interspaced Short Palindromic Repeats
CRT	Chemotactile Receptor Terpenes
DAGL.....	Diacylglycerol lipase
DD.....	Developmental Disorder
ddH ₂ O	Double distilled water
DEG/ENaC.....	Degenerin/Epithelial Sodium Channel
DG.....	Digestive Gland
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dnLof.....	<i>de novo</i> loss of function
DRG	Dorsal Root Ganglia
DSM	Diagnostic and Statistical Manual of Mental Disorders
EC50	Half maximal Effective Concentration
ECL	Extracellular Loop
EEG	Electroencephalogram
EU	European Union
FAAH	Fatty-Acid Amide Hydrolase
FABP	Fatty Acid-Binding Protein
FaNaC	FMRFamide-gated Sodium Channel
FMRFa	FMRFamide
FMRFaR.....	FMRFamide Receptor
FRET.....	Fluorescence (Förster) Resonance Energy Transfer
GALR	Galanin Receptor
GFP	Green Fluorescent Protein
GG	Gastric Ganglion
GnRH	Gonadotropin-Releasing Hormone

GO	Gene Ontology
GPCR	G-Protein Coupled Receptor
GRK.....	G protein-coupled receptor kinase
GWAS.....	Genome Wide Association Studies
HAM	Hypothetical Ancestral Mollusc
Hbss.....	Hanks' Balanced Salt Solution
Hc	Haemocytes
HCN	Hyperpolarisation-activated cyclic nucleotide-gated channel
HEK	Human Embryonic Kidney
HMM	Hidden Markov Model
HPLC	High performance liquid chromatography
IASP	International Association for the Study of Pain
IHC.....	Immunohistochemistry
Int	Intestine
ISH	<i>In situ</i> hybridisation
KCN.....	Potassium voltage-gated channel
Kid.....	Kidney
Kiss1R	Kiss peptide 1 Receptor
L1	Larval stage one
L2.....	Larval stage two
L3.....	Larval stage three
L4.....	Larval stage four
L4+1	Larval stage four worms plus one day old
LB.....	Luria Bertani (Broth/ Agar)
M.....	Molar
MAFFT	Multiple Alignment using Fast Fourier Transform
Man.....	Mantle
MCR	Melanocortin receptor
MCS	Multiple Cloning Site
min.....	minutes
MiRPs	KCNE/MinK-related peptides
mm	millimetre
N-terminal.....	Ammino-terminal
NA.....	Noradrenaline

NALCN	Sodium Leak Channel
NAM	Nicotinamide
NAPE-PLD	N-acyl-phosphatidylethanolamine-hydrolysing phospholipase D
NBRP.....	National Bioresource Project
NCBI	National Center for Biotechnology Information
NEP/MME	Neprilysin
ng.....	nanogram
NGM.....	Nematode growth medium
OCR	<i>Osm-9</i> /Capsaicin Receptor related
OL	Optic Lobe
OGFR	Opioid Growth Factor Receptor
OPR.....	Opioid Receptor
OSM-9	OSMotic avoidance abnormal-9
PABPC.....	Cytoplasmic poly(A)-binding protein
PCR.....	Polymerase Chain Reaction
PCSK.....	Neuroendocrine convertase
PEI.....	Polyethylenimine
PIEZO	PIEZO-type mechanosensitive ion channel component
PSDLH.....	Pain Suffering Distress and Lasting Harm
PSG	Posterior Salivary Gland
PUFA	Poly Unsaturated Fatty Acid
qPCR.....	Quantitative Polymerase Chain Reaction
RLU	Relative Luminescence Unit
RNA.....	Ribonucleic Acid
RT-PCR.....	Real Time Polymerase Chain Reaction
RTT	Rett's syndrome
s.....	seconds
SCNA	Sodium voltage-gated ion channel
SEM.....	Supra oesophageal Mass
s.e.m.....	Standard error of the mean
SFARI	Simons Foundation Autism Research Initiative
SNP	Single Nucleotide Polymorphism
SOC	Super Optimal broth with Catabolite repression
SSR	Somatostatin Receptor

StG	Stellate Ganglion
STOM	Stomatin
Stom	Stomach
Su	Sucker
SUB	Sub oesophageal Mass
TEVC	Two-electrode voltage clamp
TG	Trigeminal Ganglia
TK.....	Tachykinin
TKR.....	Tachykinin Receptor
TIP.....	Tip of the arm (octopus)
TM	Transmembrane elementa
TMC.....	Transmembrane Channel
TMEM	Transmembrane protein
TMHMM.....	TransMembrane Helices Hidden Markov Model
TRP.....	Transient Receptor Potential
TRPA.....	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM.....	Transient Receptor Potential Melastatin
TRPML	Transient Receptor Potential Mucolipin
TRPN	Transient Receptor Potential NompC
TRPP.....	Transient Receptor Potential Polycystic
TRPV.....	Transient Receptor Potential Vanilloid
UK	United Kingdom
UTR	Untranslated region
UV	Ultraviolet
µg.....	Microgram
µl.....	Microlitre
µm	Micrometer
µM.....	Micromolar
VEP	Variant Effect Predictor
WB	White Body
WES	Whole Exome Sequencing
WPS	Whole Plasmid Sequencing
WT	Wild Type

Chapter 1 General Introduction

1.1 Animal welfare in research: state of the art and legislations

Humans have been testing on live animals since ancient times (Greek and Greek, 2000). The value in addressing biological questions using animals has been increasingly pursued by scholars, initially with no concern at all for their well-being and without any legislations regulating their use. The increasing invasiveness and absence of boundaries permeating research in favour of remarkable scientific progress, brought criticisms and ethical debates to become accentuated, setting the stage for more active interventions which led to the restriction and controlled use of animals in research that are in place today (Festing and Wilkinson, 2007). Despite the enormous advances in developing robust *in silico* and *in vitro* techniques for clinical and exploratory studies (NC3Rs, 2025), research still heavily relies on animal model organisms (University of Cambridge, 2024) but it now needs to comply with the enforced pertinent legislations to guarantee animal welfare and rigorous scientific conclusions.

1.1.1 The question is: can they suffer? Pain as an indicator of well-being in animals

In 1789, Jeremy Bentham reflected on how non-human animals have been “neglected by the insensibility of the ancient jurists” and have been degraded into “the class of things” for our own convenience (Bentham, 1789). He criticised the arguments utilised to justify the lack of consideration when using animals, mostly based on the absence of evidence for higher cognition. Bentham concluded his thoughts with the famous sentence:

“The question is not, Can they reason? nor, Can they talk? but, Can they suffer?”

from *‘The Principles of Morals and Legislation’* (Bentham, 1789)

For the first time it was conceived that consideration for animal welfare should reside in the ability to recognise states of discomfort that imply pain in animals.

The most comprehensive legislation that was conceived to put an end to the “unnecessary suffering” to which animals were subjected to was made by the United Kingdom in 1986 with the Animals (Scientific Procedures) Act (ASPA) (1986). This legislation represented a step forward

form the previous Cruelty to Animals Act (1876) and Protection of Animals Act (1911), which enforced a licensing and inspection system for vivisection. The ASPA introduced the requirement for personal, project and establishment licences before being able to operate in a way to avoid or minimise the infliction of pain, suffering, distress and lasting harm (PSDLH) in experimental animals. Furthermore, it pioneered the introduction of the concept of three Rs in animal research (Russell and Burch, 1959). These principles:

- 1) replacement, if possible animal research should be replaced by *in vitro* studies or by employing organisms considered not to suffer;
- 2) reduction, in case animals should be employed, the number of those utilised should be minimised to the least necessary to be able to infer a statistically valid conclusion;
- 3) refinement, in case animals should be employed, the experiment should be designed in a way to reduce the possibility of inflicting pain;

represent nowadays the cornerstone of animal research worldwide.

The ASPA document set the stage for the generation of the European Union Directive 2010/63/EU (European Parliament and Union, 2010). Both the UK and EU original legislations were referring to vertebrates as these were the only ones considered complex enough to be sentient and therefore exhibit PSDLH.

1.1.2 The case of octopus: cephalopods in animal welfare legislations

In 1993, the ASPA legislation was amended to include “any invertebrate of the species *Octopus vulgaris* from the stage of its development when it becomes capable of independent feeding” (UK S.I. 1993/2103, 1993).

This was based on the increasing knowledge of this animal’s complexity in terms of neuroanatomy, learning and memory capabilities (Wells and Wells, 1957; Wells, 1959; Wells, 1964; Young, 1964; Young, 1965; Young, 1971; Young, 1983; Budelmann and Young, 1985; Young, 1991).

From the 1st of January 2013, however, the accumulating evidence of a centralised brain and the display of complex behaviours in at least all coleoid species, have been considered sufficient to conclude that cephalopods can elaborate pain-like states, leading to their inclusion as the first

(and currently only) invertebrate taxon in the UK and EU legislations regulating research animal welfare (European Parliament and Union, 2010; UK S.I. 2012/3039, 2013).

This revolutionary step was ultimately based on the precautionary principle (Bradshaw, 1998) rather than on comprehensive conclusive evidence of cephalopods ability to feel pain.

Currently, the question of pain in invertebrates still represents an open debate, as it first requires evidence of the animal's self-awareness and primary consciousness as underlying signatures of sentience. However, the limited methodologies to measure these and the bias in forcing our human and mammalian experience onto very different organisms, makes the whole process constraint and occasionally flowing into the realm of philosophy. With the precautionary principle a "better safe than sorry" approach is suggested when it comes to define consciousness in non-verbal animals. It is argued that the lack of scientific evidence, especially where a limitation of measurement exists, should not be the justification to apply a parsimony approach and conclude animals are not conscious and therefore do not merit welfare attention. Instead, respect and care should be adopted (Bradshaw, 1999).

This introduction will provide key notions that will help frame the importance of asking the question of pain in cephalopods and more in general in invertebrates. In particular, in terms of claiming pain in animals, the classical criteria set out by Bateson (1991) and their revision in 2021 (Birch *et al.*, 2021) will be extremely relevant in framing the aims of this thesis as these require animals not only to show reflexive avoidance response (nociception), but also to possess cellular and molecular pathways that could give rise to an active state of discomfort (pain) when connected to a central brain.

This thesis is structured around the overarching principle that investigation of physiological and molecular aspect of nociception, as core conserved processes in animals, will lead to a better understanding of the basis of this phenomenon, and will provide more criteria to define the higher processing of pain. In particular, the importance of studying molecular determinants of nociception in *O. vulgaris* will be emphasised given their transition status with respect to legislative and societal implications.

1.2 Nociception as a universal adaptive feature of Eumetazoa

Animals are constantly exposed to disparate stimuli coming from the interoceptive and exteroceptive environment. These are conveyed through different modalities as visual, auditory, tactile and chemical cues that once detected, are elaborated to coordinate an appropriate, evolutionary advantageous response (Julius and Nathans, 2012).

Despite the critical role in increasing the Darwinian fitness represented by finding food and mating partners, cues with a negative valence represent equally essential stimuli for the animal survival, triggering a specific reaction known as nociception.

Nociception is the ability of an animal to detect potentially dangerous stimuli in the environment and organise a reflexive or higher avoidance response to avoid any tissue damage or long-term negative effects. Given its strong adaptive value, nociception represents a feature universally present across all Metazoa, characterised by shared cellular and molecular adaptations that guarantee recognition of different modalities of noxious stimuli (Julius and Basbaum, 2001; Basbaum *et al.*, 2009).

1.2.1 Evolution of nociceptors from single-cells organisms to specialised neurons

In Bacteria it is possible to identify chemosensory receptors able to trigger movements towards or away from the source of the stimulus, according to its nature (Vaknin and Berg, 2007) or force-detecting receptors displaying mechanosensitive properties (Booth, 2014). In these organisms the detection relies on different molecules expressed at the single cell level.

The emergence of proper cellular sensory mechanisms is proposed to have happened more than 550 million years ago, predating the division between protostomes and deuterostomes (Smith and Lewin, 2009). Natural selection drove the original cellular proto receptors (e.g., ocelli and statocysts found in Cnidarians) to evolve into structures that are more complex in function and organisation. This is reflected in the evolution of nociceptors, sensory cells specialised in the detection of potentially noxious stimuli (Smith and Lewin, 2009).

1.2.1.1 Characteristics of a cellular nociceptor

Nociceptors as neuronal entities were first discovered in vertebrates when Charles Scott Sherrington observed that, when exposed to a harmful stimulus, decerebrated dogs and cats preparations showed rapid withdrawal of the limb with a sustained retracted position (Sherrington, 1906). This identified the existence of cellular structures able to specifically detect aversive stimuli. These entities were defined as nociceptors, from the Latin '*nocere*' meaning 'to harm or hurt' (Sherrington, 1906).

In mammals, nociceptors' dendrites are characterised by free nerve endings, able to detect interoceptive and exteroceptive inputs from the body and the face by relying on cell bodies distributed in the dorsal root ganglia (DRG) and trigeminal ganglia (TG) respectively (Basbaum *et al.*, 2009). In terms of their physiological activity, when compared to other sensory neurons such as photoreceptors or mechanoreceptors, nociceptors are characterised by a coupling between high threshold of activation and sustained response to avoid tissue damage (Julius and Basbaum, 2001). This is underpinned by the neuronal activity that drives the nociceptive response. A unique feature is that the nociceptive action potential is characterised by a slow adaptation and a peculiar repolarisation hump (Figure 1.1) which appears to be a conserved feature also in invertebrates (Villiere and McLachlan, 1996; Lechner *et al.*, 2009).

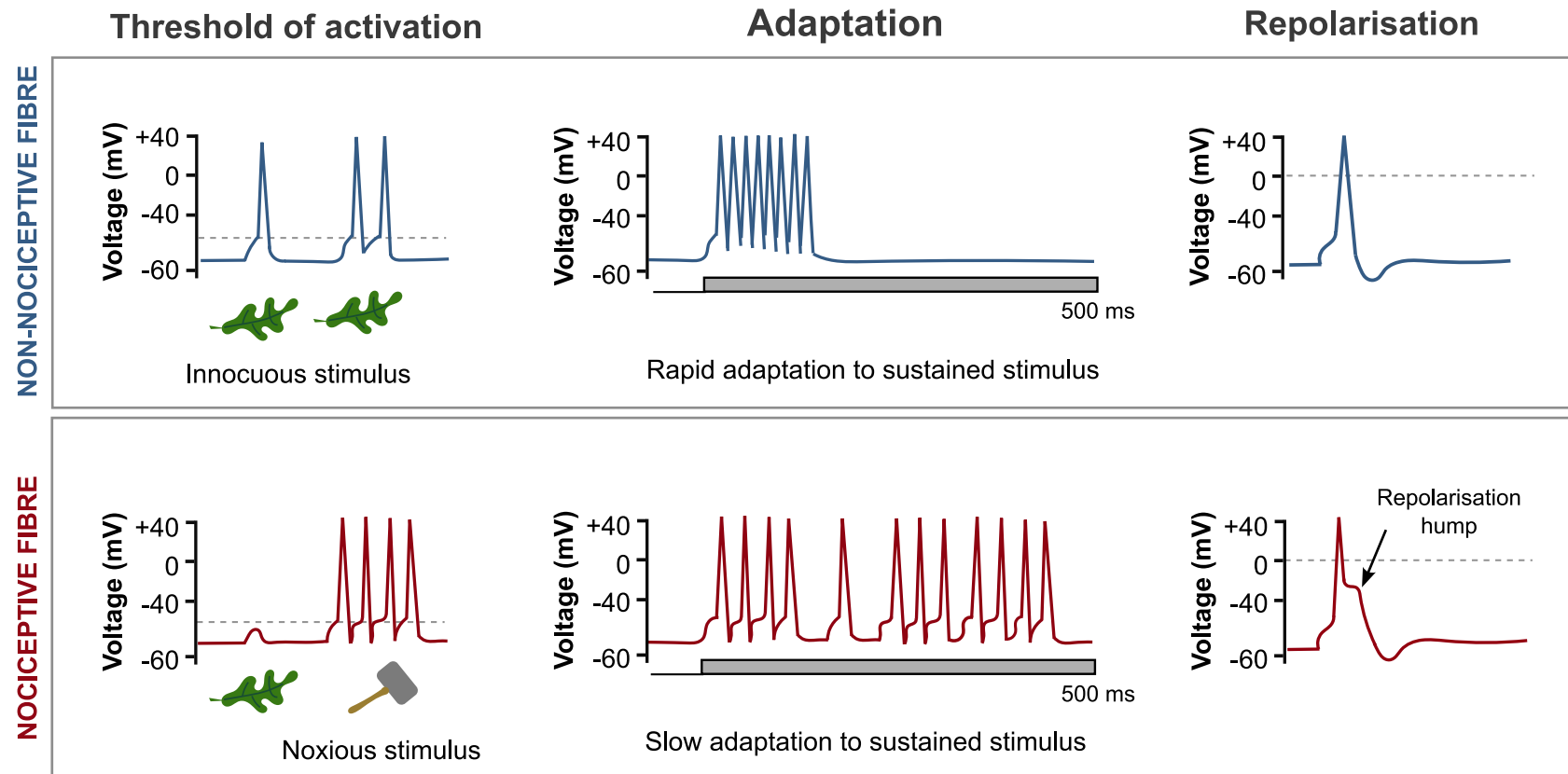


Figure 1.1 Characteristics of a nociceptor.

Cellular nociceptors and therefore nociceptive fibres have three peculiar differences with non-nociceptive fibres (e.g., non-noxious mechanosensitive fibres): 1) The threshold of activation is higher; 2) When exposed to sustained stimuli, nociceptors have slow adaptation; 3) When repolarisation starts to take place, nociceptors are characterised by an action potential with a “hump” that precedes the hyperpolarisation phase.

1.2.1.2 Invertebrate cellular nociceptors

Sensory neurons embedded in interconnected pathways that underpin nociceptive responses exist and are experimentally tractable in invertebrates.

Cellular nociceptors are found in the first bilaterian nervous systems (Figure 1.2). Classically, annelids demonstrate this principle through a body segment organisation that is endowed with a pair of ganglia with symmetrically organised sensory neurons (Bleidorn *et al.*, 2015). The leech *Hirudo medicinalis* for instance, shows three major sets of sensory cells, namely T (touch), P (pressure) and N (noxious) cells (Nicholls and Baylor, 1968). The latter ones show morphology and physiology akin to the mammalian-like nociceptors described above (Figure 1.1), in that they present high threshold of activation and the repolarisation signatures that are selectively found in this type of neuron (Schlue, 1976; Pastor, Soria and Belmonte, 1996).

The gastropod mollusc *Aplysia californica*, another classical invertebrate model organism, has nociceptive fibres characterised by nerve endings encapsulated in external body muscles. These contribute to the high threshold of activation in the mechanosensory abdominal, or pleural neurons as opposed to the buccal sensory cells (Steffensen and Morris, 1996; Walters *et al.*, 2004).

The organisation of sensory neurons of well-established model systems such as *Drosophila melanogaster* and *Caenorhabditis elegans*, has been extensively studied and experimentally manipulated to investigate different properties of animal cellular neurobiology.

Sensory neurons in *D. melanogaster* are very complex and mainly divided in four classes (I-IV) based on the functional extension of the dendrites underneath the epidermis and the cuticle (Grueber, Jan and Jan, 2002). Among these, nociceptors, belonging to class IV, are polymodal cellular receptors as they are triggered by heat, mechanical and chemical stimuli (Grueber *et al.*, 2003).

In *C. elegans*, specialised sensory neurons are distributed throughout the body with different morphology and gene expression according to their function as they convey chemical mechanical and thermal responses (Inglis *et al.*, 2007). The neurosensory system of *C. elegans* will be later discussed in more detail (see section 1.5.2).

Altogether, the data show that the cellular bases of sensory process established in mammalian systems are quite conserved, as reinforced by these historically important invertebrate model organisms (Figure 1.2).

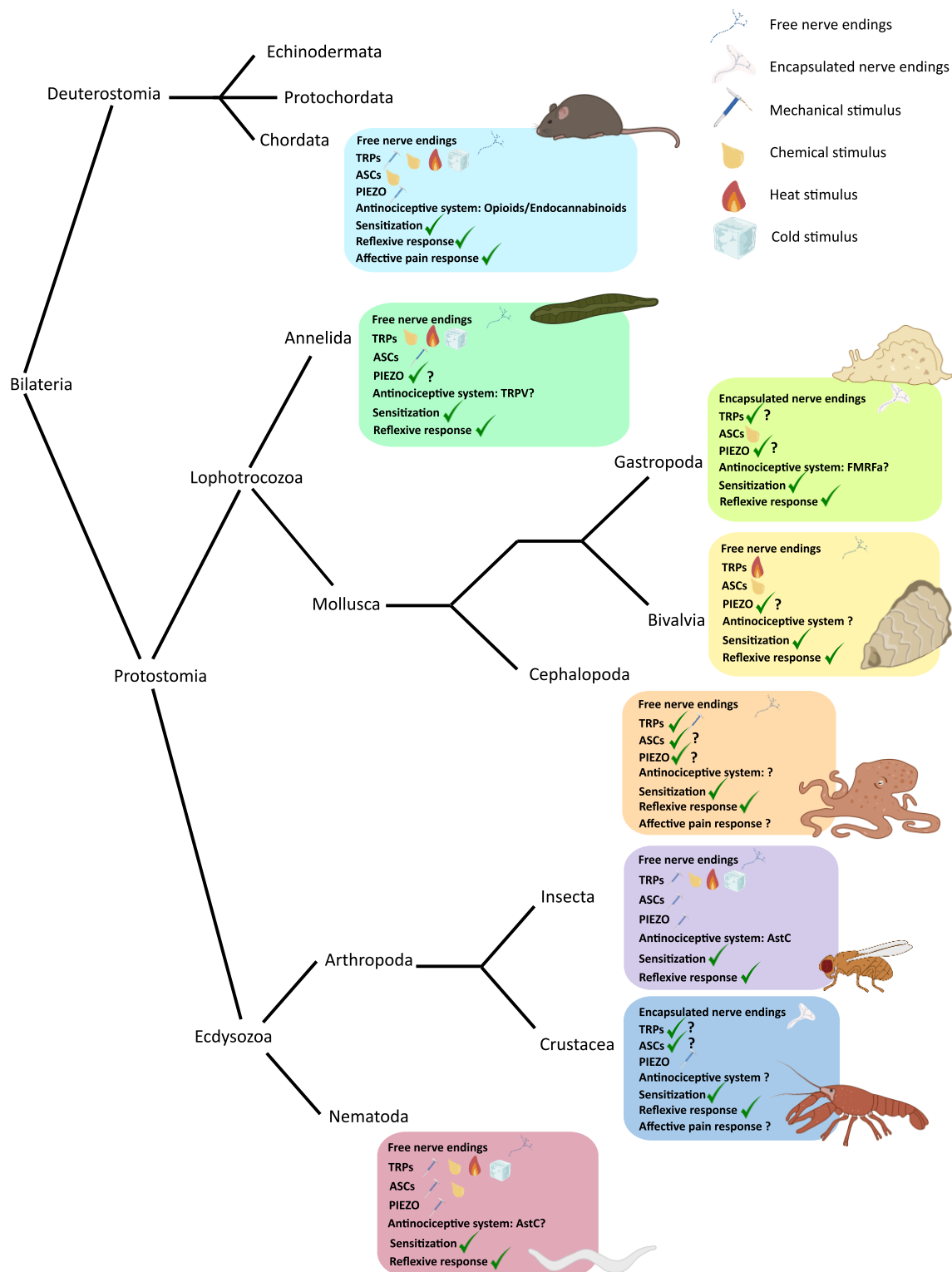


Figure 1.2 Dendrogram summarising similar and different physiological and molecular features of nociception across phyla representatives.

The reflexive nociceptive response and the sensitization of neurons to multiple exposure of the stimulus are conserved. The main molecular nociceptors are also shared across all phyla although the function is not always the same. For some of the taxa represented, the presence of an anti-nociceptive system and for a more complex pain-like response is speculated (?) but still not proven. Key is in the top-right corner.

1.2.2 Molecular determinants of nociception

As described above, cellular principles highlight conserved features of nociception cueing. This conservation extends to the molecular level where many determinants concurring to different neuron sensitivities are found across animals (Smith and Lewin, 2009). As a molecular cascade, nociception involves a sensory detection that is inputted, integrated and transduced even in most simple cases into intercellular interactions that underpin a circuit. Table 1.1(a-e) summarises major molecular functions across different phyla that characterise these nociception reflexes. The molecular functions are classified as follow: **a.** classical activators of nociception; **b.** voltage-gated ion channels and subunits; **c-d.** molecules involved in counteracting nociception and to a higher extent pain (i.e. opioid and endocannabinoid signalling); **e.** other modulators of nociception.

I will focus on examples from two of these groups: TRPV1, as a classical nociceptor and peptides, in particular enkephalins and tachykinins, as molecules involved in nociception modulation.

1.2.2.1 TRPV channels: classical polymodal detectors of noxious stimuli

The best characterised representative of the Transient Receptor Potential Vanilloid (TRPV) subfamily is the TRPV1, first discovered in primary sensory neurons of the DRG and TG for its ability of getting activated by the vanilloid substance capsaicin (Caterina *et al.*, 1997).

In vertebrates, TRPVs constitute a subfamily of the larger family of TRP ion channels (Table 1.1a). The vanilloid subfamily is formed of six members, all characterised by a typical secondary structure made up of six transmembrane elements (TMs), cytosolic N- and C- terminals and a hydrophobic short P-loop between the fifth and sixth TM that constitutes the selectivity filter when the channel is assembled in its functional tetrameric form (Figure 1.3; Rosasco and Gordon, 2017). The non-selective cationic nature of these channels is shared between all members and is characterised by Ca^{++} and Na^{+} influx (Rosenbaum and Islas, 2023). Functionally, TRPV1-TRPV4 evolved as polymodal nociceptors, capable of responding to numerous chemicals (e.g., capsaicin, anandamide, piperine, allicin etc.) as well as pH changes and high temperatures (Venkatachalam and Montell, 2007; Julius, 2013), while TRPV5 and TRPV6 are not involved in sensory detection but rather in homeostatic functions such as Ca^{++} reabsorption at the kidney and small intestine levels (Colton, 2006; Immke and Gawva, 2006; Radresa *et al.*, 2012; Shibasaki, 2024).

The first invertebrate TRPV orthologue discovered was *C. elegans* OSM-9, identified to have a role in detecting hyperosmotic stimuli (Colbert, Smith and Bargmann, 1997). Functional experiments proved the expanded polymodal nature of the ion channel, found to be implicated in high temperature, nose touch, low pH and chemical avoidance (Colbert, Smith and Bargmann, 1997). Four additional TRPV channels were later found in *C. elegans* (OCR-1-OCR-4) based on their sequence similarities with OSM-9. However, only OCR-2 seems to be implicated in sensory responses, most of which are elicited in combination with OSM-9, while OCR-1, OCR-3 and OCR-4 are found in non-nervous cells and have been associated with non-sensory functions (Tobin *et al.*, 2002; Jose *et al.*, 2007; Packer *et al.*, 2019). Similarly, in *D. melanogaster* the TRPVs Inactive (IAV) and Nanchung (NAN) are implicated in sensory responses (i.e. hearing and hygrosensation) but not necessarily in noxious responses (Gong *et al.*, 2004; Liu *et al.*, 2007; Li *et al.*, 2021a).

Altogether, TRPVs are one of the most conserved classes of sensory receptors across animals, with a versatile nature spanning homeostatic, non-noxious and polymodal aversive roles (Figure 1.2 and Table 1.1a; Tobin and Bargmann, 2004; Burrell, 2017; Himmel, Patel and Cox, 2017).

1.2.2.2 Neuropeptide signalling: turning up and down the nociceptive response

Functional neuropeptides are short size proteins processed from larger pre-pro-peptides (Figure 1.3B) through several enzymatic maturation steps, ultimately leading to their release inside large dense core vesicles in the secretory pathways (Figure 1.3C).

The organisation of the bioactive molecules within an inert larger precursor is conserved and the variety of families observed is likely the result of gene duplications from ancestor peptide forms (Strand, 1998). Given their broad physiological role, predicted neuropeptides are found in all existing animals, with representatives in very ancestral metazoans such as ctenophores (Sachkova *et al.*, 2021) and even in neuron-less organisms such as placozoans and sponges (Hauser, Koch and Grimmelikhuijzen, 2022; Yañez-Guerra, Thiel and Jékely, 2022; Yañez-Guerra *et al.*, 2025). However, while the functional significance of these peptides is well established across phyla, the depth of understanding for each predicted bioactive molecule is still at its infancy as shown by the recent large-scale de-orphanisation of *C. elegans* GPCRs receptor (Beets *et al.*, 2023).

As a neurobiological mechanism, nociception relies on classical neurotransmission (Fields, Heinricher and Mason, 1991; Yam *et al.*, 2018) and as every complex physiological response, it is also finely modulated by neuropeptides (Sweet, 1980). Peptides such as opioids and

tachykinins contribute to add a level of sophistication to the central nociceptive signalling pathway, overlapping with the negative and positive modulation of pain respectively (Table 1.1c and 1.1e).

In particular, tachykinins have been found to contribute to nociceptive sensitisation (and related hyperalgesia) through the regulation of neuronal excitability, inducing sustained activation of the primary sensory afferent (C-fibres) in mammals (Gutierrez *et al.*, 2019). A similar role has been identified in invertebrates, where researchers have found tachykinins to exert an upstream role in *Drosophila* sensitisation following noxious heat exposure (Im *et al.*, 2015).

In addition to the positive peptide-based modulation of nociception through tachykinins, a critical peptidergic signalling has evolved to convey antinociceptive responses. This is classically represented by the enkephalins system (met- and leu-enkephalin, endorphins, dynorphin, nociceptin) that underpins the pharmacological modulation of pain (Kanjhan, 1995; McQuay, 1999; Inturrisi, 2002). These molecules have been found to act on the central nervous system and more diffusely across the body, leading to a general suppression of the nociceptive signal transmission (García-Domínguez, 2024).

While physiological experiments and sometimes anecdotal descriptions report different behavioural actions of opioid substances on their nervous system (Nagabhushanam *et al.*, 1995; Dyakonova, 2001), there is no evidence that an opioid-like pathway operates in invertebrate organisms. Indeed, these molecules seem absent from any invertebrate genomes (Mirabeau and Joly, 2013; Thiel *et al.*, 2021) and all the experimental work aimed at identifying opioid molecules or their receptors within their tissues was inconclusive (Stefano, 1989; Harrison *et al.*, 1994; Stefano and Salzet, 1999). Therefore, for a long time, the exclusive presence of this signalling system in vertebrate species was interpreted as evidence for the lack of pain perception in invertebrates (see next sections).

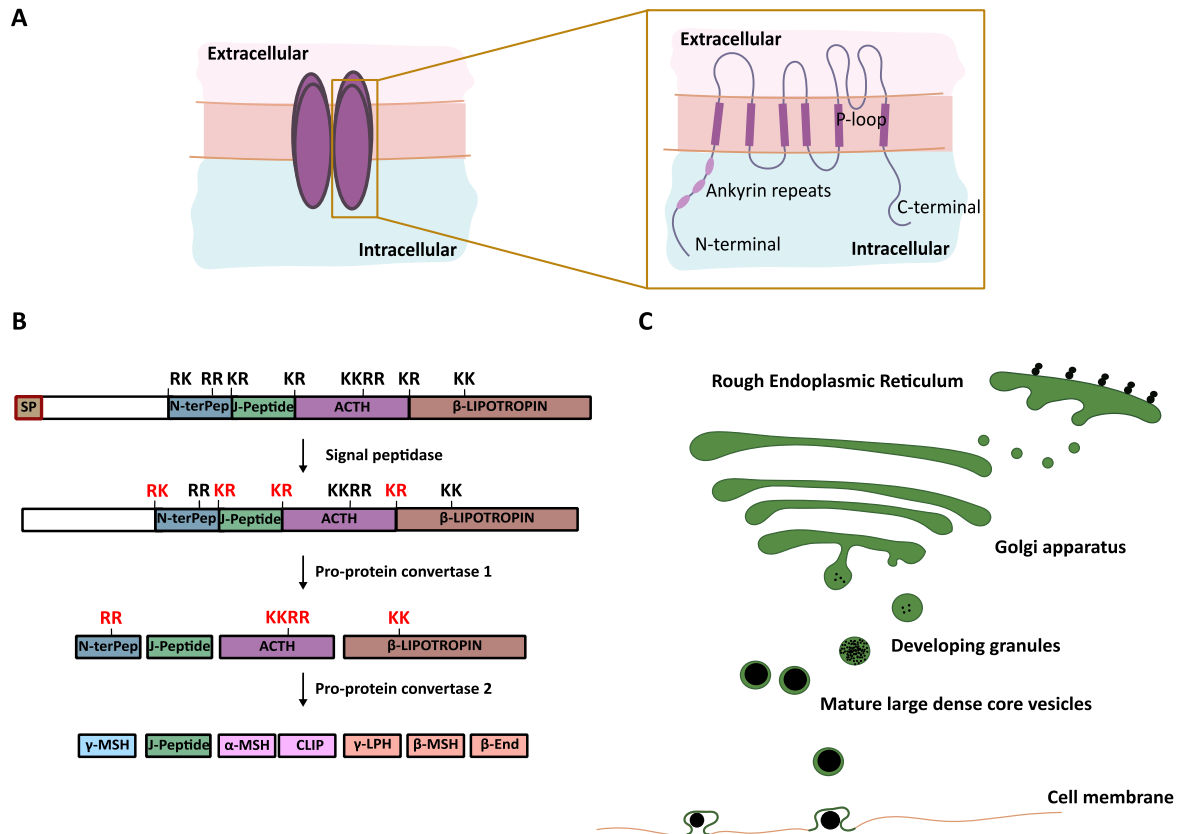


Figure 1.3 Cartoon showing TRPV and neuropeptide precursor structure and progressive maturation (adapted from Strand, 1998).

A Classical vertebrate TRPV channels are homotetrameric proteins in which each single subunit is characterised by six TMs, cytosolic N- and C- terminals, a variable number of ankyrin repeats and a hydrophobic P-loop. **B** Example of pro-opiomelanocortin (POMC) as a neuropeptide precursor and the different steps of enzymatic maturation and processing into active peptides. SP: signal peptide, ACTH: adrenocorticotrophic hormone, MSH: melanocyte-stimulating hormone, LPH: lipotrophin, β -End: β -endorphin, CLIP: Class II-associated invariant chain peptide, J-peptide: joining peptide **C** The precursor is first synthesised in the ribosomes (top), then processed into endoplasmic reticulum and Golgi apparatus, where the bioactive neuropeptides are subjected to post-translational modifications, maturation and are packaged into large dense core vesicles that are then exocytosed through fusion with the cell membrane.

Table 1.1a Summary of classical activators of nociception.

Colour legend for the background: = functional experimental evidence; = limited evidence (e.g., radioimmunoassay); = bioinformatic/phylogenetic studies only; = evidence of absence; = no data available/not investigated. M= mechanosensation; Ch= chemosensation; H= heat sensation; C= cold sensation; O=other physiological activity; U= unknown/not investigated function *Mechanical transduction involved in hearing, T= this work.

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca Cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
Degenerin /Epithelial sodium channel DEG/ENaC	ASCs	Polymodal sensory transduction receptor	DEG/ENaC FaNaC (Weber, Dannenmaier and Clauss, 1993) M	FaNaC (Perry <i>et al.</i> , 2001) Ch	FaNaC (Dandamudi and Lynagh, 2021) Ch	ASIC FaNaC (T) U	FLR-1; ASIC-1/2; MEC-4/10; UNC-8/105; DEG-1; DEL-1/4; DEGT-1; (Goodman, 2006) M, Ch	RPK PPK (Adams <i>et al.</i> , 1998) M, O	DEG/ENaC (Kozma <i>et al.</i> , 2020) U
Piezo	PIEZO	Mechanically activated ion channel	PIEZO (Heath-Heckman <i>et al.</i> , 2021) U	Uniprot U	Uniprot U	PIEZO1 (T) U	PEZO-1 (Millet <i>et al.</i> , 2021) M, O	PIEZO (Kim <i>et al.</i> , 2012) M	PIEZO (McCubbin <i>et al.</i> , 2020) M
Transient receptor potential	TRPA	Transduction of noxious cold, and irritant compounds	TRPA1 (Summers <i>et al.</i> , 2015) C, Ch	TRPA1 (Peng, Shi and Kadowaki, 2015) U	TRPA1 (Fu <i>et al.</i> , 2021) U	TRPA1 (T) U	TRPA-1 C, M TRPA-2 U (Kindt <i>et al.</i> , 2007; Chatzigeorgiou <i>et al.</i> , 2010)	TRPA1 Ch, H PAIN Ch, H PYX Ch, H WTRW O (Tracey Jr <i>et al.</i> , 2003; Lee <i>et al.</i> , 2005; Rosenzweig <i>et al.</i> , 2005)	(Kozma <i>et al.</i> , 2020; Peng <i>et al.</i> , 2015) U
Transient receptor potential	TRPC	Indirect regulation of other mechanosensitive molecules (e.g., Piezo, TMEM120)	TRPC (Northcutt <i>et al.</i> , 2018) U	TRPC (Peng <i>et al.</i> , 2015) U	TRPC (Fu <i>et al.</i> , 2021) H	TRPC (T) U	TRP-1 TRP-2 TRP-3 (Xu and Sternberg, 2003) O	TRP (Cosens and Manning, 1969) O	(Kozma <i>et al.</i> , 2020; Peng <i>et al.</i> , 2015) U
Transient receptor potential	TRPM	TRPM2 and TRPM3 are linked to heat sensation; TRPM8 to cold sensation	TRPM (Peng <i>et al.</i> , 2015) U	TRPM (Peng <i>et al.</i> , 2015) U	TRPM (Fu <i>et al.</i> , 2021) U	TRPM2; TRPM3 (T) U	GON-2 GTL-1 GTL-2 (Himmel, Gray and Cox, 2020) Ch, O	TRPM (Himmel <i>et al.</i> , 2020) Ch, C	(Kozma <i>et al.</i> , 2020; Peng <i>et al.</i> , 2015) U

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca Cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
Transient receptor potential	TRPN	Absent in mammals; Involved in mechanosensation in invertebrates (and zebrafish)	TRPN (Peng et al., 2015) U	TRPN (Peng et al., 2015) U	TRPN (Fu et al., 2021) U	NompC (van Giesen et al., 2020) M	TRP-4 (Kang et al., 2010) M	NompC (Lee et al., 2010; Yan et al., 2013) M, C, O	TRPN (Kozma et al., 2020; Peng et al., 2015) U
Transient receptor potential	TRPP	TRPP2 transduces mechanical stress in the brain with TRPC1	TRPP (Peng et al., 2015) U	TRPP (Peng et al., 2015) U	Uniprot U	TRPP PKD2 (T) U	LOV-1 M, O PKD-2 M, O (Barr and Sternberg, 1999) C, O	AMO (Gao, Ruden and Lu, 2003) C, O	TRPP (Kozma et al., 2020; Peng et al., 2015) U
Transient receptor potential	TRPV	TRPVs are polymodal receptors activated by irritants, high temperature and low pH	TRPV (Summers, Holec and Burrell, 2014) Ch, H	(Peng et al., 2015) U	(Fu et al., 2021) H	TRPV (T) Ch, M, H	OCR-1 O OCR-2 Ch, M, O, H OCR-3 O OCR-4 O OSM-9 Ch, M, H (Xiao and Xu, 2009)	IAV M*, O NAN M*, O (Gong et al., 2004)	TRPV (Kozma et al., 2020; Peng et al., 2015) U
Purinergic receptor	P2R	Involved in the modulation of pain threshold	P2R (Backus et al., 1994) M, O	P2R (Cox and Walker, 1987) O	Uniprot U	P2X4 (T) U	(Harte and Ouzounis, 2002)	(Harte and Ouzounis, 2002)	Uniprot U
Band7/mec-2	STOM	Modulates gating of ASICs and neuron mechanotransduction in association with DEG/ENaCs	Uniprot U	Uniprot U	Uniprot U	STOM (T) U	MEC-2 (Zhang et al., 2004) M	MEC2 (Liang et al., 2023) U	Uniprot U

Table 1.1b Summary of Voltage gated ion channels involved in sensory detection and nociception. Colour legend for the background: = functional experimental evidence; = bioinformatic/phylogenetic studies only; = evidence of absence; = no data available/not investigated. M= mechanosensation; Ch= chemosensation; H= heat sensation; C= cold sensation; O=other physiological activity; U= unknown/not investigated function; T=this work.

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca Cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
Na⁺⁺ voltage gated channel	Nav	Nav1.7 -Nav1.9 are implicated in pain perception	TTX-resistant Navs (Johansen, 1991) O	(Gilly, Gillette and McFarlane, 1997) O	Nav1; Nav2 (Boullot <i>et al.</i> , 2017) O	TTX-Resistant Navs in TTX bearing species (Geffeney <i>et al.</i> , 2019) O	(Bargmann, 1998)	PARA (Nav1); Ch, O DSC1 (Nav2); Ch, H (Suzuki, Grigliatti and Williamson, 1971)	(Salgado and Kem, 1992) O
Na⁺⁺ voltage gated channel	NALCN	Modulates insensitivity to algogenic substances	(Northcutt <i>et al.</i> , 2018) U	(Lu and Feng, 2011) O	Uniprot U	NALCN (T) U	NCA-2; UNC-77 (Zhou <i>et al.</i> , 2022) O	NA Flybase O	(Northcutt <i>et al.</i> , 2016) U
K⁺ voltage-gated channel	Kv	KCNA1 is a modulator of touch and mechanical pain; KCNQ plays a role in setting pain threshold	(Johansen, 1991) M	(Thompson, 1977; Jezzini and Moroz, 2004) O	(Shimatani and Katagiri, 1995) O	(Chrachri and Williamson, 2004) O	SHK-1; KQT-2; KQT-3; (Wei, Butler and Salkoff, 2005; Okahata <i>et al.</i> , 2019) O, C	SHK, SHB, SHL, SHW (Papazian <i>et al.</i> , 1988; Butler, Wei and Salkoff, 1990) O	(Harris-Warrick <i>et al.</i> , 1989; Hermann and Erxleben, 2001) O
Ca⁺⁺ voltage-gated channel	Cav	CACNA1A, CACNA1B, CACNA1E and CACNA2D3 are implicated in pain	(Kramer and Goldman, 1981) O	(Skeer, Norman and Sattelle, 1996) O	(Devlin, 1993) O	(Skeer, Norman and Sattelle, 1996) O	UNC-2 O UNC-36 M EGL-19 O (Mathews <i>et al.</i> , 2003)	STJ CAC (Kawasaki, Felling and Ordway, 2000; Neely <i>et al.</i> , 2010) H	(Fatt and Katz, 1953; Fatt and Ginsborg, 1958) O

Table 1.1c Summary of antinociceptive proteins (opioid signalling).

Colour legend for the background: = functional evidence; = limited evidence (e.g., radioimmunoassay); = bioinformatic/phylogenetic studies only; = no data available/not investigated. M= mechanosensation; Ch= chemosensation; H= heat sensation; C= cold sensation; O=other physiological activity; U= unknown/not investigated function, T= this work

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca Cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
Opioid neuropeptide precursor	POMC and derivatives	Precursor of β -endorphin, ACTH and α -MSH	(Harrison et al., 1994)	(Leung et al., 1986; Elekes et al., 1993)	(Stefano and Salzet, 1999)				(Ye et al., 2006)
Opioid neuropeptide precursor	PENK and derivatives	Precursor of Leu- and Met-enkephalin	(Harrison et al., 1994)	(Leung et al., 1986; Elekes et al., 1993)	(Stefano and Salzet, 1999)	(Martin et al., 1979)		(Pages et al., 1983; Callaerts et al., 1988)	(Mercier, Friedrich and Boldt, 2003)
Opioid neuropeptide precursor	PDYN and derivatives	Precursor of dynorphin, α - and β -neo-endorphin	(Harrison et al., 1994)	(Leung et al., 1986; Elekes et al., 1993)	(Stefano and Salzet, 1999)				
FMRFamide related peptide	FMRFa	wide variety of physiological functions	(Kuhlman, Li and Calabrese, 1985)	(Zatylny-Gaudin and Favrel, 2014)	(Price and Greenberg, 1977)	(Zatylny-Gaudin et al., 2016)	(Nelson et al., 1998)	(Nichols, 2003)	(Mercier et al., 2003)
GPCR A	OPRD1	Analgesic response	(Salzet and Stefano, 1997)	(Guo et al., 2013)	(Liu et al., 2015)	(Sha, Sun and Wang, 2012)		(Santoro, Hall and Zukin, 1990)	(Hanke, Willig and Jaros, 1996)
GPCR A	OPRK1	Analgesic response			(Liu and Sun, 2010)		NPR-17 (AstCR) (Mills, 2014)		(Hanke et al., 1996)
GPCR A	OPRM1	Analgesic response		(Dalton and Widdowson, 1989)	(Liu and Sun, 2010)				(Hanke et al., 1996)
GPCR A	OPRL1	Analgesic response			(Stefano and Salzet, 1999)				
Peptidase M2	ACE	Alters pain response	(Laurent and Salzet, 1995)	Uniprot	(Laurent, Stefano and Salzet, 1997)	ACE (T)	ACN-1 (Brooks et al., 2003)	RACE (Tatei et al., 1995)	(Chung and Webster, 2008)
Peptidase M13	MME	Catabolism of opioid peptides	(Laurent and Salzet, 1995)	(Zappulla et al., 1999)	CD10/NEP (Shipp et al., 1990)	NEP (T)	NEP (Spanier et al., 2005)	NEP (Turner, Isaac and Coates, 2001; Thomas et al., 2005)	NEP Uniprot
Peptidase M13	ECE	Processing of various neuroendocrine peptides	Uniprot	Uniprot	Uniprot	ECE (T)	NEP-1-11 Wormbase	NEP-3 (Turner et al., 2001)	Uniprot
Peptidase M14	CPE	Biosynthesis of enkephalin	Uniprot	Uniprot	Uniprot	(T)	EGL-21 (Nkambeu et al., 2019)	SVR (Settle, Green and Burtis, 1995)	Uniprot
Peptidase S8	PCSK	Maturation of enkephalins	Uniprot	Uniprot	Uniprot	(T)	KPC-1 (Salzberg, Ramirez-Suarez and Bülow, 2014)	Furin1 (CG10772), Furin2 (CG18734), Amontillado; (Aittomäki et al., 2017)	Uniprot

Table 1.1d Summary of antinociceptive proteins (endocannabinoid signalling). Colour legend for the background: ■ = functional experimental evidence; ■ = limited evidence (e.g., radioimmunoassay); ■ = bioinformatic/phylogenetic studies only; ■ = evidence of absence; ■ = no data available/not investigated. M= mechanosensation; Ch= chemosensation; H= heat sensation; C= cold sensation; O=other physiological activity; U= unknown/not investigated function; T= this work *Endocannabinoid activity was found to be exerted through TRPV1

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
GPCR A	CB1	Modulation of analgesia	(Stefano, Salzet and Salzet, 1997; Elphick, 1998)*				NPR-19 (Oakes <i>et al.</i> , 2017; Oakes, Law and Komuniecki, 2019)	*	
GPCR A	CB2	Inflammatory response and nociceptive transmission							
GPCR A	GPR55	Hyperalgesia associated with inflammatory and neuropathic pain							
GPCR A	GPR18	Receptor for NAGly							
NAPE-PLD	NAPE-PLD	Production of mature anandamide	Uniprot		Uniprot	NAPE-PLD (T)	NAPE-1; NAPE-2 (Estrada-Valencia <i>et al.</i> , 2021)	(Elphick, 2012)	(Elphick, 2012)
Lipase	DAGL	Production of mature 2-AG	Uniprot		Uniprot	DAGL (T)	NAPE-1; NAPE-2 (Estrada-Valencia <i>et al.</i> , 2021)	INAE (Leung <i>et al.</i> , 2008)	Uniprot
Amidase	FAAH	Catabolism of anandamide and oleamide	(Matias <i>et al.</i> , 2001)		(Sepe <i>et al.</i> , 1998)	FAAH1 FAAH2 (T)	FAAH-1 (Estrada-Valencia <i>et al.</i> , 2021)	(Elphick, 2012)	Uniprot
Monoacylglycerol lipase	MAGL	Catabolism of 2-AG	Uniprot		Uniprot	Uniprot	FAAH-4 (Estrada-Valencia <i>et al.</i> , 2021)	(Elphick, 2012)	Uniprot

Table 1.1e Summary of other modulators of nociception.

Colour legend for the background: = functional experimental evidence; = bioinformatic/phylogenetic studies only; = evidence of absence; = no data available/not investigated. M= mechanosensation; Ch= chemosensation; H= heat sensation; C= cold sensation; O=other physiological activity; U= unknown/not investigated function, T= this work

Family	Molecule	Function in Vertebrates	Annelida	Mollusca Gastropoda	Mollusca Bivalvia	Mollusca Cephalopoda	Nematoda (<i>C. elegans</i>)	Arthropoda Insecta (<i>D. melanogaster</i>)	Arthropoda Crustacea
Anoctamin	ANO1	Acts as a heat sensor in nociceptive neurons	Uniprot U	Uniprot U	Uniprot U	ANO (T)	ANOH-1 (Zou <i>et al.</i> , 2025) M	SUBDUED (Jang <i>et al.</i> , 2015) H, O	ANO1 (De Vos <i>et al.</i> , 2019) O
GPCR1	TACR1	Processing nociceptive stimuli	Uniprot U	(Van Loy <i>et al.</i> , 2010) U	(Van Loy <i>et al.</i> , 2010) U	(Kanda <i>et al.</i> , 2007) O	TKR-1-3 (Husson <i>et al.</i> , 2007) O	NKD (Monnier <i>et al.</i> , 1992) O	(Van Loy <i>et al.</i> , 2010) U
Tachykinin	TAC1	Sensitisation to nociception	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O	(Lundquist and Nässel, 1990) O
Transmembrane protein	TMEM63	Osmosensitive calcium-permeable cation channel						(Murthy <i>et al.</i> , 2018) M	
Transmembrane protein	TMEM87a	Component of a mechanoelectrical transduction pathway						CG17660 U	
Transmembrane protein	TMEM100	Modulator of the TRPA1-TRPV1 interaction							
Transmembrane protein	TMEM120A	Senses mechanical pain				TMEM120 (T)	TMEM-120 (Li <i>et al.</i> , 2021b) U	CG32795 U	
GPCR B	GPR83	Cooperates with TACR1 to convey thermal response					NPR-6 U		
GPCR B	CALCR	Characterises “peptidergic” nociceptors					PDFR-1 (Luo and Portman, 2021) U	PDFR (Potdar and Sheeba, 2018) U	
GPCR B	CALCRL	Characterises “peptidergic” nociceptors					PDFR-1 (Luo and Portman, 2021) U	HEC (Zink and Paro, 1989) U	

1.3 Pain perception: central brain based higher processing of nociception

1.3.1 Pain as an integrated response in the circuit of nociception

In the description above I have largely focussed on a core process in which noxious cues trigger an appropriate withdrawal that reflects a protective behaviour. Key molecules confer cue-specific or polymodal functions to specialised neurons which are strategically distributed across the body to detect visceral or peripheral sensory stimuli. For the purpose of this work, I will only focus on the skin exteroceptors.

In vertebrates, the circuit in which these two elements are inserted and partake to produce the reflexive response are well-established. We can recognise three integrated levels of action that are triggered following exposure to noxious stimuli (Figure 1.4): i. molecular; ii. physiological; iii. behavioural. The first response refers to the activation of all the molecular receptors that culminates with the generation of an action potential in the cellular nociceptors. This produces a physiological response that, through the axon of the primary sensory neurons, reaches the dorsal horn of the spinal cord where a direct synapse with motor neurons immediately activates a reflexive avoidance response (Figure 1.4). These local circuits in the spinal cord give rise to arc reflexes that don't require the processing in higher brain centres and thus allow a quick response to potential external threats to protect from tissue damage (Dubin and Patapoutian, 2010). This contributes to trigger the behavioural response, that is usually characterised by withdrawal and avoidance from the stimulus.

As anticipated, there is a more complex ascending pathway which, paired with top-down modulation allows vertebrate organisms to perceive pain (Figure 1.4). From the spinal cord in fact, the signal can be sent through the lateral spino-thalamic lemniscus which, through interneurons, projects towards the rhombo- and mesencephalic structures, and finally transmits to the thalamus (Dubin and Patapoutian, 2010). The latter acts as a relay sending its projections to the somatosensory and anterior cingulate cortices. From here, descending pathways reach the spinal cord and the brainstem and ultimately lead to the animals' associated pain perception. This include two main states, a discriminative component which allows to localise where the stimulus was applied, and an affective component which

comprehends the subjective unpleasant sensations associated with the negative experience and all the conscious active behavioural responses (Figure 1.4).

Finally, as mentioned previously, neuropeptides such as enkephalins and tachykinins can act at different levels of the whole circuit, to modify the nociceptive neurotransmission in a positive or negative direction.

The emotional aspect of pain is thus a biological state that results from a complex neurophysiological crosstalk between different central brain structures. In fact, along the spinothalamic tract, other circuits contribute to the generation of the unpleasant feeling that characterises affective pain. For instance, the spino-pontoamygdaloid and spino-hypothalamic pathways allow connections with the brainstem and the limbic structures which are engaged in pairing the noxious input with the environmental context, giving pain also cognitive connotations (Price, 2000).

The intersection of higher processing with core nociception is based on identifiable anatomical tracts and wider intercellular signalling. This top-down modulation is an important framework that can also be found in simpler model organisms (Im *et al.*, 2015; Liu *et al.*, 2023).

This justifies the attempt at extending pain investigation in more complex invertebrate organisms and provides a biological template to help address if and where nociception may express as the more sentient brain state of pain.

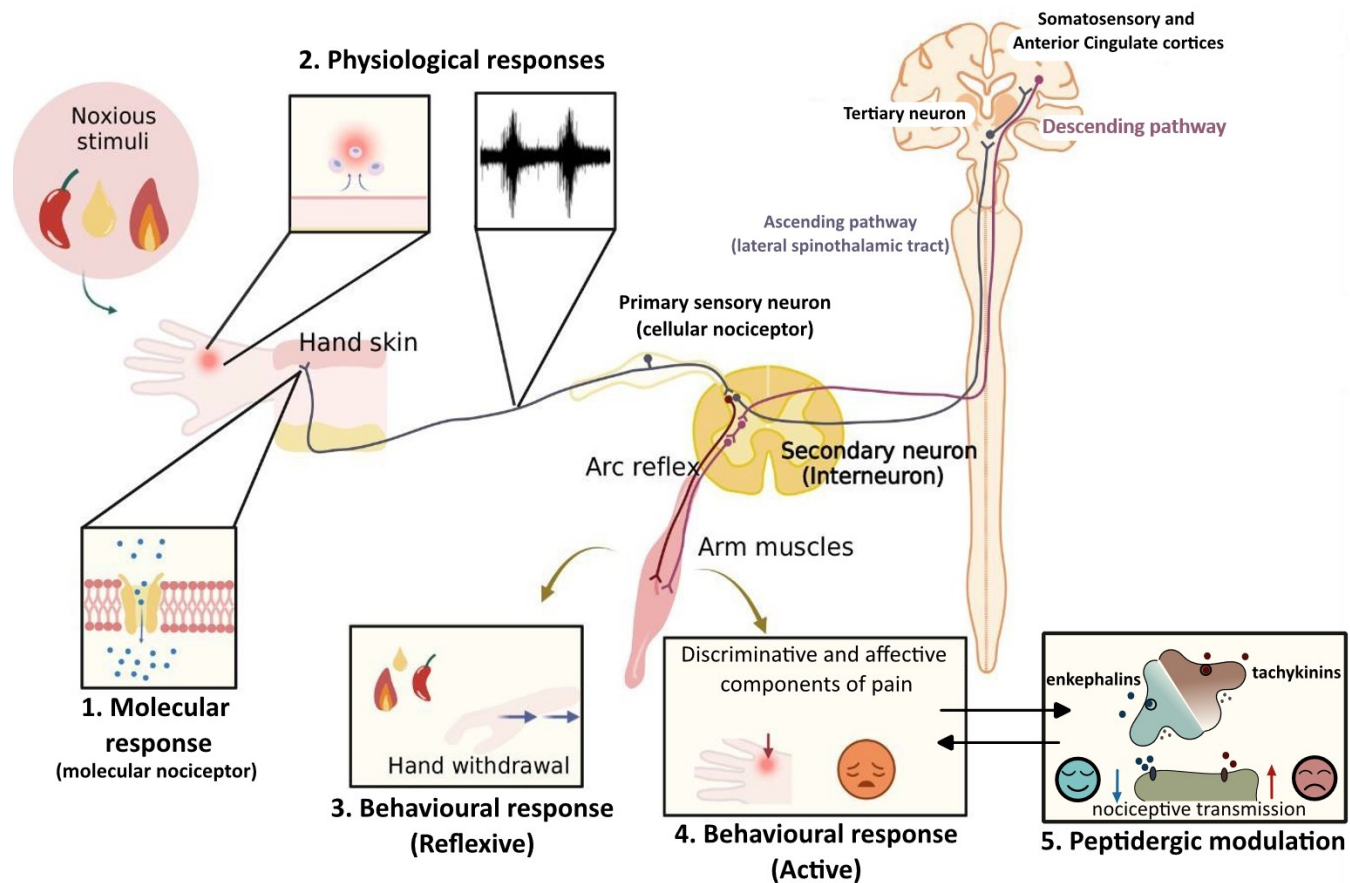


Figure 1.4 The cellular, molecular and physiological organisation of mammalian nociceptive and pain pathways.

Different molecules (1) specialised in detecting specific sensory noxious modalities lead to the activation of the cellular nociceptors. The action potential (2) of these specialised neurons reaches the spinal cord and causes the depolarisation of a motoneuron that in turn leads to the display of a reflexive withdrawal (3). A second pathway leads from the primary sensory neuron to the activation of an interneuron that reaches the central brain through ascending pathways. The information is then elaborated by different brain regions and produce the active pain response (4). The emotional components can be up- or downregulated through the contribution of peptidergic modulation (5).

1.3.2 Criteria to define the presence of pain experience in animals

Claiming pain in non-verbal animals as the emotion-based subjective experience described above, is challenging. It requires evidence of at least primary consciousness in the species, supported by unequivocal characteristics such as of EEG signatures, a crosstalk between specific anatomical structures for top-down regulation (e.g., thalamus-cortex), a recognisable widespread brain activity and behavioural responses (Seth, Baars and Edelman, 2005).

A clear attempt at providing indicators to claim pain in non-human animals was made by Bateson (1991) who summarised the markers for recognising pain capability in eight criteria (Table 1.2).

The main requirement to be able to feel pain is the possession of neurons that are specialised in the detection of potential noxious stimuli, namely nociceptors (criterion 1). Secondly, these cells must be part of a sufficiently complex structure, able to support sophisticated processing (criterion 2). The elaboration of mere inputs into a subjective experience is only possible with a pathway connecting the peripheral fibres to the central integrative structures (criterion 3). The kind of behavioural response the animal displays when exposed to threatening cues is also essential. If we observe a mere passive, avoidance response, we can only infer nociception (criterion 7) whereas active, adaptive self-directed behaviours suggest a central manipulation of the information (criterion 6). If the animal is able to internalise negative episodes and produce emotional experiences from them, it is also likely to be endowed with an anti-nociceptive pathway that allows the organism to inhibit pain sensation. Thus, the presence of opioid receptors in the central nervous system (criterion 4) and the observation that the administration of analgesics is able to modulate animal response to noxious stimuli (criterion 5) could be seen as good insights into the possibility of experiencing pain in animals. An even clearer indication is given by associative learning in the context of noxious experience (criterion 8).

1.3.3 Invertebrates and pain perception

Despite being applicable to non-human animals, Bateson's criteria are biased towards the vertebrate-base knowledge, as for instance in the case of criterion 4 where the presence of receptors responding to opioid substances is explicitly required. Nevertheless,

understanding the possibility of nociception processing into pain in invertebrates is important for the reasons mapped out previously. If we are in fact able to define that most functionally and/or structurally conserved target features are enclosed in an organism with a simpler neural organisation that does not encompass pain, we could use it as a valid model to investigate the molecular basis of nociceptive and pain related diseases while overcoming ethical issues and practical limitations that we face with mammalian models (Burrell, 2017). Conversely, if we prove that invertebrate animals are capable of integrating signals that could produce pain-like sensations, this might affect both the animal welfare and the quality of the experimental data collected (Smith, 1991).

This awareness should play a crucial role not just in research but also in other fields where invertebrate target species are employed, such as public display and commercial frameworks (i.e., fishery, aquaculture). A first step ahead has again been made by the UK in 2021, where the Animal Sentience Act (UK Public General Acts 2022 c. 22, 2022), regulating animal use in all contexts of interest for humans, included for the first time two invertebrate phyla: cephalopods and decapod crustaceans. This was achieved by applying a revised version of Bateson's criteria and thanks to the accumulating evidence, these taxa could be considered capable of processing stress and harmful responses into pain-like states (Birch *et al.*, 2021).

1.4 Octopus nociception: insights from neuroanatomy and cell physiology

1.4.1 Are cephalopod eligible to feel pain?

One of the criteria for eligibility to sense pain according to Bateson, is to have a nervous structure that supports the computational power required for processing affective experiences (Bateson, 1991). On this base, invertebrates have been excluded *a priori* from the debate as these were considered to lack a sufficiently elaborated neural architecture. This aspect, added to their relatively short life span, have been used as an argument against the possibility for invertebrates to feel emotional discomfort from negative experiences as an advantageous response to tissue damage (Walters, 2018).

Cephalopods, with their complex centralised brain capable of exhibit sophisticated behavioural responses, represent an exception among invertebrates.

1.4.1.1 Cephalopods

Cephalopoda - from the Greek *kephalós* (head) and *podós* (feet) - constitute a class of Mollusca, currently counting about 800 marine species (Sweeney and Roper, 1998; Jereb and Roper, 2005; Jereb, Roper and Vecchione, 2010) characterised by a distinct body anatomy. Cephalopods are divided in two major subclasses: Nautiloidea, the most ancestral one, currently only including the *Nautilus* genus and Coleoidea, which includes the two superorders of Decapodiformes (cuttlefishes, squids and sepiolids) and Octopodiformes (octopuses).

These animals present substantial changes from the classical *Bauplan* of the hypothetical ancestral mollusc (HAM; Lindberg and Ghiselin, 2003). A large muscular foot still conserved in gastropods, underwent reorganization into arms and tentacles in cephalopods and the saccular body shape containing the inner organs that was originally protected by a shell was lost or internalised in vestigial residues in cephalopods (Figure 1.5). An exception to this is found in the Nautiloidea order. Other anatomical features, such as the toothed tongue-like radula in the mouth are otherwise shared with the other molluscs (Hickman *et al.*, 2001).

Hypothetical Ancestral Mollusc

Octopus

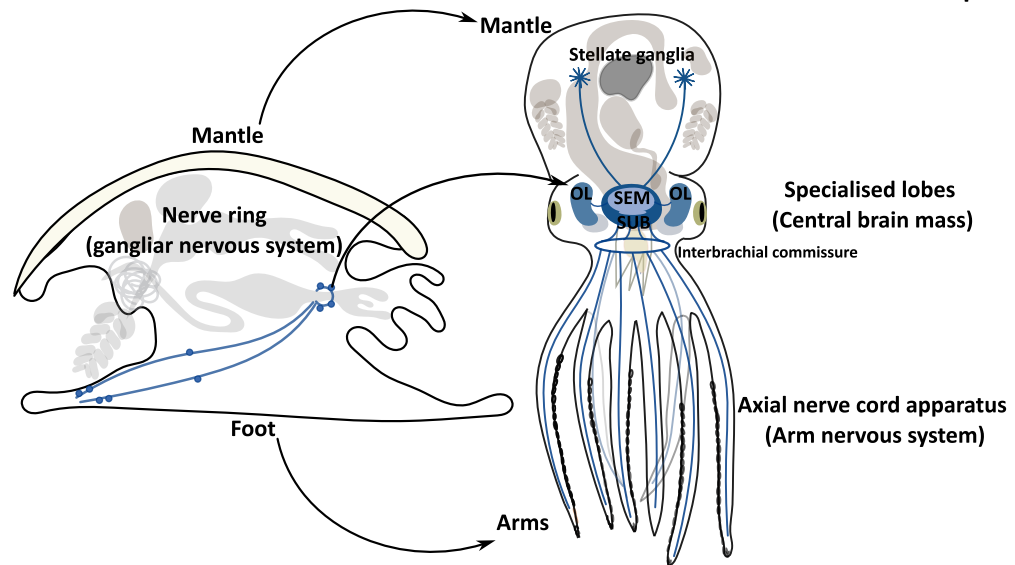


Figure 1.5 Comparison between the *Bauplan* of the Hypothetical Ancestral Mollusc and the *Bauplan* of an octopus.

Many changes happened to the body structure of the hypothetical ancestral mollusc (HAM) that led to the evolution of the octopus anatomy. Perhaps the most impressive change is represented by the neuroanatomy (in blue). The series of ganglia running through the body of the HAM, fused together to form morphologically and functionally specialised lobes. A distinction between a central nervous system in the head, and a peripheral nervous system in the mantle and an arm nervous system is also visible. SEM: supra-oesophageal mass, SUB: sub-oesophageal mass, OL: optic lobe

1.4.2 Octopus complex neuroanatomy explains elaborated behaviours that might underlie pain-like states

The most impressive evolutionary change in cephalopods happened at the level of the nervous system. In gastropods and bivalves different sets of ganglia are distributed across the whole body. In cephalopods, these underwent a significant rearrangement and have fused to give rise to a brain-like structure made up of functionally specialised lobes (Figure 1.5). This is particularly evident in octopuses where extensive studies of its neuroanatomy and the dissection of the main neural pathways have been carried out (Wang and Ragsdale, 2019).

1.4.2.1 *Octopus vulgaris* neuroanatomy

The common octopus, *Octopus vulgaris* possesses a nervous system accounting for 500 million nerve cells, organised within a central brain mass divided into two large masses by the crossing of the oesophagus, and an arm nervous system. Above the oesophagus, a supra-oesophageal mass (SEM) and two optic lobes (OL) – mainly involved in learning and memory can be recognised, while a sub-oesophageal mass (SUB), receiving and processing the inputs from the ‘peripheral nervous system’ is found ventrally to the oesophagus. The peripheral

part of the body is innervated by the semi-autonomous arm nervous system (350 million nerve cells), made up of eight axial nerve cords running across the arms, and by the mantle, innervated by the two pallial nerves (Figure 1.5; Young, 1963).

Recent tracing studies identified structural and functional correlations between cephalopod neural structures and human brain areas (Shigeno *et al.*, 2018). For instance, the octopus subvertical and dorsal basal lobes can be considered analogous to the thalamus, as they act as relay structures for the afferent inputs of the body and project to higher structures like the vertical lobe and the frontal lobe system which functionally resemble the cortex (Young, 1971; Zullo *et al.*, 2009). Other analogues of the motor, somatosensory and limbic structures were also detected in the inferior frontal lobe and the buccal and sub-pedunculate lobes respectively (Figure 1.6A). The axial nerve cords running through the arms, with a central medullary part and four lateral cords, and a bilateral symmetry, are structurally very similar to the vertebrate spinal cord and are therefore considered to some extent more as an extension of the central nervous system rather than part of a peripheral one (Figure 1.6B; Bullock and Horridge, 1965).

The size, the neuronal number outcompeting some small vertebrates (Young, 1963; Herculano-Houzel, Mota and Lent, 2006), and the complexity of the octopus brain are considered the reasons why these species are able to display a plethora of really complex behaviours such as tool-use, sophisticated problem solving, and impressive predatory strategies (Amodio and Fiorito, 2013; Amodio *et al.*, 2019).

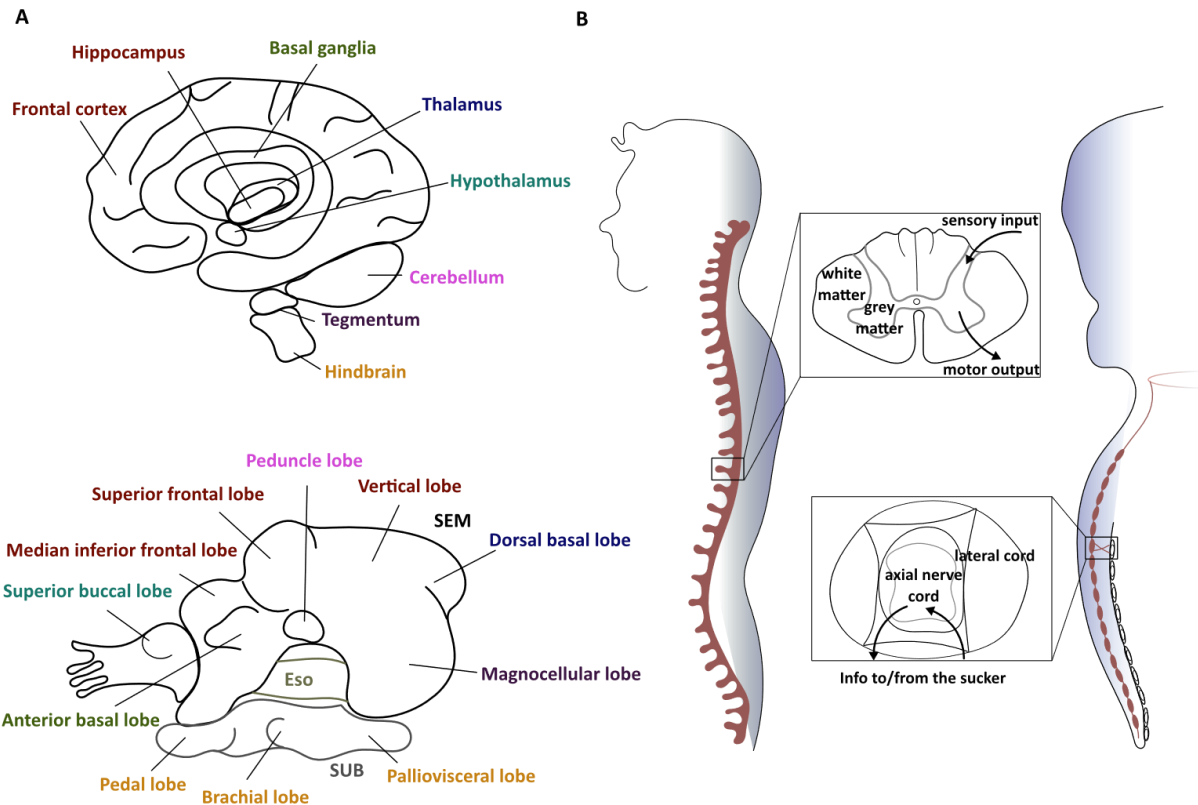


Figure 1.6 Functional analogues between mammalian and octopus nervous system.

A Human and octopus nervous systems are different but in-depth studies of the function showed that structures like the dorsal basal lobe in octopus acts as a relay to collect all the interoceptive and exteroceptive inputs and then transmits to the frontal vertical system that similarly to the frontal cortex, elaborates the information further. Matching colours between the human (top) and the octopus (bottom) areas represent the functional analogues identified (Shigeno et al., 2018). **B** Neuroanatomical organisation of the octopus axial nerve cord within a medullary centre, four lateral cords as well as the overall bilateral symmetry along the different sections of the arm, resembles the organisation of the human spinal cord, contributing to debating whether this should be considered part of the octopus central nervous system.

1.4.2.2 Neurochemical conservation and divergence in *O. vulgaris* nervous system: a largely uncharted landscape

The peculiar neuroanatomy of *O. vulgaris* is supported by complex connections across the body. Despite the independent evolution of complex brains, the core neurochemical toolkit is characterised by conserved, divergent but functionally similar and unexplored neurochemical molecules.

Although not the focus of this thesis, classical neurotransmitters are largely found in cephalopods with functions that are shared with vertebrates, as for instance the cholinergic regulation of the neuromuscular junction, despite the arm's overall anatomical organisation being different (Zullo et al., 2017; Nesher et al., 2019). Similarly GABA, glutamate, dopamine and

serotonin are present and with wide roles across the body of the animal (Andrews, Messenger and Tansey, 1983; Bellier *et al.*, 2017; Stern-Mentch *et al.*, 2022).

A point of divergence with the vertebrate system is the absence of an adrenergic system, whose functions are however most likely replaced by octopamine as in the rest of the invertebrate species (Gallo *et al.*, 2016).

Similarly, neuropeptide signalling possesses some shared molecules between vertebrates and invertebrates, such as tachykinins and gonadotropin which were also characterised in *O. vulgaris* (Kanda *et al.*, 2005; Kanda *et al.*, 2006; Kanda *et al.*, 2007) but largely these remain unexplored.

As previously mentioned, vertebrate nociception is deeply modulated by neuropeptides and therefore, knowing whether analogous peptide modulation of nociceptive circuits exists in octopus is essential to bridge the current gap between behavioural evidence of nociception and the molecular and neurochemical substrates involved.

Altogether, the neuroanatomical characteristics and its connections and the resulting elaborated cognitive skills guided the inclusion of cephalopods as currently the only invertebrate taxon among protected animals in the Directive 2010/63/EU when used in research (European Parliament and Council of the European Union, 2010; Andrews *et al.*, 2013; Smith *et al.*, 2013). This is however based on circumstantial evidence of complex nociception rather than on actual experimental proof that cephalopods feel pain, which requires more articulated investigations of nociception detection and integration in these species.

1.4.3 Evidence of nociception in cephalopods

1.4.3.1 Pioneering experimental studies of octopus nociception

Perhaps the first work interested in testing noxious chemicals in intact octopuses, was carried out in 1926. Blinded animals were exposed to different cues to assess the discrimination abilities and showed strong avoidance to hydrochloric acid, quinine sulphate, skatole and artificial musk. Aversion was still visible on isolated arm preparations, suggesting these responses were most likely reflexive (Giersberg, 1926).

Young and Wells dedicated most of their careers establishing a neural map of *O. vulgaris* brain by dissecting and describing the afferent and efferent fibres within the over 25 specialised lobes (Wells and Wells, 1957; Wells, 1959; Young, 1963; Wells, 1964; Young,

1965; Young, 1971; Young, 1983). Through ablation of the connections or entire regions of SEM or SUB and observation of the resulting behaviour, they were able to correlate these structures to their function (Wells and Wells, 1957; Young, 1965). Learning and memory capabilities in octopus were extensively studied in the context of its ability to integrate sensory inputs from the environment. This includes pathways and areas that generate a retraction from negative stimuli. Interestingly, in his works, Young referred multiple times to pain signals (Figure 1.7) associated to different areas found in the SEM: lateral inferior frontal and subfrontal, median inferior frontal and the posterior buccal lobes, collectively defined as the inferior frontal system (Young, 1964; Young, 1965; Young, 1983; Young, 1991). However, from the description of these areas as serving “reflexes to simple stimuli” it is clear he was referring to nociception rather than to a pain-like state. This is also reflected by the results of experiments which led to the identification of the role of these brain regions (Figure 1.7).

To test for their ability to discriminate between texturally different objects, blinded octopuses were rewarded if touching the correct object, or punished with a mild electric shock if touching the negative cue (Wells, 1964). The electric shock was indeed causing the animal to retract and escape, showing an avoidance response. Ablation of the entire SEM caused the animal to keep accepting negative objects despite the associated electric shock (Wells, 1959). If the inferior frontal system was not included in the ablated area, animals preserved their discrimination ability with however, severe problems in locomotion.

When, on the other hand, the inferior frontal system was removed and the SEM was left intact, animals were not rejecting the negative cue but they were still responding to the mild electric shock, thus showing a decoupling between learning avoidance and reflexive nociception.

Furthermore, the sensory afferents from the arms were found to directly synapse onto the inferior frontal system without relaying first on the SUB (Figure 1.7; Budelmann and Young, 1985).

Altogether these results suggest that the inferior frontal system seems to directly regulate arm movements rather than playing a role in the association between the stimulus and the learnt avoidance response and thus suggests a reflexive nociceptive response rather than indicating pain perception (Key and Brown, 2018).

Pure reflexive withdrawal characterisation was performed on *ex vivo* preparations of octopus arms in different occasions. One of the first works exposing the arm of an octopus to noxious

stimuli and observing the responses, was carried out by Altman in 1971. Severed axial nerve cords were put in contact with a solution of 3% quinine hydrochloride and were found to withdraw from the stimulus, even when detached from the central brain mass (Altman, 1971). Additionally, he demonstrated, consistently with the previous experiments described above, that removal of SEM destroyed the responses while the exclusion of the inferior frontal system from the ablation left the rejection reflex intact (Altman, 1971).

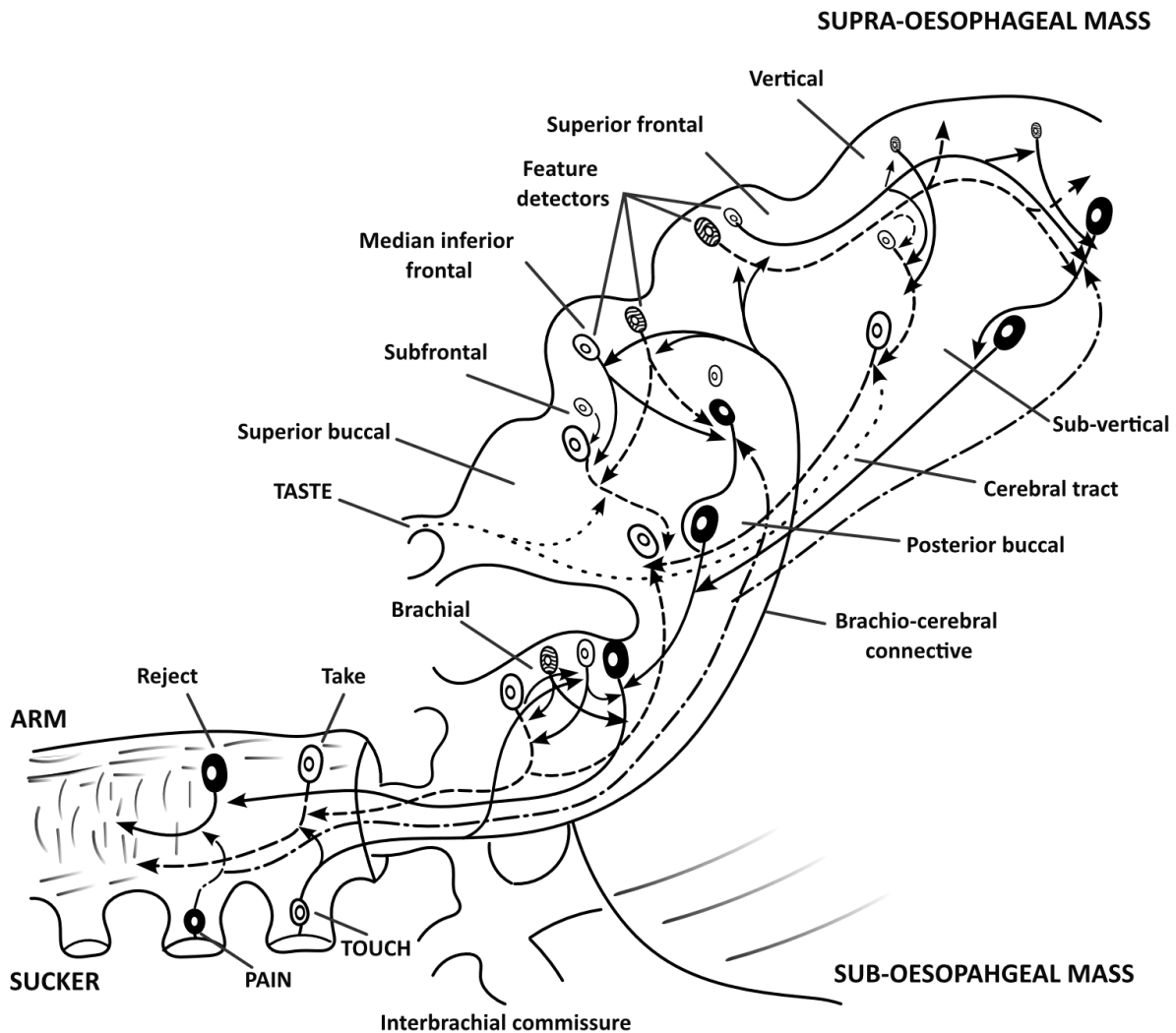


Figure 1.7 The suggested brain centres involved in sensory responses in octopus (adapted from Young, 1982). The scheme shows the main lobes constituting the inferior frontal system, namely the lateral inferior frontal and subfrontal, median inferior frontal and the posterior buccal lobes. These are found in the supra-oesophageal lobe, receiving direct synapses from the arm of octopus and organising a quick 'accept' or 'reject' response, without relaying on the sub-oesophageal mass. Noteworthy are the TOUCH and PAIN commands, showing a distinction between a normal tactile stimulation and a reflexive nociceptive one.

1.4.3.2 Follow-up experimental studies of cephalopod nociception

Many researchers in the last decade have continued to attempt at resolving the behavioural and physiological basis of nociception in cephalopods to then be able to contextualise this into a framework for investigating higher cognition.

Similarly to Altman, octopus arm preparations were exposed to mechanical (pinches), osmotic (tap water) and chemical (1% acetic acid) stimuli and showed withdrawal from all of them (Hague, Florini and Andrews, 2013). Electrophysiological recording of the arm responding to different stimuli has been recently accomplished, showing that both mechanical (pinch) and chemical stimuli (fish extract, methionine, glycine) are able to elicit a differential but clear neural response when applied to an *ex vivo* arm preparation and a distinct movement of the arm (Fouke and Rhodes, 2020).

Physiological nociceptors sensitive to mechanical stimuli were also recorded from the squid fins. Animals subjected to physical injury showed both long-term spontaneous activity and post-injury sensitization to very light von Frey filaments when compared to the sham control (Crook, Hanlon and Walters, 2013). A similar result was obtained in the octopus *Abdopus aculeatus* when the arm was crushed by forceps (Alupay, Hadjisolomou and Crook, 2014) and in the bobtail squid *Euprymna scolopes* in which a lifelong sensitization following early injury was experienced (Howard *et al.*, 2019). Sensitization and facilitated responses after exposure to potential harming stimuli are common physiological features shared across all Eumetazoa. This strengthens the hypothesis that cephalopods are able to experience nociception with an adaptive response, as showed by evidence of actual survival advantage in the context of predation risk. In fact, the same researchers showed that the minor crush injury squids group reduced the latency to display defensive behaviour when exposed to potential predators with respect to the uninjured squids group (Crook *et al.*, 2014).

Furthermore, self-directed behaviours, including focal grooming and active skin removal, were observed to be elicited in octopus after injection with acetic acid in the arm (Crook, 2021).

Finally, while chemosensory and mechanosensory cues have been primed in cephalopods, thermosensation in these invertebrates has not been experimentally described before. Only Crook and colleagues mentioned that squids subjected to high temperatures (45-55°C) did

not respond, but no data were shown in support to that statement (Crook, Hanlon and Walters, 2013).

1.4.3.3 Cellular nociceptors in relation to octopus arm anatomy

Since the 1920s, it was already clear that the chemotactile response in octopus was heavily relying on the extensive arm system as shown by the fact that most of the animals involved in the experiments were blinded prior the actual stimuli exposure (Wells and Wells, 1957; Wells, 1959; Wells, 1964). Another hint of the paramount role of octopus arm in sensing the environment and organise a coherent response, is represented by the number of nervous cells it contains. In fact, 350 million out of the total 500 million nerve cells in the octopus, are found in the arm nervous system alone (Young, 1971). Furthermore, when the interbrachial commissure between the central brain mass and the eight axial nerve cords is severed (Figure 1.5), octopuses were shown to retain a certain level of independent arm complexity (Sumbre *et al.*, 2001).

Putative cellular nociceptors in octopus arm were originally detected in the late 1950s (Rossi and Graziadei, 1956). As previously mentioned, the arm nervous system is represented by the eight axial nerve cords, each composed of a ganglionic part and non- ganglionic cerebro-brachial tracts. The ganglia present an outer layer of cells with different size and morphology, and a filament region called neuropil (Figure 1.8; Bellier *et al.*, 2017).

Each sucker is reached by a single ganglion. The suction caps are composed of an inner part called acetabulum and an external part termed the infundibulum, all surrounded by an epithelium rim (Figure 1.8). The nerve fibres from the neuropil cross the sucker musculature and start branching in small (2-5 μm) and larger (6-15 μm) diameter filaments in the acetabulum while other reach the infundibulum muscles after crossing the epithelium (Rossi and Graziadei, 1956). Graziadei was interested in characterising the cellular structures constituting octopus suckers. Through electron microscopy he was able to identify three classes of cells which share interesting resemblances with vertebrate sensory receptors: *i.* irregular cells with several dendrites were found at the base of the epithelium and were speculated to be responsive to tension; *ii.* pear-shaped cells, found in the lower half of the epithelial layer were hypothesised to be pressure receptors, *iii.* tapered cells with short cilia, located in the superficial part of the epithelium were classified as putative chemoreceptors (Graziadei, 1964).

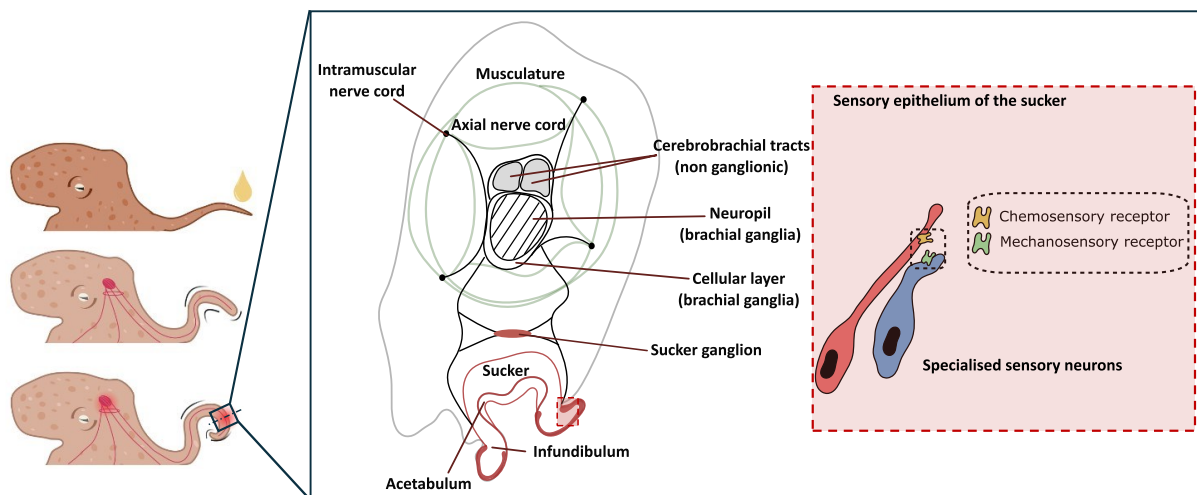


Figure 1.8 Transversal section of octopus arm and details of cellular and molecular sensory receptors.

Surrounded by three layers of musculature (green), the axial nerve cord (black) in the arm is connected to a single sucker (red) through the sucker ganglion. The axial nerve cord is formed by two non-ganglionic cerebro-brachial tracts and a ganglionic region in which a filamentous neuropil and a cellular layer are recognised (adapted from Bellier et al., 2017). A close up of the suckers, reveal two regions, an internal acetabulum and an external infundibulum. Along the extremities of the infundibulum, the red areas represent the sensory epithelium where different specialised neurons were identified to be able to respond to chemical and mechanical stimuli based on their different expression of molecular nociceptors (van Giesen et al., 2020).

1.4.3.4 Experimental evidence of cellular and molecular nociceptors in octopus arm

No functional analysis of the receptors observed by Graziadei was ever conducted until very recently. In 2020, putative sensory cells from *O. bimaculoides* suckers were isolated and their function investigated (van Giesen et al., 2020). Whole-cell patch clamp techniques revealed them to be actual receptors, a long one with thin dendritic endings responding only to certain chemicals (e.g., fish extract) and another one with short, rounded dendritic endings, firing only when mechanically stimulated (Figure 1.8). Analysis of the molecular features of these cells, identified orthologues of previously described proteins. NompC was found as a matching transcript for the mechanoreceptor, sharing genetic and pharmacological properties with the well-characterised *Drosophila* orthologue (Yan et al., 2013). No match was found for the chemoreceptors, thus revealing a cephalopod-specific category of ionotropic receptors whose structure diverged from an ancestral acetylcholine receptor (AChR) to acquire a sensory-related function (Figure 1.8). Furthermore, the analysis of these chemoreceptors across different species of Mollusca, traced back the first appearance in the Coleoid ancestor (Kang et al., 2023). In particular, three different chemotactile receptors lineages were identified, a more AChR-like receptor was found in Decapodiformes whose representatives are mainly activated by bitter substances (CRBs), and two different lineages

found in Octopodiformes, one which mainly binds terpenes (CRTs) and another orphan one whose ligand is still unknown (CRXs).

Cryo-electron microscopy structure of both CRBs and CRTs carried out respectively in the striped dumpling squid *Sepioloidea lineolata* and in the two-spot octopus *O. bimaculoides*, support the phylogenetic reconstruction (Allard *et al.*, 2023).

In fact, both CRs are responsive to prey extracts, have a mecamylamine-sensitive tonic voltage spiking and are lacking key residues for acetylcholine binding. However, squid CRBs are unresponsive to terpenes but are triggered by bitter tastants (e.g., denatonium). Conversely, octopus CRTs are unresponsive to bitter substances but are activated by terpenoids (e.g., polygodial). This difference is due to the binding site structure: squid CRBs rely on specific amino acids forming an electrostatic cage very similarly to the agonist-binding cage of AchR $\alpha 7$. However, the shorter loop C in the CRB allows to accommodate bitter substances but not ACh (Kang *et al.*, 2023).

On the other hand, the binding site of the octopus CRTs is very different from both AchR $\alpha 7$ and CRs as the short loop C and the lack of extensive hydrogen bonding network found in CRBs contribute to abolish the cage structure and form a more open pocket which is more suitable for accommodating hydrophobic compounds like terpenes and oestrogenic steroids (Allard *et al.*, 2023).

The different structures and thus triggering molecules of these CRs, likely evolved to adapt to the distinct predatory strategies and modes of sensory interaction with the environment that evolved across coleoid species (Kang *et al.*, 2023).

1.4.4 Evidence of pain in cephalopods

As mentioned earlier, Bateson's criteria were recently revised to account for the inclusion of more reliable behavioural indicators of higher-order cognition and primary consciousness which underlie the ability to process aversive stimuli into a much more sophisticated response (Birch *et al.*, 2021). In fact, not only Birch and colleagues consider self-directed behaviours and associative learning as critical hints for higher cognition, but they also include impulse control, that is trading-off between rewarding and dangerous options, as a key indicator of animal's subjective experiences (Table 1.2). Additionally, if the organism shows to choose analgesics as valuable cues after receiving harm, we can exclude a reflexive

passive response and imply top-down regulation that leads to flexible active decision-making process instead (Birch *et al.*, 2021).

A first attempt at demonstrating octopus capability of experiencing pain within a more complex experimental setup was recently made (Crook, 2021). In the study, octopuses receiving noxious stimuli (acetic acid injections) were tested in a conditioned place preference test and showed strong avoidance for the chamber in which a noxious stimulus was administered, whereas control animals receiving saline did not show any preference at all. In a second trial, the animals received acetic acid (or saline) in one chamber and a local anaesthetic (lidocaine) injection in a second one. The author observed that only the animals exposed to the aversive stimulus expressed preference for the chamber in which they received anaesthetic injection, thus showing octopus seeks a sort of relief from a pain-like sensation triggered by an actual harm. This behavioural observation was corroborated by electrophysiological recordings, showing spike firing upon acetic acid administration and inhibition when lidocaine was injected (Crook, 2021). Furthermore, self-directed behaviours that could be reminiscent of the discriminative and affective components of pain (see 1.3.1), were also observed. In fact active and focal behaviours triggered by the noxious administration such as hiding (concealment) or grooming of the injured part was reported (Crook, 2021).

These results, added to the breadth of data previously described, has been considered as sufficient evidence to include cephalopods – and on similar basis decapod crustaceans - in the UK Animal Sentience Act (UK Public General Acts 2022 c. 22, 2022). With this document, cephalopods are considered for all intents and purposes as sentient animals.

Table 1.2 Summary of Bateson's original criteria to assess pain in animals and comparison with updated criteria suggested by Birch et al., (2021).

Although to be applied to non-human animals, Bateson's criteria were very biased towards the mammalian experience and knowledge. Birch and colleagues amended these criteria to be more inclusive of unknown animals' experiences such as invertebrates. M: medium confidence; H: high confidence; VH: very high confidence

Bateson (1991) Criterion	Birch et al., (2021) Criterion	Evidence in Cephalopods	Confidence level (Birch et al. 2021)
Possession of receptors sensitive to noxious stimuli, located in functionally useful positions on or in the body	Possession of receptors sensitive to noxious stimuli (nociceptors)	(Giersberg, 1926; Altman, 1971; Crook, Hanlon and Walters, 2013; Hague, Florini and Andrews, 2013; van Giesen <i>et al.</i> , 2020)	VH (behavioural, cellular, electrophysiological, molecular)
Possession of brain structures analogous to the human cerebral cortex	Possession of integrative brain regions capable of integrating information from different sensory sources	(Sanders and Young, 1940; Boycott, 1961; Young, 1963; Wells, 1978; Shigeno <i>et al.</i> , 2018)	VH (histochemical, developmental, structural, morphological)
Possession of nervous pathways connecting nociceptive receptors to higher brain structures	Possession of neural pathways connecting the nociceptors to the integrative brain regions	(Nixon and Young, 2003; Hochner, 2012; Levy and Hochner, 2017)	H (histochemical, structural, behavioural)
Possession of receptors for opioid substances found in the central nervous system, especially the brain	a. Possession of an endogenous neurotransmitter system that reduces nociceptive responses b. Putative local anaesthetics/analgesics/anxiolytics/anti-depressants reduce nociceptive responses	a. (Deutsch <i>et al.</i> , 2023) b. (Crook, 2021)	H (behavioural, electrophysiological)
Analgesics modify response to noxious stimuli and are chosen by the animal when the experience is unavoidable	a. Self-administration of putative analgesics/anaesthetics when injured. b. Conditioned place preference to a location primed with analgesics/anaesthetics, when injured. c. Prioritisation of obtaining these compounds over other needs when injured	a. N/A b. (Crook, 2021) c. N/A	H (behavioural)
Response to noxious stimuli by avoiding them or minimizing damage to the body	Flexible self-protective behaviour (e.g. wound tending, guarding, grooming, rubbing) of a type likely to involve representing the bodily location of a noxious stimulus	(Polglase, Bullock and Roberts, 1983; Alupay, Hadjisolomou and Crook, 2014; Crook, 2021)	VH (behavioural)
Avoidance of noxious stimuli is relatively inelastic	The disvalue of a noxious or threatening stimulus is traded off against the value of an opportunity for reward, leading to flexible decision-making	(Ross, 1971; Boal <i>et al.</i> , 2000)	M (behavioural)
Response to noxious stimuli persists and the animal learns how to associate neutral events with noxious stimuli	Associative learning (pairing of a neutral stimulus with a noxious cue) produces novel ways of avoiding noxious stimuli Note: habituation and sensitisation are not sufficient to meet this criterion	(Mackintosh and Mackintosh, 1963; Fiorito and Scotto, 1992; Tokuda, Masuda and Yamashita, 2015; Kawashima <i>et al.</i> , 2020)	VH (behavioural)

1.4.5 Limitations of direct genetic tractability in octopus in the study of nociception and “pain”

The complexity of octopus neuroanatomy and the preliminary evidence showing potential for pain perception eligibility, certainly make this invertebrate an interesting organism in which studying nociceptive processing as well as the molecular players of nociception. However, cephalopods and octopuses in particular, present a set of bottlenecks that make a direct approach of investigation very difficult.

In the last few years, a great improvement in cephalopod research has been made towards the generation of transgenic lines in order to study the effect of genetic modifications directly on the target animals.

Successful CRISPR-Cas9 gene editing has been carried out both in the squid *Doryteuthis pealeii* and in the sepiolidae *Euprymna berryi*, where the enzymes responsible for the chromatophore production in these species, were deleted, leading to a nearly complete loss of pigmentation in the mutant animals (Crawford *et al.*, 2020; Ahuja *et al.*, 2023). However, the same authors argued that *D. pealeii* currently presents some difficulties, like most cephalopod species, in terms of maintenance and survival at the early life stages that will make the establishing of a direct genetic tractability challenging (Crawford *et al.*, 2020).

This is certainly true for octopus, whose paralarvae stage is quite delicate and makes it difficult for the animals to survive under the current laboratory settings (Iglesias *et al.*, 2007; Vidal *et al.*, 2014) even though significant effort has been put towards the establishment of species-specific standardised requirements (Ponte *et al.*, 2022).

To overcome these limitations, octopuses used in research are generally caught from the wild. This consequently means there is no control over the animal genetic background, ultimately affecting the standardisation of the experimental design. Furthermore, capture and transport of cephalopods for use in research is not currently regulated or standardised across countries and laboratories, despite efforts and proposals in this regard (Pieroni *et al.*, 2022; Sykes *et al.*, 2023) with the risk of impacting both animal welfare and experimental reproducibility.

Finally, cephalopods have been granted protection when used in research under the European legislations, meaning their use is strictly regulated and subjected to the same

ethical regulations and recommendations that apply to vertebrates (Andrews *et al.*, 2013; Smith *et al.*, 2013). These include the need to comply with the 3Rs principles, aiming at cautious experimental design, reduction in the animal utilised and if possible, (full or partial) replacement, through the adoption of non-animal subjects or animals that “are not considered capable of experiencing suffering”.

Evolution represents a critical key point to address these challenges. In fact, as mentioned, nociception is a phenomenon which holds a strong adaptive significance in the animals’ ability to survive. Therefore, we can imagine that successful cellular and molecular solutions have been conserved across organisms (Woolf and Walters, 1991; Smith and Lewin, 2009; Burrell, 2017). Given this premise, we can resort to simpler, more established and well-characterised models such as *Caenorhabditis elegans* to investigate the genetic basis of *O. vulgaris* nociception.

1.5 Model hopping approach: *Caenorhabditis elegans* as a platform for studying molecular nociceptors

1.5.1 Introduction to *C. elegans*

The nematode worm *Caenorhabditis elegans* is a microscopic free-living organism firstly identified in the soil where it feeds on bacteria (Brenner, 1974; Blaxter, 1998). It is formed of two cylindrical elongated structures separated by a fluid-filled chamber, the pseudocoelom, and externally protected by a collagenous cuticle (White, 1988). The Bristol N2 wild type strain has been passaged from the first isolated and cultivated *C. elegans* strain in the laboratory (Félix and Braendle, 2010). It is found as hermaphrodite protandrous, first producing sperm in the late L4 stage and then developing oocytes that are self-fertilised in the adult stage (Hodgkin, Horvitz and Brenner, 1979). After fertilisation, worms reach adulthood within approximately three days, with a life cycle and life stages that are well defined and recognisable (Figure 1.9A).

When observed under the microscope, this organism is quite simple in the behaviours displayed, but this simplicity (e.g., pharyngeal pumping, locomotion, and reproduction) offers good opportunity for an immediate analysis of physiological aspects and their biological basis (Figure 1.9B).

C. elegans and nematodes are characterised by a defined number of cells, which counts 959 somatic cells in the hermaphrodite and 1033 in the male (Sulston and Horvitz, 1977; Sulston, 1983; Alberts *et al.*, 2002; Sammut *et al.*, 2015). This provides the potential of setting up cell-specific investigations aimed at dissecting cell development mechanisms as well as different physiological and neurobiological circuits.

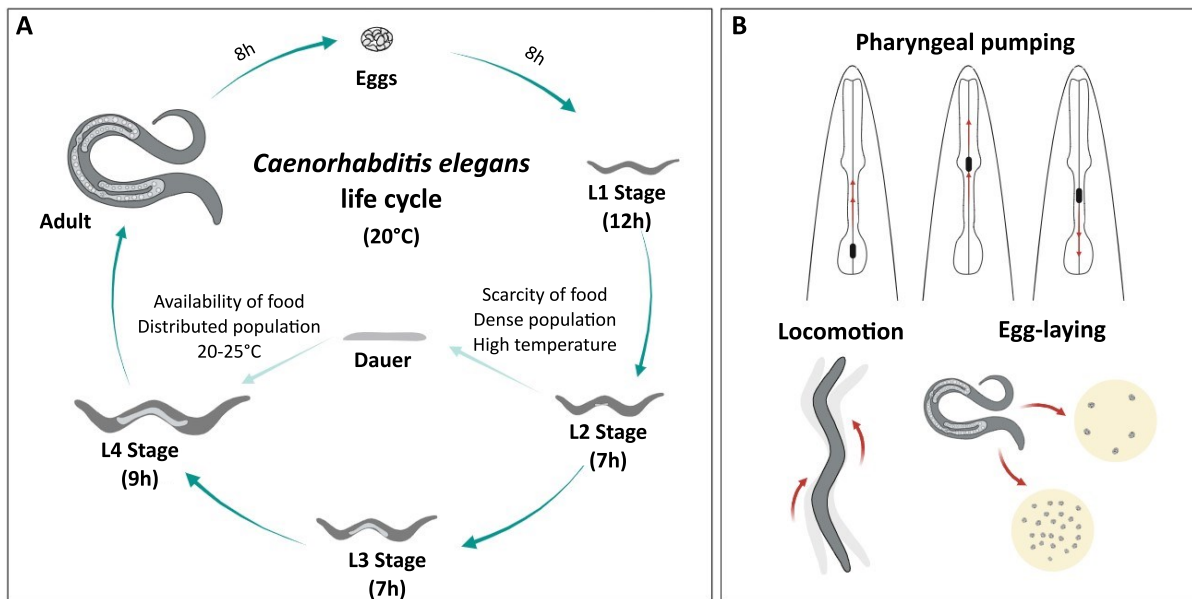


Figure 1.9 *C. elegans* life cycle and measurable physiological features.

A One of the main advantages of *C. elegans* as a model is the rapidity of its development. It takes around three days to obtain a population of adult worms starting from eggs. The transparency of its body allows identification of different developmental stages (e.g., the white crescent vulvar morphology in L4 stage). The effect of environmental stress can also be monitored by looking at normal or perturbed development (i.e., dauer stage). **B** What makes *C. elegans* experimentally convenient is the easiness of screening for drug effects or genetic mutations by looking at well-defined behaviours, such as foraging (i.e. pharyngeal pumping), locomotion and reproduction (mating and egg-laying) which can be usually measured with a counter and a timer.

1.5.1.2 *C. elegans* as a genetic tool to investigate molecular functions in other organisms

The genome of hermaphrodite *C. elegans* is characterised by six pairs of chromosomes, five autosomal pairs, and one pair of sexual chromosomes (XX), whilst males only possess one X chromosome (Hodgkin, 2005).

The genome of *C. elegans* was the first animal genome to be sequenced to near completeness, revealing around 19,000 coding genes (The *C. elegans* Sequencing Consortium, 1998). This number increased to 21,238 with the recent sequencing of the new reference genome, discovering 183 new protein-coding mRNA and 163 new ncRNA (Ichikawa *et al.*, 2025).

Transgenic lines are obtained in *C. elegans* relatively easily by targeting the gonads of adult worms with constructs containing the gene of interest under the worm orthologue- or cell-specific promoter and then investigating the behaviour of the progeny expressing the target gene (Ghanta, Ishidate and Mello, 2021). Pioneering works showing successful heterologous expression of foreign animal genes in *C. elegans* were those introducing *D. melanogaster Hsp70* gene using the fruit fly promoter, proving that the worm transcriptional machinery could recognise it and produce a functional protein in response to temperature stress (Fire, 1986), and the expression of the green fluorescent protein (GFP) from *Aequorea Victoria*, which contributed

to the development of reporter genes, one of the most powerful tool in biology (Chalfie *et al.*, 1994).

It has been estimated that only around 11% of the identified protein-coding genes are nematode-specific, whilst 83% have at least one orthologue representative in humans (Lai *et al.*, 2000), two-thirds of which are disease related genes, thus making *C. elegans* a suitable model organism for the investigation of the genetic basis of healthy and aberrant human physiological mechanisms (Zhang *et al.*, 2020; see also Appendix A).

The first successful heterologous expression of a human gene in *C. elegans* was the *bcl-2* gene that was shown to prevent worm programmed cell death (Vaux, Weissman and Kim, 1992), thus suggesting shared structural and functional domains between the two species despite the high phylogenetic distance (Wheelan *et al.*, 1999; Haerty *et al.*, 2008). On this basis, *C. elegans* has now become an established platform to insert genes of interest (e.g., disease-related genes) in mutant worms of the orthologue genes and observe phenotype rescue or changes in the behaviour (Calahorra and Ruiz-Rubio, 2011; Tissenbaum, 2015; Gaeta, Caldwell and Caldwell, 2019).

Finally, although easy to manipulate, the genome of *C. elegans* is strikingly complex in terms of the evolution it underwent, showing non-random patterns both within and between chromosomes. The reported high rates of chromosomal rearrangements, gene duplication, and intron turnover, gave rise to critical expansion in gene families which are prevalently important in sensory rather than structural functions, once again highlighting the critical role of sensory functions in the animals (Cutter, Dey and Murray, 2009).

1.5.2 The neurosensory system of *C. elegans*

Within its habitat, *C. elegans* worms are constantly stimulated by different cues and environmental perturbations that are sensed through specialised neurons, with the final aim of guiding them towards food and favourable conditions, or away from repellents and potential threats (Bargmann, Thomas and Horvitz, 1990).

1.5.2.1 Specialised sensory neurons in *C. elegans*

The nervous system of *C. elegans* adult hermaphrodites is made up of 302 neurons spread in the ventral nerve cord and head, and in the tail ganglia (Sulston, 1983; Hobert, 2018).

As other Metazoa, nematodes can detect physiological inputs from the environment and modify their behaviour accordingly. Chemical cues which indicate mating partners, presence of food or potential danger are able to trigger substantial changes in the worm, affecting their chemotaxis, the feeding behaviour and reproduction (Bargmann, Thomas and Horvitz, 1990; Hilliard, Bargmann and Bazzicalupo, 2002; Bargmann, 2006). Also, mechanical and thermal cues are responsible for the activation of specialised sensory neurons that negatively or positively impact their behaviour – as for instance, simple withdrawal in the opposite direction of the source of disturbance (Bargmann, Thomas and Horvitz, 1990; Kaplan and Horvitz, 1993; Chalfie *et al.*, 2014). In order to do so, *C. elegans* has around 60 specialised sensory neurons distributed throughout the body with different morphology and gene expression according to their function, ranging from chemosensory to light touch, proprioception, harsh touch and thermal responses (Figure 1.10 and Table 1.3).

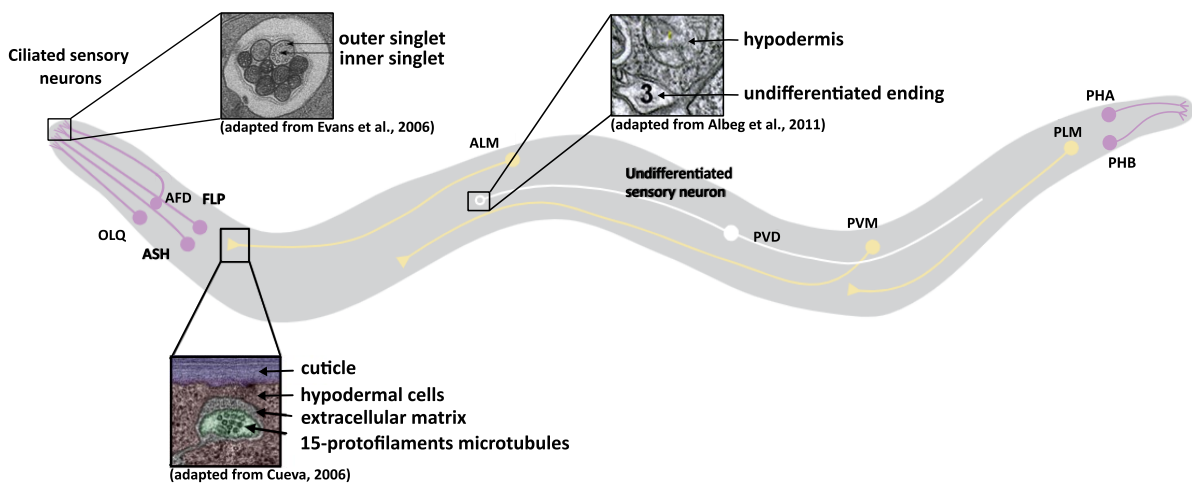


Figure 1.10 *C. elegans* cellular neurosensory system with representative cells involved in chemosensory, mechanosensory and thermosensory responses.

The main cellular sensory receptors can be classified in three categories according to their morphology: ciliated sensory neurons (magenta) constituting the amphids in the head (e.g., ASH, AFD) and phasmids in the tail (PHA, PHB); neurons characterised by endings made up of 15-protofilaments in the internal structures (yellow) distributed across the anterior and posterior part of the body (ALM, PVM); neurons with undifferentiated sensory endings (white), as in the case of the PVD neuron. For each sensory neuron type, a picture showing the microscopic organisation is provided.

1.5.2.2 Chemosensation in *C. elegans*

C. elegans heavily relies on the chemical detection of stimuli from the environment with proper distinction between gustatory and olfactory neurons triggering positive or negative chemotaxis according to the nature and concentration of the stimulus (Bargmann, 2006).

Around 32 chemosensory neurons present ciliated endings that penetrate the cuticle to directly contact the environment through socket or sheath glial cells (Figure 1.11A; Bargmann, Thomas and Horvitz, 1990). These chemosensory neurons are organised in

several groups distributed symmetrically in the head (amphids), inner labial sensilla and tail (phasmids).

C. elegans chemosensory system is organised in a redundant way, with multiple neurons sensing the same compound and multiple cues sensed by one neuron. In most of the cases, repulsive and attractive sensitivities are usually separately processed by different groups of neurons: ASH, ADL, AWB, PHA and PHB for aversive related responses and mainly AWA, AWC, ADF, ASI for attraction (Bergamasco and Bazzicalupo, 2006). Few exceptions are represented by ASE which is mostly involved in water-soluble attractants detection but gets activated by toxic levels of Cu^{++} and Cd^{++} , and ASK which transduce both lysine attraction and quinine and low pH repulsion (Table 1.3; Hilliard et al., 2004).

The neurons involved in the positive chemotaxis are found clustered together in the head with no contribution from the PHA/PHB phasmid cells. In fact, the decision on the direction relies on the constant integration of different chemical gradients throughout the movement, thus representing a “slow” response. On the other hand, neurons involved in chemical aversion are found both in the head and in the tail, activating a reflexive backward response that is more rapid and appropriate for escaping a threatening situation (Bargmann, 2006).

1.5.2.3 Genetic determinants of chemosensory signalling

The ability of *C. elegans* sensory neurons to respond to different chemical cues is due to the elevated number of genes that each neuron expresses. In fact, despite the modest number of neurons, the amount of genes encoding for chemoreceptors is comparable to the vertebrates (Troemel, 1999). Nevertheless, the complete transduction cascades and pathways that lead from the detection of the stimulus to the actual response are still largely unknown in *C. elegans*.

The downstream cascade is usually divided into two major mediators (Bergamasco and Bazzicalupo, 2006).

One is represented by the cGMP gated ion channels (CNGs) TAX-2/TAX-4. These two channels are found in neurons responding to attractants (ASE, AWC) and to volatile repellents (AWB; Coburn, 1996).

The other primary signalling is controlled by the TRPV channels OSM-9/OCR-2 (Figure 1.11B). These two act together in several amphid nociceptors such as the polymodal ASH neuron and in the phasmids (PHA, PHB) to convey chemical repulsion to low pH, water-soluble and

volatile repellents, high osmolarity, heavy metals and, in the case of ASH, also to nose touch mechanical aversive responses (Colbert, Smith and Bargmann, 1997; Tobin *et al.*, 2002). However, the upstream signalling required to detect the actual molecule is in many cases still unknown. Different studies have led to the conclusion that the receptors involved are most likely GPCRs, as suggested by the lack of response to both attractants and repellents in mutant strains of G protein orthologues such as ODR-3, GPA-3 (G α), GPC-1 (G γ) and of GPCR kinases GRK-2 (Figure 1.11B; Bergamasco and Bazzicalupo, 2006). More than 7% of *C. elegans* genes are considered to be involved in chemosensation with an estimated number of 1300 GPCRs, most of which have been associated with neurosensory tissues (Robertson and Thomas, 2006). What is making the research of the cognate receptor difficult is probably the genetic expansion that the nematode chemoreceptor genes underwent, leading to very divergent sequences from the ones recognised in the reference mammalian genomes (Cutter *et al.*, 2009).

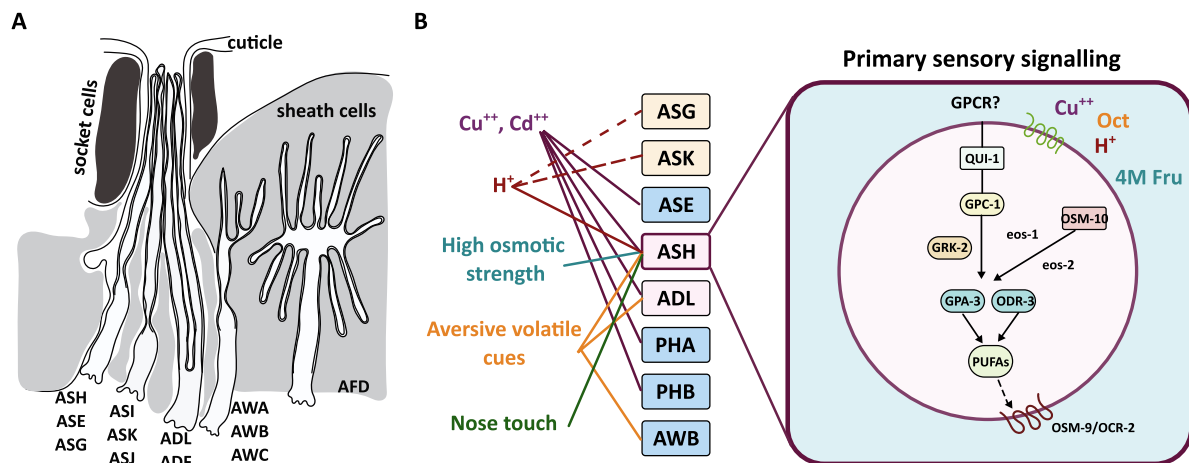


Figure 1.11 Cellular and molecular mechanisms of sensory detection in *C. elegans*.

A Schematic representation of ciliated sensory neurons of *C. elegans* (adapted from Perkins *et al.*, 1986). Detailed structure of the amphid sensory openings showing the socket cells, the sheath cells and ciliated nerve endings protruding outside the cuticle into the open environment as in the case of ASH, ASK and ADL. Other ciliated sensory neurons are not exposed but are found in the sheath cells region (AWA, AWB, AWC, and AFD). **B** Schematic representation of major sensory responses and the sensory neurons they activate, with a close up on the hypothesised signalling pathway in the polymodal neuron ASH (modified from Bergamasco and Bazzicalupo, 2006). Most of the neurons listed (on the left) express at least OSM-9, but those in blue have the primary sensory signalling of chemical cues mostly dependent on the CNGs TAX-2/TAX-4. Neurons indirectly involved in low pH response are in yellow. All three stimuli share a common downstream pathway regulated by the TRPV complex OSM-9/OCR-2 in the ASH neuron (on the right). Hyperosmolarity is controlled by a specific cascade involving OSM-10, while low pH and Cu^{++} might partially share also the upstream signalling. The transduction terminates with the activation of poly unsaturated fatty acids (PUFAs), which work as effectors towards TRPV channels and regulate their activity.

1.5.2.4 Mechanosensation in *C. elegans*

C. elegans is extremely sensitive to mechanical stimuli from the surrounding environment, as these are necessary to trigger or regulate all kind of behaviours such as locomotion, mating and egg-laying and feeding.

According to the localisation of the neurons, the worm will exhibit either backward (if touched on the anterior part of the body) or forward (if touched on the posterior part of the body) locomotion. Despite the simple readout and the low number of neurons involved the initial detection of mechanical cues, *C. elegans* relies on wide connections and integration that will allow it to express distinct behavioural outcomes depending on the strength of the cue, thus discriminating between light touch, proprioception (and interoception) and harsh touch (Table 1.3b; Goodman, 2006).

In *C. elegans*, hermaphrodites possess 45 mechanoreceptive neurons (and the males additional 42 involved in mating behaviour) that can be differently classified in three groups: *i.* neurons with ciliated sensory endings (as the chemosensory ones) such as OLQ, ASH and FLP, localised in the head, *ii.* neurons with sensory endings formed by 15-protofilament microtubules (ALM/AVM in the anterior part of the body, PLM/PVM in the posterior part of the body) and finally *iii.* neurons with undifferentiated sensory endings such as the PVD (Figure 1.10). Finally, other neurons are also involved in both proprioception and responses to different extents of mechanical pressure, such as PVD and the DVA interneuron (Table 1.3b; Goodman, 2006).

The ciliated sensory neurons (at least 26) are involved in the noxious nose touch response that triggers a quick backward response, the 15-protofilament neurons are found to respond to both light and harsh touch in the anterior or posterior part of the body, while undifferentiated sensory neurons are modulatory in proprioception and general mechanosensation (Chalfie *et al.*, 1985).

1.5.2.5 Genetic determinants of mechanosensory signalling and nociception

This fine degree of discrimination seems to be mediated at the molecular level with the activation of different sets of receptors according to the intensity of the stimulus, as in the case of *mec-10* associating with *mec-4* to respond to elicit light touch but with *degt-1* to mediate harsh touch (Li *et al.*, 2011).

Similarly to the chemosensory response, mechanosensory pathways have not been completely dissected yet, despite the large variety of genes found to be implicated in this

sensory response. Their role as directly gated sensors, tethering molecules or modulators of the mechanical response is still under study (Schafer, 2015; Al-Sheikh and Kang, 2020).

The TRPN representative *trp-4* has been associated with dopaminergic neuron-mediated basal slowing response (CEP, PDE and ADE), proprioception (DVA and DVC), and nose touch (CEP; Rivard *et al.*, 2010; Li *et al.*, 2011). Related to mechanosensation, *trp-4* has been found directly responsible for mechanosensitive currents triggered by light nose touch and posterior harsh touch and is therefore suggested as a *bona fide* sensor (Kindt *et al.*, 2007; Kang *et al.*, 2010). Other TRP channels, such as *trpa-1* and *osm-9* are also implicated in nose touch in amphid neurons. However, Ca⁺⁺ imaging in null mutants and electrical currents measured in recombinant systems expressing these receptors, showed a minor or dispensable role of these genes in evoking currents, compatible with a role as modulators rather than sensors (Kindt *et al.*, 2007; Chatzigeorgiou *et al.*, 2010). This is also in line with the proposed role of *osm-9* as a transducer described for the chemosensory responses (Figure 1.11B).

Among the amiloride sensitive ion channels, the above mentioned *mec-4* and *mec-10* have been reported to have a critical and likely direct role in detecting mechanical cues (O'Hagan, Chalfie and Goodman, 2005; Árnadóttir *et al.*, 2011). Their activity is furthermore modulated by the expression of auxiliary proteins such as *mec-2* and *mec-6* (Brown, Liao and Goodman, 2008); *deg-1* is involved in nose touch responses as shown by the strong reduction in mechanosensitive response in *deg-1* mutants (Geffeney *et al.*, 2011), but indications of residual activity, suggest other mechanosensors are probably implicated. Other DEG/ENaC receptors such as *unc-105* and *unc-8*, participate differently to muscle contraction regulation and proprioception as they are expressed in body muscle and motor neurons respectively (Tavernarakis *et al.*, 1997; García-Añoveros *et al.*, 1998). Finally, the well-characterised PIEZO family, presents only one orthologue in *C. elegans*, *pezo-1*. This channel has been found implicated in mechanically induced currents, but in association with regulation of mating, feeding and locomotion, rather than nociception (Brugman, 2020; Hughes *et al.*, 2021; Millet *et al.*, 2021; Romero *et al.*, 2022; Komandur *et al.*, 2023). More recently, the list of molecules has increased in size to include proprioceptive related *asic-2* and *acd-5*, genes expressed in the glial cells surrounding OLQ neurons (*delm-1* and *delm-2*) and the transmembrane channel-like gene *tmc-1* with proposed modulatory or tethering functions along the mechanical signalling cascade (Al-Sheikh and Kang, 2020).

1.5.2.6 Thermosensation in *C. elegans*

C. elegans is considered a generalist species, as it is able to thrive and reproduce between 12 and 26 °C (Anderson *et al.*, 2011; Schulenburg and Félix, 2017). However, steep increase or decrease of temperature below or beyond the physiological range, will produce a strong aversive response (Chatzigeorgiou *et al.*, 2010; Mohammadi *et al.*, 2013). Thermosensation in *C. elegans* has classically been attributed to the AFD ciliated sensory neuron. Laser ablation studies have found this cell to be mainly implicated in both positive and negative thermotaxis (Goodman and Sengupta, 2018). The other two main neurons later found to have a modulatory role are the AWC and ASI (Table 1.3c; Biron *et al.*, 2008; Beverly, Anbil and Sengupta, 2011).

Thermal avoidance has been tested in the context of gradient changes but also using acute stimulation with infrared lasers (Wittenburg and Baumeister, 1999; Glauser *et al.*, 2011; Mohammadi *et al.*, 2013). Apart from the above mentioned ADF and AWC, the neuron FLP and the phasmid PHC were also found to be involved in noxious heat detection (Liu *et al.*, 2012; Mohammadi *et al.*, 2013) and PVD in cold nociception (Chatzigeorgiou *et al.*, 2010).

From a molecular point of view, thermotaxis is regulated by *tax-2/tax-4* whilst the nociceptive avoidance relies on *trpa-1* (Chatzigeorgiou *et al.*, 2010), as well as again *osm-9/ocr-2* (Glauser *et al.*, 2011; Liu *et al.*, 2012) even though it is unlikely they are directly gated but rather sit at the end of a transduction cascade (Bergamasco and Bazzicalupo, 2006).

1.5.3 Assaying nociception in *C. elegans*

Assessing behaviour provides an excellent approach to quantify nociceptive responses in *C. elegans*. One of the major strengths of *C. elegans* biology that contributed to its success as a model is the straightforwardness of its response, which allows the worm to determine whether a stimulus is considered attractive or repulsive (Bargmann and Horvitz, 1990; Kaplan and Horvitz, 1993). When this is coupled to wider experimental tractability it provides mechanistic insights across biological domains.

As defined in this thesis, nociception is a reflexive response and therefore it needs to be rapid, directional and clear. Many acute aversion assays have been developed throughout the years to be able to discriminate different sensitivities to distinct stimuli (Hart, 2006).

1.5.3.1 Acute chemoaversion

For chemical stimuli there is a large plethora of available assays. For assessing acute responses, the drop assay is certainly the most effective at classifying a responsive animal from a defective

one (Hilliard, Bargmann and Bazzicalupo, 2002). This assay consists of applying a drop of a substance in front of the pathway (or on the tail) of a moving worm and recording the reaction when the contact happens. A wild type worm will exhibit a series of backward movements in the opposite direction within a short period of time (3-5 s) and will change its future trajectory. Worms that fail to display this behaviour are classified as sensory defective. This assay can be utilised to test for low pH, organic compounds, heavy metal, and hyperosmotic substances avoidance (Hilliard *et al.*, 2004).

1.5.3.2 Negative chemotaxis

Different assays that evaluate the integration of both positive and negative chemotaxis and the trade-off that the worms display are the food leaving assay, where a noxious cue can be dissolved in the liquid bacterial media and the number of worms leaving the lawn of food is scored at different time points, or the barrier assay (or food race assay) where the worms are put on one end of a large plate and are challenged to reach the patch of food on the other end by passing through a chemical noxious gradient generated at the centre of the plate (Hart, 2006).

Similarly, the quadrant aversion assay consists of an agar plate with four equally sized quadrants in which opposite pairs of quadrants have either noxious or control compounds dissolved in the agar. The worms are put in the centre of the plate and are recorded at different time points to check which quadrants are avoided and which ones are not (Wicks *et al.*, 2000).

These last three assays help identify mutant strains that fail to properly integrate aversive stimuli during the positive chemotaxis and therefore provide an understanding of which molecules are fundamental in the nociceptive circuit and, paired with laser ablation studies, which neurons and interneurons are involved.

1.5.3.3 Volatile aversion

Volatile aversion is usually evaluated similarly to water-soluble molecules, but because of the higher affinity exerted (within the nanomolar range against the micro/milli molar range for chemicals) the assays can be adapted for long-range chemotaxis (Bargmann, 2006). To measure the acute response to volatile repulsive substances, a solution of the compound (e.g., 1-octanol in ethanol) is transferred on either a pipette tip or a thin platinum wire and is then waved in front of the nose of a forward moving animal. The latency to start a reversal movement is recorded and is used as an indication of the animal sensitivity to olfactory cues (Chao *et al.*, 2004; Hart and Chao, 2010).

1.5.3.4 Mechanical aversion

Mechanosensation is elicited differently depending on the type of pressure exerted. As mentioned above, worms are able to discriminate between a gentle and a harsh noxious touch and, according to the localisation of the competent mechanosensory neurons, the worm will show a backward or forward movement. Gentle touch is usually obtained with an eyebrow that is utilised to stroke the body of the worm in a different number of serial trials in which the backward (if stroked on the anterior part) or forward (if stroked on the posterior part) movement is recorded each time (Chalfie *et al.*, 2014). For the ASH-dependent nose touch response, the eyebrow is posed perpendicularly to the path of the animal and the number of times the animal stops or starts moving backward is scored as eliciting a sensory response (Kaplan and Horvitz, 1993). Harsh touch is delivered using a thick platinum wire to tap on the worm anterior or posterior part of the body (Chalfie and Sulston, 1981). The difference between gentle and harsh touch can be seen in the number of backward movements elicited (higher for the harsh touch; Li *et al.*, 2011).

1.5.3.5 Thermal aversion

Finally, thermosensation can again be analysed through acute exposure to heat (infrared laser or heated metal wire) or through the examination of navigation in relation to differently heated sides of a plate (Wittenburg and Baumeister, 1999; Ryu and Samuel, 2002). This allows to observe that not only very cold or very hot temperatures are considered noxious, but that steep temperature changes also contribute to classify the stimulus as dangerous for the worm (Goodman and Sengupta, 2019).

Altogether, the integration of microscopy, genetics and behavioural experimental approaches in a system like *C. elegans* which allows for great malleability, represents a powerful tool for investigating other organisms' understudied genetic basis of nociception.

Table 1.3a Summary of *C. elegans* chemosensory system.

The main sensory neurons, their connections and their major functions are reported. The main molecular determinants known to be involved in sensory responses, and the behavioural outcome are also included. Purple: ciliated sensory neurons, yellow: sensory neurons with 15-prototubulin microtubules endings, white: undifferentiated/non-ciliated sensory neurons, blue: unsheathed ciliated olfactory sensory neurons, orange: interneurons. Motor neurons are not shown. GJ: gap junction.

	Neuron	Response to	Inputs from	Outputs to	Molecules involved in sensory perception expressed	References
Nose/Head	ASH (2)	hyperosmolarity (+ASE+ADF+ASI+ADL), acidic pH, volatile repellent chemicals (quinine, dead <i>C. elegans</i>), heavy metals, detergent, protons, alkaloids	GJ with AIZ, RIC, ADA, RMG	AVA, AVB, AVD, AIB, RIA, AIA	EGL-4; OSM-9, TRPA-1, OCR-2, NPR-1, TMC-1, UNC-8; KQT-3	(Bargmann and Horvitz, 1990)
	ADF (2)	chemotaxis to lysine and cAMP, biotin, Cl ⁻ , Na ⁺ (+ASE+ASG+ASI), response to hypoxic conditions (+ASG), osmolarity (+ASE+ASH+ASI+ADL)	GJ with RIH, ADA, AIA	RIA, AIZ, SMB, AUA, RIR, ADF	OCR-2, OSM-9, TMC-1, NPR-5	(Bargmann and Horvitz, 1990)
	AWA (2)	chemotaxis to diacetyl, pyrazine, trimethyl thiazole	GJ with AIA	AIZ, AFD, AIY, ASE	OCR-1, OCR-2, OSM-9, OSM-10	(Bargmann, 2006)
	AWB (2)	avoidance from 2-nonanone, 1-octanol	GJ with AUA, RMA	AIZ, ADF, RIA, AVB	TAX-2, TAX-4; KQT-3	(Bargmann, 2006)
	AWC (2)	chemotaxis to volatile odorants	AIY, AIB, AIA, ASE, ASI	AIY, AIB, AIA	OSM-9, TAX-2, TAX-4; KQT-3	(Bargmann et al., 1990)
	AFD (2)	CO ₂ sensor (+ASE+BAG)	AIY	AWA, AIN, AIB	TAX-2, TAX-4, TMC-1	(Liu, Schulze and Baumeister, 2012)
	ASE (2)	salt sensation, CO ₂ sensor (+AFD+BAG), chemotaxis to Na ⁺ , Cl ⁻ , cAMP, biotin, lysine, osmolarity response (+ASH+ASH+ADF+ADL)	AIN, AWA, ASI	AIY, AIA, AIB, RIA, AWC	TAX-2, TAX-4, DEL-3, DEL-4, NPR-1, NPR-5, NPR-15, OSM-9, TMC-1, TRPA-2, TRP-2	(Bargmann and Horvitz, 1990)
	ASG (2)	chemotaxis to Na ⁺ , Cl ⁻ , cAMP, biotin, lysine, response to hypoxic conditions (+ADF)	GJ with AIZ, AIN	AIA, AIB, AIM	TAX-2, TAX-4, NPR-1, NPR-5, NPR-15, OSM-9	(Bargmann and Horvitz, 1990)
	ASI (2)	chemotaxis to lysine and cAMP, biotin, Cl ⁻ , Na ⁺ (+ASE+ASG+ADF), osmolarity (+ASE+ASH+ADF+ADL)	GJ with AIA	AIA, AIB, AWC, ASE	TAX-2, TAX-4, NPR-5, NPR-15, NPR-17, KQT-3	(Bargmann and Horvitz, 1990)
	ADL (2)	response to chemical repellents (+ASH+ASE+ASK), heavy metals, hyperosmolarity (+ASE+ASH+ASI+ADF)	GJ with OLQ, RMG	AIA, AIB, AVD, AVB, AVA	OSM-9, OCR-1, OCR-2, TMC-1; KQT-3	(Bargmann and Horvitz, 1990)
	ASJ (2)	contributes to chemotaxis	AIM	ASK, PVQ	OSM-9	(Bargmann and Horvitz, 1990)
	ASK (2)	chemotaxis to lysine, avoidance from protons, detergents, alkaloids	ASJ, AIM (GJ with PVQ, RMG)	AIA, AIM, AIB	OSM-9, FRPR-3	(Bargmann and Horvitz, 1990)
	AQR (1)	O ₂ sensor (+BAG+URX+PQR+SDQ+BDU+ALN+PLN) mediate the responses to ambient O ₂ levels	DVA	AVB, AVA, PVC, AVD	NPR-1; TMC-1; KPC-1, TAX-2, TAX-4	(Bargmann, 2006)
	URX (2)	O ₂ sensor (+BAG+AQR+PQR+SDQ+BDU+ALN+PLN) mediate the responses to ambient O ₂ levels	URB, RIR, gap junctions with AUA, RMG, IL2	RIA, AUA, AVE, RIG, AVB, RIC	TAX-2, TAX-4, NPR-1, PDFR-1, TMC-1	(Witham et al., 2016)
Anterior	BAG (2)	O ₂ sensor (+URX+AQR+PQR+SDQ+BDU+ALN+PLN) mediate responses to ambient O ₂ levels	AIN, RIG, AQR (GJ with RIR, RIG)	RIA, RIB, AVE, RIG		(Bargmann 2006)
	AIY (2)	modulates chemosensation	AFD, ASE, AWC, AWA (GJ with RIM)	AIZ, RIA, RIB	C50F7.1, CKR-2; NPR-11, NPR-14	(Bargmann 2006)
	AIZ (2)	modulation of chemotaxis	ADF, ASE, AWA, AIY, RIR, AWB, RIH, HSN, (GJ with ASH, ASG)	RIA, SMB, AIB, RIM, AIY, AVE		(Mori, 1999)
	PQR (1)	O ₂ sensor, weakly CO ₂ responsive	GJ with PVP interneuron	AVA, AVD	TAX-2, TAX-4, NPR-1, PDFR-1	(Bargmann 2006)
Posterior	PHA (2)	regulate chemotactic avoidance	GJ with PVP interneuron	PHB, DVA	OCR-2, OSM-9, TRPA-1, NPR-1, PDFR-1; KQT-3	(Bargmann 2006)
	PHB (2)	regulate chemotactic avoidance	PHA	AVA, PVC, AVD	OCR-2, OSM-9, TRPA-1, NPR-1; KQT-3	(Bargmann 2006)

Table 1.3b Summary of *C. elegans* mechanosensory system.

The main sensory neurons, their connections and their major function are reported. The main molecular determinants known to be involved in sensory responses, and the behavioural outcome are also included. Purple: ciliated sensory neurons, yellow: sensory neurons with 15-protofilament microtubules endings, white: undifferentiated/non-ciliated sensory neurons, blue: unsheathed ciliated olfactory sensory neurons, orange: interneurons. Motor neurons are not shown. GJ: gap junction, AHT: anterior harsh touch, HT: harsh touch, PHT: posterior harsh touch, AnHT: anus harsh touch

	Neuron	Response to	Inputs from	Outputs to	Molecules involved in sensory perception expressed	References
Nose/head	ASH (2)	NT	gap junctions with AIZ, RIC, ADA, RMG	AVA, AVB, AVD, AIB, RIA, AIA	EGL-3/4; OSM-9, TRPA-1, OCR-2, NPR-1, TMC-1, UNC-8; KQT-3; FRPR-3 FLP-1	(Kaplan and Horvitz, 1993)
	OLQ (4)	NT	CEP, IL2, RIH	RIH	TRPA-1, OSM-9, OCR-4, NPR-1, PDFR-1	(Kaplan and Horvitz, 1993)
	OLL (2)	NT	CEP, ADE (GJ with IL1)	AVE, CEP	PDFR-1	(Schafer, 2015)
	IL1 (6)	NT	IL2, CEP (GJ with OLL)	RMD, URA, RIP	MEC-6, TRPA-1	(Schafer, 2015)
	IL2 (6)	NT	GJ with RMG and URX	RIP, RME, URA, RIH, OLQ, ADE	NPR-1	(Schafer, 2015)
	FLP (2)	NT, AHT (+BDU, SDQR, ADE, AQR)	ADE (GJ with RIH, AVD)	AVA, AVD, AVB, ADE	OSM-9, MEC-10, DEL-1, UNC-8, PDFR-1	(Kaplan and Horvitz, 1993)
	AQR (1)	AHT (+FLP, BDU, SDQR, ADE)	DVA,	AVB, AVA, PVC, AVD	NPR-1; TMC-1; KPC-1, TAX-2, TAX-4	(Schafer, 2015)
	ADE (2)	AHT (+FLP, BDU, SDQR, AQR)	BDU, FLP, AVM, IL1, IL2	OLL, CEP, FLP	TRP-4	(Schafer, 2015)
	CEP (4)	head mechanosensation	OLL, ALM, RIH, ADE	AVE, OLL, OLQ, IL1	TRP-4	(Schafer, 2015)
	AVA (1)	ALT, HT(+AVD+AVE)	AVD, AVE, AVB, PVC, ADE, PLM, PHB, PQR	PVC, ventral cord motor neurons	UNC-8, NEP-11, FLP-1	(Bargmann and Horvitz, 1990)
	AVD (1)	ALT, HT (+AVA+AVE)	ASH, ADL, FLP, PVC, AQR, PLM, AVB, PHB, FLP, ADA, AVM	AVA, ventral cord motor neurons	UNC-8, PDFR-1, EGL-3	(Bargmann and Horvitz, 1990)
	AVE (1)	ALT, HT (+AVA+AVD)	OLL, CEP, DVA, FLP, AVB	AVA, ventral cord motor neurons	TWK-46, FLP-1	(Bargmann and Horvitz, 1990)
Anterior	AVB (2)	mechanosensation	RIF, RIM, AVM, PVC, ASH, PVR, PVP, SDQ, ADA, AQR, FLP, AIB, AVF, ADL, URX, RIB, SIB, DVA, RID, SDQ	AVA, hypodermis	UNC-8, TWK-46, EGL-3	(Bargmann and Horvitz, 1990)
	ALM (2)	ALT, AHT	GJ with AVM	BDU, PVC, CEP	ASIC-1, DEL-1, MEC-2, DEGT-1, MEC-4/6/9/10, PDFR-1; KQT-3, ANOH-1, UNC-36	(Kaplan and Horvitz, 1993)
	AVM (1)	ALT	GJ with ALM, AVD	AVB, PVC, BDU, ADE	PDFR-1, UNC-36, EGL-3	(Kaplan and Horvitz, 1993)
Posterior	BDU (2)	AHT (+FLP, SDQR, ADE, AQR)	ALM, AVM	ADE	-	(Schafer, 2015)
	PLM (2)	posterior light touch	GJ with PVC	DVA, AVA, PDE, AVD	MEC-2/4/6/9/10, ANOH-1, PDFR-1; KQT-3	(Kaplan and Horvitz, 1993)
	PVM (1)	stretch receptor	GJ with PDE	<i>en passant</i> synapses with PDE, PVC	MEC-2/4/6/9/10, PDFR-1, ANOH-1, UNC-36, EGL-3	(Kaplan and Horvitz, 1993)
	PDE (2)	PHT (+PVD, PHA, PHB)	PVM, PLM (GJ with PVC)	DVA, hypodermal cells	TRPA-1, TRP-4	(Schafer, 2015)
	SDQR (1)	AHT (+FLP, BDU, ADE, AQR)	GJ with AVB	AVB, AVA, DVA	-	(Li et al., 2011)
	PHA (2)	AnHT (+PVD, PDE, PHB)	GJ with PVP interneuron	PHB, DVA	OCR-2, OSM-9, TRPA-1, NPR-1, PDFR-1; TMC-1	(Li et al., 2011)
Anus	PHB (2)	AnHT (+PVD, PDE, PHA)	PHA	AVA, PVC, AVD	OCR-2, OSM-9, TRPA-1, NPR-1, NLP-1, PDFR-1	(Li et al., 2011)
	DVA (1)	AnHT (+PVC)	PDE, PHC, PLM, PHA, PVD	AVE, AVA, AQR, AVB, PVC	TRP-4, UNC-36	(Li et al., 2011)
	PVC (1)	PHT, AnHT (+DVA)	ALM, AVM, AQR, AVA, PHB, PHC, PVM, DVA, PLM, ADE	AVB, AVD, AVA, AVE	PDFR-1, UNC-36, EGL-3	(Li et al., 2011)
	PVD (2)	PHT (+ PDE), AnHT (+PDE, PHA, PHB)	AVA	AVA, PVC	MEC-4/6/9/10, DEGT-1, OSM-9 TRPA-1, KPC-1	(Chatzigeorgiou et al., 2010)

Table 1.3c. Summary of *C. elegans* thermosensory system.

The main sensory neurons, their connections and their major function are reported. The main molecular determinants known to be involved in sensory responses, and the behavioural outcome are also included. Purple: ciliated sensory neurons, yellow: sensory neurons with 15-protofilament microtubules endings, white: undifferentiated/non-ciliated sensory neurons, blue: unsheathed ciliated olfactory sensory neurons, orange: interneurons. Motor neurons are not shown. GJ: gap junction.

	Neuron	Response to	Inputs from	Outputs to	Molecules involved in sensory perception expressed	References
Nose/Head	FLP (2)	head noxious temperature (35-38°C + AFD)	ADE, (GJ with RIH, AVD)	AVA, AVD, AVB, AIB, ADE	OCR-2, OSM-9	(Liu et al., 2012)
	AFD (2)	thermosensor (+AWC+ASI; 15-15°C); sense head noxious temperature (+FLP 35-38°C)	AWA, ASE, AIN (GJ with AIZ, AIB)	AIY (acting on AIZ and RIA), AWA, AIN, AIB	TAX-2, TAX-4, TMC-1, PKC-1 (TTX-4), TTX-3	(Luo et al., 2014)
	AWC (2)	head noxious temperature (+ AFD)	AIY, AIB, AIA, ASE, ASI	AIY, AIB, AIA	OSM-9, TAX-4	(Liu et al., 2012)
	ASI (2)	thermosensation response temperature	GJ with AIA	AIA, AIB, AWC, ASE,	NPR-5, NPR-15, OSM-9, OCR-1, OCR-2,	(Okahata et al., 2019)
	ADL (2)	regulation of cold tolerance	GJ with OLQ, RMG	AIA, AIB, AVD, AVB, AVA	TMC-1; KQT-2; KQT-3, KQT-2; KQT-3	
	ASJ (2)					
	AIY (2)	modulates thermosensation of physiological temperatures	AFD, ASE, AWC, AWA (GJ with RIM)	AIZ, RIA, RIB	C50F7.1, CKR-2; NPR-11, NPR-14	(Liu et al., 2012)
	AIZ (2)	thermotaxis (isothermal tracking)	ADF, ASE, AWA, AIY, RIR, AWB, RIH, HSN, (GJ with ASH, ASG)	RIA, SMB, AIB, RIM, AIY, AVE		(Bargmann, 2006)
	AIA (2)	thermosensation	ASK, ASG, ASH, ADL, PVQ, AIM, ASE, AWC, AIZ, AIB (GJ with ASI, AWA, ADF)	AIB, RIF, AWC	NPR-5, NPR-11	(Bargmann, 2006)
	AIB (2)	modulate thermosensation of noxious heat	AIA, ASE, ADL, ASH, AWC, ASG, AIZ, ASK, ASI, RIM, RIB, FLP (GJ with DVB, DVC, RIG, AFD, RIS, RIV)	RIM, AVB, RIB	-	(Bargmann, 2006)
Posterior	RIA (2)	thermotaxis	ADF, SMD, RMD, AIZ, RIH, AIY, AUA, BAG, AQR, URX, RIR, RIB, ASH, AWB, RIV	RIV, SMD, RMD	-	(Bargmann, 2006)
	RMG (2)	integrates thermal signals from AFD	ADE, CEP, RMF, (GJ with AWB, RMH, RMF, ASK, IL2, ASH, ADL, URX)	RMD, AVE	NPR-1	(Glauser et al., 2011)
Anus/Tail	PHC (2)	tail noxious temperature (35-38°C)	GJ with VA12 and DA9 (ventral cord motor neurons)	DVA, PVC	OCR-2, OSM-9	(Liu et al., 2012)
	DVA (1)	modulate tail heat noxious sensation	PDE, PHC, PLM, PHA, PVD	AVE, AVA, AQR, AVB,	TRP-4	(Liu et al., 2012)
	PVC (2)		ALM, AVM, AVJ, AQR, AVA, PHB, PHC, PVM, DVA (GJ with AVA, PLM, PDE)	PVC, AVB, AVD, AVA, AVE	PDFR-1	
	PVD (2)	cold sensation (from 20°C to 15°C)	AVA	AVA, PVC	DEGT-1, OSM-9 TRPA-1	(Chatzigeorgiou et al., 2010)

1.6 Aims of the project

Based on the available knowledge from literature developed in the introduction, complex nociception exists in octopus.

However, substantial gaps, such as the characterisation of the molecular determinants and their role in the physiological nociceptive pathway will facilitate the ability to better resolve and contextualise if a pain state exists too.

This is important on at least three levels: *i.* Scientifically, by increasing our current knowledge on a species that has a fascinating neurobiology, *ii.* Culturally, by elevating the precautionary principle to an experimentally proven status that actively maintains the debate on the importance of animal welfare *iii.* Economically, as cephalopods are currently exploited as a food source but no regulation on the capture, transport and humane killing are in place.

The current limited direct genetic tractability poses some challenges in terms of investigating the function of genes in the intact animal. However, phenomena that bare a strong value for the animal survival such as nociception, are characterised by conserved features throughout evolution. In fact, even though cephalopod-specific chemoreceptors have been identified, we can still expect cephalopods to share some of the conserved classes of proteins for sensory detection, as seen in the case of the mechanosensory TRPN orthologue of NompC (van Giesen et al., 2020).

A first survey of *O. vulgaris* transcriptome in search for conserved molecular nociceptors was carried out at the Stazione Zoologica Anton Dohrn in Naples and represents the basis on which this thesis was further developed upon (Di Cristina, 2017).

The present PhD project is built around three papers that develop the identification and functional characterisation of cellular nociceptors in octopus through a model hopping approach that utilizes *C. elegans* and is complemented by experiments carried out in recombinant systems such as HEK293 cells and *Xenopus laevis* oocytes.

In the first paper, I developed a strategy to search for *O. vulgaris* putative genes involved in nociception based on the shared homologies with other animals. A combination of literature review and *in silico* analysis was adopted to look for critical players belonging to conserved protein families known to be (positively and negatively) involved in nociceptive pathways. I then utilised *C. elegans* as a sensitive platform for shortlisting nociceptive candidates, using a

behavioural assay aimed at discriminating sensitivity and deficits in response to chemical aversive cues.

In the second paper, I focussed on one of the candidates highlighted by the work carried out in the first paper: the polymodal nociceptor TRPV. I identified two TRPV channels in *O. vulgaris* whose function is benchmarked as molecular detectors with polymodal potential (chemo- and mechano-sensory) when heterologously expressed in *C. elegans* loss of function mutants of the orthologue genes. Additionally, expression in the *Xenopus* oocyte recombinant system suggests the octopus TRPVs form a functional heteromeric channel directly gated by nicotinamide, an invertebrate bitter repellent

In the third paper, the search for potential anti-nociceptive genes in *O. vulgaris*, led to focussing on the closest orthologue of opioid receptors, the allatostatin C receptor. The recent findings demonstrating the role of allatostatin C in suppressing invertebrate nociceptive responses (Bachtel *et al.*, 2018; Liu *et al.*, 2023), justified the interest for exploring this signalling system in *O. vulgaris*. I identified two allatostatin C receptors and a single mature peptide and demonstrated its ability to differently activating them.

Altogether, these data highlight the model hopping approach as an invaluable tool in defining molecular basis of nociception. The generic role of TRPV as a polymodal molecular detector of nociceptive cues is identified. This should support further investigation of octopus utilisation through reduced recombinant experiments and translation into octopus experiments. These findings open routes to better frame questions around whether octopus nociception underpins a deeper potential to express neural substrates of pain that are emerging and have led to legislative changes.

The explicit of the current aggregated papers from this thesis are discussed in terms of how emerging molecular understanding of cephalopod biology can inform the scientific and cultural debate that impacts the experimental and economic use of these important marine organisms.

Chapter 2 Identification of molecular nociceptors in *Octopus vulgaris* through functional characterisation in *Caenorhabditis elegans*

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Supplementary Tables S1-S3 are electronic files accessible from the journal:

<https://doi.org/10.1242/bio.062268>

2.1 Abstract

Nociception, a phenomenon crucial for animal survival, deploys evolutionarily conserved molecular mechanisms. Among invertebrate species, cephalopods are of particular interest as they possess a well-developed brain speculated to be able to encode pain-like states. This has led to their inclusion in the Directive 2010/63 EU for welfare protection.

However, the molecular mechanisms of nociception in cephalopods are still poorly characterised and it is important to address this knowledge gap to better understand cephalopods' capacity to express pain states. Here we describe a bioinformatic strategy utilising conserved nociceptive genes, to identify the orthologous candidates in the *Octopus vulgaris* transcriptome. We identified 51 genes we predict to function in nociception. These add to the mechanosensory TRPN and the unique chemotactile receptors recently identified in octopus suckers, thus expanding the set of genes that merit further functional characterisation in cephalopods. We therefore selected 38 orthologues in *Caenorhabditis elegans*, a tractable experimental platform and tested loss of function mutant strains of distinct functional gene classes (e.g., *osm-9*, *egl-3*, *frpr-3*) in a low pH avoidance paradigm. This identified 19 nociceptive-related genes to be prioritised for further functional characterisation in *O. vulgaris*.

2.2 Introduction

The ability to detect potential noxious stimuli in the environment involves the triggering of specialised sensory neurons called nociceptors which activate a simple reflex response that organises a withdrawal behaviour of the animal from a potential threat (Sherrington, 1906; Dubin and Patapoutian, 2010). The number and type of modalities of noxious stimuli detected by these cells is encoded by the molecular determinants which define the transduction and integration of such environmental cues (Wood, 2006; Dubin and Patapoutian, 2010). The crucial adaptive value of nociception in contributing to increase the individual survival, made it a conserved feature across Eumetazoa (Smith and Lewin, 2009).

In cephalopods, evidence of nociception is found in *ex vivo* and *in vivo* experimental observations in which noxious cues delivered to the arms or mantle of the animal, are able to trigger withdrawal responses but also associated neurophysiological features such as post-injury sensitization (Crook, Hanlon and Walters, 2013; Alupay, Hadjisolomou and Crook, 2014; Crook *et al.*, 2014; Howard *et al.*, 2019). As an example, acetic acid exposure in *ex vivo* preparations of octopus cut arms is able to trigger a reflexive retreat of the limb (Hague, Florini and Andrews, 2013). Similarly, a complex response is suggested to be organised in the central brain to produce secondary

protective behaviours such as grooming and concealing of the arm after acetic acid exposure in alive octopuses that suggests a discriminative component of pain (Crook, 2021). The increasing interest in nociception and its processing in these cephalopods derives from the recognition of their sophisticated neuroanatomical central organization. This likely entails a top-down regulation of modulatory nerve signalling underlying the potential to exhibit pain pathways. Due to this possibility, as a precautionary principle, cephalopods are currently subjected to legislation that protects their welfare when used in research (European Parliament and Union, 2010). However, to fully address the question around pain perception, a broader understanding of the molecular determinants of nociception in cephalopods is required. To date, only two molecular classes of sensory detectors have been experimentally identified in *O. bimaculoides* (van Giesen *et al.*, 2020) and *Sepioloidea lineolata* (Kang *et al.*, 2023). These are located in the sensory epithelium surrounding the suckers which harbours putative cellular nociceptors that morphologically resemble mammalian sensory neurons (Rossi and Graziadei, 1956). One was identified as a NompC (TRPN) orthologue, activated by mechanical stimulation whilst the other class was found to be a phylum-specific ion channel, sensitive to both attractants and aversive cues (Kang *et al.*, 2023). This provides evidence of both conserved and exclusive molecular components of sensory detection in cephalopod (van Giesen *et al.*, 2020).

Most of the direct investigation of nociceptive processing in cephalopods is constrained by their limited experimental genetic tractability. These limitations derive from a series of challenges posed by culturing these animals, especially considering the very delicate early life stages (Iglesias *et al.*, 2007; Vidal *et al.*, 2014) and the strict requirements this species needs in terms of accommodation, nutrients, and temperature supplies (Ponte *et al.*, 2022). Therefore, currently most of the work carried out on *O. vulgaris* is based on animals that are caught from the wild in a non-standardised way (Pieroni *et al.*, 2022; Sykes *et al.*, 2023). This generates two major issues, one is the complete absence of control over their genetic background and the second one is represented by the variable animal welfare status after capture and transport. However, the highly conserved genetic basis of nociception across the animal phyla provides an opportunity to take an indirect, but nonetheless, informative approach.

Here we conducted an *in silico* analysis to identify molecular candidates for nociception in the *O. vulgaris* transcriptome. To provide insight into their function we identified their *C. elegans* orthologues and tested loss of function mutants for deficits in a simple assay of chemical nociception by exposing mutant nematode strains to different noxious cues, including low pH, a

common aversive cue in both species (Sambongi *et al.*, 2000; Hague, Florini and Andrews, 2013; Crook, 2021).

The approach using low pH highlighted 19 nociceptive-related genes prioritised for functional characterisation, reinforcing the molecular conservation of gene families in distinct organisms and mapping out a platform through which experimentally intractable cephalopod genes can be investigated in *C. elegans*.

2.3 Materials and Methods

2.3.1 *In silico* analysis

2.3.1.1 Resources to produce a list of conserved Eumetazoan nociceptive related genes

An in-depth literature review of conserved molecular nociceptors was performed by surveying around 400 peer reviewed journal articles on animal nociception from well-characterised vertebrate and invertebrate Eumetazoa.

This work was complemented with information taken from databases focussed on human pain genetics: 1) [Human Pain Genes Database](#) (Meloto *et al.*, 2018) a collection of nociceptive relevant genes resulted from 294 studies reporting 434 associations between genetic variants and pain phenotypes; 2) [Pain Research Forum](#), curated by the International Association for the Study of Pain (IASP) and 3) [Pain Genetics MOGILAB](#) (McGill University, Montreal, Canada).

Finally, the queries “pain perception” and “opioid activity” were searched within Gene Ontology (GO) Resource (Carbon *et al.*, 2009). The latter was included on the basis that this search term would highlight candidate molecular components that mediate or modulate anti-nociceptive pathways. The results under the “genes and gene products” label were selected for further analysis.

2.3.1.2 Gene blast against *O. vulgaris* transcriptome and manual curation of sequences

The search for orthologue nociceptive and antinociceptive candidate genes in *O. vulgaris* was performed by iterating the Uniprot amino acid sequence (UniProtKB/Swiss-Prot release 2021_04) against *O. vulgaris de novo* assembly transcriptome (Petrosino, 2015; Petrosino *et al.*, 2022). The transcriptome is based on 64,477 assembled and filtered transcripts with a calculated median length of 795 bp, average length of 1308 bp, minimum length of 201 bp and maximum of 20031 bp (Petrosino, 2015). The assembled *O. vulgaris* transcriptome is available at <https://zenodo.org/records/17820138>.

The BLAST search was carried out using the TBLASTN algorithm (BLAST+ v2.10.0+). A standard e-value of $10e^{-5}$ was used as a cut-off for the BLAST search except for neuropeptide precursors for which it was set as $10e^{-2}$ and BLOSUM62 as the scoring matrix. The rationale for the latter reduced stringency is due to the known propeptide variation relative to highly conserved and short length mature and active peptide component (Baggerman *et al.*, 2005; Akhtar *et al.*, 2011). When multiple hits were found, we considered the contig sequences with the e-value closest to 0 and a high query coverage (at least 40%) for further analysis. To check for sequence completeness we performed multiple alignments of our *O. vulgaris* predicted protein and curated sequences from other species using Clustal omega (Madeira *et al.*, 2022). Incomplete sequences from the BLAST search that we failed to reconstruct were discarded. A set of bioinformatic tools was utilised to check the quality of the pre-existing automated annotations by manually curating the sequences. The nearest species with a sequence matching the candidate provided was retrieved from NCBI Blast (Altschul *et al.*, 1990). A prediction of the protein function pathways (GO terms for “Biological Process”, “Molecular Function” and “Cellular Component”) was obtained via InterProScanSearch (Blum *et al.*, 2021) following translation of the target transcript on ExPASy Translate (Gasteiger *et al.*, 2003). The analysis of the conserved domain, the family and shared structure of the protein was also performed, using NCBI conserved domain (Lu *et al.*, 2020) and HHPRed (Hildebrand *et al.*, 2009).

2.3.1.3 Refinement of candidate *O. vulgaris* nociceptive genes

Refinement of the final *O. vulgaris* sequences to be blasted against the *C. elegans* genome was performed by taking into account the available relative gene expression of the candidates in *O. vulgaris* (Petrosino, 2015; Petrosino *et al.*, 2022). To this end, specific tissues were considered, based on their relevance in neurosensory pathways: tip of the arm (TIP), selected due to previous studies showing it is enriched in sensory receptors including those involved in nociceptive responses (Graziadei, 1964; van Giesen *et al.*, 2020); supra-oesophageal mass (SEM), sub-oesophageal mass (SUB) and optic lobe (OL), constituting the central brain mass fundamental in information processing and thus potentially implicated in the elaboration of nociceptive and anti-nociceptive responses. This process led to the selection of a specific representative for each gene when multiple plausible hits were found.

2.3.1.4 Identification of *C. elegans* orthologues for candidate *O. vulgaris* nociceptive genes

C. elegans orthologues of the hits derived from the approaches described above, were retrieved from Wormbase resource (Davis *et al.*, 2022) through the available BLASTp tool (version WS283, Bioproject PRJNA13758) using default parameters (threshold e-value: $1e+0$).

To compare the degree of molecular conservation between *O. vulgaris* and *C. elegans*, the identity of the protein functional domains was compared with Expasy-SIM tool using BLOSUM62 as the comparison matrix (Huang and Miller, 1991). Where multiple hits were found, the selection was made based on literature evidence for their implication in sensory responses or on their expression in sensory neurons (e.g., *npr-17* as allatostatin C receptor representative within its suggested 13 orthologues; Beets *et al.*, 2023).

2.3.2 *C. elegans* strains and husbandry

The following nematode strains were utilised in this study: CB1124 *che-3* (*e1124*); CB75 *mec-2* (*e75*) [STOM]; TU253 *mec-4* (*u253*) [ASIC]; CB1611 *mec-4* (*e1611*) [ASIC]; CB1515 *mec-10* (*e1515*) [ASIC]; *acd-1/deg-1* (*bz90/u38u421*) [FaNaC]; TQ225 *trp-1* (*sy690*) [TRPC]; RB1052 *trpa-1* (*ok999*) [TRPA]; TQ296 *trp-4* (*sy695*) [TRPN]; CX10 *osm-9* (*ky10*) [TRPV]; CX4544 *ocr-2* (*ak47*) [TRPV]; FG125 *ocr-2* (*ak47*), *osm-9* (*ky10*), *ocr-1* (*ak46*) [TRPV]; EJ1158 *gon-2* (*q388*) [TRPM2]; LH202 *glt-2* (*tm1463*) [TRPM3]; PT8 *pkd-2* (*sy606*) [TRPP]; AG405 *pezo-1* (*av143*) [PIEZO]; RB883 *kqt-2* (*ok732*) [KCNQ]; TM542 *kqt-3* (*tm542*) [KCNQ]; RB1392 *shk-1* (*ok1581*) [KCNA]; *twk-46* (*tm10925*) [KCNK]; CB251 *unc-36* (*e251*) [CACNA2D3]; VC48 *kpc-1* (*gk8*) [PCSK1]; MT150 *egl-3* (*n150*) [PCSK2]; MT1218 *egl-3* (*n588*) [PCSK2]; VC671 *egl-3* (*ok979*) [PCSK2]; MT1071 *egl-21* (*n476*) [CPE]; *acn-1* (*tm12662*) [ACE]; *acn-1* (*tm8421*) [ACE]; BR2815 *nep-1* (*by159*) [NEP]; JN356 *nep-2* (*pe356*) [NEP]; VC2171 *tkr-1* (*ok2886*) [TKR]; RB1340 *nlp-1* (*ok1469*) [TK]; VC2565 *frpr-3* (*ok3302*) [FMRFaR]; NY7 *flp-1* (*yn2*) [FMRFaR]; NY16 *flp-1* (*yn4*) [FMRFaR]; *npr-17* (*tm3210*) [AstCR/OPRL]; RB2030 *nlp-3* (*ok2688*) [AST/OP-like]; FX03023 *nlp-3* (*tm3023*) [AST/OP-like]; *anoh-1* (*tm4762*) [ANO]; CX14295 *pdf-1* (*ok3425*) [CGRPR]; RB1546 *tmc-1* (*ok1859*) [TMC]; FK100 *tax-2* (*ks10*) [CNG]; PR671 *tax-2* (*p671*) [CNG]; PR678 *tax-4* (*p678*) [CNG]; BR5514 *tax-2/tax-4* (*p671/p678*) [CNG]; VC9 *nca-2* (*gk5*) [NALCN]. The Bristol N2 was used as the wild-type strain.

C. elegans hermaphrodite worms were cultured and maintained as described in Brenner (1974). Single colonies of saturated LB broth cultures containing *Escherichia coli* OP50 were used to seed 15 mL nematode growth medium (NGM) agar plates (50 µL per 55 mm diameter plate). Plates containing *C. elegans* were then sealed with parafilm to prevent contamination and kept at 20°C. Three days prior to any assay, gravid adult worms were put on culturing plates to lay eggs for 4h and then removed to produce an age-synchronised population to be tested at the L4+1 day (young adult) stage. All the experiments have been carried out in compliance with the Ethics and Research Governance Online II (ERGO II) policy (nr 79739) in place at the University of Southampton.

2.3.3 Drop assay to test acidic aversion in *C. elegans*

To test acidic aversion in *C. elegans* we have used the classical acute aversion test, with modifications (Hilliard, Bargmann and Bazzicalupo, 2002; Hilliard *et al.*, 2004).

On the experimental day, ten L4+1 day old worms were transferred onto a 9 cm unseeded NGM plate (20mL) and left undisturbed for 20 min, in order to favour the transition from local area search (with high levels of reversal) to dispersal behaviour with low reversals and forward movements (Gray, Hill and Bargmann, 2005). A small drop of noxious cue was delivered in front of a moving worm through a small glass capillary (1.0mmOD, 0.78 mm ID, Harvard Apparatus, USA) attached to a 3 mL plastic syringe (Fisherbrand™). The number of reversals exhibited by the worm within 5s of exposure to the cue was recorded. A binary score was assigned with a positive response (1) scored for worms reaching the threshold displayed by the WT N2 (at least three complete reversals within 5s from the exposure to the substance), otherwise a negative response (0) was assigned. Worms for each strain for each condition were tested by exposing them to the drop only once. The resulting response score was calculated and compared to the WT N2 performance. Strains with known or observed strong impaired movements were not included in the study as they could have potentially affected the locomotory readout on which the assay is based.

To trigger low pH response, acetic acid (CAS No. 64-19-7, Fisher Chemicals™) was dissolved in M9 buffer (3 g KH₂PO₄, 6 g Na₂HPO₄, 5 g NaCl, 1 ml 1 M MgSO₄, to 1 litre H₂O; Stiernagle, 2006) to reach a final pH of 3 (M9, pH3).

Additionally, we tested aversion to classical nematode noxious cues such as a 4M fructose solution (D-Fructose, CAS No. 57-48-7, Sigma-Aldrich) to elicit a high osmolarity response and a solution of 30mM CuSO₄ (Copper(II) sulphate pentahydrate, CAS No. 7758-99-8, ThermoFisher). Each single cue was compared to the exposure of M9 buffer alone (M9, pH7). All tests were performed at 20°C and the pH of the compounds was checked before administration to the worms. The experimenter was blind to the genotype being tested and exposure to cues was randomised.

Individual worms showing impaired movements after picking on the experimental plate, or animals subjected to technical mistakes in administering the cue, were excluded from the analysis as they could have potentially affected the overall strain score.

Data were analysed using a binomial logistic regression using the WT N2 performance as a reference. Data were corrected using the original false discovery rate methods of Benjamini-

Hochberg (Q=1%). Statistics were performed with GraphPad Prism version 10 for Windows (GraphPad Software, Boston, Massachusetts, USA).

2.3.4 Volatile aversion response in *C. elegans*

In the case of volatile aversion test, using the same setup described for the drop assay, we dipped a thin platinum wire into a 30% 1-octanol solution and passed back and forth it in front of the forward moving animal. The latency (s) to start a first reversal was recorded.

Data were analysed using one-way parametric analysis of variance (ANOVA) with different strains vs WT N2 as between-subjects variable.

Post-hoc comparisons were performed using Dunnett's multiple comparisons. A level of probability set at $p < 0.05$ was used as statistically significant. Statistics were performed with GraphPad Prism version 10 for Windows (GraphPad Software, Boston, Massachusetts, USA).

2.4 Results

2.4.1 Resolution of a query set of conserved Eumetazoan nociceptive-related genes

Our bioinformatic strategy, identified a total number of 474 nociceptive related genes collectively retrieved from literature analysis (141), databases (200) and GO search (133).

A considerable number of retrieved genes (152 entries) were found multiple times across the different sources or under synonyms. These duplications were removed, resulting in a total number of 322 distinct candidates (Figure 2.1A and Table S1A).

We then filtered down this list by focusing on essential nociceptive and anti-nociceptive determinants, such as receptors directly gated by noxious stimuli or known modulators of sensory detection. This was done with a view of excluding categories of proteins involved in multiple physiological pathways or with a rather secondary/diffuse role in nociception. Among these, neurotransmitters, and their receptors (29 genes), transcription factors, large families of enzymes involved in widespread cellular activities (165 genes) and molecules contributing to the inflammatory responses were excluded (29 genes). Furthermore, given the challenge posed by some candidates in distinguishing between isoforms of the same gene or different representatives of the same subfamily we pooled related genes (e.g., TRPV1-6 have been pooled into TRPVs, ASIC1-3, into ASICs, PIEZO1-2 into PIEZO) for a total of 25 genes.

Altogether, this filtering produced a final number of 74 potential candidate genes that were blasted against *O. vulgaris* transcriptome (Figure S2.1A and Table S1B).

2.4.1.1 *O. vulgaris* shares most of the conserved protein families implicated in nociception

Each selected protein sequence from the query set was blasted against *O. vulgaris* transcriptome to find orthologue genes (Figure 2.1B).

Out of 74, we found 53 orthologues with at least one representative in *O. vulgaris* (Table S1B). This corresponded to 177 identified transcripts in the *O. vulgaris* transcriptome assembly. We manually curated each of them to remove incomplete sequences (35) and found additional, related sequences (29) which brought the final number of refined complete transcripts to 171 (Table S2). The corresponding 51 distinct genes encoded for proteins belonging to more than 30 different families (and 50 subfamilies) of receptors and modulators associated with sensory detection of chemical, mechanical and/or thermal stimuli. The results were organised into different categories based on the putative biological role of these proteins: 1) Classical activators of nociception, 2) Voltage-gated ion channels/subunits, 3) Proteins related to neuropeptide and lipid metabolism, 4) Other modulators of nociception (Table S2).

Belonging to the first category, orthologues of well-characterised proteins such as transient receptor potential (TRPs) and amiloride sensitive (ASCs) ion channels were identified with 9 and 3 representatives (within multiple isoforms) respectively.

The presence of specific subunits of ion channels implicated in nociception, such as the calcium voltage-gated channel subunit $\alpha 2\delta$ subunit 3 (CACNA2D3), known for its role in thermosensation was found, whilst other known channels specifically involved in nociception triggering such as Na_v1.7-Na_v1.9, were not clearly identified, mostly leading to Na_v1.1 (SCNA1) and Ca_v1.1 (CACNA1S) orthologues (Table S2).

2.4.1.2 Genes implicated in canonical analgesic pathways are missing from *O. vulgaris* transcriptome

Out of the 74 selected genes, 21 did not have a single representative in *O. vulgaris* transcriptome. These include opioid precursors and derivatives, classical opioid receptors, and endocannabinoid receptors. However, receptors that share strongest homologies with those receptors, such as Allatostatin C receptor (AstCR) with an assigned “opioid receptor” protein family membership (IPR001418) in InterProScan, and an Opioid growth factor receptor (OGFR) were identified. Furthermore, enzymes such as neprilysin (NEP), carboxypeptidase (CPE), neuroendocrine convertase (PCSKs) in the case of neuropeptides-related proteins or

diacylglycerol lipase (DAGL) and N-acyl-phosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) in the case of lipid modulators were found. This shows the existence of conserved pathway for the biosynthesis, processing, and metabolism of neuropeptides and lipids that have a broader physiological role that may include nociception modulation and its physiological mitigation.

2.4.1.3 *C. elegans* mutants of *O. vulgaris* orthologue genes were selected for characterisation in a chemical aversion assay

Following the identification and manual curation of *O. vulgaris* candidates, we looked at the data on available tissue distribution and prioritised the analysis of genes shown to be enriched in neurosensory tissues (TIP, SEM, SUB and OL) especially when more than one hit resulted in a plausible orthologue candidate of the putative nociceptive gene (Table S3).

As we intended to model the response triggered by these genes in *C. elegans*, each resulting *O. vulgaris* protein sequence was blasted against *C. elegans* genome database to find, where present (Table S3), the closest nematode orthologue (Figure 2.1C). The result of this final refinement led to 38 *C. elegans* genes, for which we selected 45 different strains (Table 2.1).

Based on the recurring evidence showing nociceptive responses triggered in octopuses through acetic acid administration (*O. vulgaris*, Hague et al. 2013, *O. bocki*, Crook, 2021), we selected low pH (M9, pH 3) as the exemplar cue and systematically tested against selected *C. elegans* strains using the chemoaversion drop assay.

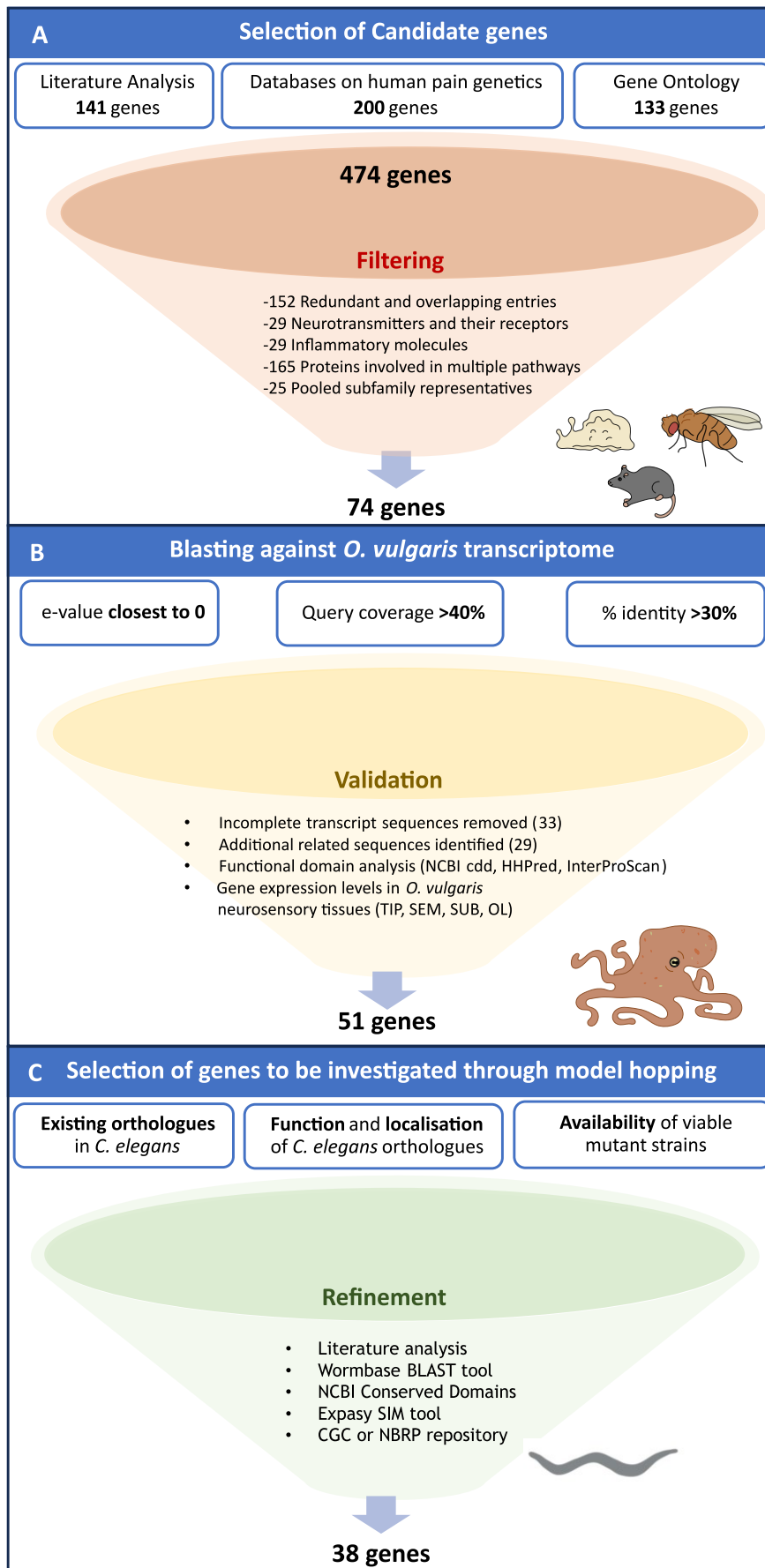


Figure 2.1 Summary of *in silico* analysis leading to the selection of *O. vulgaris* putative molecular nociceptive determinants to be modelled in *C. elegans*.

A Literature analysis of well-characterised Eumetazoan nociceptors was complemented with information from databases on human pain genetics (Human Pain Genetic Database, IASP Pain research, MOGILab) and GO “gene and gene products” to produce 474 distinct candidate genes. We manually filtered to exclude overlapping entries among resources (152), genes involved in multiple pathways (165), neurotransmitters and their receptors (29) and those involved in the inflammatory component of pain (29). We additionally pooled together (25) different representatives of the same subfamily (e.g., ASIC1-3, TRPV1-8 etc.) **B** The resulting 74 candidates were blasted against *O. vulgaris* transcriptome to retrieve the closest orthologues which were then manually curated, leading to 51 candidate genes. We prioritised genes based on available tissue expression levels in octopus sensory and nervous tissues (TIP, SEM, SUB, OL – for details see Table S2). **(C)** To promote the functional characterisation in *C. elegans*, mutant strains for the orthologue genes were selected. This filtering led to a final number of 38 genes across 45 strains.

2.4.1.4 The drop assay is a sensitive test to study chemoaversion in *C. elegans*

We analysed WT N2 and selected mutants in the assay and benchmarked the results against previously published data to validate our approach. The results agreed with published data (Sambongi *et al.*, 2000) and showed that low pH elicited a significant aversive response in N2 (M9, pH3 average number of reversals = 3.990 ± 0.115) when compared to the control (M9, pH 7, average number of reversals = 0.432 ± 0.033). This allowed us to set the threshold for measuring responsiveness (1 for avoidance, 0 for defective avoidance) to at least 3 reversals for M9 pH3 (Figure S2.1).

We then verified the sensitivity of the drop assay in discriminating the responsiveness of different strains of worms. We first tested the well-characterised *che-3* (*e1124*), a strain with ultrastructural defects in the ciliated dendritic endings of the amphid sensory neurons that play an essential role in chemosensation and compared its performance to the WT N2 (Perkins *et al.*, 1986; Wicks *et al.*, 2000).

As previously published for *che* mutant strains (Sambongi *et al.*, 2000), *che-3* (*e1124*) showed a poor avoidance performance to acidic pH when compared to the WT N2 ($p < 0.0001$) with less than 30% responsiveness to low pH (M9, pH 3 average number of reversals 2.293 ± 0.130), a difference of more than 50% with the WT N2 (around 80% responsiveness, Figure S2.1).

Additionally, we analysed *C. elegans* responsiveness to 200mM Na acetate ($C_2H_3NaO_2$) to check which chemical component of acetic acid was responsible for triggering the aversive response. Worms showed no sensitivity to sodium acetate (200 mM Na acetate vs Ctrl M9 pH7, $p = 0.9802$ in WT N2, $p = 0.9924$ in *che-3* (*e1124*)) demonstrating that the repulsive response of M9, pH3 was elicited by the protons (H^+) rather than by the acetate ion (Figure S2.1).

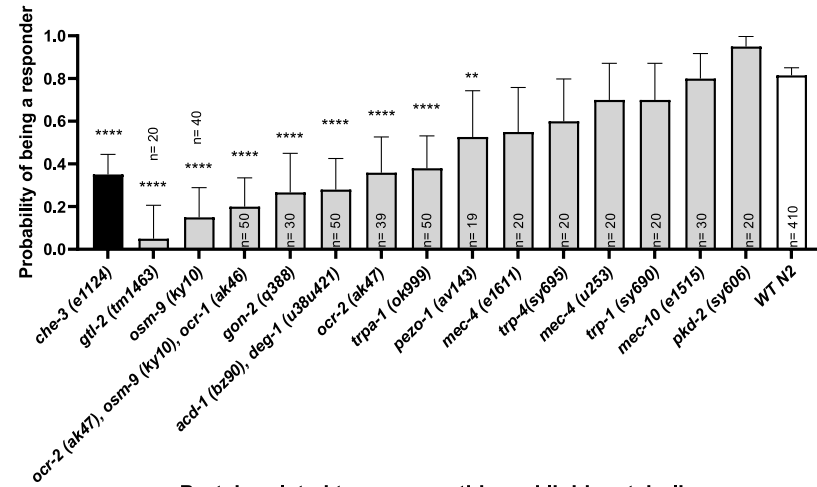
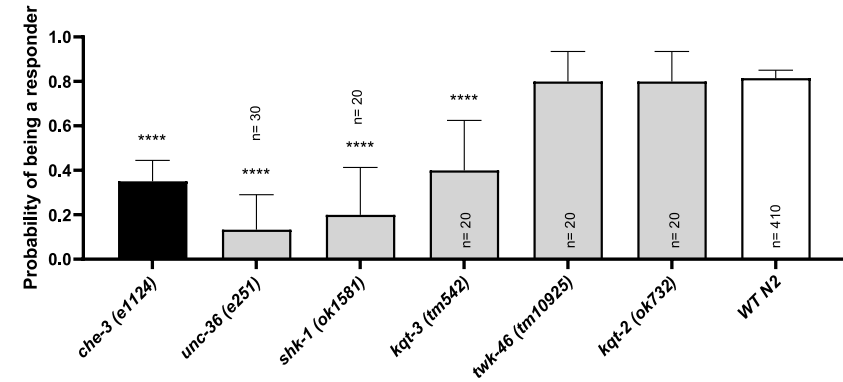
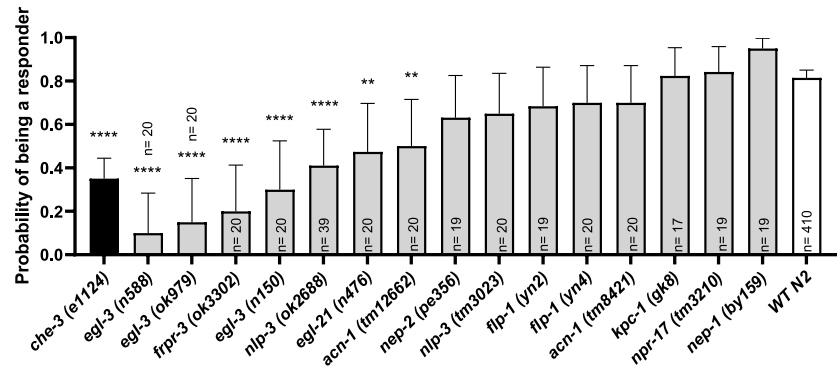
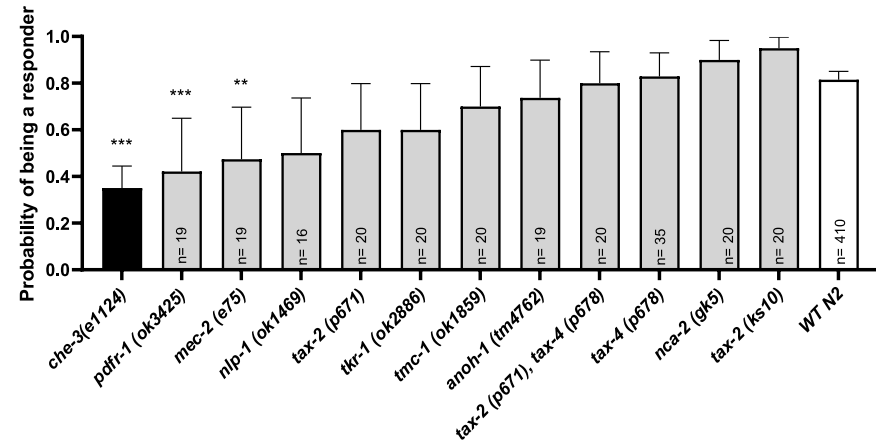
A**Classical activators of nociception****B****Voltage-gated ion channels/subunits****C****Protein related to neuropeptide and lipid metabolism****D****Other modulators of nociception**

Figure 2.2 Functional investigation of *C. elegans* orthologues of *O. vulgaris* genes implicated in nociception.

The bar graph shows the average predicted probability of being a responder (≥ 3 reversals within 5s) and 95% confidence intervals for each strain. The putative genes involved in nociception were grouped into distinct categories (A-D). Category A: “Classical activators of nociception”, refers to all the genes that were found recurringly across all the resources we interrogated (e.g., TRPs, ASCs, PIEZO), Category B: “Ion channel and subunits involved in nociception” is self-explanatory, Category C “Proteins related to neuropeptide and lipid metabolism” was originally meant to include representatives from the opioid and endocannabinoid systems but given we couldn’t find any obvious orthologues, we focused on the enzymes involved in the synthesis, maturation or catabolism of neuropeptides and lipids that might still have a fundamental role for counteracting nociception. Category D: “Other modulators of nociception” refers to a broader range of genes that encode for enzymes and receptors that have been known to be implicated in nociception processing. Significance is expressed according to the corrected p-value (q value) following Benjamini-Hochberg false discovery rate method ($Q=1\%$). n represents the number of individuals tested. Each strain was tested in at least two independent experiments. The average score and the individual data point is shown in Figure S2.2.

2.4.1.5 *C. elegans* is a discriminating platform for characterising conserved molecular nociceptors

Once we established the drop assay as a valuable test to discriminate the responsiveness of the strains, we analysed the performance of all the 45 strains of *C. elegans* mutants that came out of the list of 38 orthologues of *O. vulgaris* putative nociceptive genes (Figure 2.2 and Figure S2.2). The drop assay identified 19 distinct genes across the four different categories that seem implicated in the detection or processing of acidic pH.

Eight out of the 14 strains belong to the “classical activators of nociception” category with impaired strains, all showing an average number of reversals below the set threshold for responsiveness (Figure 2.3).

Mutant strains belonging to the TRP channel subfamilies, including the TRPVs *osm-9* ($q=0.0002$) and *ocr-2* ($q=0.0002$), the TRPA *trpa-1* ($q<0.0001$) and the TRPMs *gon-2* ($q=0.0002$) and *gtl-2* ($q=0.0002$), all showed a significant impairment in the detection of acidic pH (Figure 2.2A and Figure S2.2A). Representatives of TRPN (*trp-4*), TRPC (*trp-1*) and TRPP (*pkd-2*) TRP subfamilies, however, did not show any defect in pH 3 aversion.

Worm strains of the orthologues of other families of classical pH detectors such as ASCs, did not show any defect in chemoaversion as it might be expected by acidic sensing ion channel representatives. In fact, *mec-4* and *mec-10* strains exhibited on average more than three reversals (3.2000 *mec-4* (*u253*), 3.1000 *mec-4* (*e1611*), 5.1000 *mec-10* (*e1515*), Fig. 2.3). The only strain showing lack of response to pH 3, was the neuronal and glial ASC double mutant *acd-1* (*bz90*)/*deg-1* (*u38u421*) ($q=0.0002$). Finally, *pezo-1* (*av143*) was also found to be significantly impaired in the response ($q=0.0092$).

An impaired response to pH3 was also found among specific strains representative of the “voltage-gated ion channels/subunits” category. This included *kqt-3* ($q=0.0002$) and *shk-1* ($q=0.0002$) representatives of the KCNQ and KCNA sub families of potassium channels. This impact was selective as neither *kqt-2* ($q=0.8707$) nor *twk-46* ($q=0.8707$ with an average of 3.2500 reversals, Figure 2.3) were altered in their withdrawal response. *unc-36* ($q=0.0002$), orthologue of the CACNA2D3 subunit, showed a defective response when challenged with the noxious pH3 cue (Figure 2.2B and Figure S2.2B).

Strains with mutations in genes that encode for “proteins related to neuropeptide and lipid metabolism”, showed defects in the detection of acetic acid (Figure 2.2C and Figure S2.2C). The

nlp-3 (*ok2688*) strain showed a clear impairment in response to the repellent ($q=0.0003$), whilst mutant strains of FMRFamide-like peptide orthologues (*flp-1*) were responsive. Among the FMRFamide receptors *frpr-3*, showed a loss of response ($q=0.0003$, average number of reversals 0.9500, Figure 2.3). Considering *C. elegans* orthologues of the enzymes involved in the biosynthesis (*egl-21*), maturation (*kpc-1*, *egl-3*, *acn-1*) and degradation (*nep-1*, *nep-2*) of neuropeptides, the PCSK2 *egl-3* representatives ($q=0.0003$), the CPE *egl-21* ($q=0.0027$) and the ACE *acn-1*(*tm12662*) ($q=0.0042$) mutant strains showed a reduced response when exposed to the pH3 repellent (Figure 2.2C and Figure S2.2C).

Belonging to a broader category of “other nociception modulators”, only mutants in the stomatin-like protein *mec-2* and the calcitonin gene-related peptide receptor (CGRP) *pdf-1*, seemed to be involved in pH 3 avoidance ($q=0.0052$ and $q=0.0013$ respectively). Finally, most of the genes in this category did not show defects in this specific aversive response. This includes CNG representatives *tax-2* and *tax-4*, the anoctamin-1 orthologue *anoh-1*, all eliciting a WT-like performance (>3 reversals) when exposed to acetic acid (Figure 2.2D and Figure S2.2D and Figure 2.3).

We additionally tested the worm strains’ response to other classical aversive cues such as 4M fructose, eliciting high osmolarity, 30mM CuSO₄ and 30% 1-octanol as a volatile repellent.

Looking at the soluble cues, a consistent overlap was found for most of the mutant strains that were found to be impaired in the low pH response with a few exceptions (Figure S2.3). The mutant *mec-4* (*u253*) which was sensitive to low pH, was found to be significantly impaired to 4M fructose and 30mM CuSO₄ aversion ($q=0.0090$ and $q=0.0002$ respectively). The “voltage-gated ion channels/subunits” representative *twk-46* (*tm10925*) showed a significant reduction in the responsiveness to 30mM CuSO₄ ($q=0.0002$) as did the *anoh-1* (*tm4762*) strain ($q=0.0044$).

Few strains were found to be impaired in volatile aversion when exposed to 30% 1-octanol. These include the three TRPV representatives *osm-9* (*ky10*), *ocr-2* (*ak47*) and the triple mutant ($p<0.0001$), *gtl-2* (*tm1463*) ($p=0.0006$) among the “classical activators of nociception” category, members of the “proteins related to neuropeptide and lipid metabolism” such as *flp-1* (*yn4*), *egl-21* (*n476*) ($p<0.0001$) and *kpc-1* (*gk8*) ($p=0.0008$) and *unc-36* (*e251*) as representative of the “voltage-gated ion channels/subunits” (Figure S2.4).

Altogether, our data shows that most of the strains found to be impaired in the low pH aversion were also defective to other cue modalities (17), only three were exclusively involved in pH (*trpa-1*, *shk-1*, *pdf-1*) and only four (*mec-4*, *twk-46*, *anoh-1*, *kpc-1*) were found to be

involved in responses to non-pH related chemicals. Finally, 12 were found to not be involved at all in any aversive response.

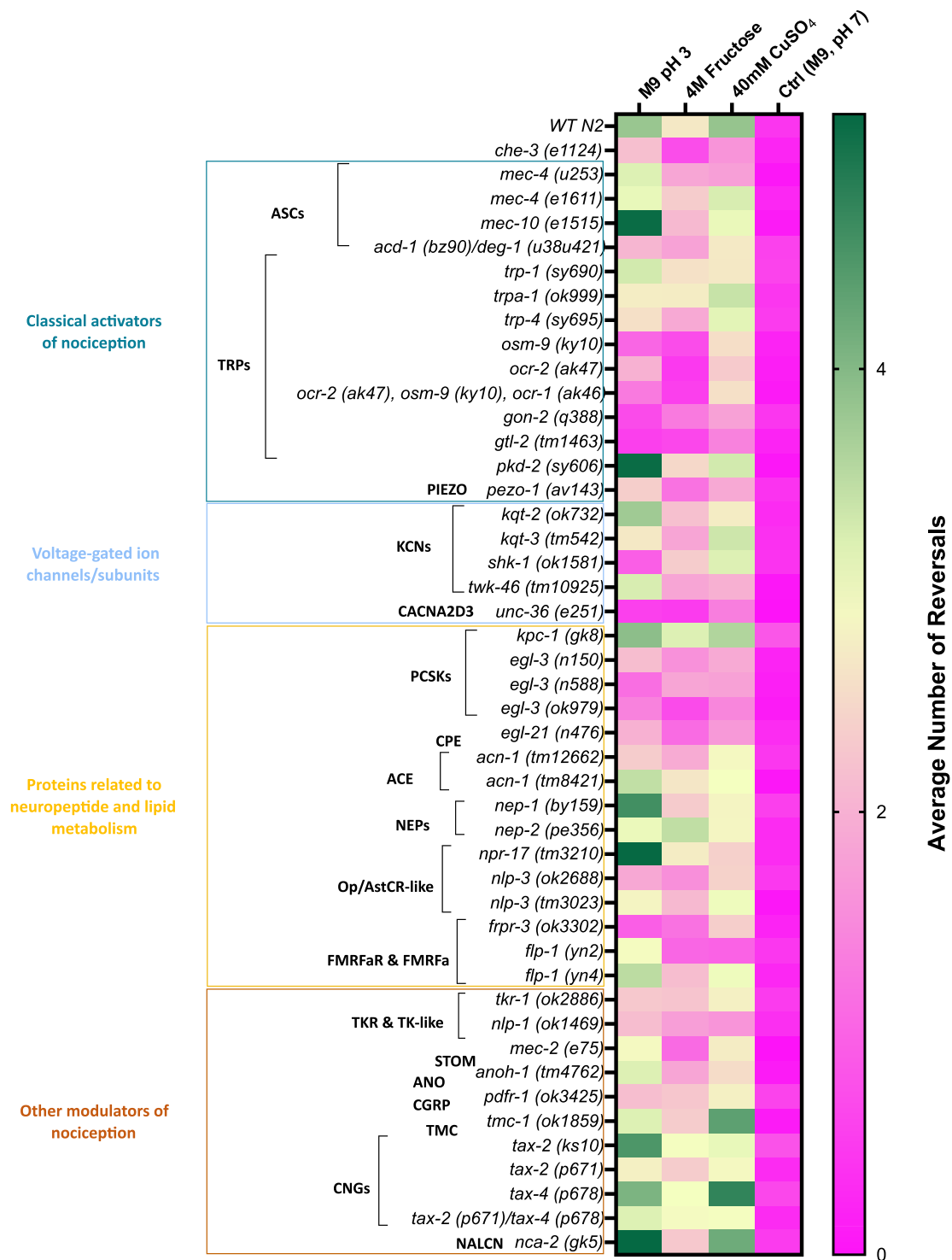


Figure 2.3 C. elegans highlights potential molecular determinants involved in O. vulgaris nociception.

Data for the drop assay is reported here as the number of actual reversals after exposure to the noxious cue (M9, pH 3). The heat map shows a colour coded indication of the strains' sensitivity to M9, pH 3 from non-sensitive *che-3 (e1124)*-like (magenta) to sensitive WT N2-like (green). Each colour block represents the average number of reversals across all the worms tested per strain. The WT N2 performance was characterised by at least 3 body reversals within 5s of contact with the drop and was used to set our criteria for responsiveness.

2.5 Discussion

2.5.1 Core conserved molecular nociceptors in *O. vulgaris* can be identified using functional characterization in *C. elegans*

The *in silico* strategy revealed that a large number of genes showing conservation to known nociceptive genes in other species are present in *O. vulgaris*. In particular, at least two representatives were found to belong to the acid sensing ion channels (ASICs), including the classical molluscan FMRFamide-gated Na⁺ channel (Cottrell, Schot and Dockray, 1983; Cottrell, Green and Davies, 1990; Cottrell, 1997) which were proven to be pH sensitive too (Perry *et al.*, 2001), two genes belonging to the PIEZO family, whilst for TRPs, 9 members (with multiple isoforms) were identified (Table S2). These include two TRPA1 channels, two TRPCs, two TRPMs, one TRPP, one TRPN and one TRPV channel, showing less variety of members when compared to vertebrates (Peng, Shi and Kadowaki, 2015). This suggests either the vertebrate TRPs underwent specialisation during evolution (e.g., gene duplication and divergence) or that the fewer invertebrate representatives play multiple physiological functions as in the original ancestral gene (Valencia-Montoya, Pierce and Bellono, 2024).

When tested in *C. elegans*, one of the strongest behavioural impairments was found in mutant strains for the TRPV channel representatives *osm-9* and *ocr-2*. (Figure 2.2A). These results are in line with previous studies showing their role in acidic pH sensation in *C. elegans* (Sambongi *et al.*, 2000) reinforced by the essential role that orthologues play in vertebrate noxious responses (Dhaka *et al.*, 2009). Additionally, in accordance with previous works, these were found to be strongly impaired to all the other cues tested (Figure S2.3 and Figure S2.4), showing a conserved polymodal role of this receptor (Radresa *et al.*, 2012) and confirming their overall importance in the primary sensory signalling of *C. elegans* (Colbert, Smith and Bargmann, 1997; Tobin *et al.*, 2002; Thies *et al.*, 2016).

Interestingly, other TRP channel orthologues were defective in chemoaversion implicating them in the acidic response. These include *trpa-1* and the TRPM representatives *gon-2* and *gtl-2*. The former has been previously found to be implicated in mechanical and thermal responses in *C. elegans* whilst a wild type response was elicited when exposed to ASH-dependent noxious chemical cues tested (i.e. copper chloride, 1M glycerol, denatonium; Kindt *et al.*, 2007) as also shown by our data (Figure S2.3 and Figure S2.4). Our results, showing for the first time a role in detecting and processing of this chemical cue, could therefore reflect the complexity of the low pH response circuitry, which does not exclusively

rely on ASH neurons but on a series of amphids response (Sambongi *et al.*, 2000; Bergamasco and Bazzicalupo, 2006). To the best of our knowledge, this work represents the first one testing chemical sensitivity in *C. elegans* TRPMs as most studies mainly focused on their role in reproduction (Sun and Lambie, 1997; Teramoto *et al.*, 2010). Despite the low expression levels of *gon-2* in amphids and the selective expression of *gtl-2* in pharynx and excretory cells, the phenotype observed might indicate some indirect effect of these TRP proteins in the chemosensation executed by exposure to low pH and other cues (Figure 2.2A and Figure S2.3).

Similarly, the PIEZO orthologue, *pezo-1*, has only been previously implicated in mechanosensory responses, as well as locomotion and mating (Brugman, 2020; Romero *et al.*, 2022; Komandur *et al.*, 2023) and our study is the first to evidence a potential implication in low pH signalling. However, the previously identified role in food intake and food sensation associated with a mechanical processing (Hughes *et al.*, 2021; Millet *et al.*, 2021), could also be the result of an interaction with the nematode chemical sensory system.

In accordance with previous investigations, the only ASC strain to show defects when exposed to acidic pH was the double mutant *acd-1/deg-1* which carries mutation in both neuronal and glial ASC proteins, suggesting a glial role in the processing of low pH in *C. elegans*, similarly to what has been previously reported in vertebrates (Chesler, 2003; Erlichman *et al.*, 2004; Wang *et al.*, 2008; Gourine and Dale, 2022). The octopus arm, the main structure involved in sensory interaction with environmental cues, includes different types of neurons and glial-like cells that might highlight the potential role of this cell type in acidic nociception as well (Young, 1971; Winters-Bostwick *et al.*, 2024). The lack of impairment in the rest of the ASC protein family members tested here is in line with previous studies showing a main role in mechanical detection (Suzuki *et al.*, 2003; Zhang *et al.*, 2004; Árnadóttir *et al.*, 2011; Schafer, 2015; Al-Sheikh and Kang, 2020).

2.5.2 *O. vulgaris* lacks canonical vertebrate opioid signalling

According to Bateson's criteria (Bateson, 1991) for eligibility to feel pain, animals must be endowed not just with nociceptors connected to a central brain mass but they should also possess a system able to counteract the inner disruption of the organism homeostasis, namely analgesic pathways. Therefore, we included candidates for opioid and cannabinoid receptors, and their ligand precursors. Blasting of 'classical' vertebrate opioid receptors (i.e. mu-, kappa-, delta-) produced two matches. The first one was the opioid growth factor

receptor (OGFR), which however has no structural resemblance to the classical opioid receptors and constitutes a different protein family (Zagon, Verderame and McLaughlin, 2002). The second and closest homology was to the allatostatin C receptor (AstCR), the protostome orthologue of the deuterostome somatostatin receptor (Koch *et al.*, 2022). Opioid receptors are thought to have evolved from a duplication of the AstCRs (Thiel *et al.*, 2021) and a few studies have reported a role of AstC in nociception and immunity in invertebrates (Bachtel *et al.*, 2018; Li *et al.*, 2021c), in addition to growth and feeding regulation (Stay and Tobe, 2007; Mizoguchi, 2016). This raises the intriguing possibility that AstC signalling might underpin anti-nociceptive signalling in phyla that lack an opioid system as supported by recent evidence in *Drosophila melanogaster* (Bachtel *et al.*, 2018; Liu *et al.*, 2023). In line with this, we selected the opioid-like/allatostatin C-like receptor *npr-17* as the best candidate to be tested in *C. elegans* (Beets *et al.*, 2023). Previous work has shown that the receptor NPR-17 and the neuropeptides NLP-3 and NLP-24 are essential in the morphine-mediated 1-octanol avoidance response and that the impaired response of the *npr-17* null mutant could be rescued by the human κ -opioid receptor (Mills *et al.*, 2016). However, in our experiment, mutant strains for *npr-17* were not impaired in low pH chemoaversion (Figure 2.2C), confirming a more complex peptidergic modulation of the aversive response. A recent large-scale de-orphanisation of *C. elegans* GPCR identified an additional 12 AstCRs which might be worth analysing in the appropriate noxious context (Beets *et al.*, 2023). Interestingly, *nlp-3* mutants showed some differences in the response: *nlp-3 (ok2688)* showed a strong impairment in low pH but also to high osmolarity and CuSO₄, the other strain *nlp-3 (tm3023)* showed a WT-like response to all cue tested. While the difference could be due to a larger deletion in the former mutant strain, affecting the phenotype more dramatically, it could also be due to background mutations that have been introduced and not removed by outcrossing. Legitimate implication in nociception, will therefore require further experimental validation.

C. elegans orthologues of enzymes involved in the biosynthesis (i.e., the CPE *egl-21*), and processing of neuropeptides (i.e., the PCSK2 *egl-3*), showed disrupted responses to low pH and other cues. Mutations in *egl-3* and *egl-21* were already known to show defects in mechanosensory and thermosensory avoidance responses (Kass *et al.*, 2001; Nkambeu *et al.*, 2019). Such defects can be related to the disrupted production of neuropeptides as highlighted by mass spectrometry experiments (Husson *et al.*, 2006; Husson *et al.*, 2007). Collectively, these data point out the importance of neuropeptide signalling in the processing of nociception (Dickinson and Fleetwood-Walker, 1998) but given the pleiotropic nature of these molecules, does not constitute evidence of the presence of the classical analgesic

pathways. Indeed, even in the case of the opioid sensitive analgesic pathway there is a broader functional consequence of this signalling extending to underpinnings of appetite, gut function and systemic homeostasis (Yuan *et al.*, 2012).

Our *in silico* findings are somewhat in line with previous phylogenetic and bioinformatic analysis suggesting opioids and endocannabinoids as vertebrate exclusive proteins (Thiel *et al.*, 2021). This does not necessarily mean that invertebrates do not possess a system to counteract noxious stimuli as they could have more ancient or unique solutions to the same problem. FMRFamide has been considered the equivalent of opioid molecules in invertebrates as the sequence (Phe-Met-Arg-Phe-NH₂) shows some similarities to the heptapeptide Met-enkephalin (Tyr-Gly-Gly-Phe-Met-Arg-Phe). Among the wider physiological functions these peptides were found to modulate in molluscs and more generally in invertebrates, there are a few showing an induced suppression of primary nociceptors in *Aplysia* (Belardetti, Kandel and Siegelbaum, 1987; Mackey *et al.*, 1987).

The mutant of the *C. elegans* FMRFa orthologue we selected, *flp-1* did not show impairment in acidic response but has been found to be involved in high osmolarity aversion (Nelson *et al.*, 1998; Li *et al.*, 1999) suggesting a more selective chemosensory role. The orthologue of the FMRFamide receptor, *frpr-3* on the other hand, showed a strong impairment in pH 3 response, and thus represents a strong candidate for further analysis.

2.5.3 Lipid signalling is an important component of nociception modulation across phyla

In the case of the endocannabinoid pathway, our *in silico* analysis identified different *O. vulgaris* genes encoding proteins implicate in fatty acid metabolism, such as DAGL, NAPE-PLD or Fatty acid-binding proteins (FABPs; Table S2.2). However, we did not find molecular homologies to sign post any classical receptors of these signalling cascades within the octopus genomes. Although we found no evidence for the canonical endocannabinoid receptors in octopus, the lipids that act on them have established physiological effects on other proteins such as allatostatin receptors and TRPVs.

Interestingly, one of the proposed “ancestors” of the endocannabinoid system is the TRPV channel, previously shown to mediate reduced nocifensive response when activated by anandamide and 2-acyl-glycerol in the medicinal leech *Hirudo verbana* (Summers *et al.*, 2017). Specifically in the case of *C. elegans*, bioinformatic analysis coupled with thermal proteomic profile, identified the AstCR *npr-32*, and the TRPV *ocr-2* as responsible for

modulating the nocifensive response in a thermal avoidance assay, confirming these molecular determinants as key players in the detection and modulation of nociceptive responses (Abdollahi *et al.*, 2024).

2.5.4 Limitations and potential confound of the study

Our *in silico* approach was biased towards the selection of pre-existing, conserved molecular determinants, thus excluding potentially phylum-specific proteins akin to the recently identified chemotactile receptors in octopus and squid (van Giesen *et al.*, 2020; Kang *et al.*, 2023). Our study found a large number of conserved candidates in the transcriptome of *O. vulgaris* (Petrosino, 2015; Petrosino *et al.*, 2022). However, the database available is not refined and therefore manual curation led to either the exclusion of incomplete or misannotated transcripts or required manual reconstruction (e.g., TRPV) to be fully investigated. The availability of the recently published chromosome-level genome could potentially improve the quality of this strategy (Destanović *et al.*, 2023). Additionally, BLAST search will limit the findings to sequences with sufficient shared similarities. Using alternative methods such as an HMM-based analysis, could help find candidates with more divergent sequences (Felsenstein and Churchill, 1996).

Despite their phylogenetic distance, with a last common ancestor between Lophotrochozoa and Ecdysozoa around 535 million years ago (Howard *et al.*, 2020), *C. elegans* represents the closest well-characterised (both genetically and phenotypically) model organism to investigate *O. vulgaris* nociceptors. However, the distinct environments (terrestrial vs marine) in which the two species evolved should be considered, as the diverse modalities and variety of ecological stimuli could have driven shared proteins to diverge and acquire different adaptations (van Giesen *et al.*, 2020; Allard *et al.*, 2023).

Additionally, our strategy could not be applied to some genes as they are missing from *C. elegans* genome. In fact, a few *O. vulgaris* gene families representative of nociception signalling could not be modelled in the nematode due to the absence of corresponding orthologues. This is exemplified by the purinergic receptors (P2X) and voltage gated sodium (Na_vs) channels (Bargmann, 1998; Harte and Ouzounis, 2002; Hobert, 2005-2018; Burnstock and Verkhratsky, 2009; Table S2.3).

2.5.5 Future directions

By performing a chemosensory assay on *C. elegans* mutants for the orthologue of *O. vulgaris* putative nociceptive genes, we have highlighted candidates that are involved in the acidic pH avoidance response. Two main categories of proteins deserve priority for further investigation: One is the classical activators of nociception, such as the TRPV channel subfamily, whose role in nociception is widely conserved across phyla, and the other is the neuropeptide-related proteins, such as FMRFa, AstC, their receptors and the enzymes involved in neuropeptide processing. A functional role of these candidates in nociception could be explored further by complementation of *C. elegans* lost sensory functions by the *O. vulgaris* orthologous gene. Overall, we highlighted an intersecting bioinformatic model hopping approach that facilitates understanding of nociception in *O. vulgaris* and which has key relevance for an evolutionary perspective on the phenomenon of pain.

Gene name	Function	<i>O. vulgaris</i> Candidate	<i>C. elegans</i> orthologue	Allele	Strain name	Mutation	Reported behavioural phenotype	Gene expression	% Domain identity
Classical activators of nociception									
Acid Sensing Ion Channel (ASIC)	Ion channel involved in low pH detection (Deval and Lingueglia, 2015; Lee and Chen, 2018)	c28071_g1_i1 MN081801	<i>mec-4</i>	e1611	CB1611	Sub (Ala442Thr). Causes aggregation of protein product (Driscoll and Chalfie, 1991)	Insensitivity to gentle touch; lower escape response to plate taps; altered NaCl chemotaxis (Chatzigeorgiou <i>et al.</i> , 2010)	Anterior and posterior mechanosensory neurons (ALM, PLM, PVM, PVD) (Lai <i>et al.</i> , 1996)	28.3% id in 127 res ov; Score: 126.0; Gap fr: 2.4%
				u253	TU253	Del (363 bp). Eliminates the first hydrophobic domain affecting the selectivity of the Na ⁺ filter (third exon) (Hong, Mano and Driscoll, 2000)	Insensitivity to gentle touch; suppressed nose touch response (Árnadóttir <i>et al.</i> , 2011)		
FMRFamide-activated sodium channel (FaNaC)	Ion channel activated by FMRFamide. Potentially involved in anti-nociceptive responses in invertebrates (Cottrell, Green and Davies, 1990; Lingueglia <i>et al.</i> , 1995)	c34210_g4_i4 MN081866	<i>mec-10</i>	e1515	CB1515	Sub (Ser105Phe). Missense in the DEG/ENaC domain, lof (Huang and Chalfie, 1994)	severe light touch insensitivity; significant reduction in the mechanoreceptor currents (Árnadóttir <i>et al.</i> , 2011)	Anterior and posterior mechanosensory neurons (ALM, PLM, PVM, PVD), (FLPL/R and PVDL/R) (Huang and Chalfie, 1994)	37.7% id in 53 res ov; Score: 105.0; Gap fr: 0.0%
			<i>acd-1/deg-1</i>	bz90/u38u421		bz90: Del (1625 bp) including the region of DEG/ENaC domain. u38: null mutation (Wang <i>et al.</i> , 2008)	Individual lof <i>deg-1</i> is moderately defective in low pH detection. However, double glial/neuronal mutant <i>acd-1/deg-1</i> is dramatically unresponsive to acidic pH (Wang <i>et al.</i> , 2008)		
Transient Receptor Potential A (TRPA)	Ion channel activated by heat, cold, mechanical and chemical stimuli (Kwan <i>et al.</i> , 2006)	c31382_g11_i1 MN081859	<i>trpa-1</i>	ok999	RB1052	Del (1334 bp). Eliminates the N ter region (Kindt <i>et al.</i> , 2007)	Defects in mechanosensory behaviours related to nose-touch responses and foraging (Kindt <i>et al.</i> , 2007)	PVD, PDE, PHA, PHB, ASH, OLQ, IL1 (Kindt <i>et al.</i> , 2007)	33.6% id in 837 res ov; Score: 871.0; Gap fr: 8.4%
Transient Receptor Potential C (TRPC)	Ion channel involved in the activation of nociceptors (Ramsey, Delling and Clapham, 2006; Julius, 2013; Sun <i>et al.</i> , 2020)	c30608_g5_i7 PV164455	<i>trp-1</i>	sy690	TQ225	Del (>2 kb). Eliminates first 4 exons including promoter region and most of N ter (Feng <i>et al.</i> , 2006)	Reduction in nicotine-response (Feng <i>et al.</i> , 2006). UIN	Interneurons, motor neurons, pharyngeal neurons, sensory neurons, muscles (Xiao and Xu, 2009)	51.9% id in 770 res ov; Score: 1935.0; Gap fr: 3.9%
Transient Receptor Potential M (TRPM)	Ion channel responsive to cold (TRPM8), heat and some irritants (TRPM2 & TRPM3) (Ramsey, Delling and Clapham, 2006; Julius, 2013; Tan and McNaughton, 2016)	c31340_g2_i1 PV164447	<i>gon-2</i>	q388	EJ1158	Sub (Glu955Lys). lof mutation (Lambie <i>et al.</i> , 2015 (Lambie <i>et al.</i> , 2015)	Gonad defective (Sun and Lambie, 1997) Hypersensitivity to Mg ²⁺ (Teramoto, Lambie and Iwasaki, 2005). UIN	pharynx, excretory cells, intestine (Xiao and Xu, 2009)	31.2% id in 186 res ov; Score: 233.0; Gap fr: 8.1%
		c31482_g3_i4 PV164444	<i>gtl-2</i>	tm1463	LH202	Del (463 bp). Eliminates from 5th to 7th exon (Teramoto <i>et al.</i> , 2010)	Hypersensitivity to Mg ²⁺ (Teramoto, Lambie and Iwasaki, 2005). UIN	pharynx, excretory cells (Xiao and Xu, 2009)	46.2% id in 262 res ov; Score: 586.0; Gap fr: 3.8%
NOMPC (TRPN)	TRPN channel involved in mechanosensation (Yan <i>et al.</i> , 2013; Turner <i>et al.</i> , 2016)	c31314_g5_i1 PV164451	<i>trp-4</i>	sy695	TQ296	Del (3kb). Eliminates sequence in the 3' region (Li <i>et al.</i> , 2006)	Abolished CEP neuron mechanoreceptor currents. Insensitivity to proprioception and posterior harsh touch (Li <i>et al.</i> , 2006; Li <i>et al.</i> , 2011)	dopaminergic ciliated sensory neurons (CEP, PDE, ADE) and in DVA, DVC interneurons (Xiao and Xu, 2009)	29.0% id in 928 res ov; Score: 556.0; Gap fr: 6.6%
Transient Receptor Potential P (TRPP)	Ion channel involved in mechanosensitive transduction signal (Giamarchi <i>et al.</i> , 2006)	c34000_g4_i1 PV164454	<i>pkd-2; him-5</i>	sy606; e1490	PT8	Null mutation producing a truncated protein from the middle of tm1 onward (Barr and Sternberg, 1999)	Chemo- and mechano-defects in male mating behaviour (Barr and Sternberg, 1999). UIN	male-specific mechanosensory/chemosensory neurons: CEM, HOB, (Barr and Sternberg, 1999)	44.7% id in 425 res ov; Score: 914.0; Gap fr: 1.6%
Transient Receptor Potential Vanilloid (TRPV)	Ion channel involved in heat and low pH perception (Caterina <i>et al.</i> , 1997; Immke and Gava, 2006; Liu and Wood, 2011)	c32354_g12_g2_g7_g14_g6 PV164572	<i>osm-9</i>	ky10	CX10	Sub (Glu/*) Nonsense mutation (Del (1960 bp) (Colbert, Smith and Bargmann, 1997; Tobin <i>et al.</i> , 2002)	Insensitivity to nose touch, hyperosmolarity, low pH and aversive volatile (Colbert, Smith and Bargmann, 1997; Sambongi <i>et al.</i> , 2000; Tobin <i>et al.</i> , 2002)	Amphid sensory neurons (ASH, AWA, AWC, ASE, ADF, ASI, ASJ, ASK, IL2s), PVD, OLQ, PHA, PHB), rectal gland cells, uterine cells (Colbert, Smith and Bargmann, 1997)	53.7% id in 214 res ov; Score: 618.0; Gap fr: 0.5%
			<i>ocr-2, osm-9, ocr-1</i>	ak47, ky10, ak46	FG125	ak47: Del (1960 bp); ky10: Sub (Q/*) ak46: Del (2026 bp) (Colbert, Smith and Bargmann, 1997; Tobin <i>et al.</i> , 2002)			
Piezo	Ion channel involved in mechanosensation (Mikhailov <i>et al.</i> , 2019; Wang and Ragsdale, 2019)	c36392_g4_i1 MN081842	<i>piezo-1</i>	av143	AG405	Del (4 kb). Eliminates from 27th to 33rd exon+ Ins of stop codon (Komandur <i>et al.</i> , 2023)	Altered crawling behaviour. UIN (Komandur <i>et al.</i> , 2023)	Pharynx, Reproductive tissues, HOB, PCB, PCC, and ray neurons (Bai <i>et al.</i> , 2020; Millet <i>et al.</i> , 2021)	56.0% id in 53 res ov; Score: 197.0; Gap fr: 0.0%

Gene name	Function	<i>O. vulgaris</i> Candidate	<i>C. elegans</i> orthologue	Allele	Strain name	Mutation	Reported behavioural phenotype	Gene expression	% Domain identity
Voltage-gated ion channels/subunits									
Calcium channel, voltage-dependent, alpha 2/delta subunit 3 (CACNA2D3)	Calcium channel associated with high heat response - orthologue of <i>Drosophila straightjacket</i> (Neely <i>et al.</i> , 2010)	c30247_g1_i1 PV164539	<i>unc-36</i>	<i>ae251</i>	CB251	Sub (Gly452*). Nonsense mutation leading to a truncated protein in the first third (Lainé <i>et al.</i> , 2011)	Defects in touch avoidance behaviour (Frøkjær-Jensen <i>et al.</i> , 2006)	mechanosensory neurons (AVM, ALM, PVM, PVQ, PVC, DUC, and DVA) (Frøkjær-Jensen <i>et al.</i> , 2006)	53.3% id in 60 res ov; Score: 111.0; Gap fr: 1.7%
Potassium Two Pore Domain Channel Subfamily K (KCNC)	TWIK-relate channel triggered by changes in the surrounding pH (Li and Toyoda, 2015)	c33106_g1_i2 PV164533	<i>twk-46</i>	<i>tm10925</i>		Del (99bp). Eliminates from the 3rd to the 7th exon (Zhou <i>et al.</i> , 2022)	Moderate/severe locomotor impairments. UIN (Zhou <i>et al.</i> , 2022)	AVE, AVB, backward (VA, DA) and forward (VB, DB) excitatory motoneurons and in few inhibitory motor neurons (VD, DD) (Zhou <i>et al.</i> , 2022)	53.5% id in 129 res ov; Score: 356.0; Gap fr: 5.4%
Potassium Voltage-Gated Channel Subfamily Q (KCNCQ)	K channels involved in modulating pain threshold (Wood, 2006)	c33477_g8_i1 PV164532 c33539_g2_i3 PV164531	<i>kqt-2</i>	<i>pk732</i>	RB883	Del (1694 bp). Eliminates most of the channel pore region (Okahata <i>et al.</i> , 2019)	Supranormal cold acclimation. Decreased temperature-dependent activity in ADL neurons (Okahata <i>et al.</i> , 2019)	Sensory neurons (ASK, ADL), fan and ray sensory neurons of male adults, and intestinal cells (Okahata <i>et al.</i> , 2019)	32.0% id in 206 res ov; Score: 221.0; Gap fr: 7.3%
			<i>kqt-3</i>	<i>tm542</i>	TM542	Del (1001 bp). Eliminates from 2nd to 4th exon (Bauer, Nebenfuehr and Golden, 2019)	Enhanced thermosensitivity (Okahata <i>et al.</i> , 2019)		68.4% id in 367 res ov; Score: 1178.0; Gap fr: 9.0%
Potassium Voltage-Gated Channel Subfamily A (KCNA)	K channel involved in the modulation of touch and pain (Hao <i>et al.</i> , 2013)	c30280_g2_i1 PV164509	<i>shk-1</i>	<i>pk1581</i>	RB1392	Ins: CTAAATAT; Del: 632 bp (Wormbase)	reduction of action potential frequency in body wall muscles and thus reduced locomotor activity (Gao and Zhen, 2011; Liu <i>et al.</i> , 2018) UIN	muscles and neurons (Gao and Zhen, 2011; Liu <i>et al.</i> , 2018)	55.9% id in 188 res ov; Score: 527.0; Gap fr: 2.1%

Gene name	Function	<i>O. vulgaris</i> Candidate	<i>C. elegans</i> orthologue	Allele	Strain name	Mutation	Reported behavioural phenotype	Gene expression	% Domain identity
Proteins related to neuropeptides and lipid metabolism									
Neuroendocrine convertase 1 (PCSK1)	Involved in the processing of enkephalins into their mature form (Johanning <i>et al.</i> , 1998)	c28798_g1_i1 PV164566	<i>kpc-1</i>	<i>gk8</i>	VC48	Del (2238 bp). Eliminates from 3rd exon to part of the 5th, resulting in a frameshift after Leu185 and a predicted stop codon after 17 non-homologous residues (Salzberg, Ramirez- Suarez and Bülow, 2014)	severely defective dendritic arbours in both PVD and FLN neurons, impaired branching and extension of other sensory neurons, interneurons and motor neurons (Salzberg, Ramirez-Suarez and Bülow, 2014; Dong <i>et al.</i> , 2016)	Broadly expressed in the nervous system (Salzberg, Ramirez-Suarez and Bülow, 2014)	50.3% id in 443 res ov; Score: 1125.0; Gap fr: 2.5%
Neuroendocrine convertase 2 (PCSK2)		c32479_g16_i1 PV164565	<i>egl-3</i>	<i>n150</i>	MT150	Sub (Gly594Glu). Missense mutation in Proprotein convertase P-domain (Kass <i>et al.</i> , 2001; Husson <i>et al.</i> , 2006)	increased sensitivity to acidic pH, heat sensitive. Drastic reduction in neuropeptides (Kass <i>et al.</i> , 2001; Husson <i>et al.</i> , 2006; Wakabayashi <i>et al.</i> , 2015)	ASH, ALM, AVM, and PVM, interneurons AVB, AVD, PVC, RIG, and SDQL; and the HSN egg-laying motor neurons (Kass <i>et al.</i> , 2001)	76.5% i in 446 res ov; Score: 1864.0; Gap fr: 0.4%
				<i>n588</i>	MT1218	Sub (Glu117Gln). Missense mutation in the Peptidase S8 pro-domain (Kass <i>et al.</i> , 2001; Husson <i>et al.</i> , 2006)	body touch sensitivity is greatly diminished (Kass <i>et al.</i> , 2001)		
				<i>ok979</i>	VC671	Del (1578 bp). Eliminates catalytic domain	Impaired odour avoidance. Drastic reduction in neuropeptides (Husson <i>et al.</i> , 2006; Yamazoe- Umemoto <i>et al.</i> , 2015)		
Carboxypeptidase E (CPE)	Involved in the biosynthesis of enkephalin (Fricker, 1993)	c30963_g3_i1 PV164485	<i>egl-21</i>	<i>n476</i>	MT1071	Del (123 bp). Out of frame deletion producing a truncated protein lacking most of the catalytic domain (Husson <i>et al.</i> , 2007)	thermal avoidance behaviour significantly hampered. Drastic reduction in neuropeptides (Husson <i>et al.</i> , 2007; Nkambeu, Ben Salem and Beaudry, 2021)	Broad nervous expression (Husson <i>et al.</i> , 2007)	46.8% id in 408 res ov; Score: 898.0; Gap fr: 1.7%
Angiotensin-converting enzyme (ACE)	Involved in the processing of opioids precursors (Choi <i>et al.</i> , 2021)	c29134_g1_i2 PV164476	<i>acn-1</i>	<i>tm12662</i>	-	Del (116 bp) + Ins (8 bp)	UIN	hypodermis, vulva and ray precursor cells (Brooks <i>et al.</i> , 2003)	26.4% id in 580 res ov; Score: 473.0; Gap fr: 5.0%
				<i>tm8421</i>	-	Del (92 bp) + Ins (6bp) (Wormbase)			
Neprilysin (NEP)	Key enzyme involved in the degradation of enkephalins (Skidgel and Erdős, 2004)	c34308_g4_i2 MN081826	<i>nep-1</i>	<i>by159</i>	BR2815	Del (1450 bp). Eliminates promoter, 1st and 2nd exon (Spanier <i>et al.</i> , 2005)	Uncoordinated pattern of locomotion (Spanier <i>et al.</i> , 2005). UIN	Pharynx, head interneuron RIH (Spanier <i>et al.</i> , 2005)	35.3% id in 326 res ov; Score: 506.0; Gap fr: 1.5%
		c32366_g10_i1 MN081824	<i>nep-2</i>	<i>pe356</i>	JN356	splicing acceptor mutation in the 1st and 2nd exon (Yamada <i>et al.</i> , 2010)	severe defects in olfactory adaptation to benzaldehyde. UIN (Yamada <i>et al.</i> , 2010)	muscle, glia, neurons (GLR, AIM, SMB) (Yamada <i>et al.</i> , 2010)	38.4% id in 664 res ov; Score: 1230.0; Gap fr: 1.5%
Tachykinin receptor (TACR)	Receptor of tachykinins (Storm <i>et al.</i> , 2013; Deng <i>et al.</i> , 2020)	c31779_g15_i4 MN081857	<i>tkr-1</i>	<i>ok2886</i>	VC2171	Ins: GGTGATCTATGT Del: 755 bp (Wormbase)	Reduced aversion-resistant ethanol seeking after pretreatment (Salim <i>et al.</i> , 2022)	Broad neuronal expression, hypodermal tissue (Barrett, 1999)	37.9% id in 293 res ov; Score: 490.0; Gap fr: 2.4%
Tachykinin (Tk)	Involved in nociceptive sensitisation (Nishimori, Naono-Nakayama and Ikeda, 2013)	c31437_g10_i1 AB037112.1	<i>nlp-1</i>	<i>ok1469</i>	RB1340	Del (700 bp) (Wormbase)	Impaired odour habituation (McDiarmid, Ardiel and Rankin, 2015)	AWC, ASI, PHB and BDU neurons and intestine (Nathoo <i>et al.</i> , 2001)	44.7% id in 38 res ov; Score: 56.0; Gap fr: 5.3%
FMRamide receptor (FMRFaR)	Potentially involved in anti-nociceptive responses in invertebrates (Kavaliers, Hirst and Teskey, 1985; Walters, 2018)	c16104_g1_i1 MN081861	<i>fpr-3</i>	<i>ok3302</i>	VC2565	Del (591 bp). Eliminates from ending part of 1st exon and the entire 2nd exon (Wormbase)	defective in locomotor arousal after tap; impairment of chemotaxis when exposed to appetitive olfactory cues (Chew <i>et al.</i> , 2018)	head neurons, including ASH, RID, ASK, AIY, and AVK (Chew <i>et al.</i> , 2018)	28.6% id in 276 res ov; Score: 326.0; Gap fr: 1.1%
FMRamide peptide (FMRFa)	Potentially involved in anti-nociceptive responses in invertebrates (Belardetti, Kandel and Siegelbaum, 1987; Mackey <i>et al.</i> , 1987)	c30173_g7_i1 MN081862	<i>flp-1</i>	<i>yn2</i>	NY7	Del (1.1 kb): Eliminates 567 bp of the promoter region up to the 4th exon (Nelson <i>et al.</i> , 1998; Buntschuh <i>et al.</i> , 2018)	nose touch defective (7% responsive), insensitive to hyperosmolarity (Buntschuh <i>et al.</i> , 2018)	Head interneurons receiving inputs from the ASH neuron (Schinkmann and Li, 1994)	33.6% id in 107 res ov; Score: 98.0; Gap fr: 9.3%
				<i>yn4</i>	NY16	Del (2.1 kb). Eliminates upstream and coding regions (Nelson <i>et al.</i> , 1998)	nose touch defective (30% responsive), insensitive to hyperosmolarity (Nelson <i>et al.</i> , 1998)		
Allatostatin C / opioid- like receptor (OPRL/AstCR)	GPCRs involved in antinociception/analgesia. (Bachtel <i>et al.</i> , 2018)	c22802_g3_i1 PV164564	<i>npr-17</i>	<i>tm3210</i>	-	Del (209 bp). Eliminates 6th and 7th exon (Wormbase)	Impairment in aversive response to aversive odorant; abolished pumping when exposed to morphine (Mills, 2014)	sensory neurons and the PVPs, PVQs, PQR (Harris <i>et al.</i> , 2010; Mills, 2014)	27.9% id in 240 res ov; Score: 171.0; Gap fr: 6.7%
Buccalin-like (Ast/Op-like peptide)	Opioids are neuropeptides involved in analgesia (Harrison <i>et al.</i> , 1994; Stefano and Salzberg, 1999)	c26559_g1_g2 PV206714	<i>nlp-3</i>	<i>ok2688</i>	RB2030	Del (1618 bp). Eliminates exons in the 3' region (Wormbase)	Locomotor impairments (Yemini <i>et al.</i> , 2013)	Pharyngeal, Ach and 5HT neurons, ADF, ASE, ASH, AWB, ASI, BAG, HSN, VNC, intestine (Nathoo <i>et al.</i> , 2001)	37.1% id in 67 res ov; Score: 19.0; Gap fr: 0.0%
				<i>tm3023</i>	FX03023	Del (354 bp). Eliminates exons in the 3' region (Wormbase)	Impaired aversive response to octanol (Harris <i>et al.</i> , 2010)		

Gene name	Function	<i>O. vulgaris</i> candidate	<i>C. elegans</i> orthologue	Allele	Strain name	Mutation	Reported behavioural phenotype	Gene expression	% Domain identity
Other modulators of nociception									
Calcitonin Gene-related Peptide Receptor (CGRPR)	Receptor characterising "peptidergic" nociceptors. Involved in pain transmission (Basbaum <i>et al.</i> , 2009; Benemei <i>et al.</i> , 2009)	c31238_g13_i1 MN081807	<i>pdfr-1</i>	<i>ok3425</i>	CX14295	Del (605 bp). Eliminates from 5th to 7th exon (Wormbase)	strong ascr#3 aversion that requires ASI but is completely insensitive to food thickness, severe disruption in crawling behaviour (Luo and Portman, 2021)	Body wall muscle cells, mechanosensory neurons (PLM, ALM, FLP, OLQD and OLQV), chemosensory neurons (PHA and PHB), ring motor neurons (RMED and RMEV) pharyngeal interneuron pair I1 (Janssen <i>et al.</i> , 2008). OLL, FLP, AVM, ALM, AVD, PLM, PVM, PVC, PHA, URX, PQR (Luo and Portman, 2021)	25.3% id in 265 res ov; Score: 200.0; Gap fr: 5.8%
Stomatin (STOM)	Protein found in mechanosensory neurons (Price <i>et al.</i> , 2004; Moshourab <i>et al.</i> , 2013)	c33141_g1_i2 PV164567	<i>mec-2</i>	<i>e75</i>	CB75	Sub (Ala204Thr). Missense mutation altering the N-ter half of the stomatin-like region (Gu, Caldwell and Chalfie, 1996)	Weak to absent light touch sensitivity due to a disruption of a protein-protein interaction (impaired activity of <i>mec-4</i> and <i>mec-10</i>). Cl ⁻ , Na ⁺ , benzaldehyde, 2-butanol, isoamyl alcohol chemotaxis defect (Gu, Caldwell and Chalfie, 1996; Goodman and Schwarz, 2003; Zhang <i>et al.</i> , 2004)	ALM, PVM PLM (Zhang <i>et al.</i> , 2004)	80.1% id in 201 res ov; Score: 335.0; Gap fr: 0.0%
Anoctamin (ANO)	Cl ⁻ channel activated by Ca, highly expressed in small sensory neurons. Reported to be activated by noxious heat over 44°C (Cho <i>et al.</i> , 2012)	c31699_g1_i2 MN081804	<i>anoh-1</i>	<i>tm4762</i>	-	Del (202 bp). Results in a frameshift and a premature stop codon after aa 17 of the predicted ANOH-1b ORF and removes the start codon of the alternatively spliced ANOH-1a ORF (Li <i>et al.</i> , 2015)	In <i>mec-4</i> and <i>anoh-1</i> double mutants the removal of necrotic touch neurons (PLML/R) is significantly delayed. UIN (Li <i>et al.</i> , 2015)	mechanosensory neurons (ALM, PLM, PVM) (Li <i>et al.</i> , 2015)	28.9% id in 477 res ov; Score: 429.0; Gap fr: 4.0%
Transmembrane Channel (TMC)	In <i>C. elegans</i> , Required for salt chemosensation and alkaline aversion (Chatzigeorgiou <i>et al.</i> , 2013; Wang <i>et al.</i> , 2016)	c31137_g8_i2 PV164552	<i>tmc-1</i>	<i>ok1859</i>	RB1546	Del (2025 bp). Eliminates from 10th to 15th exon (Wormbase)	strongly defective in the avoidance of NaCl concentrations above 100 mM, reduced avoidance response to sodium acetate and sodium gluconate; no apparent defect in nose touch avoidance (Chatzigeorgiou <i>et al.</i> , 2013; Wang <i>et al.</i> , 2016)	ASH, ADF, ASE, ADL, AQR, PQR, URX and PHA, PHC body wall muscles and cholinergic neurons (Chatzigeorgiou <i>et al.</i> , 2013; Wang <i>et al.</i> , 2016; Setty <i>et al.</i> , 2022)	41.0% id in 61 res ov; Score: 124.0; Gap fr: 0.0%
Sodium leak channel non-selective protein (NALCN)	May contribute to algogen insensitivity as seen in vertebrates (Eigenbrod <i>et al.</i> , 2019; Smith, Park and Lewin, 2020; Zhang and Wei, 2024)	c32050_g13_i1 PV164501	<i>nca-2</i>	<i>gk5</i>	VC9	Del (2970 bp). Eliminates from 8th to part of 16th exon (Wormbase)	altered response to halothane and impaired locomotion pattern. UIN (Yeh <i>et al.</i> , 2008)	Broad nervous expression (Yeh <i>et al.</i> , 2008)	52.1% id in 1070 res ov; Score: 2702.0; Gap fr: 3.8%
Cyclic Nucleotide Gated Channel (CNG)	In <i>C. elegans</i> , involved in the transduction cascade for the chemosensory detection and thermosensation (Coburn, 1996; Komatsu <i>et al.</i> , 1996)	c31964_g8_i1 PV164549	<i>tax-2</i>	<i>ks10</i>	FK100	Unknown	Unresponsive to temperature or to water-soluble or volatile chemical attractants (Coburn, 1996; Komatsu <i>et al.</i> , 1996)	AQR, AWB, AWC, AFD, ASE, ASG, ASK, ASI, ASI, PQR, URX (Coburn, 1996; Komatsu <i>et al.</i> , 1996)	35.8% id in 360 res ov; Score: 574.0; Gap fr: 2.8%
			<i>tax-2</i>	<i>p671</i>	PR671	Sub (Cys231Arg). Missense mutation in the first membrane-spanning domain, affecting the pore region (Coburn, 1996)			34.6% id in 189 res ov; Score: 584.0; Gap fr: 2.6%
			<i>tax-4</i>	<i>p678</i>	PR678	Sub (Gln/*). Nonsense mutation (Komatsu <i>et al.</i> , 1996)			
			<i>tax-2/tax-4</i>	<i>p671/p678</i>	BR5514	p671: Sub (Cys231Arg) p678: Sub (Gln/*) Nonsense mutation (Coburn, 1996; Komatsu <i>et al.</i> , 1996)			

Table 2.1. List of *C. elegans* loss of function mutant strains for the selected nociceptive related genes selected from *O. vulgaris* transcriptome after manual filtration of the in silico analysis.

For each gene, the first four columns report the gene proposed function based on literature, the *O. vulgaris* candidate gene (based on the assembled transcriptome Petrosino *et al.*, 2015 and Petrosino *et al.*, 2022) and assigned NCBI Accession number. Columns 5-10 refer to the orthologue gene of *C. elegans*, the strain and alleles selected to be tested for chemoaversion impairments and the pre-existing knowledge on the behavioural phenotype of the mutant, as well as the known localisation of the gene in worms tissues. In some cases, a mutation in more than one allele encompassing the same gene was tested. The last column refers to the degree of identity and similarity found between the two species genes when comparing their functional domains (Expasy-SIM tool, BLOSUM62 comparison matrix). Del: deletion; Ins: insertion; Sub: substitution; res ov: residues overlap; id: identity; Gap fr: gap frequency; lof: loss of function; N-ter: N-terminal; UIN: unknown involvement in nociception.

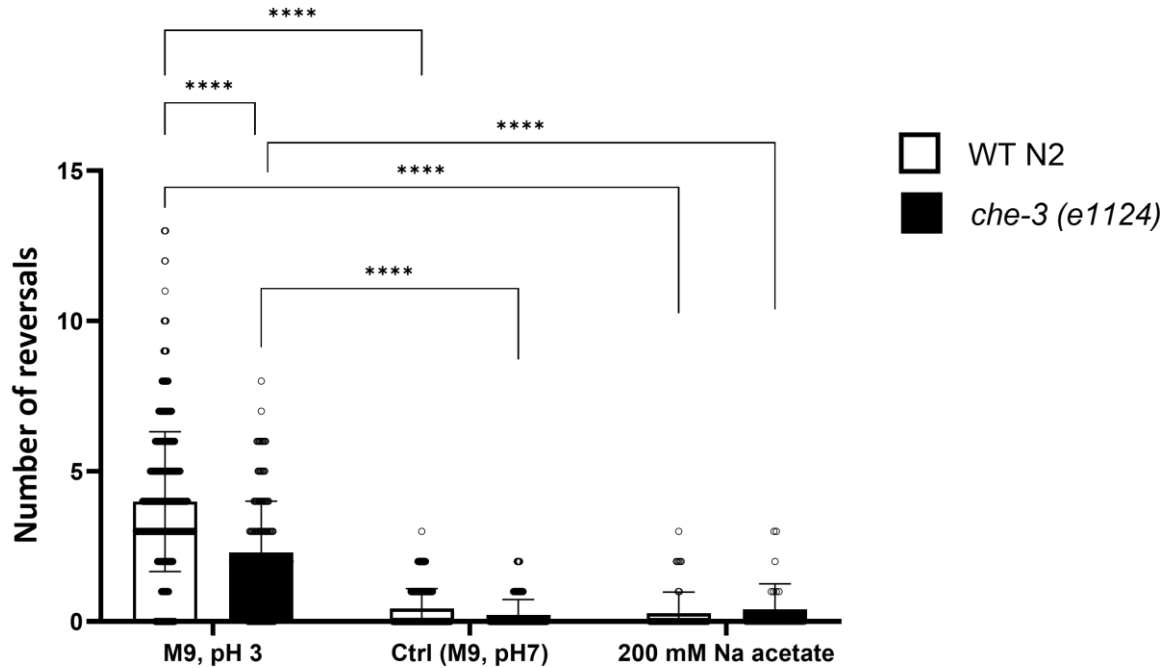


Figure S2.1 C. *elegans* elicits a clear avoidance response when in contact with acidic pH in the drop assay.

The bars represent the average number of reversals exhibited by worms $\pm$ SD. The assay highlights a clear aversive response to M9, pH3 in the WT N2 (3.990 ± 0.115 reversals) when compared to Ctrl M9, pH7 (0.432 ± 0.033 reversals, $p < 0.0001$). An effect of the repellents is still visible in *che-3(e1124)* mutants in which the ultrastructural organisation of the sensory amphids is deficient (M9 pH3 vs Ctrl M9, pH7, $p < 0.0001$) but a significant impairment in the response is evident when compared to the WT N2 ($p < 0.0001$) with an average of 2.293 ± 0.130 reversals. Additionally, worms were shown to be substantially insensitive to 200mM Na acetate (200 mM Na acetate vs Ctrl M9, pH7, $p > 0.999$), confirming that the aversive effect elicited by acetic acid is due to H^+ exposure. The mean was calculated from data collected from repeats of the same experiment conducted independently across different days. In each experiment, a single worm was exposed to the drop only once. Data was subjected to two-way ANOVA followed by Tukey's multiple comparisons test. Each dot represents a single worm. WTN2-M9, pH3 = 410 worms ; WTN2-Ctrl = 410 worms; WTN2-Na acetate = 55 worms; *che-3(e1124)*-M9, pH3 = 174 worms; *che-3(e1124)*-Ctrl = 174 worms; *che-3(e1124)*-Na acetate = 30 worms.

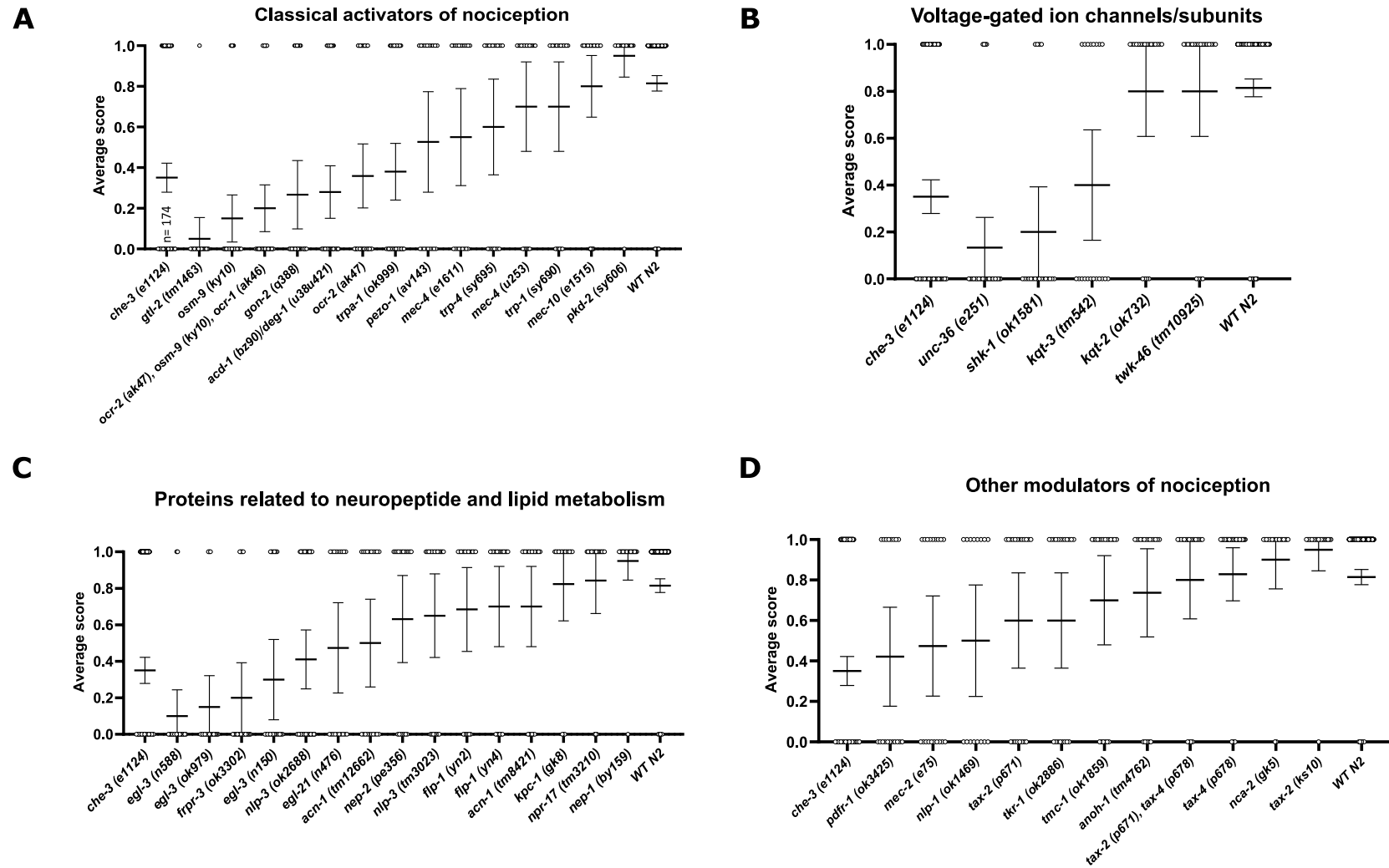


Figure S2.2 Low pH avoidance performance of *C. elegans* mutant strains for sensory detection.

Scatter dot plot showing the average score of worms tested for each strain in the drop assay. Statistical analysis is shown in Figure 2 of the main text. The shape represents the distribution of responses across the strains and each dot represents an individual worm tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ refers to the comparison against the WT N2 performance. Each strain was tested in at least two independent experiments.

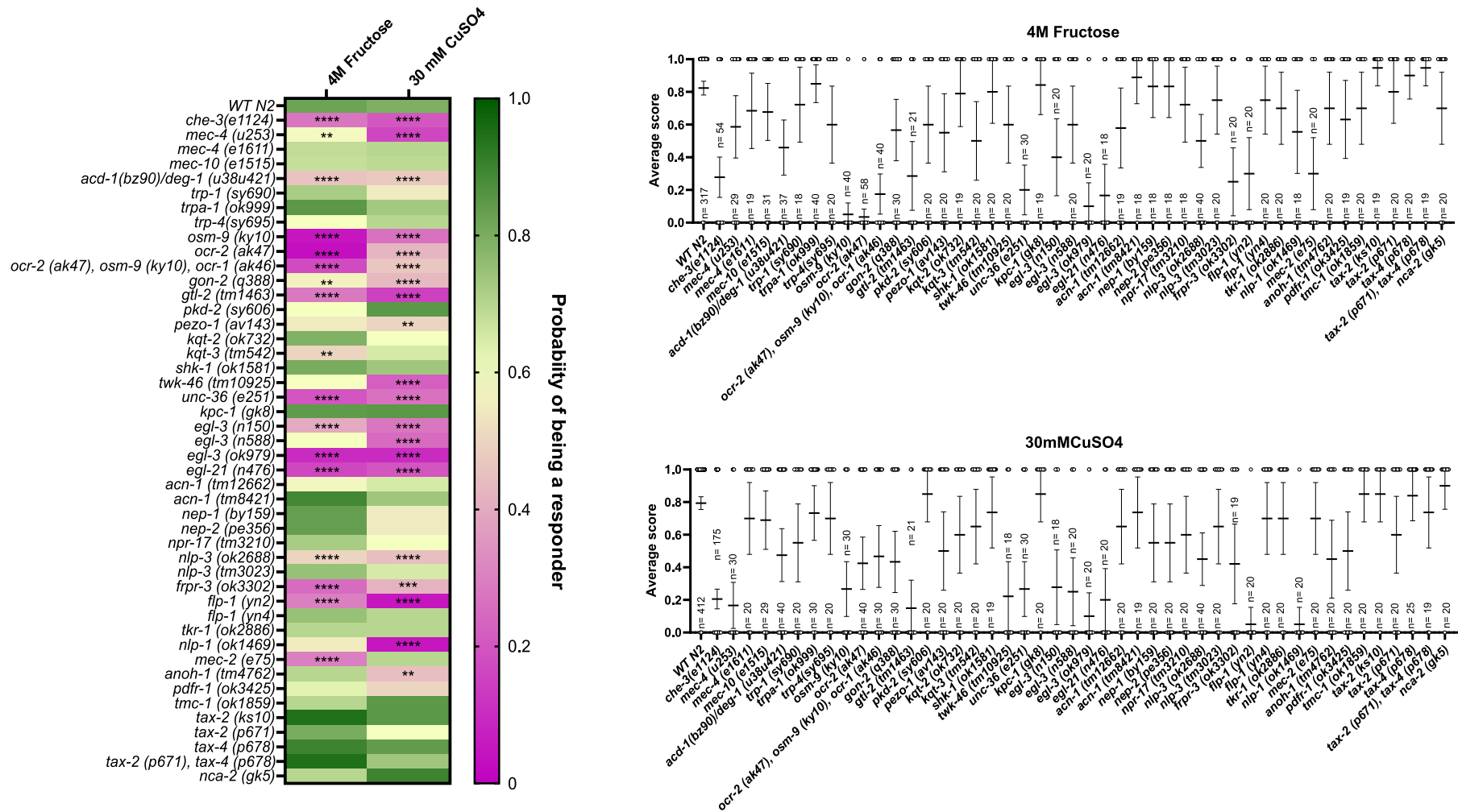


Figure S2.3 C. *elegans* mutant strains were tested for aversion to other noxious cues.

On the left, heatmap summarising the average probability of being a responder (≥3 reversals within 5s) for each strain tested compared to the WT N2 performance. Significance is expressed according to the corrected p-value (q value) following Benjamini-Hochberg false discovery rate method (Q=1%). On the right, scatter dot plots representing the average score ± SD for the same data. Each dot represents an individual worm (the total is also indicated by n). Each strain was tested in at least two independent experiments.

Chapter 3 Identification and functional investigation of *Octopus vulgaris* TRPV channels: molecular determinants of nociception in cephalopods?

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Supplementary Tables S1-S4 and Supplementary Files S1-S6 are electronic files accessible from the following link: [10.5281/zenodo.18377710](https://doi.org/10.5281/zenodo.18377710)

3.1 Abstract

Nociception is an essential response for organisms to avoid potential harm and promote survival. Its molecular determinants are largely conserved across Eumetazoa. TRPV receptors are polymodal ion channels exhibiting selective peripheral expression and functional coupling that underpins nociception and pain modulation in vertebrates. However, the execution of protective behaviours triggered by TRPVs is also found in species with a simpler nervous organisation, thus encouraging their investigation in invertebrate model organisms to increase understanding of animal nociception.

Cephalopods represent an interesting invertebrate phylum with respect to the evolution of the nervous system whose complexity suggests it might support pain-like states that exist in vertebrates. This possibility is reflected by the inclusion of cephalopods in the UK and EU animal welfare legislations. Despite this, there is poor characterisation of cephalopod molecular nociceptors.

For this reason, we used *in silico* analysis to identify two TRPV channels in *Octopus vulgaris* genome (*Ovtrpv1* and *Ovtrpv2*). We validated the putative transcript sequences and highlighted prevalent expression in sensory tissues. We investigated the functional competence of these TRPVs by heterologously expressing *Ovtrpv1* and *Ovtrpv2* cDNA into *Caenorhabditis elegans* null mutants of the orthologous genes – *ocr-2* and *osm-9* respectively. *Ovtrpvs* successfully rescued the *C. elegans* mutant lost aversive response to chemical and mechanical noxious stimuli, suggesting these receptors as polymodal invertebrate nociceptors. Additionally, complementary investigation using *Xenopus* oocytes showed *Ovtrpv1* and *Ovtrpv2* form an active heteromeric channel directly gated by nicotinamide. This study highlights *Ovtrpvs* as an important route to better understand nociceptive detection in cephalopods.

3.2 Introduction

General nociception executes function by activating a reflexive withdrawal response protecting organisms from potentially threatening stimuli. The various avoidance behaviours operate across animal phyla and advantage organism survival (Smith and Lewin, 2009). Nociception is characterised by a conserved specialised neuronal architecture and shared molecular determinants that persist across distinct nervous system organisations and largely encompasses a core reflex that triggers withdrawal avoidance responses (Julius and Basbaum, 2001). In more complex organisms, the reflex nociceptive responses are modulated by top-down neuronal signalling, leading to the elaboration of learnt and emotionally encoded pain states (Basbaum *et al.*, 2009).

Cephalopods are invertebrate species characterised by well described withdrawal responses following exposure to noxious challenges (Crook, Hanlon and Walters, 2013; Hague, Florini and Andrews, 2013; Crook, 2021). Furthermore, these molluscs possess a central nervous system that can act in a hierarchical fashion, allowing them to express complex behaviours (Amodio *et al.*, 2019; Schnell *et al.*, 2021). Whilst the details of this anatomy are different from organisms that are unequivocally known to express the emotional states defined by pain, nonetheless cephalopod central brain mass complexity provokes the possibility that their neural architecture is organised to allow brain states with components that resemble pain (Andrews *et al.*, 2013; Smith *et al.*, 2013; Shigeno *et al.*, 2018). This highlights potential ethical issues and associated welfare constraints about the experimental use of these cephalopods that have seen these animals included in research legislation (UK S.I. 1993/2103, 1993; European Parliament and Union, 2010).

This raises the scientific value of detailing the biological organisation of nociception reflexes and their modulation in this important biological and societally utilised taxon.

In our previous study, we identified *O. vulgaris* orthologues of genes with a defined role in nociception and pain to enrich understanding of the molecular determinants of these phenomena in cephalopods. We coupled this to functional studies in *C. elegans* and identified 19 potential candidates, warranting more detailed investigation for their role in *O. vulgaris* nociception (Pieroni *et al.*, 2026). Among our candidates, the previously investigated vanilloid transient receptor potential (TRPV) emerged as a priority candidate for further investigation (Caterina *et al.*, 1997).

TRP receptors are a large family of ion channels highly conserved across animal species due to their extensive role in different physiological functions (Pedersen, Owsianik and Nilius, 2005; Ramsey, Delling and Clapham, 2006; Venkatachalam and Montell, 2007). Among the seven known subfamilies, the vanilloid sensitive receptor homologues include genes encoding nociceptive detection of aversive stimuli (Colton, 2006; Radresa *et al.*, 2012; Shibasaki, 2024). The best characterised representative of TRPVs in mammals is the TRPV1, best known as the capsaicin receptor due to being gated by the chilli pepper extract (Caterina *et al.*, 1997). However, TRPV1 is a polymodal nociceptor and also responds to cell damaging pH and temperature changes (Venkatachalam and Montell, 2007; Dhaka *et al.*, 2009; Julius, 2013). TRPVs are typically tetrameric membrane receptors in which individual subunits have six transmembrane domains (TMs), cytosolic N- and C- terminals and a hydrophobic short P-loop between the fifth and sixth TMs that creates the selectivity filter (Rosasco and Gordon, 2017). This structure and the sensory related functions are largely conserved across vertebrate and invertebrate species such as *Hirudo medicinalis*, *C. elegans* and *Drosophila melanogaster*, where their contribution to

nociception is documented (Colbert, Smith and Bargmann, 1997; Tobin *et al.*, 2002; Gong *et al.*, 2004; Summers, Holec and Burrell, 2014; Ohnishi *et al.*, 2020).

In this study, we characterised two TRPV channel representatives (*Ovtrpv1* and *Ovtrpv2*) from *O. vulgaris*. These are orthologues of *C. elegans* *ocr-2* and *osm-9* receptors, respectively, enabling us to confirm function by heterologous expression in *C. elegans* null mutants and successful rescue of the aversive response in these strains. We used *in silico* and experimental analyses encompassing PCR to localise *Ovtrpvs* expression to distinct tissues, and *Xenopus* oocytes expression to characterise channel function. Taken together these data validate two *bona fide* TRPV channel representatives from *O. vulgaris* which have the functional properties and anatomical localisation to act as polymodal sensory receptors with a key role in detecting and classifying environmental stimuli.

3.3 Materials and Methods

3.3.1 In silico analysis

3.3.1.1 BLAST search criteria to identify *O. vulgaris* transcripts

The protein sequence of *Homo sapiens* TRPV1 (*Hstrpv1*) channel that encodes the human capsaicin receptor was retrieved from UniProtKB/Swiss-Prot (release 2021_04) and blasted against the previously published *O. vulgaris* *de novo* assembly transcriptome (Petrosino, 2015; Petrosino *et al.*, 2022) using the TBLASTN algorithm (BLAST+ v2.10.0+) with a standard threshold of $10e^{-5}$ used as a cut-off. Any hit with the e-value closer to 0 was selected for further analysis. The reference sequences were also used in a BLASTp search against the predicted proteome obtained from the most recent sequenced *O. vulgaris* genome (Destanović *et al.*, 2023), using the same parameters described above. The hits retrieved from both searches, were blasted and compared against *Octopus bimaculoides* ([ASM119413v2](#)), *Octopus sinensis* ([GCA_006345805.1](#)) and *Aplysia californica* ([PRJNA13635](#)) assemblies to facilitate assessment of completeness.

In the case of the *O. vulgaris* chemotactile receptor (CRTs) orthologues, *O. bimaculoides* published sequences were retrieved from NCBI and used for a BLASTp search against *O. vulgaris* genome using the parameters mentioned above (Supplementary Table S1). All the resulting candidates were then aligned against the *H. sapiens* acetylcholine receptor $\alpha 7$ subunit (NP_000737.1) prior selection, to check for the lack of the classical vicinal cysteines involved in the neurotransmitter binding, which signatures cephalopod CRTs (van Giesen *et al.*, 2020).

3.3.1.2 Exon-intron boundaries detection

The reconstructed *Ovtrpv* transcript sequences were blasted against the assembled *O. vulgaris* genome (Destanović *et al.*, 2023) to localise their chromosomal location. The closest match was

downloaded and converted into a BLAST custom database through the ncbi/suite tool container in Docker (Merkel, 2014). Alignment between the *Ovtrpv1* and *Ovtrpv2* cDNA from *O. vulgaris* or other species (i.e. *O. sinensis*) and the genomic sequence was performed using ncbi/magicblast tool container (Boratyn *et al.*, 2019). The results were sorted using biocontainers/samtools (Danecek *et al.*, 2021) and the data were processed and visualised using Tablet software (The James Hutton Institute, v1.21.02.08, Milne *et al.*, 2013).

3.3.1.3 Secondary and 3D structure prediction and modelling

The assembled contig cDNA was translated to its predicted amino acid sequence through ExPASy translate (Gasteiger *et al.*, 2003). Each octopus predicted protein of interest was analysed using DeepTMHMM - 1.0 to obtain a prediction of the secondary structure (Hallgren *et al.*, 2022). A 3D reconstruction of the proteins' single subunit was obtained using AlphaFold 3 (Abramson *et al.*, 2024) and their representation within the cell membrane was modelled through PPM3 Server (Lomize, Todd and Pogozheva, 2022).

Analysis of the conserved protein functional domains was performed using NCBI conserved domain (Lu *et al.*, 2020).

Alignment with other species orthologue proteins was carried out using Clustal Omega (Madeira *et al.*, 2022) and identity and similarities among the sequences were analysed with EMBOSS Water Pairwise Sequence Alignment (Madeira *et al.*, 2024) using a BLOSUM62 matrix.

3.3.1.4 Cluster analysis and phylogenetic investigation of OvTRPVs

Reference protein sequences for TRP channels from *H. sapiens*, *C. elegans* and *Drosophila melanogaster* as well as other cationic ion channel sequences such as voltage-gated sodium, potassium and calcium ion channels were used to perform a BLAST search (e-value $1e^{-10}$ and 40 maximum target sequences) against the complete proteome of 13 representative species from Mammalia, Cephalopoda, Gastropoda, Bivalvia, Insecta, Crustacea and Nematoda. This proteome selection was based on the best Benchmarking Universal Single-Copy Orthologs (BUSCO) value (Supplementary Table S2). All the results from each species were sorted and processed through CD-hit using a 0.95 similarity threshold to remove shorter isoforms or redundant sequences (Li and Godzik, 2006; Fu *et al.*, 2012). The resulting unique sequences from all the species (1102 sequences) were then used as a query to perform a CLuster ANalysis of Sequences (CLANS) (Zimmermann *et al.*, 2018) with the BLAST HSPs e-value of $1e^{-12}$ and BLOSUM62 scoring matrix (Supplementary File S1). Visualisation of the sequences relationship and cluster organisation was performed with the CLANS toolkit with a set p-value threshold of $1e^{-60}$ (Zimmermann *et al.*, 2018). All the sequences of the cluster which included the target *Ovtrpv1* and *Ovtrpv2* sequences (50 sequences, Supplementary File S2) were selected and used to generate a phylogenetic tree. Alignment of the sequences was performed using MAFFT v7.526, E-

INS-i method (Kato and Standley, 2013). The results were manually curated to exclude poorly aligned sequences (2) and then trimmed with TrimAl (Supplementary File S3) using gappy-out mode and default parameters (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The best-fit model of evolution was selected with ModelFinder (LG+I+G4 chosen according to the Bayesian Information Criterion) in IQ-TREE3 v3.0.1 (Wong *et al.*, 2025). Tree branches were obtained using aLRT-SH with 1,000 replicates and ultrafast bootstrap method. The final phylogeny was visualised using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>). The tree was rooted using the vertebrate TRPV channel subfamilies.

3.3.2 Animal samples

3.3.2.1 *O. vulgaris* samples collection

Young adults of *O. vulgaris* were caught by local artisanal fishermen in the Bay of Naples, Italy according to the local Animal Welfare Body authorisation (Ethical Clearance: ecACR-2302ts36). Animals were humanely killed adopting the principles described in Annex IV of Directive 2010/63/EU and following Andrews *et al.* (2013), Andrews *et al.* (2013), Fiorito *et al.* (2015), Butler-Struben *et al.* (2018) to ensure responsible and ethical use of animal-derived materials and to adhere to the principles of Replacement, Reduction, and Refinement (3Rs).

The following tissues were collected by a FELASA certified (function D) competent person, dissected, stored in 500 µL RNA later and shipped in dry ice: supra-oesophageal mass (SEM), sub-oesophageal mass (SUB), optic lobe (OL), stellate ganglion (StG), gastric ganglion (GG), arm (muscle + axial nerve cord, at 50% of its length), tip of the arm (TIP), sucker (Su), intestine (Int), stomach (Stom), white body (WB), anterior salivary gland (ASG), posterior salivary gland (PSG), digestive gland (DG), haemocytes (Hc), kidney (Kid), branchial heart (BrH), skin, mantle (Man) and gill (Supplementary Table S3). The tissues were processed following the Ethics and Research Governance Online II (ERGO II) policy (nr 68971.A1) in place at the University of Southampton.

3.3.2.2 *C. elegans* strains and husbandry

The following nematode strains were utilised in this study: CX10 *osm-9 (ky10)*; CX4544 *ocr-2 (ak47)*; the Bristol N2 was used as the wild-type (WT) strain. The strain genotype of the *osm-9 (ky10)* and *ocr-2 (ak47)* mutants was confirmed by designing a pair of primers flanking the region of mutations (Supplementary Table S4) and sequencing (Eurofins Genomics).

C. elegans were cultured and maintained as described in Brenner (1974). Three days prior to any assay, gravid adult worms were put on culture plates to lay eggs for 4h and then removed to produce a synchronised population to be tested at the L4+1 (young adult) stage. In the case of transgenic lines, L4 worms expressing GFP derived from co-marker plasmid were selected from the synchronised population and incubated overnight at 20 °C 24h prior the experiment.

3.3.3 Molecular biology

3.3.3.1 RNA extraction and cDNA synthesis from *O. vulgaris* tissues

Small resections (7-40 mg) of the indicated frozen tissues were collected in 2 mL Eppendorf tubes containing 500 µl TRIzol® (Invitrogen™), snap frozen and homogenized using a Handheld Homogeniser SHM1 (Cole-Palmer). After 5 min at room temperature, 200 µl of chloroform (Sigma-Aldrich) were added, mixed, and incubated on ice for 15 min. Samples were centrifuged (12,000 x g, 4° C) and the total RNA upper aqueous layer purified using PureLink® RNA Mini Kit (Invitrogen™). DNase I treatment (Invitrogen™) was performed to remove potential contaminating genomic DNA. Quality (260/280 ≈ 2.0) and quantity of extracted RNA were assessed through UV-visible absorption measurements (NanoDrop™ 2000/2000c Spectrophotometers). For each sample, 1 µg of RNA was used for reverse transcription following the manufacturer's protocol (SuperScript™ III Reverse Transcriptase, Invitrogen™). The cDNA samples were stored at -20°C until further use. As previously reported, the mollusc 'hidden break' does not allow a sufficient gel resolution to assess the RNA quality due to the co-migration of the 18S and 28S rRNA fragments (Natsidis *et al.*, 2019; Adema, 2021). Therefore, only the tissues from which we successfully amplified our control gene *cullin 1* (*cul1*), were selected for further use in PCR amplification of the genes of interest (Supplementary Table S3).

3.3.3.2 Primer design and end point PCR

Primer sequences of the genes of interests were designed using ApE-A plasmid Editor© v3.1.3 software and purchased from Eurofins Genomics (Supplementary Table S4). Once diluted to a working concentration of 25 pmol/µL, the primers were used to amplify the sequence in the tissues described above using 1 µL of cDNA as template in a PCR Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific™) following manufacturer's instructions.

3.3.3.3 Promoter and genes synthesis for cloning

The promoter region of the *osm-9* gene (*Posm-9*, approximately 1.6 kb – Colbert, Smith and Bargmann, 1997), the *C. elegans osm-9* (*Celeosm-9*) and the *Hstrpv1* cDNA sequences were synthesised using the Integrated DNA Technologies Gene synthesis service (IDT, Inc). The promoter region of the *ocr-2* gene (*Pocr-2*, approximately 2.5 kB - Sokolchik *et al.*, 2005) and the *O. vulgaris trpv2* (*Ovtrpv2*) were synthesised using the Genscript gene synthesis service and subcloned into the pcDNA3.1 vector (Genscript Biotech, UK). The pcDNA3.0 plasmid containing the *C. elegans ocr-2* cDNA sequence (*Celeocr-2*) was a kind gift from Prof. Cornelia Bargmann (Rockefeller University, NY, USA). All the plasmid sequences received, synthesised and cloned were verified for their authenticity using the Oxford Nanopore Whole Plasmid Sequencing (WPS) service from Eurofins Genomics. A missense mutation (AAC to AGC) in *ocr-2* cDNA leading to N695S in the protein sequence was identified in the original *pcDNA3-ocr-2* plasmid. However, the

cDNA still showed a successful behavioural rescue when expressed in *ocr-2* (*ak47*) mutant worms suggesting it is functionally silent, consistently with previous investigations (Tobin *et al.*, 2002; Ohnishi *et al.*, 2020).

3.3.3.4 Cloning for expression in *C. elegans*

The *Pocr-2/Posm-9* sequences were cloned (using *HindIII/XhoI*) into the pWormgate2 vector (Johnson, Behm and Trowell, 2005) to produce a pDEST-*Pocr-2* or pDEST-*Posm-9* vector for suitable expression in *C. elegans*. The full-length transcript sequences of *Ovtrpv1*, *Ovtrpv2* and *Celeocr-2* were amplified (Supplementary Table S4) using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific™) and the A' overhangs were added using GoTaq® G2 DNA Polymerase (Promega; 30 min at 72 °C) prior to ligation into pCR™8/GW/TOPO™ TA (Invitrogen™). This sequence was then transferred into the pWormgate2, downstream of the corresponding promoter *via* attR/attL recombination using a Gateway™ LR Clonase™ II Enzyme mix (Invitrogen™). This produced the final constructs: pDEST-*Posm-9::Ovtrpv2*, pDEST-*Pocr-2::Ovtrpv1*, pDEST-*Pocr-2::Celeocr-2*. The *Celeosm-9* cDNA sequence was cloned *via* *KpnI/SacI* downstream of *Posm-9* to produce pDEST-*Posm-9::Celeosm-9* final construct (Supplementary File S4). The plasmids were transformed into chemically competent bacterial cells (Thermo Scientific™) and the amplified plasmids purified from overnight cultures. These were then purified using the Monarch® Spin Plasmid Miniprep Kit (New England Biolabs®, UK) and authenticated by sequencing (Eurofins Genomics).

3.3.3.5 Cloning for expression in *Xenopus laevis* oocytes

The sequences of *Ovtrpv1*, *Ovtrpv2*, were cloned into the multiple cloning site of the oocyte expression vector pTB207 *via* *HindIII/NotI*. The sequence of *Hstrpv1* was cloned *via* PCR amplification to introduce the compatible cutting sites *EcoRI-NotI* (Supplementary Table S4). The pTB207 vector was a kind gift from Jean-Louis Bessereau's Lab (Claude Bernard University, Lyon, France). These plasmids were transformed into chemically competent bacterial cells (Thermo Scientific™) and the amplified plasmids purified from overnight cultures. These were then purified using the QIAGEN Plasmid Maxi Kit (QIAGEN) and authenticated by sequencing (Eurofins Genomics). The final construct sequences are available in Supplementary File S5.

3.3.3.6 *C. elegans* fosmids purification and validation

The fosmids B0212.3 *osm-9* (clone ID: WRM066bG12) and C07G1 *ocr-2* (cloneID: WRM0634bB10) were purchased from K.K. DNAFORM (Yokohama, Japan). The amplified fosmids were purified using the QIAGEN Plasmid Maxi Kit (QIAGEN). PCR verification was performed using primers designed to amplify an internal region of *osm-9/ocr-2* which was authenticated by sequencing (Eurofins Genomics). The construct sequences are available in Supplementary File S6.

3.3.4 Microinjections

The purified pWormgate2 plasmid (Johnson, Behm and Trowell, 2005) containing the indicated genes was microinjected (40 ng/μl of plasmid, 10 ng/μl in the case of fosmid) and 30 ng/μl of *Pmyo-2::gfp* or *Pmyo-3::gfp* (Addgene). These co-injection markers highlight the pharyngeal or the body wall muscle respectively. The microinjection was performed following the protocol described in Mello et al. (1991) and Mello and Fire (1991) with aluminosilicate glass capillaries (1.0 mm OD, 0.78 mm ID, Harvard Apparatus). Injections were conducted as previously described and injected worms were transferred onto a new seeded plate (Calahorra et al., 2022). Progeny from the injected worm was selected based on the fluorescence and transferred onto separate seeded plates to establish stable transgenic lines for propagation (F2 generation). The following transgenic lines were obtained: *ocr-2 (ak47) Pmyo-3::gfp*; *ocr-2 (WRM0634bB10) Pmyo-3::gfp*; *pDEST-Pocr-2::Ovtrpv1 Pmyo-3::gfp*; *pDEST-Pocr-2::Celeocr-2 Pmyo-3::gfp* in *ocr-2(ak47)* mutant background; *osm-9(ky10) Pmyo-3::gfp*; *osm-9 (WRM066bG12) Pmyo-2::gfp*; *pDEST-Posm-9::Celeosm-9, Pmyo-3::gfp*, *pDEST-Posm-9::Ovtrpv2* in *osm-9 (ky10)* mutant background.

3.3.5 Behavioural assays

3.3.5.1 Drop assay to test chemoaversion in *C. elegans*

The behavioural analyses were conducted in experimental arenas in which a microscope digital camera (MU1403B, AmScope™) was mounted and the worm behaviours in response to the indicated treatments were recorded. The captured videos were analysed through manual scoring using AmScope Software v.10.11.2024. The assays were time stamped by the digital camera and the speed and quality of the responses assessed using the criteria listed below. The experimenter and observer were blind to the strains under investigation.

Chemical aversion in *C. elegans* was performed using an adapted version of the classical acute drop assay (Hilliard, Bargmann and Bazzicalupo, 2002; Hilliard et al., 2004). Ten L4+1-day old worms from indicated lines were transferred onto a 9 cm unseeded NGM plate and left undisturbed for at least 20 min. This allows the worms to transition to roaming behaviour encompassing periods of extended forward runs (Gray, Hill and Bargmann, 2005). A small drop of noxious cue was delivered in front of a moving worm through a small glass capillary (1.0 mm OD, 0.78 mm ID, Harvard Apparatus) attached to a syringe. A binary score was assigned with a positive response (1) or negative response (0) for each compound tested within 5 s of exposure to the cue and was based on the average number of reversals being equal or higher to the average number showed by N2. The worms for each condition were tested by exposing them to the drop only once. To trigger low pH response, acetic acid (CH₃COOH; Fisher chemical) was dissolved in M9 buffer at a final pH of 3 (M9, pH 3). D-fructose (Fisher chemical) was dissolved in M9 buffer to reach a

concentration of 4M. M9 buffer (pH 7) was checked not to cause any response to N2s when administered alone.

3.3.5.2 Volatile aversive response

Following the same husbandry described above, 10 worms expressing long forward runs were individually exposed to 30% 1-octanol (Sigma-Aldrich) solution only once. A thin platinum wire ($\varnothing$ 0.1 mm, Agar Scientific) was dipped into the solution and then waived in front of a moving worm until the animal stopped and initiated a backward movement. The average latency to start a reversal (s) was recorded with a cut-off of 15 s.

3.3.5.3 Nose touch assay

Briefly, an eyebrow was placed perpendicular to the front of a moving L4+1 worm until the animal hit the hair (Kaplan and Horvitz, 1993). Worms were given a binary score indicating nose touch sensitive (1) or insensitive (0). The sensitive worms halted or/and reversed following the collision. The insensitive worms kept moving forward and tried to climb or cross the hair. The experiment was performed in a single set of 10 consecutive touches on 5 worms per strain. The final ratio of sensitive/insensitive responses per worm was calculated and compared between N2, mutant and transgenic strains.

3.3.5.4 Data analysis and statistics

In the behavioural assays the transgenic lines that showed a performance that reached two standard deviations from the mean of the mutant line were considered rescued and included in the analysis (Figure S3.1).

Data were analysed using either one-way or two-way parametric analysis of variance (ANOVA). Post-hoc comparisons were performed using the Dunnett's multiple comparisons test. A level of probability set at $p < 0.05$ was used as statistically significant. Statistics were performed with GraphPad Prism version 10 for Windows (GraphPad Software, Boston, Massachusetts, USA).

3.3.6 TRPVs ligand activation

3.3.6.1 Electrophysiology of *Xenopus* Oocytes

Defolliculated *Xenopus laevis* oocytes were obtained from EcoCyte Bioscience and maintained in ND96 solution as follows (in mM): 96 NaCl, 1 MgCl₂, 5 HEPES, 1.8 CaCl₂, and 2 KCl adjusted to pH 7.4. The plasmids of interests, *pTB207-Hstrpv1*, *pTB207-Ovtrpv1*, *pTB207-Ovtrpv2*, were linearised using *PacI*, followed by DNA purification (Zymo) and elution in RNAase free water. cRNA was synthesised using the T7 mMessage mMachine transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocol (with incubation for 6h at 37 °C). RNA was purified using the GeneJET RNA purification kit (Thermo Fisher Scientific) and quantified using a NanoDrop spectrophotometer. Oocytes were injected with 50 nl of 500 ng/ μ l RNA individually or

co-expressed (*pTB207-Ovtrpv1/pTB207-Ovtrpv2*) using the Roboinject system (Multi Channel Systems). Injected oocytes were incubated at 18 °C in ND96 solution until the day of recording, 4 days post-injection.

Two-electrode voltage-clamp (TEVC) recordings were conducted using the Roboocyte2 System (Multi Channel Systems). Measuring head electrode resistance was approximately 400-1200 kΩ, pulled on a P-97 Micropipette Puller (Sutter Instrument). Electrodes contained AgCl wires backfilled with a 1 M KCl and 1.5 M KAc mixture. Oocytes were clamped at -60 mV during continuous recording at 500 Hz. Compounds applications (capsaicin, nicotinamide, ND96) lasted for 20 s for capsaicin followed by a 60 s wash with ND96, and 600 s for nicotinamide followed by a 200 s or 1200 s wash with ND96, perfusion speed was set to approximately 3 ml/min throughout. Uninjected oocytes were also tested and did not respond to any of the compound tested. Data were analysed using the Roboocyte2+ software.

3.3.6.2 Compounds and stimuli tested

The following compounds were utilised in this study: capsaicin natural (Biosynth), acetic acid glacial (FisherChemical), 1-octanol (Sigma-Aldrich) nicotinamide (NAM, Sigma-Aldrich). Stock solutions were prepared either in ethanol or DMSO/distilled water, and the final working concentration was reached by dissolving the solution in the ND96 medium prior to performing the assay. Saline-injected oocytes did not respond in any case tested.

3.4 Results

3.4.1 *In silico* identification of *O. vulgaris trpv* transcript and its experimental validation

When interrogating the previously available *O. vulgaris* transcriptome (Petrosino, 2015; Petrosino *et al.*, 2022) using sequences of curated TRPV channels (e.g., *H. sapiens* TRPV1), we retrieved two hits (c32354_g7_i1 and c32354_g6_i1). However, translation of these sequences revealed that the retrieved hits corresponded to incomplete fragments of a larger transcript. We therefore performed an *in silico* analysis reiterating the sequence between *O. vulgaris*, *O. bimaculoides* and *Aplysia californica* transcriptome databases. This led to the identification of three additional overlapping fragments (c32354_g14_i1, c32354_g12_i1, c32354_g2_i1) generating a contig transcript encoding a putative full length TRPV channel subunit. Secondary structure prediction tools identified intracellular N- and C-terminals, six TMs and several ankyrin repeats in the N-terminal region (Figure 3.1). Using 3D modelling with AlphaFold 3 (Abramson *et al.*, 2024) and simulation of the protein structure within the membrane with PPM3 server (Lomize, Todd and Pogozheva, 2022), we revealed the presence of a P-loop between TM 5 and TM 6, another key signature of these channels that was not detectable with secondary structure tools (Figure 3.1).

We designed primers that flank the predicted full-length sequence and used PCR amplification from *O. vulgaris* reverse transcribed mRNA combined with cDNA sequencing to authenticate it as a *bona fide* sequence (transcript accession number: PV164572, Pieroni *et al.*, 2026).

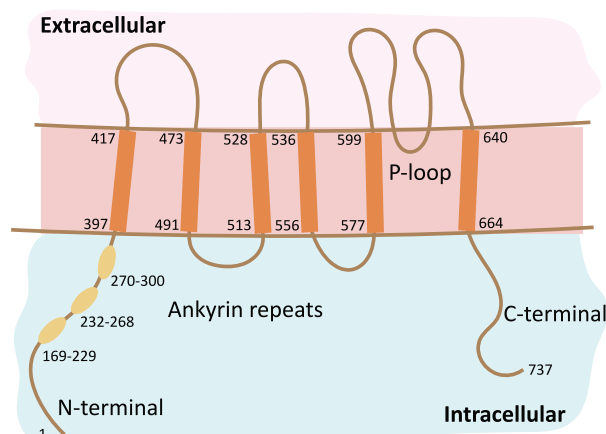


Figure 3.1 Schematic of the reconstructed *Ovtprpv1* translated protein following secondary and 3D modelling.

Typical TRPV channel secondary structure key signatures can be recognised such as intracellular N- and C- terminals, a variable number of ankyrin repeats in the N-terminal region, six transmembrane elements and a cytosolic P-loop between S5-S6 which is responsible for the pore formation and selectivity filter in the assembled tetrameric structure.

3.4.2 *Ovtprpv1* is located on chromosome 22 of *O. vulgaris* genome

The validated sequence was subsequently blasted against the most recently available *O. vulgaris* genome (Destanović *et al.*, 2023) and this led to the identification of a genomic fragment on chromosome 22 (OX597835.1). The translated protein (CAI9738794.1, OctVul6B016571P1) with an automated annotation of “transient receptor potential cation channel subfamily V member 5-like”, was shorter than the sequence predicted and validated. We therefore analysed the putative *Ovtprpv1* transcript against the genomic fragment OX597835.1 using NCBI Magic-BLAST. The result confirmed the presence of the transcript distributed in 15 exons (Figure 3.2A), with an identity of 99.6% over 100% coverage. Only nine mismatches, corresponding to single nucleotide polymorphisms were detected, but these did not affect the translated amino acids. This analysis highlights that the sequence previously tagged by the PV164572 submission (accession number of the experimentally verified sequenced transcript: PX926345) represents the correct annotation.

3.4.3 *O. vulgaris* genome analysis of *Ovtprpv1* revealed a second TRPV sequence

During our interrogation of the most recent *O. vulgaris* predicted transcriptome (Destanović *et al.*, 2023) with *Ovtprpv1*, we identified a distinct sequence located on chromosome 3 (OctVul6B028372T1). This was also automatically annotated based on sequence similarities as “transient receptor potential cation channel subfamily V member 5-like” but was not present in the previously available annotated transcriptome (Petrosino, 2015). Secondary structure prediction and AlphaFold3 modelling utilising the predicted protein sequence, showed again typical TRPV canonical features (Figure 3.1). However, the conserved domains tool highlighted

homology to the bacterial Spore_III_AF super family (cl17562; e-value = 5.81×10^{-3}) in the predicted protein sequence. To resolve this, we blasted the predicted sequence against other cephalopod transcriptomes and identified a high homology to predicted DNA sequences from *O. sinensis* and *O. bimaculoides*. However, when we aligned the proteins predicted from these DNA sequences, the apparent “Spore_III_AF super family” homology appeared not to be conserved. Therefore, we blasted both *O. vulgaris* *Ovtrpv2* and *O. sinensis* (the closest relative, *Ostrpv*) against *O. vulgaris* genome using NCBI Magic-BLAST tool. Both transcripts matched regions of chromosome 3 of *O. vulgaris*. However this matching was not as first envisaged and annotated (Figure 3.2B). The original *Ovtrpv2* predicted transcript was found to be distributed to 14 exons whilst the subsequent matching listed above found that *Ostrpv2* aligned with an additional exon. Furthermore, the N-terminal exons were differently detected between the two regions, suggesting a potential misprediction of the start codon (Figure 3.2B). We authenticated this matured prediction through PCR primers designed to flank the ends of the new prediction (Supplementary Table S4). This amplified a PCR product from mRNA derived cDNA of 2799 bp. Sequencing this product confirmed a *bona fide* sequence that does not contain the unexpected bacterial sequence (Accession number: PX926346). The depiction of the physiological gene structure of *Ovtrpv2* is represented in Figure 3.2.

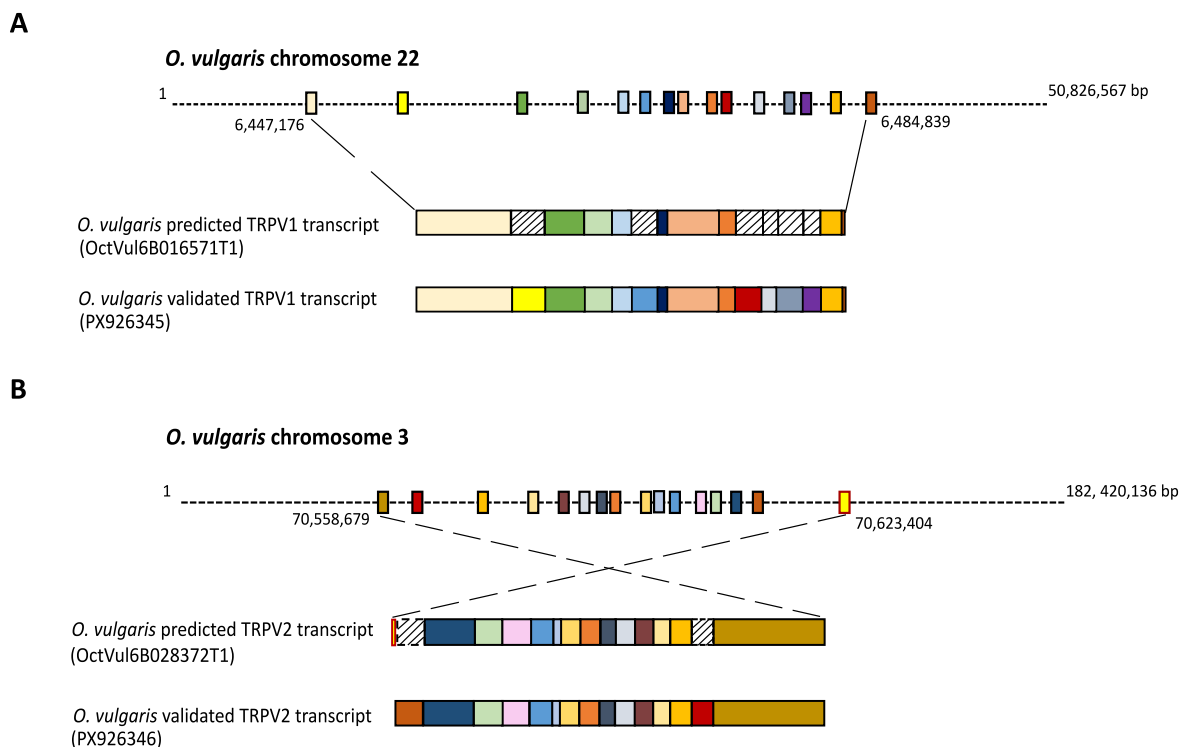


Figure 3.2 Schematic representation of *Octopus vulgaris* *trpv1* and *trpv2* gene.

A *Ovtrpv1* was found to be located on chromosome 22 with 100% coverage and 99.6% identity. **B** The second TRPV candidate gene, *Ovtrpv2*, is located on chromosome 3 and was initially mispredicted, requiring experimental validation. The red framed rectangle highlights mispredicted exons, striped pattern rectangles represent initially absent exons.

3.4.4 Cluster analysis and phylogenetic investigation confirmed OvTRPV1 and OvTRPV2 belong to the vanilloid TRPV subfamily

We next asked how these newly identified TRPV sequences related to the other TRP subfamilies. We performed a cluster analysis by blasting canonical curated TRP channel amino acid sequences against the proteome of 13 representative species (Supplementary Table S2). Our CLANS analysis showed the expected distinction among the different subfamilies of TRP channels and from other cationic ion channels as well (i.e., voltage-gated sodium, calcium and potassium ion channels, Figure 3.3A). The two newly identified sequences from *O. vulgaris* were indeed found within the same cluster that included other well-characterised vertebrate and invertebrate TRPVs. Our cluster analysis did not identify any additional *O. vulgaris* TRPV candidate. All the sequences belonging to the same cluster, were then used to build a phylogenetic tree to reveal the relationship between the sequences (Figure 3.3B). The phylogeny suggested invertebrate and vertebrate TRPV receptors share a common ancestor receptor. When looking at the invertebrates, two main branches seemed to have evolved from a duplication event that gave rise to “OCR-like” and “OSM-9-like” TRPV receptor branches, based on the homology with the *C. elegans* TRPV channels. Interestingly, the two OvTRPV sequences were found to belong to distinct branches, with OvTRPV1 belonging to the “OCR-like” and OvTRPV2 belonging to the “OSM-9 like” branch respectively (Figure 3.3B).

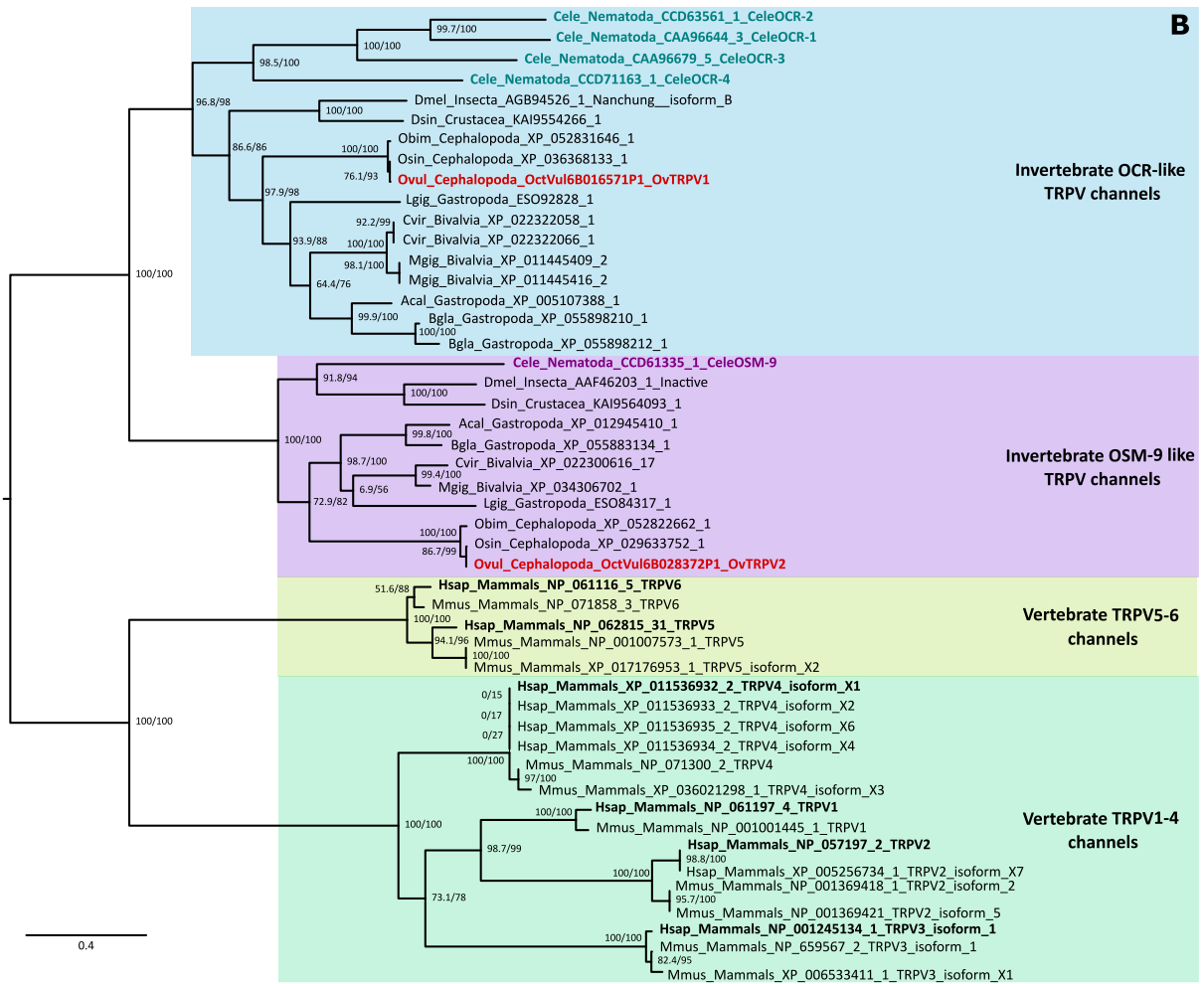
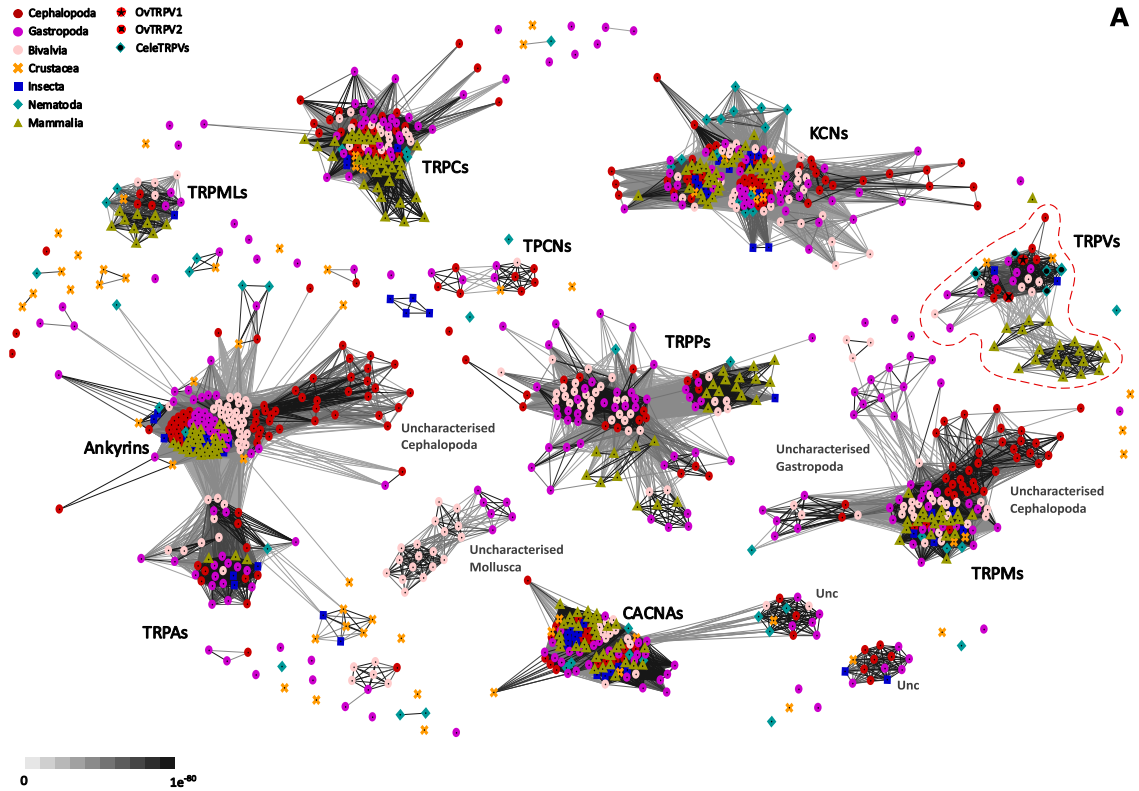


Figure 3.3 OvTRPV1 and OvTRPV2 are two distinct vanilloid transient receptor potential ion channels.

A Visual representation of the relationship among the main TRP channel subfamilies. The OvTRPVs were identified as part of the TRPV subfamily cluster (dashed red line). The two OvTRPV receptors segregate into two distinct subgroups of the cluster, thus showing some structural distinctions between each other. Same is visible with the *C. elegans* TRPVs (see legend in the top left corner). CACNA: voltage-gated calcium channels subunit alpha; KCN: potassium voltage-gated channels; TPCN: Two pore calcium channel; TRPA: Transient Receptor Potential cation channel subfamily A; TRPC: transient receptor potential cation channel subfamily C; TRPM: transient receptor potential cation channel subfamily M; TRPML: mucolipin TRP cation channel; TRPP: polycystin transient receptor potential channel interacting; TRPV: transient receptor potential cation channel subfamily V; Unc: uncharacterised. **B Phylogenetic analysis of OvTRPVs.** Phylogenetic tree showing the relationships among TRPV sequences from 13 representative species. The two *O. vulgaris* TRPV sequences (OvTRPV1 and OvTRPV2) are highlighted in bold red. *C. elegans* OCR-like sequences are shown in bold cyan and *C. elegans* OSM-9 in bold purple, while human representative TRPV sequences are indicated in bold black. The tree reveals two distinct TRPV lineages in protostomes, corresponding to OCR-like and OSM-9-like channels, with OvTRPV1 and OvTRPV2 clustering within these respective groups.

3.4.5 *Ovtrpv1* and *Ovtrpv2* are expressed in sensory tissues

Once established that the identified sequences are the only readily detected TRPV channel representatives in *O. vulgaris* genome, we investigated their relative tissue distribution using PCR of cDNA synthesised from regionally dissected *O. vulgaris* tissues (Figure 3.4A). As a comparison, we used an orthologue of the previously identified class of chemotactile receptors (*Ovcrt*), found to be selectively expressed in the sensory epithelium of the sucker (van Giesen *et al.*, 2020). Our results showed a prevalent expression of both *Ovtrpvs* in the sensory tissues such as the tip of the arm and the sucker and in the central brain mass, in a similar fashion to the *Ovcrt* (Figure 3.4B). Based on the intensity of the genes of interest relative to the control, we evidenced a lower expression in the intestine, white bodies, gill and kidney (Figure 3.4B).

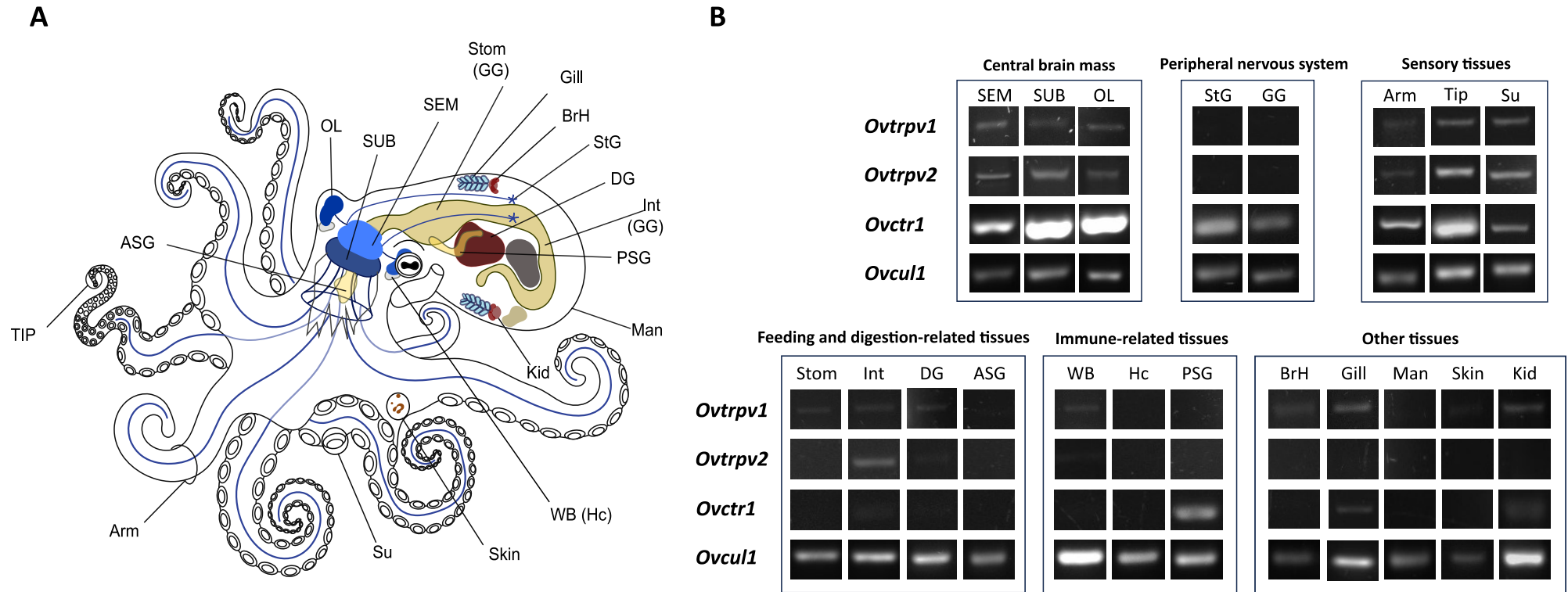


Figure 3.4. Tissue distribution of *Ovtrpv1* and *Ovtrpv2* receptors in *O. vulgaris*.

A Schematic representation of *O. vulgaris* tissues localisation in the body. **B** PCR amplification of *Ovtrpv1* and *Ovtrpv2* from a different set of tissue mRNA, grouped in the central brain mass, and the sensory, immune- and digestive-related tissues. The data are indicative of the PCR performed on mRNA extracted from the indicated tissue of 4 animals (Supplementary Table S3). Arm: muscle + axial nerve cord, at 50% of its length, TIP: tip of the arm, Su: sucker, OL: optic lobe, SEM: supra-oesophageal mass, SUB: sub-oesophageal mass, StG: stellate ganglion, GG: gastric ganglion, Kid: Kidney, Stom: stomach, Int: intestine, DG: digestive gland, ASG: anterior salivary gland, WB: white bodies, Hc: haemocytes, PSG: posterior salivary gland, BrH: branchial heart, Man: mantle (muscle).

3.4.6 OvTRPVs show polymodal sensory functions when heterologously expressed in *C. elegans*

The tissue distribution described above supports the notion that *Ovtrpv1* and *Ovtrpv2* act as determinants for sensory detection. To investigate this, we took a model hopping approach in *C. elegans*. We previously reported that a functional characterisation of *O. vulgaris* putative nociceptive genes can be carried out in *C. elegans* using behavioural readouts (Pieroni *et al.*, 2026). High sequence homology was found to the nociceptive-related TRPVs *Celeocr-2* and *Celeosm-9*. OvTRPV1 shares 55.0% amino acid similarity and 39.8% amino acid identity with CeleOCR-2, while OvTRPV2 shares 54.7% amino acid similarity and 40.5% amino acid identity with CeleOSM-9). We heterologously expressed *Ovtrpv1* and *Ovtrpv2* cDNA sequence in the corresponding *C. elegans ocr-2 (ak47)* and *osm-9 (ky10)* loss of function mutants under the control of their respective worm promoters and tested the resulting lines for rescue of avoidance to different nociceptive modalities.

We firstly investigated the ability of *Ovtrpvs* expression to restore avoidance to low pH, a cue previously identified as relevant to cephalopod nociception (Hague, Florini and Andrews, 2013; Crook, 2021). Worm lines containing the *Ovtrpvs* cDNA showed a significant rescue of the response in the mutant lines (*Pocr-2::Ovtrpv1 Pmyo-3::gfp* vs *ocr-2 (ak47) Pmyo-3::gfp*, $p < 0.0001$; *Posm-9::Ovtrpv2 Pmyo-3::gfp* vs *osm-9 (ky10) Pmyo-3::gfp*, $p < 0.0001$). More in details, *Ovtrpv1*, led to a full rescue of the phenotype (*Pocr-2::Ovtrpv1 Pmyo-3::gfp* vs WT N2 $p=0.6235$, Figure 3.5A).

Ovtrpv2 still showed a significant difference with the WT performance (*Posm-9::Ovtrpv2 Pmyo-3::gfp* vs *osm-9 (ky10) Pmyo-3::gfp*, $p < 0.0001$) suggesting a clear but incomplete rescue. However, this was also true for the corresponding *C. elegans* cDNA (*Posm-9::Celeosm-9 Pmyo-3::gfp* vs WT N2, $p=0.0004$) and genomic (*osm-9 (WRM066bG12) Pmyo-2::gfp* vs WT N2, $p < 0.0068$) rescue constructs (Figure 3.5B). Thus, the partial rescue may reflect methodological confounds of the transgenic expression (e.g., mosaic expression of the transgene, missing additional regulatory elements in the promoter region), rather than a reduced ability of the orthologue to substitute function.

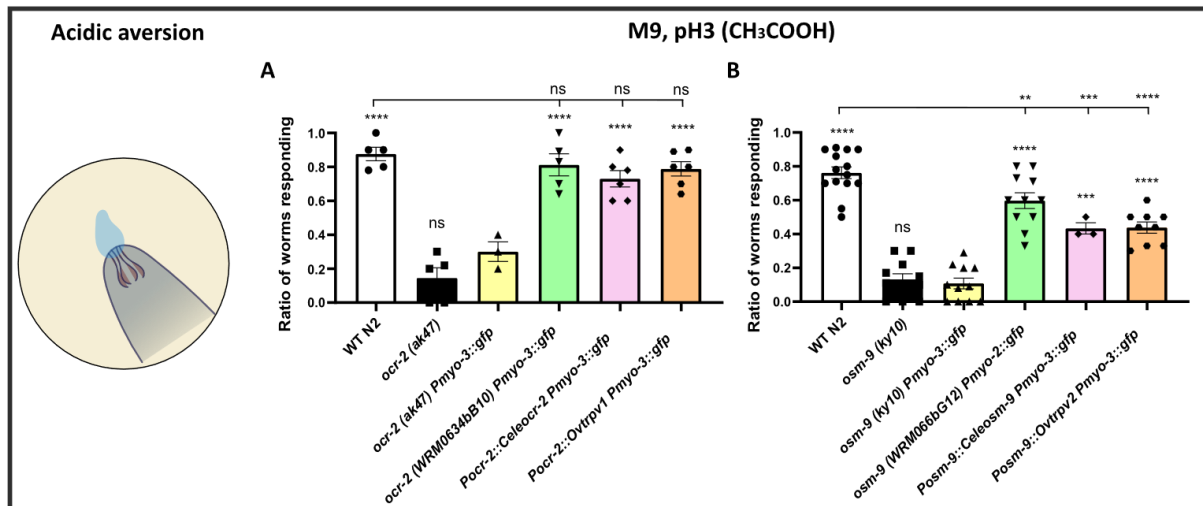


Figure 3.5 *Ovtrpv1* and *Ovtrpv2* rescue pH 3-evoked avoidance in *C. elegans ocr-2* and *osm-9* mutant strains.

The bar graphs represent the ratio of worms responding $\pm$ s.e.m. for WT N2, *ocr-2 (ak47)*, *osm-9 (ky10)*, *ocr-2 (ak47) Pmyo-3::GFP*, *osm-9 (ky10) Pmyo-3::GFP* and fosmid lines. Each dot represents a replicate experiment in which we tested 10 worms per condition. For the *C. elegans* and *O. vulgaris* cDNA constructs, each dot represents a rescue line (10 worms each), which was selected according to the threshold set at 2 standard deviations above the mean of the reference mutant line *ocr-2 (ak47) Pmyo-3::GFP* or *osm-9 (ky10) Pmyo-3::GFP* (Figure S3.1). All the average performances are here compared to the reference mutant lines. The comparisons against the WT N2 line is shown above the black line. Data were analysed using one-way ANOVA and Post-hoc comparisons have been performed with Dunnett's multiple comparisons test. *** $p < 0.001$, **** $p < 0.0001$.

Next, mechanical aversion was investigated in *C. elegans* using a nose touch assay (Figure 3.6). Again, both constructs were able to rescue the gene function by recovering aversion to mechanical insults. *Ovtrpv1* exhibited a modest rescue in *ocr-2 (ak47)* background (*Pocr-2::Ovtrpv1 Pmyo-3::gfp* vs *ocr-2 (ak47) Pmyo-3::gfp*, $p = 0.0131$, Figure 3.6A) while *Ovtrpv2* showed a full rescue of the behavioural phenotype of *osm-9 (ky10)* with more than 70% of the lines tested (12 out of 17, Figure S3.1) efficiently responding to nose touch (*Posm-9::Ovtrpv2 Pmyo-3::gfp* vs WT N2, $p = 0.1497$, Figure 3.6B).

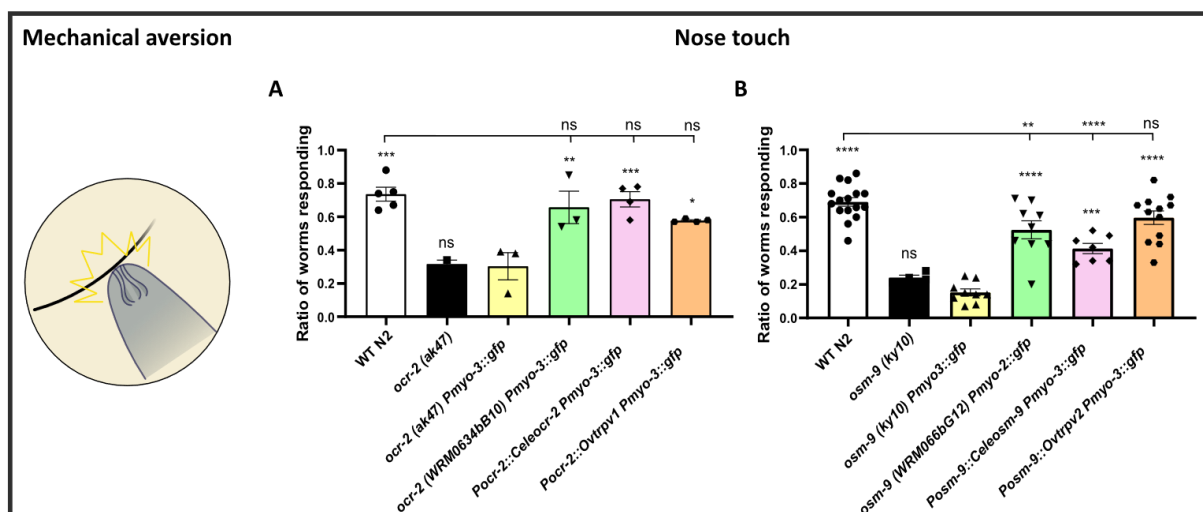


Figure 3.6 *Ovtrpv1* and *Ovtrpv2* rescue mechanical aversion in *C. elegans ocr-2* and *osm-9* mutant strains.

The bar graphs represent the ratio of worms responding $\pm$ s.e.m. for WT N2, *ocr-2 (ak47)*, *osm-9 (ky10)*, *ocr-2 (ak47) Pmyo-3::GFP*, *osm-9 (ky10) Pmyo-3::GFP* and fosmid lines. Each dot represents a replicate experiment in which we tested 5 worms in a set of 10 trials. For the *C. elegans* and *O. vulgaris* cDNA constructs, each dot represents a rescue line (5 worms per 10 trial each), which was selected according to the threshold set at 2 standard deviations above the

mean of the reference mutant line *ocr-2 (ak47) Pmyo-3::GFP* or *osm-9 (ky10) Pmyo-3::GFP* (Figure S3.1). All the average performances are here compared to the reference mutant lines. The comparisons against the WT N2 line is shown above the black line. Data were analysed using one-way ANOVA and Post-hoc comparisons have been performed with Dunnett's multiple comparisons test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

We additionally tested rescue to other chemical noxious compounds, the volatile repulsive cue 1-octanol and the water-soluble D-fructose. Only a trend towards a reduced latency to initiate reversals to diluted (30%) 1-octanol was shown by *Ovtrpv1*-expressing lines (*Pocr-2::Ovtrpv1 Pmyo-3::gfp* vs *ocr-2 (ak47) Pmyo-3::gfp*, $p = 0.0679$, Figure 3.7A), while *osm-9 (ky10)* mutants expressing *Ovtrpv2* were successfully rescued (*Posm-9::Ovtrpv2 Pmyo-3::gfp* vs *osm-9 (ky10) Pmyo-3::gfp*, $p = 0.0002$, Figure 3.7B).

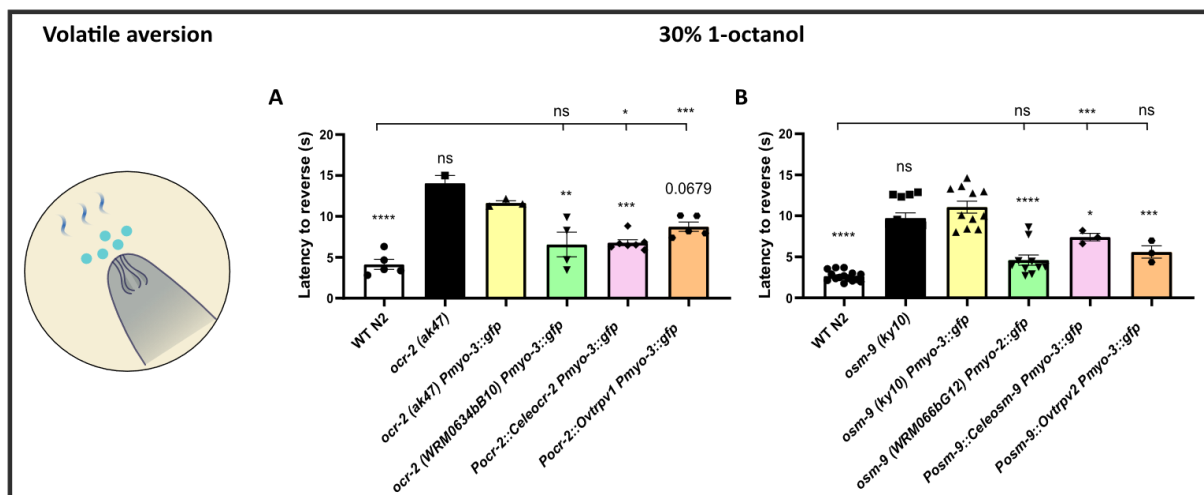


Figure 3.7 *Ovtrpv2* but not *Ovtrpv1* rescues volatile aversion in *C. elegans osm-9* mutant strains.

The bar graphs represent the ratio of worms responding $\pm$ s.e.m. for WT N2, *ocr-2 (ak47)*, *osm-9 (ky10)*, *ocr-2 (ak47) Pmyo-3::GFP*, *osm-9 (ky10) Pmyo-2::GFP* and fosmid lines. Each dot represents a replicate experiment in which we tested 10 worms. For the *C. elegans* and *O. vulgaris* cDNA constructs, each dot represents a rescue line (10 worms each), which was selected according to the threshold set at 2 standard deviations below the mean of the reference mutant line *ocr-2 (ak47) Pmyo-3::GFP* or *osm-9 (ky10) Pmyo-3::GFP* (Figure S3.1). All the average performances are here compared to the reference mutant lines. The comparisons against the WT N2 line is shown above the black line. Data were analysed using one-way ANOVA and Post-hoc comparisons have been performed with Dunnett's multiple comparisons test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

High osmolarity avoidance, represented by 4M fructose exposure, was not comparably successful as *Ovtrpv1* did not rescue *ocr-2 (ak47)* insensitivity to high osmotic strength and *osm-9 (ky10)* was only modestly rescued by *Ovtrpv2* (data not shown - Appendix B1).

3.4.7 Investigation of OvTRPVs function using recombinant systems

The successful rescue to the different modalities and cues, suggested OvTRPVs share sufficient structural similarity with their worm orthologues to replace the missing endogenous receptor in the intact organism. However, whether these act upstream as direct activators or downstream as final effectors or modulators of the primary sensory signalling is not known. To tackle this, we heterologously expressed the receptors in recombinant systems, as it was previously shown in *C. elegans* that OSM-9/OCR-2 are both required to be correctly localised at the ciliated sensory

neurons and to exert functional response (Tobin *et al.*, 2002; Ohnishi *et al.*, 2020). In order to exclude issues associated with the expression of invertebrate ion channels in mammalian cells (data not shown - Appendix B2) we used *Xenopus* oocytes to test for direct receptors activation. Having verified the ability to reconstitute a ligand activated response from the positive *Hstrpv1* control using capsaicin, *Ovtrpv*s-expressing oocytes did not respond to this vanilloid compound (Figure 3.8A). However, a long sustained response to 100 μ M NAM was observed only when *Ovtrpv1* and *Ovtrpv2* were co-expressed (Figure 3.8B), suggesting that OvTRPV subunits require co-assembly of the two subunits to reconstitute a response in recombinant system.

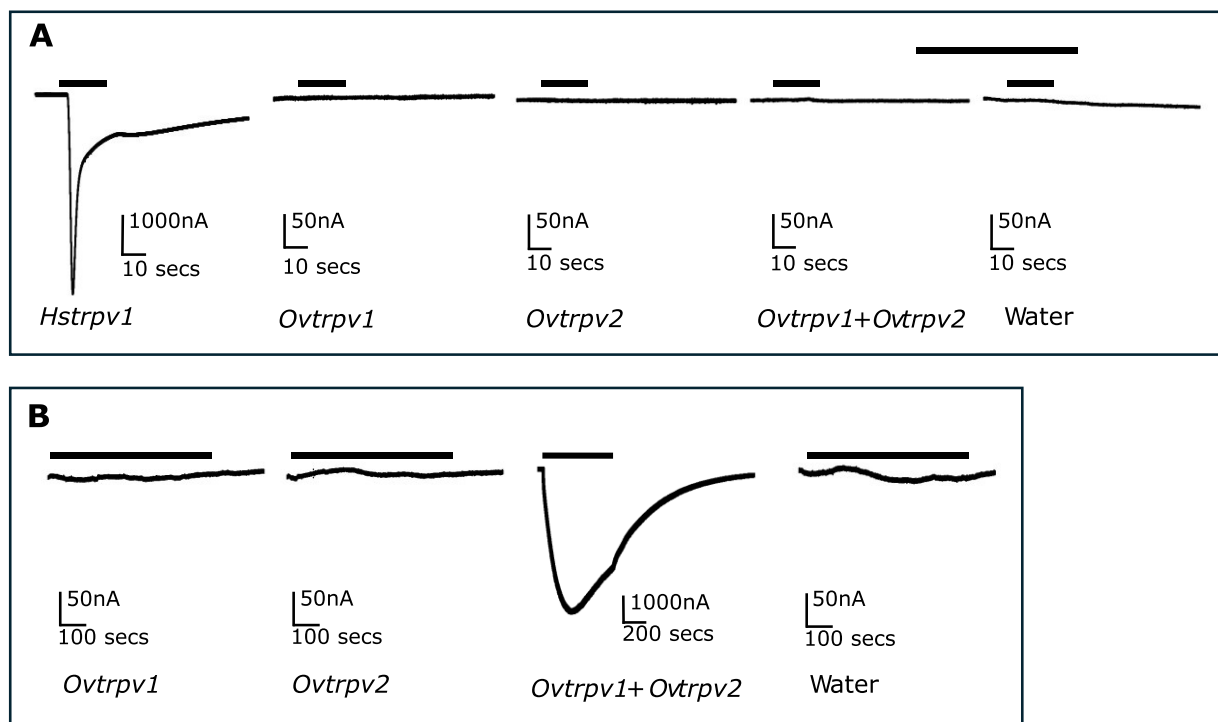


Figure 3.8 Heterologous expression of *O. vulgaris* TRPV channels in *Xenopus* oocytes.

A. Current traces from *Xenopus* oocytes injected with RNA encoding the human TRPV channel *Hstrpv1* show response to capsaicin at 100 μ M. The solid bars represent the period of capsaicin perfusion. Oocytes injected with RNA for *O. vulgaris trpv1* and *trpv2* separately or together show no response to capsaicin at 100 μ M. Oocytes injected with water show no response. $N \geq 7$ oocytes for each condition. **B.** *O. vulgaris trpv1* and *trpv2* form a heteromeric channel which responds to nicotinamide. *Xenopus* oocytes injected with RNA for *Ovtrpv1*, *Ovtrpv2* or water show no responses to nicotinamide at 100 μ M. Oocytes expressing *Ovtrpv1* and *Ovtrpv2* together show strong responses to nicotinamide 100 μ M. $N \geq 7$ oocytes for each condition.

3.5 Discussion

3.5.1 *O. vulgaris* has two distinct TRPV channel representatives

In a previous *in silico* analysis of the conserved molecular determinants putatively involved in *O. vulgaris* nociception, we highlighted candidates that could underpin the octopus's response to noxious stimuli.

In this study, we focussed on one of the candidates that emerged from these studies which has an established role in nociception in both vertebrate and invertebrate organisms. This is the

characterised molecular determinant TRPV, belonging to the vanilloid subfamily of TRP channels (Himmel and Cox, 2020). This superfamily has a wide distribution and functional contribution in biology (Pedersen, Owsianik and Nilius, 2005; Venkatachalam and Montell, 2007).

The TRPV subfamily, and particularly the TRPV1 member in vertebrates, is known to be gated by several compounds including capsaicin and anandamide but it is also known to be activated by low pH and high temperature, thus classifying it as a polymodal receptor (Caterina *et al.*, 1997; Immke and Gavva, 2006; Dhaka *et al.*, 2009). An important aspect of TRPV1 and related proteins is that this molecular sensing and signalling is placed in discrete cells so they can act as sensory receptors that initiate complex downstream signalling that allows the embedded circuits to drive judicious behavioural responses (Caterina *et al.*, 1997). Consistent with this, a prevalent expression of TRPV1 in peripheral sensory neurons that interact with the environment has been extensively reported (Caterina *et al.*, 1997; Nakagawa and Hiura, 2006; Szigeti *et al.*, 2012). In addition, a wider expression in the central brain implying distinct contributions to nociception or other functions has also been found (Yuan and Burrell, 2010; Higgins *et al.*, 2013; Marrone *et al.*, 2017).

The sensory function and the anatomical distribution of TRPV channels pertain to invertebrate and simpler organisms as well (Colbert, Smith and Bargmann, 1997; Summers, Holec and Burrell, 2014). This prompted us to investigate the TRPV subfamily in *O. vulgaris*. Our work exploited the emerging access to octopus transcriptome and genome databases and matured earlier designation of a single TRPV gene. Cluster analysis and phylogenetic investigation revealed OvTRPV1 and OvTRPV2 are indeed the only representatives of vanilloid TRP channels found in *O. vulgaris* and represent two discrete subunits (Figure 3.3A). Two representatives in fact, were also found in other cephalopods such as *O. bimaculoides* and *O. sinensis* but also in other species such as *D. melanogaster* or *A. californica* (Figure 3.3B). This indicates potential structural conservation across distinct invertebrate genres which may well reflect strong functional conservation of sensory specialisation.

An interesting exception is represented by *C. elegans*, which shows a total of 5 TRPV representatives, suggesting a specific one-to-many orthologues expansion of these receptors compared to other invertebrates (Kuzniar *et al.*, 2008). There is a clear distinction within the invertebrate TRPVs, which we are here referring to as OCR-like TRPV channels, which include all the *C. elegans* OCR receptors (OCR 1-4) to which OvTRPV1 and *D. melanogaster* Nanchung (NAN) are more closely related (55.0% similarity), and OSM-9 like channels, which include *C. elegans* OSM-9, to which OvTRPV2 and *D. melanogaster* Inactive (IAV) are related (54.7% similarity, Figure 3.3B). Invertebrate TRPVs share a common ancestor with the vertebrate TRPVs which then underwent a duplication event that gave rise to two distinct branches. One led to the TRPV5 and

6 ion channels which do not have any sensory related functions but are known to act to regulate calcium homeostasis in the kidney and small intestine and were therefore reported to be sensitive to changes such as extracellular pH (Yeh *et al.*, 2003; Nijenhuis, Hoenderop and Bindels, 2005; Lambers *et al.*, 2007). This subgroup is separated from the TRPV1-4 channels, in which further internal duplications likely led to a specialisation of these receptors in compounds and stimuli detection (Peng, Shi and Kadowaki, 2015; Morini *et al.*, 2022).

In accordance with the evolutionary analysis, secondary and 3D modelling predictions of OvTRPV1 and OvTRPV2 identified the key structural and functional regions that make the TRPV channels a very distinct category of receptors (Pumroy *et al.*, 2020). The TMs 1-4 are also distinct from the TM5 and TM6 which are characterised by having a transmembrane P-loop region, likely responsible for forming a selectivity filter in the pore region of the channel (Owsianik *et al.*, 2006). Finally, a distinctive TRP domain region was also detected as this is shared across the entire family of TRP receptors (Rosasco and Gordon, 2017).

3.5.2 Tissue expression supports the hypothesis that *Ovtrpvs* are sensory molecular determinants in octopus

As indicated, the tissue expression of the mammalian TRPV1 supports key function in sensory signalling. In the case of the *Ovtrpvs*, gross analysis of their distribution across different tissues supports an important role in sensory signalling (Figure 3.4). Both subunits appear to show relatively low expression but robust and reproducible amplification from tissues associated with sensory signalling in cephalopods. This is reinforced when we compare the expression in the sensory tissues (arm, tip of the arm and sucker) to a range of tissues encapsulating immune-related (e.g., white bodies, haemocytes) and feeding and digestion-related (e.g., stomach, intestine) tissues (Figure 3.4B). Although tissue distribution cannot define specificity of function *per se*, we identified that *Ovtrpv1* and *Ovtrpv2* have overlapping expression with the selective chemotactile receptor orthologue *Ovcrt* (Figure 3.4B). This gene is reported to be enriched in the sensory epithelium of the sucker of *O. bimaculoides* (van Giesen *et al.*, 2020). This enrichment is interesting as cephalopods, and in particular octopus, use their arms to sample the environment and classify stimuli including those that evoke aversion. These structures which express sensory receptors are primary organisers of reflexive and complex responses (Rossi and Graziadei, 1956; Zullo, Fossati and Benfenati, 2011; Hague, Florini and Andrews, 2013; Gutnick *et al.*, 2020). The prevalence of *Ovtrpv1* and *Ovtrpv2* expression in the distal part of the arm, such as the tip and more specifically the suckers, encouraged the hypothesis these receptors might be involved in sensory detection in *O. vulgaris*. The comparison with *Ovcrt* expression suggests these receptors are low abundant, and this seems in line, at least for *Ovtrpv1*, with the RNA sequencing analysis of its fragments performed in the previously assembled *O. vulgaris* transcriptome (Petrosino,

2015; Petrosino *et al.*, 2022). There are no data available for *Ovtrpv2* as this transcriptome was not identified at all in the previously published assembly.

In a similar fashion to vertebrate TRPVs, *Ovtrpvs*, have been detected in the central brain mass of *O. vulgaris* (Figure 3.4B), supporting a potential role in sensory and nociception modulation, as well as other unknown neural processes.

We additionally detected expression of both *Ovtrpvs* in the intestine and, exclusively for *Ovtrpv1*, in the stomach, digestive gland, white bodies, gill and kidney (Figure 3.4B). This broader expression in non-neuronal tissues for *Ovtrpv1* is interesting when considering it belongs to the “OCR-like” channels branch. In *C. elegans*, OCRs are not exclusively found in the main anterior and posterior sensory neurons, the amphids and phasmids, but rather show a more heterogenous expression including the rectal gland cells and intestine which play an important role in *C. elegans* digestive system (Tobin *et al.*, 2002; Packer *et al.*, 2019). Additionally, *ocr-3* and *ocr-4* seem to be co-expressed in neuroendocrine cells with *ocr-2*, to regulate egg-laying (Jose *et al.*, 2007).

3.5.3 Functional characterisation through model hopping in *C. elegans* suggests *Ovtrpvs* are polymodal nociceptors

The prevalent expression in sensory tissues of both receptors, encouraged us to functionally test them for their role in nociception. Despite the success of CRISPR-Cas9 knock out obtained in squids, the major bottleneck of creating transgenic lines in cephalopods is represented by the challenges in maintaining early life stages in captivity through generations (Crawford *et al.*, 2020; Ahuja *et al.*, 2023). This is especially true for octopus and the requirements necessary for the rearing of their delicate paralarvae stages (Iglesias *et al.*, 2007; Ponte *et al.*, 2022).

As we have already utilised *C. elegans* as a suitable model to perform functional characterisation of octopus molecular nociceptor candidates (Pieroni *et al.*, 2026), we resorted to a model hopping approach by expressing octopus receptors in the mutant strains for the orthologue genes *ocr-2* and *osm-9*. The phylogenetic distinction between the two receptors into “OCR-like” receptors for *Ovtrpv1* and “OSM-9-like” for *Ovtrpv2*, justified our approach of heterologously expressing *Ovtrpv1* under the *ocr-2* promoter in an *ocr-2 (ak47)* mutant background and *Ovtrpv2* under the *osm-9* promoter in an *osm-9 (ky10)* mutant background.

The null mutants *osm-9 (ky10)* and *ocr-2 (ak47)* reported to show defects in different sensory modalities, including chemical and mechanical aversion, effectively highlighted OSM-9 and OCR-2 as polymodal receptors (Colbert, Smith and Bargmann, 1997; Tobin *et al.*, 2002; Liedtke *et al.*, 2003; Thies *et al.*, 2016). We therefore subjected our transgenic lines expressing *Ovtrp1* and *Ovtrp2* in the *C. elegans* mutants for the orthologous gene to water soluble noxious chemical

cues such as acetic acid (pH 3) to induce acidic aversion, volatile repellents such as 1-octanol and mechanical insults such as nose touch.

Ovtrpv1 and *Ovtrpv2* successfully rescued low pH (Figure 3.5) and mechanical avoidance (Figure 3.6) in both mutant worm strains, suggesting these receptors act as polymodal nociceptors in *O. vulgaris*. These two cues have already been shown to be able to induce aversive responses *in vivo* in cephalopods. Low pH has been tested in *ex vivo* octopus arm preparations, showing a quick withdrawal when in contact with an acidic solution, and *in vivo* experiments, in which injection of acetic acid triggered grooming and protective behaviours (Hague, Florini and Andrews, 2013; Crook, 2021). Mechanical cues are also an important trigger for cephalopod sensory responses as von Frey filaments of increasing force have been found to induce nociceptive responses and also to trigger post-injury sensitisation in squids (Crook, Hanlon and Walters, 2013; Alupay, Hadjisolomou and Crook, 2014; Crook *et al.*, 2014).

However, the molecular determinants of such responses have not been investigated in *O. vulgaris* yet and the TRPV channels we described here are potential candidates. Whether OvTRPVs act as channels directly gated by pH or as indirect modulators of pH behavioural responses it is not known. Looking at the proposed key residues for proton gating in mammalian TRPV1, V538, E600, T633, E648 and F660 (Jordt and Julius, 2002; Ryu *et al.*, 2007; Aneiros *et al.*, 2011), these do not seem to be conserved in OvTRPVs or CeleTRPVs. Therefore either OvTRPV channels have a distinct mechanism for pH detection, or perhaps they possess a downstream modulatory role for this cue. As for mechanosensation, a key molecular determinant has been found in *O. bimaculoides* as part of the TRPN family, which also includes the mechanosensory *D. melanogaster* NompC (Yan *et al.*, 2013; van Giesen *et al.*, 2020). However, it is not uncommon in organisms to have multiple receptors responding to the same cue. This can allow a better resolution of the stimulus detection and its texture, by for instance specialising receptors to respond to different “tones” of the cue such as different pressures and mechanical deformation of the cellular membrane that can allow the distinction between a soft touch and a nociceptive harmful input. *C. elegans* represents a good example of this, as *osm-9/ocr-2* are not uniquely required for aversive nose touch (Kindt *et al.*, 2007; Li *et al.*, 2011), and different molecular interactions give rise to distinct sensitivities (e.g., *mec-10/mec-4* for gentle touch and *mec-10/degt-1* for harsh touch, Chatzigeorgiou *et al.*, 2010; Li *et al.*, 2011).

Our *C. elegans* experiments suggest that homologue expression gives the most potent rescue with *ocr-2* promoter driving *Ovtrpv1* and *osm-9* promoter driving *Ovtrpv2* expressions in nematode loss of function mutants of the orthologue *trpv* genes. However these functional experiments preclude insight into their transduction localisation (upstream or downstream) or the required stoichiometry that makes the receptor functional in sensory cellular signalling. The

evolutionary relationship between *Ovtrpv1* and *Ovtrpv2*, consistent with other invertebrates, suggests important divergence in function (Figure 3.3B). Furthermore, previous experiments suggested co-assembly of *osm-9/ocr-2* to exert their role (Ohnishi *et al.*, 2020; Griffin *et al.*, 2025). Finally, the shared overlapping tissue expression of *Ovtrpv1* and *Ovtrpv2* suggests they might interact together to convey sensory detection. Altogether, these pieces of evidence, encouraged our approach of using recombinant assays.

Heterologous co-expression of *Ovtrpv1* and *Ovtrpv2* in *Xenopus* oocytes produced a sustained, slow response to 100 μ M NAM, supporting the hypothesis of co-assembly between *Ovtrpv1* and *Ovtrpv2* into an active heteromeric channel that is directly gated by aversive substances (Figure 3.8). This finding is in line with previously characterised invertebrate TRPV receptors. In both *C. elegans* and *D. melanogaster*, co-expression of the pair *osm-9/ocr-2* (but more convincingly of *osm-9/ocr-4*) and *Nan/lav* respectively, produced dose-dependent responses to NAM (Upadhyay *et al.*, 2016). Behavioural and cell-studies revealed this substance is a noxious bitter cue in invertebrates, eliciting avoidance responses and accumulation of excess NAM induces cell death through desensitisation of TRPV (Upadhyay *et al.*, 2016; Ishikawa *et al.*, 2023). Our data, are consistent with a conserved TRPV role in invertebrate sensory detection and cell metabolic regulation that are worth investigating in the future.

3.5.4 Concluding remarks and future directions

Altogether, our work identified two functional TRPV channels in *O. vulgaris* and contributed to provide precise annotation and experimental validation of two important molecular determinants of nociception in cephalopods. Heterologous functional characterisation of these octopus TRPV channels in *C. elegans*, suggests they are polymodal and may have a physiological role in the octopus response to aversive chemical and mechanical cues.

The sensory role of *Ovtrpvs* is supported by our endpoint PCR analysis of a wide set of tissues, demonstrating expression in sensory and nervous tissues. However, future analysis should focus on qPCR and *in situ* hybridisation to confirm the potential selective expression in the tip of the arm and sucker relative to other non-sensory tissues.

Finally, the established phylogenetic relationship, locating OvTRPV1 and OvTRPV2 in distinct evolutionary branches of the vanilloid TRP channel subfamily, as well as the recombinant experiments showing the requirement for co-assembly of OvTRPV1 and OvTRPV2 to generate a functional channel, provides a route to experimentally investigate the pathways and the modalities in which *Ovtrpvs* operate. At the same time it fosters the development of experimental platforms that might identify important environmental sensory cues. Recent advances have in fact shown octopuses evolved species-specific receptors but that they also exploit conserved

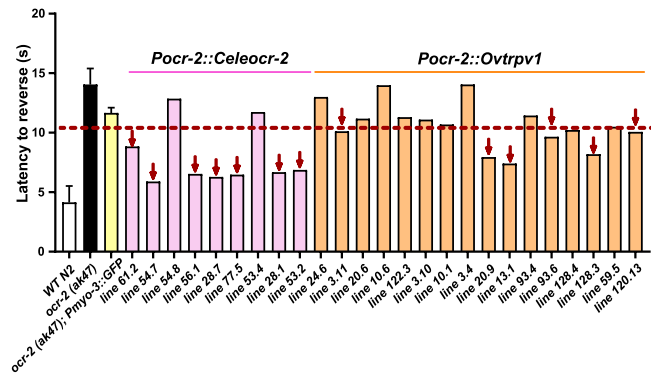
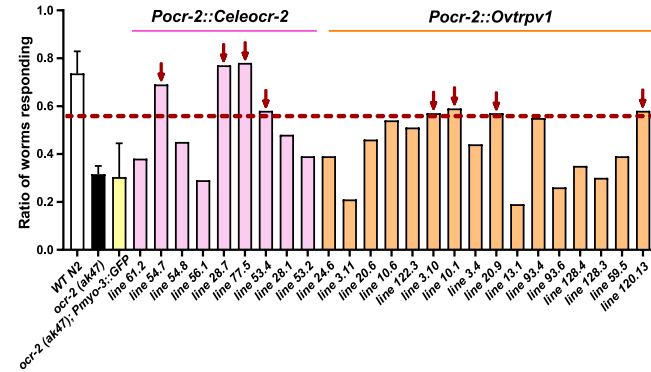
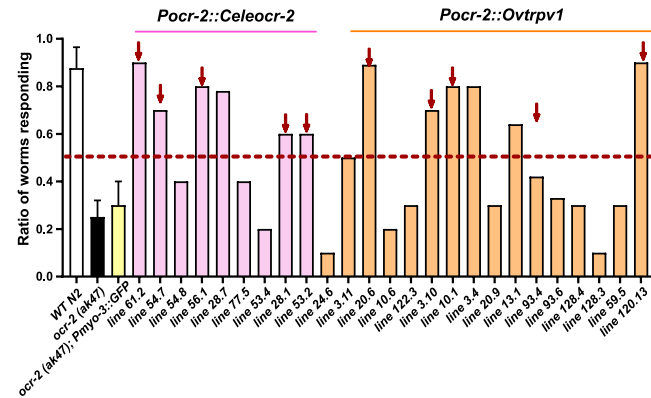
ones (van Giesen et al., 2020), showing the importance for cephalopods to recognise and classify stimuli in the environment through an elaborated sensory system able to detect, integrate and select against nociceptive cues.

M9, pH3 (CH₃COOH)

Nose touch assay

30% 1-octanol

Ovtrpv1 in ocr-2 mutant background



Ovtrpv2 in osm-9 mutant background

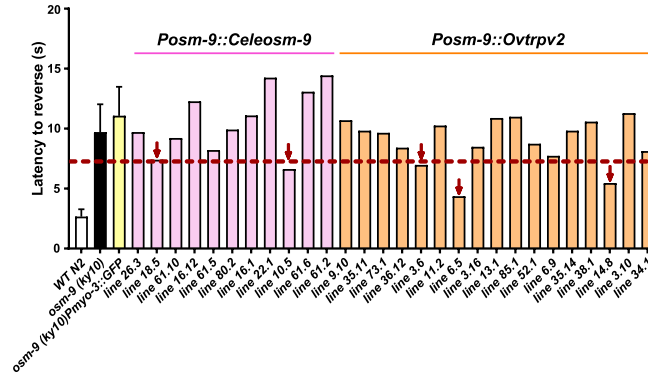
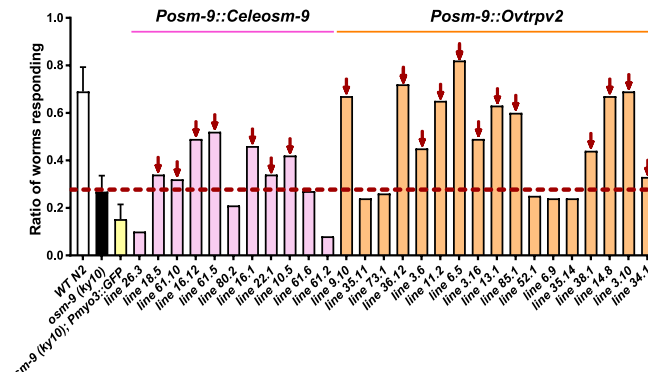
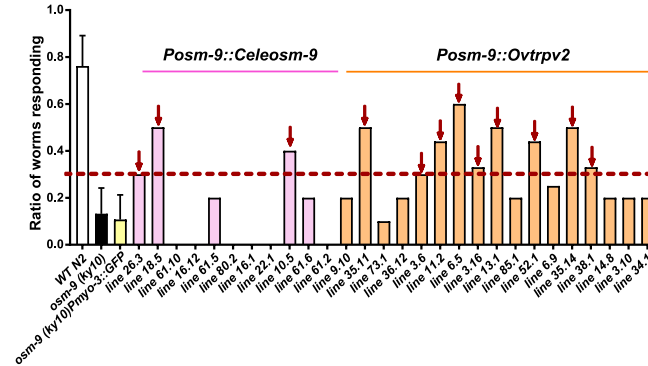


Figure S3.1 Selection of successful rescue lines for *C. elegans* and *O. vulgaris* TRPV cDNA.

The dashed red line, indicates the threshold of two standard deviation above or below the mutant GFP *C. elegans* strains performance (in yellow). The independent rescue lines reaching the threshold are indicated with a red arrow and have been further pooled for comparison in Figures 3.5-3.7.

Chapter 4 *In silico* identification and deorphanisation of an allatostatin C GPCR system in the cephalopod *Octopus vulgaris* reveal two receptors with distinct potency

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Lindy Holden-Dye: conceptualisation, methodology, writing—original draft, writing—review and editing, funding acquisition and supervision

Pamela Imperadore: tissue collection, writing—review and editing

Graziano Fiorito: conceptualisation, writing—review and editing, funding acquisition

Luis Alfonso Yañez-Guerra: conceptualisation, data curation, *in silico* analysis, experimental investigation, methodology, validation, visualization, writing—review and editing, funding acquisition and supervision

Supplementary Tables S1-S3 and Supplementary Files S1-S6 are electronic files accessible from the following link: [10.5281/zenodo.19974511](https://doi.org/10.5281/zenodo.19974511)

4.1 Abstract

Neuropeptide signalling is transversally important in all living animals as it constitutes the basis of cellular communication. The investigation of the functional roles of peptide signalling represents an important route to understanding evolution of specific physiological traits and behaviours in metazoans.

Allatostatins and their cognate receptors are classically defined as invertebrate neuropeptide hormones. Among these, allatostatin C was firstly associated with insect development. However, accumulating evidence recognises the presence of allatostatin C as a conserved signalling molecule across all invertebrate lineages, with reported pleiotropic functions. These functions span from regulation of feeding and digestion to immune responses and modulation of core nociception.

Here we combined *in silico* and experimental approaches to reveal the interacting molecular determinants of the allatostatin C signalling in the cephalopod *Octopus vulgaris*, an interesting invertebrate for its centralised nervous system capable of top-down modulation of complex behaviours. This resolved a single prepropeptide encompassing allatostatin C peptide (OvAstC), whose conserved, mature form (AVITACYFQAVSCY) was shown to differentially activate two identified cognate receptors (OvAstCR1 and OvAstCR2) when heterologously expressed in the recombinant system HEK293G5A.

PCR analysis carried out in *O. vulgaris* tissues, showed a broad distribution of OvAstC and the cognate receptors. This wide expression across tissues related to the nervous, immune and digestive systems is consistent with a pleiotropic role of this peptidergic system. Cluster and phylogenetic analysis of the receptors revealed a close relationship with somatostatin and opioid families that could underlie physiological responses resonant with emerging interest in allatostatin C signalling in invertebrate nociception.

4.2 Introduction

Neuropeptide signalling plays a pivotal role in modulating animal physiology and function, including the integration and modulation of stimuli coming from the exteroceptive and interoceptive environment cues. This broad biological significance is reinforced by evidence of peptidergic signalling across all animals, including “neuron-less” organisms such as sponges and placozoans (Hauser, Koch and Grimmlikhuijzen, 2022; Yañez-Guerra, Thiel and Jékely, 2022).

Classically, identifying evolutionary relationship among peptide families across animal phyla was rather difficult and challenged by the short, repetitive and divergent nature of these molecules

(Jékely, 2013). However, in the last decade, clustering-based approaches in conjunction with large-scale de-orphanisation have contributed to the dissection of numerous neuropeptides and their cognate receptors across different phyla, including those belonging to key phylogenetic lineages that are informative for understanding the evolution of peptidergic signalling in animals such as annelids or cnidarians (Bauknecht and Jékely, 2015; Thiel *et al.*, 2024).

Allatostatin C is a neuropeptide originally identified as an inhibitor of the juvenile hormone biosynthesis in the corpora allata of the tobacco hornworm *Manduca sexta*, leading to developmental changes such as metamorphosis and sexual maturation (Kramer *et al.*, 1991). Subsequent studies have revealed roles in insect feeding and digestion, modulation of immune responses and downstream mitigation of nociception (Ma *et al.*, 2009; Duan Sahbaz *et al.*, 2017; Bachtel *et al.*, 2018; Zhang *et al.*, 2022; Liu *et al.*, 2023). These pivotal functions have heightened pharmacological interest in the potential of allatostatin C signalling system as a pest control target (Işbilir *et al.*, 2020; Shahraki *et al.*, 2021).

Allatostatin C was found to be conserved in other invertebrates where it exerts distinct tissue-selective functional roles. Characterisation of this system in molluscs largely relied on *in silico* analyses and tissue expression studies, such as in the gastropod *Lottia gigantea*, (Veenstra, 2010) and the bivalve *Patinopectin yessoensis* (Zhang *et al.*, 2024), but these studies did not investigate the functional activation of the identified receptors by their candidate ligands.

The only allatostatin C receptor de-orphanisation study conducted on a mollusc was carried out in *Aplysia californica*, where an interaction between the identified receptor and allatostatin C peptide was experimentally demonstrated (Jiang *et al.*, 2022).

Despite their complex brain anatomy and associated physiology that suggest complex peptidergic regulation, there is limited investigation of cephalopod neuropeptides function.

Previous studies have reported functional characterisation of the Gonadotropin-Releasing Hormone (GnRH), tachykinin and octopressin/cephalotocin peptides in the common octopus *O. vulgaris* through heterologous expression in the recombinant *Xenopus laevis* system (Kanda *et al.*, 2005; Kanda *et al.*, 2006; Kanda *et al.*, 2007), but no de-orphanisation work was carried out on the allatostatins.

Immunocytochemical studies using allatostatins (although type A, not C) sequences from the arthropod *Diploptera puctata*, revealed a wide distribution in the nervous systems of a large number of animal representatives, including the octopus *Eledone cirrhosa* (Smart *et al.*, 1994). Finally, a single study investigated the neuropeptidome landscape of the cuttlefish *Sepia officinalis* using RNA sequencing, *in silico* analysis and tissue mapping through mass spectrometry but failed to identify allatostatin C (Zatylny-Gaudin *et al.*, 2016).

Here we report de-orphanisation of the putative allatostatin C system in the cephalopod mollusc *O. vulgaris*.

Using *in silico* approaches and experimental validation, we detected two *bona fide* allatostatin C receptors, OvAstCR1 and OvAstCR2, belonging to a class A GPCR cluster that includes opioid (OPR), somatostatin (SST) and melanocortin (MCR) receptors. A single allatostatin C (OvAstC) peptide (AVITACYFQAVSCY) was identified from *O. vulgaris* genome and demonstrated to act on both receptors, although with different potency. Importantly, we resolved a broad tissue distribution of all the molecular determinants of octopus allatostatin C signalling which suggests a wide range of physiological functions (e.g., immune-, digestive and sensory related) in a way that overlaps with the evolutionary close deuterostome- and vertebrate-specific somatostatin and opioid receptor families respectively (Krantic, 2000; Olias *et al.*, 2004; Sternini *et al.*, 2004). From an evolutionary perspective, these findings position cephalopods as a key comparative lineage for understanding the diversification and functional specialisation of ancient peptidergic signalling systems in bilaterians. This would provoke the investigation of physiological functions of these peptide signalling clusters that are overlooked or difficult to study but which are leveraged by characterising potential molecular determinants, as exemplified by pathways implicated in nociception. Given the significance of this biological domain in cephalopods, this work further supports attention to peptidergic signalling in cephalopods as part of a broader evolutionary and welfare context.

4.3 Materials and Methods

4.3.1 *In silico* analysis

4.3.1.1 *O. vulgaris* allatostatin C precursors search and manual curation

Allatostatin C peptide precursor sequences from known invertebrate species were retrieved from literature and complemented with those found on Uniprot within the Protostomia (id: 33317). Each query was manually filtered for non-relevant incomplete sequences and subsequently blasted against *O. vulgaris* genome (Destanović *et al.*, 2023) using $10e^{-2}$ as an e-value cut-off, due to the challenge posed by short peptide precursors sequences to be identified (Baggerman *et al.*, 2005). Sequence identity and similarity were analysed using EMBOSS Water with a BLOSUM62 matrix (Madeira *et al.*, 2024). The list of sequences used is available in Supplementary File S1.

The mature form of the peptide was predicted using SignalP - 6.0 to detect the signal peptide (Nielsen, 2025) and Neuropred to detect the cleavage site in the precursor (Southey *et al.*, 2006). We additionally checked for the presence of two cysteines that are usually involved in the formation of a disulfide bond in this class of peptides (Veenstra, 2010).

4.3.1.2 Genes exon-intron boundaries detection

Receptors and peptide precursor transcript sequences were aligned against the *O. vulgaris* chromosome scaffolds (Assembly: GCA_951406725.2) to find their genomic location using NCBI-Magic-BLAST tool (Boratyn *et al.*, 2019). The results were sorted using samtool (Danecek *et al.*, 2021) and the data were processed with samtool idxstats and visualised using Tablet software (The James Hutton Institute, v1.21.02.08, Milne *et al.*, 2013).

4.3.1.3 Cluster analysis and phylogenetic investigation of OvAstCRs

Reference protein sequences for allatostatin C, opioid and somatostatin receptors were selected from *Drosophila melanogaster*, *Aplysia californica* and *Homo sapiens* based on de-orphanised or curated sequences. Reference sequences for the outgroups galanin, Kiss1 and allatostatin A receptors were also included based on previous works showing their affinity to the target receptors that are the focus of this work (Mirabeau and Joly, 2013; Bauknecht and Jékely, 2015). The proteomes of 21 representative species from different phyla were selected based on the best Benchmarking Universal Single-Copy Orthologs (BUSCO) value (Supplementary Table S1) and used as a template for blasting the reference sequences (e-value $1e^{-10}$ and 40 maximum target sequences). The results from each species were sorted and processed through CD-hit with a similarity threshold of 0.95 to remove incomplete sequences, shorter isoforms or redundant sequences (Li and Godzik, 2006; Fu *et al.*, 2012). The results containing unique representative sequences from each species (1084, Supplementary File S2) were then subjected to a cluster analysis using the CLuster ANalysis of Sequences (CLANS) toolkit, setting a BLAST HSPs e-value of $1e^{-12}$ and BLOSUM62 scoring matrix (Zimmermann *et al.*, 2018) with an initial p-value threshold of $1e^{-60}$, to visually reveal the relationships between the sequences. However, as we were missing already annotated sequences (i.e., *C. elegans* AstCRs, Beets *et al.*, 2023), we reduced the threshold to $1e^{-40}$. All the sequences of the cluster which included the *O. vulgaris* allatostatin C receptors (190) were selected using the convex clustering option (cutoff std dev: 0.6; min sequences per cluster: 20). An additional 7 sequences from *C. elegans* were included as they showed a direct or transitive connection with the AstCRs subgroup. The final 197 sequences were used to generate a phylogenetic tree (Supplementary File S3).

The sequences were aligned with MAFFT v7.526 using the E-INS-i method (Katoh and Standley, 2013) and then trimmed with TrimAl (Supplementary File S4) using default parameters and gappy-out mode (Capella-Gutiérrez, Silla-Martínez and Gabaldón, 2009). The best-fit model of evolution was selected with ModelFinder (LG+F+R7 chosen according to the Bayesian Information Criterion) in IQ-TREE3 v3.0.1 (Wong *et al.*, 2025). Tree branches were obtained using aLRT-SH- with 1,000 replicates and ultrafast bootstrap method. The final phylogenetic relationship was

visualised using FigTree v1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>). The tree was rooted using the outgroups galanin, Allatostatin A and Kiss1 receptors.

4.3.2 Animal samples

4.3.2.1 *O. vulgaris* sample collection

Three young adults of *O. vulgaris* were caught by local artisanal fishermen in the Bay of Naples, Italy following the local Animal Welfare Body authorisation (Ethical Clearance: ecACR-2302ts36). Animals were humanely killed in compliance with the Directive 2010/63/EU (Annex IV) and following best practice protocols described in Andrews et al. (2013), Fiorito et al. (2015), Butler-Struben et al. (2018). Furthermore, animal-derived materials have been utilised in a responsible and ethical way, according to the principles of Replacement, Reduction, and Refinement (3Rs). The following tissues were collected by a FELASA certified, function D competent person: supra-oesophageal mass (SEM), sub-oesophageal mass (SUB), optic lobe (OL), stellate ganglion (StG), gastric ganglion (GG), arm (at 50% of its length), tip of the arm (TIP), sucker (Su), digestive gland (DG), anterior salivary gland (ASG), posterior salivary gland (PSG), white bodies (WB), haemocytes (Hc), stomach (Stom), Intestine (Int). Tissues were frozen in RNA later and shipped in dry ice. The tissues were processed using TRIzol-RNA extraction and cDNA synthesis (Supplementary Table S2). The tissues were processed in compliance with the Ethics and Research Governance Online II (ERGO II) policy (nr 68971.A1) in place at the University of Southampton.

4.3.3 Molecular biology

4.3.3.1 RNA extraction and cDNA synthesis from *O. vulgaris* tissues

Tissues were resected into smaller samples (4-40 mg) and placed in 2mL Eppendorf tubes containing 500uL TRIzol (Cat No. 15596026, Invitrogen™). Each sample was frozen in liquid nitrogen for a few seconds prior to mechanical homogenisation using the Handheld Homogeniser SHM1 (Cole-Palmer). After a 5 min incubation at room temperature, 200 µl chloroform (Cat No. 200-663-8, Sigma-Aldrich) was added, mixed, and incubated on ice for 15 min. Samples were centrifuged (12,000 x g, 4°C) and the supernatant was transferred to a silica-cartridge for the total RNA purification (PureLink® RNA Mini Kit; Cat No 12183018A, Invitrogen™). The purified samples were processed with DNase I, Amplification Grade (Cat No. 18068015, Invitrogen™) following the manufacturer's instructions. Quality (260/280 ratio close to 2.0) and quantity of extracted RNA was assessed through UV-visible absorption measurements (NanoDrop™ 2000/2000c Spectrophotometers, Thermo Scientific™). RNA samples were used for reverse transcription with SuperScript™ III Reverse Transcriptase (Cat. No. 18080093, Invitrogen™) in which 1µg of RNA was utilised to produce cDNA. Octopus RNA, as for all the molluscs, is characterised by the so-called 'hidden break' in 28S rRNA causing the 18S and 28S rRNA fragments to co-migrate

electrophoretically as a single band, thus challenging resolution of the RNA quality through a gel (Natsidis *et al.*, 2019; Adema, 2021). Given this, only cDNA derived from extracted tissues which successfully amplified our control gene *cullin 1* (Imperadore *et al.*, 2023) was selected to be further used in endpoint PCR to evaluate expression of the indicated genes of interest.

4.3.3.2 Primer design and end point PCR

Primer sequences of *OvAstCR1*, *OvAstCR2* and *OvAstC* were designed using ApE-A plasmid Editor® v3.1.3 software and purchased from Eurofins Genomics (Supplementary Table S3a). Once diluted to a working concentration of 25pmol/μL, the primers were used to amplify the target genes in the tissues described above. PCR amplifications were performed using Phusion™ High-Fidelity DNA Polymerase (Cat No. 10024537, Thermo Scientific™) following manufacturer's instructions and the setup was adjusted according to the amplicon length and melting temperature of each primer pair (Supplementary Table S3b). A volume of 1μL of cDNA synthesis reaction was used for each amplification.

4.3.3.3 Plasmid and peptide synthesis

The cyclic and non-cyclic forms of *O. vulgaris* mature allatostatin C peptide (AVITACYFQAVSCY) were synthesised using the custom peptide synthesis service (PeptideSynthetics, Peptide Protein Research Ltd., UK, Supplementary File S5); The predicted *O. vulgaris* Allatostatin C receptors *OvAstCR1* and *OvAstCR2* were codon optimised for *H. sapiens* expression (Supplementary File S6) and synthesised into the pcDNA3.1+ vector using Genscript gene synthesis service (Genscript Biotech, UK).

4.3.3.4 *O. vulgaris* AstCRs de-orphanisation

The cell line utilised in this study was the HEK293G5A, stably expressing the chimeric GFP-Apoeaquorin protein G5A (Cat Nr. cAP-0200GFP-AEQ-Cyto, Angio-Proteomie). Cells were grown in a T75 flask at 37 °C in 5% CO₂ atmosphere in DMEM (containing 4.5 g/L glucose, L-Glutamine, sodium pyruvate, Cat. No. 10566016 ThermoFisher™) supplemented with 10% FBS (heat inactivated, Cat. No. 10082147, ThermoFisher™) and broad-spectrum antibiotic-antimycotic (Cat No. 15240062, Gibco™). When the culture reached confluency, cells were detached from the bottom of the flask by incubation with 5 mL of TrypLE™ (Cat No. 12605010, Gibco™) for 5 min. The suspension was then centrifuged (1,500 rpm for 5 min), the pellet was resuspended in DMEM (1:5 volumes) prior transfer into Corning™ 96-Well, Cell Culture-Treated, Flat-Bottom Microplates (Cat No. 10517742, ThermoFisher™) and grown for 2 days at 37 °C in 5% CO₂ atmosphere. For the transfection, for each well, 10 μl OptiMEM (without FBS, Cat No. 11058021, Gibco™), 60 ng of GPCR-containing plasmid, 60 ng of the chimeric Gqi9 plasmid (Conklin *et al.*, 1993), and 0.30 μg per 100 ng DNA of 25 kDa linear polyethylenimine (PEI; Cat. number: 23966 PolySciences, Inc.) were mixed and incubated for 20 min at room temperature prior adding the mix to 90 μl of

Advanced DMEM (Cat No. 12491015, Gibco™) and finally replacing the old medium in the 96-well plate. The transfected culture was incubated at 37 °C and 5% CO₂ for two days. On the experimental day, the medium was removed and substituted with HBSS media (Cat. No. 20011, AAT-Bioquest) supplemented with 4 µM coelenterazine-H to reconstitute apoaequorin into functional Ca⁺⁺-sensitive aequorin (Cat. No. S2001, Promega). The plate was covered with aluminium foil to protect it from light exposure and incubated at 37°C in 5% CO₂ for 2 h. Readings were performed with a FlexStation 3 Multi-mode Microplate reader (Molecular Devices, LLC, USA) using the Flex option, for a period of 75 sec per well and ligand injection after 15-18 sec.

Each indicated peptide-receptor pair was tested in triplicates in each plate and the experiment was repeated at least three times. Cells transfected with the empty vector alone (pcDNA 3.1) were tested to check for endogenous activation. Cells viability was assessed by administering ATP and checking for a strong comparable activation across the wells.

4.3.3.5 Normalisation and statistical analysis

Data were normalised to the maximum response obtained in each experiment (100% activation) and to the luminescent background recorded in the media alone (0% activation).

An extra sum-of-squares F-test was performed to determine whether the Emax values differed significantly between cyclic and non-cyclic peptide forms for each receptor; where Emax values were significantly different, curves were fitted with independent Top parameters. For curves reaching full activation, the Bottom parameter was constrained to 0 and Top to 100; for those not reaching 100%, Bottom was constrained to 0 and Top was left unconstrained. EC₅₀ values were log-transformed and compared using a two-way ANOVA with Tukey's multiple comparisons test as post-hoc analysis. Data were analysed using GraphPad Prism version 10 for Windows (GraphPad Software, Boston, Massachusetts, USA).

4.4 Results

4.4.1 Characterisation of *Octopus vulgaris* allatostatin-C receptors reveals conserved GPCR features and evolutionary links to somatostatin and opioid receptors

In our previous *in silico* analysis of candidate *O. vulgaris* nociceptive related genes, we identified two potential allatostatin C receptors: c220802_g3_i1 ([PV164564.1](#)) and c31154_g2_i1 ([PV164563.1](#)) here after referred as OvAstCR1 and OvAstCR2, respectively (Pieroni *et al.*, 2026). When a more complete *O. vulgaris* genome became available (Destanović *et al.*, 2023), we further analysed the transcript sequences to confirm the presence of these receptors and potentially identify new candidates.

By checking the automated protein predictions associated with the new *O. vulgaris* genome (Destanović *et al.*, 2023), we found *OvAstCR1* completely matched with OctVul6B022141P1 encoding for a 410 amino acid sequence, whilst *OvAstCR2* corresponded to OctVul6B016349P which was originally incomplete (195 amino acids) due to a mispredicted start codon when compared to the c31154_g2_i1 (443 amino acids). The secondary structure of the amino acids predicted for the two receptor genes showed the classical G-protein coupled receptors (GPCRs) characteristics. This included an extracellular N-terminal, 7 transmembrane elements (TMs) and an intracellular C-terminal (Figure 4.1). The two receptors showed an overall 47.8% identity (64.5% similarity) between each other, and a 56.5% identity (71.7% similarity) when considering only the region spanned by the characteristic seven TMs.

Signature residues such as the DRY, NPILY and WLP motifs as well as the cysteines involved in a disulfide bond formation in the extracellular loop (ECL) 2 allowed the classification of these predicted receptors as members of the Rhodopsin family (class A GPCR) representatives (Figure 4.1). Interestingly, the putative cAMP- and cGMP-dependent phosphorylation motifs KKKS and xxVT, as well as conserved residues in the TM7 that are typically found in the orthologous deuterostome-specific somatostatin receptors were also identified (Figure 4.1), thus supporting the hypothesis these receptors were allatostatin C receptors (Duan Sahbaz *et al.*, 2017).

CmorAstCR	LDH-----H--EC-DTDQPTLLLLITQVLCAAVCIVGLLGNSLVIYVVLRFSSKMQTVT	90
MsexAstCR1	YDDNGTYNGTLE--NCPNINLPYVVVTVQVLYALVCVVGLLGNTLVIYVVLRYSSKMQTVT	91
MsexAstCR2	YDDNGTYNGTLE--NCPNINLPYVVVTVQVLYALVCVVGLLGNTLVIYVVLRYSSKMQTVT	73
OvulAstCR1	DMNLSLSNATEH--SIRRVFMEIVNITVMISNSLICITGLFGNCLVIYVVTMFSKMKTVT	84
OvulAstCR2	DDIDFYQNDTERKRQSNFLSHIFPLIMVACYGLVFVAGIAGNCLVIYVVTMFSKMKTVT	95
AcalAstCR	--TESNLNDTGM--PERSGYAKVLPYVMTVCYILICILGLAGNGLVIYVVLMAFKMKTVT	98
	. : : * : ** ***** : : : : *	
CmorAstCR	NMYIVNLAVADECFLLIGVPFLLATMNLRIWPFGRFMCAYMTTTSINQFTSSIFLTIMSA	150
MsexAstCR1	NMYIVNLAIADCEFLIGIPFLITMNLRIWPFGRFMCAYMISTGINQFTSSIFLCIMSA	151
MsexAstCR2	NMYIVNLAIADCEFLIGIPFLITMNLRIWPFGRFMCAYMISTGINQFTSSIFLCIMSA	133
OvulAstCR1	NTYILNLAIADVLFLVSIPLLTITIKNGFWMFGFFMCKFYLLVSINSFTGVFTLTVMISA	144
OvulAstCR2	NMYVNLAVADVLFLMSPFLMTTILKTWIFGYGMCKIYMILVSINYFTGIFTLTIMSA	155
AcalAstCR	NMYILNLALSDFIVILPMMATTTLMHWFIFGFAMCKIYFVLYSINLFGGAFNLVMSA	158
	* * : : : : * : : : : *	
CmorAstCR	DRYIAVCHPISAPKVRTPLISKVVSASAWTASALLMIPFIYANIMTN---ENVVSCNII	207
MsexAstCR1	DRYIAVCHPIAAPRLRTPFVSRIVSAAAWTASALVMTPIFYMTTLIPT---DNLGSCNIV	208
MsexAstCR2	DRYIAVCHPIAAPRLRTPFVSRIVSAAAWTASALVMTPIFYMTTLIPT---DNLGSCNIV	190
OvulAstCR1	DRYFAVCHPIKMSLRTPKVAFIITVCIWLISILMLPALMYSTTVRN--SKNKDCMMV	202
OvulAstCR2	DRYMAVCHPVQSVSIRTPKLAFLCLCLWTASVLTMMPTFLYSKTVLKS--KKGITCAIL	214
AcalAstCR	DRYLAVCHPIRSLKYRTPRIALFLCLCVWSLFLVMLPJILYSRTEKHRRFPKGKSCSIF	218
	*** : : : : *	
CmorAstCR	WPESENVSSGAFITLYFILGFSIPLMLISVFYILVIRKLKTVGPKNKSKEKKKSHRKVT	267
MsexAstCR1	WPKRDFNKGQTSFTLYSFALGFAAPLTLIFVFYCLVIRKLKTVGPKNKSKEKKKSHRKVT	268
MsexAstCR2	WPKRDFNKGQTSFTLYSFALGFAAPLTLIFVFYCLVIRKLKTVGPKNKSKEKKKSHRKVT	250
OvulAstCR1	MPDHLGISPDKIFIWYNFIFSYAVPVPLISVFYLSVVHKLRTTGPAKKSSEKKKSQRVT	262
OvulAstCR2	WPSDKHTEGKAFILYCFFLSYAIPMLVLSFFYIMVILRLSKLGPANKQKEKKKSRRVT	274
AcalAstCR	WPKDQLIPPDKAFIWSYFILGFAIPVSLISVFYLVILRLRHVGPAKKSKEKKKSHRRVT	278
	* . * * * : : : : *	
CmorAstCR	KLVLTVITVYVLCWLPYWTQMAIIFTPPKQCQSRVSTVSLLAGCLSYNSAMNPILYA	327
MsexAstCR1	KLVLTVIAVYVLCWLPYWAFOVALIYSPPTTECASRITITVFLVAACFSYSNSAMNPILYA	328
MsexAstCR2	KLVLTVIAVYVLCWLPYWAFOVALIYSPPTTECASRITITVFLVAACFSYSNSAMNPILYA	310
OvulAstCR1	RLVLTVIGVYIICWLPYWAFOQDIIWVFPTPLT--EGRILMFQTFNLTYSNMLNPILYN	320
OvulAstCR2	KMVLAIIGVYMMCWLPYWTQIIIIIAKASFWRPSFWGITAFHLFTFLTLANSMMNPFLYN	334
AcalAstCR	RLVLTVITVYVLCWLPYWCFOVHIVFNDVKIT-SDAMTLVFNGFTVLSFANSMLNPFLYA	337
	: : : : * : : : : *	
CmorAstCR	FLSDNFKKSFLKACTCATGQDVNNVLMENSVPFRRHRRQGGSERQRPNKMA-----	379
MsexAstCR1	FLSDNFKKSFLKACTCAAGKDVNATLHVENSVIPRKR--GGAARAQARAIES-----	379
MsexAstCR2	FLSDNFKKSFLKACTCAAGKDVNATLHVENSVIPRKR--GGAARAQARAIES-----	361
OvulAstCR1	FLSDNFRKSFTKAFKCTSRFEVNRSLRAENSIYPKGREAYSQTTVIEKQEL----QTINH	376
OvulAstCR2	FLSDNFRKSFSKAFKCVSKFEVSKSLTAENSMCPRRNQCTELTKFDEDECQNYEMKVTKQ	394
AcalAstCR	FLSDNFRKSFVKAFKCLSNVEMNKSVCNENSVPRTSQTYSRSGVMGEERM----ELSAV	393
	***** : : *	
CmorAstCR	-----TLETGEGGKGPLVSKADSSVTMTMTSRNITVSTDAKDQASKDAAAMETAL	432
MsexAstCR1	-----RGGLSVAVGGSRSE----ASTALTSRVAASEVLPL--EARPP----TLAPLIA	423
MsexAstCR2	-----RGGLSVAVGGSRSE----ASTALTSRVAASEVLPL--EARPP----TLAPLIA	405
OvulAstCR1	CATENKVQEDFVNGETQTEDIDADG-----PEAVVTELE-----	410
OvulAstCR2	QNTIVKVENTFAQAEKETNIESEK-----NVKQIVNDIEETSGDK----EIL	438
AcalAstCR	DT-NPTASPNVAEQGSIKSPQDEHG-----FLKPPVQL-----	425
	. : :	

Figure 4.1 Sequence alignment highlights class A GPCR conserved residues in *O. vulgaris* putative allatostatin C receptors.

Alignment of OvAstCR1 and OvAstCR2 with AstCRs of indicated invertebrate species. The alignment revealed key GPCR A signatures: the seven transmembrane elements (dash-pattern frames), conserved functional residues (in bold), the residues for functional activation of the receptor (in magenta) and the cysteine residues for the disulphide bond formation (highlighted in yellow). Predicted cAMP and cGMP-dependent phosphorylation sites (KKKS, RKVT) found in somatostatin receptors are underlined. The proposed key residues implicated in the ligand binding region Duan Sahbaz *et al.*, 2017) in the third extracellular loop are shown in green and the conserved motif IxPP is framed in red. Interestingly, a similar but slightly different VxxPP was found in the OvAstCR1 but no conserved residues are found in OvAstCR2. Cmor: *Carausius morosus* (KX255655.1); Msex1: *Manduca sexta* AstCR1 (A0A921ZMA1); Msex2: *Manduca sexta* AstCR2 (XP_030033910_1); Acal: *Aplysia californica* (XP_005095139_1).

Next, we carried out a cluster and phylogenetic analysis using reference sequences from well-characterised and curated invertebrate allatostatin C receptors and vertebrate somatostatin (SSRs) and opioid receptors (OPRs), as these classes of GPCRs were previously shown to be related (Mirabeau and Joly, 2013). We performed an initial BLAST search against the complete proteome of 21 representative species from different phyla (Supplementary File S1) and visualised the sequences relationship through a cluster analysis using CLANS (Zimmermann *et al.*, 2018). The CLANS map showed numerous representatives of the Rhodopsin GPCR class, such as monoamine receptors, opsins, and neuropeptide F and FF receptors (Figure 4.2). The two *O. vulgaris* sequences of interest OvAstCR1 and OvAstCR2 were found to be the only *O. vulgaris* receptors within a cluster with strong connections with AstCRs, SSRs and OPRs (Figure 4.2). Additionally, these sequences showed connections within the cluster with melanocortin receptors (MCRs), galanin receptors (GALRs), Kiss peptin 1 receptors (Kiss1Rs) and the invertebrate allatostatin A receptors (AstARs). All the sequences connected within the same cluster (197 sequences), were used to build a phylogeny tree and resolve their relationship more in detail.

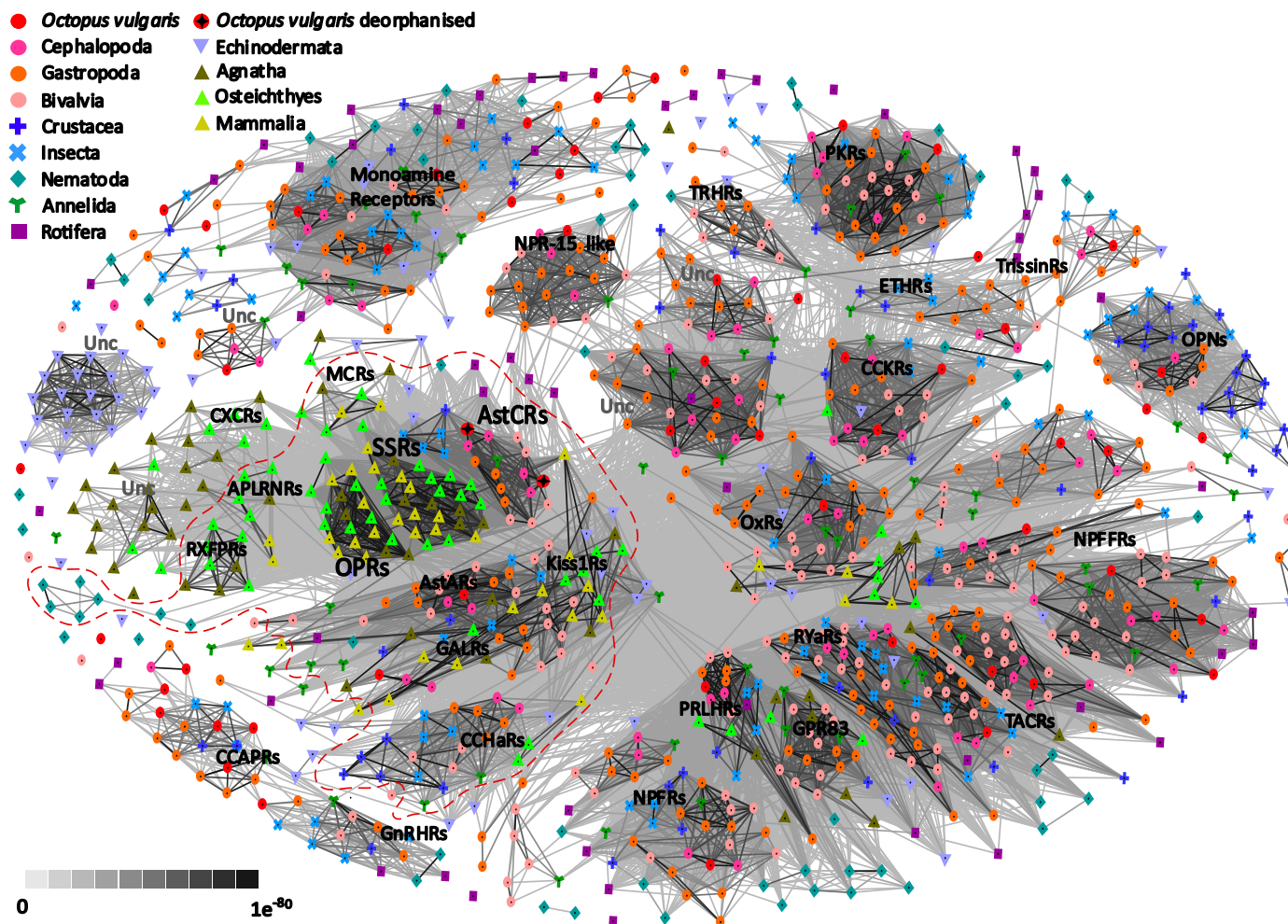


Figure 4.2 OvAstCR1 and OvAstCR2 are allatostatin C receptors with evolutionary relationship with somatostatin and opioid receptors.

Following a BLAST search for AstCRs, SSRs, OPRs, and related class A GPCRs across different representative species, a CLANS analysis has been carried out to reveal the relationship between each other. Each dot represents a species-specific receptor sequence (see legend in top left corner). The gradient of the connection represents the degree of similarities between sequences (indicated in the bottom left corner). The analysis shows the identified *O. vulgaris* receptors are indeed Allatostatin C receptors and share the same cluster with somatostatin and opioid receptors (dashed red frame).

The phylogenetic analysis showed that AstCRs are an evolutionary related but distinct class of invertebrate receptors from the vertebrate-exclusive OPRs. Additionally, as previously shown, the phylogeny is consistent with a duplication event that led to the AstCR and the deuterostome-specific SSRs branches (Figure 4.3). Specifically looking at *O. vulgaris*, the two sequences of interest were identified as part of the allatostatin C branch, confirming their identity as allatostatin C receptors (Figure 4.3).



Figure 4.3 Phylogenetic analysis reveals OvAstCRs are allatostatin C receptors.

Allatostatin C receptors (red section) are the closest protostome orthologues of the vertebrate exclusive OPRs (blue section) and paralogue of the deuterostome-specific SSRs (yellow section). The OvAstCRs are highlighted in bold red and are clearly part of the AstCRs branch. The phylogenesis has been carried out using the 197 sequences belonging to the same identified cluster (Figure 4.2). Tree has been rooted using the outgroup sequences such as GALRs, AstARs, Kiss1Rs. All sequences used are available in Supplementary File S3.

4.4.2 Two receptors, one peptide: *O. vulgaris* has one putative endogenous AstC peptide

Next, we performed a BLAST search against *O. vulgaris* genome in search of the candidate endogenous activators of the OvAstCRs (Destanović *et al.*, 2023) using AstC precursor sequences from different protostomes (Uniprot Taxon id: 33317). The search produced a single hit (OctVul6B022881P1) with an identified 22 amino acid signal peptide sequence and a typical dibasic KR cleavage site with a single embedded 14 amino acid peptide sequence (Figure 4.4A). When aligned with other invertebrate AstC precursor sequences, highly conserved residues within the predicted mature peptide sequence such as the two cysteines, known to classically cyclise AstC peptides via the formation of a disulfide bond, were revealed (Figure 4.4B). The amino acid residues surrounding the cysteines shared the strongest conservation. The only exception was the third residue after the first cysteine, in which a conserved asparagine (N) is found in all protostome phyla except for cephalopods. In this group, this position is occupied by a glutamine (Q), with 100% identity observed in the mature peptide sequence (Figure 4.4B).

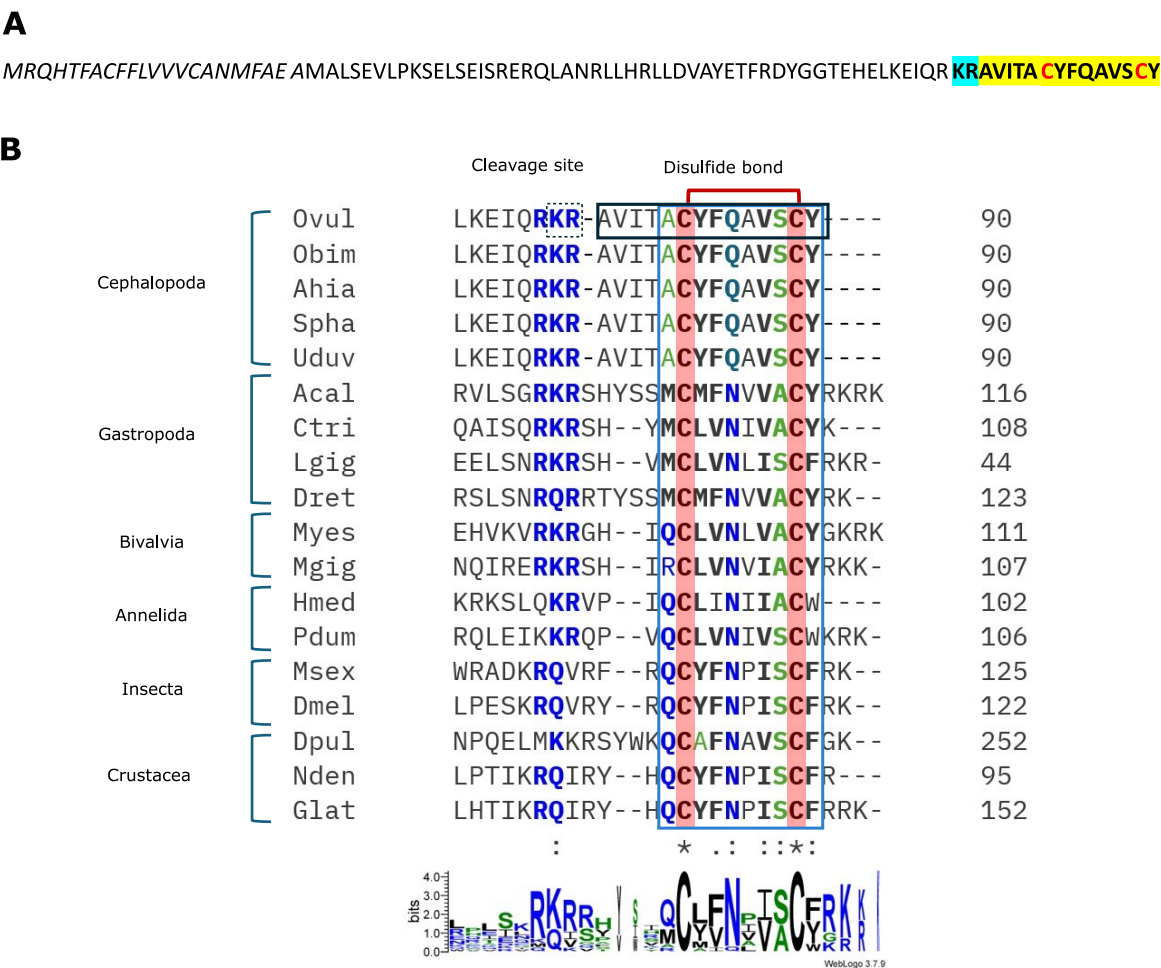


Figure 4.4 Precursor sequence of *O. vulgaris* allatostatin C peptide and alignment with allatostatin C precursors from different invertebrate phyla.

A *In silico* analysis predicted the presence of one *O. vulgaris* AstC precursor with a single embedded mature peptide. The signal peptide is in italics. The classical dibasic KR cleavage site is highlighted in cyan, and the putative mature peptide is highlighted in yellow. The two cysteine residues, typically forming a disulfide bridge in the bioactive form of

the peptide, are in bold red. **B** The mature AstC shows a high level of conservation across selected representative invertebrate species, particularly in the cysteine residues (here highlighted in red) forming a disulfide bond. Key conserved residues have been highlighted in different colours within the light blue rectangle according to their relative prevalence as depicted by WebLogo 3.7.9 below. The cleavage site KR in the OvAstC (Ovul) sequence is shown by the dashed rectangle, the mature form of the peptide is highlighted by the solid black rectangle. Ovul: *Octopus vulgaris* (CAI9731309.1); Obim: *Octopus bimaculoides* (Ocbimv22019504m, mRNA); Ahia: *Argonauta hians* (GAB1606153.1); Spha: *Sepia pharaonis* (GEIE01009944.1, from TSA); Uduv: *Uroteuthis duvaucelii* (GKZI01071814.1, TSA) Acal: *Aplysia californica* (XP_005112794.1); Myes: *Mizuhopecten yessoensis* (XP_021356393.1); Mgig: *Magallana gigas* (XP_011412814.1); Ctrit: *Charonia tritonis* (AQS80487.1); Lgig: *Lottia gigantea* (Veenstra, 2010); Dret: *Deroceras reticulatum* (ARS01353.1); Hmed: *Hirudo medicinalis* (FP643552.1 –mRNA sequence); Pdum: *Platynereis dumerilii* (AHB62362.1); Msex: *Manduca sexta* (XP_030040870.1); Dmel: *Drosophila melanogaster* (NP_523542.1); Dpul: *Daphnia pulex* (XP_046455835.1); Nden: *Neocaridina denticulata* (AIY69122.1); Glat: *Gecarcinus lateralis* (A0AA50EVT2 – Uniprot).

4.4.3 The putative OvAstC system is broadly represented across tissues suggesting different physiological functions

The expression of the authenticated receptors and peptide precursor transcripts was investigated by designing primers spanning the entire length of the transcripts. The amplified products were validated through sequencing.

We additionally aligned the OvAstCRs and OvAstC transcript sequences against the *O. vulgaris* genome to find their correct genomic location (Destanović *et al.*, 2023). *OvAstCR1* (1233 bp) was found on chromosome 23 (Figure 4.5A) and *OvAstCR2* (1332 bp) was located on chromosome 24 (Figure 4.5B). Both genes were found to be intronless, whilst the *OvAstC* peptide precursor sequence was found on chromosome 12 and was identified as formed by two exons (102 + 171 bp) and one 24,304 bp intron (Figure 4.5C).

Following the *in silico* identification and experimental validation of the sequences of the candidate representatives of the *O. vulgaris* AstC system, we proceeded to characterise their expression across a broad set of tissues associated with different physiological functions in octopus (Figure 4.5D). To this end, we used primers encompassing either the whole (*OvAstC*) or internal (*OvAstCRs*) sequence of the transcript and performed an endpoint PCR using tissues from three individual octopuses.

Our data showed the endogenous peptide precursor is expressed broadly across different tissues, including the central brain mass (i.e., SEM, SUB, OL), the peripheral nerves (StG, GG) and the sensory-motor tissues (Arm, Tip, sucker). Expression was also found in representative tissues for the feeding and digestion-related (except for the ASG) and immune-related (WB, Hc, PSG) systems (Figure 4.5D).

The PCR carried out on *OvAstCR1* and *OvAstCR2* amplified bands of the predicted size (538 bp and 497 bp) which showed an overlapping and broad expression. They were amplified in all the tissues investigated with variable low expression in ASG (Figure 4.5D).

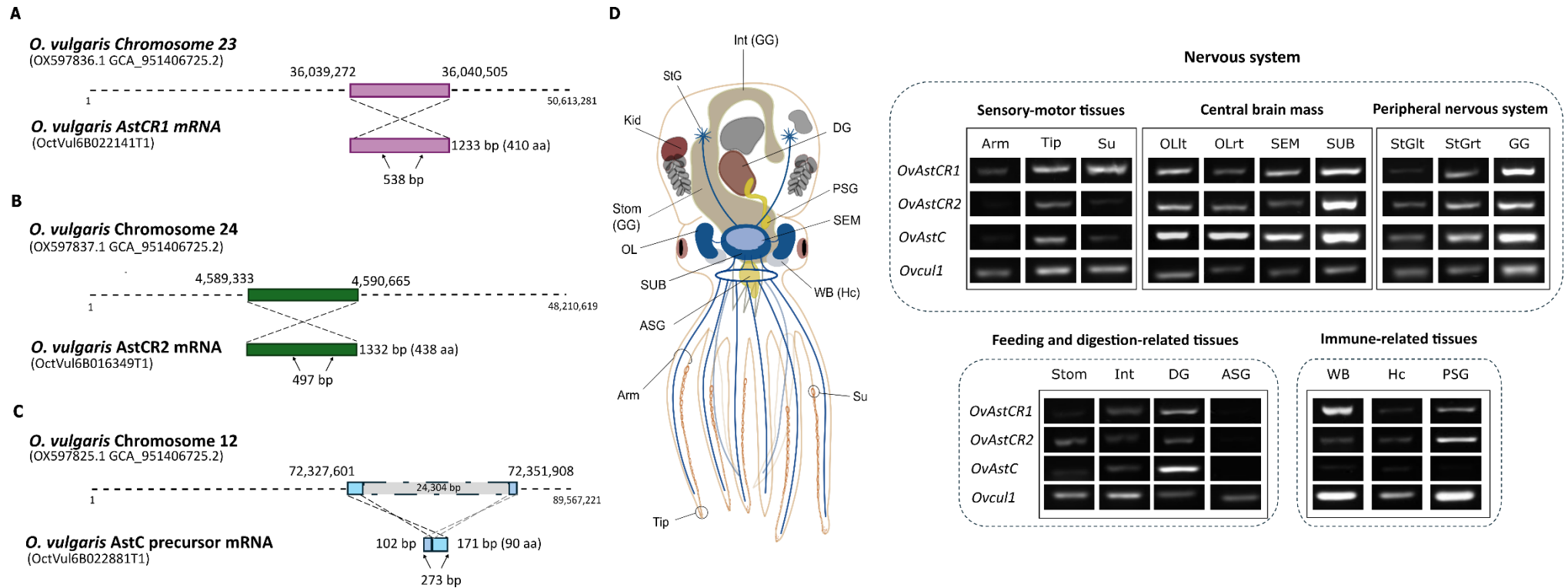


Figure 4.5 Genomic location and tissue distribution of *OvAstCRs* and *OvAstC* sequences.

A-C Schematic representation of the *O. vulgaris* allatostatin C receptors and related peptide precursor transcripts and their position on the matching chromosome. *OvAstCR1* and *OvAstCR2* are intronless genes found on chromosome 24 and 23. The *OvAstC* precursor transcript is found on chromosome 12 and is composed of two exons and one intron. The arrows underneath the mRNA sequence indicate the region amplified by the designed primers to analyse the tissue distribution. **D** Schematic anatomy of *O. vulgaris* showing the tissues from which RNA was extracted, reverse transcribed and used in the PCR reactions to show the distribution of the genes of interest across key physiological systems. The representative bands shown, refer to PCR performed at least once for each animal tissue (for a total of three animals utilised, Supplementary Table S2) that successfully amplified the control gene *cul1*. This gene was previously shown to be broadly expressed across different *O. vulgaris* tissue types (Imperadore et al., 2023). The amplified bands shown were all at the size expected based on the primer pair selected (see Supplementary Table S3). Arm: arm muscle and embedded axial nerve cord at 50% of its length; Tip: tip of the arm; Su: sucker, OL_lt: left optic lobe; OL_rt: right optic lobe; SEM: supra-oesophageal mass; SUB: sub-oesophageal mass; StG_lt: left stellate ganglion, StG_rt: right stellate ganglion; GG: gastric ganglion; Stom: stomach; Int: intestine; DG: digestive gland; ASG: anterior salivary gland; WB: white body; Hc: haemocytes; PSG: posterior salivary gland.

4.4.4 OvAstC is the putative endogenous agonist of the OvAstCRs

We next asked whether the candidate genes identified interacted together in a way that would be consistent with a physiological *O. vulgaris* AstC system. To investigate this, we carried out a de-orphanisation of the receptors using the predicted endogenous mature OvAstC peptide. We heterologously expressed *OvAstCR1* and *OvAstCR2* separately in the recombinant cell system HEK293G5A, stably expressing the Ca⁺⁺-sensitive aequorin (Baubet *et al.*, 2000). This approach is based on exogenous agonist-induced activation of the chimeric G_{q19} protein (Conklin *et al.*, 1993) from the heterologously expressed candidate receptor that leads to an increase in the intracellular Ca⁺⁺ levels which subsequently activates Ca⁺⁺ sensitive aequorin to produce a luminescence signal (Thiel *et al.*, 2024; Yañez-Guerra *et al.*, 2025).

We synthesised the mature peptide (AVITACYFQAVSCY) both in the open (*OvAstC_nc*) and cyclised structure (with a disulfide bridge between the cysteines, *OvAstC*) and tested recombinant *OvAstCR1* or *OvAstCR2* activation.

The synthetic *OvAstC* peptides elicited activation of *OvAstCR1* and *OvAstCR2* in a dose-dependent manner (Figure 4.6A). A 10-fold shift in the EC₅₀ value was observed between the two forms of the mature peptide, with the cyclised version showing higher affinity for both receptors (*OvAstCR1*: *OvAstC* EC₅₀ = 1.1 x 10⁻⁸ M, 95%CI: 8.3x10⁻⁹ – 1.5 x 10⁻⁸ M vs *OvAstC_nc* EC₅₀ = 8.7 x 10⁻⁸ M, 95%CI: 7.0 x 10⁻⁸ – 1.1 x 10⁻⁷ M; *OvAstCR2*: *OvAstC* EC₅₀ = 3.7 x 10⁻⁶ M, 95%CI: 2.8 x 10⁻⁶ – 4.8 x 10⁻⁶ M vs *OvAstC_nc* EC₅₀ = 4.0 x 10⁻⁵ M, 95%CI: 2.0 x 10⁻⁵ – 2.1 x 10⁻⁴ M).

When comparing the receptors' affinity for the peptide, a 100-fold difference in the EC₅₀ value between *OvAstCR1* and *OvAstCR2* was observed for both peptides (Figure 4.6B) with *OvAstCR1* showing higher affinity when compared to the *OvAstCR2* (*OvAstC*: *OvAstCR1* EC₅₀ = 1.1 x 10⁻⁸ M, 95%CI: 8.3x10⁻⁹ – 1.5 x 10⁻⁸ M vs *OvAstCR2* EC₅₀ = 3.7 x 10⁻⁶ M, 95%CI: 2.8 x 10⁻⁶ – 4.8 x 10⁻⁶ M, ****p<0.0001).

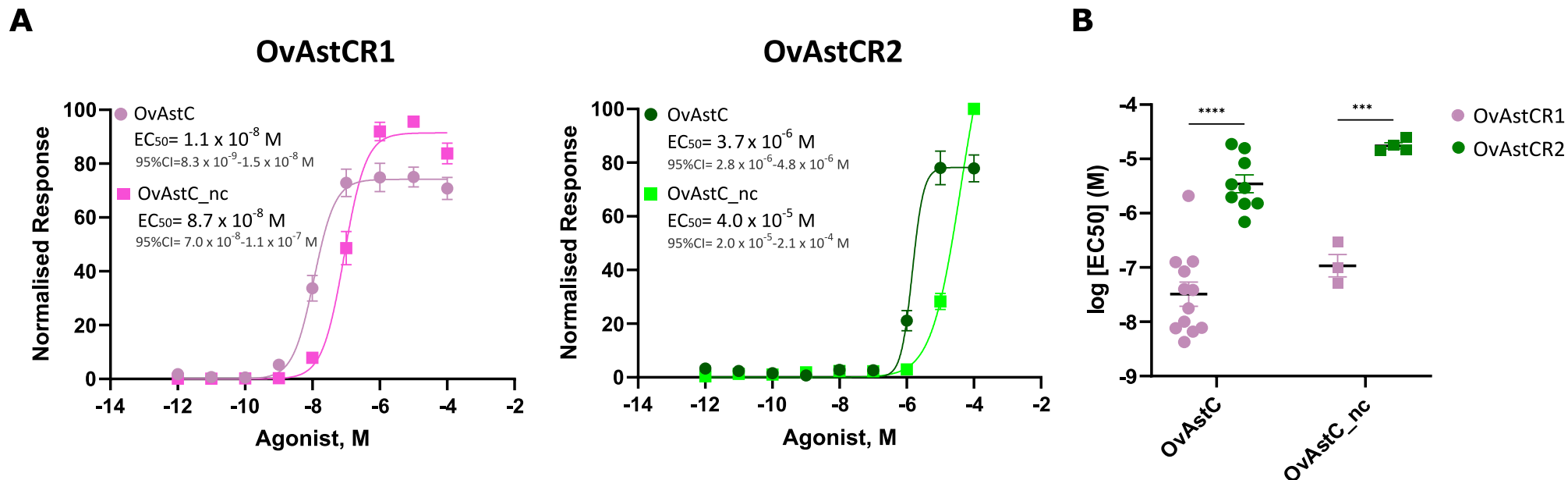


Figure 4.6 OvAstC activates both OvAstCRs in a dose-dependent manner.

A Dose response curves of OvAstCRs in the presence of the mature cyclic (AVITACYFQAVSCY) and non-cyclic (AVITACYFQAVSCY) AstC peptide expressed as normalised response $\pm$ s.e.m. The EC₅₀ value and related 95%CI for each receptor-peptide pair is shown. OvAstC: cyclic form of allatostatin C mature peptide; OvAstC_nc: non-cyclic form of allatostatin C mature peptide. Each indicated peptide-receptor pair was tested in triplicates in each plate (OvAstCR1-OvAstC, n= 12; OvAstCR1-OvAstC_nc, n=3; OvAstCR1-OvAstC, n= 9; OvAstCR1-OvAstC_nc, n=4, where n is the number of independent transfections). **B** Plot showing the average EC₅₀ values $\pm$ s.e.m of each receptor with the different forms of AstC peptide. There is a 100-fold difference in the EC₅₀ between OvAstCR1 and OvAstCR2 that is visible with both the cyclic and non-cyclic form of the peptide. Comparison between the EC₅₀ values of the different AstCRs with the non-cyclic and cyclic form of the peptide was performed with a two-way ANOVA and Tukey's multiple comparisons test as a post-hoc analysis. The level of significance was set at $p < 0.05$. ***= $p < 0.001$; ****= $p < 0.0001$.

4.5 Discussion

4.5.1 *O. vulgaris* possess two allatostatin C receptors with a tissue distribution consistent with a broad physiological role

In this study we identified for the first time, a putative allatostatin C signalling system in the cephalopod mollusc *O. vulgaris* using *in silico* and functional approaches.

GPCRs are characterised by a very conserved membrane-spanning structure, and the specific receptor identity can only be resolved by complementing multiple alignments, genome structural analysis and phylogenetic investigation with functional studies that ultimately demonstrate the interaction with the predicted ligands (Yañez Guerra and Zandawala, 2023). Our previous bioinformatic analysis of the *O. vulgaris* transcriptome, identified two putative AstCRs (Pieroni *et al.*, 2026). However, while sequence alignment and secondary structure prediction analysis showed unequivocal conserved signatures of class A GPCRs, such as DRY and NSxxNPxxY motifs, cluster-based and phylogenetic investigations were carried out to confirm their classification as AstCRs.

Indeed, our phylogenesis showed OvAstCR1 and OvAstCR2 to be the only two *O. vulgaris* representatives identified in the AstCR invertebrate-specific branch. In line with what has been previously shown, AstCRs are the closest protostome orthologues of the vertebrate-specific OPRs and are paralogous receptors of the deuterostome-specific somatostatin (Mirabeau and Joly, 2013; Elphick, Mirabeau and Larhammar, 2018; Zhang *et al.*, 2022). Interestingly, our phylogenesis suggests that AstCRs have undergone a series of internal duplications leading to at least two types of receptors in different lineages. We found a single receptor in Crustacea, Annelida and Gastropoda, but two receptors in Rotifera, Bivalvia, Cephalopoda and Insecta (Figure 4.3). In particular, the nematode *C. elegans* shows exaggerate duplication and expresses 8 different AstCRs, characteristic of a one-to-many orthologues expansion event (Kuzniar *et al.*, 2008).

The shared phylogeny of AstCRs with members of SSRs and OPRs makes the case for asking whether the molecular similarity is also shared at the functional level. Despite the conserved class A GPCR features, such as the intracellular elements involved in the recruitment and interaction with the cellular G-protein, the extracellular elements that are involved in the formation of the ligand binding site are extremely different across the three subfamilies and seem to have evolved to provide discrete differences in agonist recognition. This is supported by

experiments showing that opioid peptides and somatostatins are unable to activate *D. melanogaster* AstCRs and that replacing the fruit fly ligand binding site with residues from the rat SSR2 impaired the activation of DmelAstCR by the endogenous peptide (Kreienkamp *et al.*, 2002).

Although the specificity of the ligand binding site impedes cross reactivity between the different receptors, an overlap in the functions can indeed be found.

The main role identified for AstC is the inhibition of the synthesis of the juvenile hormone in some insects corpora allata (Kramer *et al.*, 1991). To some extent this hormonal regulatory role is retained in vertebrates, where somatostatins have been found to inhibit the release of the growth hormone from the pituitary gland (Brazeau *et al.*, 1973). Furthermore, for both signalling systems and to some extent the opioid system too, the broad tissue distribution has been found to be associated with promiscuous functions in the organism physiology. AstCRs have been found expressed across the digestive system and the musculature of intestine and buccal mass in invertebrates, supporting the identified function in feeding and digestion, with a role both at the motility (with a myo-modulatory role) and at the secretory level (Ma *et al.*, 2009; Stemmler *et al.*, 2010; Alzugaray, Hernández-Martínez and Ronderos, 2016; Kubrak *et al.*, 2022; Zhang *et al.*, 2022). Similarly, SSRs have been found to be implicated in gut motility, pancreatic regulation and digestive secretions (Olias *et al.*, 2004; Tostivint *et al.*, 2014) and OPRs have been reported to be involved in the modulation of fluid and electrolytes transport in the gastrointestinal tract (Sternini *et al.*, 2004). The expression of both OvAstCRs in tissues related to the digestive tract such as stomach, intestine, gastric ganglion and secretory glands like the digestive gland, supports this potential role is conserved in cephalopods as well (Figure 4.5D).

An immune-related role has been proven for the AstC system in bivalves and suggested by the broader distribution found in the haemolymph of different invertebrate species (Skiebe, 1999; Li *et al.*, 2021c). A similar role has been reported for somatostatin and opioids as well (Krantic, 2000; Ferone *et al.*, 2004; Liang *et al.*, 2016). We have been able to amplify the genes of interest from immune-related tissues of octopus, including the haemocytes and the white bodies, where they are produced, or the posterior salivary gland where substances (e.g., venom, enzymes or other neuropeptides) used as a defence or protection are secreted from (Gestal and Castellanos-Martínez, 2015).

Lastly, and with welfare implications in cephalopods, is the potential role of this peptidergic signalling in antinociceptive response that is again conserved across AstCRs, OPRs and SSRs

(Carlton *et al.*, 2001; Pintér, Helyes and Szolcsányi, 2006; Yaksh and Wallace, 2011). Indeed, the lack of convincing opioid peptides and receptors from invertebrate genomes and tissues has prompted the view that these animals do not possess the capacity to experience pain as defined by the Bateson's criteria (1991). However, recent evidence showed how AstC is involved in the suppression of thermal responses in *D. melanogaster*, thus suggesting the presence of a more ancestral analgesic system, predating the emergence of the actual vertebrate-exclusive one (Bachtel *et al.*, 2018; Liu *et al.*, 2023). We have detected the presence of *OvAstCR1*, *OvAstCR2* and *OvAstC* in the central brain regions (SEM, SUB, OL) and in peripheral tissues usually associated with the sensory responses such as arms and suckers. In cephalopods, the central brain mass comprises interconnected lobes responsible for the integration of sensory information, motor coordination and higher-order processing, including learning and behavioural modulation (Young, 1971). The detection of *OvAstCR1*, *OvAstCR2* and *OvAstC* within these regions therefore suggests that AstC signalling may act centrally to modulate neural circuit activity, rather than being restricted to peripheral effector tissues, suggesting a top-down action through the stimulation of potential descending pathways that is worth exploring in the context of nociceptive stimulation.

Altogether, our gross analysis of the tissue distribution supports a conserved pleiotropic, physiological role of the *O. vulgaris* allatostatin C system, but more detailed studies on the actual localisation of the receptors within the cells, combined with functional and behavioural investigations in the whole animal, are needed to confirm this (Figure 4.5D).

From a genomic point of view the *OvAstCRs* were found to be intronless genes, similarly to most of the human SSRs (Tostivint *et al.*, 2014). The most recently published genome from *O. vulgaris* reports 87% of transcripts are multi-exonic (Destanović *et al.*, 2023); this is in line with a large occurrence of introns reported in invertebrate genomes, including GPCR A receptors (Bryson-Richardson *et al.*, 2004). When compared to other species in which a genomic analysis has been performed, *OvAstCRs* are similar to the mosquito *Aedes aegypti* AstCRs and other insects from coleoptera, Hymenoptera and lepidoptera which are intronless (Mayoral *et al.*, 2010). However this is different from *Drosophila* AstCRs which have two introns each (Kreienkamp *et al.*, 2002).

The evolutionary significance of such occurrence would be interesting to investigate in association with a proposed scenario of local gene duplication and subsequent “dispersion” into the genome. This was also suggested in a previous large scale investigation of cephalopod GPCRs evolution where the AstCRs are found to belong to the unexpanded group of genes which therefore derive from conserved GPCR families (Ritschard *et al.*, 2019).

4.5.2 Cephalopod AstC mature peptide shows a strong conservation across species and strong similarity across phyla

Even though we found two AstCRs in *O. vulgaris* genome, our *in silico* analysis led to the detection of a single peptide precursor. Apart from two forms of the peptide previously characterised in insects (Kramer *et al.*, 1991; Kreienkamp *et al.*, 2002), our results are in line with findings from other molluscan species with two receptors, where a single peptide was identified as well (Li *et al.*, 2021c).

The only study reported in cephalopods aimed at surveying the neuropeptidome of *S. officinalis* and it did not report the presence of AstC (Zatylny-Gaudin *et al.*, 2016). However, this could be ascribable to challenges in interpreting cyclised peptides with mass spectrometry rather than the actual absence of the peptide (Chaturvedi, Bawake and Sharma, 2024). In support of this, an *in silico* analysis using our identified OvAstC precursor sequence revealed the presence of a single predicted peptide in the transcript assembly of other cephalopods including *Sepia* (Figure 4.4B). Interestingly, in all cases analysed, despite slight changes in the signal peptide region, the mature peptide shared a 100% identity across all the species (Figure 4.4B). When compared to other protostome peptides, a strong conservation of the cysteines that are notably involved in the formation of a disulfide bridge and of the surrounding residues could be found, except for the third residue following the first cysteine, which was changed from a highly conserved N into a Q in Cephalopoda (Figure 4.4B). It would be interesting to experimentally investigate whether this might represent a key cephalopod-specific functional residue in the activation of/interaction with the receptors.

The OvAstC precursor sequence was found widely expressed across the tissues analysed, supporting the potential promiscuous role, for instance as a neuromodulator in the sensory tissues and brain, or with hormonal-like function as suggested by the presence in the haemocytes or in the salivary glands (Figure 4.5E).

4.5.3 Distinct activation profiles of *O. vulgaris* allatostatin-C receptors revealed by Ca^{++} -based deorphanisation

The use of a Ca^{++} -sensitive aequorin-based assay demonstrated that the predicted mature form of OvAstC could activate the two OvAstCRs in a dose-dependent manner, leading to the identification of a putative AstC system in *O. vulgaris* (Figure 4.6A). An interesting result was the finding of a very different affinity for the endogenous peptide between the two receptors. Several

reasons could explain this 100-fold difference in their EC₅₀ between OvAstCR1 and OvAstCR2 (Figure 4.6B).

The high conservation of the key intracellular amino acids in class A GPCRs, suggests this difference is unlikely to be found in the G-protein binding site, but rather at the level of the ligand binding region. To this regard, in most of the rhodopsin GPCRs, including SSRs and AstCRs, the ligand binding site is formed by the interaction between the N-terminal region, the ECL2 and ECL3 (Peeters *et al.*, 2011). A recent study carried out in the Indian insect *Carausius morosus*, showed that the N-terminal is rather variable and seems to be involved in maintaining the conformational structure of the binding site, while the ECL3 is actually mediating the key peptide interaction (Işbilir *et al.*, 2020). In insects, a conserved IxTPP motif has been found to be conserved, and mutating this motif leads to a strong reduction in potency and efficacy of the peptide action (Duan Sahbaz *et al.*, 2017; Işbilir *et al.*, 2020). Interestingly, this motif is not particularly recurrent in other phyla such as annelids, gastropods and bivalves, but OvAstCR1, which displayed a physiological affinity for the peptide (Figure 4.6A), does show a similar VxxTPP motif (Figure 4.1). As this pattern is missing in OvAstCR2, it would be interesting to verify whether in cephalopods these residues might play a significant role, similarly to insects, in the ligand-mediated activation of the receptor.

Another hypothesis is that OvAstCR2 is only able to work efficiently if heterodimerising with OvAstCR1. The overlapping expression of both receptors in the same tissues, although not indicative of the actual cellular distribution, might be supportive of the potential functional association. Fluorescence Resonance Energy Transfer (FRET) studies have reported that a series of class A GPCRs in *D. melanogaster*, including DmelAstCR2, are subjected to the formation of homodimers (Rizzo and Johnson, 2020). Heterodimers within the same family (i.e., with DmelAstCR1) were not tested but interaction with different receptor families did not show any significant FRET associated luminescence (Rizzo and Johnson, 2020).

Data on the vertebrate counterparts, show that SSRs often associate to form heteroligomers with other family members or even other GPCRs such as OPRs and dopamine receptors, with resulting significant changes in their pharmacological properties (Olias *et al.*, 2004).

An alternatively explanation for the receptors' differential response to the putative endogenous peptide might be a direct consequence of the action of the peptide. As proposed above and as also suggested by other works, OvAstC might have a dual action depending on the tissue: it may act as a neuromodulator, therefore displaying a neurotransmitter-like release, or as a secreted

compound, therefore displaying a more hormone-like release. As a consequence, this might require the receptors to exert a different sensitivity to different secreted concentrations, or to have a different cellular distribution (Mayoral *et al.*, 2010; Muscato *et al.*, 2021). Spatiotemporal activity of the peptide relative to the developmental stage of the organism could also be interesting to investigate in this context (Kreienkamp *et al.*, 2002).

Finally, technical limitations of heterologous systems in recapitulating *in vivo* milieu, could explain the differences of affinity observed between the two receptors. In fact, OvAstCR1 and OvAstCR2 may express with different density at the surface. Therefore, utilising the same amount of plasmid during transfection might explain the requirement of more ligand to achieve the same response, artificially shifting the EC₅₀ rightward. Furthermore, we utilised a chimeric G_{q19} protein that may couple with different efficiency to OvAstCR1 vs OvAstCR2, thus translating into poor signal amplification and shift in EC₅₀. Finally, looking at the non-cyclic vs cyclic form of the peptide, there is no selective activation of the receptors that might suggest a specialisation of both receptors and peptide, and despite the possibility of both forms co-existing in the organism, it is most likely that the cyclised peptide make up the prevalent bioactive physiological form.

4.5.4 Concluding remarks and future directions

We here presented the discovery of a putative endogenous allatostatin C signalling system in *O. vulgaris*. The detection of a functional neuropeptide system, known to be implicated in many biologically relevant functions, constitutes an important contribution to the understanding of the physiology of this class of molluscs in relation to their evolutionary context.

Of particular interest is the potential role this system might have in the organisation of octopus antinociceptive signalling, a pathway that has been for long neglected in invertebrates and only predicted in cephalopods, ultimately leading, following the precautionary principle, to their inclusion in UK and EU legislations that regulate their welfare when used in research (Bradshaw, 1999; European Parliament and Union, 2010; UK Statutory Instruments 2012 No. 3039, 2012). The evolutionary relationship with the vertebrate-exclusive OPRs and the recent experimental evidence of the AstC role in suppressing noxious responses in insects (Bachtel *et al.*, 2018; Liu *et al.*, 2023), make this family of receptors a good candidate for further investigation in this behavioural framework, thus contributing to our understanding of the potential molecular contributors of complex nociception in these species.

Chapter 5 General Discussion

This project focussed on finding molecular candidates potentially involved in *O. vulgaris* nociception on the premise that nociception is a conserved adaptive phenomenon essential for animal survival. The results of this research led to the investigation of the functional properties of two distinct determinants, namely TRPV channels and allatostatin C. These analyses platformed an approach documented in the three papers presented and provided a systematic pipeline and novel reagents whose utilisation is evidenced in two additional publications (see Appendix A and Appendix C).

I will here review how the findings of this thesis represent new opportunities to open a discussion around cephalopod welfare, offering potential measurable biomarkers for animal stress and substrates for investigating nociception. I will finally focus on aspects that could be further considered and should be addressed ultimately requiring us to question the sole use of our mammalian-centric view.

5.1 Understanding molecular determinants of nociception in *O. vulgaris*: progress and perspectives

5.1.1 Tissue and cellular distribution of *O. vulgaris* determinants in relation to nociception

A natural consequence of the suggested functional role of *O. vulgaris* TRPV channels in nociception is to look at their prevalent tissue distribution. The work carried out in Chapter 3 evaluated the presence of the genes of interest *Ovtrpv1* and *Ovtrpv2* within a defined range of tissues that were used as representatives of specific physiological pathways in *O. vulgaris* (Figure 3.4). Even though PCR is suggestive of a selective expression of the TRPVs in the peripheral sensory tissues (i.e., arms, tip and sucker), it is not indicative *per se* of any function within the associated tissues and cannot be used to quantify the abundance of the gene and make conclusions on whether it is more enriched in sensory tissues when compared to others.

In this regard, isolation of discrete sensory structures such as arms or suckers should be investigated with techniques that provide better quantitative and cellular resolution. While immunohistochemistry (IHC) represents a critical tool to identify the location of expressed proteins, it is still quite challenging to routinely apply in cephalopod research, as the strong divergence in protein sequence between vertebrates and molluscs requires the use of customised antibodies rather than the commercially available ones. Despite being achieved

several times with success, the costs and time required in antibody generation limits the systematic use of this experimental approach (Baden *et al.*, 2023). *In situ* hybridisation (ISH) to detect RNA levels within tissues, paired with cellular functional investigations, offers an approach that could be systematically adopted. The advances in mRNA probes and ability to multiplex multiple gene detection afford an exciting approach to detect gene expression in specific tissues that could then be correlated to their function as detectors in the octopus's arm and/or transducers within the central brain mass (Rajesh *et al.*, 2020).

Anatomical, cellular and functional investigations in *O. bimaculoides* have resolved insight into the functional relevance of the cephalopod-specific CRTs and the conserved mechanosensory receptor TRPN (van Giesen *et al.*, 2020). Directing similar approaches to investigate cellular localisation of OvTRPV1 and OvTRPV2 in relation to OvCRT and OvTRPN offer an obvious route into developing the studies of this thesis. These should include characterisation of the isolated sensory cells in terms of the target cues and the threshold of activation. This would help address the issues discussed in Chapter 3 and clarify whether the identified receptors have a main, accessory or redundant role in specific *O. vulgaris* modalities.

A similar approach could be adopted for the allatostatin C system. The analysis I carried out indicates a widespread expression of OvAstCR1, OvAstCR2 and OvAstC. This is consistent with the pleiotropic role of allatostatin C in other invertebrate species and more in general with the nature of neuropeptide signalling (Figure 4.5). However, the identification of enriched localisation of the genes of interest within specific tissues might be an indication of a modulatory role of neuropeptidergic signalling in a potential descending nociceptive pathway that is worth exploring further.

5.1.2 Molecular candidates of nociception as biomarkers of stress in octopus: a proxy to animal welfare

Cephalopods are organisms whose use in research is protected by European legislations. However, the features that have led to this protected welfare raises concerns in the context of fishery and public display in aquaria. As mentioned, there are currently no standardised regulations controlling the impact that capture and transport have on cephalopod welfare despite the increasing public demand and ensuing efforts in addressing these limitations (Pieroni *et al.*, 2022; Sykes *et al.*, 2023).

Many researchers have tried to define biomarkers that indicate stress in these organisms with a view of improving the husbandry associated with the capture and fishing methods. To do this,

haemocytes withdrawal, skin swab and collection of small tissue samples (e.g., tip of the arm, muscle) are usually followed by measurements of gene expression changes of common stress biomarkers such as glucocorticoids (e.g., cortisol, corticosterone), oxidative stress modulators (e.g., catalase and superoxide dismutase) and heat shock proteins (Sillero-Ríos *et al.*, 2018; Barragán-Méndez *et al.*, 2019; Maselli *et al.*, 2025). All these studies succeeded in identifying significant changes in the expression levels of these genes when the animals are exposed to either temperature (acclimation) or physical stress (fishing method adopted), establishing these molecules as reliable indicators of stress.

5.1.2.1 Gene expression changes as an indicator of nociceptive implications

Particularly relevant in the welfare context, real time PCR (RT-PCR) could help analyse expressional changes in the genes of interest following exposure of individual animals to a noxious challenge to identify any increase or decrease in their expression profile (Shakeeb, Varkey and Ajit, 2021).

TRPV channels represent good candidates, as TRPV1 has been previously characterised as a biomarker of oxidative stress and brain inflammation in mammals and has been associated with increased levels of cytokines and reactive oxygen species (Pinho-Ribeiro *et al.*, 2015; Marrone *et al.*, 2017). Somatostatin, the closest deuterostome orthologue of allatostatin C, has been also found to be a candidate for the modulation of the stress response. Studies conducted in murine models, showed that the intracerebroventricular administration of SST is able to increase anxiolytic- and antidepressant-like effects (Engin *et al.*, 2008). Furthermore, alterations of SSTRs expression in rat models of chronic mild stress have been reported (Faron-Górecka *et al.*, 2016). This mirrors findings in human patients diagnosed with major depressive disorders, who have been found to have significantly low levels of SST in the cerebrospinal fluid paired with an increase in cortisol that is typical of impaired hypothalamic-pituitary-adrenal axis in depressed subjects (Molchan *et al.*, 1991). Allatostatin C receptors and peptide genes, have already been used to some extent as markers of stress in bivalves, showing through RT-qPCR how immune challenges with *Vibrio harveyi*, induces upregulation of AstC and downregulation of the receptors in *Mytilus galloprovincialis* at different time points (Li *et al.*, 2021c). Similarly, an upregulation of different genes including *AstAR1* and both *AstCR1* and *AstCR2*, was found in *D. melanogaster* when challenged with bacterial strains that mimic inflammatory responses (Bachtel *et al.*, 2018).

Therefore, the further characterisation of OvTRPVs and OvAstC following noxious exposure might be facilitated by the work conducted in this thesis.

The success of this approach will depend on identifying the neuropeptides and related peptide genes that are associated with the organism response to noxious cues. This line of investigation is preceded by the peptide transcripts that are subjected to large levels of regulation following nociception triggering as exemplified by tachykinins and enkephalins.

Finally, on a larger scale, tissues of animals subjected to injuries could be analysed through RNA sequencing to reveal a wider set of genes that are potentially triggered or modulated by up- or downregulation, some of which are supported by the *in silico* and functional characterisation carried out in Chapter 2.

The absence of evidence for a classical opioid signalling system in *O. vulgaris* and the accumulating evidence that a very divergent peptide, allatostatin C, mediates suppression of other invertebrate nociceptive responses directs attention to the *O. vulgaris* specific AstC signalling system in the context of nociception and also suggests the need to extend the research into the characterisation of the cephalopod neuropeptidome. Advanced bioinformatic tools for the identification of neuropeptides such as NeuroPeptide-HMMer (Zandawala *et al.*, 2024), and experimental approaches such as mass spectrometry from octopus tissues, coupled with large scale de-orphanisation methods, would provide new insights into conserved and potentially cephalopod- or octopus- specific molecules that could then be functionally investigated (Zatylny-Gaudin *et al.*, 2016; Beets *et al.*, 2023; Thiel *et al.*, 2024).

Many studies have reported cephalopod sensitisation to molecular stimuli following injury or harsh mechanical forces with electrophysiological evidence (Crook, Hanlon and Walters, 2013; Alupay, Hadjisolomou and Crook, 2014; Crook *et al.*, 2014; Howard *et al.*, 2019). Pairing this investigation with transcriptome profiling, posing particular attention to neuropeptide changes, would be therefore interesting to explore.

The criteria to define animal capability to feel pain described in the introduction focus on the importance of the presence of analgesic pathways. However, molecules that sensitise the animal to noxious responses are equally critical for its survival as they promote protective behaviours aimed at reducing tissue damage. Peptides such as tachykinins, which also emerged from the *in silico* analysis carried out in this thesis, were found to be implicated in vertebrate and invertebrate sensitisation to noxious stimuli (Im *et al.*, 2015; Gutierrez *et al.*, 2019). Although not investigated in the context of a noxious challenge, tachykinins have been identified in *O. vulgaris* transcriptome and Southern blot analysis showed tachykinin receptors sequences in DNA

preparations from different central brain regions (Kanda *et al.*, 2007). These findings might be worth pursuing with functional and *in vivo* behavioural experiments.

5.1.3 Complementation of recombinant systems to investigate ion channels and GPCRs activation by noxious cues

The work carried out in Chapter 2 and 3 confirms *C. elegans* as a suitable candidate for model hopping from a more challenging and less experimentally established species such as *O. vulgaris*.

This approach allowed me to provide evidence consistent with functional conservation between OvTRPV and CeleTRPV proteins. However, it had limited understanding of the role of these receptors, as directly gated, mediators or transducers, within the sensory neuronal circuit *in vivo* or on the quaternary structure required to make the channel functional (homomeric or heteromeric subunits). Therefore, I adopted different experimental tools such as mammalian HEK293 cells and vertebrate *Xenopus* oocytes for expression of TRPVs to assess their response to known TRPV activators.

The aequorin-based calcium assay carried out in HEK293G5A cells has been successfully employed to de-orphanise the OvAstCRs (Chapter 4), showing this system provides a good physiological context to successfully express invertebrate GPCRs. However, although it was able to reconstitute functional responses with the human receptor HsTRPV1, it failed to resolve OvTRPV ion channels (Appendix B2). The reporting that other researchers failed to obtain a response with *osm-9* and *ocr-2* in HEK293 cells (Tobin *et al.*, 2002) but succeeded with *Xenopus* oocytes (Upadhyay *et al.*, 2016; Ohnishi *et al.*, 2020), suggests this system as more flexible for expression of invertebrate ion channels. In line with this, when *Ovtrpv1/Ovtrpv2* were co-expressed in *Xenopus* oocytes, evoked currents were recorded when exposed to NAM (Figure 3.8B). When testing cues that were causing a response in the *Ovtrpv*-expressing rescue strains, such as low pH, no response was detected even in the *Xenopus* system (data not shown), suggesting either technical limitations or a stimuli-specific direct gating and an additional action as downstream transducer for other cues.

Furthermore, the data indicate that the recombinant systems might be differently suited according to the species and the nature of the invertebrate receptor studied (ion channel vs GPCR). In principle, both systems allow the expression of the receptors of interest and provide functional characterisation through the recoding of their activation either by measuring calcium dependent luminescence or evoked electrical current. However, different limitations and

strengths depending on species- and receptor-specific contexts might require the adoption of both to reach a final answer.

To overcome the time and cost consuming cloning effort that is required when switching between the two systems, I collaborated in the optimisation of experiments that led to the development of a dual expression plasmid, pXeDNA3.1-LacZ (Guan *et al.*, 2025, see Appendix C). The construct presents both a cytomegalovirus promoter for mammalian transcription and a *Xenopus* β -globin UTR to enhance oocyte translation. This system thus effectively lends itself for expressing target receptors in both systems with a single cloning.

5.1.4 When conventional is not enough: exceptional *cis*- and *trans*-regulatory rearrangements in octopus genome as a source of novel sensory receptors

As mentioned in the General Introduction (1.5.2.3), the lack of known upstream receptors in the sensory pathway of *C. elegans* is likely due to the difficulties of identifying highly divergent proteins in a phylum in which several genes, especially those encoding for GPCRs, have been evolutionarily subjected to expansion through massive duplication events, that led to neo- or sub-functionalisation (Troemel, 1999; Bargmann, 2006; Cutter, Dey and Murray, 2009).

In a similar fashion, cephalopods, and particularly coleoids, are a taxon which underwent strong environmental pressure that led to the outstanding level of genomic modification researchers observe today relative to other invertebrate and vertebrate species (Albertin *et al.*, 2015). Interesting expansions in this phyla include the protocadherins and the C2H2 superfamily of zinc-finger transcription factors, both known to be involved in neuronal development and synaptic organisation (Wang *et al.*, 2006; Garrett and Weiner, 2009; Peek, Mah and Weiner, 2017; Al-Naama, Mackeh and Kino, 2020; LaMassa *et al.*, 2021). This molecular substrates have been central to supporting the evolution of a nervous system with this increased complexity (Albertin *et al.*, 2015; Albertin *et al.*, 2022). Additionally, expansion in large families of proteins such as GPCRs have been also previously detected with a higher frequency in tissues associated with the nervous and sensory systems (Ritschard *et al.*, 2019). Lineage-specific genes, such as reflectins (coleoid-specific proteins involved in skin iridescence) and suckerins (decapod-specific proteins expressed in the tentacles) and the already mentioned CRTs (coleoid-specific ion channels) highly expressed in the suckers have been observed and associated with sensory functions (Albertin *et al.*, 2022). These novelties and peculiarities have been correlated with interesting

genome rearrangements such as duplication from in tandem repeats but also exceptional high frequency of transposable elements activity (Albertin *et al.*, 2015).

Even more interesting is the high level of post-transcriptional modification that has been recorded in coleoids genomes. In fact, high incidence of A to I (inosine) changes found in the StG, OL, SUB and SEM, is indicative of the activity of the adenosine deaminase acting on RNA (ADAR) activity. RNA editing has been reported in other organisms, including mammals, but is typically a rare phenomenon (Kleinman, Adoue and Majewski, 2012; Zhan *et al.*, 2023). Cephalopods, conversely, seem to have sacrificed conventional DNA-level evolution (the surroundings of the regions subjected to recoding must remain conserved) in order to maintain RNA editing levels that provide transient and flexible phenotypic plasticity that ultimately guarantees useful adaptation in a pressuring environment (Liscovitch-Brauer *et al.*, 2017).

This outstanding genome remodelling might represent an additional challenge but also an invaluable opportunity to identify novel sensory receptors in *O. vulgaris* that act alone or in shared pathways with conserved conventional receptors, resulting in the sophisticated sensory responses that are exhibited by cephalopods.

5.2 How far are we from knowing whether a pain-like state can exist in cephalopods?

5.2.1 Integration of peripheral sensory signals into the central brain

Considering the criteria for eligibility to feel pain proposed by Bateson (1991) and Birch *et al.* (2021) presented in the general introduction (Table 1.2), this thesis deepens the knowledge on *O. vulgaris* nociceptive-related molecules. This was achieved through an amalgamation of different experimental approaches that allowed me to confirm that structural conservation underpins potential functional conservation too (criterion 1). This is well emphasised by the identification and follow up investigation of *Ovtrpv1* and *Ovtrpv2*. As described above, targeting those genes by analysing their tissue and cellular localisation, as well as their change through development or following stress-specific events, would be important for characterising mechanisms of cephalopod sensory detection and nociception.

Higher brain structures and their complex interactions (criterion 2) in receiving inputs and elaborating them downstream, is universally recognised by anatomical and behavioural studies. Evidence of any enrichment of the genes of interest in these regions or of alteration

of expression in these areas following noxious challenges, would suggest a complex modulatory role of nociception that is usually only associated with vertebrate organisms.

While these two criteria are necessary for the generation of pain responses, they are sufficient only when functionally organised in ascending and descending pathways that constitute a functional crosstalk (criterion 3). The evidence of neural connections between the nociceptors and the integrative higher brain centres requires investigations. Many connections were found between the arm sensory system and the central mass, but none of the available studies provide a comprehensive answer on the functional correlation between them. Ablation studies described in the general introduction (1.4.3.1), suggested a role of the inferior frontal system that is receiving direct afferent fibres from the arms (Young, 1983). However, when looked at in more detail, the gross effect of these fibres seems to be related to physical avoidance rather than in the establishment of a nociceptive association and learnt avoidance (Key and Brown, 2018). This, added to the evidence of semi-autonomous activity of the arm nervous system when the interbrachial commissure is severed (Sumbre *et al.*, 2001), can represent a challenge worth investigating. More studies, including developing appropriate fibre tracing studies (Gonzalez-Bellido and Wardill, 2012; Imperadore *et al.*, 2019), that might represent a less harsh and more controlled manipulation of the nervous system when compared to removal of large areas of brain and their connections, are needed.

5.2.2 Evidence of absence or absence of evidence? Why imposing a mammalian view is not always a good idea

5.2.2.1 Allatostatin C as a distinct molecular determinant of antinociception in invertebrates

According to Bateson, animals should be sensitive to substances that interact with opioid receptors in order to be eligible to feel pain (criterion 4). This mammalian-centric vision represents a limitation that was also adopted at the beginning of this project. However, while it is reasonable to assume that an antinociceptive system exists in *O. vulgaris*, the *in silico* analysis I carried out, as well as evidence from other invertebrates, suggest it is not to be found in a conserved opioid system but rather in a pharmacologically distinct but evolutionarily related one, with evidence highlighting a role for allatostatin C.

While genetic variations across species have translated into a structural evolution of receptor-peptide interactions (Kreienkamp *et al.*, 2002), allatostatin C was found to have a role in counteracting noxious responses in invertebrates. In fact, an upregulation of five

genes, *trpa1*, *ppk* (DEG/ENaC), *AstAR1*, and both *AstCR1* and *AstCR2*, was found in *D. melanogaster* when immune challenged with bacterial strains (Bachtel *et al.*, 2018). Using a heat escape assay and a withdrawal latency assay to measure hypoalgesia and hyperalgesia respectively, it was revealed that *trpa1* null mutants displayed hypoalgesia whereas *AstCR1* and *AstCR2* mutants display hyperalgesia, thus suggesting AstC is implicated in nociception reduction (Bachtel *et al.*, 2018). Consistent with this study, a novel thorough investigation of the heat response in the fruit fly has led to the identification of two neurons, the Epi neurons which express the *trpa1* channel found to be involved in the direct detection of heat (Liu *et al.*, 2023). More interestingly, the study showed that these neurons release AstC which then acts downstream to suppress the jump response in flies exposed to 45 °C on a hotplate (Liu *et al.*, 2023).

An allatostatin C receptor, *npr-17* has been identified to have a role in reducing *C. elegans* response to aversive volatile cues. The work reported that morphine and salvinorin A, two strong opioid receptor agonists, were able to produce a serotonin-dependent initiation of aversive responses which however was quickly replaced by forward movements, suggesting a suppression of nociception (Mills, 2014; Mills *et al.*, 2016). This phenotype is lost in *npr-17* null mutants but is rescued by the heterologous expression of *H. sapiens* kappa opioid receptor. As shown by the phylogenetic analysis carried out in this thesis (Figure 4.3) and supported by recent large scale de-orphanisation of *C. elegans* GPCRs (Beets *et al.*, 2023), *C. elegans* represents a peculiar example of one-to-many orthologues when it comes to AstCRs, with a dozen identified receptors and plenty of putative peptides found (Golinelli *et al.*, 2024). Its genetic robustness and straightforward behavioural readout could be used to quantify changes in worms' threshold of responsiveness to noxious cues with a similar approach utilised for *D. melanogaster* jump behaviour with the hotplate (Liu *et al.*, 2023). It would be interesting to investigate whether this function is redundantly found in other CeleAstCRs or if these receptors are part of a neo or sub-functionalisation in the worm and whether *O. vulgaris* AstCRs can rescue the *npr-17* mutant phenotype as in the case of the human opioid receptors.

These studies on well-characterised model organisms, confirm the existence of an antinociceptive system in invertebrates without the need of a classical opioid system but through an orthologue receptor and a peptide with a very divergent sequence.

Altogether, applying the structural conservation, the evolutionary relationship with signalling systems that conveys antinociceptive responses (OPRs and SSRs), and the functional studies demonstrating a role in nociception suppression carried out in other invertebrates, I

would prioritise the AstC signalling system as a potential candidate for antinociception in cephalopods.

5.2.2.2 Does peptide sequence similarity equal function similarity? FMRFa as an opioid-like candidate

Another strategy applied when the identification of known vertebrate neuropeptides is missing in invertebrate genomes, is to look at peptide residues that might have some similarities and conservation. This is the case of the peptide FMRFamide. FMRFa (sequence: Phe-Met-Arg-Phe) has been originally tested as a good opioid-like candidate based on its partial sequence similarity with vertebrate met-enkephalin (Tyr-Gly-Gly-Phe-Met-Arg-Phe). However, despite some suggestions that opioids and FMRFamide-related neuropeptides originate from the same ancestral gene (Taussig and Scheller, 1986), when FMRFa were tested on human opioid receptors, very low affinity was reported, and when tested on mollusc their action was not counteracted by naloxone, suggesting convergent evolution rather than common origin (Greenberg *et al.*, 1983). Discovered for the first time in the bivalve *Macrocallista nimbosa* (Price and Greenberg, 1977), FMRFa led to the identification of numerous RFamide peptides that play an important role in molluscan physiology. These peptides have been identified as regulators of the animal cardiac activity and enzyme regulation and only one of them has been related to nociception in *Aplysia*, where a suppression of central and peripheral responses has been reported following exposure to noxious stimuli (Belardetti, Kandel and Siegelbaum, 1987; Mackey *et al.*, 1987). A few representatives from FMRFa and their cognate receptors have been identified in *O. vulgaris* transcriptome in this thesis (Table 2.1). Tissue localisation, gene expression changes in response to noxious cues as well as whole animal experiments could contribute to test their potential contribution in facilitating or counteracting nociception.

5.2.2.3 TRPV receptors as precursors of the endocannabinoid system?

Endocannabinoid receptors were also among the nociceptive gene candidates not identified in my investigation of *O. vulgaris* transcriptome. Even though enzymes involved in the maturation and catabolism of cannabinoid precursors (e.g., AEA, 2-Arachidonoylglycerol, 2-AG) were identified, this does not represent *per se* evidence of the presence of an endocannabinoid signalling as these enzymes act on multiple physiological pathways. It is however a good indication that a conserved regulation of lipids operates in *O. vulgaris*.

Fatty acids are known to play important functional roles in animal physiology, and evidence from both vertebrates and invertebrates studies indicate they might act to modulate nociception. An example of this is again the TRPV channel.

In mammals, activation of TRPV1 in sensory neurons mediates nociception and contributes to the ascending pathway resulting in pain. However, TRPV1 is also expressed in central brain regions such as the periaqueductal gray and the rostral ventral medulla that mediate a descending antinociception pathways (Louis-Gray, Tupal and Premkumar, 2022). This latter effect seems to be the result of an AEA-mediated interaction with the cannabinoid receptors (CBRs) at the spinal level (Horvath *et al.*, 2008; Starowicz *et al.*, 2012).

In invertebrates, endocannabinoids such as 2-AG have been shown to suppress the electrical activity of leeches' noxious responsive N-cells (Higgins *et al.*, 2013). These effects were also supported by behavioural evidence showing how AEA and 2-AG reduced nocifensive responses when the animal was exposed to noxious chemical (Summers *et al.*, 2017). Again, the effects were demonstrated to involve the TRPV receptor, as its pharmacological inhibition blocked the behavioural outcome. Whether lipids act directly on the TRPV receptor or this acts as a transducer within the pathway is unknown but represents an interesting point to investigate.

Similar results were found in *D. melanogaster* where CBRs are absent, but AEA and 2-AG seem to act on the TRPV and TRPA channels (Jacobs and Sehgal, 2020).

These findings may suggest that while CBRs could be absent in invertebrates, endocannabinoid metabolism is present and sustained by the signalling triggered by TRPVs. In addition, this could suggest that the endocannabinoid system originally exerted its functions through TRPs and raises the possibility that early lipid signalling evolved distinct receptor classes, such as CBRs in vertebrates.

In this thesis, I have functionally characterised two TRPV channels in *O. vulgaris* as polymodal receptors. However, this does not preclude a wider role as interactors or modulators of nociceptive responses and it raises the interest in functionally investigate the role of fatty acid metabolism.

5.2.2.4 Can AI-based tools facilitate candidate gene search and functional characterisation?

This work was carried out with the use of pre-existing bioinformatic tools and automated predictions that although constituting a critical starting point, required manual curation and experimental validation (*Ovtrpv1* and *Ovtrpv2* in Chapter 3 and *OvAstCR2* in Chapter 4). The advent of artificial intelligence (AI) and machine learning is however bringing those computer-based models and prediction a step further, as new approaches for classifying genomic DNA, and therefore identifying intron-exon boundaries are emerging (Chandrashekar *et al.*, 2024;

Sharif *et al.*, 2025). It is therefore expected that these advanced techniques will definitely facilitate drafting previous and new genome assemblies with an unprecedented level of accuracy, thus improving the quality of the current datasets and of the derived searches.

As a consequence, it is reasonable to think that genes and derived protein sequences that are currently challenging to identify with high accuracy, such as neuropeptides, will become more accessible and less influenced by the background noise previously originated by their short length (Rahmani, Kalankesh and Ferdousi, 2025).

At the protein structural level, many tools have been improved or developed by integrating deep learning methods to predict secondary structure (Hallgren *et al.*, 2022) but also 3D and quaternary assembly of protein complexes (Evans *et al.*, 2022; Abramson *et al.*, 2024).

These not only grant good insight into the amino acid interaction and the overall conformation of the protein, but are now critical to locate potential binding sites and to predict interactions with natural or appropriately designed synthetic ligands. This hugely helps in performing *in silico* screening of pharmacological compounds or in simulating interactions between orphan receptors and potential ligand or between two proteins, thus reducing experiment-related cost and time (Ferguson *et al.*, 2025).

Altogether, AI methodologies can indeed improve the *in silico* aspect of this and future research at each step of the analysis: speed up the identification of complete transcript from genome assembly and facilitate protein prediction at the structural level, offering good insight into their interactions with the external or internal organism environment.

5.2.3 Behavioural criteria

The identification, experimental validation and functional characterisation of TRPVs and AstC receptors and peptide in *O. vulgaris*, represent a good opportunity to now investigate the remaining criteria (criteria 5-8) which are based on behavioural evidence of higher cognitive features, such as flexible self-protected behaviours, trade-off and associative learning (Table 1.2).

The detection of increasing or decreasing levels of the target genes following RT-PCR will determine if they are implicated in the noxious response, but will not provide information on whether they are involved in sensitisation or suppression of nociception transmission.

This will therefore ultimately require observation of the behaviour elicited by the octopus when presented with different noxious cues. Understanding the behavioural changes in relation to administration of agonists or antagonists of the target receptors, would give a

direct empirical measure of the specific or redundant role of those gene candidates in facilitating or counteracting nociception. Therefore having identified some of the targets, provides a platform that could be pharmacologically manipulated to characterise the gene's role when the animals are injured or stressed. In this regard, observation of flexible self-protective behaviours (e.g., grooming, rubbing, concealing of the affected body part) following administration of aversive cues, is indicative not just of a reflexive response but also of a discriminative component of pain described in the general introduction (Crook, 2021). Furthermore, experiments such as the conditioned place preference described in the general introduction (1.4.4), could be modified using agonists of AstC, rather than vertebrate local anaesthetics or opioids, to analyse whether a positive association with the substance is established following the exposure to noxious stimuli, thus speculating also on the existence of an affective component of pain.

5.2.4 Concluding remarks

This thesis identified candidate conserved molecules that have been filtered to highlight potential roles in organising core nociception in *O. vulgaris*. However, a cautious approach should be adopted when using evolutionary arguments about function with organisms that have undergone exceptional environmentally driven evolutionary changes. I acted against this by investigating functional ability of candidate genes. My data strongly support the supposition that OvTRPV receptors are a conserved family that will directly act in nociception. Although good arguments can be made for the AstCRs and AstC, the evolutionary relationship with OPRs and SSR alone and the limited tissue specificity, temper the conviction. However, with this reservation one also recognises that applying a vertebrate or mammalian-biased view, could really limit the investigation of novel or distinct players acting on invertebrate sensory detection. The outcome of this thesis and the future experimental directions proposed with respect to the criteria for eligibility of pain in animals previously described, are summarised in Table 5.1.

Rigorous genetic and functional characterisation of *O. vulgaris* nociceptive related genes, the application of a model hopping approach utilising *C. elegans* and the development of successful culturing solutions that allow direct genetic tractability in these species, all contribute to the next steps in tackling the question of pain in cephalopods.

Table 5.1 Summary of Bateson's and Birch and colleagues' criteria for assessing pain in cephalopods analysed following contribution of this thesis and future work proposed

Criterion (Bateson, 1991 + Birch et al., 2021)	Comments based on this thesis's results	Future work proposed
Possession of receptors sensitive to noxious stimuli (nociceptors) found in peripheral areas of the body	<i>In silico</i> analysis revealed the presence of 51 orthologues of nociceptive related genes in <i>O. vulgaris</i>, including <i>Ovtrpvs</i>; - Functional characterisation in nociception through model hopping in <i>C. elegans</i> revealed 19 potential candidates - Endpoint PCR suggests presence of <i>Ovtrpvs</i> in sensory-related tissues such as suckers and arms - Cell assay showed direct or indirect activation of <i>Ovtrpvs</i> by repulsive compounds	ISH and qPCR are required to effectively localise the genes and understand their relative enrichment in peripheral tissues (suckers, tip of the arm)
Possession of integrative brain regions capable of integrating information from different sensory sources	Endpoint PCR showing expression of <i>Ovtrpvs</i> and <i>OvAstCRs</i> and <i>OvAstC</i> in the octopus central brain mass could indicate a more complex modulatory role of these molecules.	RNAseq and RT-PCR can help identifying changes in genes expression following noxious exposure and can help identify novel potential stress biomarkers
Possession of neural pathways connecting the nociceptors to the integrative brain regions	-	Requires modern and optimised methods of mapping and tracing peripheral nervous fibres to their brain areas
a. Possession of an endogenous neurotransmitter system that reduces nociceptive responses b. Putative local anaesthetics/analgesics/anxiolytics/ anti-depressants reduce nociceptive responses a. Self-administration of putative analgesics/ anaesthetics when injured. b. Conditioned place preference to a location primed with analgesics/ anaesthetics, when injured. c. Prioritisation of obtaining these compounds over other needs when injured	- No opioid or endocannabinoid receptors or opioid peptides have been found with <i>in silico</i> analysis - Identification of a receptor evolutionary related to the OPRs, AstCR and a divergent peptide AstC - Other potential molecules involved in antinociception suggested: FMRFamide, TRPV	- Expressional changes (RT-PCR, RNAseq), - Rescue experiments using model organisms (e.g., <i>C. elegans</i> AstCRs mutant strains) - Behavioural experiments such as observation of flexible self-directed behaviours or development of positive association with allatostatin C agonists or change in threshold of nociceptive activation following a noxious challenge could help identifying the direction of their modulatory central role
Flexible self-protective behaviour (e.g. wound tending, guarding, grooming, rubbing) of a type likely to involve representing the bodily location of a noxious stimulus		
The disvalue of a noxious or threatening stimulus is traded off against the value of an opportunity for reward, leading to flexible decision-making		
Associative learning (pairing of a neutral stimulus with a noxious cue) produces novel ways of avoiding noxious stimuli Note: habituation and sensitisation are not sufficient to meet this criterion		

Appendix A Sensory and developmental phenotyping of *C. elegans* parses autism associated genes into behavioural classifications

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A.1 Abstract

Global sensory processing changes in individuals with autism spectrum disorder (ASD) has been a growing area of study in recent years. Emerging literature suggests there is value in grouping ASD cohorts phenotypically and then investigating the genetic basis for such groupings. Limited standardised sensory testing in human cohorts has limited the input of sensory phenotypes into the emerging literature of sub-categorising ASD cohorts. *C. elegans* have robust readouts for developmental and sensory phenotypes, allowing these behaviours to be systematically tested and compared. A large subset of ASD associated genes, almost 50% of the highest confidence risk genes listed on the Simons Foundation Autism Research Institute database, are epigenetic modifiers.

52 epigenetic modifiers (65 strains) were selected for study in *C. elegans* based on gene function, presence of orthologues in *C. elegans* and the availability of viable putative null strains. Experimental investigation highlighted significant changes to reproduction, gross development and sensory processing across the range of epigenetic modifiers assayed. Hyper and hyposensitivity to stimuli was observed mirroring similar phenotypes seen in human ASD cohorts. When analysing each strain for their selective phenotypic profiles three primary groups emerge, those with sensory perturbations but unaffected gross development (6), developmentally affected genes with intact sensory function (6) and finally genes with impaired gross development and sensory function (7). Thus, this study provides a link between sensory and developmental outcomes in ASD associated mutant strains and suggests that more regular sensory testing should be performed in human cohorts to further refine sub-categorisation of ASD cohorts.

A.2 Introduction

Autism spectrum disorder (ASD) is a pervasive developmental disorder clinically identified by two behavioural features encompassing social communication/interaction and restricted/repetitive patterns of behaviour (American Psychiatric Association, 2013). These behavioural traits manifest during early development and then persist with varying levels of severity (American Psychiatric Association, 2013). Evidence for the perturbation of sensory processing in ASD cohorts has been observed across multiple modalities (Balasco, Provenzano and Bozzi, 2019). Typically, these sensory studies are performed on ASD cohorts with unknown genetic backgrounds and focus primarily on tactile cues with few studies focussing on other modalities (Balasco, Provenzano and Bozzi, 2019). Sensory testing ranges from parent's self-reporting behaviours to matched cohort sensory testing in laboratory conditions (Balasco, Provenzano and

Bozzi, 2019). All these studies have contributed to define clear diagnostic criteria but are insufficient to confidently assign phenotypic outcomes to specific genetic perturbations.

The genetic architecture of ASD is becoming better understood, and the increasing quantitative nature of these investigations opens a platform to better resolve the gap in our knowledge. An abundance of genetic determinants has been discovered through Genome wide association studies (GWAS; Grove *et al.*, 2019), Whole exome sequencing (WES; Yu *et al.*, 2013) and twin studies (Tick *et al.*, 2016). Identified genetic risk factors are catalogued and ranked on the Simons Foundation Autism Research Initiative (SFARI) gene database with over 1200 (Abrahams *et al.*, 2013, last accessed Jan 2026) genes implicated in the aetiology of the disorder. The genetic architecture contains multiple genetic risk factors of different severities including copy number variants (CNVs), *de novo* loss of function (dnLoF) mutations, missense mutations and common variants (i.e., single nucleotide polymorphisms, SNPs; Choi and An, 2021). With heritability rates ranging from 75%-95% (Colvert *et al.*, 2015), the contribution of genetic mutation to the aetiology of ASD is dominant but is unable to provide a wholistic explanation of the disorder.

Despite the strong influence of genetic determinants there is some evidence that environmental factors such as maternal age, viral infection and exposure to heavy metals have all been shown to have a positive association with autism (Karimi *et al.*, 2017). This is likely complicated by the now established environment as a secondary cause that is mediated by secondary regulation of the genome. The most likely candidate for environmental-genome interactions is through epigenetic modifiers which are found abundantly in the SFARI gene database (Abrahams *et al.*, 2013). This makes these genes particularly interesting to study in the aetiology of ASD as they modulate the environmental impact and have broad transcriptional regulation consistent with context dependent outputs and are likely to underpin the spectrum observed in ASD.

The behavioural outcomes and the variance in genetic underpinnings are reflected in the complexity and spectrum of severities that fall under the autism diagnosis criteria and raises the possibility that despite being complex, the intersection between genetics, development and environment will enable classifications that parse the autism diagnosis into distinct sub-cohorts. A recent study has validated that ASD cohorts can be statistically parsed by combining phenotypic presentation of clinical symptoms including developmental milestones, repetitive behaviours, social communication and child behaviour, and associated genetic signatures (Litman *et al.*, 2025). The four key groups were sub-categorised as moderate challenges, broadly affected, social and/or behavioural and mixed ASD with developmental disorder (DD). Findings from another study seeking to investigate possible subgroupings of Autism cohorts found three subgroups of autistic individuals (Leyhausen *et al.*, 2026). These results were correlated based on imaging phenotypes and transcriptomic profiles (Leyhausen *et al.*, 2026). This approach

yielded the result of mapping transcriptomic profiles to neuroanatomical changes which give rise to different clinical measures (Leyhausen *et al.*, 2026).

While helping to drive clearer clinical classification with obvious values, these studies are less precise in explaining the driving mechanisms. Interestingly they also are built without clear use of the association of neuroatypical sensory sensitivity. Hyper or hyporeactivity to sensory input commonly observed in individuals with ASD, originates in altered neural pathways (Palau-Baduell *et al.*, 2012), atypical sensory modulation (Giacardy *et al.*, 2018) and sensory gating dysfunction (Chien, Hsieh and Gau, 2019). A definitive explanation for sensory perturbations has yet to be uncovered but has excellent potential to refine the emerging cohorts.

Based on this background we wanted to use *C. elegans* to investigate the intersection between environmental and genetic determinants that impact on sensory signalling that might underpin facets of autism. *C. elegans* has the advantage of genetic tractability along with a diverse range of quantitative assays available in which the environmental cues that drive sensory sub-behaviours can be quantified. The cost-effective nature of *C. elegans* provides a suitable model for large scale screening of behavioural phenotypes (García-Garvía and Sánchez-Salmerón, 2025). Previous studies have identified ASD-like phenotypes in mutants of ASD associated genes with diverse functions ranging from synaptic signalling to metabolic pathways (Wong *et al.*, 2019; McDiarmid *et al.*, 2020; Rawsthorne *et al.*, 2021). Developmental delays have been shown to be linked to greater severity of other ASD behaviours in human cohorts (Shan *et al.*, 2021) making an analysis of both developmental and sensory behaviours in *C. elegans* worthwhile to determine whether developmental delays manifest as sensory processing deficits.

Here we use quantitative measures of *C. elegans* development and sensory responses across autism associated gene expression regulators. This was designed to directly profile associated gene function in mutants that likely express a pleiotropic shift in gene expression. Quantifying outcomes across strains with an understanding of developmental profile and sensory responses in the mature nervous system allowed us to develop comparative criteria. This led to the development of a pipeline that readily segregated major sub-categorisations. These were “sensory”, “developmental and reproduction”, “development and reproduction and sensory”. This conclusion is consistent with the emerging evidence base that supports the prevalent perception that there is no single cause. Interestingly the study highlights that quantitative measures of sensory responses and associated phenotypes can facilitate useful subclassification of criteria that model key developmental and sensory phenotypes associated with autism related genes. Given the value of driving the analysis through sensory assays, *C. elegans* promotes better collation of such measures in human cohorts. In addition, although

more restricted, the identification of distinct genes promotes mechanistic investigation of the underlying molecular pathways.

This outcome will hopefully platform quantitative investigation of gene expression to better define if distinct molecular changes converge on discrete behavioural sub-categories that may inform clinical investigation of ASD.

A.3 Materials and Methods

A.3.1 *C. elegans* strains and culturing

The N2 (Bristol Strain) was obtained from the *Caenorhabditis* Genetics Center (CGC, University of Minnesota, USA. Funded by NIH Office of Research Infrastructure Programs (P40 OD010440)). The remaining strains were obtained from the CGC and National Bioresource Project and made by S. Mitani (NBRP; Yamazaki *et al.*, 2010). All strains were cultured at 20 °C on standard nematode growth medium (NGM). The worms were maintained on *Escherichia coli* strain OP50 (Brenner, 1974).

A.3.2 Bioinformatics

Category 1 and category S genes listed on SFARI (Accessed, Jan 2026, Ver 2025 Q4, <https://gene.sfari.org/>), were the basis for candidate gene selection. Gene expression modifiers were identified by review of the literature using the descriptor ‘gene expression modifying activity’ as the criteria to functionally annotate the genes selected from the SFARI database. The key GO descriptors were ‘transcriptional regulation’, ‘translational regulation’ and ‘regulation of gene expression’. Ortholist2 (<http://ortholist.shaye-lab.org/>) and STRING (<https://string-db.org/>) were used to determine whether *C. elegans* contains an orthologue of the gene expression modifiers identified in the previous step (Kim *et al.*, 2018; Szklarczyk *et al.*, 2023).

Viable mutants were identified using Wormbase (Version: WS297; https://wormbase.org/species/c_elegans#401--10) which contains JBrowse2 for strain identification and reported phenotypic information on available strains such as the effect of the mutation allowing for those with a high variant effect predictor score (VEP). This enabled exclusion criteria such as sterility or lethality to be applied to each candidate gene strain. Strains identified were purchased from the CGC or NBRP. Additional information on human expression of candidate genes was collated using Bgee (<https://www.bgee.org/>) (Bastian *et al.*, 2021) and *C. elegans* transcript number data using CeNGEN (<https://cengen.shinyapps.io/CengenApp/>) (Taylor *et al.*, 2021). Sequence alignments of amino acids for identity and similarity comparison

of orthologues was performed with EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) (Madeira *et al.*, 2024).

A.3.3 Egg Laying and Development Analysis

A synchronised population of hermaphrodite worms was generated by allowing 3 L4+1-day (reproductive young adults) worms to lay eggs for 3 hours before removal. This was then converted to an egg laying rate by dividing by the number of worms and time allowed for egg laying. Developmental analysis was performed using the same plates with which egg laying was recorded by allowing the eggs to develop in culture incubated at 20°C. At set time points, 0 hours, 20 hours, 25 hours, 43 hours, 48 hours and 67 hours the plates were observed under a stereo microscope (Nikon SMZ800N) to count the number and developmental stage of the worms. These stages are defined as either eggs, L1 or L2 for early development and L3, L4 and young adults for late development as described (Thummel, 2001). All strains were compared to N2 and the distribution and variation of the distinct stages used to determine the developmental timings. Additionally, *che-3 (e1124)* was included to benchmark sensory deficits on development in *C. elegans*. Strains were rank ordered for developmental delays vs N2 and a correlation of early and late developmental rank orders for each strain was performed. Experimenters were blind to the strains being investigated during the assay and statistical analysis.

A.3.4 Confocal Microscopy and Dil Labelling

A synchronised population of L4+1-day (72 hours old) worms were washed once with 1 ml M9 before being resuspended in 1ml Dil in M9 (2mg/ml Invitrogen, Catalogue: D282) and left on a shaker for 3 hours at room temperature. Worms were washed 3 times post staining and re-plated onto an OP50 seeded NGM plate for 15 minutes prior to imaging. Individual worms were picked into single drops of 10mM sodium azide (CAS 26628-22-8, Sigma-Aldrich) solution on microscope slides containing 1% agar pads to paralyse them for imaging (Schultz and Gumienny, 2012). Worms were then imaged using a Leica DMI8-CS microscope with excitatory laser DPSS 561 nm and collection of emitted light at 565 nm – 660 nm. Following staining, sensory amphids ASK, ADL, ASI, AWB, ASH and ASJ and phasmids PHA and PHB were identified using their anatomical location and morphology (Andrews *et al.*, 2025). The above protocol successfully stained the 6 amphids and 2 phasmids in all N2 worms. N2 staining was run in parallel to the experimental strains and was used as a reference to assess the number and morphology of their sensory neurons. Strains were deemed to be significantly different if at least one amphid or phasmid did not label. Experimenters were blind to the strains being investigated during the assay and image analysis.

A.3.5 Quadrant Assay

The arena consisted of a 6cm petri dish with 13ml NGM. On the plastic base of the dish the plate was split into 4 equal sized quadrants. A dot was placed in each quadrant 2 cm from the intersect point of the quadrant lines. An immobile worm zone was also outlined at 0.5 cm from the intersect point in all directions. The quadrants were split into 2 test and 2 control diagonally mirrored. On the day of the assay, 1 μ l 0.5 M sodium azide was pipetted on the dot in each quadrant first and allowed to dry. This was followed by either 1 μ l M9 in the control quadrants or 1 μ l 1% v/v diacetyl (Sigma-Aldrich, Catalogue: 8.03528) in M9. A synchronised population of worms was generated by allowing 7 L4+1-day worms to lay eggs for 4 hours. The population of L4+1-day worms generated were washed twice using 1ml M9 solution before being transferred to the assay plate with a glass capillary onto the intersect point of the quadrant in $<15 \mu$ l M9. The assay plates were then left for 50 minutes and scored based on the number of worms in each quadrant. A chemotaxis coefficient was obtained by taking the differential of worms on the test vs control quadrants divided by the total number of worms in the assay. Worms still within 0.5 cm of the start point were counted as non-moving and the percentage of non-moving worms per mutant was recorded and compared to N2. Experimenters were blind to the strains being investigated during the assay and statistical analysis.

A.3.6 Droplet Avoidance Assay

We adapted the classical acute aversion assay first described by (Hilliard, Bargmann and Bazzicalupo, 2002; Hilliard *et al.*, 2004). 9 cm plates were poured with NGM (20ml) three days prior to the assay. 7 L4+1day old worms were picked onto an unseeded NGM plate and allowed to roam for 30 seconds to remove residual bacteria. These worms were then transferred onto a fresh unseeded 9 cm NGM plate and left for at least 20 minutes to allow the transition from local area search to escape behaviour (Gray, Hill and Bargmann, 2005). One drop ($<20 \mu$ l) of the indicated aversive cue was placed on the trajectory of a forward moving worm. Soluble aversive cues used in this study included copper sulphate (CuSO_4 , 30mM), M9 (pH 3.0), and fructose (4M) in water and were controlled against M9 (pH 7.0). The volatile aversive cue used was 30% v/v 1-octanol in ethanol and presented on a platinum wire in the trajectory of a forward moving worm.

The performance of the selected autism associated gene mutant strains was recorded and compared to the N2 and a classically defined sensory mutant *che-3 (e1124)* (sensory deficient control). *che-3 (e1124)* lacks all sensory amphids and phasmids (Wicks *et al.*, 2000). Video recordings were made of the worm's response to the sensory cue and analysed *a posteriori*. For soluble aversive cues (water soluble cues), the total number of reversals initiated within 5 seconds of compound exposure was counted. For the volatile aversive cue 1-octanol (30% v/v),

the latency from cue introduction to initiating a reversal was recorded. Experimenters were blind to the strains being investigated during the assay and video analysis.

A.3.7 Significance Heatmap

Data from statistical tests of each ASD associated mutant in all assays conducted were compiled. Statistically determined p values for each strain, from post-hoc multiple comparison tests, in every condition (excluding Dil labelling) were compiled into a single database. From this dataset each p value was converted into a binary value of 1 or 0 for significant ($p < 0.05$) or non-significant ($p > 0.05$) respectively. These binary values were then plotted to show each individual strains significant perturbations in each assay compared to N2.

A.3.8 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 10.4.2. All data expressed as mean $\pm$ SEM. Statistical tests and post-hoc analysis are outlined in figure legends. Significance defined at $p < 0.05$.

A.4 Results

A.4.1 A bioinformatic pipeline for selection of ASD associated gene expression modifiers: comparative phenotypic analysis in *C. elegans*

The SFARI database was used and those listed as category 1 genes were selected for the current pipeline. Our intention was to bias the investigation to select for genes that encode modulators of gene expression.

Of the 232 category 1 genes, 111 (47.8%), met the indicated definition of being gene expression modifiers (See Methods). Orthologues were identified using Ortholist 2 (Kim *et al.*, 2018), STRING (Szklarczyk *et al.*, 2023). Further criteria refined the candidate gene pool to those with an orthologue in *C. elegans* (Figure A.1A, 76 genes).

C. elegans strains with putative null mutations in orthologous genes were identified (Supplementary Table SA.1). Mutant alleles were either large deletions or point mutations with a high variant effect predictor (VEP) score (Szklarczyk *et al.*, 2023) indicating the probable loss of protein function by instability or nonsense mediated decay. Mutant strains for 52 (68.4%) of the 76 orthologous genes identified were selected for further downstream analysis. Whilst mutant strains were available for the other 24 orthologous genes, they were not selected based on the

mutation generating lethal or sterile strains being unsuitable for our downstream phenotypic analysis. In the case of some genes, up to two strains with distinct alleles were analysed.

Each gene was assigned based on molecular function identified from primary literature searches for the human orthologue. The molecular function of the 52 candidate genes can be classified into two primary groups; chromatin remodelers and transcription factors which make up 40% and 35% of the total respectively (Figure A.1B). RNA binding proteins and transcription co-factors contribute 13% and 8% of the total. Finally, a single phosphatase and kinase emerged through the selection pipeline described above. These were included in this analysis despite not directly driving transcription or translation functions due to phosphorylating or dephosphorylating gene expression modifiers (Atas-Ozcan *et al.*, 2021; Smolen *et al.*, 2023).

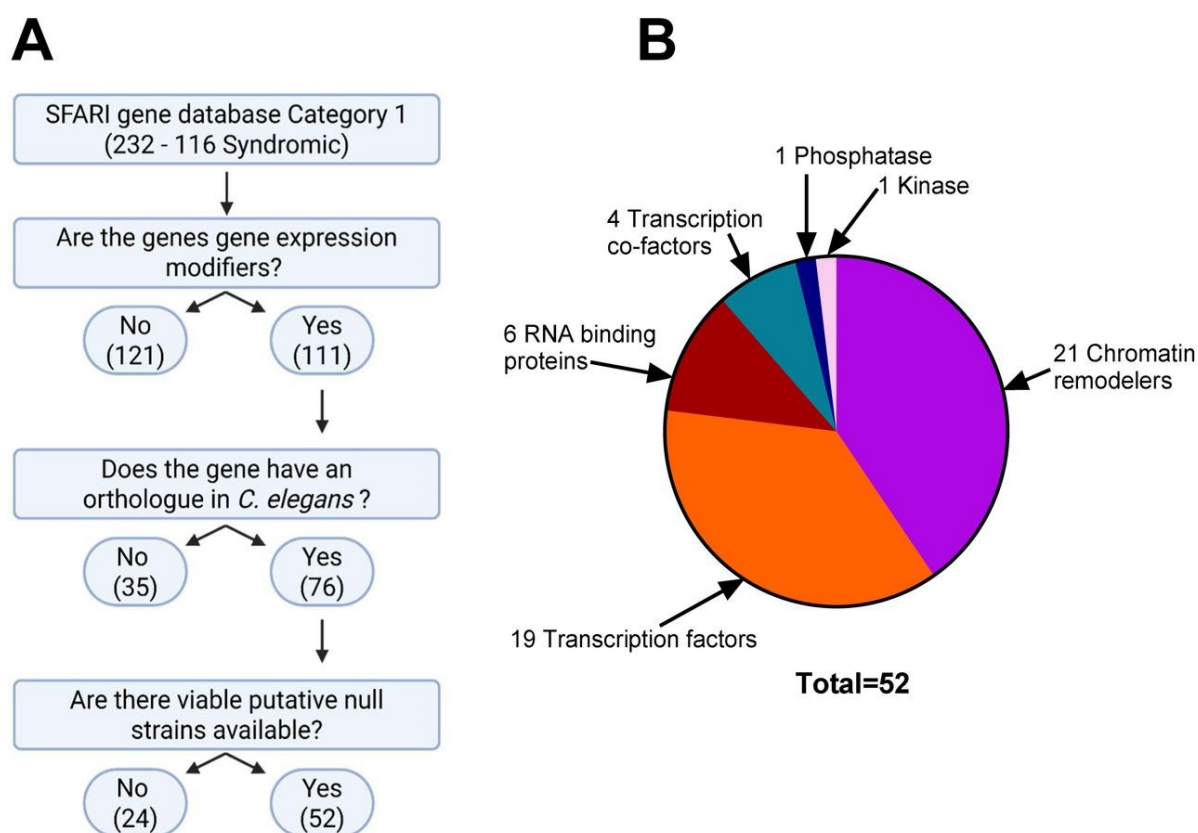


Figure A.1 Schematic process and diagrammatic outcome of selected ASD-linked gene expression modifiers conserved in *C. elegans*.

A Schematic diagram showing the bioinformatic filtering process of ASD related gene expression modifiers found in the SFARI gene database. Numbers in brackets are the number of genes for each group. **B:** The breakdown of the 52 candidate genes based on their molecular function. Includes: chromatin remodelers, RNA binding proteins, transcription co-factors, phosphatase, kinase, and transcription factors. Molecular function was defined by primary literature search or gene ontology (GO) with additional verification using available structural motif analysis.

A.4.2 Experimental pipeline for the phenotypic analysis of selected *C. elegans* mutants

A combination of developmental, anatomical and sensory driven behavioural assays were conducted on the selected *C. elegans* strains (Figure A.2). These were based on robust assays which have already been validated for measuring either sensory or developmental phenotypes (Hilliard, Bargmann and Bazzicalupo, 2002; Bargmann, 2006; Altun and Hall, 2009). Sensory assays were performed using the aversive cues low pH, fructose, copper sulphate and octanol, whilst diacetyl was used as an attractive cue. These are established sensory cues known to drive behavioural responses in *C. elegans* that can be readily quantified.

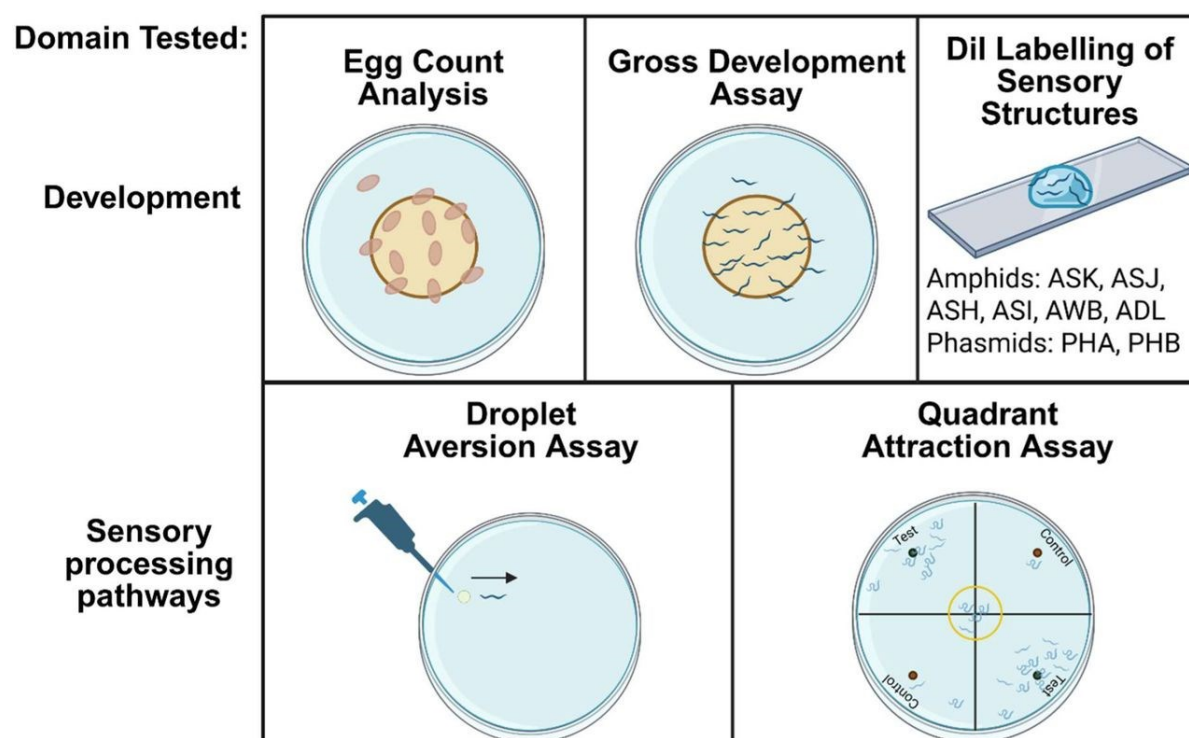


Figure A.2 Assay schematics for interrogating the developmental and chemosensory capabilities of *C. elegans*.

Assay schematics of assays used within this pipeline to interrogate the developmental and chemosensory phenotypes of *C. elegans* strains with ASD associated mutations. For a readout of reproduction an egg count analysis was used. For determining developmental capabilities a gross developmental assay and Dil labelling of sensory amphids and phasmids were used. For determining sensory capabilities, a droplet aversion assay (aversive chemosensory) and quadrant attraction assay (attractive chemosensory) were used.

A.4.3 ASD associated mutants show significant disparate effects on egg laying

28 mutants (43%) spanning all the functional subgroupings had a significantly lower egg laying rate than N2 (Figure A.3). The lowest egg laying rate was observed for the mutant *egl-27(ok1670)*, a phenotype that has been previously described (Trent, Tsung and Horvitz, 1983). Only one mutant, *baz-2(tm235)* encoding a chromatin remodeller had a significantly higher egg laying rate compared to the N2. Interestingly, chromatin remodellers were the least affected group with only 6 strains (19.4%) showing a significant difference compared to N2. By contrast, 57.9% of

transcription factors and 85.7% of RNA binding proteins. This assay can therefore highlight molecular functional specific phenotypes based on the integrative behaviour of egg laying, suggesting dysregulation of reproductive systems is more likely to have a negative impact on fecundity. Additionally, although sensory processing including detection of food cues impact this behaviour, this reduction in egg laying is not easily explained by perturbations in sensory processing. This is evidenced by the fact that the chemosensory deficient mutant *che-3 (e1124)* egg laying rate was not significantly reduced (Figure A.3).

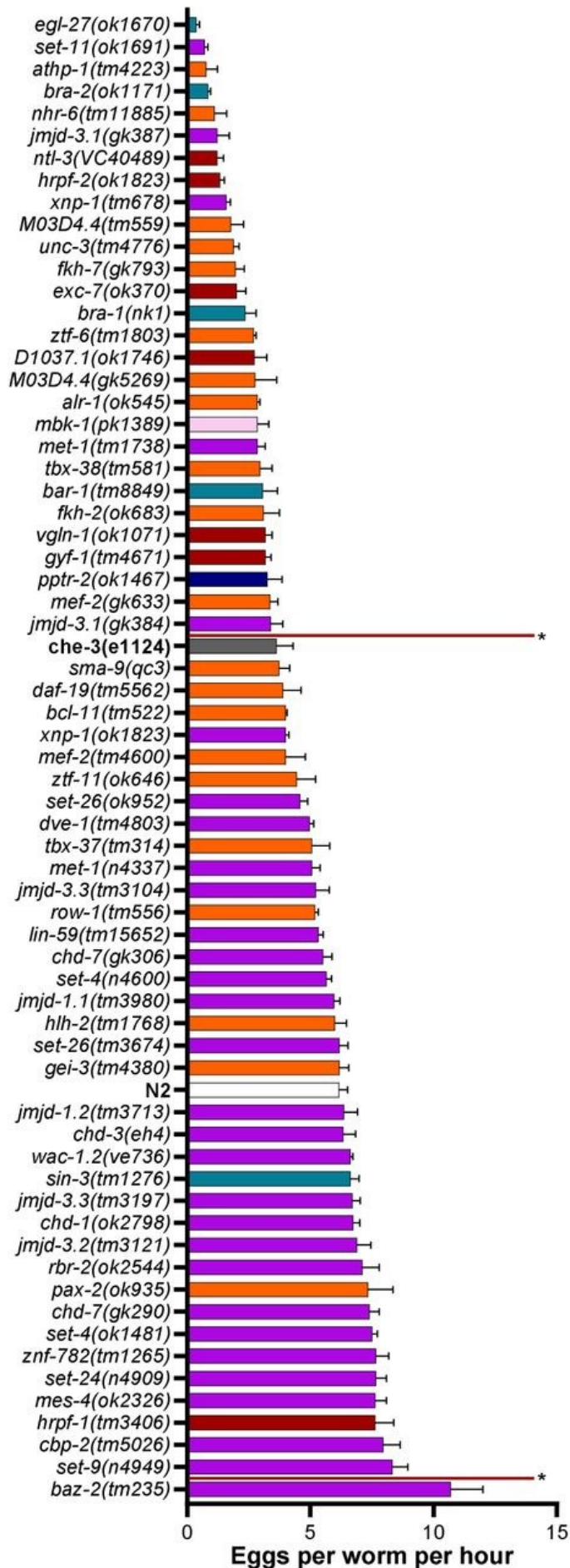


Figure A.3 Egg count analysis of N2 vs ASD associated mutants.

Egg count analysis comparing the egg laying rate of 3 worms over 3 hours between N2, ASD associated mutants and chemosensory deficient mutant *che-3* (e1124). All data shown as mean±SEM Statistical analysis performed using one-way ANOVA with Dunnetts's multiple comparison test. n = 41 for N2 and n = 3 for all other mutants. Vertical red lines indicate significance compared to N2, strains not contained within the red lines are significantly different. Chromatin remodelers, transcription factors, RNA binding proteins, transcription co-factors, phosphatase, kinase.

A.4.4 Pervasive and transient developmental delays observed in ASD associated mutants

Precise co-ordination of gene expression is important for tissue development and function. We measured the developmental progression of the selected autism associated mutant strains by recording the proportion of developmental stages at timepoints in the *C. elegans* developmental lifecycle that corresponds to larval moults during early, mid and late development (Figure A.2). At 20 hours, the approximate time of the L1 moult to L2, the proportion of L1 worms was 7% for the N2 as the majority of the population had undergone the first larval molt to L2 (Figure A.4A). The chemosensory deficient mutant, *che-3 (e1124)* displays significantly delayed development across all life stages. Thus, sensory deficits can give rise to developmental delays which are pervasive providing a benchmark for solely sensory based developmental delays. Conversely to the egg count analysis, there was no clear association between a specific molecular function and phenotypic output (Figure A.3 and Figure A.4A-C).

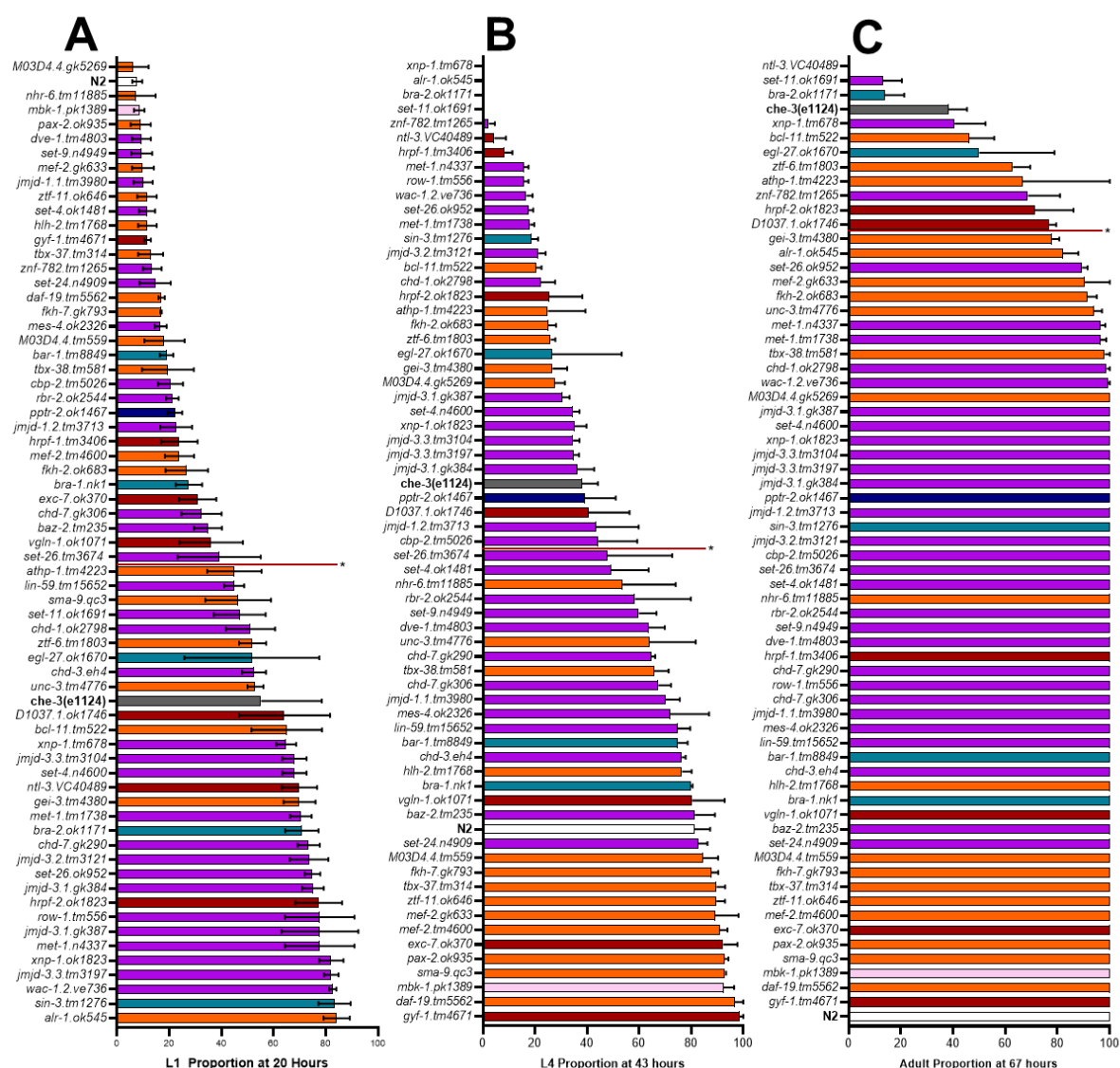


Figure A.4 Gross developmental delays identified in ASD associated mutants vs N2.

A. L1 proportion at 20 hours (approximate time of L1 moult to L2) of N2 compared to ASD associated mutants and chemosensory deficient mutant *che-3(e1124)*. **B.** L4 proportion at 43 hours (approximate time of L3 moult to L4) of N2 compared to ASD associated mutants and *che-3(e1124)*. **C.** Adult proportion at 67 hours (Sufficient time for N2 worms to reach adulthood) of N2 compared to ASD associated mutants and *che-3(e1124)*. $n = 18$ for N2 and $n = 3$ for all other mutants. All data shown as Mean $\pm$ SEM. Statistical analysis performed using one-way ANOVA and Dunnetts's multiple comparison test. Vertical red lines indicate significance compared to N2, anything above the red line is significantly different. Chromatin remodelers, transcription factors, RNA binding proteins, transcription co-factors, phosphatase, kinase.

Over half of the strains (34 of 65) investigated displayed an early developmental delay. A significant delay in early development was observed in the mutant strain *alr-1(ok545)* (Figure A.4A), which had the highest proportion of L1s compared to N2 (12-fold increase). A significant delay in mid and late development was observed in 33 of the 65 strains (Figure A.4B and 4C). Plotting a rank order comparison of early and late development for each strain can show how pervasive the developmental delays are. There was a high concordance of strains with significant early and late developmental delays. This is indicated by 80% of strains with an early developmental delay also satisfied the criteria for delayed later stages of development (Figure A.5). This shows the majority of strains fit the pattern observed in human cohorts of a pervasive developmental disorder which is present early in development and sustained (Miniscalco and Carlsson, 2022). However, 18% of the strains identified with a late developmental delay had no observed early developmental delay. Conversely, 15% that had an early developmental delay showed amelioration by the time these strains reached late development. In this case it implies potential compensatory mechanisms within these strains.

Finally, a large proportion encompassing 27 strains had no identified developmental delay. These results suggest that the two most common outcomes are pervasive developmental delays or strains with mutant autism associated genes that were unaffected in development.

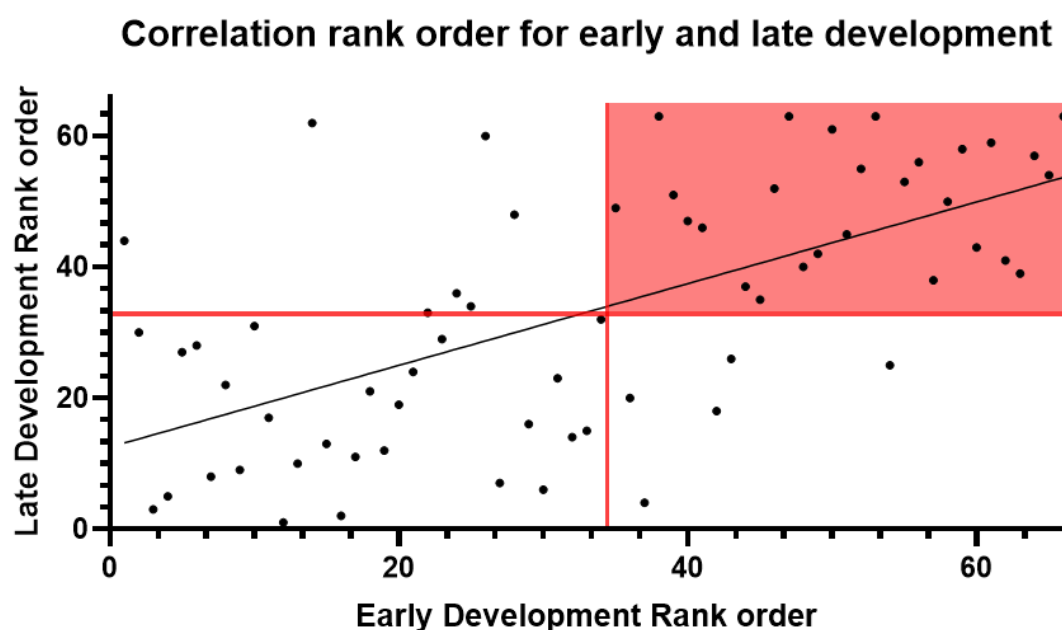


Figure A.5 Correlation of early and late developmental rank orders.

Correlation of each strains developmental rank order identified by developmental rate analysis. Early development is defined as significant difference from wildtype (N2) at L1/L2 moult occurring at ~20 hours. Late development is defined as a significant difference from the wildtype (N2) at the L3/L4 moult time of ~43 hours or delayed reaching adulthood ~67 hours. The red lines mark the significance cutoff vs N2, and the red square highlights the area which is significantly different in both developmental categories.

A.4.5 Neuroarchitectural changes in key sensory neurons are rare in ASD associated mutants

In the context of autism, the interaction between developmental programming and structural changes in the nervous system are well documented (Hashem *et al.*, 2020). To address if there was a detectable difference we focussed on the core sensory structures that mediate the environmental cues. These are the amphid and phasmid structures. Given the growing emphasis of research on sensory systems in ASD, we wanted to develop the analysis around assays that quantified sensory responses to distinct modalities. These environmental inputs are mediated primarily by ASH for aversive modalities (Krzyzanowski *et al.*, 2016) and AWA for diacetyl (Zhang *et al.*, 1997). When dysfunctional, they underpin disrupted sensory responses. Before initiating this, we investigated the structural integrity of these sensory neurons, where possible, using Dil labelling.

Dil staining was used to test for the presence of anatomical features primarily associated with sensory detection that lie upstream of the integrating and output aspect that lead to integrated behaviour. As previously described, Dil was sufficient to stain the 6 amphid sensory neurones ASK, ASJ, ASH, ASI, AWB and ADL and both phasmids, PHA and PHB of the N2 strain (Figure A.6A-C; Collet *et al.*, 1998). The same staining protocol was applied to mutants and then imaged for the presence or absence of amphids and phasmids (*che-3 (e1124)* used as a positive control).

Only two of the ASD associated mutants had a significant alteration to their amphid/phasmid structure, as previously described. These mutants were *alr-1 (ok545)* (Tucker *et al.*, 2005) and *egl-27 (ok1670)* (Solari, Bateman and Ahringer, 1999). *alr-1 (ok545)* showed a complete loss of dye filling by adulthood in the six sensory amphids (Figure A.6D). However, this was selective as phasmids were still capable of dye filling (Figure A.6E) in two of the six worms imaged. *egl-27 (ok1670)* showed the opposite pattern with both phasmids being unlabelled but all six sensory amphids accounted for. Thus, the gross sensory structures were unaffected in the vast majority of the strains when compared to N2. This is shown by *jmjd-3.1 (gk387)* which showed all their amphids and phasmids (Figure A.6F,G,H) but as we describe later exhibited both pervasive developmental delays as well as sensory deficits. Based on this, the mutants in the pipeline showed no gross disruption in structures that initiate sensory processing, thus informing interpretation of subsequent behavioural analysis.

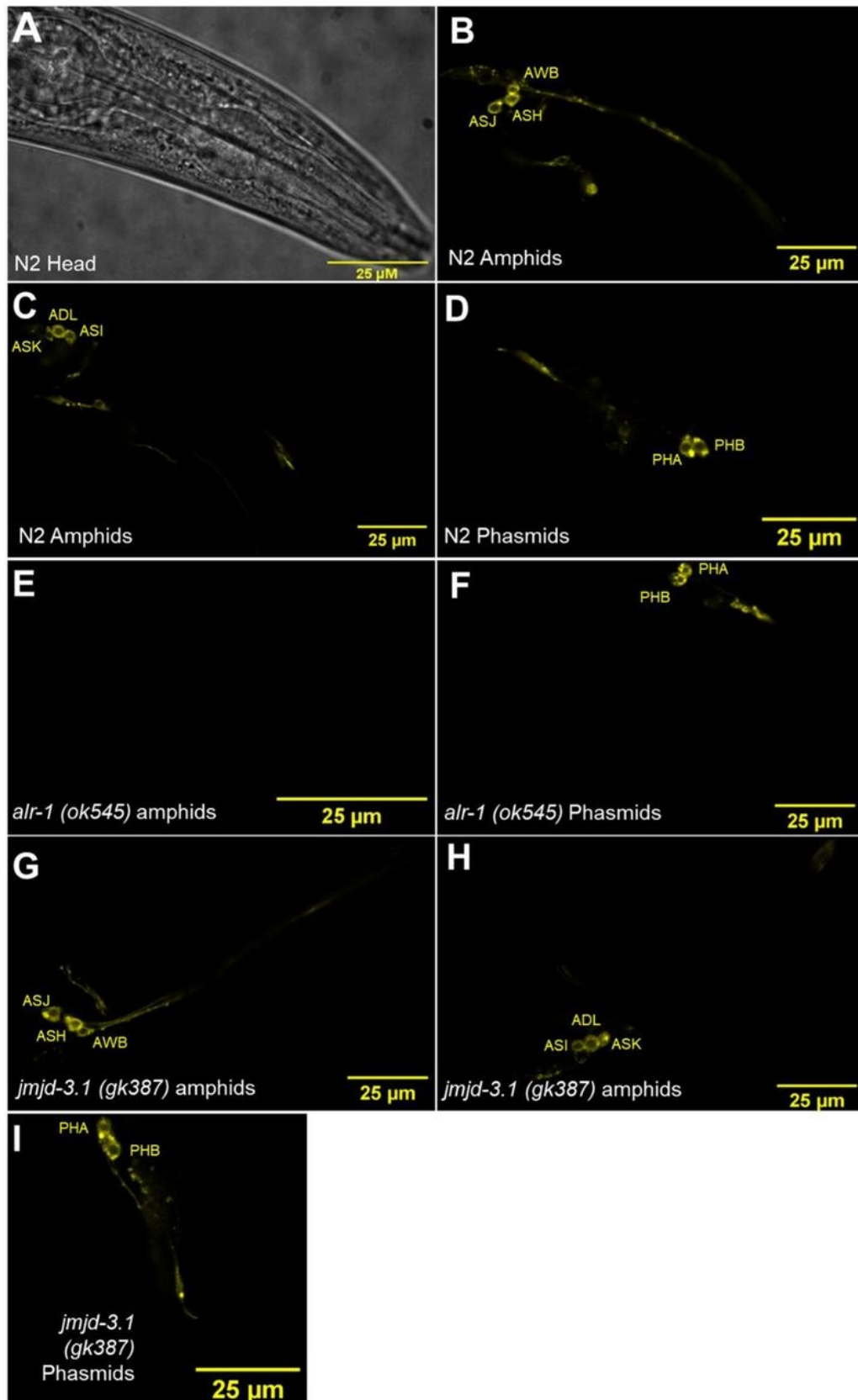


Figure A.6 Sensory processing architecture in ASD associated *C. elegans* mutants.

A. Brightfield image of N2 head showing the area imaged for analysing amphid presence. **B.** N2 sensory amphids, ASH, AWB and ASJ. **C.** N2 sensory amphids ASK, ADL and ASI. **D.** N2 sensory phasmids PHA and PHB. **E.** *alr-1(ok545)* missing sensory amphids. **F.** *alr-1(ok545)* showing presence of both PHA and PHB. **G.** *jmjd-3.1 (gk387)* sensory amphids ASJ, ASH and AWB. **H.** *jmjd-3.1 (gk387)* sensory amphids ASI, ADL and ASK. **I.** *jmjd-3.1 (gk387)* sensory phasmids PHA and PHB.

A.4.6 ASD associated mutants cause hypo or hypersensitive behavioural responses to chemosensory stimuli

We evaluated sensory processing capabilities within each strain using chemoattractant and aversive cues. A diacetyl (1% v/v) quadrant assay (Figure A.2) was utilised to measure attractive chemosensory processing. In this assay each worm had to detect the positive cue and integrate that signal into a locomotory response and move in the direction of the cue (chemotaxis). The observed chemotaxis of the mutants was compared to the N2 performance to determine any modified sensitivity to the attractive cue.

In addition, aversive chemosensory processing was assayed by quantifying reversals to both water soluble and volatile aversive cues. Sensory processing of attractive and aversive chemosensory cues requires the functionality of different sensory amphids (ASH or AWA), but downstream interneuron and motor neuron circuits are shared. Thus, by comparing strains across multiple modalities it is possible to distinguish between strains impacted at the level of sensory detection versus those impacted in downstream circuitry.

A.4.6.1 Chemotaxis response perturbed in 25% of ASD associated mutants

N2 showed a reproducible attraction to the chemosensory cue diacetyl (1% v/v) as evidenced by the performance index of 0.77 (Figure A.7A). 16 mutants showed a significant reduction in their performance index compared to N2. There was no clear bias for any of the molecular functional groups with each group represented proportionally. Additionally, only one strain, *M03D4.4* (*tm559*), showed a performance index lower than the negative control *che-3* (*e1124*) of 0.05 with the remaining significantly impaired strains scoring between 0.07 and 0.42. This suggests an incomplete loss of sensory detection or sensory processing as most mutants fall between normal processing by N2 and complete loss of sensory processing by *che-3* (*e1124*).

A.4.6.2 Aversive cue responses show hypo and hypersensitivity

We measured responses to different aversive cues which utilise distinct molecular determinants to trigger the initiating response that drives the behaviour of reversing. In addition, we extended this to aversive volatile cues. Hypersensitivity, defined in this study as an enhanced aversive behavioural response compared to the wild-type was observed in *xnp-1* (*ok1823*), *jmjd-3.3* (*tm3197*) and *wac-1.2* (*ve736*) in response to low pH and copper sulphate (Figure A.7B and 7C) and 1-octanol (Figure A.7E). Hyposensitivity, defined as a reduced aversive behavioural response was exhibited by 17 mutants, to one or more of the soluble aversive cues. 6 of the 9 strains that were hyposensitive to 1-octanol demonstrated hyposensitivity to at least one soluble cue (Figure A.7B-E).

Of the 65 strains tested 25 reached the criteria that indicate they show significant changes in response to the sensory cues (Figure A.7B-E) compared to N2. Hyposensitivity to the aversive cues was more commonly observed at 30 instances compared to just 5 instances of hypersensitivity.

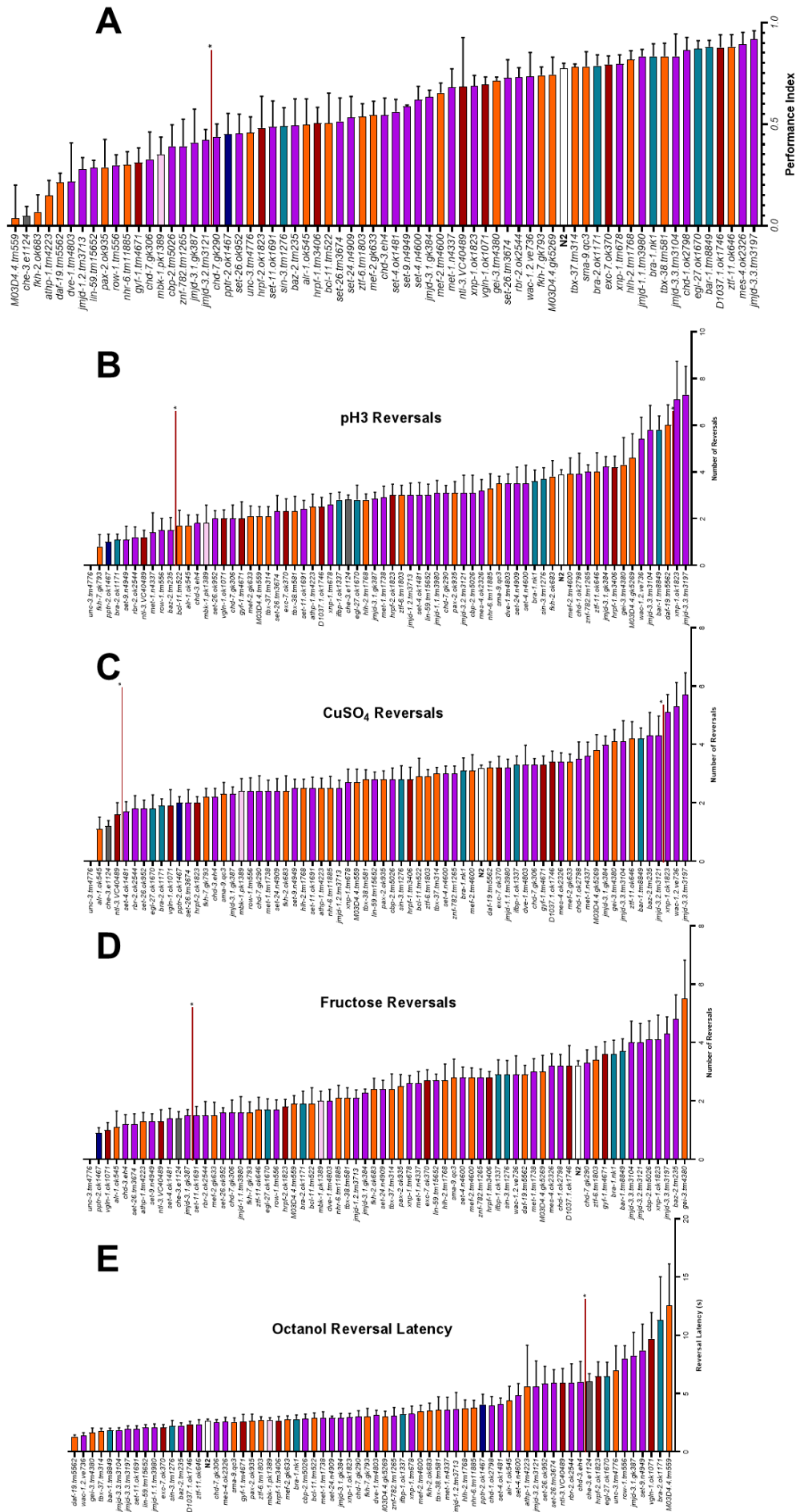


Figure A.7 Sensory processing capabilities modified in *C. elegans* ASD associated mutants.

A. Performance index of N2 vs ASD associated mutants in diacetyl quadrant assay. $n=48$ for N2. $n = 3$ for all other strains. Statistical analysis performed using one-way ANOVA and Dunnett's multiple comparison test. **B, C, D.** Average number of reversals of N2 vs ASD associated mutants in response to aversive stimuli (low pH, copper sulphate and fructose). **E.** Latency to initiating reversals of N2 vs ASD associated mutants in response to 30% octanol. **B, C, D, E.** Statistical analysis performed using one-way ANOVA and Dunnett's multiple comparison test. $n = 180$ for N2 and $n = 10$ for all other mutants. All data shown as Mean $\pm$ SEM. Chromatin remodelers, transcription factors, RNA binding proteins, transcription co-factors, phosphatase, kinase. Significance shown by red horizontal line, in A and D strains above the red line are significant, in B, C and E strains outside of those contained with the red lines are significant vs N2.

A.4.7 Significance heatmap of developmental and sensory assays of ASD associated mutants show functional segregation

The results of the developmental, anatomical and sensory phenotypic analysis are summarised in Figure A.8. To aid in the identification of patterns within the dataset each mutant's p value from each assay was converted to a binary value indicating significant or not significant and then plotted into the heatmap. Each row therefore is the specific phenotype observed in a particular strain and can be directly compared to other strains to highlight phenotypic overlap within the dataset. Furthermore, each gene is associated with a specific molecular functional grouping allowing for comparisons across individuals within a group as well as across groups.

Egg laying appears very sensitive to mutant perturbation, with over 50% of transcription factors and over 80% of RNA binding proteins showing significance compared to less than 20% of chromatin remodellers studied. Early and late developmental phenotypes are not associated with any one molecular functional grouping as evidenced by a consistent percentage of strains in each group showing developmental delays. Furthermore, there are no genes that are significantly affected across all phenotypes suggesting that there is no fully penetrant sensory deficient mutant which is also impaired in development and neuroarchitectural changes.

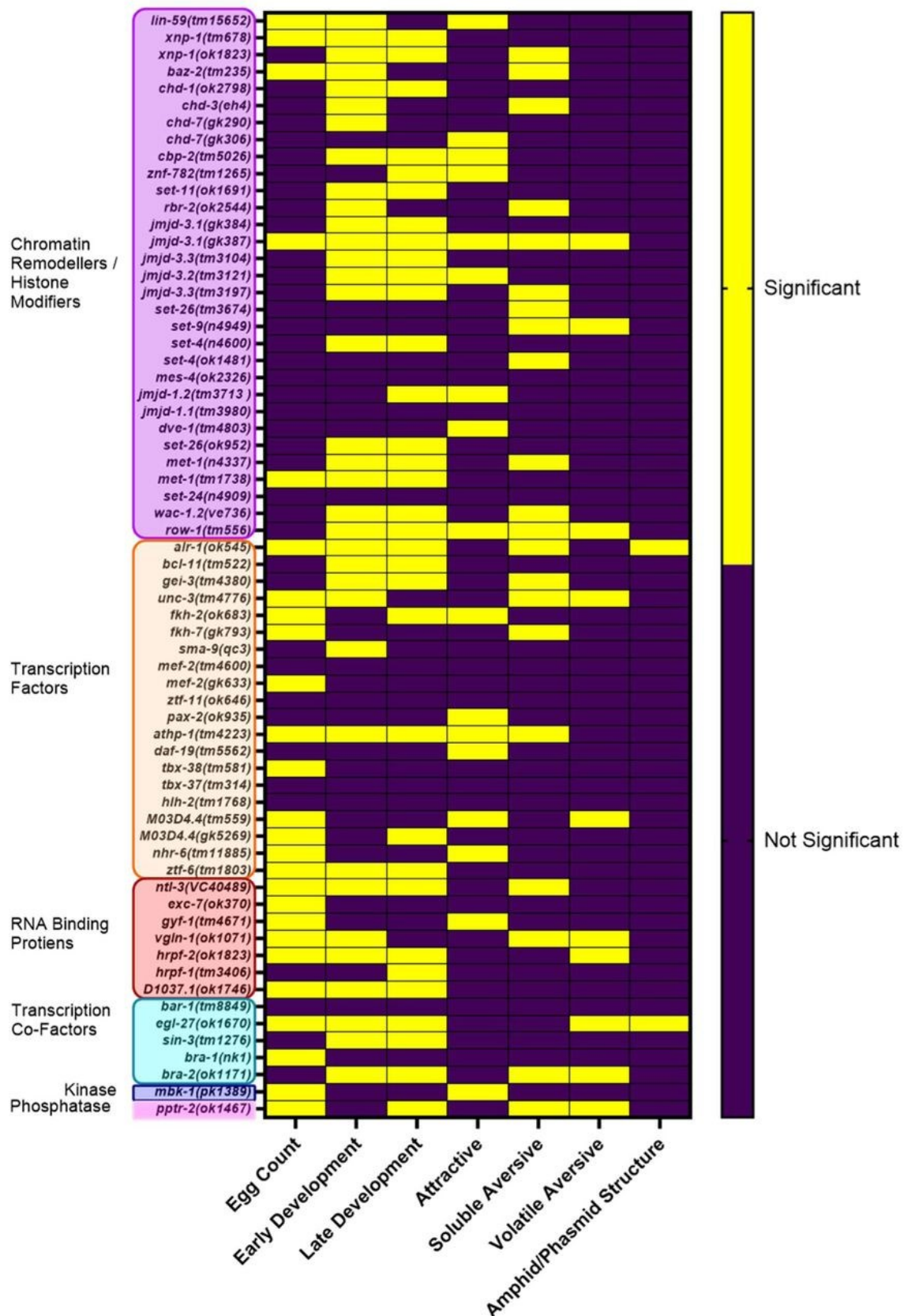


Figure A.8 Significance heatmap of ASD associated mutant's vs N2 for developmental and sensory phenotypes. Significance heatmap showing the seven phenotypes assessed in the experimental pipeline including, egg count, early and late development, attractive chemosensory processing, soluble aversive chemosensory processing, volatile aversive chemosensory processing and sensory amphid/phasmid structure. Yellow boxes show significant difference from N2, and purple are non-significant vs N2, in each respective phenotype. Significance is defined at $p \leq 0.05$. For egg count, early and late development, attractive, soluble aversive and volatile aversive chemosensory processing one-way ANOVAs and Dunnett's multiple comparison test were performed to determine significance. For amphid/phasmid structure any deviation from the expected six amphids and two phasmids was noted as significant.

A.4.8 Three phenotypic groupings emerge from developmental and sensory screen of ASD associated mutants

The integration of our analysis from genes that emerged in our pipeline provided an opportunity to assess associations between the distinct domains from the analysis summarised above. We conducted a phenotypic grouping analysis to parse the cohort of mutants that passed through the comparative pipeline. This approach that integrates phenotypic score with each gene established 3 groups of genes (Figure A.9). The classification was driven by ascribing normal or perturbed phenotypic response based on multiple comparison statistical tests of each gene in each assay (Statistical tests in figure legends). This is indicated on each graph and best reflected in the summary table (Figure A.8) by a binary scoring in which a measured trait is defined as normal or perturbed.

The subgroups derived from Figure 8 were classified as being sensory deficient, developmental deficient or showing a combined sensory and developmental deficiency relative to controls. Group 1, deficient in both sensory responses and development, includes 2 chromatin remodelers; 3 transcription factors an RNA binding protein and a phosphatase (Figure A.9A, D). Group 2, impaired in only development, includes 3 chromatin remodelers, 2 transcription factors and an RNA binding protein (Figure A.9B, D). Group 3, impaired in only sensory responses, includes 4 chromatin remodelers and 2 transcription factors (Figure A.9C, D). Group 1 genes show a significant difference across all phenotypes investigated except neuroarchitectural changes which provides a subset of genes with pleiotropic effects. Group 2 and 3 genes show more selective effects in development and sensory processing respectively.

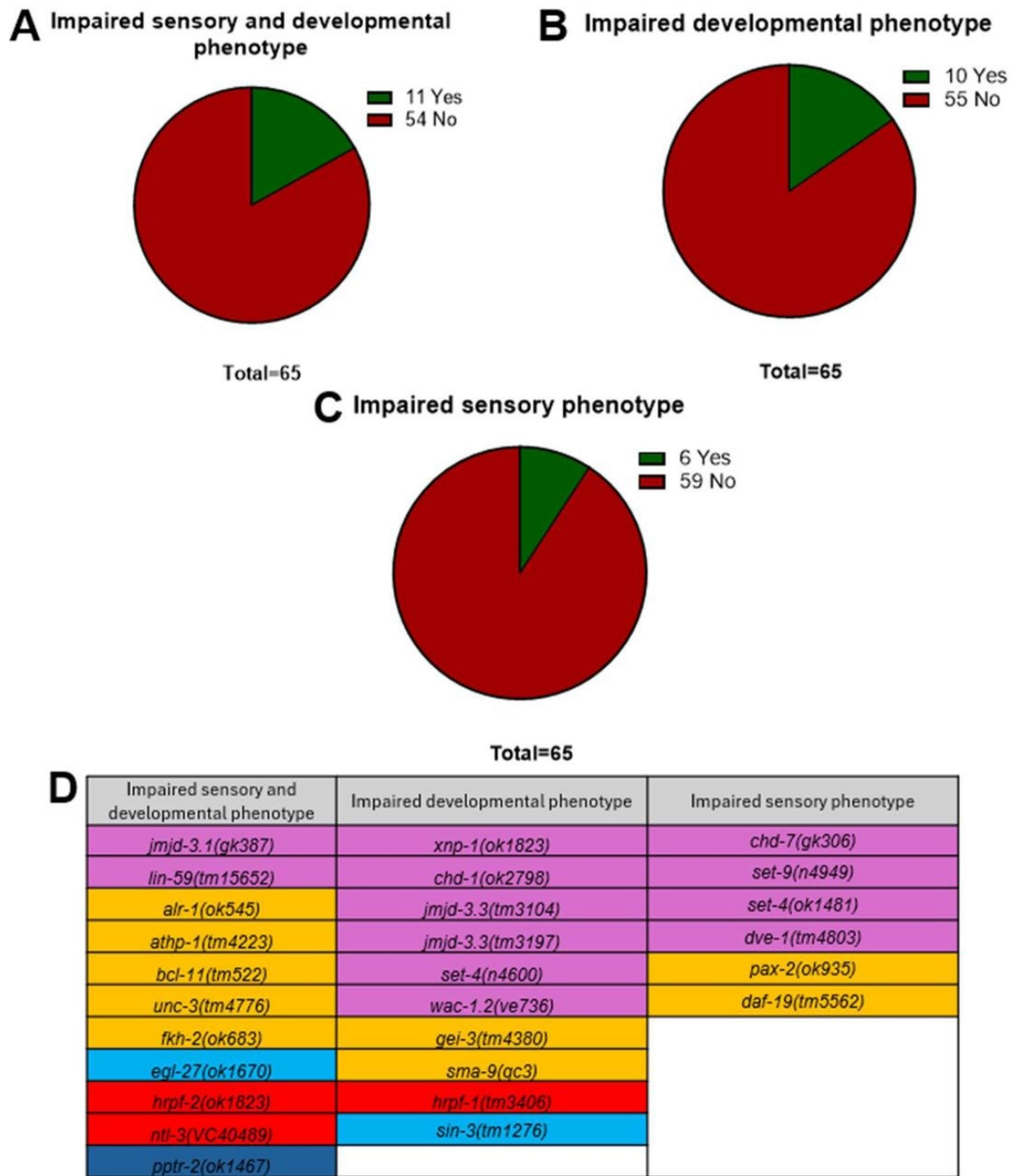


Figure A.9 Filtering for significant developmental or sensory affected mutants highlights three distinct groupings.
A. Subclassification of mutant strains for deficits in sensory processing, gross developmental delays and egg laying deficits. **B.** Phenotypically filtered genes for deficits in developmental programs and egg laying but without any sensory function deficits. **C.** Phenotypically grouped genes for deficits in sensory function without any gross developmental delays or fecundity deficits. **D.** Genes filtered from the significance heatmap for impaired sensory, impaired developmental and mixed phenotypes. The phenotypes grouped as developmental for the purpose of filtering were egg count, early development and late development. The phenotypes grouped as sensory were attractive chemosensory, soluble and volatile aversive chemosensory.

A.5 Discussion

A widely recognised phenotypic trait associated with autism is the atypical sensory processing. This is readily observed in clinical cohorts and has emerged as a listed characteristic in the updated DSM V (American Psychiatric Association, 2013). This supplements the established criteria around social and repetitive motor behaviours and the signature early life emergence of the neuroatypical traits. In clinical cohorts the reported changes span multiple sensory modalities from visual, auditory to tactile and chemosensory (Bennetto, Kuschner and Hyman, 2007; Sivapalan *et al.*, 2024), although they are not systematically recorded. However, individuals with ASD have shown an increased severity in behavioural issues because of sensory processing changes requiring routine therapy to try and alleviate the problematic behaviours (Sivapalan *et al.*, 2024). This highlights the severity of real-world consequences for individuals with ASD and the lack of genetic or pharmacological targets available to ameliorate the changes.

This recognized significance of the sensory component led us to design a pipeline in *C. elegans* that sort to exploit approaches that inform on this aspect. We designed a pipeline to resolve epigenetic and wider genetic modulators in which there is good evidence for intersection with environmental cues that go through sensory pathways. In addition, this pipeline utilizes epigenetic regulators that contribute to polygenic transcriptional programs that may represent a distributed polygenic landscape like those that represent the most significant genetic determinants of autism. The pipeline is supplemented by additional transcriptional regulators. In restricting our analysis to category 1 SFARI genes it allowed the comparison of key biological traits encompassing developmental and sensory responses in the highest confidence gene expression modifiers.

Furthermore, developmental delays in ASD cohorts have correlated with a greater likelihood of other ASD symptoms such as impairments in fine motor skills, language ability and personal social activities (Shan *et al.*, 2021). For this analysis, it is important to note that intrinsic changes in gross development represented in the mutations are confounded by the potential for gross sensory disruption which is sufficiently penetrant to execute developmental changes as exemplified by the positive control *che-3 (e1124)*. Therefore, understanding the intersection of developmental processes and sensory processing changes in ASD is vital to develop pharmacological treatments.

When investigating bioinformatically selected autism associated genes, we identified that development and by extension underpinning programs were especially susceptible to perturbation with 61% of strains being impacted in either early or late development. Between early and late a number of subtle transitions occur between early larval stages but, by their fourth

molt, 400 epidermal, gonad, muscle and neuronal cells have been added to a total of 959 somatic cells (Rougvie and Moss, 2013). This process is susceptible to perturbation at any point which could lead to a developmental delay.

Mutants that showed perturbations early in development were largely pervasive with over 80% concordance of strains with significant early/late developmental delays suggesting delays in early development were not easily overcome. However, this was not universal and 15% of strains with an early developmental delay showed no significant delay in later development, suggesting a possible mechanism by which these strains were able to expedite their later development. This is not unprecedented as early perturbation of developmental programs have been corrected by late expression of the affected gene (Luikenhuis *et al.*, 2004).

An association to the human disorder can be made here as there have been documented examples of spontaneous recovery from an autistic phenotype suggesting the possibility that recovery of developmental delays is not strictly a worm phenotype (Sitholey, Agarwal and Pargaonkar, 2009). Another example is MeCP2 the main driver of Rett's Syndrome (RTT) in which expression of functional MeCP2 in mutant mice lines rescue the RTT phenotype (Luikenhuis *et al.*, 2004). Despite this phenotypic rescue originating from transgenic expression of the affected gene, it does not preclude the possibility for endogenous mechanisms being present within *C. elegans* that are capable of self-rescuing developmental delays. Additionally, environmental cues from other worms on the same plate have been shown to affect developmental rates, providing an example of environmental impact on development which could also contribute to the developmental phenotypes we observed (Ludewig *et al.*, 2017).

The criteria used to define sensory dysfunction was based on being significantly perturbed compared to N2 in either attractive, soluble aversive or volatile aversive chemosensory processing. However, when considering mutants that had impaired development, the potential impact becomes clearer when highlighting a more severe sensory phenotype. Increased severity here is defined as significant perturbations across multiple sensory modalities. Severity of sensory perturbation overlaps with developmental delays. This adds credence to our approach of scoring development alongside behavioural outcomes as 27.5% of strains with an impairment in early or late development show an increase in severity of impact to sensory processing.

Interestingly, it is only the severity of the sensory perturbations which correlates with developmental delays, as developmentally delayed strains had a 55% overlap with sensory perturbations compared to 60% of non-developmentally delayed strains. This highlights that the worm has a distinct relevance to modelling this condition as the same effect of developmental delays correlating with greater severity of ASD symptoms in human cohorts (McDiarmid, Ardiel

and Rankin, 2015). Furthermore, it shows that sensory perturbations in the adult can arise without a developmental delay being present, highlighting the possibility for selectivity in the genetic underpinnings of different subsets of ASD mutants.

Our analysis led to three groups of mutants which can be aligned with dysfunction in different areas of ASD symptomology investigated, namely Group 1: sensory, gross development and reproduction; Group 2: gross development and reproduction; Group 3: sensory. These sub-groups which are criteria-based, contain orthologues spanning diverse molecular functions. One speculation is that these represent distinct but definable grouping in which the flagged molecular perturbation generates shared mechanistic behaviours. This view expresses outcomes from genetic interrogation of symptomology from human cohorts (Litman *et al.*, 2025; Leyhausen *et al.*, 2026). These studies highlight the preferred view that distinct subcategories relate to distributed mechanistic underpinnings cohorts (Litman *et al.*, 2025; Leyhausen *et al.*, 2026). Our criteria-based approach in which the intrinsic developmental programs and associated behavioural outcomes in the adult do a similar thing in a biologically simpler organism demonstrates the capacity to model the ASD spectrum in *C. elegans*. This provides a basis for further analysis to investigate the transcriptional profile within each grouping identified in our study to determine if shared mechanistic pathways underpin behavioural changes within each group and differentiate the groups from each other.

These groups suggest that genes with molecularly distinct functions converge upon specific pathways which can be both independent in the case of sensory or development specific groups or combined. Comparing the molecular consequences in each group to identify shared determinants would provide direct genotype/phenotype information to evidence if there is a shared core component in these artificially created sub-groupings. Investigating this in gene expression regulators associated with autism might highlight if specific cohort of genes or convergent functional biology define mechanisms that explain the spectral basis of autism (Figure A.9).

These notions are consistent with a recent studies evidence for selective pathways altered in ASD individuals creating a unique presentation of the disorder (Litman *et al.*, 2025). However, one limitation is the absence of sensory phenotypes in any of the groups they identify. This work informs on the value of quantitative sensory assays, and the data supports the need for a more systematic sensory testing of ASD cohorts. One reason for this is the lack of reported quantitative sensory testing in ASD cohorts with known genetic backgrounds. Using the 3 gene groups identified from our investigation we attempted to identify sensory phenotypes in human and mouse cohorts (Supplementary Table SA.2) but due to limited sensory information it was difficult to determine if our identified genes correlated with human or murine findings.

In conclusion, investigation of ASD associated gene expression modifier orthologues in *C. elegans* led to a criteria-based grouping with shared phenotypic disruptions. These groupings encompass genes with distinct divergent biological functions with a shared ability to modulate wider gene expression. We drove this by considering the phenotypic overlap between developmental timing and quantifiable and systematic measurement of sensory processing. The latter is an important and underreported component of ASD pathology with a direct impact upon an individual's behaviour and quality of life (Giacardy *et al.*, 2018). Therefore, further investigation into the molecular basis of sensory processing perturbation is necessary and our robust experimental pipeline can identify candidate genes for further analysis within this domain.

Table SA.1 ASD associated genes phenotypically filtered for sensory and developmental phenotypes in *C. elegans*.

Phenotypic group refers to the phenotypic filtering performed as part of this experimental pipeline. The strains and human genes are defined by orthology as outlined in the bioinformatic pipeline. Each gene's status as syndromic is defined by SFARIs syndromic annotation for each gene. Sensory phenotypes in human cohorts and mouse models defined by primary literature search with subject descriptions and phenotypic databases. Sensory phenotypes defined as "Not Identified" lacked reference to specific sensory phenotypes but does not preclude their existence which could be due to a lack of sensory testing. Sensory phenotypes defined as "Yes" had a patient specific or mouse model with reports of sensory changes in any modality but most commonly hearing or vision. Finally sensory phenotypes defined as "N/A" are due to no mouse model existing for a specific gene.

Phenotypic Group	Strain	Human Gene	Syndromic?	Sensory phenotype in human cohorts?	Sensory phenotype in mouse models?
Impaired Sensory	<i>chd-7(gk306)</i>	CHD8	YES	Not Identified	YES
	<i>set-9(n4949)</i>	KMT2E, SETD5	YES x2	Not Identified	YES
	<i>set-4(ok1481)</i>	KMT5B	NO	Not Identified	Not Identified
	<i>dve-1(tm4803)</i>	SATB1	YES	YES	N/A
	<i>pax-2(ok935)</i>	PAX5	NO	YES	Not Identified
	<i>daf-19(tm5562)</i>	RFX3	NO	YES	Not Identified
Impaired development	<i>xnp-1(tm678)</i>	ATRX	NO	Not Identified	Not Identified
	<i>baz-2(tm235)</i>	BAZ2B	NO	Not Identified	Not Identified
	<i>met-1(tm1738)</i>	SETD2	NO	YES	Not Identified
	<i>M03D4.4(gk5269)</i>	VEZF1	NO	Not Identified	Not Identified
	<i>ztf-6(tm1803)</i>	ZBTB20	YES	YES	Not Identified
	<i>D1037.1(ok1746)</i>	SON	YES	YES	Not Identified
Mixed	<i>lin-59(tm15652)</i>	ASH1L	NO	YES	Not Identified
	<i>jmjd-3.1(gk387)</i>	KDM6B	NO	Not Identified	Not Identified
	<i>alr-1(ok545)</i>	ARX	YES	YES	YES
	<i>unc-3(tm4776)</i>	EBF3	YES	YES	YES
	<i>athp-1(tm4223)</i>	PHF12	NO	Not Identified	Not Identified
	<i>hrpf-2(ok1823)</i>	HNRNPH2	NO	Not Identified	YES
	<i>pptr-2(ok1467)</i>	PPP2R5D	YES	Not Identified	YES

Table SA.2 All ASD associated mutant strains used in this study as well as additional information including human gene name, protein function, *C. elegans* orthologue, allele, strain name, number of times outcrossed and mutation type.

Colour scheme is as follows: Green – chromatin remodellers/ histone modifiers, Orange – transcription factors, Red – RNA binding proteins, Light Blue – transcription co-factors, Dark Blue – Kinase, Yellow – Phosphatase.

Human gene	Gene name	Protein function	<i>C. elegans</i> orthologue	Allele	Strain name	Outcrossed	Mutation
ASH1L	ASH1 like histone lysine methyltransferase	Histone methyltransferase	<i>lin-59</i>	tm15652	FX25652	x0	Deletion
ATRX	ATRX chromatin remodeler	SWI/SNF chromatin remodeler	<i>xnp-1</i>	tm678	FX00678	x0	Deletion
				ok1823	VC2088	x1	Deletion
BAZ2B	Bromodomain adjacent to zinc finger domain 2B	Component of chromatin remodeling complexes	<i>baz-2</i>	tm235	FX00235	x0	Deletion
CHD2	Chromodomain helicase DNA binding protein 2	SNF2 related chromatin remodeler	<i>chd-1</i>	ok2798	RB2613	x0	Deletion
CHD3	Chromodomain helicase DNA binding protein 3	SNF2 related chromatin remodeler	<i>chd-3</i>	eh4	HX103	x4	Deletion
CHD8	Chromodomain helicase DNA binding	SNF2 related chromatin remodeler	<i>chd-7</i>	gk290	VC606	x0	Deletion
				gk306	VC676	x0	Deletion
CREBBP	CREB binding protein	Histone acetyltransferase and transcriptional regulator	<i>cbp-2</i>	tm5026	FX05026	x0	Deletion
CTCF	COCTC-binding factor	Component of histone acetylase and deacetylase complexes and transcriptional regulator	<i>znf-782</i>	tm1265	FX01265	x0	Deletion + Insertion
EHMT1	Euchromatic histone lysine methyltransferase 1	Histone methyltransferase	<i>set-11</i>	ok1691	VC1219	x0	Deletion
KDM5B	Lysine demethylase 5B	Jumonji/ARID family lysine demethylase	<i>rbr-2</i>	ok2544	RB1941	x0	Deletion
KDM6B	Lysine demethylase 6B	Jumonji/ARID family lysine demethylase	<i>jmjd-3.1</i>	gk384	VC936	x0	Deletion + Insertion
				gk387	VC912	x0	Deletion
				tm3121	FX03121	x0	Deletion + Insertion
				tm3104	FX03104	x0	Deletion
				tm3197	FX34427	x2	Deletion
KMT2E	Lysine methyltransferase 2E	Histone methyltransferase	<i>set-26</i>	tm3674	FX03674	x0	Deletion
KMT2E			<i>set-9</i>	n4949	MT16426	x1	Deletion
KMT5B			<i>set-4</i>	n4600	MT14911	x2	Deletion
KMT5B				ok1481	VC997	x0	Deletion
NSD1	Nuclear receptor binding SET domain	Histone methyltransferase	<i>mes-4</i>	ok2326	VC1874	x1	Deletion + Insertion
PHF2	PHD finger protein 2	Histone demethylase	<i>jmjd-1.1</i>	tm3980	FX17844	x2	Deletion
PHF2			<i>jmjd-1.2</i>	tm3713	FX17361	x2	Deletion
SATB1	SATB Homeobox 1	Chromatin remodeling scaffold	<i>dve-1</i>	tm4803	FX04803	x0	Deletion
SETD1A	SET domain containing 1A	Histone lysine methyltransferase	<i>set-26</i>	ok952	RB1025	x0	Deletion
SETD2	SET domain containing 2	Histone lysine methyltransferase	<i>met-1</i>	n4337	MT16973	x4	Deletion
SETD2				tm1738	FX01738	x0	Deletion
SETD5	SET domain containing 5	Histone lysine methyltransferase	<i>set-9</i>	n4949	MT16426	x1	Deletion
SETD5			<i>set-24</i>	n4909	MT16133	x0	Deletion
WAC	WW domain containing adaptor with coiled-coil	Signalling protein	<i>wac-1.2</i>	ve736	RG3236	x0	Deletion
POGZ	Pogo transposable element derived with ZNF domain	Chromatin remodeling complex protein and transcription factor	<i>row-1</i>	tm556	FX00556	x0	Deletion
ARX	Aristaless related homeobox	Homeobox transcription factor	<i>alr-1</i>	ok545	RB762	x0	Deletion
BCL11A	BCL11 Transcription Factor A	Transcription factor	<i>bcl-11</i>	tm522	FX0522	x0	Deletion

CIC	Capicua Transcriptional Repressor	Transcriptional repressor	<i>gei-3</i>	<i>tm4380</i>	FX04380	x0	Deletion
EBF3	EBF Transcription Factor 3	Transcriptional activator	<i>unc-3</i>	<i>tm4776</i>	FX04776	x0	Deletion
FOXG1	Forkhead Box G1	Transcriptional repressor	<i>fkf-2</i>	<i>ok683</i>	RB853	x0	Deletion
FOXP1	Forkhead Box P1	Transcriptional repressor	<i>fkf-7</i>	<i>gk793</i>	VC1646	x0	Deletion
FOXP2	Forkhead Box P2	Transcriptional repressor					
HIVEP2	HIVEP Zinc Finger 2	Transcriptional activator	<i>sma-9</i>	<i>qc3</i>	CS67	x1	Point Mutation (Stop Gained)
MEF2C	Myocyte Enhancer Factor 2C	Transcriptional activator	<i>mef-2</i>	<i>tm4600</i>	FX04600	x0	Deletion
MEF2C				<i>gk633</i>	VC1402	x0	Deletion
MYT1L	Myelin Transcription Factor 1 Like	Transcription factor	<i>ztf-11</i>	<i>ok646</i>	RB824	x0	Deletion
PAX5	Paired Box 5	Transcription factor	<i>pax-2</i>	<i>ok935</i>	RB1013	x0	Deletion
PHF12	PHD Finger Protein 12	Transcriptional repressor	<i>athp-1</i>	<i>tm4223</i>	FX04223	x0	Deletion
RFX3	Regulatory Factor X3	Transcription factor	<i>daf-19</i>	<i>tm5562</i>	FX05562	x0	Deletion
TBR1	T-Box Brain	Transcriptional repressor	<i>tbx-38</i>	<i>tm581</i>	FX0581	x0	Deletion
TBR1	Transcription Factor 1		<i>tbx-37</i>	<i>tm314</i>	FX0314	x0	Deletion
NR4A2	Nuclear Receptor Subfamily 4 Group A Member 2	Transcriptional regulator	<i>nhr-6</i>	<i>tm11885</i>	FX011885	x0	Deletion
TCF4	Transcription Factor 4	Transcription factor	<i>hlh-2</i>	<i>tm1768</i>	FX01768	x0	Deletion
VEZF1	Vascular Endothelial	Transcription factor	<i>M03D4.4</i>	<i>tm559</i>	FX0559	x0	Deletion
VEZF1	Zinc Finger 1			<i>gk5269</i>	VC4183	x0	Deletion (Balanced)
ZBTB20	Zinc Finger And BTB Domain Containing 20	Transcription factor	<i>ztf-6</i>	<i>tm1803</i>	FX01803	x0	Deletion
CNOT3	CCR4-NOT Transcription Complex Subunit 3	mRNA deadenylase	<i>ntl-3</i>	<i>gk944863</i>	VC40489	x0	Deletion
ELAVL3	ELAV Like RNA Binding Protein 3	RNA-binding protein	<i>exc-7</i>	<i>ok370</i>	VC176	x0	Deletion
GIGYF2	GRB10 Interacting GYF Protein 2	Repressor of translation initiation	<i>gyf-1</i>	<i>tm4671</i>	FX04671	x0	Deletion
HDLBP	High Density Lipoprotein Binding Protein	RNA-binding protein	<i>vgh-1</i>	<i>ok1071</i>	RB1093	x0	Deletion
HNRNP2	Heterogeneous Nuclear Ribonucleoprotein H2	Heterogeneous nuclear ribonucleoprotein	<i>hrpf-2</i>	<i>ok1823</i>	VC2088	x1	Deletion
HNRNP2			<i>hrpf-1</i>	<i>tm3406</i>	FX03406	x0	Deletion
SON	SON DNA And RNA Binding Protein	mRNA splicing cofactor	<i>D1037.1</i>	<i>ok1746</i>	RB1489	x0	Deletion
CTNNB1	Catenin Beta 1	Transcription cofactor	<i>bar-1</i>	<i>tm8849</i>	FX08849	x0	Deletion
IRF2BP1	Interferon Regulatory Factor 2 Binding Protein Like	Transcription corepressor	<i>ifbp-1</i>	<i>ok1339</i>	VC812	x0	Deletion
RERE	Arginine-Glutamic Acid Dipeptide Repeats	Transcriptional corepressor	<i>egl-27</i>	<i>ok1670</i>	VC1217	x1	Deletion
SIN3A	SIN3 Transcription Regulator Family Member A	Transcriptional corepressor	<i>sin-3</i>	<i>tm1276</i>	FX01276	x0	Deletion
ZMYND8	Zinc Finger MYND-Type Containing 8	Transcriptional corepressor	<i>bra-1</i>	<i>nk1</i>	NU1	x10	Deletion
ZMYND8			<i>bra-2</i>	<i>ok1171</i>	VC869	x1	Deletion (Balanced)
DYRK1A	Dual Specificity Tyrosine Phosphorylation Regulated Kinase 1A	CTD kinase of RNAP II	<i>mbk-1</i>	<i>pk1389</i>	EK228	x6	Deletion
PPP2R5D	Protein Phosphatase 2 Regulatory Subunit B' Delta	Ser/Thr Phosphatase	<i>pptr-2</i>	<i>ok1467</i>	RB1338	x0	Deletion

Appendix B Additional data from Chapter 3

B.1 *Ovtrpv2* and *Ovtrpv1* did not successfully rescue hyperosmolarity aversion in *C. elegans osm-9* mutant strains

High osmolarity avoidance, represented by 4M fructose exposure, was not rescued by *Ovtrpv1* (*Pocr-2::Ovtrpv1 Pmyo-3::GFP* vs *ocr-2 (ak47) Pmyo-3::GFP*, $p=0.3278$, Figure B.1A) and only modestly rescued by *Ovtrpv2* (*Posm-9::Ovtrpv2 Pmyo-3::GFP* vs *osm-9 (ky10) Pmyo-3::GFP*, $p=0.0003$ 8B) which in fact still shows a significant difference with the WT N2 (*Posm-9::Ovtrpv2 Pmyo-3::GFP* vs *osm-9 (ky10) Pmyo-3::GFP*, $p<0.0001$, Figure B.1B).

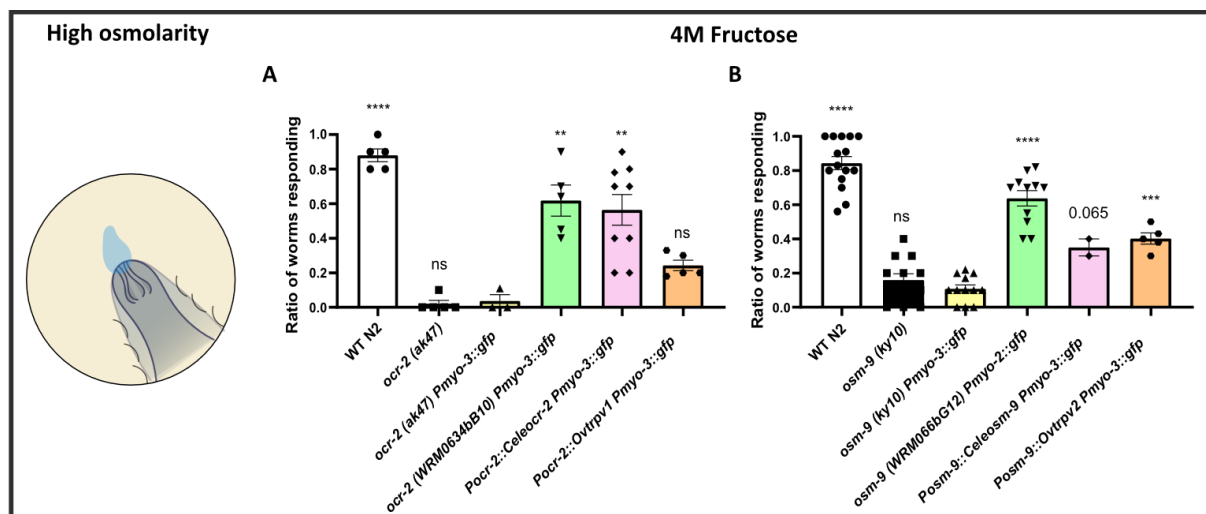


Figure B.1 *Ovtrpv2* but not *Ovtrpv1* rescues hyperosmolarity aversion in *C. elegans* TRPV mutant strains.

The bar graphs represent the average ratio of worms responding $\pm$ s.e.m. For the control lines WT N2, *ocr-2 (ak47)*, *osm-9 (ky10)*, *ocr-2 (ak47) Pmyo-3::GFP*, *osm-9 (ky10) Pmyo-2::GFP* and fosmid lines, each dot represents a replicate experiment in which we tested 10 worms. For the *C. elegans* and *O. vulgaris* cDNA constructs, each dot represents a rescue line (10 worms each), which was selected according to the threshold set at 2 standard deviations below the mean of the reference mutant line *ocr-2 (ak47) Pmyo-3::GFP* or *osm-9 (ky10) Pmyo-3::GFP*. All the average performances are here compared to the reference mutant lines. Data were analysed using one-way ANOVA and Post-hoc comparisons have been performed with Dunnett's multiple comparisons test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

B.2 OvTRPVs expression in HEK293G5A cell line

B.2.1 Cloning for expression in mammalian cells

The full-length *Ovtrpv1* and *Ovtrpv2* cDNA sequences were codon optimised for expression in *H. sapiens* and directly synthesised into pcDNA3.1(+)-N-Myc Vector using Genscript gene synthesis service (Genscript Biotech, UK). The *Hstrpv1* and *Celeosm-9* sequences were synthesised (Integrated DNA Technologies, USA) and cloned into pcDNA3.1 vector through sticky end cloning (EcoRI/XbaI and XhoI/ApaI). The plasmids were transformed into chemically competent bacterial cells (Thermo Scientific™) and the amplified plasmids purified from overnight cultures. These

were then purified using the QIAGEN Plasmid Maxi Kit (QIAGEN) and authenticated by sequencing (Eurofins Genomics). The final construct sequences are available in Supplementary File S5.

B.2.2 Cell growth, transfection, and calcium assay

As TRPV channels are notably non-selective cationic ion channels, known to pass calcium, we adopted a modified version of the protocol described in Thiel *et al.* (2024) to adapt it to calcium ion channels. The cell line utilised was the HEK293G5A, stably expressing the chimeric GFP-Aequorin protein G5A (Cat No. cAP-0200GFP-AEQ-Cyto, Angio-Proteomie). Cells were grown in a T75 flask at 37 °C in 5% CO₂ atmosphere in DMEM (containing 4.5 g/l glucose, L-Glutamine, sodium pyruvate, Cat. No. 10566016, ThermoFisher™) supplemented with 10% FBS (heat inactivated, Cat. No. 10082147, ThermoFisher™). When reaching confluency, cells were detached with 5mL of TrypLE™ (Cat No. 12605010, Gibco™), centrifuged (5 min at 1,500 rpm) and the pellet was then resuspended (1:5 dilution) in fresh DMEM before transferring into Corning™ 96-Well, Cell Culture-Treated, Flat-Bottom Microplates (Cat No. 10517742, ThermoFisher™) and grown for 2 days at 37°C in 5% CO₂ atmosphere. Transfection was carried out by exchanging the cell medium with 90 µl of Advanced DMEM (Cat No. 12491015, Gibco™). For each well, 10 µl OptiMEM (without FBS, Cat No. 11058021, Gibco™), 60 ng of cDNA-containing plasmid, 0.30/100 ng DNA µl 25 kDa branched polyethylenimine (PEI; Cat No. 9002-98-6, Sigma-Aldrich) were mixed and incubated for 20 min at room temperature prior cell transfection in the 96-well plates. Two days post transfection (28°C), the medium was removed and substituted with Hbss medium (Cat. No. 20011, AAT-Bioquest) supplemented with 4 µM coelenterazine-H (Cat. No. S2001, Promega) and incubated for a period of 2 hours. Readings were performed with a FlexStation 3 Multi-mode Microplate reader (Molecular Devices, LLC, USA), for a period of 45 sec per well, ligand injection after 15-18 sec, and the whole plate was read with the Flex option.

Each compound-receptor pair was tested in three independent triplicates, and each receptor was paired with the corresponding empty vector (pcDNA 3.0) to check for potential endogenous activation and to the positive control (pcDNA 3.0-Hstrpv1). As further controls, we used ATP to check for cell viability. Relative luminescence units of cells expressing the genes of interest were normalised to the pcDNA3.0 response and each receptor-compound response was compared to the corresponding receptor-medium response to check for significant differences indicative of activation. Data were analysed using two-way ANOVA. Post-hoc comparisons were performed using the Tukey's multiple comparisons test. A level of probability set at $p < 0.05$ was used as statistically significant. Statistics were performed with GraphPad Prism version 10 for Windows (GraphPad Software, Boston, Massachusetts, USA).

B.2.3 Invertebrate ion channels do not show activation in mammalian recombinant systems

We recorded the luminescence upon calcium release of the cells when exposed to different TRPV relevant cues, namely, capsaicin, acetic acid (pH 5), 1-octanol and anandamide using the *Hstrpv1*-expressing cells as a positive control (Figure B.2A). Cells were all viable and in no case significant endogenous responses were recorded (Figure B.2B) but except for HsTRPV1, no signal was detected to be associated with the administration of any of the compounds neither alone nor when co-expressed (Figure B.2A).

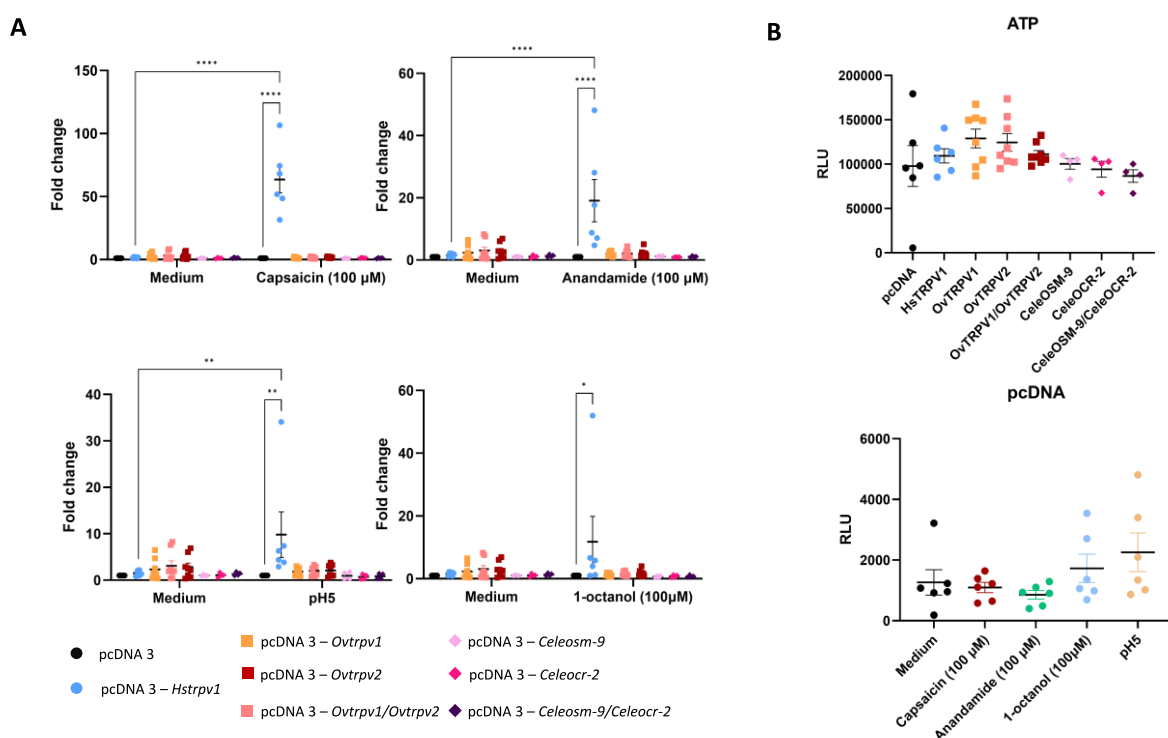


Figure B.2 Invertebrate TRPV receptors do not get activate by any TRPV-relevant stimuli when expressed in HEK293 cells.

A *C. elegans* and *O. vulgaris* TRPV expressing cells did not show any significant increase in luminescence upon administration of a relevant stimulus in the cell medium neither when individually expressed nor when co-transfected. This might suggest absence of key residues for direct gating of these stimuli at the receptors but might also be indicative of issues in expressing these receptors in the HEK293 cells. Data are expressed as fold change with respect to the pcDNA response to which data were normalised. Each receptor-compound response was compared to the corresponding receptor-medium response to check for a significant difference indicative of receptor activation. Statistical analysis was carried out using two-way ANOVA and post-hoc comparisons have been carried out using Tukey's multiple comparison analysis; $p < 0.05$ was set as the threshold for statistical significance. **B** Cells were all viable as shown by the strong signal detected upon ATP administration (top) and the stimuli were checked not to cause any significant endogenous response in the cells by checking activation of empty pcDNA-expressing cells (bottom). Data are represented as relative luminescence unit (RLU) $\pm$ s.e.m.; $p < 0.05$ was set as the threshold for statistical significance.

Appendix C Development of pXeDNA3.1-LacZ, a Dual-System Plasmid for Membrane Protein Expression in *Xenopus* and Mammalian Cells

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C.1 Abstract

Cross-species expression of proteins in mammalian cells and *Xenopus* oocytes remains a powerful strategy for functional studies. This can be facilitated by plasmids with dual promoters, enabling use in both systems without subcloning. We developed pXeDNA3.1-LacZ, a plasmid that supports expression in mammalian cells and oocytes. The construct combines a cytomegalovirus promoter for mammalian transcription with *Xenopus* β -globin untranslated regions to enhance oocyte translation, and incorporates XcmI-mediated TA cloning and blue/white selection. To validate it, we used human TRPV1 (HsTRPV1). The vector drove robust expression in HEK293G5A cells and *Xenopus* oocytes. pXeDNA3.1-LacZ streamlines assays and facilitates studies of membrane proteins.

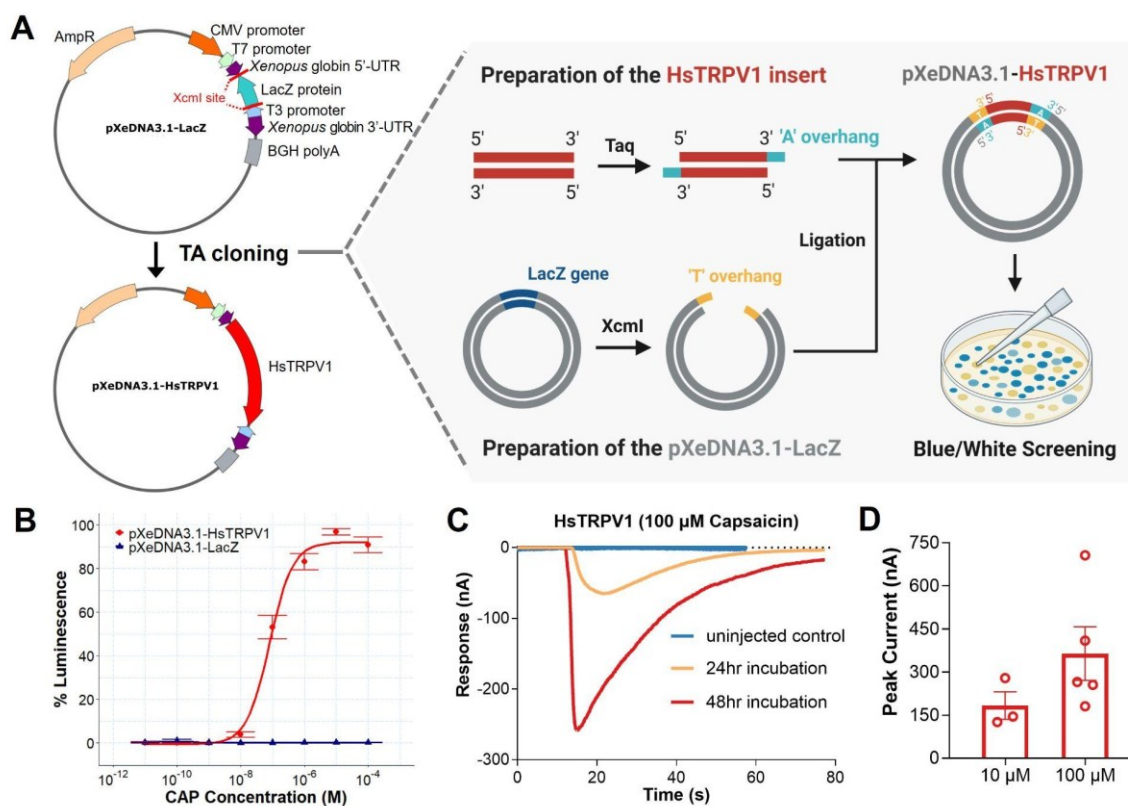


Figure C.1 A. Schematic representation illustrating how the TA cloning strategy can be used to insert gene of interest (HsTRPV1) into the dual-expression vector pXeDNA3.1-LacZ.

The plasmid was linearized by digestion with XcmI to generate T-overhangs, while the HsTRPV1 insert was amplified from U373-MG cell-derived cDNA and subsequently incubated with Taq DNA polymerase to generate complementary A-overhangs. Ligation products were mixed and following ligation the mix transformed into pXeDNA3.1-LacZ and plated onto selective media for blue/white screening. **B.** Dose-response curve obtained from bioluminescence assays in HEK293G5A cells transiently transfected with pXeDNA3.1-HsTRPV1, demonstrating dose dependent receptor activation by capsaicin (CAP). The half-maximal effective concentration (EC₅₀) for capsaicin was determined as 0.103 μ M. Data points represent mean $\pm$ SEM from three independent transfections, each performed in technical triplicates (n = 9 per concentration). **C.** Representative electrophysiological recordings obtained by two-electrode voltage clamp (TEVC) in *Xenopus* oocytes expressing HsTRPV1 24h and 48h after injection, showing characteristic inward currents upon application of 100 μ M capsaicin (CAP). **D.** Peak currents from oocytes expressing HsTRPV1, 48 h post-injection, evoked by 10 μ M and 100 μ M capsaicin. Error bars represent the S.E.M. of three and five replicates, respectively.

C.2 Description

The study of membrane proteins, particularly ion channels and G-protein coupled receptors (GPCRs), requires systems that allow integrated investigation of their structural, functional, and pharmacological properties. These proteins are central to cellular signalling, homeostasis, and disease mechanisms (Hilton *et al.*, 2015; Caterina and Pang, 2016; Balse and Boycott, 2017; Sheng *et al.*, 2017). *Xenopus* oocytes have served as a gold standard for functional expression, offering exceptional utility in electrophysiology, robustness in protein translation, and suitability for high-throughput screening of ion channels and ligand-gated receptors (Dascal, 1987; Stühmer, 1998; Hardege *et al.*, 2023). By contrast, mammalian cell systems, such as HEK293 cells, provide a more physiologically relevant environment for investigating vertebrate signalling pathways, post-translational modifications, and subcellular trafficking (Thomas and Smart, 2005). Relying on a single system can be misleading because of species-specific contexts. For ion channels, mammalian cells better capture human-relevant trafficking and accessory subunit interactions. For example, in *Xenopus* oocytes, endogenous KCNE/MinK-related peptides (MiRPs) can modulate mammalian K⁺ channels, and trafficking-defective hERG mutants may appear functional in oocytes yet are retained in mammalian cells (Ficker *et al.*, 2000; Anantharam *et al.*, 2003; Lin *et al.*, 2010). For GPCRs, G protein-coupled receptor kinase (GRK)/ β -arrestin-dependent desensitisation and internalisation are most faithfully studied in mammalian cells, whereas oocytes often require co-expression of GRKs and β -arrestins to reconstitute these pathways (Kovoor *et al.*, 1997; Kovoor *et al.*, 1998; Celver *et al.*, 2001). However, mammalian systems also have caveats. HEK293 cells exhibit an endogenous proton-gated conductance attributable to hASIC1a, which can confound studies of proton-gated receptors (Gunthorpe *et al.*, 2001). They also express endogenous voltage-gated K⁺ channels (Yu and Kerchner, 1998). These channels recover from closed-state inactivation during the hyperpolarising prepulses used to activate hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels; the subsequent positive tail step evokes outward K⁺ currents that can be misattributed to HCN channels, leading to inaccurate estimates of exogenous activity (Varghese *et al.*, 2006). Together, these examples argue for complementary use of *Xenopus* oocytes and mammalian cells.

Transitioning between these models can be hindered by the need to subclone into separate vectors. Dual-expression plasmids overcome this by enabling side-by-side use of the same construct in *Xenopus* oocytes and mammalian cells. The pXOOM vector established this concept (Jespersen *et al.*, 2002). Unfortunately there is no publicly accessible repository entry for an empty pXOOM backbone, nor a complete, downloadable plasmid map and sequence. Existing Addgene records contain only insert-bearing derivatives of pXOOM with limited documentation.

To mitigate this accessibility gap, we developed a dual-promoter plasmid for seamless expression in mammalian cells and *Xenopus* oocytes. The vector pairs a CMV promoter for robust mammalian transcription with *Xenopus* β -globin 5'/3' UTRs to enhance oocyte translation and mRNA stability (Jespersen et al., 2002). An adjacent T7 promoter positioned immediately upstream of the β -globin 5' UTR enables *in vitro* transcription of RNA for oocyte injection. For *in vitro* transcription, the plasmid can be linearised downstream of the β -globin 3' UTR and its 17 nucleotide polyA stretch, using unique restriction sites (AgeI, ApaI, or PmeI). This ensures that capped transcripts include the complete 3' UTR and its encoded 17-adenosine polyA tail. The bovine growth hormone polyadenylation signal, located further downstream of the *Xenopus* regions, functions specifically in mammalian cells to ensure proper mRNA processing. Whole-plasmid sequencing confirmed the final construct, designated pXeDNA3.1-LacZ (Figure C.1). The plasmid and whole sequence are available in Addgene (ID; 242746).

To validate cross-system performance, we cloned HsTRPV1 into pXeDNA3.1-LacZ (Figure C.1A) and assayed function in the two systems. In the mammalian cell line HEK293, HsTRPV1 activity was monitored using an aequorin-based calcium reporter, in which Ca^{2+} binding to the photoprotein triggers oxidation of coelenterazine and emission of bioluminescence. The stable aequorin-expressing HEK293 cell line used for these assays is referred to as HEK293G5A (Baubet et al., 2000; Thiel, Yañez-Guerra and Jekely, 2023). Since HsTRPV1 is a non-selective cation channel permeable to Ca^{2+} , capsaicin application produces Ca^{2+} influx that is captured by calcium reporters, as shown before (Starkus et al., 2019). Capsaicin was tested over a concentration range of 10^{-4} to 10^{-11} M, producing graded bioluminescent signals corresponding to Ca^{2+} influx, from which a concentration–response curve with an EC_{50} of 0.103 μM was derived (Figure C.1B). This value falls within the range of those previously reported for HsTRPV1 expressed in HEK293 cells ($\text{pEC}_{50} \approx 6.8$; $\text{EC}_{50} \approx 0.158 \mu\text{M}$; Lam, McDonald and Lambert, 2005) and comparable to values obtained in CHO cells where HsTRPV1 was expressed using a mammalian-only expression vector ($\text{EC}_{50} = 0.53 \mu\text{M}$; McIntyre et al., 2001). The addition of capsaicin in the empty-vector transfected HEK293G5A cells produced no signal (Figure C.1B). To assess function in *Xenopus laevis* oocytes, we used two-electrode voltage clamp (TEVC). The addition of capsaicin (100 μM) to uninjected oocytes produced no detectable response (Figure C.1B). However, robust inward currents were observed upon application of 10 μM and 100 μM capsaicin, 24 and 48 hours post-injection (Figure C.1C-D). At 48 hours, the mean peak current amplitudes were $0.18 \pm 0.05 \mu\text{A}$ (10 μM) and $0.36 \pm 0.09 \mu\text{A}$ (100 μM ; values represent mean $\pm$ SEM). These values fall within the 0.1–15 μA range previously reported for rat TRPV1 expressed in oocytes and activated with 15 μM capsaicin (Rivera-Acevedo et al., 2013). Because no equivalent quantitative data exist for human TRPV1 in oocytes, we used the Rivera-Acevedo et al. dataset as a comparison, as it defines the expected range of TRPV1 current amplitudes across expression

levels under systematically varied mRNA concentrations and incubation times. Our amplitudes are near the lower threshold of this range, corresponding to moderate expression levels, as reported by Rivera-Acevedo *et al.* for rat TRPV1, but remain well within the expected variability.

Our plasmid enables direct expression of identical constructs in both *Xenopus* oocytes and mammalian cells, avoiding additional subcloning and allowing functional differences to be attributed to the biological systems rather than to vector design. It also includes practical features such as TA cloning and blue/white screening, and we provide the full sequence and annotated map. Both the empty backbone and validation plasmid are freely available through Addgene (ID 242746), ensuring transparency and broad accessibility for future use.

A potential limitation of this study is that we tested the plasmid with only one receptor; HsTRPV1. However, our plasmid contains all the necessary regulatory elements for expression in both systems, as described above: a CMV promoter and bovine growth hormone (BGH) polyadenylation signal for mammalian expression, and *Xenopus* β -globin 5' and 3' untranslated regions (UTRs) for oocyte expression, followed by a 17-adenosine poly(A) stretch downstream of the 3' UTR. This 17-adenosine tail lies above the minimal length required for high-affinity binding of cytoplasmic poly(A)-binding protein (PABPC), which typically needs a minimum of ~12 adenosines for stable interaction through RNA-recognition motifs 1 and 2 facilitating the translation (Mangus, Evans and Jacobson, 2003; Kühn and Wahle, 2004). However, in *Xenopus* oocytes, extending poly(A) tails has been shown to further enhance mRNA stability and translation; for example, artificial elongation of the *c-mos* tail directly activated its translation and meiotic maturation (Barkoff, Ballantyne and Wickens, 1998). Moreover, extending poly(A) tails on synthetic RNAs enhances their persistence after injection into *Xenopus*: capped, polyadenylated transcripts show markedly greater stability, with long (~200 nt) tails roughly doubling mRNA half-life compared with short-tailed or non-adenylated RNAs (Drummond, Armstrong and Colman, 1985; Colman and Drummond, 1986; Harland and Misher, 1988). While *in vitro*-transcribed RNA generated directly from our plasmid was sufficient to support robust TRPV1 expression in *Xenopus*, users who observe low expression in their own constructs should consider post-polyadenylating the transcripts using bacterial poly(A) polymerase (e.g. NEB M0276 or Thermo Fisher Poly(A) Tailing Kit), as extending the poly(A) tail can enhance mRNA stability and translation efficiency.

C.3 Methodology

C.3.1 Construction of a Dual-Expression Vector

A synthetic cassette was designed and synthesised by Genscript, consisting of a T7 promoter followed by the *Xenopus* β -globin 5' UTR, a first XcmI site, a reverse-oriented LacZ cassette containing a multiple cloning site (MCS) with several restriction enzyme sites, a second XcmI site to enable TA cloning, and the *Xenopus* β -globin 3' UTR. This cassette was initially cloned into the pUC57 vector. The insert was then excised using SacI and AgeI and subcloned into the pcDNA3.1(+) backbone, which provides a CMV promoter upstream of the MCS and a bovine growth hormone (bGH) polyadenylation signal downstream. Colonies with successfully cloned LacZ fragments produced blue colonies on LB agar plates supplemented with ampicillin and 40 μ g/mL X-gal. Following bacterial culture and plasmid miniprep, the construct (pXeDNA3.1-LacZ) was verified by whole-plasmid sequencing (Eurofins). The complete plasmid sequence is provided as an Extended Data file in FASTA format, and annotated maps were generated using SnapGene software (GSL Biotech, version 8.1.1).

C.3.2 Cloning of Human TRPV1 Gene into pXeDNA3.1-LacZ

The HsTRPV1 gene was amplified from cDNA derived from U373-MG cells. The primers used for amplification were: forward primer (5'-ACCATGAAGAAATGGAGCAGC-3') and reverse primer (5'-TCACTTCTCCCCGGAAGCG-3'). PCR was performed using VeriFi™ Red PCR Master Mix (PCRBIO, Cat. No. PB10.41). Following amplification, 1 μ L of Taq DNA polymerase (NEB, Cat. No. M0273) was added directly to the PCR reaction mixture and incubated at 72 °C for 15 minutes to add adenine (A) overhangs required for subsequent TA cloning. The pXeDNA3.1-LacZ vector was linearised using XcmI (NEB, Cat. No. R0533), generating complementary thymine (T) overhangs. Digestion was confirmed by agarose gel electrophoresis. The linearised vector was purified using the Monarch® DNA Gel Extraction Kit (NEB, Cat. No. T1020), following the manufacturer's protocol. The PCR-amplified HsTRPV1 insert was then ligated into the linearised pXeDNA3.1-LacZ vector using T4 DNA ligase (NEB, Cat. No. M0202). The ligation mixture was transformed into competent *E. coli* cells and plated onto LB agar plates supplemented with ampicillin and X-gal for blue/white screening. We named this vector "pXeDNA3.1-HsTRPV1".

C.3.3 Bioluminescence Assay in HEK293G5A cells

The full protocol for receptor deorphanisation in HEK cells has been described previously and scaled for high-throughput GPCR screening (Thiel, Yañez-Guerra and Jékely, 2023; Thiel et al., 2024, Yanez-Guerra et al., 2025). Here, we adapt that workflow for ion-channel assays and summarise the protocol used in this study below. HEK293 cells stably expressing the chimeric

GFP–Aequorin protein G5A (HEK293G5A) were cultured in DMEM supplemented with 4.5 g/L glucose, L-glutamine, sodium pyruvate, and 10% FBS under 5% CO₂ at 37 °C (Gibco. Cat No. 11995073). Cells were seeded into white, clear-bottom 96-well plates and grown for two days. At ~90% confluency, transfection was performed using polyethyleneimine 1mg/ml (PEI, MW 40,000; Polysciences, Cat. No. 24765). For transfection, the culture medium was exchanged for 90 µL of Advanced Medium supplemented with 5% FBS. A DNA–PEI complex was prepared by mixing 60 ng of the pXeDNA3.1-HsTRPV1 plasmid DNA with 0.35 µL PEI in 10 µL Opti-MEM (without serum or phenol red) per well, incubated for 10 minutes at room temperature, and then added to the wells. Cells were returned to the incubator and allowed to express for 48 h. Two days post-transfection, the medium was replaced with 50 µL per well of 1× HBSS (140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 6 mM glucose, 4 mM NaHCO₃) without phenol and without FBS, supplemented with 4 µM coelenterazine H (Promega Cat. No. S2011). Cells were incubated in the dark at 37 °C for 2 h to allow aequorin loading. During this incubation, a separate reagent plate was prepared containing capsaicin serially diluted in 1× HBSS across the concentration range 10⁻⁴ to 10⁻¹¹ M. Following the incubation period, bioluminescence measurements were performed on a FlexStation 3 Multi-Mode Microplate Reader, which automatically dispensed the prepared capsaicin dilutions into the wells containing aequorin-loaded cells. Luminescence signals, corresponding to Ca²⁺ influx, were recorded in real time. Raw luminescence data were converted into tibble format and analysed in R using the drc package to fit dose–response curves and estimate EC₅₀ values. Data were generated from three independent transfections (biological replicates), each performed on separate plates, and for each concentration three wells were assayed (technical replicates). Thus, each concentration point reflects the mean of nine measurements. Data processing followed the publicly available R script published before [\(Yañez Guerra and Zandawala, 2023\)](#), which can be accessed via GitHub at

https://github.com/Imnotabioinformatician/Branchiostoma_Dose_response_curves.

C.3.4 Electrophysiology of *Xenopus* Oocytes

Defolliculated *Xenopus laevis* oocytes were obtained from EcoCyt Bioscience and maintained in ND96 solution as follows (in mM): 96 NaCl, 1 MgCl₂, 5 HEPES, 1.8 CaCl₂, and 2 KCl. The pXeDNA3.1-HsTRPV1 plasmid was linearised using *PmeI* (Thermo Fisher Scientific Cat. No. ER1341), followed by DNA purification (Zymo Cat. No. D4013) and elution in RNAase free water. cRNA was synthesised using the T7 mMessage mMachine transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocol (with overnight incubation at 37 °C). RNA was purified using the GeneJET RNA purification kit (Thermo Fisher Scientific) and quantified using a NanoDrop spectrophotometer. Oocytes were injected with 50 nl of 500 ng/µl RNA using the

Roboinject system (Multi Channel Systems). Injected oocytes were incubated at 18 °C in ND96 solution until the day of recording, either 1 or 2 days postinjection.

Two-electrode voltage-clamp (TEVC) recordings were conducted using the Roboocyte2 System (Multi Channel Systems). Measuring head electrode resistance was approximately 400-1200 k Ω , pulled on a P-97 Micropipette Puller (Sutter Instrument). Electrodes contained AgCl wires backfilled with a 1 M KCl and 1.5 M KAc mixture. Oocytes were clamped at -60 mV during continuous recording at 500 Hz. 100 mM stock of capsaicin was made in ethanol. Capsaicin application lasted for 7 s, followed by a 60 s wash with ND96, perfusion speed was set to approximately 7 ml/min throughout. Two batches of oocytes were used: one batch for recordings after 1 d incubation and another batch for recordings after 2 d incubation. Uninjected oocytes were also tested after 1 d incubation and did not respond to capsaicin. Data was analysed using the Roboocyte2+ software and plotted in GraphPad Prism.

C.3.5 Reagents

STRAIN/CELL LINE	DESCRIPTION	AVAILABLE FROM	CAT NO.
<i>Xenopus laevis</i> oocytes	Stage V-VI oocytes for TEVC recordings	EcoCyte Bioscience	
HEK293G5A cells	Human embryonic kidney cells stably expressing GFP-Aequorin chimera	Angio-proteomie	cAP-0200AEQ-Cyto
U373-MG cells	Human glioblastoma astrocytoma cell line (source of HsTRPV1 cDNA)	ATCC	HTB-17
REAGENT/ENZYME	DESCRIPTION	AVAILABLE FROM	CAT NO.
XcmI	Restriction enzyme used for linearization	NEB	R0533
SacI	Restriction enzymes used for cloning	NEB	R0156
AgeI	Restriction enzymes used for cloning	NEB	R3552
PmeI	Restriction enzyme used for plasmid linearisation	Thermo Fisher Scientific	ER1341
T4 DNA ligase	Ligation of HsTRPV1 insert	NEB	M0273
Taq DNA polymerase	Used to add A-overhangs for TA cloning	NEB	M0202
VeriFi™ Red PCR Master Mix	High-fidelity PCR mix	PCRBIO	PB10.41
Monarch® DNA Gel Extraction Kit	DNA purification	NEB	T1020
DNA Clean & Concentrator-5	DNA clean up	Zymo	D4013
mMESSAGE mMACHINE T7 Kit	cRNA synthesis	Thermo Fisher Scientific	AM1344
GeneJET RNA Purification Kit	RNA purification	Thermo Fisher Scientific	K0791
REAGENT/CHEMICAL	DESCRIPTION	AVAILABLE FROM	CAT NO.
Polyethylenimine (PEI)	Transfection reagent	Polysciences	24765
Coelenterazine.H	Aequorin substrate for bioluminescence assay	Promega	S2011
Capsaicin	HsTRPV1 agonist	Sigma-Aldrich	M2028
X-gal	Substrate for blue/white screening	Sigma-Aldrich	B4252

Ampicillin	Selection antibiotic	Sigma-Aldrich	A9518
PLASMID	CONSTRUCT	AVAILABLE FROM	ID
pXeDNA3.1-LacZ	CMV promoter, <i>Xenopus</i> beta-globin UTRs, LacZ XcmI sites	Addgene	242746
pXeDNA3.1-HsTRPV1	Human TRPV1 cloned into pXeDNA3.1-LacZ	Addgene	244454

C.3.5 Data availability

Annotated SnapGene map; and analysis code are deposited at Addgene (pXeDNA3.1-LacZ, #242746; pXeDNA3.1-HsTRPV1, #244454). Raw data for the traces is available upon request.

C.3.6 Ethics

Defolliculated *Xenopus laevis* oocytes were purchased from EcoCyte Bioscience and used in accordance with institutional and national regulations. No procedures on live animals were performed at our institution.

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