University of Southampton

Faculty of Environmental and Life Sciences

Biological Sciences

The nicotinic acetylcholine receptor EAT-2, a novel target for mitigating parasitic nematode infections

by

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Abstract

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Plant parasitic nematodes (PPNs) are worms that infect all major crops and cause yield declines valued above US$150 billion annually. To mitigate the damage caused by these parasites, control strategies have relied on synthetic chemical nematicides that are effective in keeping infection below disease thresholds.

The determinants of the nervous system that underpin key parasitic behaviours have been successful targets for pest mitigation. This includes nicotinic acetylcholine receptors (nAChRs), fast acting ligand gated ion channels that underpin key nerve and nerve-muscle physiology. They offer selective targets for naturally occurring and synthetic chemicals that effectively control pests in agriculture and veterinary medicine.

In this study I investigated EAT-2, a nAChR that regulates feeding in *Caenorhabditis elegans* as a target to mitigate PPNs. EAT-2 plays an important role, it has a non-classical agonist binding site that makes it unique among members of the acetylcholine gated cation channels, and it has a unique dependence for association with the EAT-18 auxiliary protein for function. Thus, I hypothesized that EAT-2 is potentially a selective pharmacophore for mitigating PPNs

I initiated the study by establishing the presence of EAT-2 in PPNs and then utilized pharmacological evidence to suggest that EAT-2 regulates stylet thrusting in the PPN *Globodera rostochiensis*. Stylet thrusting is a parasitic behaviour required for hatching of infective juveniles (J2s), root invasion, and the establishment of feeding sites. This highlights that pharmacological modulation of EAT-2 affords a route to disrupt the parasitic behaviour and potentially break the lifecycle.

I used *C. elegans* as an experimental platform to identify EAT-2 modulators by screening 192 compounds. This identified monepantel and 3 analogues, an analogue of epibatidine and rotenone as inhibitors of pharyngeal function. I validated this using functional expression of the EAT-2/EAT18 receptor in *Xenopus* oocytes and showed that these hits were antagonists of the receptor. Moreover, when exposed to J2s of *G. rostochiensis*, these hits inhibited stylet thrusting. Although these lead compounds bind EAT-2 they have broader efficacy across sub-types of nAChRs. However, the hits resolved by the screen and subsequent validation are new EAT-2 compounds that occupy this unique agonist binding site. Overall, the screening approach identified compounds that suggest that EAT-2 is a bona fide target for PPN mitigation. EAT-2 pharmacology holds promise for novel nematicidal potential.

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Research Thesis: Declaration of Authorship

Print name: Henry Atemnkeng Nvenankeng

Title of thesis: The nicotinic acetylcholine receptor EAT-2, a novel target for mitigating parasitic nematode infections

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. None of this work has been published before submission

Signature: ..... Date: .....

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Definitions and Abbreviations

5-HT ..... 5-Hydroxytryptamine/ serotonin

ACh ..... Acetylcholine

AChE..... Acetylcholinesterase

ANOVA ..... Analysis of variance

BAPTA-AM..... 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester)

BSA ..... Bovine serum albumin

^{o}C ..... Degrees celsius

CGC ..... *Caenorhabditis elegans* genetics centre

CN ..... Cyst nematode

ddH_2O ..... Double distilled water

dH_2O ..... Distilled water

DMSO ..... Dimethyl sulfoxide

EU ..... European Union

EPG ..... Electropharyngeogram

FLP ..... FMRFamide-like peptide

G-protein ..... Guanine nucleotide-binding proteins

GABA ..... Gama-aminobutyric acid

GFP ..... Green fluorescent protein

GluCl ..... Glutamate-gated chloride channel

GPCR ..... G-protein-coupled receptor

HEPES ..... 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HPLC ..... High performance liquid chromatography

J1 ..... First-stage juvenile

J2 ..... Second-stage juvenile

J3 ..... Third-stage juvenile

J4 ..... Fourth-stage juvenile

L1 ..... Larval stage one

L2 ..... Larval stage two

L3 ..... Larval stage three

L4 ..... Larval stage four

LB ..... Luria Bertani (Broth/ Agar)

L4+1 ..... Larval stage four worms plus one day old

M ..... Molar

mm ..... Millimeter

mM ..... Millimolar

mV ..... Millivolt

nAChR ..... Nicotinic acetylcholine receptor

NGM ..... Nematode growth medium

NLP ..... Neuropeptide-like protein

nM ..... Nanomolar

NMJ ..... Neuromuscular junction

PCN ..... Potato cyst nematode

PPNs ..... Plant parasitic nematodes

RKN ..... Root knot nematode

RNAi ..... RNA interference

SEM ..... Standard error of the mean

Spp. ..... Species

µm ..... Micrometer

µM ..... Micromolar

WHO ..... World health organisation

WT ..... Wild type

Chapter 1 General Introduction

1.1 Nematodes, a menace to crops and animals

Nematodes are among the most successful animals because of their biochemical adaptations that allow them to exist in extreme environments (Sapir, 2021). There are over 27,000 described nematode species made up of free-living, predators, mutualists, animal parasites and plant parasitic nematodes (Kiontke and Fitch, 2013). Animal parasitic nematodes have a global distribution, causing disease in companion animals, livestock and humans, whilst plant parasitic nematodes (PPNs), the focus of this project, are a serious problem world-wide to agricultural productivity (Dold and Holland, 2011).

Plant parasitic nematodes (PPN) are microscopic nematode pests that attack the roots of host plants, impacting their viability and in the case of crop plants their productivity. Their global economic and financial losses are estimated to be around $US157 billion annually (S. Singh et al., 2015). Since PPNs are microscopic, soil-borne parasites that cause rather unspecific symptoms, they are often misdiagnosed as other plant diseases (Coyne et al., 2018). Expert knowledge of their signs and symptoms is needed for correct diagnosis and treatment (Mokrini et al., 2023).

Addressing the threat posed to food security by PPNs is an increasingly urgent problem as the methods that are used to control them typically involve the application of chemicals, i.e., nematicides, that have detrimental effects to the environment. In this regard, regulatory authorities are removing approval for the use of some of the most effective control methods leaving crop production at risk (EU Commission, 2023). This provides an impetus to discover new, more selective and environmentally compatible chemicals to control PPNs.

To contextualize the problem, I will present a broad introduction to the Nematoda and then describe the biology and ecology of PPNs. It will elaborate on the interaction of PPNs with their host plants and the damage that they can cause, with a particular focus on the potato cyst nematode *Globodera rostochiesis* which is used experimentally in this project. The history and current state of PPN control through nematicides and other treatments will also be discussed. The use of the model nematode *Caenorhabditis elegans* as a tractable system to investigate more broadly the physiology and function of nematodes and as a platform for nematicide discovery and mode of action studies will be introduced. As many anthelmintics and

nematicides are known to target neural signalling pathways (see Holden-Dye and Walker (2014) for review), PPN and *C. elegans* neurobiology will be discussed. Finally, a nicotinic acetylcholine receptor EAT-2 will be introduced as a potential new target for nematicide discovery to mitigate PPNs providing the overarching rationale for this project.

1.2 The phylum Nematoda

The phylum Nematoda, commonly referred to as roundworms, is a diverse group of bilaterally symmetrical invertebrate worms that are unsegmented. They are found occupying a wide range of environments from marine and freshwater habitats to soils. There exist over 27,000 described nematode species made up of free-living, predators, mutualists, animal parasites and plant parasitic nematodes (Kiontke and Fitch, 2013). Their diverse reproductive strategies (hermaphroditism, parthenogenesis, and amphimixes), high fecundity rates, short reproductive cycles, cryptobiotic abilities, and their presence in all major trophic levels of the soil food web make them successful (Sorribas et al., 2020; Neher and Powers, 2022).

Arguably, most of our knowledge concerning the physiology, pharmacology and biochemistry of nematodes has been provided by extensive investigations of just two species of nematode. The large ~30 cm long gastrointestinal animal parasite *Ascaris suum* can be obtained from abattoirs and maintained in the laboratory for up to one week. It has been widely used in physiological investigations and is not discussed further here, but the interested reader is referred to this review by Wolstenholme et al. (2024).

The other most informative model is the free-living nematode *C. elegans*. Free-living nematodes are non-parasitic roundworms that feed on bacteria, fungi, algae and other dead organisms and are the most abundant nematodes in aquatic and terrestrial environments (Iqbal and Jones, 2017). *C. elegans* is a bacterial feeder that lives in the soil and on rotting fruit. In the early 1960s, Sydney Brenner chose *C. elegans* as a model animal to study animal development, genetics and behaviour (Ahringer, 2006).

1.3 General perspectives on nematode body plan

All nematodes have a general basic plan, composed of two concentric tubes that are separated by a fluid-filled space known as a pseudocoelom (Riddle et al., 1997). Their cylindrical body is covered by a tough cuticle that they moult in different stages of their lifecycle (Blaxter and Koutsovoulos, 2014). The inner concentric tube is made up of the pharynx, the alimentary system, the nervous system, and a reproductive system with gonad arms that run from the mid-body towards the anterior and posterior ends in an adult hermaphrodite (Scharf et al., 2021).

The outer tube, also known as the body wall, consists of a hypodermis that secretes the cuticle, as well as secretory-excretory systems, neurons, and muscles. Separating both tubes is a pseudocoelomic cavity, which is fluid-filled and contains scavenger cells called coelomocytes. Coelomocytes are six non-muscular mesodermal cells known to endocytose low molecular weight dyes and proteins, accumulate macromolecules from body cavity fluids, absorb and concentrate soluble materials and destroy invasive pathogens (Yanowitz and Fire, 2005; Tahseen, 2009). In general, food gets into the worm through the pharyngeal system, where it is broken down and passed through the alimentary system for digestion, absorption and defecation. Together, these systems, play important roles in the life of the worm.

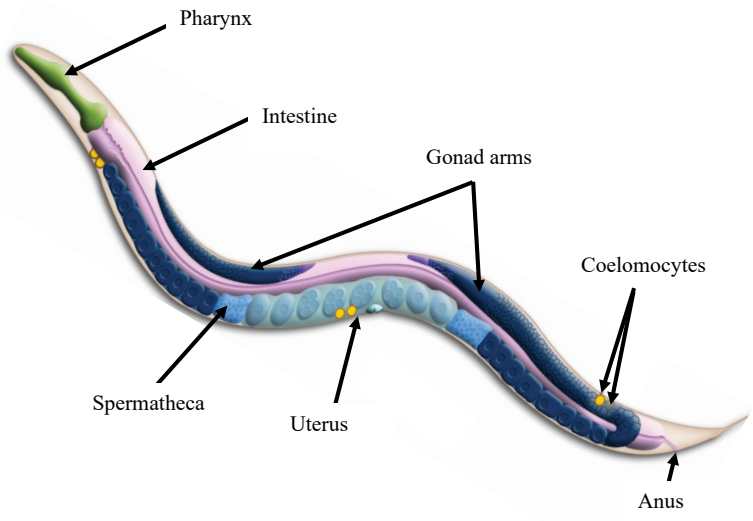

Fig. 1. 1 Basic anatomy of an adult hermaphrodite *C. elegans*. This cartoon exemplifies the body plan, showing the pharyngeal region, digestive tract and reproductive organ. Adapted from (WormAtlas)

1.4 General perspectives on nematode life cycle

The general organisation of the life cycle of nematodes shows a very similar pattern despite their very different lifestyles and habitats (Haag et al., 2018). The precise timings and life span may vary significantly but it is useful to use the model organism *C. elegans* to introduce the basic concepts. Young L1 juveniles hatch out of eggs, feed and develop through four larval stages (L1-L4) to adults. The end of each developmental stage is characterized by a period of quiescence called lethargus, when a new cuticle is made, and it terminates with the moulting of the old cuticle (Raizen et al., 2008). Adult hermaphrodites can lay over 300 self-fertilized eggs during the first 5 days of adulthood, after which only oocytes can be produced. Hermaphrodites can produce approximately 300 spermatocytes during their lifetime (Bahrami and Zhang, 2013). However, when mated by males, they can produce up to 1000 more fertilized

eggs. *C. elegans* has a relatively short life cycle, which takes about 2.5 to 4 days depending on environmental conditions (Porta-de-la-Riva et al., 2012). In normal populations they are mostly hermaphrodites, although males can occur at a frequency of 0.2%. During fertilization in adult hermaphrodites, an oocyte passing through the spermatheca may be fertilized by a spermatocyte and the resulting zygote temporarily resides in the oviduct until about the 24-cell stage, when the egg is laid (Ahringer, 2006). In adverse conditions like food depletion, overcrowding, and high temperatures, L1 larva can moult to a pre-dauer (L2d) larva, then to a dauer larva (Hu, 2007). A dauer is a special facultative stage when the juvenile becomes more resistant to environmental stressors, has an altered metabolism (reduced) and a special morphology (Hu, 2007). During this period, the cuticle that surrounds the young juvenile blocks the buccal cavity and prevents the animal from feeding, thereby arresting development (Riddle et al., 1979). When favourable conditions are restored, dauers shed their mouth plugs, commence feeding and exit the dauer stage through a moult into L4 larvae (Ahringer, 2006). Notably, all nematodes progress through four larval stages to reach adult, and many appear to have a dauer-like stage similar to *C. elegans*.

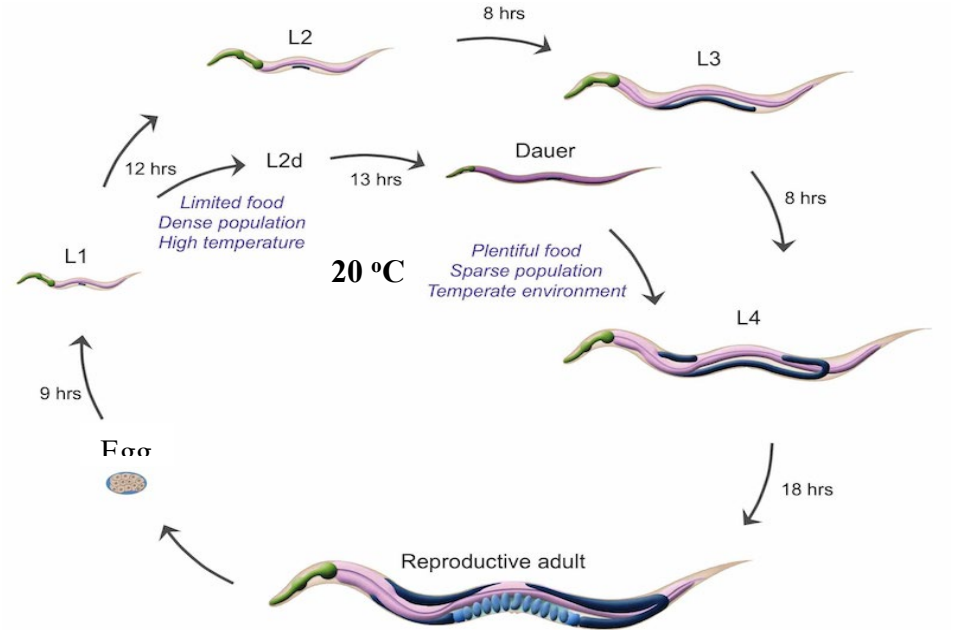

Fig. 1. 2 The nematode life cycle using *C. elegans* as an example. Egg develops through four larval stages (L1 through L4) into a reproductive adult. The end of each larval stage is characterised by a moult into the next developmental stage. In stress conditions like overcrowding, food depletion or elevated temperatures, L1 larvae may enter an alternative developmental pathway, resulting to dauer formation. Dauers have adapted to survive these unfavourable conditions. When suitable conditions return, dauer larvae may exit this diapause

state and re-enter reproductive development through a moult into L4 larvae. Adapted from (Wolkow and Hall, 2015).

1.5 Animal parasitic nematodes

Animal parasitic nematodes infect animals, including millions of humans globally (Dold and Holland, 2011). They infect muscles, eyes, the alimentary canal and several other body tissues (Iqbal and Jones, 2017). Human parasitic nematodes such as ascaris, hookworms, pinworms, filarial worms, and whipworms, gain access to human bodies through consumption of infected meat, the skin, or from bites of infected vectors like mosquitoes that carry filarial worms (Iqbal and Jones, 2017). About 3.5 billion people in developing and tropical countries are infected by nematodes annually (Iqbal and Jones, 2017). Depending on the nematode species, different life stages may be interrupted to disrupt parasitism. In the case of *A. suum*, a parasite of pigs, the infective stage is the embryonated egg carrying the L3 larval stage. *Ascaris* spp develops from eggs, through four larval stages to adults and unlike other nematodes that hatch during the second juvenile stage and moult into other life stages, *Ascaris* spp hatch at the third juvenile stage (L3) (Dold and Holland, 2011). Several anthelmintic drugs have been used to control these parasitic worms. There are four main anthelmintic drugs used to control nematode parasites of humans: pyrantel pamoate, albendazole, mebendazole, levamisole and ivermectin with some newer anthelmintics, like emodepside, derquantel and tribendimidine (Abongwa et al., 2017; Liu et al., 2020).

1.6 Plant parasitic nematodes

Plant parasitic nematodes (PPNs) are obligate parasites of plants that feed on most vascular plant parts and inflict a significant burden to crops if they are not properly managed (Fuller et al., 2008). There are over 4,100 described species of PPNs (Decraemer and Hunt, 2006) and collectively, they present an important constraint on the delivery of global food security (Hassan et al., 2013; Coyne et al., 2018). They invade host plants via the roots or other below ground structures like tubers and bulbs. Once in the plant, they draw off water and nutrients meant for the plant and cause severe stress and growth disruptions, root galling, scabbing of tubers and yellowing of leaves (chlorosis) (Sikder and Vestergård, 2020).

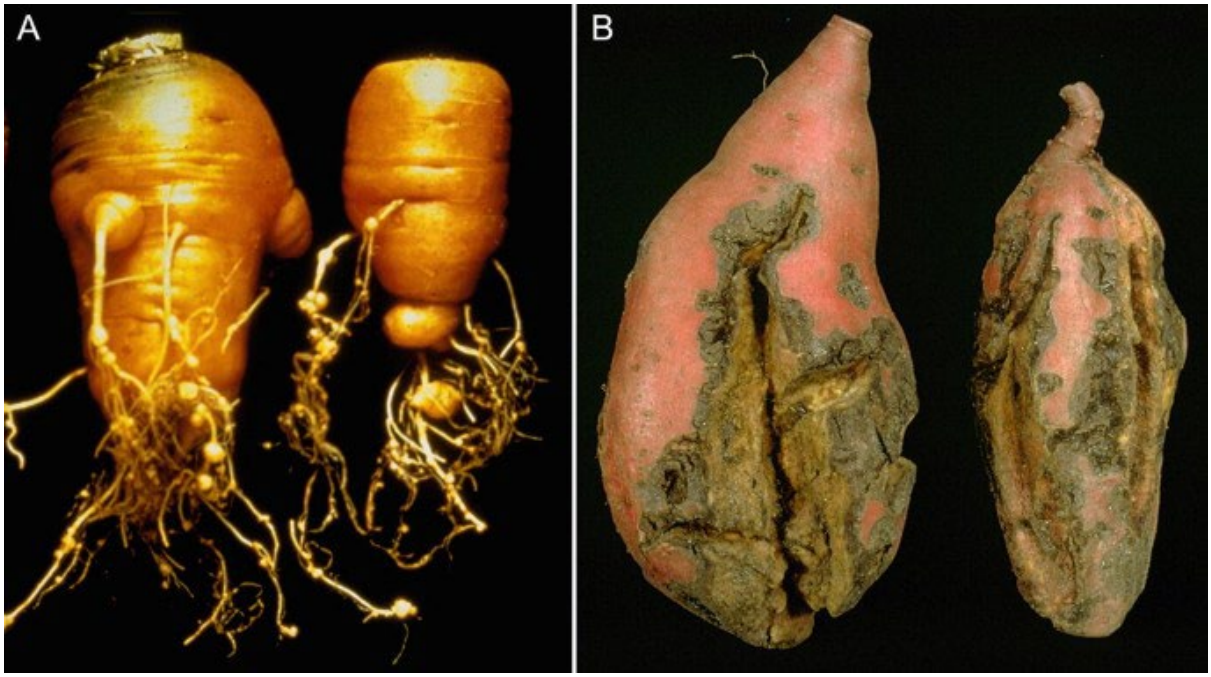

Fig. 1. 3 Root and tuber damage caused by plant parasitic nematodes. A. Carrot root forking caused by *Meloidogyne hapla* forking and **B.** cracking and scabbing of sweet potato tuber by *Rotylenchulus* spp. From (Chitambar et al., 2018)

The main symptom of PPN presence is stunted growth due to the disruption of osmotic pressure and nutrient diversion to the nematode (Fuller et al., 2008). The distinct lifestyles of PPNs classify them as ectoparasitic, migratory endoparasitic, sedentary endoparasitic and semi-endoparasitic (Topalović and Vestergård, 2021).

1.6.1 Ectoparasites

Ectoparasitic PPNs feed from the outside of host plants (**Fig. 1. 5**) by thrusting their very long withdrawable stylets and, in some cases the head and neck region into the root system of their host, as they can access food (Bogale et al., 2020). Some examples are *Xiphinema* (dagger nematodes), *Longidorus* (needle nematodes), *Trichodorus* (stubby-root nematodes), *Helicotylenchus* (spiral nematodes), *Criconemoides* (ring nematodes), *Paratylenchus* (pin nematodes) and *Tylenchorhynchus* (stunt nematodes). They spend their entire lives on the outside of the plant and cause severe damages at higher population densities (Branch and Brenneman, 2015). Besides draining the host plant of valuable nutrients, some of these ectoparasites are vectors of economically important plant viruses. Nepoviruses and tobraviruses are reportedly transmitted by the Longidorids, Trichodorus and Paratrichodorus (Singh et al., 2020).

1.6.2 Migratory endoparasites

Migratory endoparasitic PPNs are facultative or obligate parasites that invade plant roots and move freely through the cortical tissues, feeding destructively as they move (**Fig. 1. 5**) (Espada et al., 2025). They are incapable of forming feeding sites within their hosts, so they use their robust stylets to continuously burrow through root tissues, puncturing cells and extracting nutrients (Perrine-Walker, 2019). Cell wall modifying enzymes secreted from their pharyngeal glands facilitate the softening of cell walls, making migration easy within intracellular spaces and rupturing of cells to make their content more accessible (Mathew and Opperman, 2020). Nematodes in this group include: *Pratylenchus* spp., *Ditylenchus* spp., *Bursaphelenchus* spp. Endoparasitic feeding behaviours have been classified into 3 distinct stages in *Pratylenchus penetrans.* First is the migration to apical regions of the root, probing of local epidermal cells and initiation of stylet thrusting (Sirpa and Thierry, 1985; Zunke, 1990). In the second stage, the nematode penetrates the root by puncturing through the cell wall with the stylet and pressure from the labial region facilitates this penetration. The final stage involves feeding and the release of saliva containing effectors and cell wall-modulating enzymes to ease the process (Mathew and Opperman, 2020). These activities cause severe necrosis and lesions that disrupt root function, making the plant more susceptible to fungal and bacterial infections (Espada et al., 2025).

1.6.3 Sedentary endoparasites

Sedentary endoparasitic PPNs are highly specialized PPNs that invade host roots and establish permanent feeding sites. Root-knot nematodes (RKNs) of the genus *Meloidogyne* and cyst nematodes (CNs) of the genera *Globodera* and *Heterodera* are the most well-known. Based on the damages caused by these parasites, they are considered the most economically important

Meloidogyne spps. widely referred to as RKNs are obligate parasites of plants known to parasitize almost every vascular plant species (Jones et al., 2013). Second-stage juveniles (J2s) penetrate root tips and migrate intracellularly to the vascular cylinder where they establish feeding sinks consisting of several multinucleated giant cells (Jones et al., 2013). Increased metabolic activities in giant cells mobilize products of photosynthesis from the shoots, making more nutrients available for the parasite (Hofmann and Grundler, 2007). The tissues surrounding these giant cells undergo hyperplasia and hypertrophy, giving the appearance of knots (Eisenback, 2020). They get their name from the galls (root-knots) they induce on roots of their host plants (**Fig. 1. 4**).

Fig. 1. 4 Damage on roots caused by *Meloidogyne* spp. A). Galls on roots of a tomato plant grown in Ethiopia (Photograph courtesy of Seid Awol, Ghent University, Belgium) **B).** Galls on parsley (Photograph courtesy of Grahame Jackson, Bondi Junction NSW, Australia).

As the nematodes feed and develop, females swell, become sessile and release egg masses into the soil (**Fig. 1. 6**B) (Karssen and Moens, 2006). Males do not swell but exit the gall into the soil where they mate with females, for species that reproduce sexually (Eisenback and Triantaphyllou, 2020).

CNs, primarily *Globodera* and *Heterodera* species, are obligate biotrophs and economically important pests of potato, cereal, and soybean (Jones et al., 2013). Originating from South America, CNs have spread and become prevalent in most potato-growing regions globally (Hockland et al., 2012). Infective juveniles invade hosts through the roots, migrate intercellularly and establish specialized feeding structures called syncytia in the vascular tissues. Effectors secreted by the nematodes induce the fusion of neighbouring cells to form a large multinucleate food sink called a syncytium. The nematode remains stationary, feeds from the specialized structure and develops into adults. Females grow till their bodies break out of the root, while males remain vermiform and exit the root to find female sex mates.

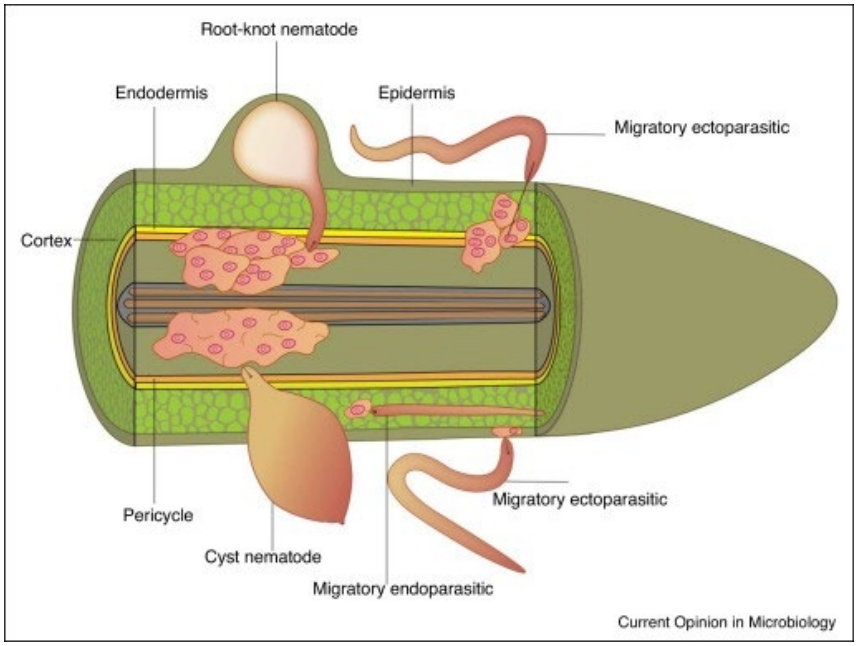

Fig. 1. 5 Different lifestyles of plant parasitic nematodes. Migratory endoparasites burrow through the root as they feed. Semi-endoparasites embed a third of their bodies into plant roots and obtain nutrients from endodermal cells. Sedentary endoparasites are sessile with their stylets wedged into a giant cell from which they obtain nourishment. Migratory ectoparasites live in the soil and parasitize plant roots by inserting their long stylets into the cells of their hosts from the outside. From (Siddique and Grundler, 2018).

1.6.4 Semi-endoparasites

Unlike ectoparasites, semi-endoparasitic PPNs retain part of their bodies on the outside of the host plant. This group includes the reniform nematode *Rotylenchulus* spp., a pest of cotton, soybean and vegetables. Reniform nematodes have a unique lifecycle. J2s hatch from eggs and undergo three successive moults to adult males or females without feeding (Jones et al., 2013). Only young females are infective. They insert a third of their bodies into the roots of host plants and establish feeding sites similar to the syncytium in endodermal and pericycle cells. These cells undergo hypertrophy with increased cytoplasmic densities and supply the parasite with all its nutrients.

1.7 Life cycle of cyst and root-knot nematodes

The second-stage juvenile is the most important stage for CNs and RKNs. J2s hatch from eggs, find suitable plant roots to invade and develop through 3 juvenile stages (J2-J4) to adults.

In CNs, sex is dimorphic. The common assumption is that sex is not genetically predefined but is attributed to the quality of the respective feeding site, the genetic makeup of the host plant and other favourable or unfavourable conditions (Grundler et al., 1991; Abad and Williamson, 2010). During overcrowded conditions where nematodes compete for resources, males stop feeding at the J3 stage and moult to J4s. After a short J4 phase, adult males hatch out of the juvenile cuticle and make their way into the soil in search of females to mate with (Bohlmann, 2015). After a successful mating encounter, females die, and the cuticle tans to form a cyst that holds and protects hundreds of eggs (**Fig. 1. 6A**) (Jones et al., 2013). These tanned cysts can remain dormant in the soil when conditions are unfavourable for over 20 years

Most RKN species reproduce via parthenogenesis, as females produce viable offspring without male fertilization (Castagnone-Sereno, 2006). In the classic lifecycle, J2s hatch in response to factors like soil temperature, moisture, soil organic matter content, pH and in several cases, hatching factors (root exudate) from host plants (Ngala et al., 2021). Like CNs, J2s of RNKs invade host plants and develop through 3 juvenile stages to adult females that carry hundreds of eggs. Females remain embedded in the root, while any developed males migrate into the soil (**Fig. 1. 6B**).

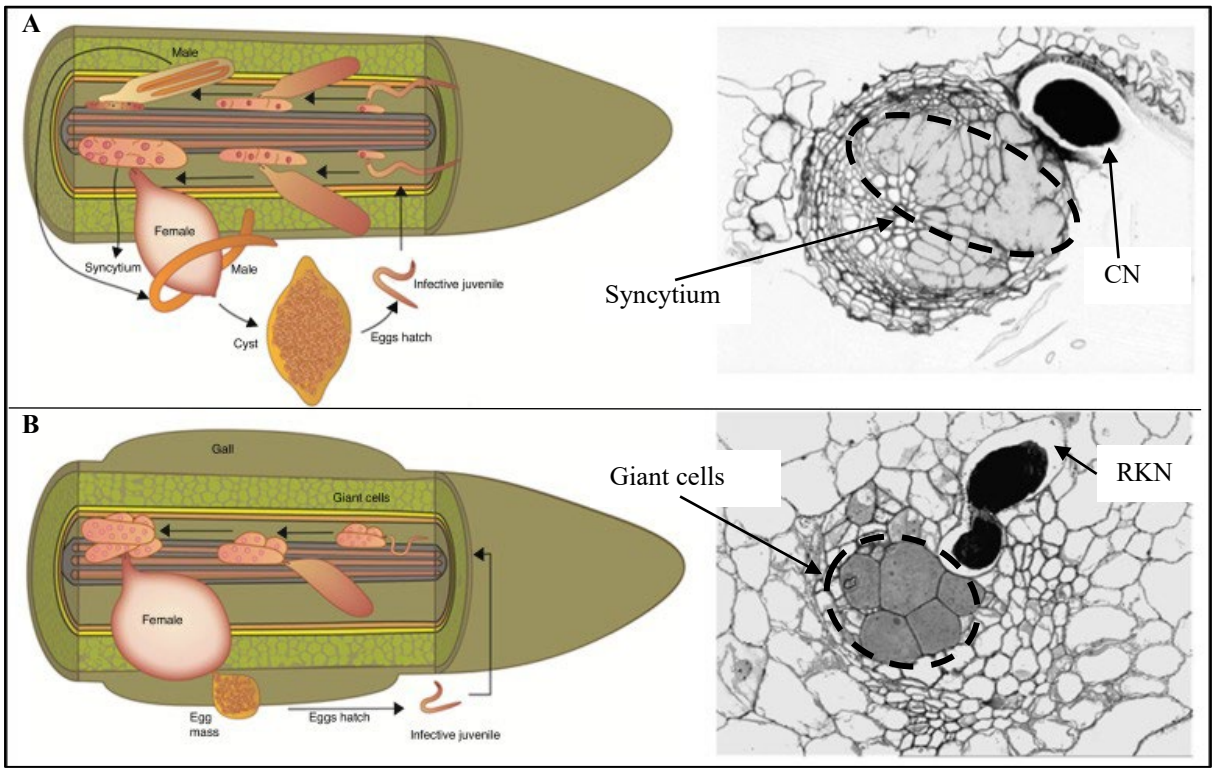

Fig. 1. 6 Life cycle of cyst and root-knot nematodes. J2s hatch from eggs and burrow into the roots of plants. **A**. Within the root, CNs establish feeding sites called syncytia from where they feed. They moult through three juvenile stages to adult males or females. Females swell and break their way out of the root while males remain vermiform, exit the root and locate sex mates. **B.** After three successive moults, RKNs develop into adult females that produce and lay eggs in a gelatinous matrix that is secreted out onto the root surface in egg masses. New J2s hatch, infect now roots and the cycle continues. Modified from (Siddique and Grundler, 2018).

1.8 The free-living nematode *C. elegans,* a model organism for PPN studies

Clearly to have a better opportunity to control PPNs it would be helpful to understand the mechanisms underpinning their behaviours that enable them to enact a complex life cycle to ensure their viability and reproduction. However, there are limitations to investigating PPN biology encompassing the limited genomic resources, genetic intractability, poor anatomical and neurobiological characterizations (Coke et al., 2024). Therefore, it is helpful first to review the understanding and the neural basis of behaviour in *C. elegans* and then to discuss to what extent this may be extrapolated to PPNs.

1.8.1 The nervous system

The nervous system has an essential role in executing the complex behaviours evolved to allow parasitic lifestyle. In *C. elegans* the nervous system is required for most processes in the animal,

like feeding, locomotion, reproduction and underpins systemic regulatory outcomes like aging (Alcedo et al., 2013; Gjorgjieva et al., 2014). It receives several sensory modalities that coordinate and generate distinct phenotypic and behavioural outputs (Emmons, 2005; Avery and You, 2012; Chew et al., 2013; Thapliyal and Babu, 2018). In terms of numbers and diversity, the nervous system is the most complex of tissues in *C. elegans* (Hobert, 2010). It is comprised of 302 neurons, which have been assigned to 118 distinct neuronal classes based on their topology and synaptic connection patterns and 56 glial cells that make up 37% of somatic cells in hermaphrodites (White et al., 1986; Hobert, 2010). Hermaphrodites have two independently distinct nervous systems: a large somatic nervous system made up of 282 neurons, and a smaller pharyngeal nervous system, which has 20 neurons (Altun and Hall, 2005). These neurons communicate through approximately 6400 chemical synapses, 1500 neuromuscular junctions (NMJs) and 900 gap junctions. Males, however, have a larger nervous system with a total of 387 neurons (93 of which are cell-specific) and 90 glia (Molina-García et al., 2020).

1.8.2 The pharynx

The pharynx is an organ made of a syncytium of radial muscles that are responsible for feeding in *C. elegans* and described later as an important regulator of pharyngeal behaviours in other parasitic nematodes. This neuromuscular pump is approximately 100 μm long and 20 μm in diameter. It sucks and crushes bacteria before passing them to the intestine. A total of eight pharyngeal muscle groups (PM1-PM8), divided into three main parts, the corpus, isthmus, and terminal bulb, play key roles in the feeding process (Trojanowski et al., 2016). In *C. elegans,* feeding is achieved through pharyngeal pumping and isthmus peristalsis, two processes that are controlled by the pharyngeal nervous system, through pharyngeal neurons and receptors (Avery and You, 2012).

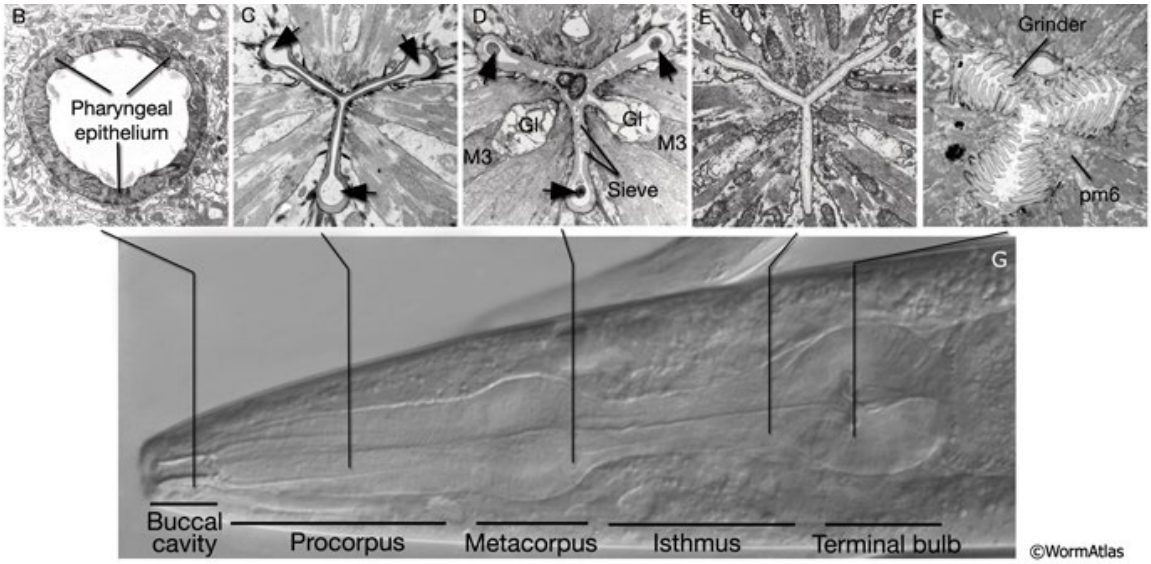

Fig. 1. 7 Cross (top) and transverse (bottom) sections of *C. elegans* pharynx. From (WormAtlas)

1.8.3 Feeding in *C. elegans*

The pharyngeal pumping is controlled by contraction-relaxation cycles that open and close the lumen sucking bacteria from the outside into the lumen of the animal. This is followed by peristalsis (once every four pumps), a process that transfers the bacteria through the isthmus to the terminal bulb, where it is crushed and passed into the intestine (Seymour et al., 1983). Out of the 20 known pharyngeal neurons, three are known to be particularly important for these processes. The MC and the M3 neurons are excitatory and inhibitory neurons responsible for the start and the end of pharyngeal pumping respectively. Upon stimulation, these neurons release neurotransmitters acetylcholine (ACh) and glutamate that act through EAT-2 and AVR-15 receptors, respectively, in the contraction-relaxation cycles during feeding (Raizen et al., 1995).

During a pharyngeal pump cycle, muscles of the corpus and terminal bulb contract to open the lumen and bacteria rush into the buccal cavity and lumen. This is followed by muscle relaxation to close the mouth and trap bacteria in the lumen. Ablating the MC and M3 neurons results in severe feeding disruptions that reduce the amount of food intake, a phenotype that is phenocopied by mutants of the EAT-2 receptor (Raizen et al., 1995; Dent et al., 1997; Avery and You, 2012). To transfer food accumulated in the lumen to the terminal bulb, the M4 neuron induces waves of muscle contractions in the isthmus, which force bacteria from the corpus through the isthmus to the terminal bulb, where they are macerated by the grinder. Ablating

the M4 neuron completely abolishes feeding (Avery and Horvitz, 1989). Interestingly, worms are capable of proper growth and development, with subtle feeding abnormalities when 17 pharyngeal neurons are killed, but the MC, M3 and M4 neurons (Avery and You, 2012). Other factors such as mating, moulting, satiety, food depletion, heat stress, overcrowding, aging and chemosensation are other determinants that influence feeding rates of nematodes (You et al., 2008).

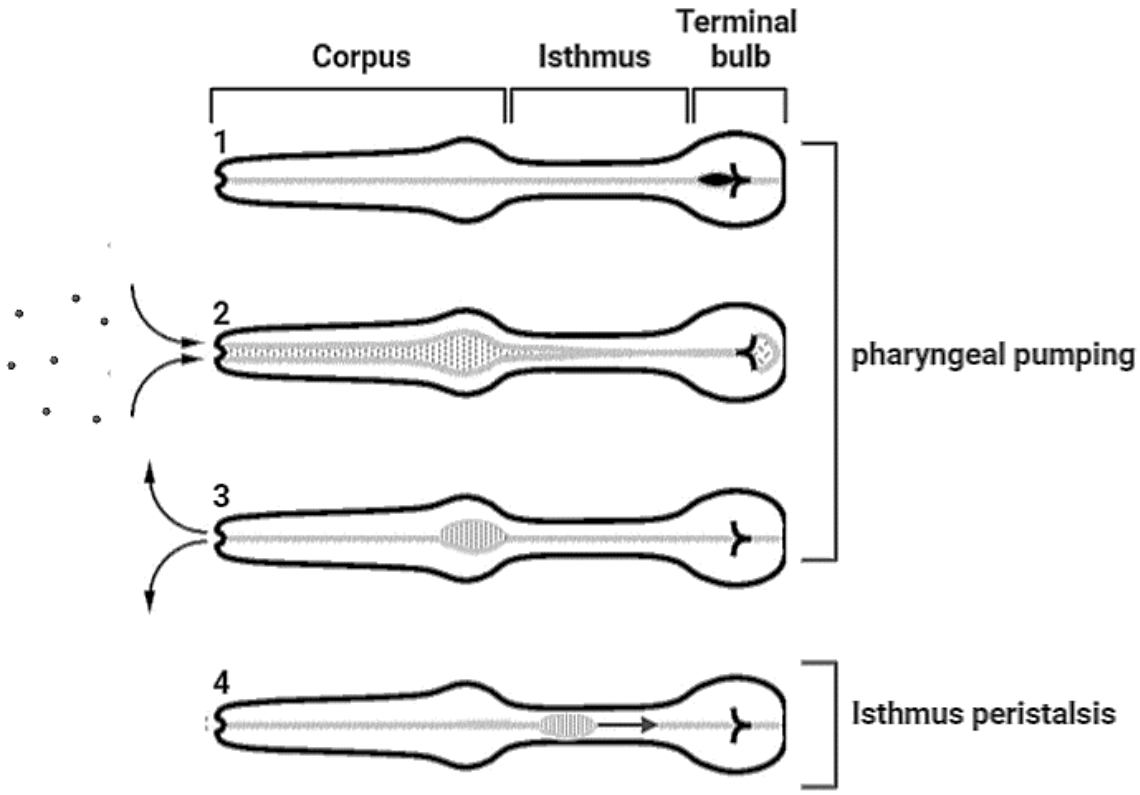

Fig. 1. 8 Main processes involved in *C. elegans* feeding. **1.** The process begins with the simultaneous contraction of muscles of the corpus, anterior isthmus, and terminal bulb. **2.** As pharyngeal muscles contract, the mouth and lumen open, creating a cavity into which liquids and suspended bacteria fill. **3.** After contraction, pharyngeal muscles relax, closing the mouth and lumen, trapping bacteria and expelling water. **4.** Following every 3-4 pumps is a peristaltic contraction of the posterior muscles of the isthmus, which moves bacteria from the corpus through the isthmus to the terminal bulb, where it is ground and passed into the intestine. Adapted from (Avery and Horvitz, 1989).

1.8.4 Nematode feeding types (trophic groups)

Feeding mechanisms that utilize nerve muscle coordination have evolved differently across nematode species and have acquired specialized mouth parts to aid them in feed (Blaxter and Koutsovoulos, 2014). The pharynx is a conserved organ in nematodes. Although different in structure, neuromuscular connections in the pharynx that drive feeding in these nematodes are thought to be similar (Fairweather et al., 1995). Nematodes are classified into different feeding types mainly based on the morphology of their buccal cavity (mouthparts) and associated structures, which reflect their feeding strategies (Hodda, 2022). But they all possess a pharyngeal musculature that plays an important role in feeding.

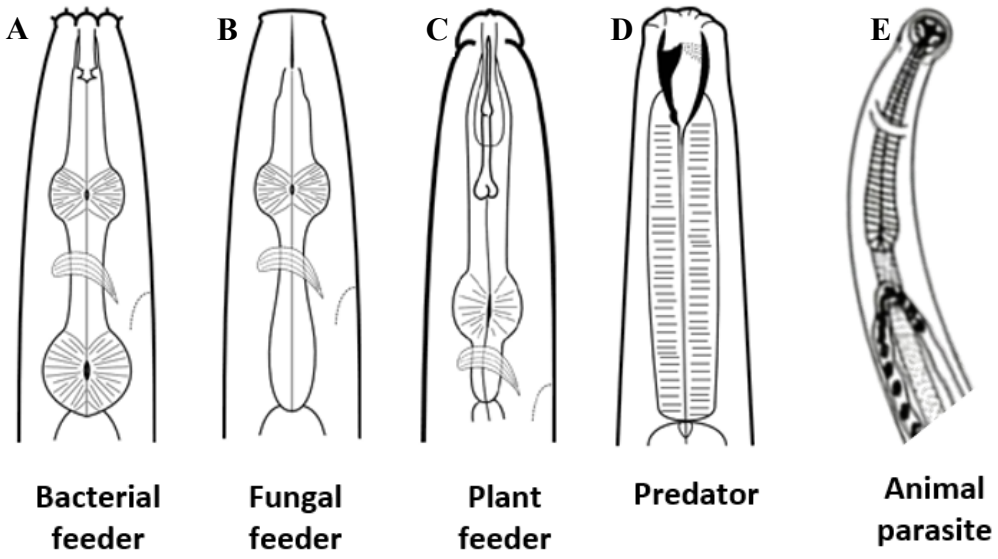

Fig. 1. 9 Diagrammatic representation of the pharyngeal region of different nematode species. A. Bacterial feeder with a large, toothless buccal cavity and lumen which accumulates food when the worm feeds, **B.** fungal feeder with a false stylet, less sclerotized, less rigid and efficient for only piercing into lower plants **C.** plant parasitic nematode with a well sclerotized and robust stylet capable of borrowing into roots and above ground parts of higher plants, **D.** predator with a large dorsal tooth in the buccal cavity and **C.** animal parasitic nematode with a muscular pharynx and cutting mouth structures in some species. Adapted from (Ed Zaborski, University of Illinois)

1.8.5 The body wall muscle in *C. elegans*

In *C elegans*, the body wall muscle (BWM) is made of 95 striated muscle cells. These muscles are required for sinusoidal movements on solid surfaces and thrashing in liquids. Fibrous

organelles anchor BWMs to the cuticle and the contraction/relaxation of these muscles distorts the cuticle and enables locomotion (Gieseler et al., 2017).

1.8.6 Locomotion in *C. elegans*

Locomotion is a foundation for many behaviours like foraging, mating, defecation (Ahringer, 2006; Piggott et al., 2011). The four basic movement patterns are: forward, backward (reversals) and omega turns, fine-scale head swings, and locomotion pause (Flavell et al., 2020) In the presence or absence of food, worms can change their locomotory behaviours, switching between dwelling and roaming respectively (Fujiwara et al., 2002; Hills et al., 2004; Flavell et al., 2020). In the absence of food, *C. elegans* tend to move around their environment, searching for new food sources. During off food foraging, worms make long forward movements with few intermittent reversals. Upon locating a suitable food source, they reduce their speed and tend to switch to a dwelling mode. Dwelling is characterised by reduced speed, frequent short reversals and turning. This is to facilitate the worm explore its immediate food environment and consume as much as possible (Flavell et al., 2020).

In addition to this solid phase motility, *C. elegans* moves in liquid by rhythmic flexion of the body around a mid-point in a behaviour termed 'thrashing' (Buckingham and Sattelle, 2009).

1.9 Neuromodulation of pharyngeal function and locomotion in *C. elegans* and *G. rostochiensis*

As introduced above, neuronal control is key for muscle-dependent behaviours like feeding and locomotion.

1.9.1 Cholinergic transmission

ACh synthesis is catalysed by the enzyme choline acetyltransferase (ChAT) during the transfer of the acetyl group from acetyl-coenzyme A (Acetyl-CoA) to choline in the cytoplasm (Rand, 2007). A total loss of function mutation in the gene *cha-1* which encodes for ChAT results in developmental arrest and death shortly after hatching, as they do not feed (Rand, 1989, 2007; Miller et al., 1996). Reduced function mutations result in mutants that are slow to develop, have locomotory defects and are resistant to acetylcholinesterase inhibitors (Rand, 2007; Treinin and Jin, 2021). Synthesized ACh is loaded into vesicles by vesicular acetylcholine transporters (VAChT) for pre-storage before stimulated release through exocytosis. Following synaptic vesicle fusion ACh is released into the synaptic cleft where it diffuses and subsequently activates acetylcholine receptors. Following release ACh is broken down by

acetylcholinesterase (AChE) into choline and acetate. This choline is taken up into the presynaptic neuron via the high-affinity choline transporter (HAChT or ChT) (Meriney and Fanselow, 2019).

1.9.2 Cholinergic regulation of pharyngeal function in *C. elegans*

As highlighted above, ACh is essential in the pharyngeal behaviour that regulates feeding (Trojanowski et al., 2016; Treinin and Jin, 2021). The intercellular signalling of ACh is mediated through the activation of nAChRs expressed in neurons and muscle cells. These receptors can be classified into the two major subtypes, G-protein metabotropic muscarinic acetylcholine receptors (mAChRs) or ionotropic nicotinic acetylcholine receptors (nAChRs), which are ion channels (Resende and Adhikari, 2009). Based on bioinformatic comparisons, *C. elegans* expresses about 30 AChRs, four of which are muscarinic and at least 4 ACh-gated chloride channels (Meriney and Fanselow, 2019).

G-protein muscarinic acetylcholine receptors (mAChRs) in *C. elegans* belong to a heptahelical transmembrane G-protein coupled receptor class that consists of five distinct subtypes (M1-M5) (Kudlak and Tadi, 2021). They are activated by the neurotransmitter ACh and play a role in pharyngeal activity through a slow downstream cascade that either stimulates or inhibits muscle contraction (Kozlova et al., 2019). In the *C. elegans* genome, there are three genes that encode mAChRs, *gar-1*, *gar-2*, and *gar-3*. Of these three genes, only *gar-3* is expressed in pharyngeal muscles (Lee et al., 2000; Dittman and Kaplan, 2008).

All ionotropic receptors are pentameric ligand-gated ion channels that have an agonist binding site (ABS) for neurotransmitters and an ion-conducting pore to allow for the exchange of ions across the plasma membrane (Meriney and Fanselow, 2019). In the case of nAChR that influx cations, the membrane undergoes depolarization which can activate a neighbouring neuron or depolarize muscle leading to muscle contraction (Meriney & Fanselow, 2019). Cholinergic pharyngeal neurons MC and M4 are important in feeding, and release ACh that acts on EAT-2 and GAR-3 receptors expressed in the nematode pharyngeal muscle (see chapter 1.15).

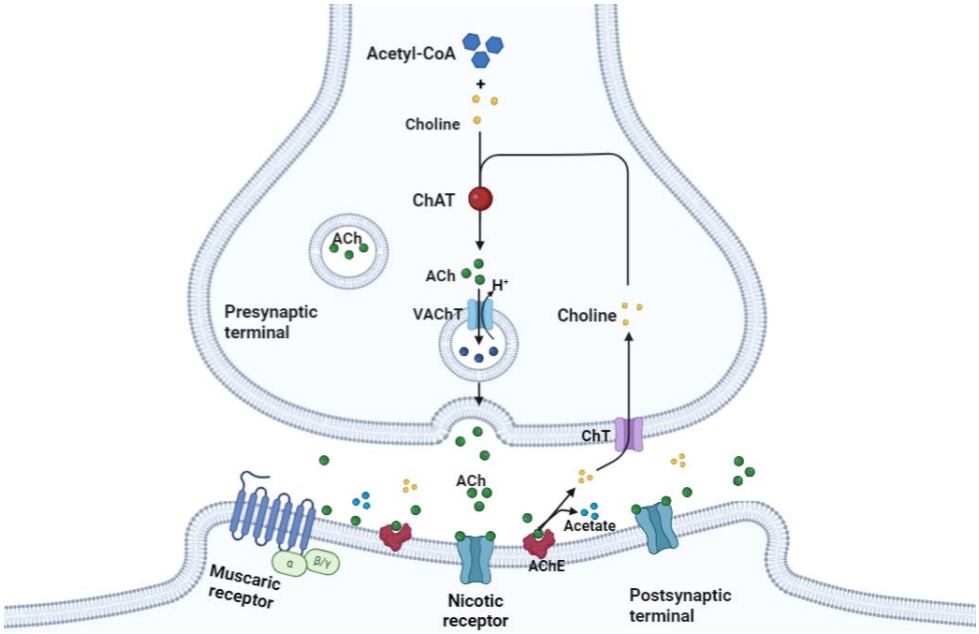

Fig. 1. 10 Schematic representation of the cholinergic pathway in *C. elegans*. Acetylcholine (ACh) is synthesized from acetyl-CoA and choline in a reaction catalysed by choline acetyltransferase (ChAT). Synthesised ACh is transferred into vesicles by the vesicular acetylcholine transporter (VaChT). ACh released into the synaptic cleft binds to muscarinic or nicotinic receptors of the postsynaptic cell, leading to either a stimulatory or inhibitory response. In the synaptic cleft, ACh is rapidly degraded by the enzyme acetylcholinesterase (AChE) to acetate and choline. Through the high-affinity choline transporter (ChT), choline is retaken up into the presynaptic cell and reused as a precursor for the synthesis of more ACh. Cartoon made with Biorender.

1.9.3 Cholinergic regulation of locomotion in *C. elegans*

Several other behavioural pathways other than feeding in *C. elegans* are ACh-dependent. Approximately 40% of its neurons cholinergic (Rand, 2007; Treinin & Jin, 2021). With a total of 75 BWM motor neurons belonging to eight groups, four groups innervate the dorsal side (11-AS, 9-DA, 7-DB, and 6-DD), and the other four (12-VA, 11-VB, 6-VC, and 13-VD) the ventral side (Gjorgjieva et al., 2014; Zhen and Samuel, 2015). These neurons are essential for the sinusoidal posture and movement of the worm. In adult worms, ACR-2 receptors (ACR-2R) expressed in the ventral cord cholinergic motor neurons provide major excitatory inputs required by BWMs involved in locomotion (Gjorgjieva et al., 2014).

1.9.4 Cholinergic regulation of pharyngeal function in PPNs

The details of neurotransmission are not well investigated in PPNs relative to *C. elegans*. Presently, the nematode species *C. elegans* has the largest number of nAChRs (Yu et al., 2017). There is no direct evidence that confirms the role of cholinergic signalling in PPNs however, there are speculations about cholinergic signalling involvement in the pharyngeal behaviour that regulates stylet thrusting since the cholinesterase inhibitors aldicarb and oxamyl at very low concentrations induced stylet thrusting in *M. incognita* (McClure, 1987). Parasitic nematodes generally have fewer nAChR subunits. In the PPN *Bursaphelenchus xylophilus*, nAChR genes have been identified and successfully cloned in *Xenopus* oocytes to construct functional nAChRs which showed agonist responses to acetylcholine and imidacloprid, a neonicotinoid insecticide (Yu et al., 2017).

1.9.5 Cholinergic regulation of locomotion in PPNs

Some studies have inferred that ACh is the principal excitatory neurotransmitter in PPNs that plays a central role in locomotion and host invasion (Wright et al., 1989). The paralysis of PPNs exposed to carbamates and organophosphate pesticides, acetylcholinesterase inhibitors, suggests there to be cholinergic signalling at neuromuscular junctions of the body wall muscle (Wright et al., 1989). There have been observations of hyperactivity in *M. incognita* worms exposed to oxamyl, a potent acetylcholinesterase inhibitor (Wright, 1981; Wright and Robert, 1984).

1.9.6 Serotonergic regulation of pharyngeal function in *C. elegans*

The neurotransmitter 5-hydroxytryptamine (5-HT), also known as serotonin, is a conserved neuromodulator that regulates pharyngeal function through processes like pharyngeal pumping in *C. elegans* and stylet thrusting in plant parasitic nematodes (Cunningham et al., 2012; Han et al., 2017; Crisford et al., 2020). 5-HT is synthesized from the precursor amino acid L-tryptophan through a short metabolic pathway that is catalysed by the enzyme tryptophan hydroxylase (TPH) to 5-hydroxy-L-Tryptophan (5HTP) and the aromatic L-amino acid decarboxylase enzyme to 5-hydroxytryptamine (5-HT).

Several pharyngeal neurons and muscles express 5-HT receptors that play a role in modulating pharyngeal pumping (Szø et al., 2000; Ishita et al., 2020). The ADF (amphid neuron) and NSM (neuro-secretory motor neuron) are the two neurons responsible for serotonergic signalling at the nematode pharynx and modulating pharyngeal pumping (Ishita et al., 2020). The ADF neuron coordinates the response to the bacterial food cues by releasing 5-HT that activates

SER-7 receptors in MC, M2, M3, and M4 pharyngeal neurons. This modifies the pharyngeal pumping and supports efficient feeding (**Fig. 1. 11A**) (Song et al., 2013; Ishita et al., 2020).

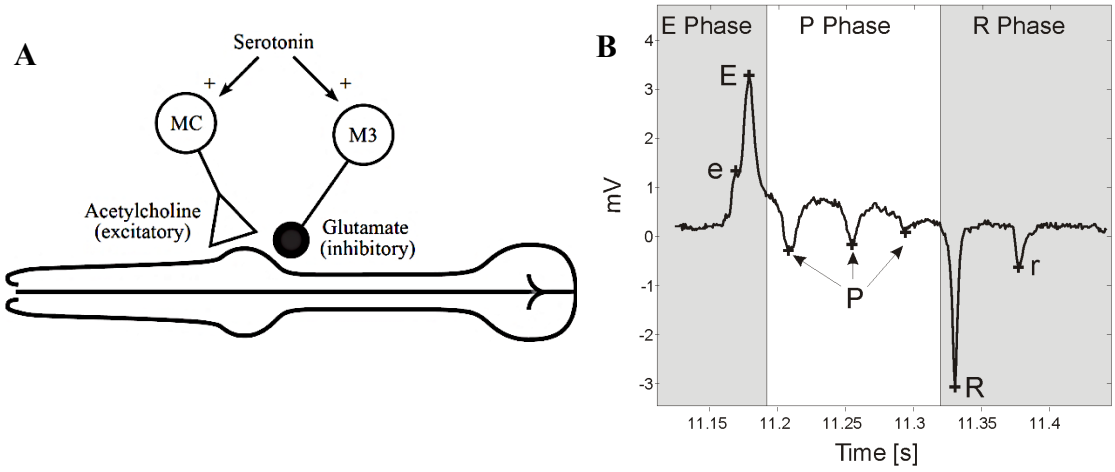

Fig. 1. 11. An overview of neurotransmitters regulating feeding in *C. elegans*. Serotonin, acetylcholine, and glutamate are the main regulators of pharyngeal pumping**. A.** The MC and M3 neurons play major roles in pharyngeal pumping through action potentials that cause the depolarization and repolarization of pharyngeal muscles, respectively. **B.** The electropharyngogram shows current movement across the pharyngeal muscle membrane, where depolarization and the onset of pharyngeal muscle contraction are indicated by the large positive transient (E), and repolarization and the end of the action potential are indicated by the large negative transient (R). The action potential is the time difference between spikes E and R. The portion of the trace between spikes E and R represents the plateau phase of the action potential. The negative transients during this plateau phase (P) are pharyngeal muscle inhibitory postsynaptic potentials (IPSPs) caused by the firing of the M3 motor neurons. Adapted from (Niacaris and Avery, 2003; Dillon et al., 2009).

Sensory cues like novel food odours inhibit the ADF neurons via mechanisms that are unknown (Ishita et al., 2020). On the other hand, attractive food odours activate NSM neurons, resulting in similar downstream effects as the latter (Flavell et al., 2020; Ishita et al., 2020). An ADF-specific knockdown of *tph-1,* the gene that encodes the enzyme TPH-1 produces viable mutants that do not synthesize serotonin and show abnormal behaviours with reduced pumping compared to wild-type animals (Cunningham et al., 2012). Conversely, the ablation of NSM neurons in wildtype worms only showed a suppressed duration of fast pharyngeal pumping

when the feeding behaviour was observed over long periods. This suggests that ADF neurons play major roles in regulating pharyngeal pumping in response to the presence of food, and NSM neurons may have subsidiary feeding regulatory contributions (Ishita et al., 2020).

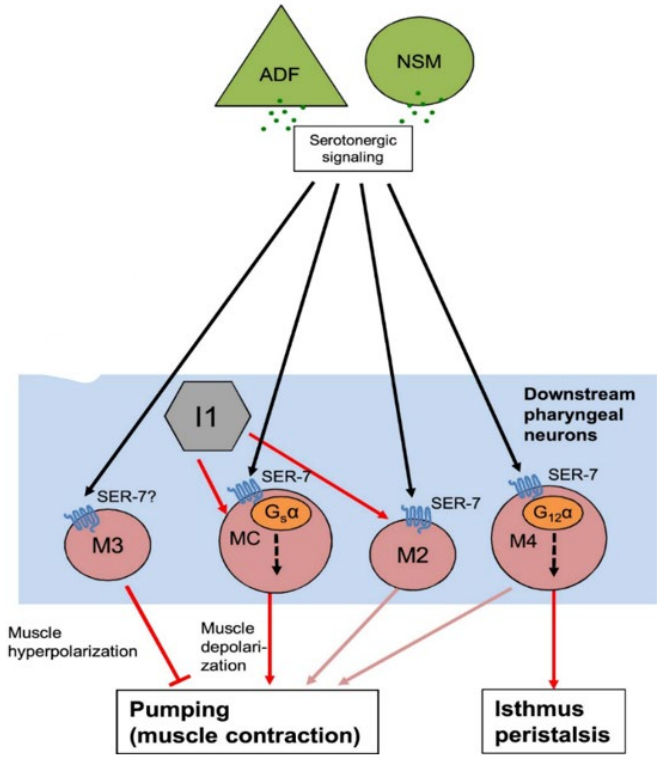

Fig. 1. 12. Serotonergic neural pathway in *C. elegans* feeding. In the presence of environmental cues, ADF and NSM neurons are triggered to release serotonin. This activates the M3, MC, M2 and M4 neurons in the nematode pharynx through the G-protein coupled receptor, SER-7. I1 is the only neuron with electrical connections to the somatic nervous system and can stimulate pumping via the MC and M2 neurons. When activated, the MC and M3 neurons release acetylcholine and glutamate, respectively, causing a phasic action potential in pharyngeal muscles. The presence of acetylcholine and glutamate in pharyngeal muscles simultaneously induce fast rhythmic contraction and relaxation cycles that result in pumping. Adapted from (Ishita et al., 2020).

1.9.7 Serotonergic regulation of locomotion in *C. elegans*

The role of 5-HT in *C. elegans* is not limited to pharyngeal function. It regulates other behaviours such as locomotion and egg laying (Horvitz et al.,1982; Segalat et al.,1995). In the presence of food, 5-HT suppresses locomotion and enhances egg laying. This behavioural observation in the presence of abundant food suggests the importance of serotonergic signalling (Rand, 2007). The serotonin-chloride gated anion channel, MOD-1 is suggested to be involved

with the inhibitory enlaced slowing of worms via secondary messenger pathways (Ranganathan et al., 2000; Ji et al., 2021). The SER-1 and SER-4 receptors have been implicated as the core 5-HT receptors involved in this food-induced slowing (Gürel et al., 2012). Mutants of *ser-1* were defective in slowing in the presence of food and during food search, mutants changed directions more frequently than wildtype animals. Dernovici et al. (2006) suggested that both MOD-1 and SER-1 were required to modulate these locomotory behaviours. The locomotory circuit analysis has shown that serotonergic release mainly from the NSM is required to initiate slowing down and dwelling of worms (Ji et al., 2021)

1.9.8 Serotonergic regulation of pharyngeal function in PPNs

The role of 5-HT is highly conserved and its involvement in the pharyngeal behaviour, stylet thrusting and other reproductive behaviours in PPNs has been investigated (Perry et al., 2004; Han et al., 2017; Crisford et al., 2020). Although the entire neural pathway involved with stylet thrusting is not yet known, disrupting 5-HT signalling interrupts stylet thrusting in *G. pallida.* In *C. elegans* the pharyngeal response to 5-HT is dependent on SER-7 receptors (Crisford et al., 2020). In *G. pallida*, the ortholog of the *C. elegans* receptor SER-7 has been identified and investigated for its potential modulatory properties. The *G. pallida ser-7* restored sensitivity to 5-HT when used to transgenically rescue *ser-7* deficits in *C. elegans*. This restored sensitivity was blocked by methiothepin, a potent antagonist of the SER-7 receptor. Based on the potent methiothepin block of 5-HT induced stylet thrusting in *G. pallida* suggests that *ser-7* also functions in stylet thrusting. It has also been reported that *tph-1* mutants of *M. graminicola* had impaired root invasion potential due to the disruption of stylet thrusting activities. However, stylet thrusting was partially rescued by the exogenous application of 5-HT and improved root invasion successes (Yang et al., 2024).

1.9.9 Serotonergic regulation of locomotion in PPNs

5-HT's role in locomotion of PPNs is not well researched; however, it has been identified that without proper signalling, the chances of J2s locating host roots are reduced. It is reported that silencing the tryptophan hydroxylase gene *tph-1*, required for the synthesis of serotonin in *Meloidogyne graminicola* reduces host invasion, as fewer J2s reach the root tips (Yang et al., 2024). 5-HT signalling impacts how far or fast PPNs move. Crisford et al. (2020) show that reserpine the inhibitor of the vesicular monamine transporter (VMAT) disables 5-HT signalling in *Globodera pallida,* resulting in the disruption of nematode dispersal and movement.

At very high concentrations (33 µM), exogenously applied 5-HT induces paralysis in wild-type *C. elegans.* Crisford et al. (2020) observed that *C. elegans* mutants of *mod-1,* the gene that encodes MOD-1, a serotonin-gated chloride gated channel were irresponsive to this 5-HT induced paralysis. They successfully rescued the paralysis with *Ce.mod-1* and its orthologue in *G. pallida* and concluded that serotonergic signalling was important in locomotion. Rodriguez Araujo et al. (2022) showed using *C. elegans* that 5-HT rapidly decreased worm motility and this was reversible by lowering 5-HT concentrations. However, mutants of *mod-1* were resistant. This provided supporting evidence that MOD-1 has modulatory effects on locomotion.

The exogenous exposure to 5-HT has also been reported to induce reproductory behaviours like spicule eversion in cyst nematodes (Jonz et al., 2001). Taken together these observations support a critical role for 5-HT signalling in PPN viability.

1.9.10 Dopaminergic regulation of pharyngeal function in *C. elegans*

The effect of dopamine on pharyngeal pumping has not been widely investigated although Dallière et al. (2016) identified a modulation of the pumping in an off-food context. Although there is no evidence that the pharyngeal nervous system synthesizes dopamine, some pharyngeal neurones express dopamine receptors that implicates dopaminergic signalling from extra-pharyngeal circuits to act neurohormonally on pharyngeal muscles and regulate pumping (Sugiura et al., 2005; Dallière et al., 2016).

1.9.11 Dopaminergic regulation of locomotion in *C. elegans*

Sawin et al., (2000) have shown that the basal slowing response of *C. elegans* to food is mediated by dopamine. This is evidenced by well-fed mutants defective in the synthesis of dopamine showing much reduced basal slowing in the presence of food compared to N2 worms (Sawin et al., 2000). The slowing down of worms when they get to a food source is thought to be an adaptive mechanism by worms to spend more time on food. Dopamine also plays a role in local area search behaviours, like dwelling in the presence of quality food (Hills et al., 2004). It is suggested that multiple dopamine neurones coordinate to modulate area restricted search.

1.9.12 Dopaminergic regulation of pharyngeal function and locomotion in PPNs

The evidence of dopaminergic control of pharyngeal behaviour in PPNs is largely inferential and highlights a gap in the understanding of the dopaminergic pathway in PPNs. In *M. incognita*, positive immunoreactivity for dopamine was detected through high performance

liquid chromatography, suggesting the presence of dopaminergic signalling (Stewart et al., 2001). Biogenic amines including dopamine are known modulators of feeding circuits in *C. elegans* implying that by extension, dopamine could be involved with regulating the neuronal program that drives pharyngeal function and feeding behaviours in PPNs.

1.9.13 Glutamatergic regulation of pharyngeal function in *C. elegans*

Glutamate is an important central transmitter that as described plays an essential role in pharyngeal function. In the presence of food, 5-HT activates the pharyngeal glutamatergic neuron M3, which then releases glutamate that acts on the AVR-15 glutamate receptor. In this context, glutamate is an inhibitory neurotransmitter which hyperpolarises the pharyngeal muscle membrane through inhibitory postsynaptic potentials that result in muscle relaxation (Niacaris & Avery, 2003). The *eat-4* gene, known to load glutamate into presynaptic vesicles of the M3 neuron, is essential for this glutamatergic transmission. Mutants of *eat-4* have reduced pump rates in the presence of food, as the M3 neuron fails to release glutamate and terminate the pharyngeal muscle action potential (Dallière et al., 2016; R. Lee et al., 1999; Niacaris & Avery, 2003). Interestingly, glutamate's role in regulating feeding is food dependent. In the presence of food, it has a stimulatory function on the behaviour (through a quick repolarization and relaxation of pharyngeal muscles). However, in the absence of food, its role represses pumping, resulting in a constitutive inhibitory tone that clamps the pump rate, as evidenced by the enhanced pumping rates observed with *eat-4* mutants in the absence of food (Dallière et al., 2016).

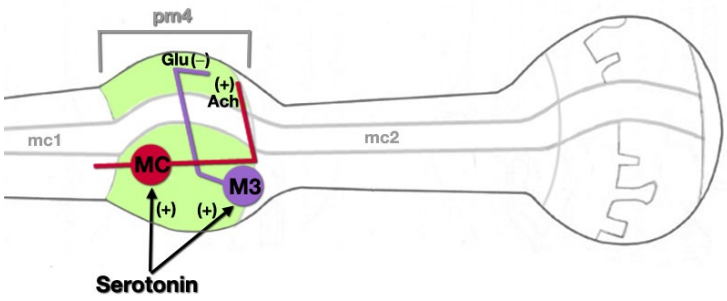

Fig. 1. 13. The M3 neuron innervates fast muscle hyperpolarisation in *C. elegans*. Through the receptor AVR-15 expressed in pm4 muscles of the nematode pharynx, glutamate released from the M3 neuron terminates the muscle action potential initiated by MC neurons. This shortens the duration of the action potential. From (Niacaris and Avery, 2003).

1.9.14 Glutamatergic regulation of locomotion in *C. elegans*

Glutamate also has a significant role in locomotion and foraging behaviours. During foraging, wildtype worms perform an intensive search in the area to find food. This strategy, known as 'local area search', is characterised by numerous sharp turns that keep the worm in its target search area (Hills et al., 2004). When food is identified, worms reduce their speed and start dwelling with increased turning frequencies. On the contrary, *eat-4* mutants tend to roam, and are hyperactive in foraging with reduced turning frequencies since glutamatergic signalling is required to modulate turning frequencies in response to food deprivation (Hills et al., 2004; Chalasani et al., 2007).These generic roles of glutamate are mediated by several distinct glutamate receptors that include ligand-gated chloride channels (GluCls) and metabotropic glutamate receptors (Pemberton et al., 2001; Dillon et al., 2015).

1.9.15 Glutamatergic regulation in PPNs

As highlighted in *C. elegans* glutamate acts as an important neurotransmitter which executes modulatory roles in complex behaviours. In *G. pallida* genes responsible for the production and utilization of glutamate has been identified in very similar complements to *C. elegans* (Cotton et al., 2014). In addition, some putative glutamate-gated chloride channels, metabotropic and ionotropic glutamate receptors have been identified in the *G. pallida* genome (Cotton et al., 2014). Physiological and pharmacological studies reveal that macrocyclic lactones stimulate glutamate chloride channels on nematode neurons and pharyngeal muscle cells of *A. suum* and *Haemoncus contortus*, inducing hyperpolarization blocks that result in paralysis and mitigation of these animal parasitic nematodes (Wolstenholme and Rogers, 2005; Wolstenholme, 2012). These pharmacological findings evidence the wide role of glutamate but its discrete role in PPN is largely unexplored despite clear evidence for the expression of the key transmitter determinants (Cotton et al., 2014).

1.9.16 Gama-aminobutyric acid (GABA) regulation in *C. elegans*

In *C. elegans*, GABA is an important neurotransmitter that acts primarily at body wall neuromuscular synapses. The neurotransmitter plays a major role in relaxing BWMs during locomotion and in contracting enteric muscles during defecation. During exploratory periods, there are narrow range head swings of the worms' head, which facilitates the finding of favourable environmental cues. Interestingly, when all four GABA-containing neurons are ablated, the range of the head swing movements during foraging becomes more exaggerated (Dallière et al., 2016). Mutant *unc-25,* with a defect in glutamic acid decarboxylase, an essential

enzyme for GABA synthesis, shows reduced pumping in the presence and absence of food, suggesting GABA's stimulatory role during feeding (Dallière et al., 2016).

1.9.17 Gama-aminobutyric acid (GABA) regulation in PPNs

GABAergic neurons have been mapped in in several PPN species. Immunohistochemical analyses detected GABA neurons in *M. incognita*, *H. glycines* and *P. penetrans,* although their arrangement patterns differ from *C. elegans* (Han et al., 2018; Reed et al., 2023). Pharmacological evidence shows that the exogenous exposure of some PPN species to GABA, GABA-agonist and antagonist significantly altered locomotion, signifying GABAergic regulatory role in motor activity of PPNs (Reed et al., 2023). GABA regulation appears to change across different life stages. In the case of *H. gylcines,* sedentary females showed a reduced expression of *hg-unc-25*, the gene that encodes for the GABA synthesizing enzyme and fewer GABA-immunoreactive neurons compared to motile J2s (Han et al., 2018).

Currently, there is limited evidence of GABA signalling in the pharyngeal behaviours, stylet thrusting and pumping in PPNs.

1.10 The pharynx of plant parasitic nematodes

Whilst PPNs possess a pharyngeal system similar to *C.elegans*, unlike *C. elegans*, the pharynx has an associated stylet. This is regulated by a specialized musculature that plays a central role in parasitism.

1.10.1 The stylet, structure and evolution

The stylet is a hollow, spear-like, protrusible structure located in the anterior end of the nematode head. Primarily, it is an essential tool for hatching, invasion and feeding (Bohlmann and Sobczak, 2014; Bozbuga et al., 2018). Structurally, the stylet can be broken down into three main parts: The cone, which is the sharp anterior portion that breaks through plant cell walls, the shaft, which provides rigidity and the knobs, which are located at the posterior end and serve as attachment points for procorpus and protractor muscles that control stylet movements (Siddiqi, 2000). Stylets of PPNs have independently evolved in three different occasions into three different stylet types, namely stomatostylet, onchiostylet and odontostylet. The stomatostylet from the name is derived from the stoma and belongs to members of the order Tylenchida, which include *Globodera* and *Meloidogyne* species. Onchiostylets are curved with no stylet knobs and belong to the nematode order Tryplonchida, with *Trichodurus* species as an example. Odontostylets are different from stomatostylets in both structure and

origin. Odontostylets are derived from the nematode's tooth odontium, rather than the stoma. They are solid, long, needle-like, usually with a guiding ring that anchors the stylet within the nematode's mouth. Ordontostylets belong to members of the order Dorylimida, which include *Longidorus* and *Xiphinema* species. Movement of the different stylet types is controlled by the stylet protractor muscles, which attach to the basal knobs of the stomatostylet, the odontophore of the odontostyle, and the onchiophore of the onchiostylet. Some nematode species have protractor and retractor muscles that control stylet movements in and out of the stoma, respectively. However, Tylenchids like *Globodera* have no retractor muscles (Decraemer and Hunt, 2013b). Tension in the alimentary tract causes the retraction of protruded stylets when the protractor muscles relax.

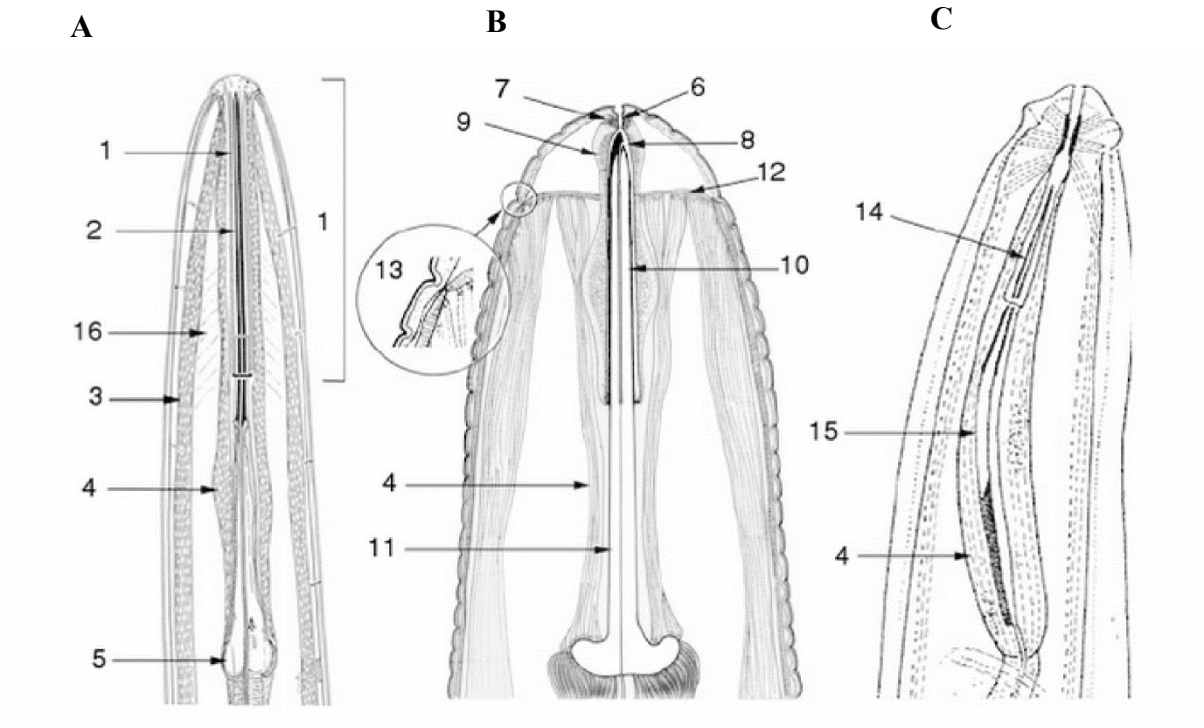

Fig. 1. 14 Variations in the stoma region of plant parasitic nematodes. A. Odontostyle for Longidorids, **B.** stomatostylet for Tylenchids and **C.** onchiostylet for Triplonchids. The numbers indicate the following morphological features: 1, cheilostome, 2) odontostyle, 3) somatic musculature, 4) stylet protractor muscles, 5) odontophore, 6) pre-stoma, 7) cuticular thickening around pre-stoma, 8) stylet opening, 9) stoma, 10) stylet conus, 11) stylet shaft and stylet knobs, 12) basal cephalic framework, 13) body cuticle, 14) onchium of the onchiostyle, 15) onchiphore of onchiostyle, 16) dilatores buccae muscles. From (Perry et al., 2024).

1.10.2 Pharyngeal muscles in plant parasitic nematodes

As in *C. elegans*, the pharyngeal muscles are a group of triradial symmetrical muscles that power the movement of the stylet and pharyngeal pumping of the median bulb. The structure of the pharynx relates to the feeding mode of the nematode and represents key adaptations that link the mechanisms of feeding to their success as obligate parasites. In Tylenchomorpha, pharyngeal muscles are divided into three main parts: The anterior corpus, an isthmus and the basal bulb. The corpus is subdivided into an anterior, muscular procorpus, which attaches to the stylet knobs and, a muscularly larger and more robust metacorpus or median bulb (Decraemer and Hunt, 2013b). The position of the median bulb is considerably different, relative to the position of the pharyngeal gland. The median bulb is a cuticularized triradiate valvate apparatus that is attached to a well-developed musculature that generates strong suction through contraction-relaxation cycles to suck and pump fluid into the intestine. The isthmus is non-muscular, sometimes short or absent and guides the passage of fluid into the intestine, and the basal bulb regulates secretions from the dorsal and sub-ventral glands (Decraemer and Hunt, 2013b).

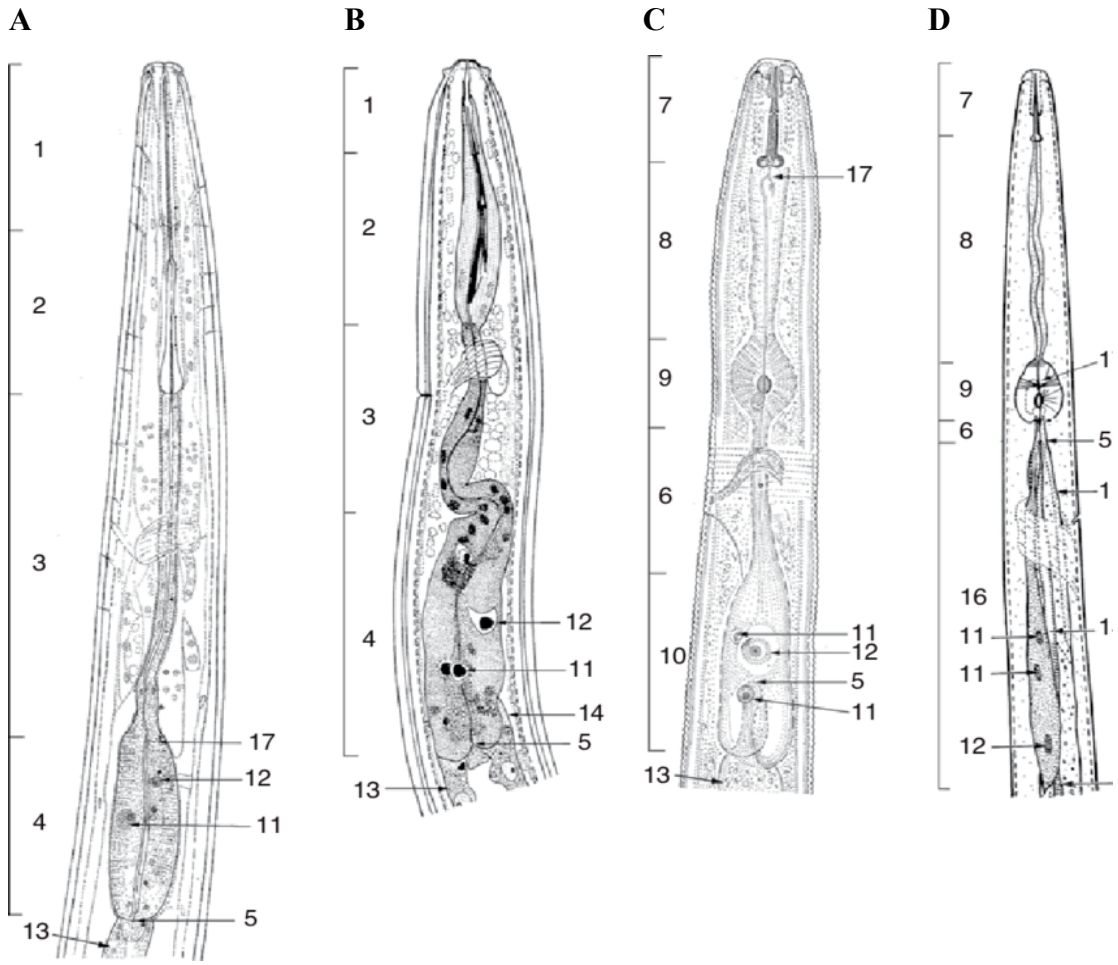

Fig. 1. 15 Pharyngeal regions of some plant parasitic nematode taxa showing the digestive system. A. Paraxiphidorus (Longidoridae). **B.** Paratrichodorus (Trichodoridae). **C.**, Pratylenchoides (Pratylenchidae). **D.** Aphelenchoides (Aphelenchoididae). 1, cheilostome; 2, pharyngostome; 3, narrow anterior region of pharynx; 4, pharyngeal bulb; 5, pharyngeal–intestinal junction; 6, isthmus; 7, stomatostylet; 8, procorpus; 9, metacorpus; 10, post-corpus; 11, ventrosublateral pharyngeal gland nuclei; 12, dorsal pharyngeal gland nucleus; 13, intestine; 14, intestine dorsally overlapping pharynx; 15, pharyngeal–intestinal junction valve cell; 16, pharyngeal gland lobe; 17, dorsal pharyngeal gland orifice. From (Perry et al., 2024).

1.11 Disrupting the neural network of plant parasitic nematodes

The organization of the neurobiology of PPNs is essentially conserved and information from the well-studied free-living nematode *C. elegans* and animal parasite *A. suum* can be used to predict a similar organization in other nematode species (Stretton and Maule, 2013). Homologs of well-characterized serotonergic neurons in *C. elegans* look to be conserved in *P. penetrans* based on neurotransmitter phenotype and location of the neuronal cell body (Han et al., 2017). Despite the conservation of neuronal types between different nematode species, there can be differences in neuronal connectivity, suggesting differences in the processing of sensory information as exemplified by studies on *Pristionchus pacificus* (Bumbarger et al., 2013). The central nervous system of nematodes is a circumpharyngeal nerve ring, dorsal and ventral nerve cords that run longitudinally or circumferentially, as commissures (Perry et al., 2024). This entire system coordinates sensory inputs that mediate locomotion, feeding, and reproductive behaviours. Upon stimulation of a nerve ending, a neurotransmitter is released to trigger downstream events that eventually result in behavioural changes.

Small molecule neurotransmitters, neuropeptides, ion channels and gap junctions are central to the regulation of nematode behaviour. There is good evidence that classic small molecule neurotransmitters, acetylcholine (ACh) and serotonin modulate locomotion and pharyngeal behaviours like stylet thrusting in PPNs. FMRFamide-like peptides (FLPs) and neuropeptide-like proteins (NLPs) modulate parasitic behaviours (Kimber and Fleming, 2005; Warnock et al., 2017). This important role of the nervous system for nematode viability makes it an ideal target for anthelminthics and nematicides.

1.12 Approaches to find selective pharmacology for plant parasitic nematodes

Progress in the pharmacology of parasitic nematodes uses *C. elegans* as a tool in high-throughput anthelmintic drug discovery studies (Burns, et al., 2015a). Using *C. elegans* as a

model organism, researchers have not only improved the pace of drug screening assays but also focused on drug specificity by implementing forward and reverse genetics to identify new anthelmintics. Mathew and others (2016), demonstrated that mutant strains could be used to screen for drug resistance and susceptibility. This screening approach led to the discovery of new compounds acting through pathways that were different from the well-known benzimidazole, levamisole and ivermectin pathways and perhaps represent novel targets acting through different mechanisms (Mathew et al., 2016). Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cation channels expressed at nematode neuromuscular junctions that mediate fast cholinergic transmissions and are critical for locomotion and feeding. Several classes of anthelmintics exploit these receptors as pharmacological targets. Pyrantel and morantel were first identified as agonists that elicited spastic muscle paralysis due to prolonged activation of the excitatory nAChRs on body wall muscle of nematodes, contributing their efficacy in both veterinary and agricultural nematode control (Aubry et al., 1970; Harrow and Gration, 1985). Similarly, levamisole is an example of a compound that acts as a selective agonist at a subset of nematode nAChRs of the body wall muscle, producing prolonged depolarization, spastic paralysis, and expulsion of worms from the host (Martin et al., 2012). Recent chemotypes exploit the diversity of nAChR subtypes. The amino-acetonitrile derivative, monepantel was a breakthrough as it selectively targeted the nematode-specific receptor ACR-23 of the DEG-3/DES-2 subfamily (Hansen et al., 2022).

1.13 Structure of nAChRs

nAChRs are well-characterized pentameric ligand gated-gated ion channels (LGICs) that belong to the Cys-loop receptor family (Udgaonkar and Hess, 1986; Dougherty, 2008). Subunits that make up the functional receptor are encoded by the same or different genes comprised of α-subunits or combinations of α- and non-α subunits (Treinin and Jin, 2021). Nematodes studies have increasingly revealed nAChRs, that contribute to parasitology. A non-canonical pharyngeal nAChR composed of non-α subunits called EAT-2 was characterized as a functional receptor in *C. elegans* with potential as a novel drug target (Choudhary et al., 2020).

The hallmark of receptor subunits in this family is the presence of a Cys-loop, a conserved structural motif in the extracellular ligand binding domain. This structural loop exists in all five subunits that associate in the membrane and for the functional receptor. It is comprised of two cysteine residues that are separated by 13 amino acids and are linked by a disulphide bond at the N-terminus of the receptor subunits (Treinin and Jin, 2021). The Cys-loop position at the

entrance of the ABS and close to the transmembrane domain, plays an important role in linking the extracellular domain and transmembrane domains (Tsetlin et al., 2011). Each receptor subunit is made up of the large Cys-loop containing extracellular amino-terminus, four transmembrane regions (TM1-TM4) with a relatively longer intracellular loop between TM3 and TM4 and a short extracellular carboxyl terminus (Iturriaga-Vásquez et al., 2015). In the extracellular domain is a specialised region called the agonist binding site (ABS) The ABS has conserved amino acid residues that facilitate transmitter binding onto the receptor (Corringer et al., 2000).

The pentameric nAChRs can be homomeric, where the same gene encodes for all five receptor subunits, or heteromeric, in which case different genes encode the different receptor subunits. The pentameric structure is symmetrically arranged around a central pore with five symmetry axis perpendicular to the membrane (Cecchini et al., 2024). A side view of the receptor reveals three main domains: 1.) a large extracellular domain at the N-terminus with the ABS 2.) a water-filled transmembrane domain that opens up a hydrophilic ionic pore through the lipid bilayer and spans about 30 Å and tappers from 80 Å in the extracellular domain, to less than 10 Å in the transmembrane domain (Karlin, 2003; Tsetlin et al., 2011; Dani, 2015), 3.) an intracellular domain made of sites for modification and interaction with cytoplasmic elements, about 25 Å and 4.) a relatively short extracellular C-terminus (**Fig. 1. 16 A&B**). In a homomeric receptor, the orthosteric ABS spans between two alpha subunits or an interface between an alpha subunit (principal face) and a non-alpha subunit (complementary face) (**Fig. 1. 16 C&D**)(Changeux and Paas, 2009; Shahsavar et al., 2016). In early studies, the crystalized structure of a soluble acetylcholine binding protein (AChBP) was thought to mimic the receptors ABS domain and identified key amino acid residues for ligand-receptor interactions as aromatic residues in the ABS in loop A (Tyr), B (Trp) C (2 Tyr) and D (Trp) that make up the aromatic box and an additional disulphide cysteine pair (vicinal cysteines) (Corringer et al., 2000; Hansen et al., 2004).

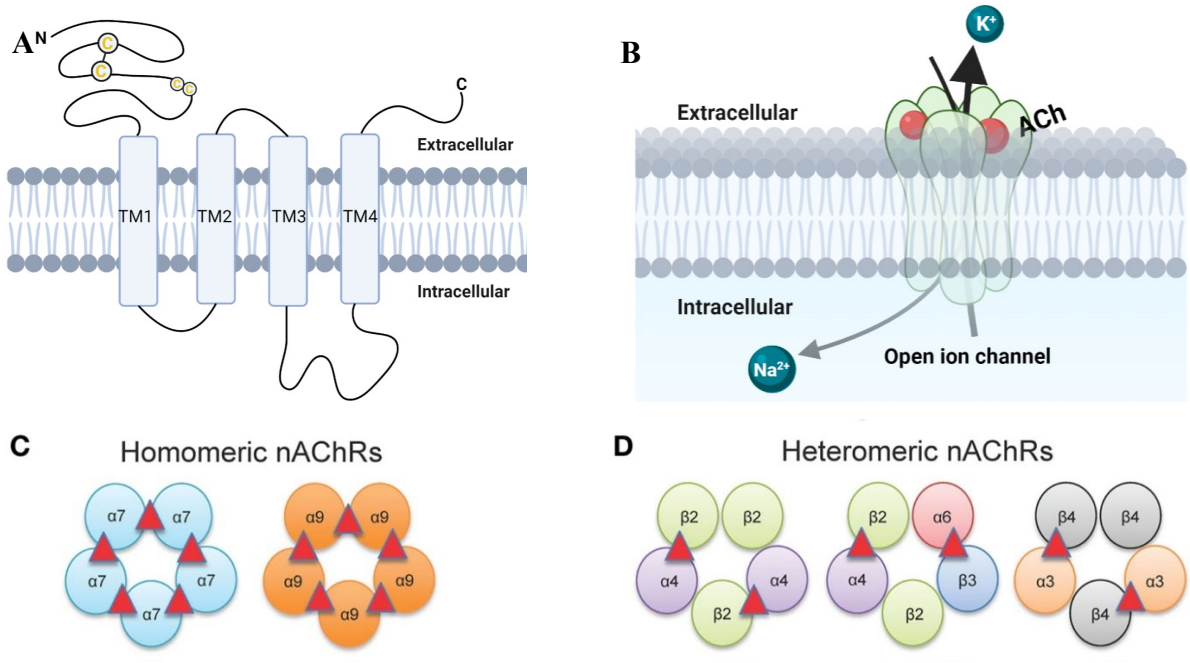

Fig. 1. 16. nAChR structures A. Membrane topology of a receptor subunit. Each subunit consists of an extracellular amino and carboxylic terminus, four transmembrane domains (TM1-TM4), and a variable-length intracellular loop connecting TM3 and TM4. **B.** Five subunits can co-assemble to form a functional pentameric nAChR activated by the neurotransmitter ACh, to open an ion channel at the centre of the receptor. **C.** Five alpha subunits assemble to make a homo-pentameric receptor with ABSs between subunits. **D.** Hetero-pentameric receptors are made up of alpha and non-alpha subunits that co-assemble to form a functional receptor, with ABS usually between an alpha and a non-alpha subunit. Cartoon made with Biorender.

1.13.1 The significance of vicinal cysteines in agonist binding to nAChRs

The main characteristic of the α-subunit in nAChRs is the presence of adjacent cysteines called vicinal cysteines in the C-loop of the ABS that facilitate ligand binding onto the ABS (Albuquerque et al., 2009; Blum et al., 2011; Dani, 2015). Based on the presence or absence of these vicinal cysteines, nAChR subunits are classified as alpha or non-alpha (β) subunits, respectively (Dani, 2015). There are varying findings on the importance of these vicinal cysteines in ligand binding. Early studies discussed the possible role of these vicinal cysteines through mutagenetic studies on *Torpedo* nAChR, where mutations in both cysteines, C192S and C193S, resulted in irresponsive receptors even at high ACh concentrations (Mishina et al., 1985). Contrary to previous studies, Blum et al. (2011) reported that mutations at C192/193 in mouse and human alpha-7 nAChRs produced functional receptors with reduced functional

responses compared to wildtype receptors. They proposed that the role of the vicinal cysteines is to shape the structure of the β-turn between Tyr-190 and Cys-193 and is not necessarily involved in agonist binding (Blum et al., 2011). However, other studies suggest that vicinal cysteines are important for ligand binding specificity, as they provide a rigid hydrophobic surface where the sulphurs in the disulphide bridge can make weak van der Waals contact with ligands (Ruggles et al., 2009; Richardson et al., 2017). Mutations in all four cysteine residues of the Cys-loop (C128-C142) and the loop-C (C190-C191) of the human α7 receptor resulted in no receptor activity when expressed in *Xenopus* oocytes (Tillman et al., 2020). Receptor activity was, however, restored when all cysteine pairs were present. Cryo-EM structures of muscle-type nAChRs reveal the involvement of vicinal cysteines in an extended hydrogen-bond network that constrains loop-C mobility during agonist binding, thereby facilitating the conformational changes required for efficient channel opening (Zarkadas et al., 2022). Review of mammalian and insect nAChRs confirms that only alpha subunits possess the conserved cysteine pairs, making it a defining structure of the principal face of the binding site (Papke, 2024; Ito et al., 2025). These structural peculiarities guide the explanation of why loop-C interactions vary among receptor subtypes and could underlie the selective recognition of endogenous agonists and exogenous ligands.

1.14 Organization of ACh receptors in *C. elegans*.

C. elegans possesses a large family of nAChRs subunit genes that are expressed in both neuronal and muscle tissues. Major ionotropic ACh-activated channels in body wall muscles include the classical levamisole sensitive receptor assembled from α-subunits UNC-38, UNC-63, and LEV-8 together with non-α subunits UNC-29 and LEV-1(Boulin et al., 2008), and homo-pentameric receptors ACR-23 and ACR-16 (Treinin and Jin, 2021). Pharyngeal muscles express EAT-2, a homo-pentameric receptor that regulates pharyngeal function. In motor neurons, the ACR-2 receptor is a well characterized ACh receptor for cholinergic neurons. Others like ACR-12 are reported expressed in GABAergic neurons. There are multiple subunits of nAChRs that show homology to vertebrate and invertebrate receptors but do not match the five core groups (UNC-18, UNC-29, ACR-16 DEG-3 and Chloride channels) and so are designated as orphan subunits (**Fig. 1. 17A**) (Jones et al., 2007). Recently some of these uncharacterized subunits were deorphanized and classified as polymodal channels activated by cholinergic and aminergic ligands (**Fig. 1. 17B**) (Hardege et al., 2023a).

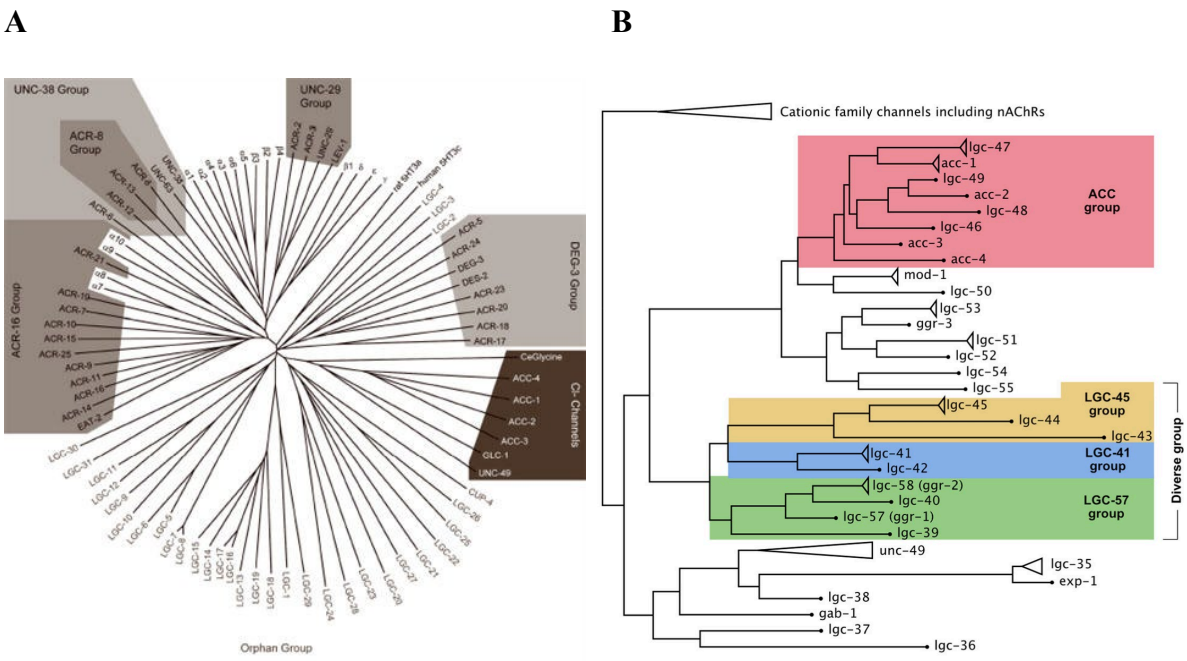

Fig. 1. 17. Phylogenetic tree of *C. elegans* nAChR genes. A. Five core gene groups, UNC-38, UNC-29, DEG-3, ACR-16, Chlorine channels and an Orphan group. **B.** The deorphanized ACC group of ACh-gated channels, LGC-45 group, LGC-41 group, and LGC-57 group. (From Jones et al., 2007; Hardege et al., 2023a)

1.15 Pharyngeal cholinergic receptors and determinants of feeding in *C. elegans*

In *C. elegans*, cholinergic inputs from cholinergic neurons are key for pharyngeal function. The EAT-2 receptor is an ionotropic Cys-loop ligand-gated nAChR expressed in the neuromuscular junction of pm4 and pm5 muscle of the nematode pharynx and binds acetylcholine released by the MC neuron (Altun and Hall, 2009). The G-coupled receptor GAR-3 is the major metabotropic receptor in pharyngeal muscles. GAR-3 signalling prolongs pharyngeal and modulates pumping frequencies, especially during conditions of high food availability or cholinergic excitation (Steger and Avery, 2004). Other putative cholinergic receptors whose contribution to pharyngeal pumping are not yet fully investigated include: the ACR-6, ACR-10, DEG-3, and ACR-9 (Holden-Dye et al., 2013), the acetylcholine-gated chloride channel subunit ACC-3 (Putrenko et al., 2005).

1.15.1 The EAT-2 Receptor

C. elegans EAT-2 nAChR is a homo-pentameric receptor made up of five non-α EAT-2 subunits that form a key part of the pharyngeal neuromuscular junction. Unlike most nAChRs, which are pentameric complexes of solely α-subunits, or a mix of α- and β-type subunits, EAT-2 is made from the assembly of non α-subunits with functional properties (McKay et al., 2004).

Genetic interaction studies revealed that EAT-2 requires the auxiliary protein EAT-18 for its functional expression (McKay et al., 2004; Choudhary et al., 2020). EAT-18 is a small, single-pass transmembrane protein and unlike classical auxiliary proteins of vertebrate nAChRs, EAT-18 is nematode-specific. This highlights the evolutionary divergence of cholinergic systems in invertebrates.

EAT-2 is fundamental in sustaining high pharyngeal pumping rates in *C. elegans* wildtype worms on food (about 250 pumps/minute), under physiological conditions (Izquierdo et al., 2022). Mutants of the *eat-2* phenocopy the feeding behaviours of nematodes with the MC neuron ablated with an average of 60 pumps/minute, highlighting its important role in pharyngeal pumping (McKay et al., 2004). Using *eat-2* and *eat-18* mutants, Raizen et al., (1995) showed genetic and pharmacological evidence suggesting that the MC neuron, acting through the neurotransmitter ACh, activated the EAT-2 receptor, resulting in a depolarisation of the plasma membrane, muscle excitation, and contraction. Interestingly, despite this major deviation in the structural determinants of the ABS, EAT-2 still interacts with ligands (Choudhary et al., 2020). EAT-2 still possesses most of the residues that form the binding site (aromatic box) as in the α-7 nAChR (Choudhary et al., 2020). These residues include: a W149 (loop-B) and Y197 (loop-C) contributed by the "principal face" of one subunit and W54 (loop-D) provided by the "complimentary" face of the adjacent subunit (Dougherty, 2008). Possibly, this is how a receptor missing a signature determinant for ligand binding was able to constitute a functional cation receptor. This difference could imply a distinct pharmacology from other nAChRs.

1.15.2 EAT-2 Pharmacology

Choudhary et al., (2020) investigated EAT-2 receptor pharmacology by recording the concentration-response relationship of selected agonists and antagonists in recombinantly expressed receptors. The EAT-2 receptor was shown to meet the criteria for a suitable anthelmintic drug target previously outlined by Wever et al., (2015): 1) can perform neuromuscular functions that are essential for parasite biology; 2) is druggable, with a distinct pharmacology from other somatic muscle receptors and is insensitive to several currently used cholinergic anthelmintics like morantel, tribendimidine and pyrantel; 3) is present in other relevant nematode species; 4) has a low identity (only 36%) compared to human α7 nAChR. Coupled with the fact that the EAT-2 receptor requires an auxiliary protein that has no known mammalian homologs for its functional expression (**Fig. 1. 18**), it makes it a suitable selective

pharmacological target. Electrophysiological and biochemical analyses suggest that EAT-18 is not required for EAT-2 trafficking but is essential for receptor activity (Choudhary et al., 2020).

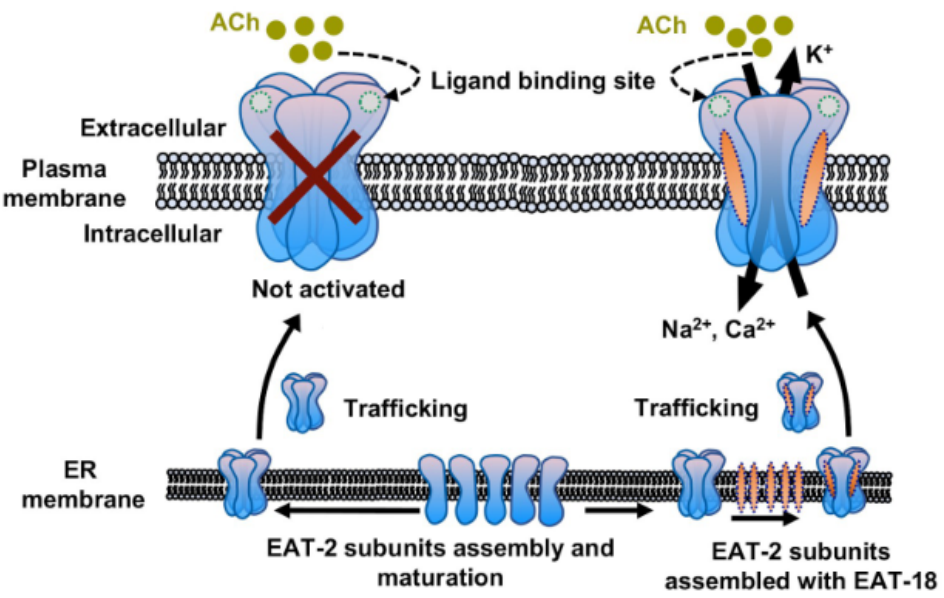

Fig. 1. 18. Formation of an EAT-2/EAT-18 receptor complex. A schematic representation of the interaction between both proteins. In the presence of the auxiliary protein EAT-18, mature EAT-2 receptors are trafficked and functionally expressed in the plasma membrane. In the absence of EAT-18, the receptor fails to be activated by ligands. From (Choudhary et al., 2020).

The role of the EAT-2 receptor in *C. elegans* pharyngeal pumping and the neurotransmitters involved has been reported (Avery, 1993a; McKay et al., 2004; Avery and You, 2012; Kozlova et al., 2019). In *eat-2* and *eat-18* mutant pharyngeal pumping rate is significantly reduced (**Table 1**). In wildtype worms, pharyngeal pumping is coupled to peristalsis, which usually occurs every three to four pumps. From observing *eat-2/eat-18* mutants, this coupling is different, as peristalsis follows every pump. The role of peristalsis is to concentrate and transport food from the buccal cavity, through the lumen, to the terminal bulb (Avery and Horvitz, 1989; Ishita et al., 2020).

Table 1. 1 EAT-2 and EAT-18 regulate the peristalsis/pharyngeal pump ratio

Strain	Pharyngeal pump/minute	Number of peristalsis/minute	Peristalsis/pump ratio
Wildtype (N2)	259.4 ± 12.1	60	1:4
lev-1(x427)	253.7 ± 17.8	60	1:4
eat-2(ad465)	50.3 ± 10.1	60	1:1
eat-18(ad1110)	65.9 ± 8	60	1:1

1.16 Extra pharyngeal crosstalk in *C. elegans*

In recent findings, there has been a report of cross communication between the distinct muscle tissues of the body wall muscle (BWM) and the pharynx. The L-type receptor of the body wall neuromuscular junction (NMJ) is the main excitatory postsynaptic nAChR (Izquierdo et al., 2022). The receptor is composed of 3 α-subunits (UNC-38, UNC-63 and LEV-8) and 2 non-α subunits (UNC-29 and LEV-1). It is characterized by its sensitivity to levamisole and loss-of-function mutations on any of the receptor subunits result in resistance to the drug (Lewis et al., 1980). Izquierdo et al., (2022) reported that the pharmacological activation of the BWM receptor allowed for the distal inhibition of the pharynx. Even though a full understanding of the mechanism that regulates the crosstalk between these two discrete muscles is not fully understood, it is a useful backdrop when considering pharmacological investigations of pharyngeal function. With this insightful premise, techniques around a more selective and target-specific pharmacology can be developed.

1.17 Using *C. elegans* as a model for screening approaches

There is the argument that *C. elegans* are not parasitic and therefore lack adaptations that may be vital for parasitism and possibly the druggable targets that regulate these processes (Geary and Thompson, 2001). Furthermore, *C. elegans'* evolutionary difference could mean that genes and gene families linked with parasitism could be completely absent or play different biological roles in the free-living worm (Viney, 2018). Conversely, although the lifestyle of a free-living nematode is very different from that of parasitic nematodes, in terms of body plan and genome, species of the phylum Nematoda exhibit many biological similarities and high levels of sequence conservation (Jex et al., 2011). The comparative physiology and pharmacology for members of this phylum justifies the use of *C. elegans* as a suitable model for anthelmintic and nematicide studies. An analysis of the *C. elegans* genome identified 2,958 lethal genes, 1083 of which have orthologues in the PPN *Meloidogyne incognita* (Holden-Dye and Walker, 2014).

Molecules that kill *C. elegans* are more than 15 times likely to be lethal to PPNs (Burns et al., 2015). The above points, coupled with difficulties with using PPNs in drug developmental studies, present pre-screening with *C. elegans* as a more attractive alternative, especially in high-throughput assays. Screening assays with *C. elegans* may miss some potential hit compounds due to factors like the concentration and exposure time to the compound however, the benefits of using them in primary screening systems outweigh the disadvantages (Burns et al., 2015).

It is worth noting that not all chemicals that have suppressive effects on *C. elegans* translate to PPNs. This is the case with the newly developed nematicide flauzaindolizine a carboxamide that has no effect on *C. elegans* but shows good activity with PPNs (Lahm et al., 2017). Though its mode of action is not yet known, this finding underpins arguments for species-selective pharmacology. The similarity in the wiring of the neuromuscular system for *C. elegans* to the animal parasitic nematode *A. suum,* further underpins their use as model organisms (Angstadt et al., 1989).

1.17.1 Nematicide development in *C. elegans*

In nematicide studies, once the effect of a drug is defined, different strategies may be adopted to investigate the molecular basis for its biological activity. The first is a reverse genetics approach. Using the genetic tool RNA interference (RNAi), mutant strains for genes of known function are tested for altered drug sensitivity. The alternate strategy is the forward genetic screen where strains obtained through random mutations are observed for new phenotypes (Miller et al., 1996; Sieburth et al., 2005). As early as the 1980's, *C. elegans* was already used as primary screening tool in anthelmintic screening assays (Simpkin and Coles, 1981). In the early years of using *C. elegans* in screening assays reasons like, the low-throughput nature of screening protocols and the absence of substantial lead results negated testing in targeted animals. Over decades, technological advancements like automated liquid handling systems that standardize the addition of chemicals and worms in assay plates, automated image acquisition and analysis have enabled the development of high-throughput screens with *C. elegans* (Burns & Roy, 2012). Using these advancements, in a screen for resistance, mutant strains have been successfully used to find protein targets for novel anthelmintics (Burns and Roy, 2012). With forward genetic screens Kaminsky et al. (2008a), identified the *acr-23* gene as the major contributor to amino-acetonitrile derivatives (AAD) resistance in *C. elegans.* Monepantel a member of the novel anthelmintic class AAD was found to control major gastro-intestinal nematodes, including those resistant to classical anthelmintics like the

benzimidazoles, imidazothiazoles and macrocyclic lactones (Kaminsky & Rufener, 2012; Rufener et al., 2013).

Table 1. 2 Current nematicides and their modes of action

Nematicide	Mode of Action
Cyclobutrifuram	Broad-spectrum succinate dehydrogenase inhibitor (SDHI) of mitochondrial respiration (Complex II), disrupting energy production.
Salibro	Novel non-systemic sulfoamide that disrupts nematode fitness by causing paralysis, loss of mobility and ability to infect roots.
Aldicarb	Inhibits acetylcholinesterase (AChE), causing paralysis due to acetylcholine accumulation at nerve synapses.
Carbofuran	Inhibits acetylcholinesterase (AChE), causing nerve dysfunction and nematode paralysis.
Fenamiphos	Inhibits acetylcholinesterase (AChE), leading to nerve overstimulation, paralysis, and nematode death.
Oxamyl	Inhibits acetylcholinesterase (AChE), causing nerve dysfunction and paralysis.
Methomyl	Inhibits acetylcholinesterase (AChE), disrupting nerve and muscle function, causing nematode paralysis.
Fosthiazate	Inhibits acetylcholinesterase (AChE), causes nerve dysfunction, paralysis, and is systemic, affecting root-feeding nematodes.
1,3-Dichloropropene	Soil fumigant that disrupts nematode cellular metabolism, affecting respiration and energy production.
Chloropicrin	Soil fumigant that disrupts cellular respiration and inhibits normal cell processes in nematodes.
Emamectin Benzoate	Binds to glutamate-gated chloride channels, causing an influx of chloride ions, hyperpolarization, and paralysis in nematodes.
Nimitz (Fluensulfone)	Interferes with nematode movement and feeding, disrupting signaling and causing paralysis.
Pyridaben	Inhibits mitochondrial electron transport, disrupting cellular respiration in nematodes.
Abamectin	Binds to glutamate-gated chloride channels, causing paralysis by disrupting neurotransmission and cellular function.

1.18 Justification of Studies

The limitations in selective toxicity for many chemical pesticides have resulted in pesticide bans and embargoes in several countries. In the case of four of the world's largest users and exporters of synthetic pesticides, USA, EU, China and Brazil, there are distinct pesticide regulatory systems tailored towards human and environmental protection (Donley, 2019). To

act on these constraints, by 2030, the European Union made plans to halve the use of chemical pesticides and implement a full ban on pesticide use in public parks, gardens, playgrounds, and ecologically sensitive spots (Regulation EU, 2020).

These concerns justify the need for selective toxicity and although this is hard to achieve (Huggins et al., 2012), in this research work, I seek to exploit the EAT-2 receptor, a key determinant for pharyngeal function in the model nematode *C. elegans*, as a selective drug target. Factors that qualify EAT-2 as a potentially selective pharmacophore include:

1. EAT-2 is important for quick pharyngeal pumping in the worm. Disrupting the receptor function would impede the animal's ability to feed properly, as observed in mutants missing the receptor
2. The absence of vicinal cysteines, in the ABS of the receptor. Despite this difference, the receptor can still effectively bind acetylcholine like other classical nAChRs. We hypothesis that this structural difference presents a distinct pharmacology from other members of the nAChR group.
3. EAT-2's requirement of EAT-18 for its functional expression. Although the role of EAT-18 is not well understood, it is thought to be involved with receptor gating. Interestingly, no homologs of this protein have been identified in other animal species. This presents a second arm of targeting the EAT-2 receptor in a selective and/or specific manner.

1.19 Research Aims

The above identifies a rich neurochemistry between transmitters and cognate receptors that co-ordinate the behaviour of *C. elegans*. The core transmitters are used in nematodes and shared with other non-pest species. In this study, I focused on the feeding behaviour to ascertain whether it can be controlled by chemicals that selectively bind and disrupt the EAT-2 receptor. To do this I had to:

1. Establish the presence and role of EAT-2 in plant parasitic nematodes
2. Develop a drug screening assay to identify drugs that selectively target EAT-2 and inhibit pharyngeal pumping
3. Take advantage of a heterologous expression approach to validate identified EAT-2 modulators and demonstrate how they impact PPNs

Overall, I seek to integrate outcomes from these distinct approaches to develop EAT-2's credibility as a bona fide target for the chemical mitigation of PPNs.

2 Chapter 2 Investigation of the nicotinic receptor EAT-2 as a novel target to prevent plant parasitic nematode infections

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Jim Goodchild– conceptualization, investigation, methodology, writing–original draft, writing–review and editing, funding acquisition and supervision

Philippa Harlow – conceptualization, investigation, methodology, writing–original draft, writing–review and editing, funding acquisition and supervision

Lindy Holden-Dye – conceptualization, investigation, methodology, writing–original draft, writing–review and editing, funding acquisition and supervision

Vincent O'Connor– conceptualization, investigation, methodology, writing–original draft, writing–review and editing, funding acquisition and supervision

2.1 Abstract

Plant parasitic nematodes (PPNs) exploit crops as hosts, using their stylets to hatch, invade plants, and establish feeding sites. Thus, stylet function should be a good behaviour to target, and for developing approaches designed to disrupt their lifecycle. However, knowledge of pharyngeal regulation in PPNs is limited. In the model nematode *Caenorhabditis elegans*, the nicotinic receptor EAT-2 stimulates the pharyngeal contraction that underpins feeding. Here we hypothesize that EAT-2 orthologues may regulate similar functions in PPNs. This study investigated EAT-2 in the potato cyst nematode *Globodera rostochiensis* using phylogenetics, localization, and pharmacological bioassays. Genomic analysis confirmed EAT-2 is nematode-specific and localized to the pharyngeal tissue. Functional assays in *C. elegans* and *G. rostochiensis* revealed that acetylcholine and nicotine triggered pharyngeal and stylet activity and this was blocked by mecamylamine and tubocurarine. This data is consistent with a conserved cholinergic pathway mediated by EAT-2. This highlights EAT-2 as a potential determinant of stylet thrusting and a promising pharmacological target to selectively mitigate PPN infections.

2.2 Introduction

Plant parasitic nematodes (PPNs) are global economic pests that account for significant annual losses in agriculture (Lilley et al., 2024). Current control strategies rely on cultural practices, use of resistant crop varieties and chemical strategies that disrupt the parasitic life cycle (Li et al., 2015; Mesa-Valle et al., 2020; Pires et al., 2022). Unfortunately, several available nematicides are either restricted or banned because of their broad actions and negative environmental impacts on soil and water quality (Ngala et al., 2021). Thus, finding novel pesticide targets and recommending new products that meet the regulatory standards is of high importance in the fight against these parasites (EPA, 2023). Generally, the nervous system of pests is an attractive target for pesticides because they are comprised of several biological structures and targets that are rapidly and readily druggable (Hirata, 2016). Voltage sensitive sodium channels and nicotinic acetylcholine (ACh) receptors (nAChRs) are primary targets in the nervous system whose disruption impairs signal transduction resulting in muscle paralysis, spasms and death of invertebrate insect pests (Hirata, 2016; Raisch and Raunser, 2023). Although not widely used, integrating the biological understanding of mechanisms unique to behaviours that support parasitism might refine the focus on pesticide selectivity and reduce the limiting negative environmental impacts.

Investigating the molecular determinants of core behaviours in plant parasitic nematodes is challenging given the lack of genetic tools. In this regard, studies in the model nematode *Caenorhabditis elegans* can be informative, despite its different lifestyle (Coke et al., 2024). The nervous system receives several sensory modalities that co-ordinate and generate distinct phenotypic and behavioural outputs which regulate essential processes supported in tissues and organs. This means that behaviours like feeding, locomotion and reproduction can be templated in model organism like *C. elegans* but are highly similar in other nematodes (Avery and You, 2012; Alcedo et al., 2013; Chew et al., 2013; Gjorgjieva et al., 2014; Thapliyal and Babu, 2018). The pharynx acts as a neuromuscular pump that regulates ingestion of bacteria. This pump is under nervous control and largely controlled by the pharyngeal nervous system (McKay et al., 2004; Avery and You, 2012). Its muscular organization is comprised of 20 radial muscle cells organized into 8 pharyngeal muscles. In a two-part process involving pharyngeal pumping and peristalsis, the presence of food triggers serotonergic neurons to release 5-HT resulting in elevated pharyngeal pumping rates (Dallière et al., 2016; Ishita et al., 2020). The biogenic amine 5-HT signals via cholinergic neurons MC and M4 to induce essential muscle contractions that are necessary for feeding (Ishita et al., 2020). A core determinant for this response is the EAT-2 receptor that utilizes ACh released from the MC neuron to initiate pharyngeal muscle contraction, opening of the lumen and influx of bacteria (McKay et al., 2004). EAT-2 was classified as a non-alpha nicotinic receptor subunit because it is missing the signature vicinal cysteines amino acid residues known to be a major molecular determinant of agonist binding (Kao and Karlin, 1986). Surprisingly, despite lacking this motif, both the *C. elegans* EAT-2 and its orthologue from the animal parasitic nematode *A. suum* have been shown to function as a homo-oligomeric receptors made up of the co-assembly of five identical EAT-2 receptor subunits (Choudhary et al., 2020). Moreover, functional expression of EAT-2 requires co-expression with EAT-18, a single transmembrane domain protein that functions as an auxiliary subunit. Intriguingly, and of relevance with respect to potential as a target for selective nematicides, EAT-18 has no known homology to previously described proteins (McKay et al., 2004). EAT-18's unique and essential contribution is proposed to be via an association with the EAT-2 pentamer at the muscle plasma membrane (Choudhary et al., 2020).

In PPNs pharyngeal function controls critical behaviours using specialized pharyngeal structures called the stylet, a lance-like hollow structure important for their lifestyle. The neurobiology underpinning this pharyngeal function is poorly understood (Grundler and Böckenhoff, 1997). In one modality pharyngeal muscles co-ordinate the protraction and

retraction of the stylet by J2s to hatch from the egg (Perry and Clarke, 2000; Mkandawire et al., 2022a). This biology is used later in the life cycle by hatched J2s to invade roots of host plants (Bernard et al., 2017; Pulavarty et al., 2021a). Additionally, a distinct pharyngeal function involving stylet thrusting and median bulb pumping allows protruded stylets to ingest nutrient or release effectors and cell-wall degrading enzymes into feeding sites of invaded host plants (Koga, 2014).

Despite the difference in the feeding style and habits of *C. elegans* and *G. rostochiensis*, the organization of their pharyngeal muscles includes the corpus, isthmus and terminal bulb. Furthermore, the exposure of the whole organism to 5-HT drives pharyngeal responses from averages of about 60 pumps/minute to 250pumps/minute in *C. elegans* and about zero to 80 thrusts/minute in *Globodera* (Kudelska et al., 2018; Crisford et al., 2020). A putative model based on the limited comparisons of pharyngeal structure and pharmacological evidence suggests that serotonergic signalling regulates pharyngeal function upstream of EAT-2 (Kearn et al., 2017; Ishita et al., 2020). Based on this information, we have investigated the molecular details of EAT-2 in PPNs, its expression pattern and developed pharmacological assays to resolve its function. The data obtained describe a scenario in PPNs whereby cholinergic modulation of pharyngeal function via EAT-2 regulates stylet function. This provokes the idea that EAT-2's function and its distinct molecular features make it a suitable target for new nematicide development.

2.3 Materials and methods

2.3.1 *C. elegans* maintenance

C. elegans, N2 (Bristol strain) were obtained from the *Caenorhabditis* Genetics Centre (CGC), grown and maintained under standard conditions (Brenner, 1974) and used as wild type (WT) worms.

2.3.2 *G. rostochiensis* maintenance

Infective juveniles (J2s) of *G. rostochiensis* were hatched by incubating nematode cysts in potato root diffusate (PRD) over 7 days at room temperature (Gaihre et al., 2019). Freshly hatched J2s (1 day old) were washed in M9 buffer containing 0.01% w/v Bovine Serum Albumin (BSA) prior to experimentation. Batch hatchings were performed for each experiment to obtain freshly hatched juveniles. Unused worms were collected in batches and stored at -20 ºC before being used for RNA extraction. Nematode cysts used were from the James Hutton

Research Institute, Scotland and provided by Vivian C. Blok. The PRD used for hatching was provided by Catherine Lilley, University of Leeds, UK.

2.3.3 Drugs and chemicals

Serotonin creatinine sulphate monohydrate, Nicotine hydrogen tartrate salt, Mecamylamine hydrochloride and Tubocurarine chloride were purchased from Sigma Aldrich, UK. To make stock solutions that were used in experiments the drugs were dissolved in sterile ddH_2O and stored at -20 ºC. These were used no longer than 1 month after preparation.

2.3.4 Characterizing *C. elegans* and *G. rostochiensis* response to 5-HT, ACh and nicotine

2.3.4.1 *C. elegans* behavioural assay

These assays were performed on solid NGM plates by supplementing molten NGM with 5-HT, nicotine or ACh to achieve the indicated final concentration. A *C. elegans* pharyngeal pump dose-response to 5-HT was established by testing concentrations up to 5mM. ACh and nicotine were tested at 10 mM. 3 ml NGM containing vehicle or the indicated concentration of drug was transferred into clear Corning Coster 6-well plates. After setting the NGM plates were stored at 4 ºC and used the following morning. Before experimenting, these plates were equilibrated to room temperature for 30 minutes. Well-fed young adult *C. elegans* (L4+1s) were washed in M9 buffer to remove bacteria and then picked onto test plates. Pharyngeal pump rates were measured by making visual observations using a Nikon SMZ800 binocular microscope. A pump was defined as a forward and backward movement of the grinder and was recorded for a minute per worm (Raizen et al., 1995). All *C. elegans* behavioural assays were performed on solid NGM plates because in liquid *C. elegans* thrash and it is impossible to make valid measurements.

2.3.4.2 G. *rostochiensis* behavioural assay

Hatched J2s were pipetted into 96 well-plates. Stylet thrusting dose-response was established by supplementing 20 mM HEPES solution, pH 7.4 with indicated concentrations of 5-HT. Ten freshly hatched J2s washed 3 times with ddH_2O were pipetted in 2 µl and added into wells containing 200 µl of 20 mM HEPES with or without the indicated 5-HT concentration. The number of stylet thrusts were measured for 30 seconds per worm, 30 minutes after incubating G. *rostochiensis* in 5-HT and represented as number of thrusts per minute. A forward and backward movement (extension and retraction) of the stylet was counted as one stylet thrust

(Kearn et al., 2017). All *G. rostochiensis* behavioural assays were performed in liquid assays unless specified otherwise.

2.3.4.3 Acute responses to selected drugs

We investigated the acute effects of 10 mM nicotine, 200 µM mecamylamine and 300 µM tubocurarine on 5-HT induced pharyngeal pumping and stylet thrusting. This was performed by adding respective modulatory drug or requisite vehicle after establishing a steady-state stylet thrusting response for 30 mins in 1mM 5-HT. The acute response to these selected drugs on 5-HT induced pharyngeal pumping and stylet thrusting was measured for up to 2 hours

2.3.4.4 Chronic responses to selected drugs

The chronic effects of the modulating drugs were investigated by pre-incubating *C. elegans* or *G. rostochiensis* for 24 hours in 10 mM nicotine, 200 µM mecamylamine and 300 µM tubocurarine prior to transferring them onto assays plates.

2.3.5 Molecular biology

2.3.5.1 Phylogenetic analysis

We used the basic alignment search tool (BLAST) (Altschul et al., 1990) and the query *Ce*.EAT-2 (Uniprot accession number: Q9U298) to search for EAT-2 homologues on Uniprot and WormBase ParaSite. The resulting putative protein sequences for nematodes, insects and the closest human homologue were imported into the MEGAx (Molecular Evolutionary Genetics Analysis) tool and aligned via MUSCLE alignment (multiple sequence alignment by log-expectation) (Stecher et al., 2020) with default parameters. The aligned sequences were used to generate a phylogenetic tree using the Maximum Likelihood (ML) statistical method, a Jones-Taylor-Thornton (JTT) substitution model and 500 bootstrap replications to assess node support. The tree interference options were set to default parameters. The phylogenetic tree output was exported in a Newick format and visualized using iTOL (interactive tree of life) (Letunic and Bork, 2024). The following protein sequences were used in the analysis to build the phylogenetic tree: Q9U298 (*C. elegans*); A0A261C1U6 (*C. latens*); A0A9P1MVH6 (*C. angaria*); A0A158PA63 (*A. cantonensis*); A0A0N4XX74 (*N. brasiliensis*) A0AA36C8X6 (*M. spiculigera*); A0AAF3EAP6 (*M. belari*); A0AA39GWV7 (*S. hermaphroditum*); A0A7E4V0M4 (*P. redivivus*); A0A2A6C485 (*P. pacificus*); A0A0K0DZ20 (*S. stercoralis*); A0A811LPF4 (*B. okinawaensis*); A0A1I7S0H9 (*B. xylophilus*); A0A914M6R1 (*M. incognita*); A0A6V7VQB4 (*M. enterolobii*); A0A914I8V5 (*G. rostochiensis*); A0A8T0A120 (*M. graminicola*); A0A914XFN7 (*P. sambesii*); A0A0N4UD55 (*D. medinensis*);

A0A0B2W3Z2 (*T. canis*); A0A4E9FW31 (*B. malayi*); W2TG30 (*N. americanus*); A0A4U8V1R9 (*S. carpocapsae*); A0A2G9V6F1 (*T. circumcincta*); A0AA36MEL8 (*C. nassatus*); A0A0N4VD61 (*E. vermicularis*); A0A1I7V6L7 (*L. loa*); A0A0V0XKJ1 (*T. pseudospinralis*); A0A9C6WDJ3 (*D. albomicans*); A0A8B8HQB2 (*V. tameamea*); M9PFD8 (*D. melanogaster*); A5H031 (*M. domestica*); A0A9P0BVQ3 (*C. includens*); A0A8R2AB14 (*A. pisum*); A0A8B7PB12 (*H. azteca*); A0A8I6SIA1 (*C. lectularius*); AgR003_g027 (*A. suum*); A0A915M3T9 (*M. javanica*); tig00002135.g43658.t1 (*M. arenaria*); A0A1I8BSG2 (*M. hapla*); Hsc_gene_20958.t1 (*H. schachtii*), A0A183C532 (*G. pallida*); KAH7704458.1 (*A. avenae*); A0A158PA63 (*H. sapiens*).

2.3.5.2 RNA extraction

Total RNA was extracted from one 1000 *C. elegans* young adults and 2000 infective *G. rostochiensis* juveniles respectively. Nematodes were washed with diethyl pyrocarbonate (DEPC) treated M9 buffer, centrifuged at 500 g and the supernatant discarded and leaving the worms in minimal volumes of DEPC in the 2 ml eppendorf tubes. 1 ml of TRIzol™ was added to each sample and homogenized for a minute using the hand-held VWR® VDI 12 homogenizer (VWR Collection). Homogenization was monitored via visual inspection and was continued until all intact organisms were broken down. Total RNA was isolated using the sequential TRIzol™ (Invitrogen) Qiagen RNA clean up protocol (Metzger, 2024). Total RNA extracted was stored at -20 ºC or used to synthesize cDNA using the SuperScript® III First-Strand Synthesis System protocol (Invitrogen).

2.3.5.3 PCR amplification

The sequences that encode the open reading frame (ORF) for *Ce*.EAT-2 (Uniprot accession number: Q9U298) was used as input to perform a protein BLAST search on Uniprot and WormBase ParaSite. We identified orthologues in sequenced parasitic nematodes and the predicted ORF for *Gr.*EAT-2 (accession number: A0A914I8V5). Primers designed and synthesized with Integrated DNA Technologies were used to amplify the predicted coding sequence of both organisms. The following primers were used to amplify EAT-2 transcripts for *C. elegans* and *G. rostochiens;*

FwCe.eat-2 5'- CGCAC***gaattc***ATGACCTTGAAAATCGCATT -3'

RvCe.eat-2 5'- CAGGCTAACAACTATAACTTATT***cccggg***CG - 3'

FwGr.eat-2 5'- GC***ggtacc***ATGTTTTTGCGA -3'

Rv.Gr.eat-2 5'- GGTGGAGAGATT***cccggg***GA -3'

Restriction sites (underlined sequences) were added at primer ends to flank the amplified sequence. The authenticity of the amplified sequence was confirmed by sequencing the forward and reverse strands of the amplified gene (Eurofins Genomics) (see supplementary 1).

2.3.5.4 Hybridization Chain Reaction RNA-Fluorescence in-situ hybridization

The expression patterns for the target mRNA *Gr.*EAT-2 (Uniprot accession number: A0A914I8V5), *Gr.*MYO-3 (Uniprot accession number: A0A914HJT9) and *Gr.*UNC-17 (Uniprot accession number: A0A914GTZ9) were investigated using a modified HCR™ RNA-FISH (v3.0) protocol for whole-mount nematode larvae (Choi et al., 2016).

2.3.5.5 Probe sets, amplifiers and buffers

Probes against target transcripts, probe hybridization buffers, probe wash and amplifier buffers were purchased from Molecular Instruments. The number of probe binding sites for *Gr.eat-2* was set to 40 to mitigate likely low and restricted expression levels based on exemplar data in *C. elegans* (McKay et al., 2004). Binding sites for *Gr.unc-17* and *Gr.myo-3* were standard 20. Amplifiers and hairpins used were: B1H1, B1H2 for EAT-2 and B2H, B2H2 for UNC-17 and MYO-3. Other reagents used included: M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 20.5 mM NaCl, 1 mM $MgSO_4$), PBST (1X PBS, 0.1% Tween), 4% paraformaldehyde (PFA), glycine solution (2 mg/mL glycine, PBST), proteinase K solution (100µg/mL).

2.3.6 Preparation of fixed whole-mount nematode larvae

Approximately 1000 *G. rostochiensis* J2s were washed three times with 1 mL of M9. During each wash the tube were centrifuged at 500 g for 2 min to bring larvae to the bottom and the supernatant carefully removed. Washed J2s were aliquoted in minimal volumes of M9 buffer and incubated 1 mL of 4% paraformaldehyde (PFA) before immediately freezing at -80 ˚C overnight. The following day the fixed J2s were thawed at room temperature for 45 minutes washed twice in 1 mL of PBST and treated with 1 mL proteinase K (100 µg/mL) (Sigma Merck) for 10 min at 37 ˚C. The proteinase K treated J2s were washed with PBST and incubated in 1 mL of glycine solution (2 mg/mL) for 15 min on ice followed by 2 washes with PBST.

2.3.7 Probe hybridization, amplification and detection

Fixed and permeabilized J2s were incubated in 1 mL of 50% PBST / 50% probe hybridization buffer for 5 min at room temperature and centrifuged at 500 g for 2 min before removing the supernatant. These processed J2s were then pre-hybridized in 300 µL of HCR™ probe

hybridization buffer at 37 ˚C for 1 h. The indicated probe solutions were prepared just before use by adding 2 µL of the 1 µM stock to 500 µL of probe hybridization buffer at 37 ˚C (See the HCR^{TM} RNA-FISH (v3.0) protocol for more detail) (Choi et al., 2016). The pre-hybridized J2s were incubated overnight (>12 h) at 37 ˚C in 500 µL freshly made probe solution. After the overnight incubation the probe solution was removed by washing J2s 4 times in 1 mL of probe wash buffer at 37 ˚C for 15 minutes. These washes were followed by two further 5 mins washes at room temperature in 1 mL of 5× SSCT. To facilitate pelleting of worms between washes prepared worms were centrifuged at 800 g. Hybridized J2s were incubated in 300 µL of amplification buffer for 30 min before adding 200 µL of the hairpin solution and incubating overnight (>12 h) in the dark at room temperature. Following this incubation the worms were washed 5 times with 1 mL 5× SSCT at room temperature. These samples were treated with 0.01% DAPI (v/v) and stored at 4 ˚C protected from light before being imaged.

2.3.8 Mounting and imaging

The probe treated specimens were pipetted in 20 µL of SSCT (about 30-50 nematodes), placed on a glass slide, secured with a cover slip and sealed with nail varnish. Slides for imaging were mounted on the Olympus/Yokogawa Spinning Disk confocal microscope using the Alexa fluor 488 for GFP and 594 for mcherry. Images were prepared using image J, with minor adjustments on the brightness and contrast of the DIC images (Schindelin et al., 2012).

2.3.9 Statistical analysis

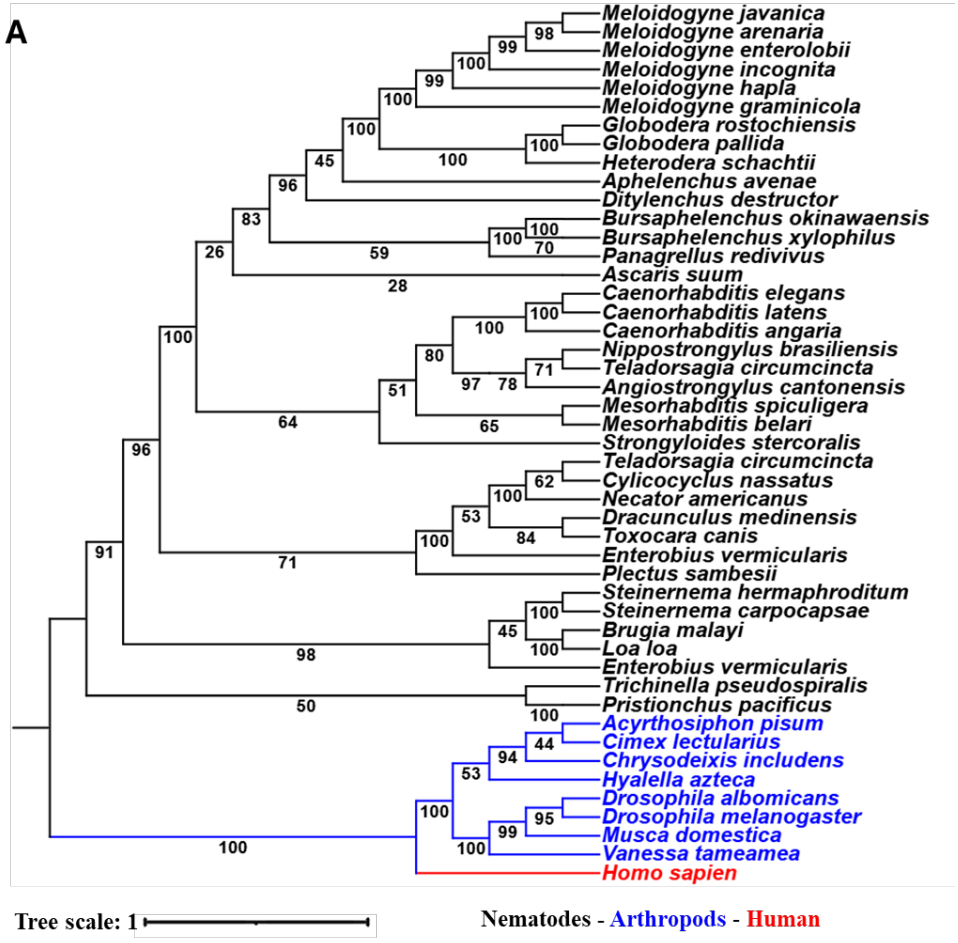
Data analysis was performed with GraphPad Prism 10.4 and presented as the mean ± standard error of the mean (SEM) for a number of observations (n). Statistical significance was tested either by unpaired student's t-test, one-way or two-way ANOVA followed by Bonferroni multiple comparison where appropriate. Significance levels were set at $p<0.05$. Every experiment was repeated in 3 or more independent occasions, unless stated otherwise. EC_{50} values with 95% confidence intervals were determined by plotting log (agonist) vs. normalized response-variable slope and fitted to the equation; Y=100/(1+10^((LogEC_{50}-X)*HillSlope))

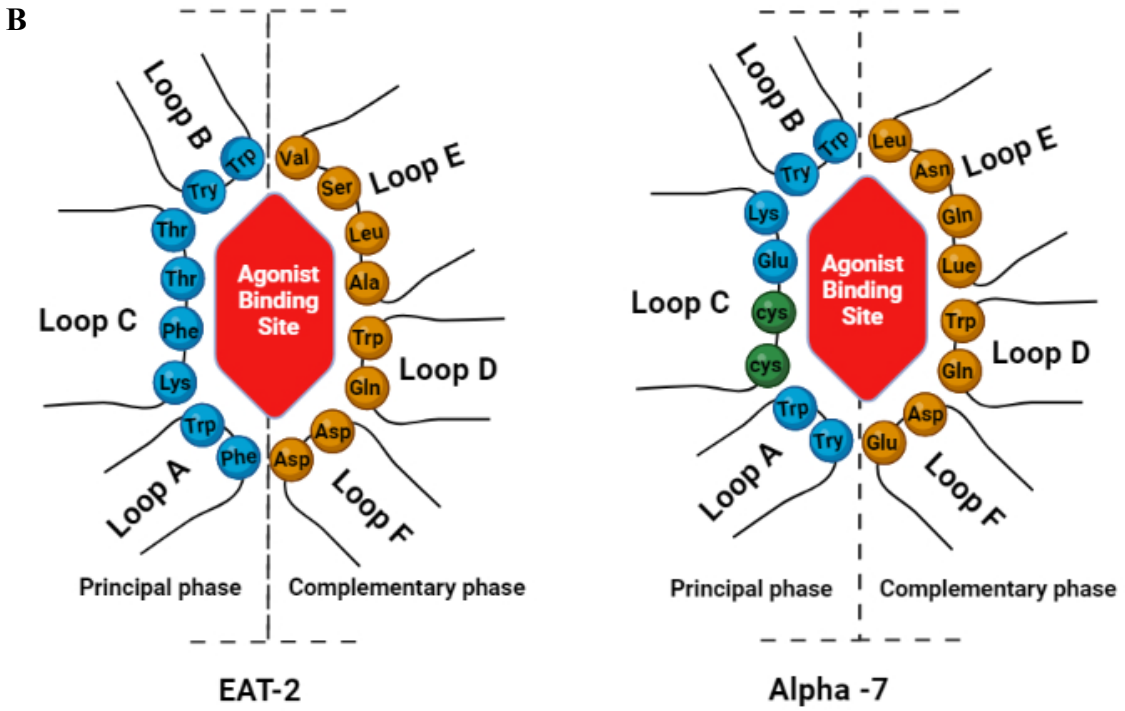
2.4 RESULTS

2.4.1 Phylogenetic evidence reveals EAT-2 unique expression in the Nematoda

Amino acid sequence for *C. elegans* EAT-2 was used as a query to run a protein blast on Uniprot (all animal species) and WormBase ParaSite databases (specifically for all parasitic helminths). The phylogenetic analysis revealed *Ce.*EAT-2 and orthologues in other nematode

species cluster as a distinct group that suggests the EAT-2 orthologues are exclusive of other animal species (**Fig. 2. 1A**). The EAT-2 orthologues predict the classical nicotinic acetylcholine receptor transmembrane topology encompassing a large extracellular N-terminal domain and four intervening transmembrane helices leading to a short extracellular C-terminus. In the context of a nAChR the acetylcholine binding site lies at the interface between the extended N-terminal extracellular domains of neighbouring subunits. In homomeric receptors like alpha-7 and EAT-2 this is at the principal (front) and complementary (back) end of each subunit. The six-loop fold that comes together to contact the activating ACh has a major and minor contact made up of discontinuous loops ABC and DEF respectively. Comparing amino acid residues that make up the principal and complementary phases of the ligand binding domain for *Ce.*EAT-2 and its closely related human orthologue the nicotinic receptor alpha-7 (Choudhary et al., 2020), revealed a 50% conservation of amino acid residues (**Fig. 2. 1B**) in these loops. A multiple sequence alignment for *Ce*.EAT-2 and some selected nematode species with alpha-7 revealed the absence of vicinal cysteine residues in the loop-C region of these receptor subunits (**Fig. 2. 1C**). Interestingly these pivotal vicinal cysteines were originally identified as an absolute requirement for nicotinic receptor alpha subunits acetylcholine binding, but emerging data recognizes that activation can occur in the absence of this motif (Blount and Merlie, 1990; Blum et al., 2011; Choudhary et al., 2020).


A
Meloidogyne javanica
Meloidogyne arenaria
Meloidogyne enterolobii
Meloidogyne incognita
Meloidogyne hapla
Meloidogyne graminicola
Globodera rostochiensis
Globodera pallida
Heterodera schachtii
Aphelenchus avenae
Ditylenchus destructor
Bursaphelenchus okinawaensis
Bursaphelenchus xylophilus
Panagrellus redivivus
Ascaris suum
Caenorhabditis elegans
Caenorhabditis latens
Caenorhabditis angaria
Nippostrongylus brasiliensis
Teladorsagia circumcincta
Angiostrongylus cantonensis
Mesorhabditis spiculigera
Mesorhabditis belari
Strongyloides stercoralis
Teladorsagia circumcincta
Cylicocyclus nassatus
Necator americanus
Dracunculus medinensis
Toxocara canis
Enterobius vermicularis
Plectus sambesii
Steinernema hermaphroditum
Steinernema carpocapsae
Brugia malayi
Loa loa
Enterobius vermicularis
Trichinella pseudospiralis
Pristionchus pacificus
Acyrthosiphon pisum
Cimex lectularius
Chrysodeixis includens
Hyalella azteca
Drosophila albomicans
Drosophila melanogaster
Musca domestica
Vanessa tameamea
Homo sapien
Tree scale: 1
Nematodes - Arthropods - Human


B
Loop B
Loop C
Loop A
Loop E
Loop D
Loop F
Agonist Binding Site
Principal phase
Complementary phase
EAT-2
Alpha -7

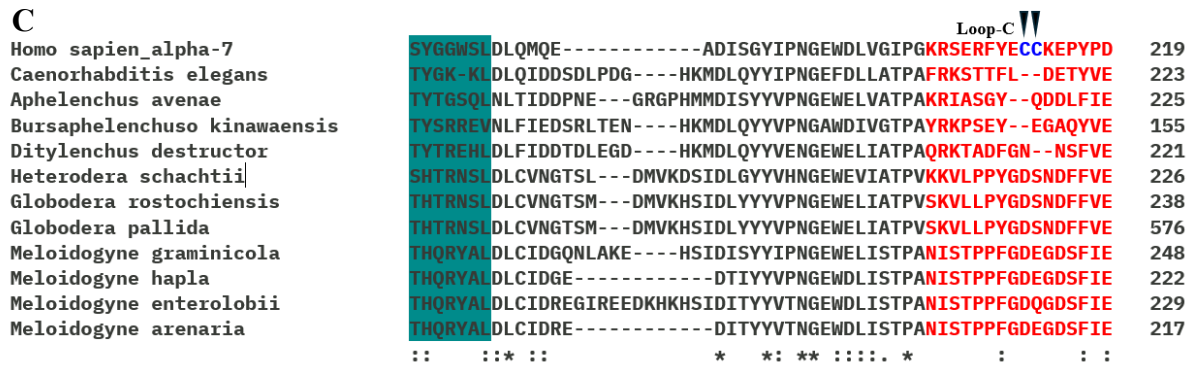


Fig. 2. 1 Maximum likelihood phylogeny of EAT-2. A. The tree was rooted with α-7, the closest human orthologue of *C. elegans* EAT-2. Phylogenetic analysis groups EAT-2 of nematodes (green) in a common branch separate from orthologues of arthropod and humans. **B.** A schematic representation showing a 50% conservation of amino acid residues that make the agonist binding site (ABS) of EAT-2 and α7. Loops A, B and C make up the principal phase of the binding domain and loops D, E and F, the complementary phase. **C.** A multiple sequence alignment for *C. elegans* EAT-2 and orthologues in some economically important plant parasitic nematodes shows the absence of vicinal cysteines in the loop-c when compared to the human α7.

2.4.2 EAT-2 expression pattern in *G. rostochiensis*

2.4.2.1 Spatial expression of Gr.EAT-2 HCR RNA-FISH

To resolve the organization of cholinergic transmission and the molecular features of *Gr.eat*-2 we first authenticated the predicted sequence. This involved extracting total RNA from *G. rostochiensis,* reverse transcribing it to cDNA and then using specific primers to amplify the predicted ORF for *Gr.eat-2.* The amplification product was authenticated through sequencing. The sequencing revealed that the amplified product fully aligned with the predicted sequence. This amplified sequence was used to design probes for HCR RNA-FISH, to investigate the spatial expression of *Gr.eat-2* transcripts using whole-mount in-situ hybridization. We extend this to the wider cholinergic system using the predicted orthologues for *Gr.unc-17* which encodes the vesicular ACh transporter in *C. elegans* (Alfonso et al., 1993) and is found in all cholinergic releasing neurons and *Gr.myo-3* which encodes the myosin heavy chain protein highly expressed in longitudinal muscles of the body wall muscle (Meissner et al., 2009). Previously developed methods for getting small molecules like DAPI into worms suggested the need to physically sever the cuticle (Lilley et al., 2018). However, we used the HCR™ RNA-FISH (v3.0) protocol for whole-mount nematode larvae (Choi et al., 2016) and achieved a 30% success rate (n=100). With probes for *Gr.eat-2* and *Gr.unc-17* in a co-localization

hybridization reaction we identified a discrete expression pattern for *Gr*.EAT-2 in pharyngeal muscles, specifically in the median bulb and further discrete staining in what looked to be the pharyngeal glands (**Fig. 2. 2A**). *Gr*.UNC-17 was identified as punctate staining in the anterior region and along the ventral side of the worm, highlighting the possible locations of cholinergic motor neurons (**Fig. 2. 2A**). These specific signals in the pharynx likely highlight the neuronal cell bodies of the cholinergic neurons that innervate pharyngeal muscles. Using *Gr*.MYO-3 as a marker to access the effectiveness of the hybridization reaction, I showed the specificity of *Gr*.EAT's pharyngeal spatial expression pattern (**Fig. 2. 2B**). Worms fixed and prepared in the absence of target probes showed no signal for our target genes. The sclerotized head and stylet were auto fluorescent and very bright for both control and probe treated specimen (**Fig. 2. 2C**).

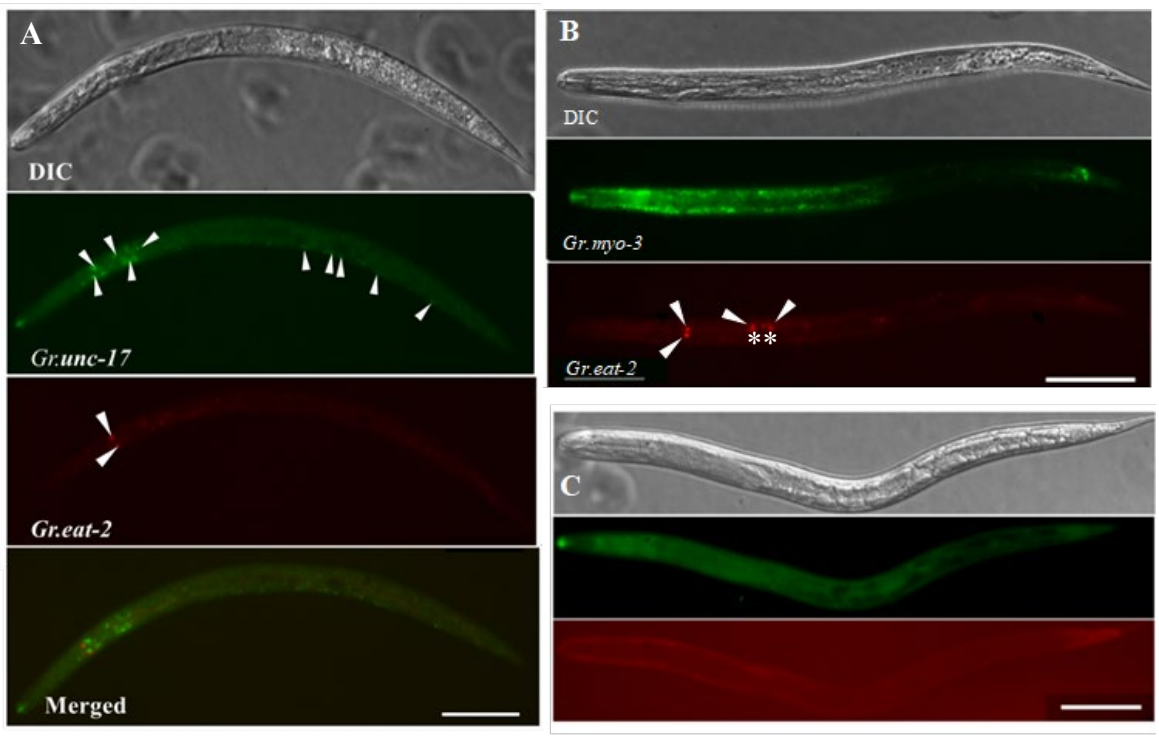


Fig. 2. 2 Spatial expression of EAT-2 in *G. rostochiensis* visualized with FISH. A. Whole-mount nematode visualizing the expression of *Gr.eat-2*, seen as red puncta and *Gr-unc17* seen as green puncta in the anterior region, consistent with pharyngeal expression. **B.** *Gr.myo-3* expressed in longitudinal muscles of the body wall muscle (green), **C.** Worms treated without target probe show no cellular expression but identify a non-specific auto fluorescence in the head region and the stylet. Arrow heads point to the localization indicated hybridization probe, the asterix represents an additional puncta pattern not always observed in worms that otherwise always show the non-asterix arrow heads. The images are representative images from 2k prepared J2s in two independent histology runs. Scale bar, 50 µm.

2.4.3 5-HT induces pharyngeal pumping and stylet thrusting

The functional control of pharyngeal behaviour is regulated by modulatory neurons that can be probed by external exposure to high concentrations of 5-HT (Horvitz et al., 1982; Perry et al., 2004; Hobson et al., 2006). We investigated the dose dependence of 5-HT on *C. elegans* pharyngeal pumping and *G. rostochiensis* stylet thrusting to benchmark pharmacological comparisons between these nematode species. Increasing concentrations of 5-HT induced increased pharyngeal activity for both nematode species. In *C. elegans* 5-HT had an EC_{50} of 237 µM and 5 mM induced the highest pharyngeal pumping rate (**Fig. 2. 3A&C**). Similarly, in *G. rostochiensis* increasing 5-HT concentrations induced increasing stylet thrusting responses with an EC_{50} of 409 µM that was maximal at 1mM (**Fig. 2. 3B&C**).

In solutions like M9 or 20 mM HEPES buffer, *C. elegans* motility is represented by a coordinated thrash (Buckingham and Sattelle, 2009). In contrast, movement in *G. rostochiensis* is variable and relatively uncoordinated (see supplementary 2). Interestingly, incubations in 5-HT to measure pharyngeal function identified that 1 mM and 30 mM immobilized *G. rostochiensis* and *C. elegans* respectively. *C. elegans* assumed a steady position with elevated pharyngeal pump rates while *G. rostochiensis* adopted a kink position that was associated with the induced stylet thrusting (see supplementary 3).

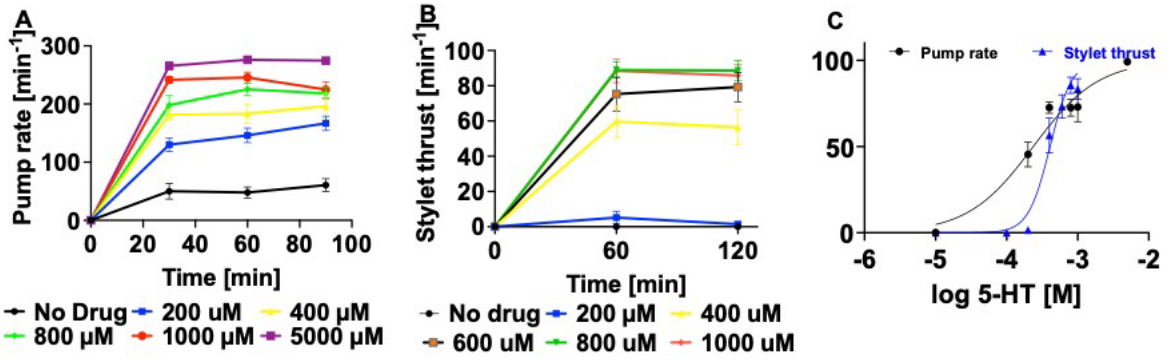


Fig. 2. 3. Dose dependent effects of 5-HT on pharyngeal pumping and stylet thrusting. A. Pharyngeal responses to 5-HT was measured as described in the materials and methods. Exposing *C. elegans* to increasing doses of 5-HT resulted in increasing pharyngeal pump response, n<10**. B.** Stylet thrusting in *G. rostochiensis* increased with increasing 5-HT concentrations and peaked at 1 mM beyond which the response dropped, n=15. **C.** A dose response curve for 5-HT identified EC_{50} values of 237 µM (95% confidence limit 168.5 to 315 µM) and 409 µM (95% confidence limit 365.0 to 457.5 µM) for *C. elegans* pharyngeal pumping and *G. rostochiesis* stylet thrusting respectively. Data represented as mean ± SEM

2.4.4 Acetylcholine and nicotine induce pharyngeal functions

We applied known agonists of cholinergic systems, ACh and nicotine at 10 mM and observed that these ligands activated pharyngeal pumping and stylet thrusting behaviours. ACh and nicotine induced stronger responses in *C. elegans* compared to *G. rostochiensis* (**Fig. 2. 4A**). The stylet thrusting response to ACh and nicotine was weak compared to 5-HT induced responses in *G. rostochiensis* (**Fig. 2. 4B**). This reported increase in pharyngeal function due to exogenous application of nicotinic receptor agonists supports an underlying contribution of cholinergic transmission.

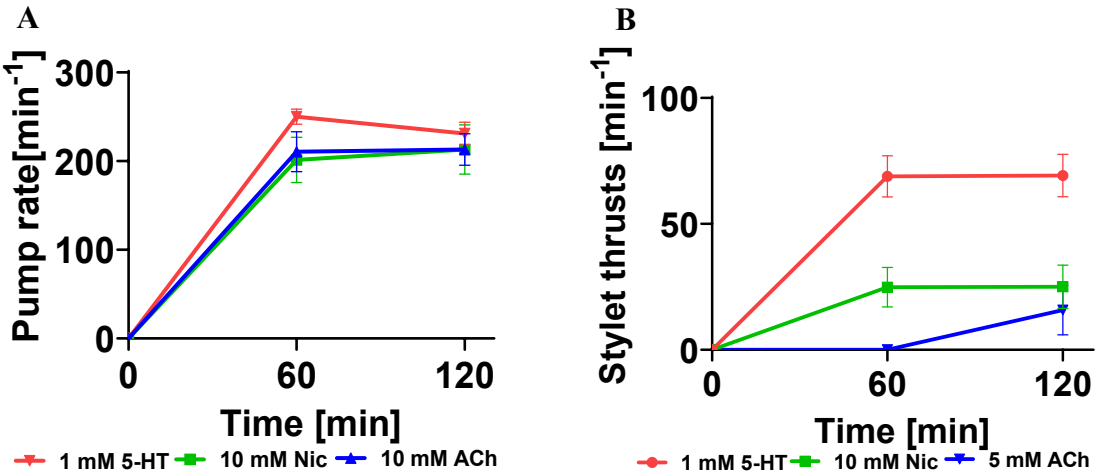


Fig. 2. 4 EAT-2 agonists induce feeding behaviours in *C. elegans* and *G. rostochiensis*. The exogenous exposures to nicotine and ACh induced **A.** pharyngeal pump responses in *C. elegans* (n>8) and **B.** stylet thrusting in *G. rostochiensis* (n=10). Data represented as mean ± SEM

2.4.5 EAT-2 modulators inhibit 5-HT induced pharyngeal function in *C. elegans* and *G. rostochiensis*

Having established the 5-HT dependence of stylet thrusting and pharyngeal pumping, we tested other distinct classes of drugs that might inform on the intersection and potential organization of pharyngeal function. To do this we investigated how these selected drugs impacted pharyngeal behaviours. This involved comparing acute (short-term) and chronic (long-term) exposure effects of these selected drugs on 5-HT induced pharyngeal responses. We tested how the EAT-2 agonist nicotine and antagonists mecamylamine and tubocurarine affected pharyngeal function of *C. elegans* and *G. rostochiensis* pre-stimulated with 5-HT or how long term overnight pre-exposures to these drugs effected the subsequent ability of 5-HT to stimulate pharyngeal responses.

2.4.5.1 Acute exposure to nicotine inhibits pharyngeal function

Short term exposure of *C. elegans* to nicotine had a rapid inhibitory effect on 5-HT stimulated pharyngeal pumping. A 30 minute pre-exposure to nicotine inhibited 5-HT induced pharyngeal pumping (**Fig. 2. 5A**). Similarly, *G. rostochiensis* pre-exposed to 10 mM nicotine prior to 5-HT stimulation observed reduced stylet thrusts (**Fig. 2. 5B**). This inhibitory nicotine effect was sustained in both nematodes over the 2 h experimental period. This was interesting because nicotine activated these pharyngeal behaviours in the absence of 5-HT pre-stimulation (**Fig. 2. 3**)

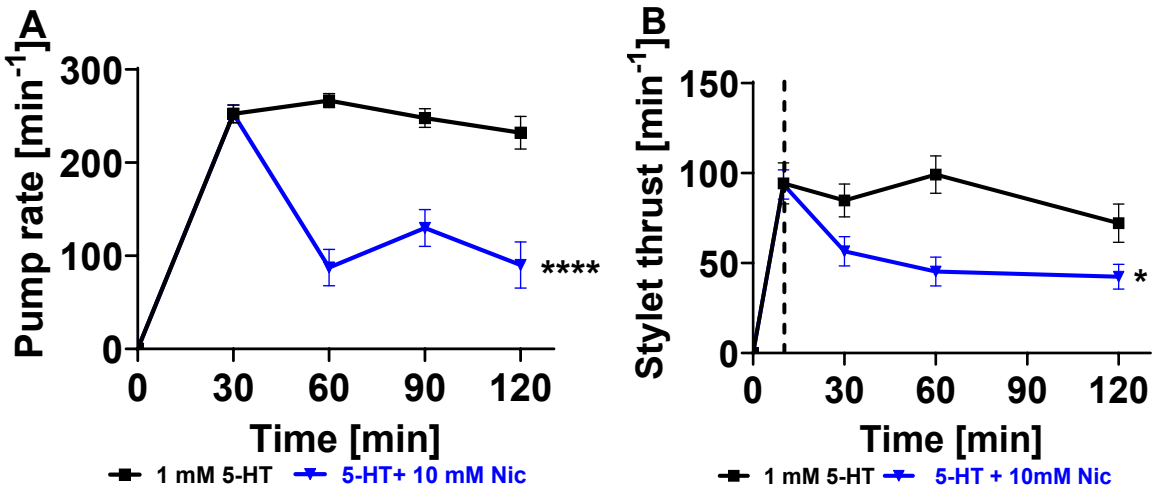


Fig. 2. 5 Nicotine inhibits pharyngeal function in 5-HT stimulated *C. elegans* and *G. rostochiensis*. A. 5-HT induced pharyngeal pump rates in *C. elegans* were inhibited by 10 mM nicotine, n=10 **B.** In *G. rostochiensis*, the same concentration had inhibitory effects on the stylet thrusting, n=13. Broken line indicates when nicotine was added. Data represented as mean ± SEM. The analysis uses the steady state rate of 5-HT as control or 5-HT + ligand and significance tested by two-way ANOVA with Bonferroni's multiple comparison (*p<0.05, ****p<0.0001).

2.4.5.2 Acute exposure to EAT-2 antagonists impact pharyngeal function differently

Short-term exposure to mecamylamine and tubocurarine had varying effects on the pharyngeal pumping and stylet response to 5-HT stimulation. In *C. elegans*, mecamylamine had a sustained inhibitory effect on 5-HT stimulated pharyngeal pumping 30 minutes post exposure. Tubocurarine had no effect on pharyngeal pumping (**Fig. 2. 6**). In *G. rostochiensis* short-term exposure to mecamylamine and tubocurarine failed to inhibit 5-HT stimulated stylets.

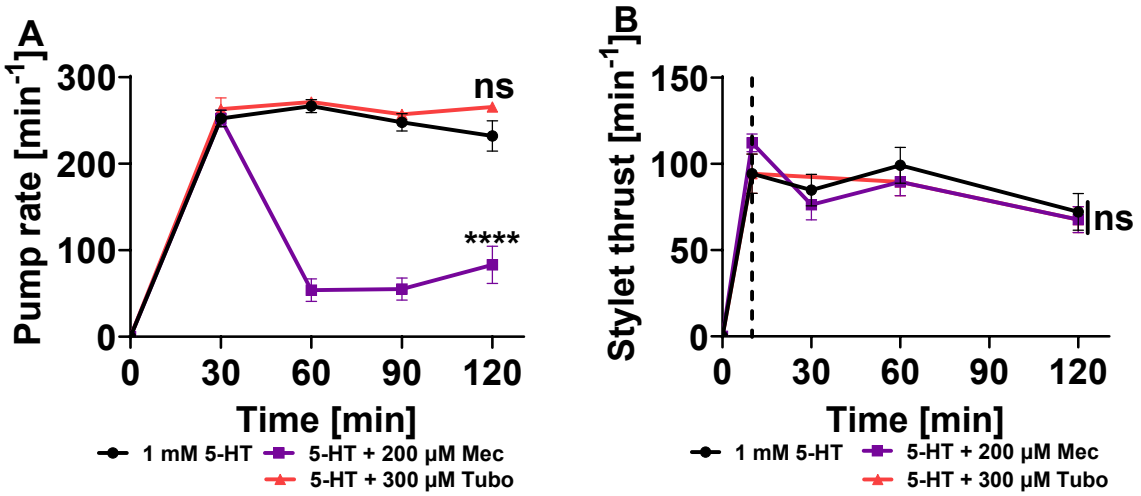


Fig. 2. 6 The acute effect of nAChR antagonists on 5-HT stimulated pharyngeal function. **A.** 5-HT stimulated pharyngeal pumping in *C. elegans* following a short-term exposure to 200 µM mecamylamine or 300 µM tubocurarine, n=10. **B.** stylet thrusting of *G. rostochiensis* following a short-term exposure to mecamylamine and tubocurarine on 5-HT stimulated, n=10. Broken line indicates when antagonists were added. The data are shown as mean ± SEM the response to 5-HT or 5-HT + ligand and significance tested by two-way ANOVA with Bonferroni's multiple comparison ($^{ns}p>0.05$, ****$p<0.0001$).

2.4.5.3 Chronic exposure to EAT-2 nicotine inhibits 5-HT stimulated pharyngeal function

A challenge of whole nematode drug exposure is the rate of accumulation of drug largely restricted by the cuticle. Prolonged incubation can help overcome this limitation. We explored prolonged pre-incubation of *C. elegans* and *G. rostochiensis* in modulatory drugs prior to 5-HT exposure. Long-term pre-incubation in nicotine had inhibitory effects on 5-HT stimulated pharyngeal function. In *C. elegans,* a 24 h incubation in nicotine markedly inhibited 5-HT induced pharyngeal pumping (**Fig. 2. 7A**). In *G. rostochiensis*, this prolonged incubation completely abolished stylet thrusting behaviours (**Fig. 2. 7B**).

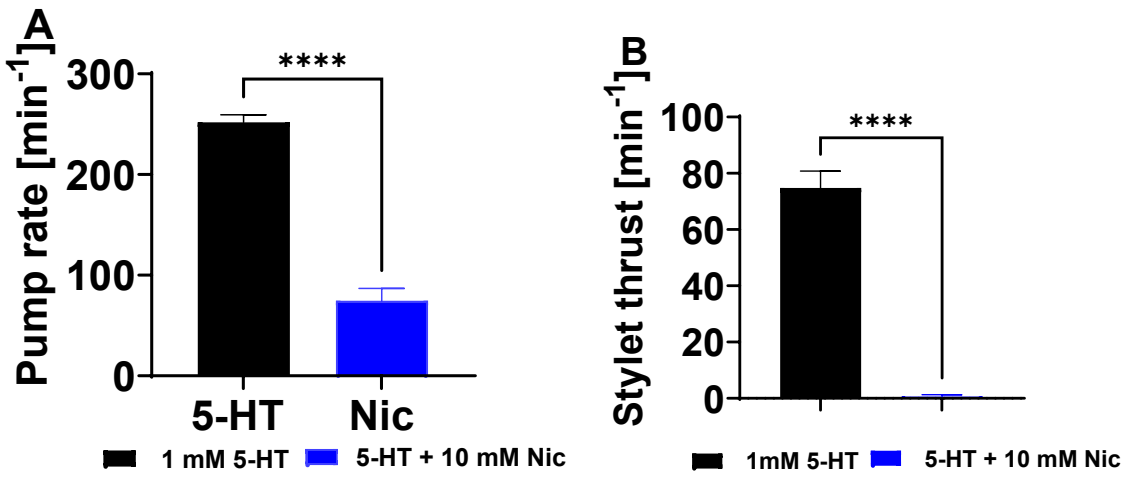


Fig. 2. 7 Protracted nicotine pre-exposure inhibits 5-HT induced pharyngeal behaviours in *C. elegans* and *G. rostochiensis*. A. Pharyngeal responses in *C. elegans* pre-exposed to 10 mM nicotine prior to 5-HT (n=8). **B.** 5-HT stimulated stylet thrusting in *G. rostochiensis* pre-incubation in nicotine (n=40). The data are shown as mean ± SEM and the analysis correspond to 5-HT as control or 5-HT + ligand and significance tested by unpaired t-test (****p<0.0001).

2.4.5.4 Chronic exposure to EAT-2 antagonists impact pharyngeal function

There is a limited pharmacology around the EAT-2 receptor but mecamylamine and tubocurarine have shown good potency against recombinantly expressed EAT2 receptors (Choudhary et al., 2020). A 24 h pre-incubation of *C. elegans* in mecamylamine blocked 5-HT stimulation pharyngeal responses. This inhibitory effect was similar to the response observed with acute exposure to the drug. In contrast, pre-incubating *C. elegans* overnight in tubocurarine had no effect on the subsequent ability of 5-HT to induce pharyngeal pumping (**Fig. 2. 8A**).

Interestingly in the case of *G. rostochiensis* the overnight incubation in tubocurarine saw a significant reduction in the subsequent 5-HT induced stylet thrusting. Thus, long-term pre-exposure to both EAT-2 antagonists inhibited 5-HT stimulatory effects on stylet thrusting (**Fig. 2. 8B**).

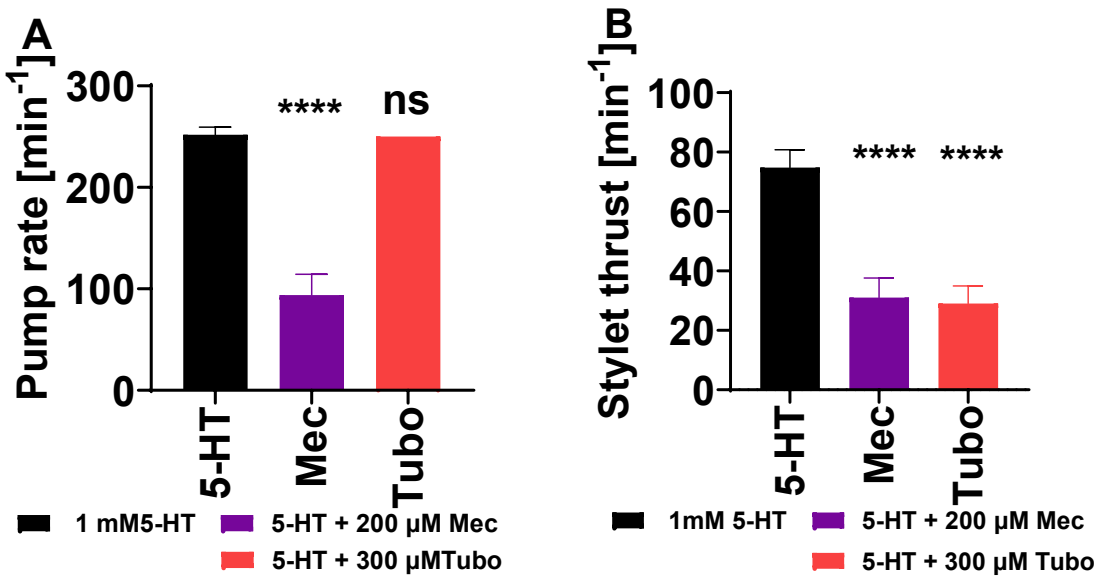


Fig. 2. 8 The effect of EAT-2 antagonists mecamylamine and tubocurarine on 5-HT induced pharyngeal function in *C. elegans* and *G. rostochiensis*. A. Mecamylamine and tubocurarine effects on 5-HT induced pharyngeal pump rates in *C. elegans* (n=8). **B.** Effects of mecamylamine and tubocurarine on 5-HT induced stylet thrusting responses in *G. rostochiensis,* (n=30). The data are shown as mean ± SEM. The analysis corresponds to 5-HT as control or 5-HT + ligand and significance tested by one way ANOVA with Bonferroni's multiple comparison ($^{ns}p>0.05$, ****$p<0.0001$).

2.4.6 Stylet thrusting and body posture in *G. rostochiensis*

As indicated above, in addition to inducing pharyngeal function the exogenous application of 5-HT induced changes in *G. rostochiensis* motility and body posture. When incubated for longer than 30 minutes in 1 mM 5-HT, G. *rostochiensis* became immobile and assumed a kink posture in the head region with elevated stylet activity (**Fig. 2. 9A**). Worms incubated in the control solution (20 mM HEPEs) did not kink and did not stylet thrust. *G. rostochiensis* pre-incubated for 24 h in nicotine prior to 5-HT exposure failed to adopt this kinked posture and this coincided with the complete block of 5-HT induced thrusting. Interestingly, prolonged pre-incubations in mecamylamine and tubocurarine did not disrupt kinking but had a selective inhibitory effect on the stylet thrusting (**Fig. 2. 9B**). In summary, the comparison of the consequence of 5-HT on posture and stylet function in face of distinct nicotinic acetylcholine receptor agents suggests a potential uncoupling of the stylet thrusting and associated body postures.

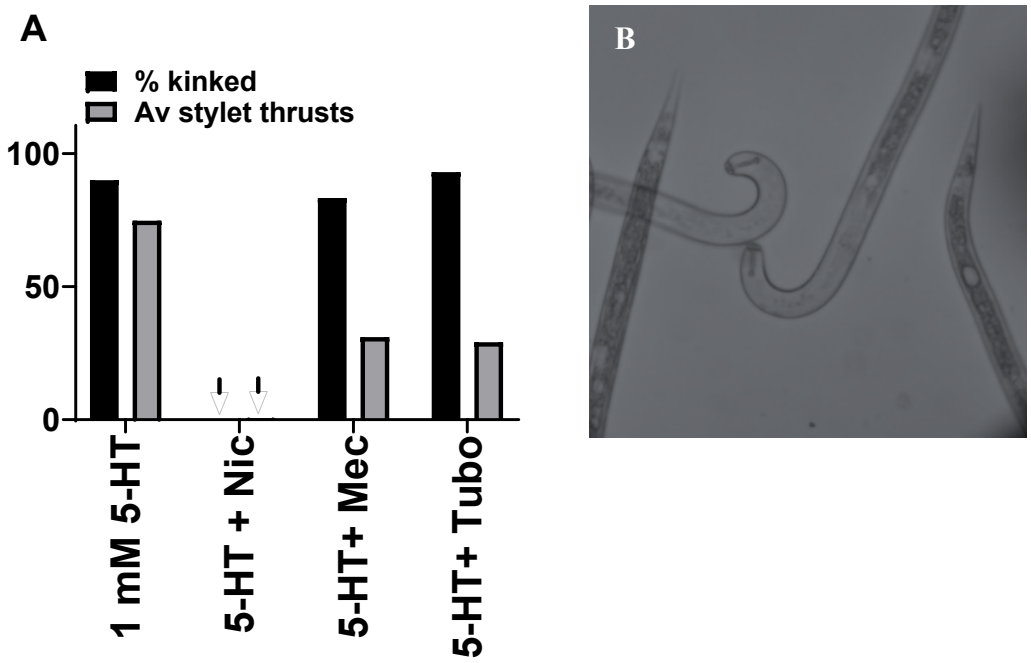


Fig. 2. 9 Comparing effect of nicotinic acetylcholine drugs on 5-HT induced stylet and posture states. A. The effects of 10 mM nicotine, 200 µM mecamylamine and 300 µM tubocurarine on 5-HT induced stylet thrusting and associated kinked posture on *G. rostochiensis* (5-HT n= 13, nicotine n=12, mecamylamine n=12 and tubocurarine n=30). J2s subjected to the indicated treatments were scored for their kinked body posture and stylet thrusting activity and represented as a percentage of kinked J2s and average (Av) stylet thrusts. **B.** Grayscale image of *G. rostochiensis* in a kinked posture when incubated in 1mM 5-HT**.**

2.5 DISCUSSION

The targeting of several distinct molecules and functional loci of the cholinergic transmitter system has been a successful strategy for invertebrate pest control (Raisch and Raunser, 2023). These pesticides impact acetylcholinesterase, the vesicular acetylcholine transporter or distinct classes of acetylcholine receptors (Costa et al., 2008; Čadež et al., 2021; Goodchild et al., 2024) but their use is confounded by the off-target effects on other organisms (Wan et al., 2025). As a mitigation to this, nAChRs offered a more selective approach to inhibiting the nervous system of invertebrate pests (Bradford et al., 2020). However, despite phyla selective potency for this receptor class it is still challenging to overcome the issue of non-selective targeting of pests relative to non pest organisms (Li et al., 2025a).

In this study we looked at specialized pharyngeal functions in two nematodes: stylet thrusting in the plant parasitic nematode *G. rostochiensis* and pharyngeal pumping in the free-living

model nematode *C. elegans*. This could offer a tissue-selective target underpinning specialized life cycle behaviours as an approach to improve pesticide selectivity. We showed that these pharyngeal functions in both nematode species were controlled by cholinergic and serotonergic signalling. This is thought to be driven by the rather distinct, nematode-specific nAChR EAT-2 which has previously been shown to be required for normal pumping in *C. elegans* (McKay et al., 2004). It should be noted that whilst this role is important to *C. elegans* it is non-essential as null mutants are merely retarded in development (Avery, 1993b; McKay et al., 2004). However, considering the multiplicity of life cycle selective functions like hatching, host root invasion and feeding behaviours that the pharyngeal muscles play in the PPNs it can be envisaged that targeting this receptor will be particularly pernicious for this nematode class. Our study probed the molecular and structural determinants of pharyngeal muscles that regulate pharyngeal function in *G. rostochiensis*. We investigated the possibility of translating existing understanding of EAT-2 function in *C. elegans* to PPNs.

Initially, we established EAT-2 presence in other nematode species through a protein blast and a phylogenetic compilation that revealed its conservation within Nematoda. This was confirmed in the PPN *G. rostochiensis* by successfully amplifying the cDNA for the predicted *Gr*.EAT-2 ORF. In *C. elegans*, EAT-2's functional expression requires EAT-18, an auxiliary protein that is under investigated. EAT-18 is a short single transmembrane domain protein with an intracellular N-terminus and an extracellular C-terminus. Evidence suggests that in the absence of EAT-18, EAT-2 receptor is made, trafficked and localized in the plasma membrane but is non-functional (Choudhary et al., 2020). This makes EAT-18 a potential target to indirectly disrupt EAT-2 function. The heterologous expression of EAT-2 in *Xenopus* does not require any other nAChR subunit suggesting that EAT-2 is a homo-pentameric receptor *in vivo.* Orthologues of EAT-18 was found in other nematode species and a multiple sequence alignment of *Ce*.EAT-18 with other orthologues revealed a high degree of conservation with a 59.09 % identity to the putative *Gr*.EAT-18 (see supplementary 4). *Ce*.EAT-18 is imbedded within the first intron of *Ce*.LEV-10 and perhaps there is a requirement to investigate LEV-10 in other nematode species to identify and better annotate the gene.

2.5.1 Fluorescence in-situ hybridization reveals *Gr*.EAT-2 expression in the pharynx

Given the molecular conservation of EAT-2 across Nematoda, we probed its expression pattern and function. Such histology has been challenging in nematode species even with the increased availability of molecular information (Sperling and Eves-van den Akker, 2023). Our study took advantage of multiplex probes that facilitate the detection of gene expression in whole mount

nematodes to provide primary evidence on the expression pattern of EAT-2 to underpin key signalling in stylet thrusting and possibly the median bulb pulsation. Most FISH studies on PPNs have focused on effector genes (De Boer et al., 1998, 1999; Lilley et al., 2018; Sperling and Eves-van den Akker, 2023). Here, we show visual evidence of the expression pattern for cholinergic neurons using probes for *Gr.unc-17,* the transporter gene required for loading synthesized ACh into vesicles (Alfonso et al., 1993), *Gr.eat-2*, a cholinergic receptor and putative regulator for pharyngeal function in PPNs and *Gr.myo-3* a highly expressed gene in longitudinal muscles of the body-wall. With probes for *Gr.eat-2* we identified discrete and reproducible expression in pharyngeal structures. The tissue in which we consistently found a hybridization pattern across several stained specimen was the median bulb (metacorpus). In addition, there was a robust but less frequent associated staining in discrete structures located in a position associated with the oesophageal glands (**Fig. 2. 2**). These observations resonate with recent transgenic work Cao et al. (2023) reporting *Ce*.EAT-2 expression in all pharyngeal muscles but favours a more restricted expression observed by McKay et al. (2004). Sense probes for *Gr.unc-17* identified neurons in the pharynx and around the ventral side of the worm. The expression pattern for *Gr*.UNC-17 was similar to transgenic expression studies in *C. elegans* where expression patterns for *Ce.*UNC-17 were observed in cholinergic neurons in the head region and the ventral nerve cord motor neuron (Mathews et al., 2012; Haque and Nazir, 2016). Probes for *Gr.myo-3* localized on the longitudinal muscles of the body wall muscle. The selective distribution of cholinergic determinants is consistent with the key role in body wall muscle transmission, pharyngeal transmissions and supports a discrete role for EAT-2 in PPN pharyngeal function.

2.5.2 A common pathway may regulate pharyngeal pumping and stylet thrusting

2.5.2.1 5-HT indirectly regulates pharyngeal pumping and stylet thrusting

We developed this molecular organization to investigate transmitter signalling in nematode pharyngeal function. Consistent with other findings, we show that exogenous exposure to 5-HT induced the pharyngeal behaviours, pharyngeal pumping and stylet thrusting in *C. elegans* and *G. rostochiensis* respectively (Horvitz et al., 1982; Perry et al., 2004; Hobson et al., 2006). Although this happens, it is worth mentioning that 5-HT has no direct effect on EAT-2, the cholinergic regulator of pharyngeal pumping in *C. elegans.* The biogenic amine activates a cholinergic pathway through the MC neuron to initiate pharyngeal pumping through EAT-2 (McKay et al., 2004; Song and Avery, 2012). Beyond pharyngeal function, 5-HT concentrations that induce pharyngeal behaviours in *G. rostochiensis* rendered them immotile

with a characteristic kinked-shape posture around the midbody or neck region. A supporting interpretation for this kink is that worms generate enough hydrostatic pressure and tension to act like a flex point for protractor muscles to drive stylet movements (Doncaster, 1966).

2.5.2.2 ACh and nicotine directly induce feeding behaviours

The neuromuscular system within Nematoda shows a high degree of conservation, which allows for generally valid hypotheses and conclusions to be made on physiological behaviours like motility, egg laying, and feeding behaviours across different nematode species (Hahnel et al., 2020). *C. elegans* requires cholinergic signalling to achieve muscle contractions that drive pharyngeal functions like pharyngeal pumping and peristalsis. By utilizing the cholinergic compounds ACh and nicotine, we induced stimulatory effects on pharyngeal function with *C. elegans* and *G. rostochiensis*. Our findings complement the findings of Kozlova et al. (2019) who observed that WT *C. elegans* and *cha-1* mutants deficient in choline transferase activity (Rand and Russell, 1984) exposed to nicotine showed induced pharyngeal pumps whilst *eat-2* mutants were not significantly affected, suggesting that nicotine's stimulatory effect on pharyngeal pumping may be EAT-2 dependent. ACh and nicotine have also been reported as agonist of recombinantly expressed EAT-2 receptors (Choudhary et al., 2020). Per se, comparing the pharmacologically induced pharyngeal behaviour in *G. rostochiens* with the well-researched pharyngeal pump pathway in *C. elegans,* we propose that a cholinergic involvement via EAT-2 drives pharyngeal function that can be manifested as stylet thrusting (**Fig. 2. 10**). The role of sensory inputs that innervate pharyngeal muscles and possibly controlling stylet protractor muscles have long been recognized (Doncaster, 1966). Here we provide insights into the downstream pharmacological and molecular determinants of this action.

2.5.2.3 Nicotine inhibits 5-HT stimulated pharynx

Nicotine's inhibitory effect on 5-HT stimulated pharyngeal responses was interesting because nicotine in solution induced pharyngeal responses but in combination with 5-HT was inhibitory suggesting that the overstimulation of pharyngeal muscles resulted in an activation block and a subsequent inhibition in pharyngeal responses. Acute and chronic pre-exposure of *C. elegans* and *G. rostochiensis* to nicotine inhibits 5-HT induced pharyngeal effects and this may occur because the extended incubation leads to an inhibitory block. This interpretation is in support of the notion that nicotinic responses act down stream of 5HT (Kudelska, 2019).

2.5.2.4 Mecamylamine and tubocurarine directly modulate pharyngeal function

We designed experiments to help decipher the intersection between pharyngeal pumping and stylet thrusting. The stimulatory effects of ACh and nicotine supports a role of EAT-2 in these responses. We further probed this by investigating the effect of the potent EAT-2 antagonists mecamylamine and tubocurarine on 5-HT stimulated pharyngeal response and observed that for both *C. elegans* and *G. rostochiensis* mecamylamine significantly inhibited 5-HT stimulated pharyngeal responses. Importantly we identified that tubocurarine, an independent antagonist with known selectivity for specific subclasses of nAChRs also inhibited stylet thrusting. Thus, based on two chemically distinct inhibitors we can suggest that EAT-2 is an important mediator of stylet thrusting. Interestingly, exposing *C. elegans* to tubocurarine in overnight incubations had no effect on 5-HT induced pharyngeal pumping possibly, reflecting a differential access based on the distinct cuticles (Decraemer and Hunt, 2013a). In other studies, we identified that tubocurarine had excellent potency in *C. elegans* preparations in which the cuticle was opened to expose the pharyngeal muscles to the exogenously applied drug (**Fig. 2. 10**). In addition, tubocurarine has been reported to completely block ectopically expressed *Ce*.EAT-2 receptors at concentration 10 folds lower than that used in our whole animal assay (Choudhary et al., 2020).

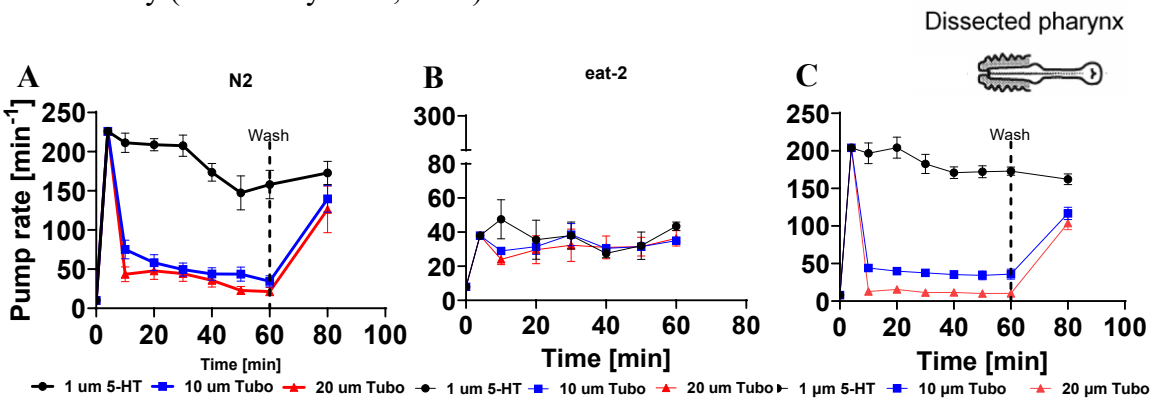


Fig. 2. 10 Effect of tubocurarine on exposed *C. elegans* pharyngeal muscles. A. Tubocurarine blocked 5-HT induced pharyngeal pumping in cut-head N2s, **B.** had no effect on *eat-2* mutants and **C.** the effect was restored in *eat-2* rescued (*pmyo-2::eat-2*) mutants. Pharyngeal pumping recovered after a 10 minute wash and re-exposure to 5-HT.

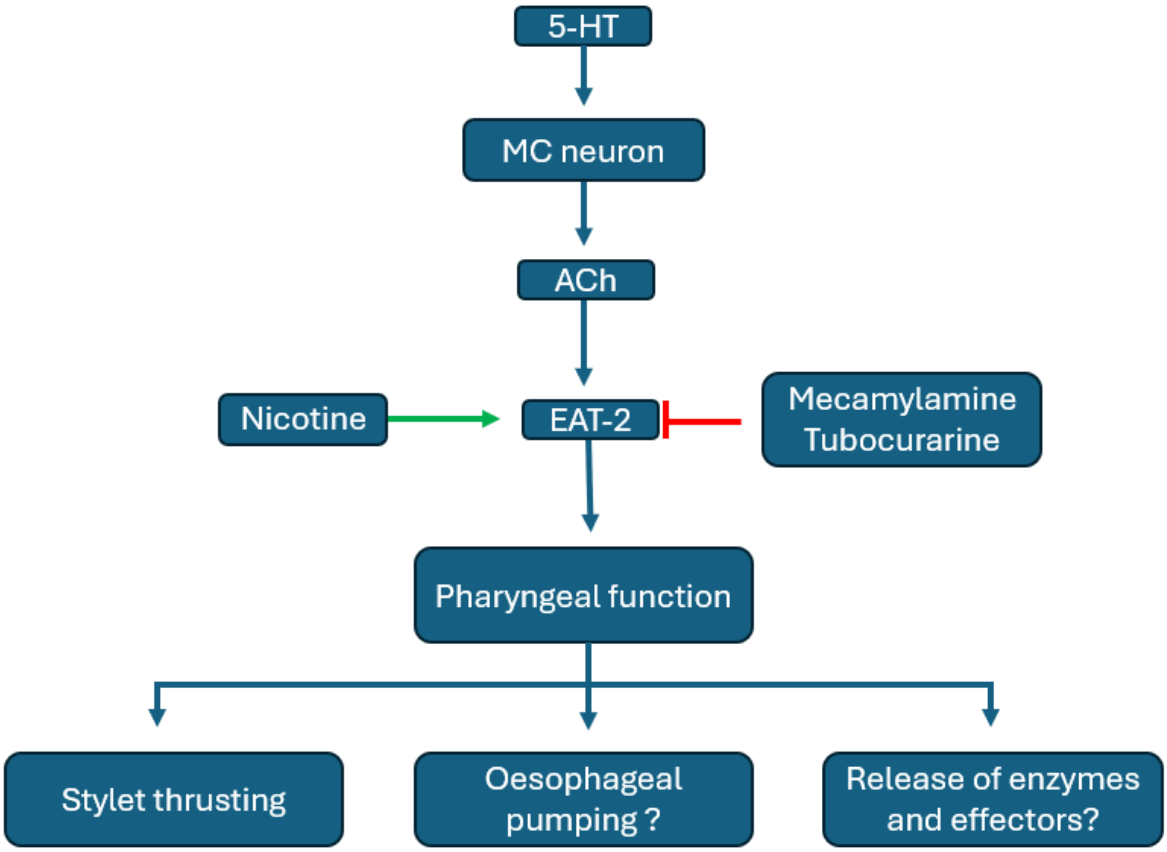


Fig. 2. 11 A putative signalling pathway for pharyngeal function in *G. rostochiesis*. 5-HT modulates pharyngeal function through the MC neuron, activating a cholinergic release that acts directly on EAT-2 and triggering pharyngeal function. Nicotine and ACh are agonists of EAT-2 that induce stylet thrusting. Mecamylamine and tubocurarine block EAT-2 and inhibit stylet thrusting functions.

To summarize, we set out to understand the physiological and pharmacological determinants that regulate the pharyngeal behaviours involved with stylet thrusting behaviours in PPNs. Firstly, we showed that EAT-2, the cholinergic regulator of pharyngeal pumping in *C. elegans*, was uniquely expressed in the Nematoda with a conserved absence of key vicinal cysteine residues required for agonist binding. Secondly, using *G. rostochiensis* as our PPN model we successfully identified the expression pattern of *Gr.eat-2* in pharyngeal muscles. Finally, we presented pharmacological evidence for EAT-2's involvement in regulating stylet behaviours. With EAT-2 agonists nicotine and ACh we induced pharyngeal responses that were blocked by antagonists mecamylamine and tubocurarine in *C. elegans* and *G. rostochiensis.* Whilst these antagonists are not EAT-2 specific, they were useful pharmacological tools for this study. Our findings implicate neuronal involvement in the stylet thrusting behaviour and suggest that *Gr*.EAT-2 plays an important role in the signalling pathway that drives pharyngeal function

(**Fig. 2. 11**). Considering the biological significance of stylet thrusting for PPNs, we propose *Gr*.EAT-2 to be a valuable target to disrupt the lifecycle of this global economic agricultural pest. As such the EAT-2 pharmacophore merits further investigation to resolve potential selective channel modulators.

3 Chapter 3 Optimizing a *C. elegans* whole organism screen biased for chemicals that target the nematode clade specific receptor EAT-2

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3.1 Abstract

Pesticides are important resources in the control of pests and pathogens of plants and animals. Unfortunately, there are concerns around their broad impacts on non-target organisms and the environment. Over the past three decades the free-living nematode *Caenorhabditis elegans* has been used in anthelminthic and nematicide studies to address this problem. Their genetic experimental tractability and relatively simple biology makes them good models for investigating novel nematicide targets. Moreover, despite their different lifestyles, key elements of the physiology of *C. elegans* and parasitic nematodes are conserved. In this study we optimize a platform biased for the nicotinic acetylcholine receptor EAT-2, a physiological regulator of feeding in *C. elegans.* EAT-2 has molecular signatures different from other nicotinic receptors which suggests it could be a novel, selective, druggable target. We show that by screening for chemicals that inhibit *C. elegans* pharyngeal pumping in a specific genetic background, *lev-1 (x427)*, we can identify lead compounds that are modulators of EAT-2. Finally, we show that EAT-2 can be selectively modulated to disrupt *C. elegans* pharyngeal function and bring pharyngeal pumping (feeding) to a halt. This provides a motivation for further studies to investigate the role of EAT-2 in parasitic nematodes and their potential as a target for novel nematicides.

3.2 Materials and Methods

3.2.1 *C. elegans* maintenance

C. elegans, WT (N2) (Bristol strain), *eat-2(ad465)* and *lev-1(x427)* mutants were obtained from the Caenorhabditis Genetics Centre (CGC), grown and maintained under standard conditions (Brenner, 1974). Synchronised *C. elegans* L4 worm were identified by the crescent-shaped, vulva saddle clearing made by the maturing vulva (Divekar et al., 2021), and picked onto OP50 seeded plates, incubated overnight prior to performing behavioural assays with them as young adults (L4+1).

3.2.2 Drug stocks

Aldicarb, levamisole, nicotine, mecamylamine were purchased from Sigma-Aldrich Company Ltd, UK. Monepantel was provided by Syngenta, UK. Nicotine, levamisole, aldicarb and mecamylamine were dissolved in sterile double distilled water to make stock solutions. Monepantel was dissolved in 100% DMSO and tested at 0.1% (v/v) to negate adverse effects of DMSO on multiple aspects of *C. elegans* biology (Calahorro et al., 2021). All drug stocks stored at 4°C were used within a month of preparation.

3.2.3 Drug screening plates

The protocols developed to interrogate EAT-2 function was setup in 24-well plates. 3 ml of molten NGM was added into each well and left in the laminar flow for 30 minutes to solidify. Each well was then seeded with a 25 µl drop of OP50 laced with 3 µl of drug or DMSO as control. The drop of OP50 was spread over the whole surface of NGM in each well and left for 30 minutes in the laminar flow to dry. These plates were then stored overnight at 4°C with the expectation that the drug equilibrates over the entire 3 ml NGM. Before experimenting, plates taken out of the fridge were set on the bench for at least 30 minutes to equilibration to the room temperature (rtp). Three L4+1 worms were picked into each well and incubated for 24 hours at rtp. The following day, phenotypic observations on pharyngeal pumping, motility, progeny development, averseness and potential bactericidal effects scored (**Table 3. 1**). These observations were made using the Nikon SMZ800 binocular dissecting microscope (60x)

Table 3. 1 Qualitative and quantitative measurements of drug induced effects on *C. elegans*

Phenotype	Observation and Quantification	Score (0 or 1)
Pharyngeal pumping	One pharyngeal pump is a complete cycle of one forward and backward movement of the grinder. Using a counter and an electronic stopwatch, pump rates were measured for one minute, per worm.	Pump <200 = 1
Motility	Motility effect was quantified mainly by looking for paralysis. Worms were considered paralysed if they failed to move when prodded 3 times with an aluminium picking needle. Measuring the body length of paralysed worms informed on the type of paralysis (spastic or flaccid).	No movement = 1
Progeny	Number of eggs and juveniles were counted 24 hours post incubation.	Eggs and Juveniles < 150/3 = 1
Aversiveness	Worms crawling off plates were scored for drug aversiveness by counting the number of worms on the food lawn 24 hours post incubation.	< 2/3 worms = 1
Bacterial effect	By visual observations, drugs that affected the establishment of the food lawn compared to the control were considered to have an effect on bacterial growth.	Unestablished bacteria lawn = 1

Scoring was binary. There was either an effect (1) or no effect (0).

3.2.4 Dose-time response assays

Dose-time response assays for hit compounds that emerged from the screen were performed on 6-well plates containing NGM. OP50 was laced with a range of different drug concentrations and then used to seed NGM plates. Drug dose induced effects on pharyngeal pumping was recorded at scheduled times over 24 hours.

3.2.5 Statistical analysis

Data was analysed using GraphPad Prism 10 and displayed as mean ± standard error of the mean (SEM) of the number of worms used for each assay. The number of worms accessed for each experiment was specified on the corresponding figure. Statistical significance was assessed using two-way ANOVA followed by a post hoc analysis with Bonferroni corrections.

3.2.6 Description

Nematicides are an important group of pesticides used to control parasitic nematodes of plants and animals, however there are several concerns around their environmental impact, safety and sustainability (Desaeger et al., 2020a). Demonstrating that a pesticide does not cause adverse effects to humans, the environment and is within reasonable economic and social costs, justifies that the said pesticide meets desired qualities for production and marketing (US EPA). There have been several approaches used to find novel chemicals that are effective and void of the aforementioned concerns. In the context of nematode control, studies in parasitic nematode pharmacology have used C*aenorhabditis elegans* as a useful tool in high throughput anthelmintic drug discovery assays (Burns et. al 2015). By using *C. elegans* as a model system, researchers have improved on the pace of drug screening assays while focusing on drug selectivity by implementing forward and reverse genetics to identify new anthelmintic targets (Mathew et al., 2016). *C. elegans* is a powerful genetic tool that has played a significant role in defining the mode of action for anthelmintics and nematicides (Holden-Dye and Walker, 2014; Burns et al., 2015d, 2017). More targeted approaches to nematicide discovery are exploring the nematode nervous system for target sites like ion channels and receptors that have been underexploited for their pharmacological potentials (Holden-Dye and Walker, 2014).

A key factor in phenotypic assays is to establish a specific endpoint phenotype. Several behavioural experiments after exposing *C. elegans* to toxicants rely on phenotypes like motility, pharyngeal pumping, head thrash, body bends, forward/reversal movements, omega/U-turn, chemotaxis, aversiveness (Sobkowiak et al., 2011). This methods paper

describes the rationale for setting up the screening platform and using pharyngeal pumping as our primary endpoint phenotype. In *C. elegans,* the cholinergic receptor EAT-2, drives pharyngeal pumping and has been reported to be a potential drug target for mitigating parasitic nematodes (McKay et al., 2004; Choudhary et al., 2020). This study demonstrates how we leveraged pharyngeal pumping in a model hopping approach to investigate a novel target for parasitic nematode control. Specifically, we optimized the use of *C. elegans* in a whole organism assay to screen compound libraries and find selective modulators of EAT-2. We hypothesise that pharmacologically modulating EAT-2 will disrupt pharyngeal muscle function, impede feeding and starve parasitic worms.

Prior studies that used *C. elegans* as model organisms in screens for novel anthelmintics or nematicides demonstrated the importance of using appropriate genetic backgrounds (Burns et al., 2015c). To achieve our aim, we used *lev-1(x427)* null mutants of the levamisole-sensitive (L-type) receptor in the body-wall muscle (BWM) as our genetic background. This mutant is viable and has no phenotypic disadvantages compared to wild-type worms. It was selected for the study because we needed to eliminate confounding cholinergic inputs from the BWM that could introduce false positive results in our screen for modulators of pharyngeal pumping. Izquierdo et al. (2022) observed that over activating the BWM L-type receptor paralysed wild-type worms and disrupted pharyngeal muscle function, suggesting a cross-communication between BWM and pharyngeal muscle i.e., hypercontraction of BWM in response to cholinergic agonists indirectly causes an inhibition of pharyngeal pumping. This interpretation is supported by the observation that levamisole fails to inhibit pharyngeal pumping in *lev-1(x427)* loss of function mutants (Izquierdo et al., 2022). Besides the L-type receptor of the BWM, there is also the nicotine sensitive receptors (N-type receptors) consisting of the ACR-16 group whose over activation results in the hypercontraction and paralysis of *C. elegans* and other nematodes (Charvet et al., 2018; Choudhary et al., 2019b).

We characterised the chronic effects of 24-hour exposure to the selected drugs; aldicarb, levamisole, nicotine, mecamylamine and monepantel on *lev-1(x427)* mutants and then compared the data with those from N2 and *eat-1(ad465)* mutants. These selected ligands were assessed for their effects on; pharyngeal pumping. In parallel, we assessed their effects on body wall muscle contraction, motility, progeny development and aversiveness. To ensure the effects observed were not due to an effect of the compounds on the bacterial lawn rather that a direct effect on *C. elegans* behaviour we also tested their effects on *E. coli*, growth (**Table 3. 1**).

In agreement with published observations, levamisole, agonist of the L-type receptor in *C. elegans* BWM had no effect on *lev-1* mutants compared to N2 and *eat-2* mutants where pharyngeal pumping was totally inhibited and the worms were paralysed (**Fig. 3. 1A** & **B**). Pharyngeal pump inhibition observed with N2 and *eat-2* worms is due to hypercontraction of the BWM (**Fig. 3. 1B**) (Izquierdo et al., 2022) and in agreement with the observation that levamisole is not a modulator of EAT-2 (Choudhary et al., 2020). A similar result was observed with aldicarb, a cholinesterase inhibitor that disrupts cholinergic pathways in the BWM and pharynx, resulting in paralysis and pharyngeal pump inhibition. Aldicarb inhibits ACh breakdown at the neuro-muscular junctions of BWMs and the pharynx chronically elevating ACh levels. This induces a spastic paralysis, immobilises the worms and inhibits pharyngeal pumping (Mahoney et al., 2006; Izquierdo et al., 2023). Monepantel induced paralysis in N2, *eat-2* and *lev-1* mutants also resulting in an indirect inhibition of pharyngeal pumping due to BWM contraction (**Fig. 3. 1A** & **B**). Previously, monepantel has been reported as an agonist of ACR-23 receptor of the BWM, inducing a spastic paralysis (Rufener et al., 2013). Interestingly, mecamylamine and nicotine had inhibitory effects on pharyngeal pumping void of paralysis and although nicotine induced a modest inhibitory effect on motility this did not impact pharyngeal pumping in *eat-2(ad465)* mutants suggesting EAT-2 to be a target for the drug. Mecamylamine had a more selective effect as the only phenotypic disruption observed was pharyngeal pump inhibition in N2 and *lev-1(x427)* mutants. Mutants of EAT-2 were resistant to this drug induced inhibition suggesting it to be a target of the drug. In recombinant assays nicotine and mecamylamine are reported to be an agonist and antagonist of EAT-2 respectively (Choudhary et al., 2020).

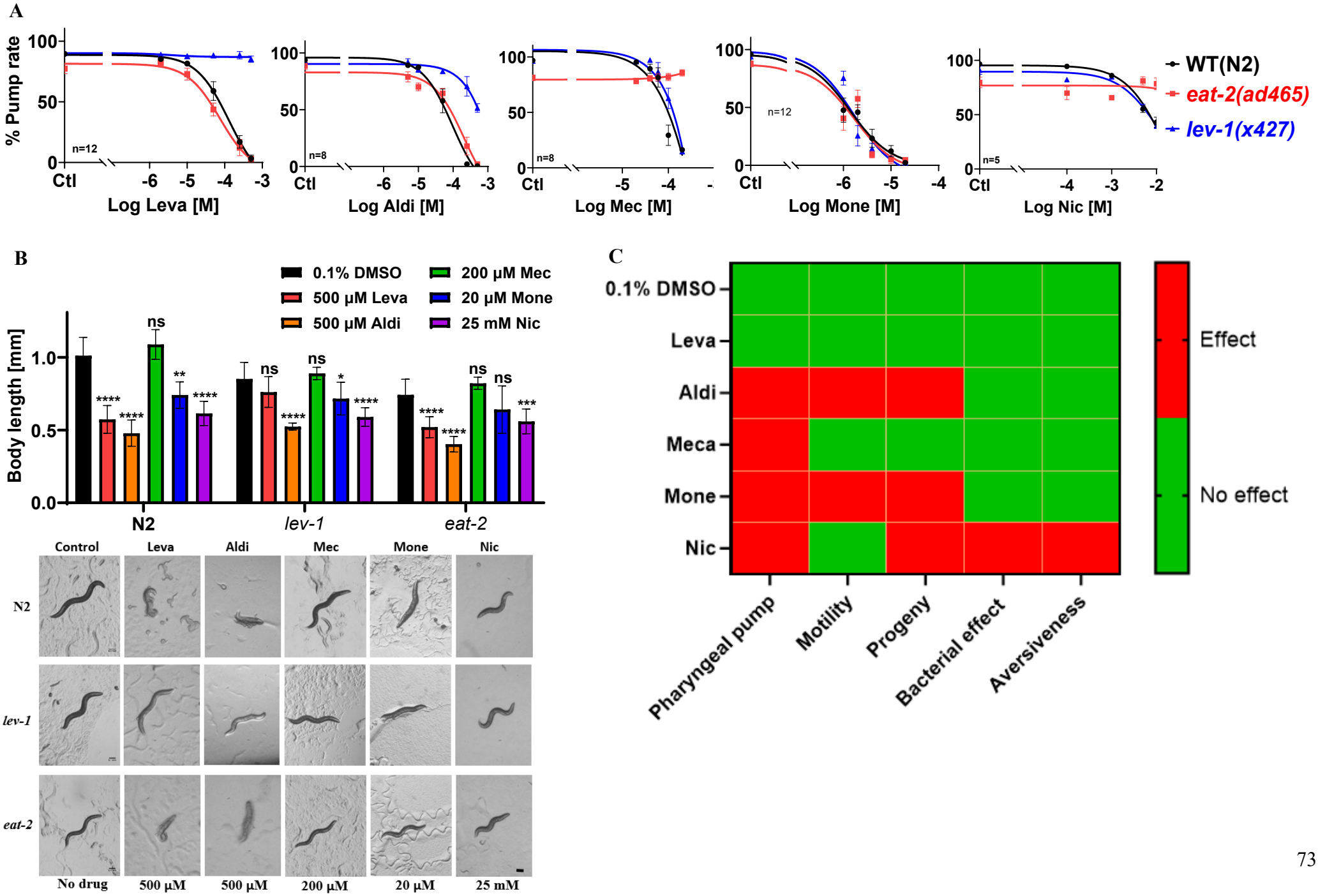
A
% Pump rate
100
50
0
n=12
Ctl
-6
-5
-4
-3
Log Leva [M]
n=8
Log Aldi [M]
n=8
Log Mec [M]
n=12
Log Mone [M]
n=5
-2
Log Nic [M]
WT(N2)
eat-2(ad465)
lev-1(x427)
B
0.1% DMSO
500 µM Leva
500 µM Aldi
200 µM Mec
20 µM Mone
25 mM Nic
Body length [mm]
1.0
0.5
0.0
ns
**
****
*
***
N2
lev-1
eat-2
Control
Leva
Aldi
Mec
Mone
Nic
N2
lev-1
eat-2
No drug
500 µM
500 µM
200 µM
20 µM
25 mM
C
0.1% DMSO
Leva
Aldi
Meca
Mone
Nic
Pharyngeal pump
Motility
Progeny
Bacterial effect
Aversiveness
Effect
No effect

Fig. 3. 1 A comparison of the dose-dependent effects of cholinergic compounds on *C. elegans* behaviours in N2 *eat-2(ad465)* and *lev-1(x427)* resolves EAT-2 selective effects. A. The concentration-dependent effects of cholinergic compounds levamisole (Leva), aldicarb (Aldi), mecamylamine (Mec), monepantel (Mone) and nicotine (Nic) on pharyngeal pumping (pump rate) of N2 (●black), eat-2(■red) and lev-1(▲blue) worms. A 24-hour chronic exposure of nematodes to these chemicals induced concentration and strain dependent effects on pharyngeal pumping. 0.1% DMSO (control) had no effect on behaviours. At maximal concentrations, levamisole and aldicarb completely abolished feeding in N2 and *eat-2* mutants with complete and partial resistance in *lev-1* mutants respectively. Mecamylamine fully inhibited pharyngeal pumping in N2 and *lev-1* mutants but *eat-2* mutants were fully resistant. Monepantel elicited complete inhibition in all strains. In contrast, the inhibitory effect of nicotine was *eat-2* dependent as these mutants were resistant to nicotine induced pump inhibition observed with N2 and *lev-1* mutants. **B.** The effects of the compounds on body wall muscle was assessed by measuring body length from images captured of worms exposed to the drugs for 24 hours (Image J). Levamisole and aldicarb completely paralysed N2 and *eat-2* mutants but *lev-1* mutants showed full or partial resistant to the drug induced effect respectively. Mecamylamine did not paralyze exposed worms, monepantel spastically paralysed them and nicotine induced a modest reduction in motility. Scale bar, 100 µm. **C.** A phenotypic readout of drug effects on *lev-1* mutants. A binary scale was used to quantify the effect drug induced responses 24 hours post exposure. Ligands were tested at maximal concentrations; aldicarb 500 µM, levamisole 500 µM, mecamylamine 200 µM, monepantel 20 µM and nicotine 25 mM.

Statistical analysis was performed with GraphPad Prism 9, and the data are shown as mean ± SEM. The analysis corresponds to the absence or presence of drug and significance tested by two-way ANOVA with Bonferroni's multiple comparison ($^{ns}p>0.05$, $*p<0.05$ $**p<0.01$, $****p<0.0001$)

3.3 Conclusion

We optimized the screening platform to investigate modulators of the nAChR EAT-2 and showed that it was also suitable for obtaining secondary data of drug effect on egg laying and development, motility, aversiveness and bacterial growth. We demonstrated that pumping in *C. elegans* could be directly inhibited by modulating EAT-2 function as seen with nicotine and mecamylamine or indirectly as in the case of levamisole monepantel and aldicarb. Care must be taken when interpreting the data as pump inhibition does not directly imply an EAT-2 effect.

Lead compounds identified must be tested in other paradigms to ensure that a true interaction exists with the target. EAT-2 receptor function can be compromised through over activation by agonists resulting in an activation block or by an antagonist in which case, blocks the receptor activation keeping pharyngeal muscles extendedly relaxed.

4 Chapter 4 Identifying modulators of the nicotinic acetylcholine receptor EAT-2 for the control of plant parasitic nematodes

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4.1 Abstract

Pesticide development studies consider multiple factors aimed at establishing efficacy along with safety profiles that achieve selectivity and reduce negative environmental impacts. In this regard we have proposed that EAT-2 a phylum selective nicotinic acetylcholine receptor affords an opportunity to act as a target with good efficacy and potential selectivity. This is based on the central role that this receptor plays in pharyngeal function including activities that underpin key stages of the plant parasitic nematode life cycle. In this study, we used the model nematode *Caenorhabditis elegans* to initiate a medium throughput screen aimed at defining chemicals that inhibit pharyngeal function through EAT-2. This *in vivo* screen identified 6 among 192 compounds as potential EAT-2 modulators. These compounds were from the chemical classes isoflavonoids, aminoacetonitrile derivatives and nicotinoids represented as rotenone, analogues of monepantel and epibatidine, respectively. To better define the mode of action we tested their efficacy *in vitro* against recombinantly expressed EAT-2 in oocytes. All the compounds identified in the *in vivo* screen showed micromolar antagonist potency at the recombinant receptor. These compounds add to the limited pharmacology of EAT-2 and the distinct mode of action of these chemical classes indicate that the receptor has a discrete organization that may allow selectivity. Importantly, these compounds inhibit the stylet thrusting behaviour in the plant parasitic nematode *Globodera rostochiensis,* validating their potential to disrupt the parasitic lifecycle. Currently these lead compounds have broad modes of action against sub-types of nicotinic acetylcholine receptors. However, the screening method used, and the compounds identified validate approaches by which EAT-2 potencies can be refined to develop EAT-2 pharmacology for better nematicidal potential.

4.2 Introduction

There is an urgent need to find safer synthetic nematicides, that are sustainable and selective, to balance the growing demand for and increase in regulatory pressures on traditional nematicides (Desaeger et al., 2020b). This regulatory control is driven by recognition that older chemical treatments have limited selective toxicity. Plant parasitic nematodes (PPNs) pose a serious threat to global food security and account for an estimated US$ 125 billion/year in economic damage (Iqbal and Jones, 2017). Synthetic pesticides used in crop protection have been effective in controlling these parasites by disrupting key facets of their life cycle. However, they are increasingly restricted and even banned in several regions because of these environmental impacts and other use related impacts. These problems include persistence in the environment, pest resistance, and off-target effects (Fru and Puoti, 2014; Chen et al., 2020).

In several recent anthelmintic and pesticide development studies, *C. elegans* has been a resourceful model organism in classic anthelmintic screens, heterologous expressions and molecular characterization of major drug targets (Crisford et al., 2015; Hahnel et al., 2020b). Although not a parasitic nematode, there is a high degree of conservation in the Nematoda, from physiological characteristics to neuromuscular systems that qualify them as suitable models (Holden-Dye and Walker, 2014; Hahnel et al., 2020b).

Most insecticides are neurotoxins that disrupt ion channels or enzymes in neurons, causing lethal neurotoxic effects (Costa et al., 2008; Raisch and Raunser, 2023). Neuronal signalling is largely shared across animal species with many cases of overlapping and conserved function (Edsinger and Dölen, 2018) thus making high level drug selectivity challenging. Nonetheless, there are several anthelmintics and pesticides that show reasonable levels of target selective (Martin and Robertson, 2007; Hu et al., 2009; Rufener et al., 2013). Nicotinic acetylcholine receptors (nAChRs) have a good coverage in drug discovery research as their diversity, wide expression, and quick action time open up rich possibilities for novel drug target research. (Williamson et al., 2009; Wolstenholme, 2011; Charvet et al., 2018). Despite their broad expression, target selectivity has been achieved for some cholinergic anthelmintics like levamisole and monepantel (Kaminsky and Rufener, 2012; Martin et al., 2012). These execute agonists activity and selective toxicity by activating functionally important acetylcholine receptors at the nematodes body wall muscle that control movement. The selective activation of distinct body wall muscle receptors independently causes muscle paralysis. This disruption of worm movement underpins their successful nematicidal activity.

In this study, we extend our hypothesis that modulators for the nAChR EAT-2, a physiological determinant for nematode pharyngeal function could provide a platform for identifying nematicidal chemicals. We take advantage of the pharyngeal muscle contraction and relaxation cycles that drive the pumping that underpins feeding in *C. elegans*. Interestingly the pharyngeal structure in parasitic nematodes also performs important feeding functions (Fairweather et al., 1995; Lambert and Bekal, 2002). In the case of PPNs this structure supports stylet thrusting which has distinct functional modalities (see chapter 2.4.3). Stylet thrusting is necessary for juveniles hatching from eggs, penetrating host cells, and establishing conduits for sucking essential nutrients or releasing effectors and degrading enzymes into host cells (Molloy et al., 2024). Pharmacological regulations indicate that the anatomical evolution of the pharyngeal neuromuscular junction has a shared dependence for cholinergic transmission and by extension

implicates EAT-2 (see chapter 2.4). This raises the potential of finding EAT-2 selective chemicals with excellent potential to halt the life cycle of parasitic nematodes.

On a molecular level EAT-2 expresses a surprising molecular specificity around key determinants of its agonist site binding (ABS), rather different from classic nAChRs. EAT-2 lacks signature cysteine residues in the ABS (McKay et al., 2004). This highlights unique features of the receptor that is essential for pharyngeal function in *C. elegans* and parasitic nematodes. The gross structure of the receptor subunit also conforms to the classic four transmembrane organization with a large external N-terminus harbouring the acetylcholine (ACh) binding site and a short extracellular C-terminus (Dani, 2015). Based on the functional importance of EAT-2 and the potential to making it a more selective target to mitigate parasitic nematodes, we devised complementary approaches to identify EAT-2 drugs (see chapter 3). Our study identified a series of lead compounds that show excellent potential for development and use in treatment of PPNs.

4.3 Materials and Methods

4.3.1 *C. elegans* maintenance

All the nematode strains used in this study were obtained from *Caenorhabditis* Genetic Centre (University of Minnesota, Minneapolis, MN, USA) and were maintained under standard conditions (Brenner, 1974). Synchronised *C. elegans* L4 worms identified by the presence of a vulva saddle (Divekar et al., 2021) were picked onto OP50 seeded plates an, incubated overnight prior to performing the indicated behavioural assays as young adults (L4+1).

4.3.2 *Globodera rostochiensis* maintenance

Infective juveniles (J2s) of *G. rostochiensis* were hatched from cysts incubated in potato root diffusate (PRD) over 7 days at room temperature. One day old freshly hatched J2s were washed in M9 supplemented with 0.01% Bovine Serum Albumin (BSA) prior to experiments. Freshly hatched juveniles were used for every experiment. Nematode cysts used were from the James Hutton Research Institute, Scotland and provided by Vivian C. Blok and were utilized under the Department for Environment, Food and Rural Affairs license No: 13209/194911-4. The PRD used in hatching assays was provided by Catherine Lilley, University of Leeds, UK.

4.3.3 Drug stock

All compounds tested were provided by Syngenta, UK. These compounds were dissolved in 100% DMSO at stock concentrations of 30 mM, stored at -20 ºC and tested at a 0.1% (v/v) to

negate adverse effects of DMSO on multiple aspects of *C. elegans* biology (Calahorro et al., 2021).

4.3.4 Drug summary

192 compounds were dispensed and dispatched in two 96-well microtiter plates and shipped on dry ice. These compounds represented distinct classes of drugs including insecticides, nematicides or nematicide related compounds. These included representatives of nicotinoids, neonicotinoids, spinosyns, tropanes, tropane ethers, cyanotropanes, cynopyrisines, tropenes, and nereistoxins whose known activity warrants investigation in a bioassay designed to take advantage for existing nicotinic pharmacology. More details of these compounds are presented in Table 1 in the supplementary section.

4.3.5 *C. elegans* drug *in vivo* screening assays

The protocol developed to interrogate EAT-2 function was setup in 24-well plates as previously described in chapter 3. In brief, the screened compounds were unknown to the investigator and tested at 30 μM. The 192 compounds tested were labelled based on their corresponding positions on the 96-well plates, from A1-H12 or AA1-HH12 for plates 1 and 2 respectively. *C. elegans* mutants of the L-type receptor *lev-1(x427)* were synchronised and used as for this purpose. We used *lev-1* mutants to negate the crosstalk between the body wall muscles and the pharynx (Izquierdo et al., 2022) that could introduce false positive results to the screen. Three L4+1 worms were picked into each well and incubated for 24 hours at room temperature. The following day, phenotypic observations of pharyngeal pumping, motility, progeny development, averseness and potential bactericidal effects scored. Drug effects were scored as binary, as effect (1) or no effect (0). (See **Table 3. 1**)

4.3.6 *In vivo* dose-time response assays

Dose-time response effects for designated hit compounds were performed on 6-well plates containing NGM. Aliquots of stock concentrations of the hit compounds were added to OP50 to provide the desired concentration range for testing and then used to seed NGM plates. Plates were dried, stored at 4°C and used the following day. Drug dose induced effects on pharyngeal pumping was recorded at scheduled times over 24 hours (Mulcahy et al., 2013).

4.3.7 Body-length

After a 24 hour incubation of worms on hit compounds or no drug controls, body-length measurements were made as previously described (Mulcahy et al., 2013). Images were

acquired with the Nikon SMZ800 binocular zoom microscope, skeletonized on the image J software and the length of the skeleton used to determine the body-length of the worm.

4.3.8 *G. rostochiensis* behavioural assays

Freshly hatched J2s washed 3 times with ddH_2O were incubated in 30 µM of the indicated compound in ddH_2O over 24 hours. Post incubation, ten J2s were pipetted in 2 µl and transferred into 96 well-plates containing 200 µl of 20 mM HEPES solution (pH 7.4) supplemented with or without 5-HT and a requisite compound. A steady state stylet thrusting response was established in 20 mM HEPES solution (pH 7.4) supplemented with 1 mM 5-HT. After an hour in 5-HT the number of stylet thrusts per worm was measured for 30 seconds and represented as the number of thrusts per minute. A forward and backward movement (extension and retraction) of the stylet was counted as one stylet thrust (Kearn et al., 2017).

4.3.9 Molecular biology

A proof-reading DNA polymerase enzyme was used in PCR reactions with primers that generated amplicons with selected restriction sites to the 5' and 3' ends of *C. elegans eat-2* and *eat-18d* transcripts purchased from Integrated DNA Technologie, Belgium. The primer sequences listed on **Table 4. 1** were used for the PCR reaction. Standard DNA recombination methods were used to ligate sticky-end PCR fragments for *eat-2* and *eat-18* into EcoI/ApaI and KpnI/ApaI restriction sites respectively downstream of the T7 promoter in the pTB207 *Xenopus laevis* expression vector. The vector also contained multiple linearization sites and a beta-globin sequence downstream of the promoter region (Boulin et al., 2008). The ligation product was transformed in DH5-α competent cells (Invitrogen). Successful transformants were sequenced using the pTB207 vector forward primer T7 and reverse primer M13R (Choudhary et al., 2019b). Positive clones were used to synthesize capped mRNA using the mMessage, mMachine T7 invitro transcription kit (Invitrogen) and quantified with agarose gel electrophoresis and absorption spectroscopy.

Table 4. 1 Primer sequences used to amplify EAT-2 and EAT-18 cDNA

Gene	Sample	Primers	Annealing temperature °C
Eat-2	cDNA	5' CGCAC***gaattc***ATGACCTTGAAAATCGCATT 3' 5' CAGGCTAACAACTATAACTTATT***cccggg***CG 3'	60
eat-18	cDNA	5' GC***ggtacc***ATGCGAAGCCTGGAGCGAATC 3' *5'* GCCCTAGCTCCGTCGCACATT***cccggg***GA 3'	60

Underlined sequences represent restriction sites flanking 5' and 3' ends of the cDNA.

4.3.10 Oocyte electrophysiology

The recombinant EAT-2 receptor was heterologously expressed in *Xenopus laevis* oocytes and assayed using the two-electrode voltage clamp (TEVC) technique (Ranganathan et al., 2000). Oocyte lobes purchased from the European Xenopus Resource Centre, University of Portsmouth, UK were teased apart with forceps, transferred into a falcon tube containing collagenase (Worthington Biochemical) (1 mg/ml) in calcium free solution and incubated at room temperature, on a rocker for 30 minutes. Digested oocytes were washed at least 10 times with calcium free solution followed by 5 washes with the storage solution. Oocytes were transferred into 9 cm petri dishes and defolliculated using a modified 200 µl pipette tip. Defolliculated oocytes were co-injected with a 50 ng mix of EAT-2 and EAT-18 mRNA (ratio 1:1) in a total volume of 50 nl (Choudhary et al., 2020) using the SMARTouch nanolitre injector (World Precision Instruments). Some oocytes were uninfected, others with EAT-2 or EAT-18 mRNA. Oocytes were incubated at room temperature for 5-7 days in storage solutions containing gentamicin (100 units/mL) (**Table 4. 2**) (Choudhary et al., 2020).

Table 4. 2 Oocyte working solutions

Calcium free oocyte ringer 2 (OR2) buffer	**Recording Solution (ND96)**	**Modified Barth's saline solution (Storage solution)**
82.5 mM NaCl	96 mM NaCl	96 mM NaCl
2.5 mM KCl,	2 mM KCl,	2 mM KCl,
1 mM $MgCl_2$	0.3 mM $MgCl_2$	1 mM $MgCl_2$
5 mM Hepes	5 mM Hepes	5 mM Hepes
pH 7.6	pH 7.6	1.8 $CaCl_2$
		Gentamicin (100 units/mL)
		pH 7.6

4.3.11 Two electrode voltage clamp recording

TEVC was used to record currents generated from the activation of EAT-2 receptors expressed in oocytes. Prior to electrophysiological recordings oocytes were incubated in 100 µM 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) (Sigma-Merck), a selective calcium chelator, for 3 hours to prevent the activation of intracellular calcium ion channels (Choudhary et al., 2019a). Clark borosilicate thin wall (1.2 mm outer diameter) filament glass capillaries (Warner Instruments) were pulled with a laser-based micropipette puller (P-2000) (Sutter Instruments) and slightly broken at the tip prior to

using as current and voltage recording electrodes. Electrodes were back-filled with 3 M potassium chloride to get final resistances of 0.1- 0.5 MΩ. Oocytes were paced into a recording chamber, impaled with the voltage and current electrode and then clamped at a holding cell membrane potential of -60 mV, for all experiments (Sarkar et al., 2024). Membrane currents in response to applied compounds were recorded by the Oocyte Clamp OC-725C amplifier connected to a Digidata 1550B converter and analysed with Clampex 11.3 software (Molecular devices). All tested compounds and wash solution were made in ND96 and applied to oocytes using a gravity-assisted perfusion system (Ranganathan et al., 2000).

4.3.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism. Significance was assessed using ANOVA followed by a post hoc analysis with Bonferroni corrections where applicable.

4.4 Results

4.4.1 Phenotypic readout of the effects of screened compounds

We used a screening assay to identify drug modulators of the EAT-2 receptor in *C. elegans lev-1(x427)* mutants. We initially investigate 192 compounds (see table 1, supplementary section) named by the coding described in the materials and methods section. Initially, we used nicotine as a positive control to define a selective inhibitory effect the of drug on pharyngeal pumping. The screen revealed compounds where the primary readout, reduced pharyngeal pumping (**Table 3. 1**), was scored as a positive effect. In addition, drug effects on motility, progeny development, bacterial growth and aversiveness were scored. This identified 12 compounds that inhibited pharyngeal pumping, 6 that inhibited motility by inducing a hyper-contraction and paralysis, 18 that inhibited egg laying or hatching, 4 that inhibited the formation of a bacterial lawn and 8 that had aversive effects on the worms (**Fig. 4. 1**)

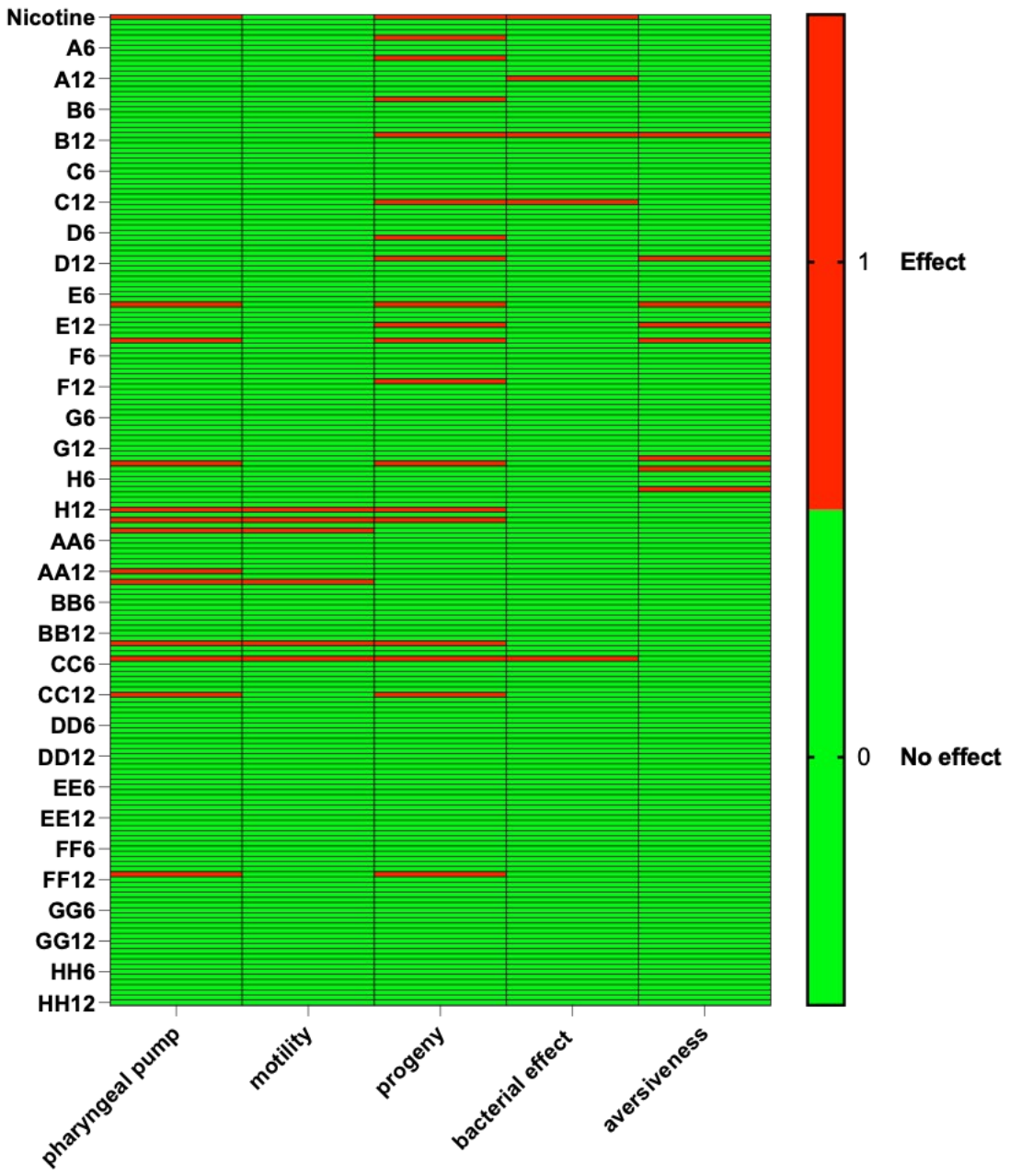


Fig. 4. 1 The effect of compounds on measured behaviour and assay parameters: A colour coded summary of the binary scoring effects of screened compounds on *C. elegans lev-1(x427)* mutants, n=3. Red bars indicate that an effect was observed while green bars indicate that no effect was observed.

4.4.2 Time and dose dependence of hit compounds on pharyngeal pumping of wildtype worms

A retest of the 12 inhibitors of pharyngeal pumping in *lev-1* mutants failed to reproduce the effect 6 of them. The remaining 6 compounds coded as AA2, AA4, BB2, CC2, CC12 and H12 that retained their potent inhibitory effect on pharyngeal pumping were revealed to be

analogues of monepantel (AA2, BB2 and CC2), an analogue of epibatidine (AA4), rotenone CC12 and monepantel (H12). We established a dose-time dependence for them on WT (N2), *lev-1* and *eat-2* mutants and showed that they all had dose dependent effects that were sustained over 24 hours.

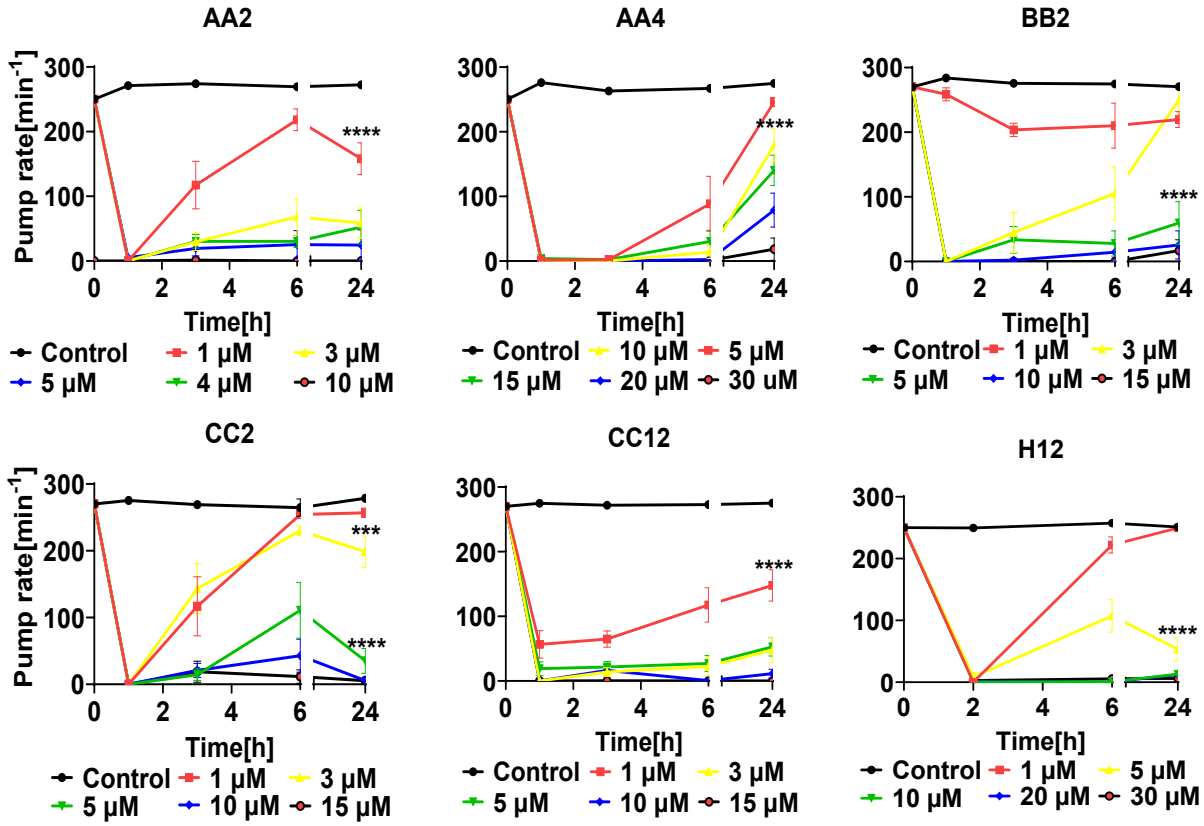


Fig. 4. 2 Dose-time-dependent effects of hit compounds on pharyngeal pumping in *C. elegans*. Pharyngeal pump rates were measured on N2 worms exposed to hit compounds AA2, AA4, BB2, CC2, CC12 and H12 over 24 hours. Control worms incubated on just food maintained stable high pumps rates per minute while the compounds induced dose dependent inhibition on pharyngeal pump rates, n≥7. Data are presented as mean ± SEM and the analysis uses the steady pump rate as control or control + tested compound and significance was tested by two-way ANOVA with Bonferroni's multiple comparison (****p<0.0001).

We recognised that these identified compounds could act at a wide number of receptors. Given this, we compared their potencies in N2 and *eat-2* mutants and recorded similar potent effects as in *lev-1* mutants (**Table 4. 3**). Mutants of *eat-2* already defective in pharyngeal pumping with averages of 60 pumps/min saw a further reduction in the presence of these hit compounds suggesting that the compounds were not EAT-2 selective.

Table 4. 3 Inhibitory effects of hit compounds on pharyngeal pumping in N2, *lev-1* and *eat-2* mutants.

Compounds	N2			*lev-1(x427)*			*eat-2(ad465)*		
	Pumps/min	EC50 (µM)	N	Pump/min	EC50 (µM)	N	Pump/min	EC50 (µM)	N
Control	274.6 ± 1.7	-	34	269.7 ± 2.2	-	25	64.2 ± 2.5	-	24
10 µM AA2	10.4 ± 8.5	1.4	7	20.8 ± 15.4	1.5	4	5.3 ± 4.6	3.5	4
30 µM AA4	18.3 ± 17.2	1.2	10	0.0 ± 0.0	1.1	8	17.0 ± 5.7	1.2	7
15 µM BB2	8.4 ± 8.4	4.0	8	14.5 ± 8.6	5.1	8	2.8 ± 2.0	3.6	8
15 µM CC2	5.4 ± 3.3	3.5	8	1.9 ± 1.4	3.3	8	18.0 ± 5.9	3.6	8
15 µM CC12	0.0 ± 0.0	1.1	12	0.0 ± 0.0	0.65	12	0.0 ± 0.0	1.0	12
30 µM H12	6.4 ± 3.2	1.4	12	3.5 ± 1.5	1.5	12	6.6 ± 2.1	1.4	12

4.4.3 Effect of hit compounds on *C. elegans* motility

All 6 compounds induced paralyses and a subsequent inhibition in pharyngeal pumping. Figure 3 compares the body length of control N2 worms incubated on food to worms on food dosed with a specified compound. All drug treated worms were significantly shorter that the control (**Fig. 4. 3A**). These compounds induced a spastic paralysis that resulted in impaired motility of treated worms (**Fig. 4. 3B**)

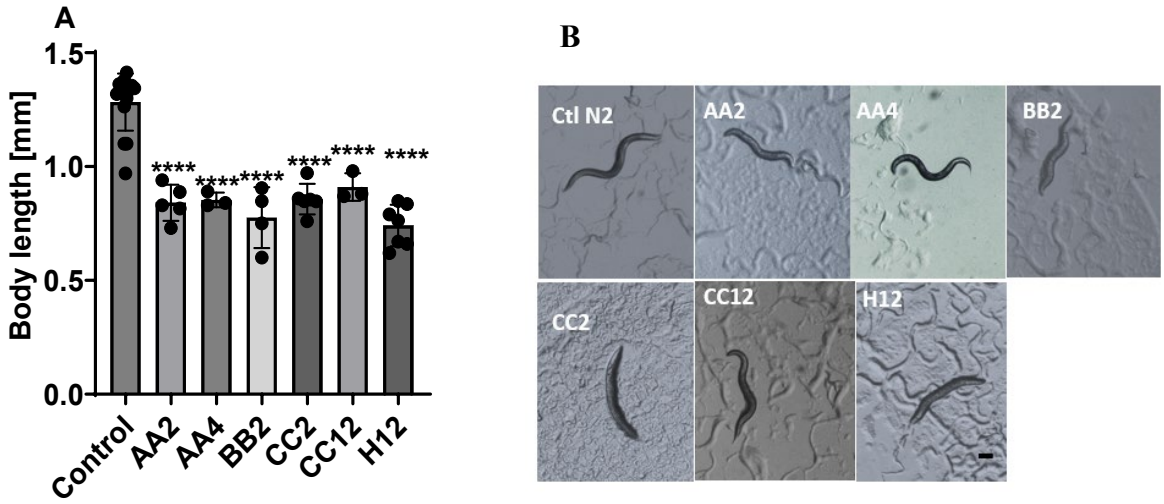


Fig. 4. 3 Hit compounds induce paralysis of body-wall muscles in *C. elegans*. A. N2 worms exposed to AA2, AA4, BB2 CC2, CC12 and H12 had a shorter body length compared to control worms, n≥3 for all treatments**. B.** Images show normal looking control worms and paralyzed worms exposed to hit compounds, scale bar represents 0.1 mm. Data are presented as mean ± SEM and the analysis compares control + tested compound, and significance was tested by one way ANOVA with Dunnett's multiple comparison (****p<0.0001).

4.4.4 Investigating hit compounds in *Xenopus laevis*

To achieve functional expression in *Xenopus*, we co-injected mRNAs for EAT-2 and EAT-18 We then used TEVC to investigate the functional expression of the receptor by exposing the oocytes to acetylcholine (ACh), the known endogenous agonist for EAT-2 (McKay et al., 2004). To test this, we exposed oocytes for 5s to increasing doses of ACh followed by a 1 minute wash between each exposure. We measured large amplitudes of current in oocytes expressing EAT-2 and EAT-18 when exposed to ACh with an EC_{50} of 6.1 µM, which was very similar to the 4.8 µM recorded in literature (Choudhary et al., 2020) (**Fig. 4. 4A**). Oocytes that were injected with either EAT-2 or EAT-18 mRNA alone had no response to 100 µM ACh (data not shown) 2). Nicotine and cytisine were tested at 100 µM and they induced smaller currents compared to normalised ACh currents (**Fig. 4. 4B**). To test the effects of antagonists, 100 µM ACh was first applied for 5s as control, followed by a 1 minute wash, then perfusion with just the antagonist for 30s, after which ACh applications were repeated in the presence of the antagonist. Inward currents induced by 100 µM ACh were completely blocked by 10 µM mecamylamine, a known antagonist of nicotinic receptors (**Fig. 4. 4C**). 10 µM tubocurarine and 30 µM hexamethonium were also potent antagonists of EAT-2 (**Fig. 4. 4D**).

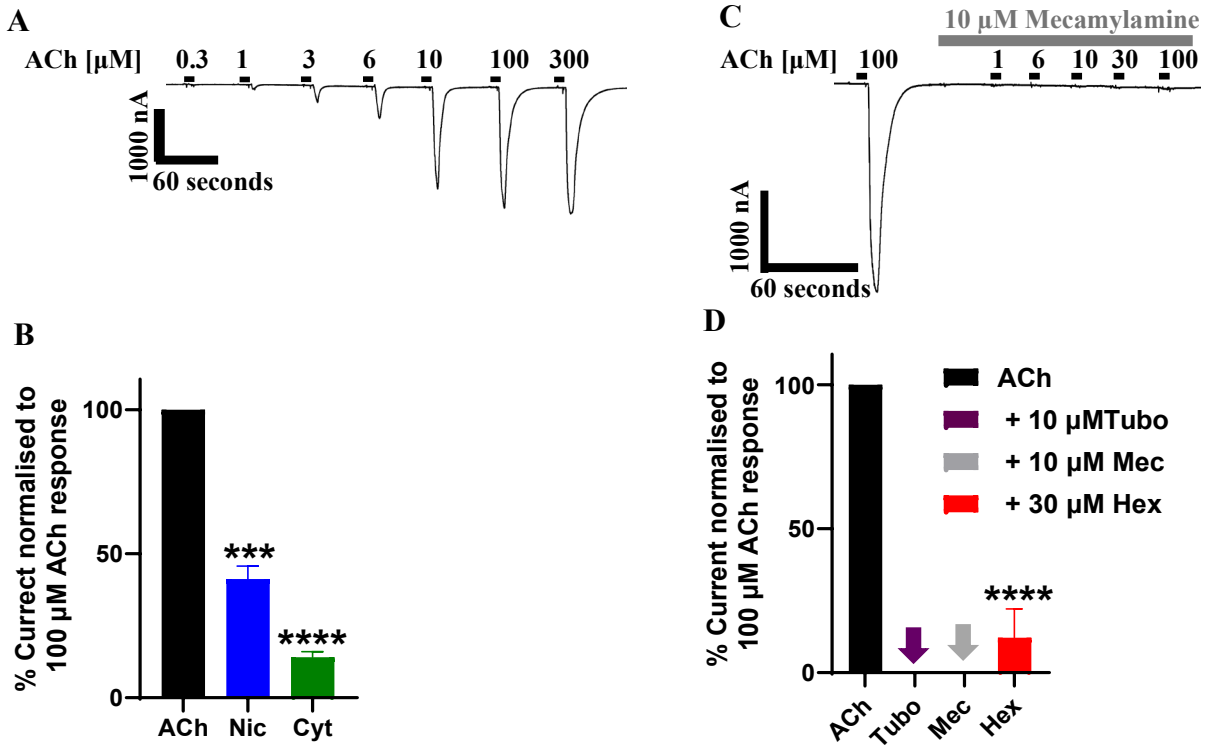


Fig. 4. 4 The modulation of recombinantly expressed EAT-2 A. Sample traces for ACh concentration-response relationships for *Ce*-EAT-2 co-expressed with the auxiliary protein *Ce*-EAT-18. **B.** Ranked EAT-2 response to selected agonists normalised to 100 µM ACh current

(expressed as mean ±SEM, n≥15): Acetylcholine (ACh; 100 ± 0.0) > nicotine (Nic; 41.2 ± 4.5) > cytisine (Cyt; 14.1 ± 1.9). **C.** Sample traces for ACh concentration–response relationships in the presence of 10 µM mecamylamine (Mec). **D.** EAT-2 response to selected antagonists normalised to 100 µM ACh current (expressed as mean ±SEM, n≥8): ACh; 100 ± 0.0, Tubo; 0 ± 0.0, Mec; 0 ± 0.0, Hex; 12.1 ± 3.2. Data are presented as mean ± SEM and the analysis compares ACh + test compounds. Significance tested by one way ANOVA with Dunnet's multiple comparison (***p<0.001, ****p<0.0001).

4.4.5 A subset of the hit compounds are EAT-2 antagonists

To get a characterisation of the identified hit compounds, we investigated their ability to modulate EAT-2 in the recombinant system by testing them at 30 µM. Initially, we exposed oocytes for 30 seconds to these compounds and recorded no responses. Having failed to induce currents we tested them for antagonistic effects (**Fig. 4. 5A**). When the analogue of monepantel (AA2), epibatidine AA4 and rotenone (CC12) were co-applied with 100 µM ACh they inhibited agonist induced currents by 30.8%, 63.7% and 49.6% respectively (**Fig. 4. 5B**).

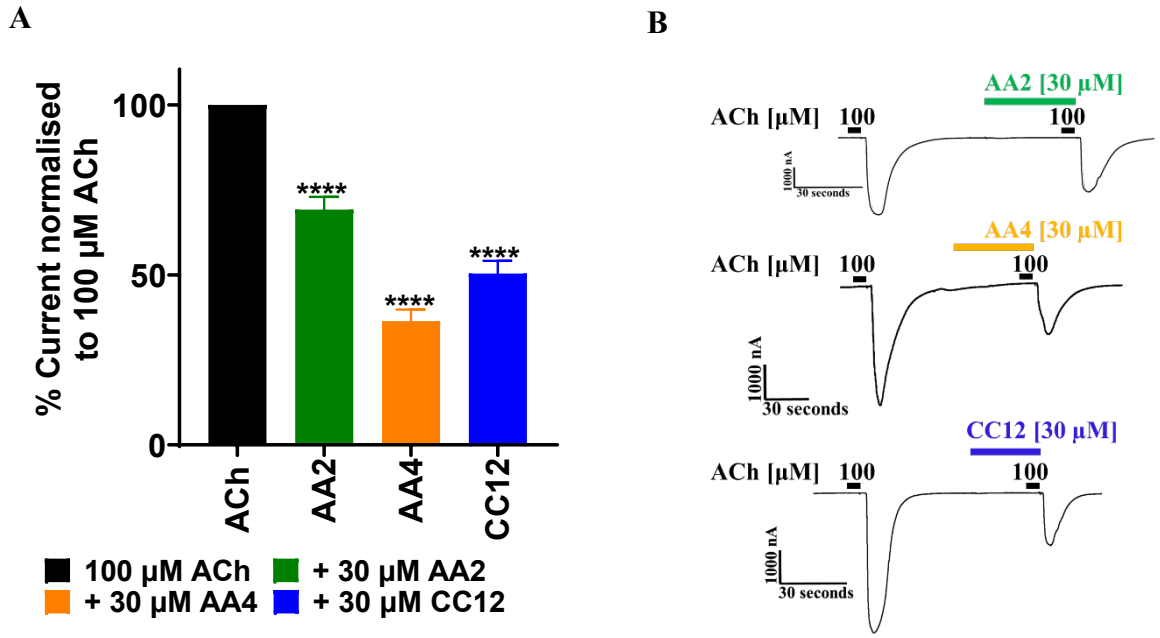


Fig. 4. 5 Comparative effects of hit compounds on EAT-2 expressing oocytes. A. Sample trace responses of AA2, AA4 and CC12 tested at 30 µM in the presence of 100 µM ACh. **B.** Responses normalised to 100 µM ACh current (expressed as mean ± SEM, n≥13): Acetylcholine (ACh; 100 ± 0.0) > AA2 (69.2 ± 3.7) > CC12 (50.4 ± 3.7) > AA4 (36.4 ± 3.4). Data are presented as mean ± SEM and the analysis compares ACh + test compounds. Significance tested by one way ANOVA with Dunnet's multiple comparison (****p<0.0001).

4.4.6 Hit compounds inhibit stylet thrusting in *G. rostochiensis*

Plant parasitic nematodes use their stylets to hatch from eggs, invade host plants and establish feeding sinks through which they obtain nourishment (Pulavarty et al., 2021b; Mkandawire et al., 2022b). In essence, interrupting this behaviour could potentially break their parasitic lifestyle. We used the biogenic amine 5-HT to pharmacologically induce stylet thrusting in freshly hatched *G. rostochiensis* J2s (Crisford et al., 2020) and then investigated the effect of our hits, the analogues of monepantel, epibatidine and rotenone on this 5-HT induced behaviour. We also investigated the effects of mecamylamine and tubocurarine due to their very potent effects on the recombinantly expressed EAT-2 receptor. To do this we incubated freshly hatched J2s in these compounds for 24 hours prior to 5-HT exposure. This extended incubation period was to enhance bioaccumulation of drugs which is known to be impeded by the cuticular barrier (Burns et al., 2010). We observed that incubating J2s in all hit compounds prior to 5-HT exposure significantly inhibited stylet thrusting. Rotenone (CC12) at 30 µM was the most potent inhibitor of stylet thrusting. At the same concentration, the monepantel analogue was a less potent inhibitor. The analogue of epibatidine was the least potent inhibitor. Mecamylamine and tubocurarine were also significantly potent inhibitors of 5-HT induced stylet thrusting (**Fig. 4. 6**).

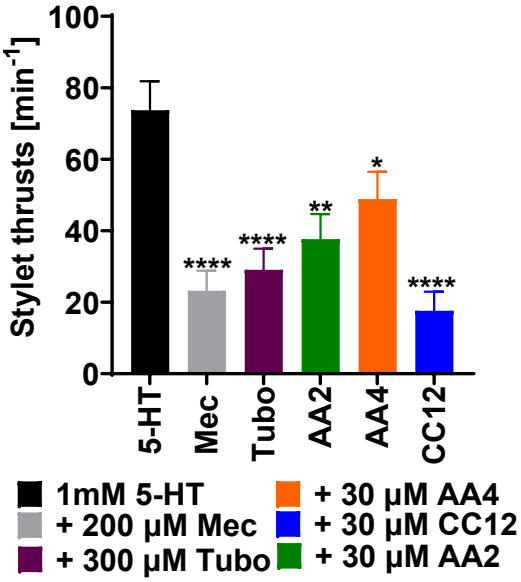


Fig. 4. 6 Inhibitory effects of mecamylamine, tubocurarine and hit compounds monepantel analogue (AA2), analogue of epibatidine (AA4) and rotenone (CC12) on stylet thrusting, n=30. Data are presented as mean ± SEM and the analysis compares 5-HT + test compounds. Significance tested by one way ANOVA with Dunnet's multiple comparison (*p<0.05 **p<0.01, ****p<0.0001).

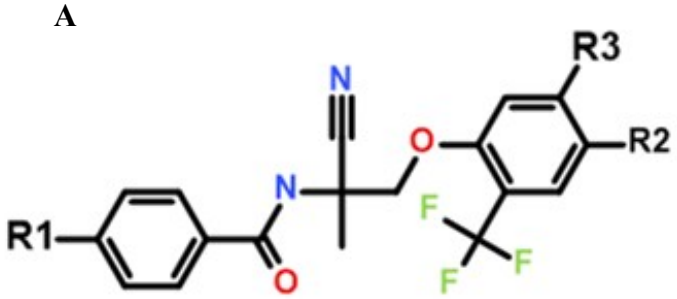
4.5 Discussion

Considering the challenges involved with finding pesticide targets that are selective, identifying receptor targets that are not evolutionarily conserved between the pest and non-target organisms can open up a new focus for investigation. The *C. elegans* nicotinic receptor EAT-2 has been proposed as a pharmacophore with potential for selectivity based on its unique structure, function and pharmacological profile (Choudhary et al., 2020). In a recent study we have reported EAT-2's conservation within Nematoda and its involvement with the parasitic behaviour stylet thrusting (see chapter 2.4). In this study we screened 192 compounds selected based on their chemical structure and known mode of action for possible modulators of the nAChR EAT-2. The screen identified some compounds with potential to disrupt parasitic lifestyle.

4.5.1 Monepantel indirectly inhibits pharyngeal function in nematodes

The inhibitory observations of monepantel (H12) and its analogues (AA2, BB2 and CC2) on pharyngeal pumping and stylet thrusting in *C. elegans* and *G. rostochiensis* respectively suggests that its mode of action is conserved across nematode species. Monepantel is implicated as a target modulator in neuromuscular junctions of several nematodes. Hansen et al. (2022) observed that monepantel was an allosteric modulator of *C. elegans* DEG-3/DES-2 receptors expressed in pharyngeal neurones and associated with motility, nociception and possibly chemotaxis. Rufener et al. (2013) reported monepantel to be an agonist of the ACR-23 receptor expressed in body wall muscles in *C. elegans* and *Haemonchus contortus*. Abongwa et al. (2018) found monepantel to be a non-competitive antagonist of the *A. suum* ACR-12 receptor expressed in body wall muscles. In this study we observed that monepantel induced paralysis associated with a spastic contraction of the body wall muscle but also inhibited pharyngeal pumping in *C. elegans*. Izquierdo et al. (2022) reported that spastic paralysis introduced an inter-muscle communication between pharyngeal muscles and body wall muscles that resulted in pump inhibition. In our study, although the paralysis induced by monepantel and its analogues did not implicate EAT-2, electrophysiological evidence suggest that these compounds blocked receptor function. This finding suggests that monepantel and its analogues may inhibit pharyngeal function via EAT-2 in addition to the inter-muscle communication and could provide leads for developing EAT-2 selective antagonists.

A



B

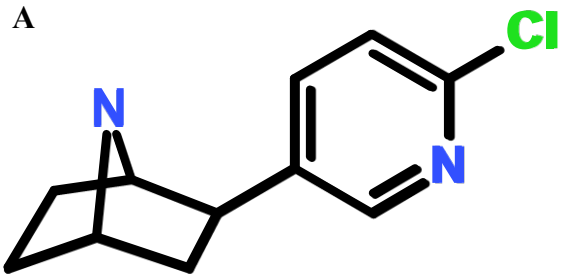
CODE / ID	R1	R2	R3
H12	SCF3	H	CN
AA2	SCF3	CN	H
BB2	OCF3	CN	H
CC2	CF3	H	CN

Fig. 4. 7 A. Core chemical structure for monepantel and **B.** R-group substitutions**.**

4.5.2 Epibatidine analogue (AA4) is an inhibitor of EAT-2

Epibatidine is an alkaloid that acts as an agonist of nAChRs. Due to its high toxicity in mammals, epibatidine is unsuitable for practical anthelminthic field applications but it has found significant use as a pharmacological tool in both insecticide and nematicide research (Matsuda et al., 2000; Hansen et al., 2021). In insect studies, epibatidine has been used to probe nAChR subtypes and binding interactions, helping to inform on the development of safer, more selective neonicotinoid insecticides. In *C. elegans*, epibatidine (**Fig. 4. 8A**) has been important in characterizing cholinergic signalling pathways and receptor pharmacology. This has enhanced the understanding of nAChR-mediated behaviours and enabled comparative studies across species (Hansen et al., 2021). Choudhary et al. (2020) reported epibatidine as an agonist of recombinantly expressed EAT-2. In contrast AA4 an analogue of epibatidine (**Fig. 4. 8B**) had an antagonistic effect on the receptor. It blocked the recombinantly expressed receptor in *Xenopus* and also inhibited pharyngeal function in *C. elegans* and *G. rostochiensis.* This highlights how substitution groups around the core structure of a compound can alter its activity and/or mode of action

A



B

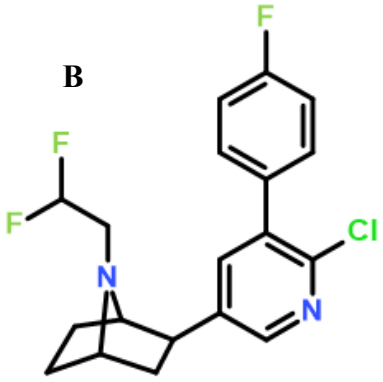


Fig. 4. 8 A. Epibatidine and **B.** analogue AA4

Rotenone (CC12) (**Fig. 4. 9A**) is a naturally occurring flavonoid with insecticidal properties. It has been widely used as a broad-spectrum pesticide against insect pests of crops and animals

(Gupta and Milatovic, 2014a). Its potency is based on its high-affinity as inhibitor of electron-transport at complex I of the respiratory chain. This impairs mitochondrial function by significantly decreasing mitochondrial membrane potential in *C. elegans* (Romero-Sanz et al., 2023). There is limited evidence on rotenone's use as pesticide. Findings from our screen suggest that rotenone is a potential inhibitor of pharyngeal function in nematodes. Electrophysiological data show that this inhibition could be EAT-2 dependent but not specific. Rotenone shares structure with monepantel (**Fig. 4. 9B**) however, its activity is unlikely to be based on structural overlap with monepantel as the closed ring structure of rotenone forces a very different orientation of the backbone compared with monepantel.

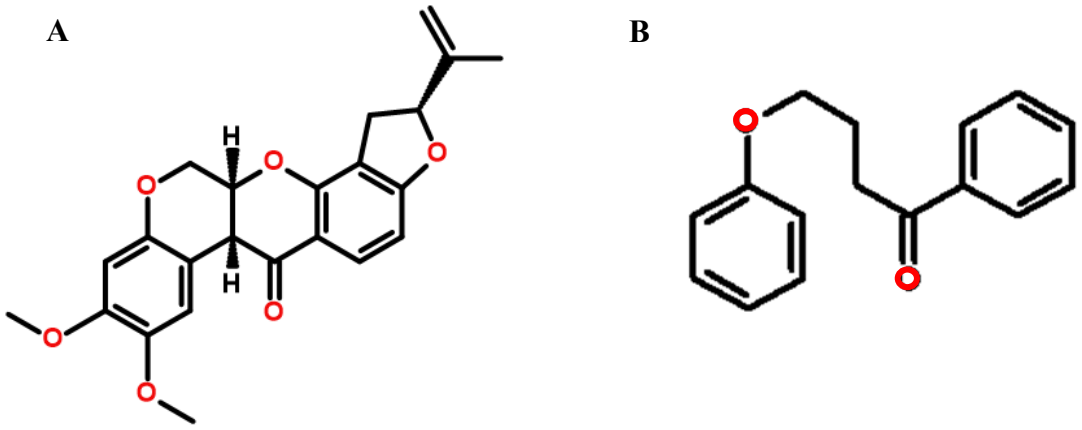


Fig. 4. 9 A. Rotenone and **B.** substructure shared with monepantel

In summary *C. elegans* is a useful tool in pesticide screening studies. The ease to replicate, track and scale up assays in ways that are otherwise challenging in their parasitic counterparts makes them invaluable models for this purpose. We devised a screening platform that took advantage of the *C. elegance* mutant phenotypes and biased our screen to successfully identify modulators of pharyngeal function. Notably, our screen identified rotenone, analogues of monepantel and epibatidine as inhibitors of pharyngeal pumping. We validated these compounds as antagonists of the target receptor EAT-2. Lastly, we show these compounds inhibit stylet thrusting function in *G. rostochiensis*. Whether or not this directly results from EAT-2 dependence of stylet thrusting remains to be determined. These results add to the pharmacological characterisation of the EAT-2 receptor. Importantly, the identification of these ligands for EAT-2 provides a platform for resolution of a pharmacophore that has potential as a selective nematicide target.

5 Chapter 5 General Discussion

The overall aim of this research was to investigate the nAChR EAT-2 as a potential selective target for plant parasitic nematode control. In *C. elegans*, EAT-2 plays a pivotal role in regulating feeding (McKay et al., 2004). Three main factors motivated the investigation of EAT-2 as a selective pharmacophore. 1.) Its pivotal role in feeding implied that it could be pharmacologically disrupted to impair feeding 2.) EAT-2 lacks signature vicinal cysteine residues that are important for ligand binding implying that it has potential for a distinct pharmacology and 3.) EAT-2's functional expression being dependent on an auxiliary protein with no known homologues in other animal species makes it a unique target. To investigate these hypotheses; First, I established the expression and likely role of EAT-2 in plant parasitic nematodes. Second, I designed approaches that used *in vivo* techniques to investigate chemical modulators of EAT-2. Third, I established the consequences of chemically disrupting EAT-2 function in plant parasitic nematodes.

5.1 Cholinergic regulation of pharyngeal function in plant parasitic nematodes

Initially, to support the hypothesis that EAT-2 is a potential drug target (Choudhary et al., 2020), I needed to ascertain its presence and function in parasitic nematode species. A functional role is often reflected in specialization of expression. The phylogenetic evidence obtained suggested that EAT-2 was selectively expressed in nematodes implying that it might have evolved a phylum selective function connected to feeding. A comparison of the molecular determinants that make up the ligand binding site of *C. elegans* EAT-2 and other economically important nematode species also showed a conserved absence of the signature vicinal cysteine residues in the loop-C domain (see chapter 2.4.1). These initial findings are significant requirements for a selective drug target and reinforce the hypothesis that the receptor may be a distinct pharmacophore for agonists and antagonists. Examples where pharmacological selectivity was defined by molecular composition of nAChRs include studies on levamisole receptors (Fleming et al., 1997; Culetto et al., 2004; Boulin et al., 2008) and the development of the first amino-acetonitrile derivative (AAD) anthelmintic monepantel (Kaminsky et al., 2008b; Kaminsky and Rufener, 2012). The absence of ACR-23 homologues in non-target species made monepantel a selective nematicide without off-target toxicity in mammals (Kaminsky et al., 2008b).

Having phylogenetically identified EAT-2 as a unique receptor conserved in the Nematoda and expressed in key parasitic nematodes, I progressed to establish its expression pattern in the

plant parasitic nematode *G. rostochiensis*. For a pesticide to be effective and selective, its target needs to be essential for survival of the pest and absent or lowly expressed in non-target organism (Li et al., 2025b). We successfully showed the spatial expression of *eat-2* in the anterior region of the parasite and based on this we probed its function as a regulator of the pharyngeal behaviour stylet thrusting. There is little evidence describing the pathway that regulates this parasitic behaviour. However, serotonergic signalling has long been established to drive pharyngeal behaviours in nematodes (Fairweather et al., 1995; Perry et al., 2004; Crisford et al., 2020). Our pharmacological experiments proposed a putative model depicting a pathway that requires serotonergic signalling to activate a cholinergic pathway that drives pharyngeal muscle contractions via EAT-2 (**Fig. 2. 10**). To support this claim, we used agonists and antagonists of nAChRs to activate pharyngeal behaviours in *C. elegans* and *G. rostochiensis* using *in vivo* and *in vitro* assays (**Fig. 2. 5 & 2. 10**). These findings set the foundation for investigating 192 compounds in a medium throughput screening platform that used *C. elegans* to find modulators of EAT-2. The proof of principle screen was based on blind analysis of coded compounds that were included in the compound library for screening based on knowledge of their activity on nAChR, structural derivatives of these chemical classes and inclusion of compounds with other nematocidal activities. From the screen, hit compounds were identified in the chemical classes isoflavonoid, aminoacetonitrile derivatives and nicotinoids. These hits were characterised on EAT-2 expressing oocytes and revealed to be antagonists of the recombinantly expressed receptor. Mecamylamine and tubocurarine were also potent EAT-2 antagonists. Although the screen was successful in identifying lead compounds it is important to acknowledge that the nematode cuticle impedes drug accumulation by preventing drugs with low permeability from reaching their target (Burns et al., 2010). Additionally, a caveat of the screening process is a confounding effect introduced by paralysis of the body wall muscles in *C. elegans* (Izquierdo et al., 2022). This paralysis which hyper-contracts body wall muscles induces a distal inhibition of pharyngeal muscles and impedes pharyngeal pumping. In a screening assay this can introduce false positive results. Thus, data must be carefully interpreted because pump inhibition does not directly imply an EAT-2 effect. Pharyngeal inhibition independent of body wall muscle paralysis would be a phenotypic gold standard for identifying EAT-2 selective compounds in a drug screening assay. An example is seen with mecamylamine which potently inhibits pharyngeal pumping in *C. elegans* and does not paralyse the body wall musculature (**Fig. 3. 1**).

To increase the probability of finding more hit compounds, it is recommended to increase the compound library screened and the throughput of the platform used (Galloway et al., 2009).

5.2 The consequences of disrupting key functions in plant parasitic nematodes

Pesticide development studies are designed to target and disrupt key behaviours that are essential for the pest (Gupta and Milatovic, 2014b). In nematicide studies, these key behaviours have been around motility, reproduction and other physiological mechanisms (Jeschke, 2016; Tocco et al., 2017; Schleker et al., 2022).

Amides like fluopyram that was originally developed as a fungicide, and cyclobutrifluram selectively inhibit complex II of the mitochondrial respiratory chain, causing rapid depletion of energy in *C. elegans* (Jeschke, 2016; Flemming et al., 2025). Exposure of *Meloidogyne* sp and *Heterodera schachtii* to fluopyram prevented their infection and development in the roots of lettuce and *Arabidopsis thaliana* plants (Schleker et al., 2022).

Fluensulfone, a sulfone nematicide with little impact on the environment and non-target organisms does not cause paralysis by inhibiting acetylcholinesterase of nematodes (Kearn et al., 2014). Although its target is unclear, a suggestion about its mode of action is that it gradually causes metabolic damage on nematodes to a point where the nematode fails to obtain lipid storage and dies (Kearn et al., 2017).

Tioxazafen is broad-spectrum systemic nematicide that was identified with the help of molecular field models to provide control for nematodes of corn, soy and cotton (Jeschke, 2016). The chemical structure gives it a new mechanism of action by interaction with a nematode-specific insertion of the L3 subunit of the mitochondrial ribosome and is proposed to cause death in nematodes by inhibiting ribosomal function (Jeschke, 2016; Lewis et al., 2016). Some studies report the inhibitory effects of organometallic complexes on the reproduction of *C. elegans* and their potential in nematicide research (Attar et al., 2011).

All these studies report several chemical effects on different physiological behaviours but until now there are limited studies around regulating one of the most important behaviours in plant parasitic nematodes, stylet thrusting.

Considering that the stylet function is central to a number of key parasitic behaviours in plant parasitic nematodes, understanding how it works is crucial to facilitate ways of disrupting it and interrupting the parasitic life cycle. Currently, no nematicide studies have investigated how to selectively block stylet activity in PPNs, although its loss of function is identified to be

associated with a broad inhibition of the worms' neurochemistry and metabolism (Kearn et al., 2017; Crisford et al., 2020).

The medium throughput screening performed in this study identified an analogue of monepantel, an analogue of epibatidine and rotenone as disruptors of pharyngeal behaviour that blocked stylet thrusting in *G. rostochiensis*. Among these compounds, the analogue of epibatidine was the most potent and significantly inhibited stylet thrusting by 63.7%. Rotenone induced about a 50% reduction in stylet thrusting activity and the analogue of monepantel was the least potent with a 30% inhibition. These results provide primary evidence to suggest that stylet thrusting can be interrupted by pharmacologically targeting the pharyngeal nAChR EAT-2. The structure-activity relationships of these lead compounds, especially the analogue of epibatidine, can be drawn on to improve on quality and build bigger libraries to refine specificity. Incentivising the development of more compounds around the core structures of these identified compounds could improve the chances of finding EAT-2 selective compounds. The presence of EAT-2 in other economically important nematode species including the animal parasite *A. suum*, suggests that EAT-2 modulation can be extended to animal parasitic nematode control. The pharyngeal muscle in *A. suum* through contraction-relaxation cycles acts like a pump to suck up food (del Castillo and Morales, 1967; Fairweather et al., 1995). This pharyngeal behaviour has been pharmacologically induced by serotonin and ACh in *in vitro* assays (Fairweather et al., 1995; Elwyn et al., 1996) suggesting a conserved pathway drives pharyngeal behaviours in nematode.

5.3 Improving the probability of finding novel hit compounds

5.3.1 Larger library screens

The approach to drug discovery and development has long seen a shift from serendipitous strategies to more rational approaches (Thorat et al., 2023). Some screens yield few hits that may not be selective or very potent. Screening larger libraries with high-quality chemicals and biasing the screen to identify compounds which induce restricted endpoint phenotypes, can greatly improve on the chances of finding more selective or potent compounds for target proteins (Nishiguchi et al., 2021). Larger libraries cover broader sets of chemical scaffolds and functional groups. This diversity in the molecular scaffold reduces restrictions to particular known scaffolds and improves on the likelihood of finding novel chemotypes (Galloway et al., 2009). Studies have shown that only a small fraction of compounds in a library potently bind a target and big libraries improve the chances of finding molecules with distinct binding modes.

Larger number of hits identified from a screen, provide more information on structure-activity relationship which can be relevant for improving on qualities like binding affinity, stability and absorption, distribution, metabolism, and excretion (ADMET) (Wawer et al., 2010; Klingler et al., 2019)

5.3.2 Hight throughput *in vivo* and *in vitro* screening assays

Traditional *in vivo* screens relied on parasitic species of worm, but these were costly, laborious and less successful (Burns et al., 2015c). *C. elegans* has been a valuable alternative in novel anthelmintic and nematicide studies (Leung et al., 2013; Calahorro et al., 2022). Advances in liquid cultures, imaging and data analyses techniques in automated high-throughput assays have made these possible (O'Reilly et al., 2013). High throughput screens are well established hit generation methods however, they are usually complemented by knowledge-assisted approaches like medium-throughput screens of selected targets (Weigelt and Dorange, 2016; Collie et al., 2024). It is important to note that high-throughput screens mostly identify chemical starting points or clusters of chemical analogues around which lead chemistry can be designed (Wildey et al., 2017).

5.3.3 Incorporating artificial intelligence, machine learning and molecular docking in agrochemical research

Artificial intelligence (AI) is gaining traction in the agrochemical industry. An advantage AI offers over traditional pesticide development methods is that it can be incorporated across all stages in the development pipeline, from mining omics datasets to compound design, optimization, formulation and delivery (Singh et al., 2023).

Machine learning can highlight comparative expression patterns of essential genes in target and non-target organisms to improve on target selectivity (Ashenden, 2021). Deep learning models can be used to design or prioritize compounds, AI-guided docking to screen millions of compounds in silico and make efficient predictions about binding affinities (Zhou et al., 2024a; Zhu et al., 2025).

Molecular docking also known as virtual screening is a rapidly emerging technology that has become an important tool for predicting the binding modes of small molecules (Zhang et al., 2025). The technology uses computer algorithms to study intermolecular interactions and make predictions on how, where and the strength of these interactions (Singh et al., 2023). This technology is recently being applied in agrochemical research because it is relatively cheaper and allows for the rapid screen of large libraries of compound *in silico* to identify potential hits

even before drug synthesis and bioassays (Zhao et al., 2022). Ultra-large AI-assisted virtual screens are already yielding successes in biomedical research (Liu et al., 2025) and this approach can be translatable in nematicide or insecticide studies. Even more exciting, docking can also be used in 'reverse-docking' to check for possibilities of a candidate compound binding to non-target proteins (McCarthy et al., 2022).

Pesticide informatics presents enormous potential that can be exploited to speed up the discovery process of EAT-2 selective modulators. Investing resources into developing a crystal 3-dimensional structure for EAT-2 would be a valuable starting point. With a crystal structure, the identified EAT-2 modulators could be docked to establish their binding affinities with the receptor, the type and strength of the interaction and the orientation of these compounds in the binding pocket. The data obtained could be visualized and used to design chemical structures that satisfy strong interactions and build libraries around them. A crystal structure for EAT-2/EAT-18 will the first 3D visualization of the receptor. Having this will improve the general understanding of interaction between these proteins and could open new possibilities.

5.4 What does the future of pesticide development look like?

5.4.1 Nanobody biotechnology in pesticide discovery

Nanobodies are specialised antibodies obtained from camelids and are considered to be the smallest intact antigen-binding fragments (Muyldermans, 2013). Unlike antibodies that have heavy and light chains, camelids also produce a class of heavy-chain-only antibodies (HCAbs) from which small single-domain fragments called nanobodies are isolated (Muyldermans, 2013). The potential of nanobody use has extended into agriculture and biotechnology studies (Steeland et al., 2016; Kourelis et al., 2023; Njeru et al., 2024). Their high stability, solubility, binding affinity, small size (15 kDa) and shape makes them readily accessible to hidden or cryptic epitopes, receptor clefts or binding pockets that are inaccessible to larger compounds (Muyldermans, 2013; Steeland et al., 2016).

As highlighted, functional EAT-2 is dependent on the cell surface association of a small auxiliary subunit. We probed the nematode specific auxiliary protein EAT-18 for possibilities as a target to disrupt EAT-2 function. The topology of the small single transmembrane protein suggests that EAT-18 is a type II transmembrane protein with an intracellular N-terminus and an extracellular C-terminus (Chou and Elrod, 1999; Choudhary et al., 2020).

To explore EAT-18 as a secondary target to disrupt EAT-2 function, I inserted ALFA-tag or GFP to the C-terminus of the small auxiliary subunit and used it to functional reconstitute EAT-2 in *Xenopus laevis* (**Fig. 5. 1D**). EAT-2 expression was successful with EC50 responses to ACh of 7.2 µM and 9.2 µM for GFP tagged and ALFA-tagged receptors respectively. The tagged receptors showed reduced efficacies, with smaller current amplitudes in response to ACh compared to the wildtype receptor (**Fig. 5. 1C**). There however seemed to be an increase in the sensitivity of the ALFA tagged receptor as the response to ACh peaked at 30 µM (**Fig. 5. 1B**). This suggests that a free hanging C-terminus on EAT-18 is important for the receptor function When these oocytes were exposed to nanobodies, no effect was observed (data not shown). This preliminary study introduces the concept of using nanobodies for nematicidal purposes.

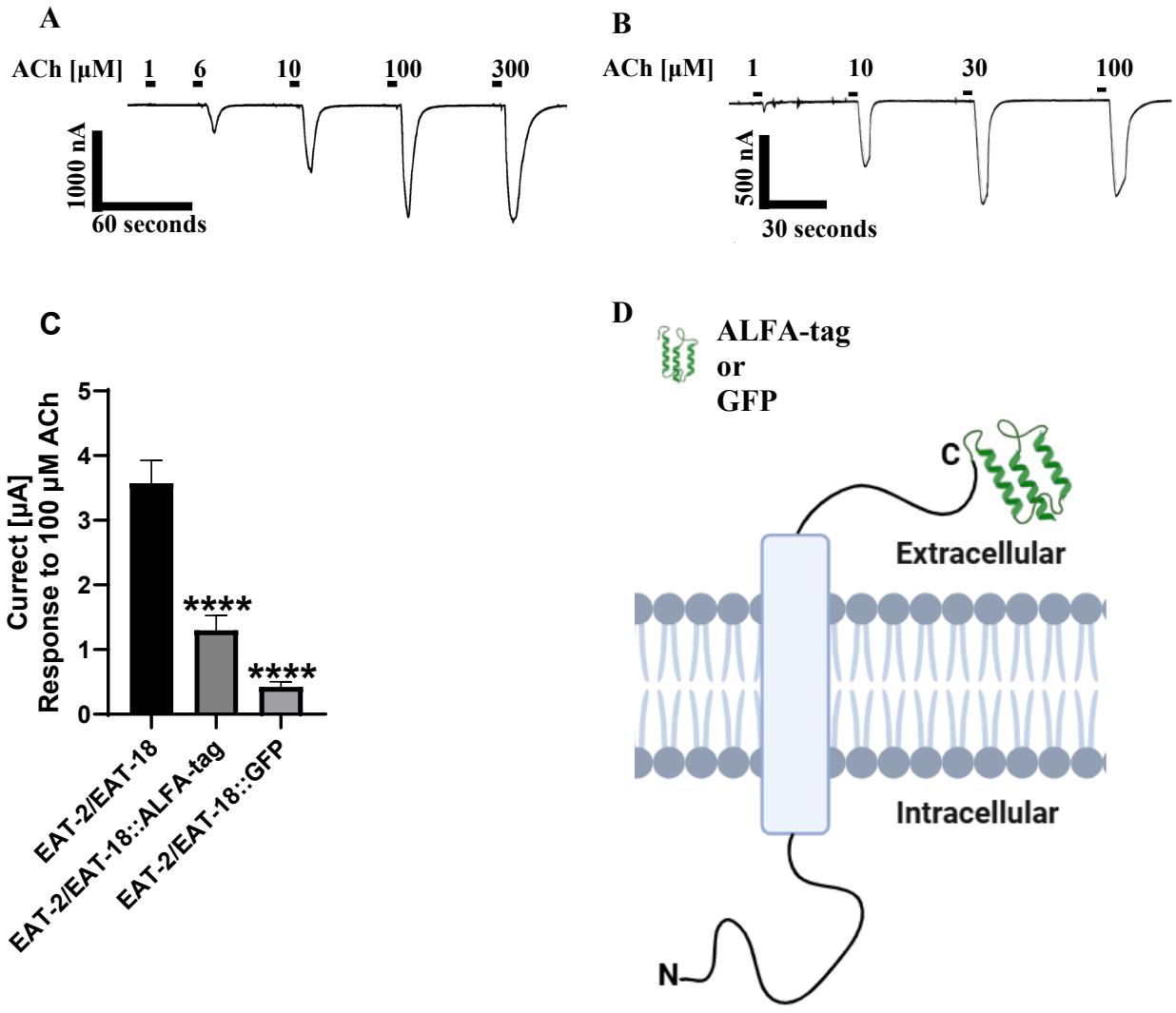


Fig. 5. 1 ALFA-tag and GFP fused to the C-terminus of EAT-18 disrupts its function. A and **B** Sample trace of response to ACh for EAT-2/EAT-8::ALFA-tag and EAT-2/EAT-18::GFP respectively. **C.** Responses to 100 µM ACh current (expressed as mean ± SEM, n≥11).

D. EAT-18 membrane topology showing ALFA-tag and GFP attachment. Data are presented as mean ± SEM and significance was tested by two-way ANOVA with Bonferroni's multiple comparison (****p<0.0001).

5.4.2 RNA interference-based potential nematicide

RNA interference (RNAi)-based pesticides are an emerging biotechnology incorporated into crop protection strategies (Ahmad et al., 2016; Bachman et al., 2016). Double stranded RNAs (dsRNAs) are designed to target genes unique to a pest and minimizing harm to non-target organisms. There is also a reduced chance for resistance but in case of resistance build up, this can be mitigated by designing dsRNA for multiple targets. Their impact on the environment is very low as RNA molecules degrade relatively quick and do not persist like synthetic chemicals. The diverse ways of application, (as sprays, seed coatings, or even engineered in crops), their compatibility with integrated pest management and broad applicability makes them quite attractive. To investigate this technology in plant parasitic nematode control, engineering crops with dsRNA for EAT-2 and/or EAT-18 could be a sustainable, safe and selective way of controlling these parasites. This control strategy could be revolutionary as it would have zero consequences on non-plant feeding nematodes. Also, because EAT-2 and EAT-18 are conserved within Nematoda, the chances of off-target effects would also be near zero.

Some challenges with this control approach could be around delivering dsRNA to the target in the nematode, the cost of production of dsRNA, regulatory and commercial barriers. Just like with conventional pesticides, the nematode cuticle would impede dsRNA getting into the nematode and since they are easily degradable the activity period of the product is reduced. The cost of producing these dsRNAs are currently more expensive than conventional pesticides making them less attractive options for clients. As this is a relatively new area, regulatory structures are not standard globally and this may place embargos to trading. Users will be cautious about RNAi-based pesticides since there is limited data that evaluate the long-term implications of their use.

In summary, there are several variables to consider before deciding on what is or is going to be a more sustainable option to mitigate plant parasitic nematodes. Biological and cultural control strategies provide compelling evidence of their successes however, there are concerns around resistance development, slow action time and other socio-economic issues like high costs of establishment. Chemical control strategies are very effective, cheaper with quicker action

times, but are also globally condemned for their high off-target toxic effects and accumulation in the environment. Pesticide selectivity is achievable and has been shown in several instances (Boulin et al., 2008; Kaminsky et al., 2008b). This thesis provides evidence that EAT-2 may add to viable targets that could be investigated via our proposed strategy. Identifying unique targets in pest organisms and building pesticide development programs around such targets improves the chances of finding new chemicals that are not only effective, but also selective.

However, a holistic approach that combines two or more strategies has shown in several instances to improve the success rates of pest control regiments (Zhou et al., 2024b). The control of plant parasitic nematodes cannot be looked at as a "one size fit all" strategy but perhaps investing more into finding how to combine multiple approaches through an integrated management strategy is still the best way to target parasites.

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