University of Southampton

Faculty of Environmental and Life Sciences
School of Biological Sciences

Investigation of the selective toxicity of neonicotinoids using the nematode worm *Caenorhabditis elegans*

by

Monika Magdalena Kudelska

Thesis for the degree of Doctor of Philosophy
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Abstract

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An ability of insecticides to selectively target pests without affecting non-target species is a key determinant of success of compounds used in agriculture. Neonicotinoids which encompass seven different types of chemical representing three distinct chemical classes, namely the cyanoamidines, nitroguanidines and nitromethylenes, are a major class of insecticides. They effectively control a wide range of insect pests and have low toxicity against mammals, however they can also negatively impact on non-target species of bees, threatening food safety. Neonicotinoids act by targeting insect nicotinic acetylcholine receptors (nAChRs), which are major excitatory receptors in the insect central nervous system. Difficulties in heterologous expression of these proteins hinders their pharmacological characterisation and identification of the molecular determinants of neonicotinoid-toxicity. This thesis describes efforts into developing *Caenorhabditis elegans (C. elegans)* as a platform in which the mode of action and selective toxicity of neonicotinoid-insecticides can be studied.

We determined the effects of neonicotinoids on *C. elegans* behaviours governed by the cholinergic neurotransmission. The cyanoamidine represented by clothianidin, the nitroguanidine represented by thiacloprid and the nitromethylene represented by nitenpyram showed low efficacy on locomotion, pharyngeal pumping, egg-laying and egg-hatching of young adult wild-type *C. elegans*. Exposure of mutant worm with enhanced cuticular permeability showed increased susceptibility of worms to all three neonicotinoids tested, suggesting an adult cuticle limits drug access. The role of the cuticle in neonicotinoids susceptibility was investigated in *C. elegans* cut-head preparation, in which the cuticle is removed and the effects of compounds on pharyngeal pumping are scored. Out of the three neonicotinoids applied, clothianidin showed the greatest efficacy. It stimulated pharyngeal pumping at ≥ 75 μM (18.75 ppm). Generally, the concentrations effective against the function of the pharynx are an order or magnitude lower than the residual, average concentration of neonicotinoids in the soil, suggesting *C. elegans* is not impacted in the field, and at least several fold lower than lethal doses in insect-pests. The difference in neonicotinoid-susceptibility between adult *C. elegans* and insects precludes the use of *C. elegans* pharynx as a platform for the mode of action studies, but highlights its potential as a suitable background for the heterologous expression of insect nAChRs.

Further experiments showed that *C. elegans eat-2* nAChR mutant is a suitable genetic background, in which the expression of heterologous nAChRs can be scored. Expression of *C. elegans* nAChR EAT-2 in the pharyngeal muscle rescued the blunted feeding phenotype and 5-HT insensitivity of the *eat-2* mutant.

Expression of the exogenous receptor, human *α*7 in the pharynx of *eat-2* mutant led to a cell-surface expression, as shown by staining with labeled *α*-bungarotoxin (α-bgtx). However the feeding and pharmacological phenotypes of the mutant were not rescued. *C. elegans* strain in which human *α*7 is expressed in the wild-type genetic background was also generated to determine whether the pharmacology of the human receptor can be imposed on the *C. elegans* pharynx. No difference in the pharyngeal response to nAChR agonists cytisine, nicotine or acetylcholine were noted. The lack of apparent functionality of *α*7 receptor could be due to the incorrect cellular localisation of this protein. *α*-bgtx staining showed that *α*7 receptor is expressed in the specific cells of the pharyngeal muscle, however this localisation does not overlap with the localisation of native EAT-2 receptors. A transgenic strain in which exogenous proteins are expressed using EAT-2 native promoter should be made.

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Definitions and Abbreviations

5-HT - 5-hydroxytryptamine

ACE - Acetylcholinesterase

AChBP - Acetylcholine Binding Protein

bgtx - Bungarotoxin

BSA - Bovine Serum Albumin

BWM - Body Wall Muscle

Ch - Choline

ChAt - Choline Acetyltransferase

DMSO - Dimethyl Sulfoxide

ECD - Extracellular Domain

ELIC - *Erwinia* Ligand Gated Ion Channels

ER - Endoplasmic Reticulum

GABA - Gamma Aminobutyric Acid

GFP - Green Fluorescent Protein

GLIC - *Gloebacter* Ligand Gated Ion Channels

GluCl - Glutamine-Gated Chloride

GPCR - G-protein Coupled Receptor

HSNs - Hermaphrodite Specific Neurons

IPTG - Isopropyl beta-d-1-Thiogalactopyranoside

LB - Luria-Bertani

nAChR - Nicotinic Acetylcholine Receptor

NGM - Nematode Growth Medium

NMJ - Neuromuscular Junction

NSM - Neurosecretory Motor Neurons

MBP - Maltose Binding Protein

PCR - Polymerase Chain Reaction

PVDF - Polyvinylidene Pifluoride

RIC-3 - Resistant to Inhibitors of Cholinesterase-3

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TM - Transmembrane

VAChT - Vesicular Acetylcholine Transferase

VC - Ventral Cord

Research Thesis: Declaration of Authorship

Monika Magdalena Kudelska

Selective toxicity of neonicotinoids using the nematode worm *Caenorhabditis elegans*

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. Part of this thesis has been published as:
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Chapter 1

General introduction

1.1 Selective toxicity of neonicotinoid insecticides

Selective toxicity is a key term in pharmacology and refers to the ability of a drug to explicitly act on the target. For many pest-controlling agents released into the environment, including insecticides, an ability to kill a target pest, without impacting humans, or beneficial animal species is a key determinant of their marketing success (Sánchez-Bayo 2018). The understanding of selective toxicity has a long history of studies and has been an important driver in development of novel insecticides (Casida and Quistad 1998).

1.1.1 The history of insecticides

Insecticides are compounds utilised in agriculture, medicine, industry and private households to protect crops, life-stock and human health from pest infestation (Anadón, Martínez-Larrañaga, and Martínez 2009; Dryden 2009; Oberemok et al. 2015). Until late 1800s organic, natural compounds contained within the plant or animal matter were utilised (Casida and Quistad 1998). The first record of agricultural application of nicotine-containing *Tobacco* (David and Gardiner 1953; Steppuhn et al. 2004) dates back to 1690 (McIndoo 1943). *Tobacco* plant was used in France, England and in the U.S. to protect orchards and trees against a wide range of pests including aphids, caterpillars and plant lice (McIndoo 1943). *Chrysanthemum* plants containing pyrethrum were used against worms and insects in America and Europe (Elliot 1995). These treatments were however suitable only for small scale agricultural treatment, due to the limited availability.

Arsenic compounds were the earliest inorganic insecticides. Although their history dates back to 5th century (Kerkut and Gilbert 1985), they did not gain popularity until the 19th century. Aceto-arsenite Paris Green was used in controlling Colorado potato beetles and mosquitoes (Cullen 2008; Peryea 1998), whereas lead arsenate was an effective insecticide for apple and cherry orchards (Peryea 1998). Although effective against pests, these

substances are toxic to humans (Nelson et al. 1973; Gibb et al. 2010; Argos et al. 2010) thus their current use is marginal (ECHA 2017).

In the last century, several synthetic compounds became available, including dichlorodiphenyltrichloroethane (DDT), and members of the carbamate, organophosphate and pyrethroid class of compounds. DDT was one of the most popular insecticides in the 1900s, with the peak annual use of over 85 000 tonnes in the U.S. alone (U.S. Department of Health and Human Services 2002). DDT's potent insecticidal activity was discovered 60 years after its synthesis in 1874, by the Swiss chemist Paul Hermann Muller, who was later awarded a Nobel prize in Medicine "for his discovery of the high efficiency of DDT as a contact poison against several arthropods." (NobelPrize.org 2019). DDT became commercially available in the 1940s in Europe and the U.S., and it was used to suppress potato beetles, mosquitoes, fleas and lice. Since 1970s, the use of DDT has been progressively phased out due to its propensity to bio-accumulate in the adipose tissues of animals resulting in the environmental persistence (U.S. Environmental Protection Agency 1975).

Diminishing popularity of DDT, created a market space for organophosphates, carbamates and pyrethroids (Table 1.1). By the 1990s, the respective market share of members of these three classes of insecticides was: 43%, 15% and 16% and the annual sales of 3.42, 1.19 and 1.17 billion Euro, respectively (Jeschke et al. 2011). The main issue associated with the use of organophosphates and carbamates is their ability to cause serious human poisoning, which can lead to death (King and Aaron 2015). The lack of selectivity combined with increasing resistance (Bass et al. 2014) instigated new management strategies aimed to combat these negative effects. In the 1990s research activities concentrated on finding new insecticides which have greater selectivity and better environmental and toxicological profiles.

Table 1.1: Synthetic insecticides

Class	Chemical	Mode of action
Organophosphates	parathion, malathion, azinphosmethyl	Acetylcholinesterase inhibitor
Carbamates	aldicarb, carbamyl, carbofuran	Acetylcholinesterase inhibitor
Pyrethroids	allethrin, cypermethrin	Voltage gated sodium channel blocker

1.1.2 Structural diversity of neonicotinoid insecticides

In 1970s, the scientists of Shell Development Company Biological Research Centre in California identified α-DBPN (2-(dibromonitromethyl)-3-(methylpyridine)), first synthesised by Prof. Henry Feuer (Feuer, Bevinakatti, and Luo 1986). This lead compound showed low insecticidal activity against aphid and house fly (Tomizawa, M. and J.E. Casida 2003; Tomizawa, M. and J. E. Casida 2005). Structural alterations of DBPN resulted in production of nithiazine (Figure 1.1). Nithiazine showed improved insecticidal activity and was particularly effective as a new housefly repellent (Kollmeyer et al. 1999). Further replacement of the thiazine ring by chloropyridinylmethyl (CPM) group, addition of the imidazolidine or its acyclic counterpart, and retention of the nitromethylene group resulted in generation of more potent compounds, one of which, nitenpyram, exhibited particularly high efficacy. Regrettably, both nithiazine and nitenpyram are not useful in fields, as they are unstable in light. The latter however is successfully used in veterinary medicine as an external parasite treatment for cats and dogs.

To solve the issue of photo-instability, nitromethylene group ($CCHNO_2$) was replaced by nitroguanidine ($CNNO_2$) and cyanoamidine (CNCN) (Figure 1.1; Kagabu and Medej (1995)). These chemical moieties have absorbance spectra at much shorter wavelengths hence do not degrade upon exposure to sunlight. Further alterations, such as replacement of imidazolidine by thiazolidine or oxadiazinane, and/or chloropyridinylmethyl by chlorothiazole or tetrahydrofuran (THF) did not hinder insecticidal activity (Yamamoto and Casida 1999). As a result of these modifications, 6 most commonly used neonicotinoids were synthesised. They are grouped according to their pharmacophore into N-nitroguanidines, nitromethylenes and N-cyanoamidines (Figure 1.1). Generally compounds with acyclic- guanidine or amidine and with nitromethylene are more efficacious against moth- and butterfly- pests than those with cyclic counterparts or nitroimine respectively (Ihara et al. 2006), nevertheless all are commonly used in agriculture. Imidacloprid, currently the most widely used neonicotinoid, was synthesised in 1970 in Bayer Agrochemical Japan and introduced to the EU market in 1991. Its trade names include confidor, admire and advantage. Together with thiacloprid (calypso), imidacloprid is marketed by Bayer CropScience. Thiamethoxam (actara) is produced by Syngenta, Clothianidin (poncho, dantosu, dantop) and Nitenpyram (capstar) by Sumitomo Chemical, acetamiprid (mospilan) by Certis, whereas dinotefuran (starkle) by Mitsui Chemicals company. Last neonicotinoid, dinotefuran, was launched in the EU in 2008.

Research into novel neonicotinoids continues (Shao, Swenson, and Casida 2013). In the last decade, several novel insecticides have been characterised and approved for use in the EU. Sulfoxafrol (Zhu et al. 2011; The European Commission 2019) and flupyradifurone (Nauen et al. 2015; The European Commission 2019) have been classified as representatives of new chemical classes, namely sulfoximines and butenolides. However, due to their mode of action and similar biochemical properties, some argue that they are in fact neonicotinoids, whereas their mis-classification has been deliberate to avoid association with neonicotinoids (Pest Action Network Europe 2016).

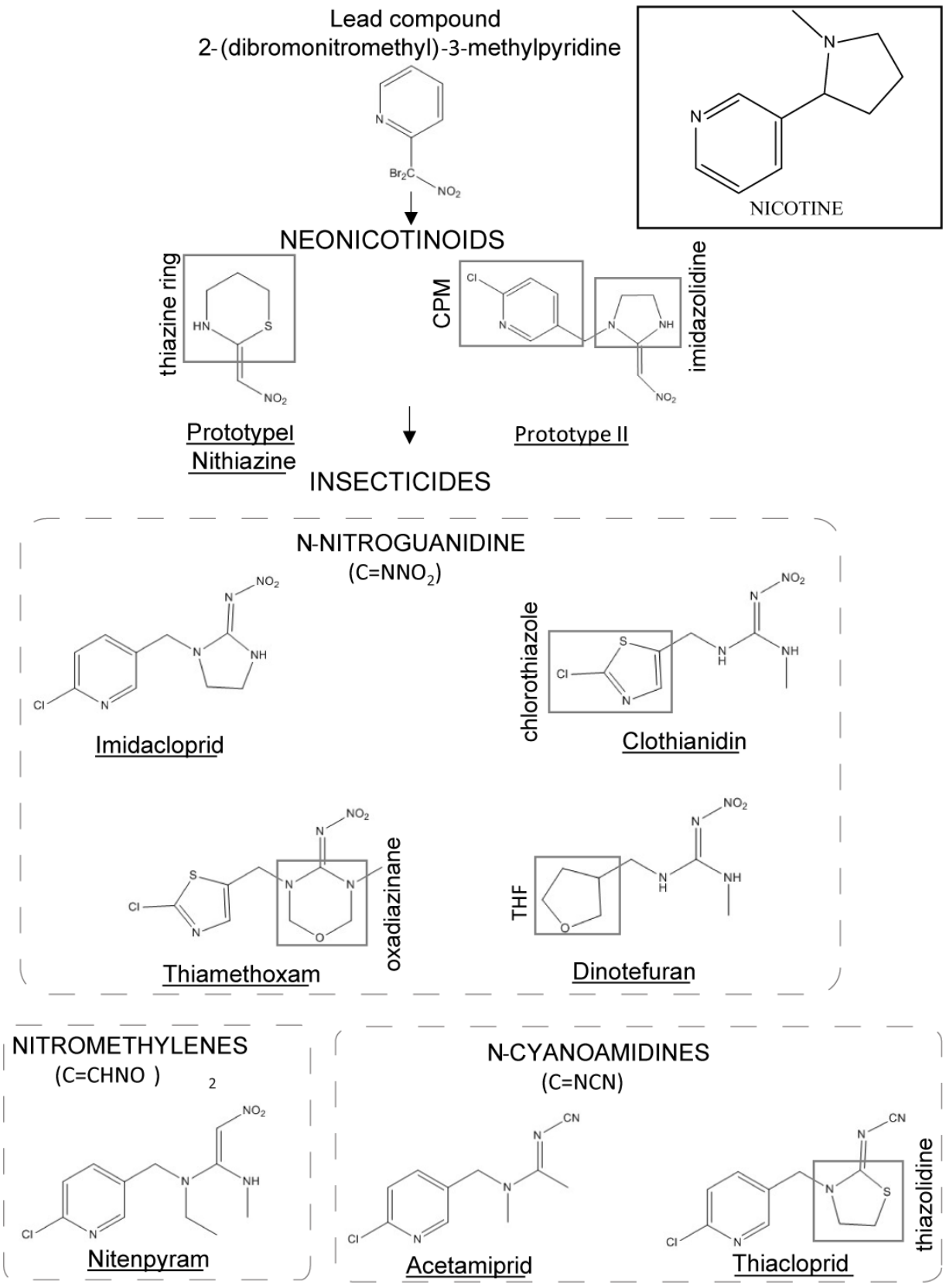


Figure 1.1: **Development and chemical structures of the synthetic insecticides, the neonicotinoids.** Systematic modification of the lead and prototype compounds led to the discovery of seven neonicotinoids currently used in agriculture and animal health. They are structurally related to nicotine (shown in top right corner) and classified according to the pharmacophore moiety into N-nitroguanidines, N-cyanoamidines and nitromethylenes.

1.1.3 Economical status of neonicotinoids

The use of neonicotinoids in agriculture has been increasing steadily since their launch in the early 1990s. By 2008, they became major chemicals in the agriculture, replacing organophosphates and carbamates (Jeschke et al. 2011). Continual increase in popularity of neonicotinoids is reflected in the total usage data. In Great Britain, the yearly use of neonicotinoids increased by over 10-fold from 10 tonnes/year in 1996 to over 105 tonnes/year in 2016 (The Food and Environment Research Agency. Department for Environment, Food and Rural Affairs 2019). Similar trends are observed in the U.S. (United States Geological Survey 2019), Sweden and Japan (Simon-Delso et al. 2015). Continual increase in usage coincides with the rise in their economical impact. In 2008, the estimated global market value of neonicotinoids was 1.5 billion dollars (Jeschke et al. 2011). This increased to 3.1 billion dollars in 2012 (Bass et al. 2015).

The widespread usage and monetary value of neonicotinoids is a reflection of their many advantages.

1.1.4 Physicochemical properties of neonicotinoids grant versatile methods of application

One of the major benefits of neonicotinoids are their physical and chemical profiles (Table 1.2). Due to relatively high water solubility, neonicotinoids act as systemic insecticides (Westwood et al. 1998). This means that once applied on crops, they dissolve in the available water and are taken up by the developing roots or leaves. Upon plant entry, they are then distributed to all parts of the plant (Westwood et al. 1998; Stamm et al. 2016), providing protection against herbivorous pests (Stamm et al. 2016). This property of neonicotinoids means they can be used as a seed coating, reducing the required frequency of application. Indeed, seed dressing is the most commonly used method, accounting for 60% of all neonicotinoids applications worldwide (Jeschke et al. 2011) and particularly popular to protect potatoes, oilseed rape, cereal, sunflower and sugar beet. In addition, neonicotinoids half-life in soil is from several weeks to years (Cox, Koskinen, and Yen 1997; Sarkar et al. 2001; Gupta and Gajbhiye 2007), hence seed-dressing creates a continual source for re-uptake by plants. Neonicotinoids are also suitable for ground treatment and are used as soil drenching for the protection of citrus trees and vines, granules for amenity grassland and ornament flowers and as a trunk-injection to protect trees against herbivores. They are not volatile, therefore can be also applied as spray. This method is used in the gardens for flowers and vegetables and in agriculture on soft fruits and greenhouse crops. Low lipophilicity, indicated by octanol/water partition coefficient value (log Pow), suggest they do not bio-accumulate in the adipose tissues of animals (Turaga et al. 2016). However, moderate water solubility combined with low lipophilicity means they may have a potential to accumulate in water.

Table 1.2: Physicochemical properties of neonicotinoids

Compound	log Pow pH=7.4 24°C	pKa at 20°C	Water solubility mg / L 20°C pH=7	Henry's law Pa x m^3 x mol^{-1} 20°C	Water sediment DT50 (days)
Nitenpyram	-0.64 (1)	3.1 and 11.5	590 000 (3)	4 x 10^{-13} (5)	NA (3)
Clothianidin	0.70 (1)	11.09 (5)	340 (3)	3 x 10^{-11} (5)	56.4 (3)
Thiacloprid	1.26 (1)	NA (5)	184 (3)	5 x 10^{-10} (5)	28.0 (3)

log Pow = octanol/water partitioning, DT50 = half-life for degradation, 1 = Jeschke and Nauen 2008, 2 = Sangster 1997, 3 = Bonmatin et al., 2015, 4 = Maeda et al., 1978, 5 = Pesticide Properties Database (PPDB), 2019

1.1.5 Neonicotinoids are highly potent against insect pests

Neonicotinoids are highly potent against insect pests, (Table 1.3), including a wide range of piercing-sucking pests such as cotton and peach aphids (*Aphis gossypii, A. gossypii* and *Myzus persicae, M. persicae*) (Nauen et al. 1996; Mota-Sanchez et al. 2006; Bass et al. 2011), house- (*Malus domestica, M. domestica*) and may-flies (*Epeorus longimanus, E. longimanus*) (Tomizawa, Lee, and Casida 2000; Alexander et al. 2007) as well as planthoppers (*Nilaparvata lugens, N. lugens*) (Zewen et al. 2003). Their LC_{50} (the concentration needed to kill 50% of pests) in generally in the region of 2 μM. Although all neonicotinoids are highly effective against insect pests, their potency depends on the chemical structure. The rank order of insecticidal potency on the cotton aphid *A. gossypii* and the Colorado potato beetle, *Leptinotarsa decemlineata* (*L. decemlineata*) was clothianidin $>$ nitenpyram $=$ thiacloprid, suggesting nitroguanidines are generally more potent than nitromethylenes and cyanoamidines (Shi et al. 2011; Mota-Sanchez et al. 2006).

The potency also depends on the route of exposure. LC_{50}s are lower upon systemic or oral administration in comparison to the topical exposure (Alexander et al. 2007). Imidacloprid injected into the abdomen of American cockroaches *Periplaneta americana* (*P. americana*), killed 50% of animals at 1 nM (Ihara et al. 2006). Concentrations of 285.49 nM and 1.83 μM were required to observe the same effect upon oral or contact exposure, respectively in the peach aphid *M. persicae* (Nauen et al. 1996). Effective doses obtained from oral and topical studies are most relevant, since these are the two main routes of exposure of pests in the agriculture. The $LC(D)_{50}$ values of neonicotinoids are at least 6-fold higher than those of structurally related nicotine, highlighting the superiority of neonicotinoids as pest controlling agents.

Table 1.3: Toxicity of nicotine and neonicotinoids

Drug	Species	Common name	Taxon	LD50	LC50	Bioassay	Ref
Thia	*A. gossypii*	Cotton aphid	Insect pest	-	9.35 μM	Topical	16
Clo	*A. gossypii*	Cotton aphid	Insect pest	-	7.29 μM	Topical	16
Nit	*A. gossypii*	Cotton aphid	Insect pest	-	9.12 μM	Topical	16
Imi	*M. persicae*	Green peach aphid	Insect pest	-	1.83 μM	Topical	1
Imi	*M. persicae*	Green peach aphid	Insect pest	-	285.49 nM	Oral	1
Nic	*M. persicae*	Green peach aphid	Insect pest	-	1.85 mM	Topical	1
Nic	*M. persicae*	Green peach aphid	Insect pest	-	27.74 mM	Oral	1
Imi	*M. persicae*	Green peach aphid	Insect pest	-	3.87 μM	Topical	3
Thtx	*M. persicae*	Green peach aphid	Insect pest	-	2.19 μM	Topical	3
Imi	*M. persicae*	Green peach aphid	Insect pest	-	257.52 nM	Topical	3
Thtx	*M. persicae*	Green peach aphid	Insect pest	-	1.64 mg/L	Topical	3
Nic	*L. decemlineata*	Colorado beetle	Insect pest	0.35 ng/mg	-	Topical	2
Dino	*L. decemlineata*	Colorado beetle	Insect pest	0.05 ng/mg	-	Topical	2
Thia	*L. decemlineata*	Colorado beetle	Insect pest	6.8 ng/beetle	-	Topical	2
Imi	*L. decemlineata*	Colorado beetle	Insect pest	0.34 ng/beetle	-	Topical	2
Nit	*L. decemlineata*	Colorado beetle	Insect pest	0.20 ng/beetle	-	Topical	2

Table 1.3: Toxicity of nicotine and neonicotinoids *(continued)*

Drug	Species	Common name	Taxon	LD50	LC50	Bioassay	Ref
Thia	*L. decemlineata*	Colorado beetle	Insect pest	0.18 ng/mg	-	Topical	2
Clo	*L. decemlineata*	Colorado beetle	Insect pest	0.15 ng/mg	-	Topical	2
Ace	*L. decemlineata*	Colorado beetle	Insect pest	0.14 ng/mg	-	Topical	2
Imi	*N. lugens*	Brown planthopper	Insect pest	0.82 ng/mg	-	Topical	4
Thia	*M. domestica*	Housefly	Insect pest	3 ng/mg	-	?	5
Nic	*M. domestica*	Housefly	Insect pest	>50 ng/mg	-	?	5
Imi	*E. longimanus*	Mayfly	Insect pest	-	82.13 nM (24 hrs)	Topical	6
Imi	*E. longimanus*	Mayfly	Insect pest	-	2.54 nM (96 hrs)	Topical	6
Imi	*A. mellifera*	Honey bee	Insect pollinator	-	6.88 μM	Oral	14
Imi	*A. mellifera*	Honey bee	Insect pollinator	0.81 ng/mg	-	Oral	14
Imi	*A. mellifera*	Honey bee	Insect pollinator	0.81 ng/mg	-	Topical	15
Clo	*A. mellifera*	Honey bee	Insect pollinator	0.44 ng/mg	-	Topical	15
Thtx	*A. mellifera*	Honey bee	Insect pollinator	0.24 ng/mg	-	Topical	15
Clo	*C. virginianus*	Bobwhite quail	Bird	>200 mg/kg (acute)	-	Oral	7
Clo	*C. virginianus*	Bobwhite quail	Bird	>5040 mg/kg (5 days)	-	Oral	7
Clo	*A. platyrhynchos*	Mallard duck	Bird	>5230 mg/kg (5 days)	-	Oral	7
Clo	*M. musculus*	Mouse	Mammal	389-465 mg/kg	-	Oral	7

Table 1.3: Toxicity of nicotine and neonicotinoids *(continued)*

Drug	Species	Common name	Taxon	LD50	LC50	Bioassay	Ref
Clo	*O. mykiss*	Rainbow trout	Fish	-	424.51 μM	?	7
Clo	*L. macrochirus*	Bluegill	Fish	-	468.60 μM	?	7
Imi	*E. fetida*	Redworm	Earth worm	-	4.81 μM (24 hours)	Topical	8
Imi	*E. fetida*	Redworm	Earth worm	-	2.74 μM (48 hours)	Topical	8
Clo	*E. fetida*	Redworm	Earth worm	-	62.08 μM (14 days)	Topical	9
Clo	*E. fetida*	Redworm	Earth worm	-	24.24 μM (7 days)	Topical	10
Clo	*E. fetida*	Redworm	Earth worm	-	24.27 μM (14 days)	Topical	10
Imi	*E. fetida*	Redworm	Earth worm	-	11.93 μM (14 days)	Topical	11
Ace	*E. fetida*	Redworm	Earth worm	-	12.08 μM (14 days)	Topical	11
Nit	*E. fetida*	Redworm	Earth worm	-	26.75 μM (14 days)	Topical	11
Clo	*E. fetida*	Redworm	Earth worm	-	3.72 μM (14 days)	Topical	11
Thia	*E. fetida*	Redworm	Earth worm	-	10.60 μM (14 days)	Topical	12
Thia	*M. incognita*	Root-knot nematode	Nematode	-	143. 24 μM (6 hours)	Topical	13

Note:

References (Ref) 16: Shi et al. 2011, 1: Nauen et al. 1996, 2: Mota-Sanchez et al. 2006, 3: Bass et al. 2011, 4: Zewen et al. 2003, 5: reported in Tomizawa et al. 2000, 6: Alexander et al. 2007, 7: De Cant and Barrett 2010, 8: Luo et al. 1999, 9: De Cant and Barrett 2010, 10: Wang et al. 2012, 11: Wang et al. 2015, 13: = Dong et al. 2017, 14: Cresswell 2011, 15: = Godfray et al. 2015

1.1.6 Neonicotinoids are selectively lethal to insect pests

The key determinant of success of agrochemical compounds is their ability to selectively target pests over non-target species. Neonicotinoids are generally effective at $\sim$ 2 μM concentrations against piercing-sucking pest infestations, whereas their LD_{50s} are in the region of 0.2 - 0.3 ng/mg of body weight (Mota-Sanchez et al. 2006; Zewen et al. 2003; Tomizawa, Lee, and Casida 2000; Alexander et al. 2007). The $LC(D)_{50}$ values for non-target species are at least 2 times higher (Table 1.3). Honeybees (*Apis mellifera, A.mellifera*) are among the most susceptible non-targets, with the average LC_{50} and LD_{50} values for imidacloprid of 7.04 μM and 4.5 ng per mg of body weight, respectively (Cresswell 2011). Some studies report high potency of neonicotinoids on earth worms, with the LC_{50} as low as 2.74 μM on redworm *Eisenia fetida* (*E. fetida*) (Luo et al. 1999). Fish and birds are hundred fold less susceptible (De Cant and Barrett 2010), whereas mammals are the least susceptible with LD_{50} doses higher than 130 mg/kg of body weight (De Cant and Barrett 2010; Legocki and Polec 2008). This differential susceptibility between target and non-target species, is expected to enable an environmental release of neonicotinoids at concentrations which will exterminate pests without killing the non-targets.

Indeed, field realistic concentrations of neonicotinoids are higher than those causing lethality of the most susceptible species of honey bee and worms. Residues of neonicotinoids can be found in the nectar, wax and pollen and nectar, with the highest concentration generally present in the former (Goulson 2013). Cresswell (2011) determined that imidacloprid is present in most commonly bee-consumed nectar at 2.3 - 20 μM. He also estimated that the average realistic amount of imidacloprid in a nectar load is 0.024-0.3 ng. This is higher than the reported honeybee LC_{50} and LD_{50} values of 7.04 μM and 4.5 ng, respectively (Cresswell 2011).

The concentration of neonicotinoids in soils with several years of history of treatment by seed coating were also investigated. Samples were collected 10 months after sowing (Botías et al. 2015) just before (Jones, Harrington, and Turnbull 2014; Schaafsma et al. 2016) or after planting (Perre, Murphy, and Lydy 2015). The average reported concentrations of neonicotinoids in the centre of the field are in the region of 20 μM, which is higher than the concentrations effective against earth worms and nematodes; the LC_{50} against the most susceptible species is 2.74 μM (Luo et al. 1999). A differential between the concentration of neonicotinoids in the field and the LD_{50} values, suggest that neonicotinoids are not expected to kill bees or worms, however, a substantial body of evidence from lab- and field- based experiments suggest that they can impair on the behaviour and the cognitive function of these animals.

1.1.7 Sub-lethal effects of neonicotinoids on non-target species

1.1.7.1 Effects on insect pollinators

Pollinating services are provided by many species of bees, flies, beetles and bats (Thapa 2006). Eighty percent of the total pollinating activity is carried out by bees (Thapa 2006). There are over 20 000 species of bees, 267 species live in the UK (Breeze, Roberts, and Potts 2012). Among them are honey bees (*A. mellifera*), bumblebees and over 220 species of solitary bees. Honeybees and bumblebees served as platform to determine toxic effect of neonicotinoids on biological pollinators.

1.1.7.1.1 Reduced olfactory learning and memory

Honeybees are social insects, living in colonies where a clear division of labor exists. Worker bees account for up to 95% of the entire colony (Sagili and Burgett 2011). These non-reproductive females are responsible for finding, collecting and transporting nectar or pollen from the flowering plants to the hive. Their ability to process, learn, memorise sensory cues and navigate through the environment is crucial for the survival and overall success of the entire hive. It is these essential processes that are disrupted by neonicotinoids. Bees exposed to 93 nM of imidacloprid in the sugar solution showed reduced ability to olfactory learn, as showed by the Proboscis Extension Reflex (PER) (Decourtye et al. 2004) - a paradigm used to measure olfactory learning of bees (Takeda 1961). Imidacloprid also compromised foraging activity of honeybees (Decourtye et al. 2004; Gill, Ramos-Rodriguez, and Raine 2012) and bumblebees. Four-week exposure of early-developmental stages to imidacloprid at 23 nM in pollen reduced the foraging efficiency and duration (Gill, Ramos-Rodriguez, and Raine 2012). Neonicotinoids at low nM concentrations, also reduced the number of bees returning to hives (Henry et al. 2012; Feltham, Park, and Goulson 2014).

1.1.7.1.2 Impaired reproduction

Neonicotinoids have been also shown to negatively impact on various aspects of bees' fecundity. Reproduction of bees is performed by a single member of the colony - the queen. She lays fertilised and unfertilised eggs into cells of the comb. These eggs develop into larva, pupa and adult male drones and female workers. Fourteen day exposure of bumblebees to imidacloprid at 2, 4 and 23 nM, increased the number of empty pupal cells (Whitehorn et al. 2012). Imidacloprid has been shown to reduce the total size of treated colonies, reduce the brood production (Laycock et al. 2012) and the number of born queens (Whitehorn et al. 2012) and workers (Gill, Ramos-Rodriguez, and Raine 2012) of bumblebees. Exposure of drones to thiamethoxam at 15.5 nM and clothianidin at 6 nM led to shortening of life-span and hindered sperm vitality and quantity (Straub et al. 2016). Although these effects were observed in the lab- and semi- lab conditions, the negative impact of neonicotinoids was also seen in the field-studies. Bumblebees foraging on oilseed rape coated with clothianidin, exhibited decreased queen production, colony growth and reduced bumblebee density (Rundlof et al. 2015).

More recently, international field studies confirmed negative effects of neonicotinoids on overwinter success and reproduction of honey and wild bees (Woodcock et al. 2017).

Insect pollinators play an important ecological, economical and evolutionary role. They pollinate wild plants (Kwak, Velterop, and Andel 1998), food crops (Klein et al. 2007) and promote plant sexual reproduction (Gervasi and Schiestl 2017). The emerging evidence of the negative impact of neonicotinoids on bees and honeybees, restricted their use in Europe in 2013 (The European Commission 2013) and is likely to lead to a complete ban of neonicotinoids in the future (The European Food Safety Authority 2018).

1.1.7.2 Effects of neonicotinoids on worms

Worms can be divided into several phyla, including Platyhelminthes (flatworms), Annelida (segmented worms) and Nematoda (roundworms, pinworms, etc.). These animals can live on land, in the ocean, in freshwater, in or on animals and plants. They can be further divided into parasitic and free-living, non-parasitic worms. In the soil, non-parasitic earth worms and nematodes have an important biological role whereby they are a significant biomass contributors and nutrient cyclers (Ingham et al. 1985; Neher 2001; Lecomte-Pradines et al. 2014) (Section 3.1.1). They have also an important role in shaping the soil structure (Blouin et al. 2013). These functions are heavily reliant on worms ability to burrow or move in the soil, feed and reproduce (Medina-Sauza et al. 2019), however it is these behaviours that have been shown to be impacted by neonicotinoids.

1.1.7.2.1 Earth worms

Clothianidin and thiacloprid at concentrations $\geq$ than 1.2 μM and the EC_{50} of 5.1 μM and 3.4 μM, respectively reduced the reproductive potential of redworm *E. fetida*, as measured by the cocoon production (Gomez-Eyles et al. 2009). Neonicotinoids showed a negative impact on the reproduction of other species, including *Lumbricus rubellus* (*L. rubellus*) (Baylay et al. 2012), *Dendrobaena octaedra* (*D. octaedra*) (Kreutzweiser et al. 2008) and *Eisenia andrei* (*E. andrei*) (Alves et al. 2013). Reduction of body weight of *E. fetida* and *D. octaedra* were observed after a 14 day treatment with imidacloprid at 27.08 and 54.75 μM (Kreutzweiser et al. 2008). Imidacloprid at 488.85 nM to 7.82 μM increased avoidance of *E. andrei* (Alves et al. 2013), whereas at 782 nM it reduced the *A. caliginosa* burrowing depth and length (Dittbrenner et al. 2011). Burrowing of *L. terrestris* was also impacted, but at higher imidacloprid concentrations (Dittbrenner et al. 2011).

1.1.7.2.2 Soil nematodes

Neonicotinoids also induce sublethal effects on the the free-living nematode *C. elegans*. Thiacloprid and imidacloprid impaired on the reproduction of *C. elegans* with EC_{50} of 1.14 nM and 2.09 mM, respectively (Gomez-Eyles et al. 2009). Thiacloprid at 37 nM has an effect on chemosensation, whereas at 18 μM it impairs motility of this free living nematode (Hopewell et al. 2017). Impaired motility of *C. elegans* in

response to $\geq$ 120 μM imidacloprid was also recorded (Mugova et al. 2018). Taken together, neonicotinoids have sublethal effects on earth worms and soil nematodes at concentrations as low as nM. Most of the doses effective against worms are higher than the average doses of neonicotinoids in the field. However, the presence of clothianidin, imidacloprid and thiamethoxam has been detected at lower than average levels, such as 80.10 nM for imidacloprid, 23.01 nM for imidacloprid and 68.56 nM for thiamethoxam (Jones, Harrington, and Turnbull 2014). This suggests that the environmentally relevant concentrations of neonicotinoids may negatively impact on the the well-being of soil dwellers.

1.1.7.3 Effects of neonicotinoid on birds

Environmentally relevant concentrations of neonicotinoids may also have a negative effect on birds (Hallmann et al. 2014). In particular, granivorous and insectivorous birds may be at risk, should they consume neonicotinoid-contaminated seeds and/or insects (Goulson 2013). Environmental neonicotinoids may impair their migratory ability (Eng, Stutchbury, and Morrissey 2017) and negatively impact on their growth and reproduction (Sanchez-Bayo, Goka, and Hayasaka 2016).

The environmental ecotoxicity of neonicotinoids highlights the importance of selective toxicity of agrochemical compounds in successful pest management programs. The development of new insecticides, effective against pest and not beneficial insects or other species requires a detailed knowledge of their mode of action.

1.2 Neonicotinoids act by binding to nicotinic acetylcholine receptors (nAChR)

1.2.1 nAChR structure

Nicotinic acetylcholine receptors (nAChRs) are members of the pentameric ligand-gated ion channels which are found in a diversity of species from bacteria to human. They are the representatives of the Cys-loop superfamily of channels which also include γ -aminobutyric acid (GABA) type A receptors, 5-hydroxytryptamine type-3 receptors (5-HT3), and glycine receptors. Structural studies of the nAChRs from the muscle of the electric fish *Torpedo* (Figure 1.2a) shed light on the the stoichiometry, the shape and the size of Cys-loop receptors.

The identity of the neuromuscular junction (NMJ) nAChR was first investigated using indirect, biochemical approaches. Membrane bound NMJ receptors were isolated by *in-situ* cross-linking with a radiolabelled antagonist and a subsequent purification. sodium dodecyl sulfate (SDS) -resolved fragments pattern suggesting the pentameric nature of these receptors (Hucho, Oberthur, and Lottspeich 1986; Schiebler, Bandini, and Hucho 1980) of the total size 270 000 kDa composed of 4 different subunits namely α, β, δ and γ arranged into a pentamer. The SDS- polyacrylamide gel electrophoresis (PAGE) pattern and the analysis of nAChR complexes purified with the use of non-denaturing buffer led to a suggestion that the stoichiometry is: $\alpha 1$, $\beta 1$, δ, $\alpha 1$, γ (clockwise) (Reynolds and Karlin 1978). Heterologous expression in *Xenopus* oocytes confirmed that 4 subunits are needed to achieve expression. In the absence of any other one of the subunits, the responses to acetylcholine (ACh) were either absent or greatly reduced, therefore 4 subunits are required for the normal function of this protein (Mishina et al. 1984).

The stoichiometry and structural details of muscle type nAChRs were confirmed by more direct structural approaches: cryo- and electron- microscopy. The receptor protein is in the shape of an elongated, 125 Å funnel (Unwin, N. 1993; Toyoshima and Unwin 1990). It consists of large, extending to the synaptic space (Toyoshima and Unwin 1990) N-terminal ligand binding domain (Sigel et al. 1992), the membrane spanning pore-domain (Eisele et al. 1993), intracellular MA helix (Toyoshima and Unwin 1990; Unwin, N. 1993), and C-terminus positioned extracellularly. Constituent nAChR subunits are arranged pseudosymmetrically, around the central ion conduction pore (Brisson and Unwin 1985). The subunit composition of the neuromuscular nAChR follows the strict order of $\alpha 1$, $\beta 1$, δ, $\alpha 1$, γ (clockwise). Each subunit of the nAChR contains 4 transmembrane helices (Noda et al. 1982, 1983) named M1, M2, M3 and M4, as moving from N- to C- terminus. M1, M3 and M4 are exposed to the plasma membrane (Blanton and Cohen 1994), shielding pore-forming M2 helices (Imoto et al. 1986; Hucho, Oberthur, and Lottspeich 1986) from the hydrophobic environment of the bilayer.

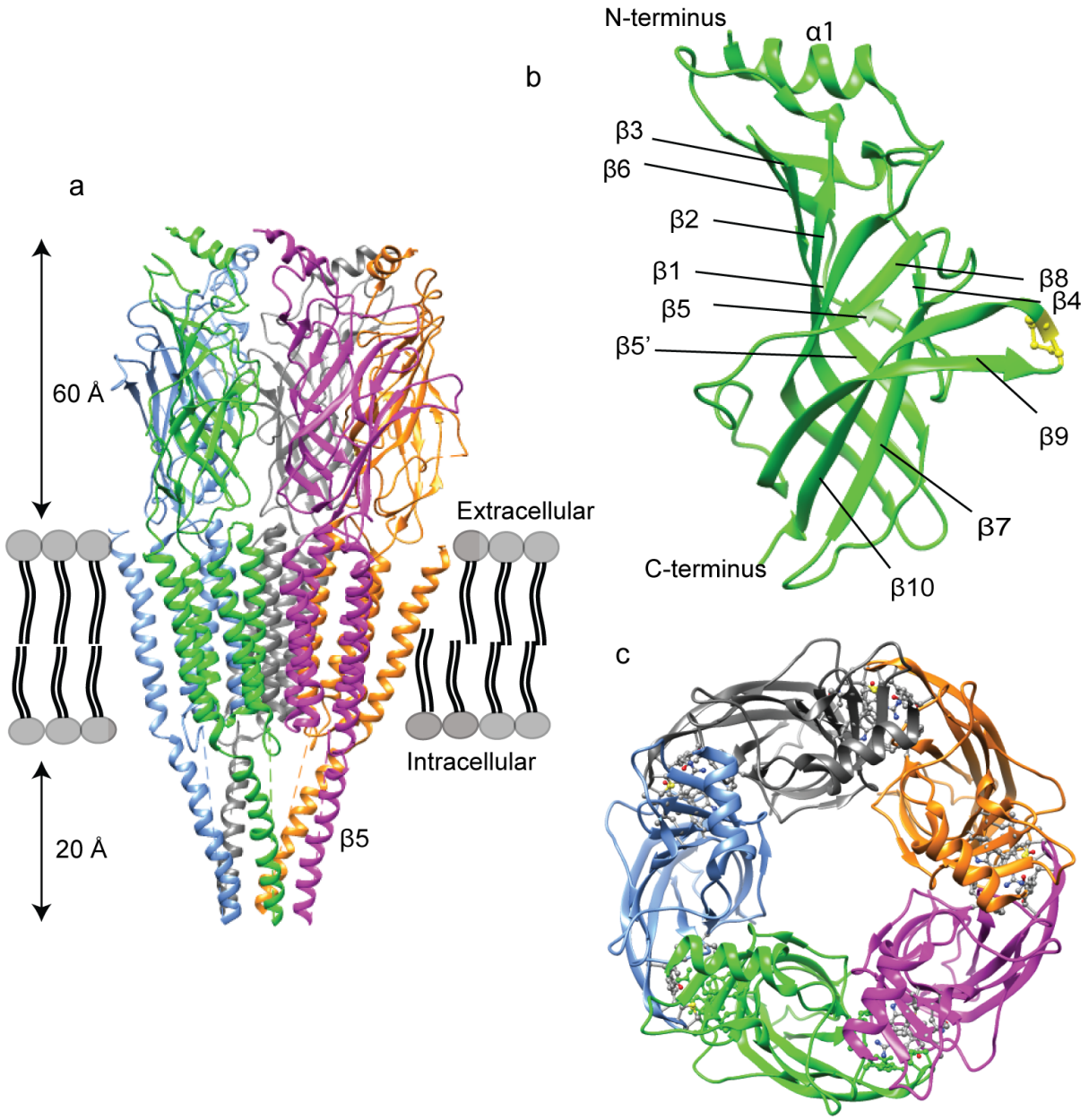


Figure 1.2: **Structural features of the nicotinic acetylcholine receptor.** Torpedo nAChR is a transmembrane protein, made up of 5 subunits (colour-coded), arranged around the ion conductivity pore. Each subunit consists of extracellular ligand-binding, transmembrane and intracellular domain (a) (PBD code:2BG9). Extracellular domain of a single subunit consists of 10 β-strands and N-terminal α-helix. It contains a disulphide bridge between Cys192 and Cys193 (highlighted in yellow) (b). Fully formed receptors have five ligand binding pockets formed by the contributions from the neighboring subunits (A-B, B-C, C-D, D-E and E-A), named the principle and the adjacent components, respectively. Top view of the molluscan AChBP (PDB:1I9B) with amino acids forming the agonist binding site in ball and stick representation (c). Images generated with the UCSF Chimera software.

As the outer helices progress from the outer to the inner leaflet of the membrane, they tilt inwards (Miyazawa, Fujiyoshi, and Unwin 2003), narrowing down the width of the channel. M2 on the other hand, bends roughly in the middle of the bilayer (Unwin N. 1995), where it forms the most restricted part of the ion conductivity pathway. There are hydrophobic interactions between the outer helices, which stabilise the outer wall of the receptor and hence limit the conformational changes adopted by the inner helix. In contrast there are no extensive bonds between the inner and outer helices (Miyazawa, Fujiyoshi, and Unwin 2003). As lining pore structures, the inner helix and flanking sequences contain molecular determinants for ion selectivity, permeability, the rate of conductance and gating. These were investigated by pharmacological, biochemical and electrophysiological approaches. Imoto et al. (1988); Imoto et al. (1991); Konno et al. (1991) investigated the function of several rings of anionic and neutral amino acids with side chains facing towards each other in the centre of the pore. The so called intermediate ring (constituting of αE241 and equivalent), and the adjacent to α E241 in helical configuration central ring, (formed by α L244 and equivalent) form a narrow constriction of the ion pore, hence have the strongest effect on the conductance rate (Imoto et al. 1991, 1988). In addition, the negatively charged side chains of intermediate ring are crucial for ion selectivity (Konno et al. 1991). The gating of the channel is governed by conserved leucine residues, slightly towards the extracellular side from the centre of the bilayer with side chains projecting inwards (Unwin N. 1995), hence occluding the passage for ions.

1.2.2 Model of the nAChR binding site

Determination of the crystal structure of the molluscan acetylcholine binding protein (Brejc et al. 2001, Figure 1.3b and c)) provided a platform to study the ligand binding domain of nAChRs. Acetylcholine binding protein (AChBP) is asoluble protein, secreted by snail glial cells into the cholinergic synapses to bind released ACh and modulate neurotransmission (Sixma and Smit 2003). It shares 24% sequence identity with mammalian $\alpha 7$ homopentameric receptor. It has similar structure to the extracellular domain of the nAChRs mammalian $\alpha 1$ (Dellisanti et al. 2007) and $\alpha 7$ (Li et al. 2011). It is a homopentamer with N-terminal helix and 10 β sheets. It also shares similar pharmacological properties to this receptor. AChBP binds to dassical nAChR agonist and antagonists: nicotine, acetylcholine and α-bungarotoxin (Smit et al. 2001). Therefore AChBP is considered a good model for the nAChR ligand-binding domain structural studies. The structures of AChBP inactive (Brejc et al. 2001), bound to agonist and antagonist (Celie et al. 2004; Hansen et al. 2005), chimera $\alpha 1$ (Dellisanti et al. 2007) and $\alpha 7$ are known (Li et al. 2011). The common structural features of the ligand binding site emerge from all available data. Here data from the great pond snail *Lymnaea stagnalis* are discussed.

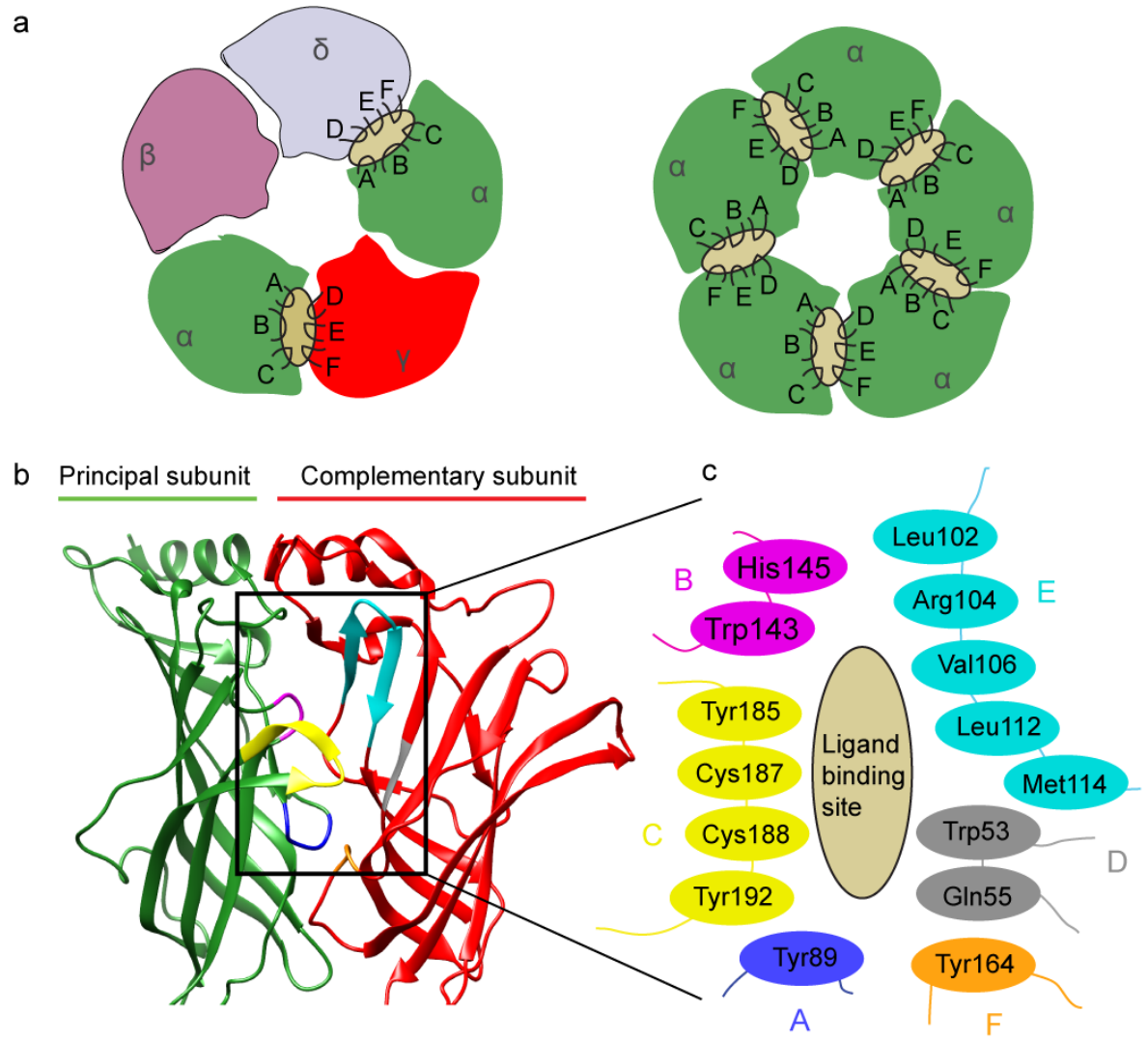


Figure 1.3: **The ligand binding domain of acetylcholine binding protein.** Agonist binds to the loops situated in the adjacent subunits of the nAChR. In muscle type receptor, there are 2 binding sites, and there are 5 in homopentameric receptor (a). The ligand binding pocket of the AChBP (PDB:1I9B) is formed from loops of the neighboring subunit (b). Principal and complementary subunits contributed amino acids from loops A, B, C and D, E, F, respectively (c). Crystal structure of the AChBP generated with the USCF Chimera software.

1.2.3 Agonist binding site of nAChRs

The nicotinic acetylcholine receptor binding pocket is formed on the interface of the adjacent subunits (Brejc et al. 2001; Middleton and Cohen 1991; Blount and Merlie 1989, Figure 1.3 a, b). In case of the neuromuscular heteropentameric receptor, it constitutes of α and non-α subunit contributions, whereas in homopentameric or α heteropentameric receptors it is made up of neighboring subunits. The principal, α-subunit site subsides amino acid side chains originating from discontinuous loops A (loop β4-β5), B (loop β7-β8) and C (loop β9-β10), whereas the complementary (non-α) subunit contributes amino acid side chains originating from loop D (loop β2-β3), E (loop β5-β6) and F (loop β8-β9). Specific residues involved in the formation of the ligand binding pocket were depicted by the molluscan AChBP (Figure 1.3 c). Amino acids of the principal component are: Tyr93, Trp147, Tyr188 and Tyr195, whereas non-α component contributes Trp53, Gln55, Arg104, Val106, Leu112 and Met114, Tyr164.

1.2.4 Pharmacophore of nAChR agonists

Crystal structure of the AChBP bound to acetylcholine, carbamylcholine, nicotine (Celie et al. 2004) and its analogue epibatidine (Hansen et al. 2005) provided some general features of the nAChR binding pocket (Figure 1.4). More recently, structures of mammalian receptors: α9 (Zouridakis et al. 2014) bound to methyllycaconitine, the artificially expressed α2 extracellular domain bound to epibatidine (Kouvatsos et al. 2016) and α4β2 receptor bound to nicotine (Morales-Perez, Noviello, and Hibbs 2016) have been obtained. These structures provide details of how structurally varied agonists bind to nAChRs.

The agonist binding site is buried on the interface of the neighboring subunits. They are stabilised in the binding pocket by 5 conserved aromatic residues from A, B and C loops of the principal site (known as the aromatic box), which engulf the cationic atom of the quaternary ammonium atom of bound agonist. There are two major and conserved features: cation-π interaction and hydrogen bond.

Cation -π interactions are formed between the cationic nitrogen and aromatic side chain of tryptophan in loop B (143 in AChBP) of the principal side of the binding pocket. Whereas hydrogen bond is formed between the bond acceptor and amino acids of the complementary side of the binding pocket via water molecule (Celie et al. 2004; Olsen et al. 2014). In ACh and nicotine bound to AChBP structures, water bridges to the oxygen of the carbonyl group of Leu112 and amide group of Met114 in loop E (Olsen et al. 2014; Celie et al. 2004).

Choline is an agonist lacking the hydrogen bond acceptor, which is likely contributing to its lower efficacy and affinity. Heterologously expressed α7 are activated with choline with the EC_{50} between 0.4 and 1.6 mM, whereas the EC_{50} of nicotine is between 49 and 113 μM (Wonnacott and Baric 2007). Radiolabelled studies report up to 500 times lower binding affinity of choline in comparison to nicotine (Wonnacott and Baric 2007).

Cation-π interactions and a hydrogen bond are the staple features of the ligand-receptor interactions, however there are also some less conserved characteristics. For example, in AChBP-nicotine structures, there is a

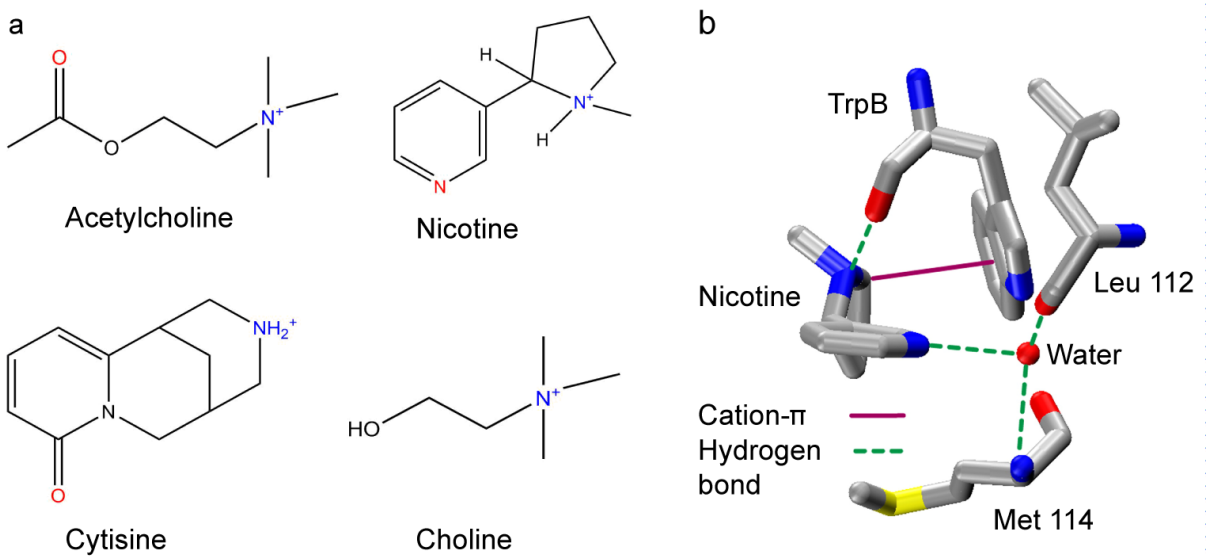


Figure 1.4: **Nicotinic acetylcholine receptor agonist pharmacophore.** Agonists of the nAChRs contain hydrogen bond acceptor (red) and cationic nitrogen (blue) (a). Interactions with the receptor based on the crystal structure of the AChBP and nicotine (PB:1UW6) (b). Image b) is taken from Blum, Lester, and Dougherty (2010).

hydrogen bond between cationic nitrogen of the agonist and the carbonyl of TrpB in the principal site of the receptor (Celie et al. 2004). Similarly, in human $\alpha 2$ structures a hydrogen bond between the cationic nitrogen of epibatidine and carbonyl of TrpB or Tyr in loop A is formed (Kouvatsos et al. 2016). In contrast, cationic nitrogen of ACh forms cation-π with Trp53 in loop D of AChBP and $\alpha 2\beta 2$ proteins (Morales-Perez, Noviello, and Hibbs 2016; Olsen et al. 2014).

1.2.5 Pharmacophore of neonicotinoids

Structure of AChBP proved to be also valuable in determining structural elements which may account for neonicotinoids' selectivity. Ihara et al. (2008); Talley et al. (2008); Ihara et al. (2014) derived crystal structures of the great pond snail (*L. stagnalis*, Ls) and California sea slug (*Aplysia californica, A. californica*) AChBP complexed with neonicotinoids (imidacloprid, clothianidin, thiacloprid), and non-selective nAChR ligands- nicotinoids (nicotine, epibatidine and desmotroimidacloprid). Comparison of these structures revealed differences in binding modes between nicotinoids and neonicotinoids (see Appendix A.1 for sequence alignment), which allowed for predictions of the binding interactions between neonicotinoids and insect receptors (Figure 1.5).

Structures of wild-type and mutant AChBP with increased affinity to neonicotinoids revealed no differences in the interactions between imidacloprid, clothianidin and thiacloprid (Figure 1.6) (Ihara et al. 2008; Talley et al. 2008; Matsuda et al. 2009; Ihara, Sattelle, and Matsuda 2015). Thus, to describe the differences between neonicotinoids and nicotinoids, crystal structures of *L. stagnalis* AChBP complexed with nicotine and imidacloprid are compared (Figure 1.5). The positioning of the pyridine ring of imidacloprid and nicotine is

virtually the same. The nitrogen forms identical interactions: hydrogen bond with the amide group of Met114 and carbonyl group of Leu102 of loop E, via water molecule (Celie et al. 2004; Ihara et al. 2008; Talley et al. 2008). In addition, chlorine atom of imidacloprid makes van der Waals interactions with oxygen of Ile106 and oxygen of Met116 of AChBP (Talley et al. 2008).

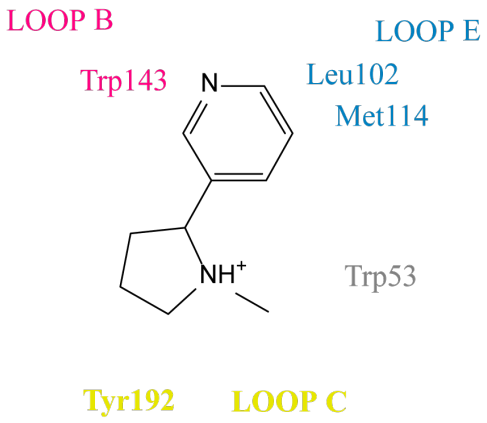
Regarding 5-membered ring interactions, in nicotine-bound structures, the cationic nitrogen forms 3 interactions when bound to AChBP: the cation-π with the ring of Trp143 (TrpB), the hydrogen bond with the backbone carbonyl of TrpB (Celie et al. 2004), as well as the cation-π interaction with Tyr192 in loop A (Matsuda et al. 2009). In imidacloprid bound structures, the ring stacks with aromatic residue Tyr185 of loop C (Ihara et al. 2008). These stacking interactions result in the formation of CH-π interactions between the methyline bridge (CH_2-CH_2) of imidacloprid and TrpB. All residues described so far are conserved in other agonist-bound nAChR structures, therefore do not account for neonicotinoids-selectivity.

The differences come to light when one begins to dissect the interactions between imidacloprid ring substituents and the AChBP. Partially positive nitro group (NO_2) of imidacloprid bridges to glutamine of loop D (Gln55) via a hydrogen bond. This interaction was also seen in thiacloprid bound AChBP and in the Gln55Arg mutant of AChBP bound to clothianidin (Ihara et al. 2014). It is interesting that in some nAChR subunits, such as *M. pyrsicae* $\beta 1$, honeybee $\beta 1 - 2$ and $\alpha 7$, glutamine corresponds to basic residue (lysine/arginine). Basic residues electrostatically attract nitro group, possibly forming a hydrogen bond, which in turn would strengthen the stacking and aromatic CH/π hydrogen bond interactions between the ring and the protein. In contrast, other subunits such as human $\alpha 7$ or *C. elegans* ACR-16 and EAT-2 contain either acidic or polar amino acids in the exact position, repulsing or forming no electrostatic interactions with imidacloprid, which could at least in part explain low sensitivity of nematodes and mammals to neonicotinoids (Section 1.1.7.2.2).

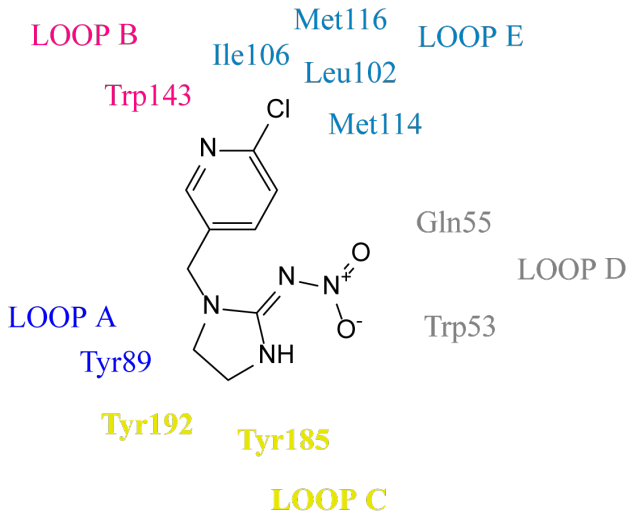
Analysis of the structure of Gln55Arg AChBP mutant complexed with neonicotinoids revealed another residues with a potential to confer high binding affinity of these compounds. Basic residue of loop G, namely Lys34, forms electrostatic interaction with the NO_2 group of clothianidin and CN group of thiacloprid, but does not interact with imidacloprid (Figure 1.6) (Ihara et al. 2014).

1.2.5.1 Selectivity of neonicotinoids

Based on the structural data, it has been proposed that the basic residue in loop D and G interacting with the nitro or cyano group of neonicotinoids is important in confirming neonicotinoid selectivity in insect nAChR subunits. This is supported by genetic studies. Loop D arginine to threonine mutation naturally occurring in $\beta 1$ subunit of peach aphid *M. persicae*, and cotton aphid *A. gossypii* (Hirata et al. 2015, 2017; Bass et al. 2011) gives rise to neonicotinoid resistance. Additionally, Shimomura et al. (2002) showed that mutation of glutamine in loop D of human $\alpha 7$ to basic residue, markedly increases sensitivity of the $\alpha 7$ homopentamer to nitro-containing neonicotinoids, whereas mutation of loop D threonine to acidic residues in chicken $\alpha 4\beta 2$



NICOTINE



IMIDACLOPRID

Figure 1.5: **Pharmacophore of nicotine and imidacloprid**. Schematic representation of the agonist binding site of acetylcholine binding protein, highlighting residues interacting with nicotine and imidacloprid.

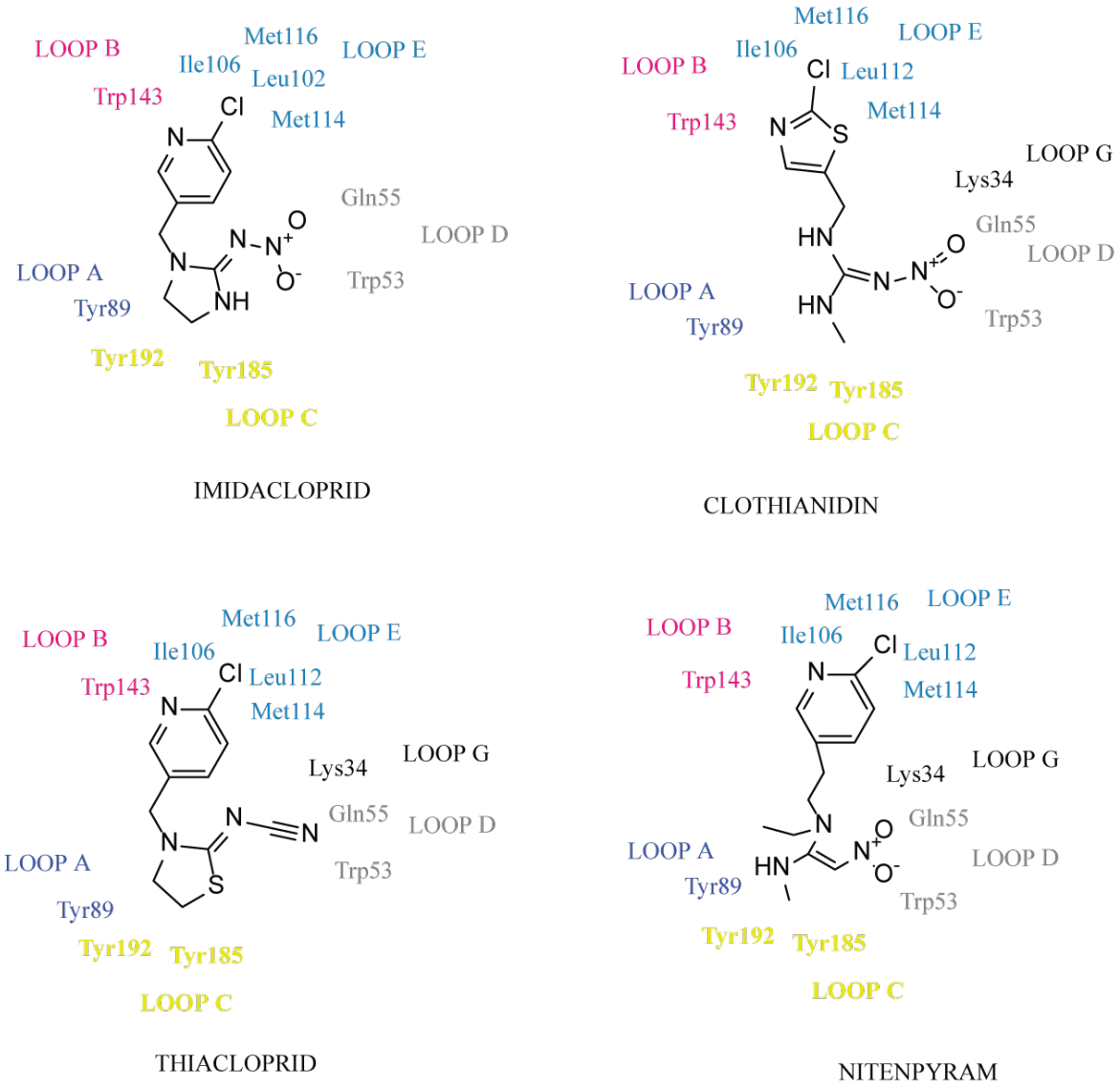


Figure 1.6: **Pharmacophore of neonicotinoids**. Schematic representation of the agonist binding site of acetylcholine binding protein, highlighting residues interacting with imidacloprid, thiacloprid, thiacloprid and nitenpyram. For nitenpyram, the interactions are predicted based on other structures.

and hybrid chicken/*Drosophila* $\alpha 2\beta 2$ receptor had an opposite effect (Shimomura et al. 2006). Interestingly, described mutations did not influence the efficacy to nicotinoids, suggesting this interaction is specific to neonicotinoids. In addition, double mutant of avian $\alpha 7$ nAChR in which amino acids equivalent to Gln55 and Lys34 were mutated to basic residues showed increased binding affinity of thiacloprid and clothianidin, but not nicotine or acetylcholine (Ihara et al. 2014), providing further evidence that these residues are important in conferring high binding affinity of neonicotinoids.

Genetic studies identified other amino acids of potential importance. Imidacloprid-resistant strain of planthopper *N. lugens* has been found to have Y151S mutation in loop B of $\alpha 1$ and $\alpha 3$ nAChR subunits (Liu et al. 2005). This residue corresponds to LsAChBP H145 of the loop B. Loop B, D and G originate from the complementary site, but the principal site may also play a role. Studies on *Drosophila*/chicken $\alpha 2\beta 2$ hybrid and chicken $\alpha 2\beta 4$ receptors showed that the presence of nonpolar proline in YXCC motif of loop C enhances affinity, whereas mutation of proline to glutamate markedly reduces affinity of neonicotinoids to these receptors (Shimomura et al. 2005). The importance of C-loop regions was also demonstrated by Meng et al. (2015) who showed that chimera receptors are deferentially sensitive to imidacloprid at least partly due to the difference in loop C region, equivalent to Ls184-191.

Taken together, AChBP is a useful model in which potential molecular determinants of neonicotinoid-selectivity were investigated. Of particular interest is basic residue in the principal site of the biding pocket, however many other amino acids, including those in the complementary binding site and those away from the binding site may also play a role. This highlight the complex nature of the receptor-agonist binding and creates a challenging environment for the *in-silico* structure-based design of novel neonicotinoids.

1.3 Neonicotinoids act on the cholinergic neurotransmission as a nicotine mimic

1.3.1 Cholinergic system in insects

Cholinergic neurotransmission is the process of signal propagation between neurons at the synapse. Cholinergic synapse is characterised by the presence of several proteins which mediate the breakdown, the synthesis, the processing and the function of the neurotransmitter ACh (Figure 1.7).

Choline acetyltransferase (ChAT) is an enzyme synthesising ACh (Greenspan 1980), by a transfer of acetyl-CoA onto choline. There are at least two isoforms in *Drosophila*, which are produced by alternative splicing from the ChAT gene (Slemmon et al. 1982). One is membrane bound, whereas the other is soluble (Pahud et al. 1998). A soluble isoform of ChAT was also isolated from the locust *Schistocerca gregaria* (*S. gregaria*) (Lutz, Lloyd, and Tyrer 1988). In *Drosophila*, the soluble isoform performs the majority of enzymatic activity (Pahud et al. 1998).

Vesicular acetylcholine transferase (VAChT) mediates ATP-dependent transport (Varoqui and Erickson 1996), which packs ACh into the synaptic vesicles for release (Song et al. 1997). In *Drosophila*, a single VAChT gene was identified (Kitamoto, Wang, and Salvaterra 1998).

Acetylcholinesterase (ACE) is a soluble enzyme that catalyses breakdown of ACh (Chao 1980; Hsiao et al. 2004). In the fruit fly *Drosophila melanogaster* (*Drosophila*), it is encoded by the Ace locus (Hall and Kankel 1976). Acetylcholinesterase is a homodimer covalently bonded by the disulphide bridge (Chao 1980; Hsiao et al. 2004). Each monomeric subunits is ~67 kDa, folded into 4-helix bundle (Harel et al. 2000).

Insect nAChRs are expressed at the post-synaptic membrane (Kreissl and Bicker 1989; Gu and O'Dowd 2006) and mediate fast synaptic transmission (Callec and Sattelle 1973). Upon binding of neurotransmitter acetylcholine, nAChRs open, allowing the flux of cations down their concentration gradient (Goldberg et al. 1999), leading to depolarisation of the post-synaptic neuron and signal propagation.

1.3.1.1 Localisation of the cholinergic neurons in insects

Enzymes, transporters and receptors present at the cholinergic synapse have been used as markers for detection of cholinergic neurons in insects. (1) Immunocytochemistry with monoclonal antibodies specific to ChAt and ACE, (2) *in-situ* hybridization using sequences complementary to the ChAT mRNA (3) colorimetric technique for detection of AChE activity (Karnovsky and Roots 1964) (4) and reporter gene fused to the ChaT gene regulatory elements, outlined the presence of cholinergic pathways in *Drosophila* (Buchner et al. 1986; Gorczyca and Hall 1987; Barber et al. 1989; Yasuyama 1999), honeybee (Kreissl and Bicker 1989) and locust *Locusta migratoria* (Lutz and Tyrer 1987; Geffard et al. 1985). Based on these data, cholinergic neurons are in almost all regions of the brain and in the peripheral nervous system, namely the visual system and the antenna. They are also present in the thoracic, abdominal and the terminal abdominal ganglia involved in the regulation of movement of wings, abdomen and legs, as well as the regulation of the anal and reproductive muscles in insects (Smarandache-Wellmann 2016).

Cholinergic neurons have also been mapped using radiolabelled ligand, specific for nAChRs. α-bungarotoxin (α-bgtx), is a 74-amino acid long, 8 kDa proteins isolated from the venom of a snake *Bungarus multicinctus*. It binds with high affinity to nAChR (Lee, Tseng, and Chiu 1967) and blocks synaptic responses evoked by acetylcholine and other nAChR agonists (Chang and Lee 1963). Incubation of the honeybee brain with α-bgtx led to staining in the optic lobes, antenna lobes, ocellar system and mushroom bodies (Scheidler et al. 1990). This correlated with the staining in the central nervous system of *Drosophila* (Schmidt-Nielsen et al. 1977), moth *Manduca sexta* (Hildebrand, Hall, and Osmond 1979) and *cockroach* (Orr, Orr, and Hollingworth 1990). Incubation of α-bgtx with the ganglia of the American *cockroach* (Sattelle et al. 1983) and cricket *Acheta domesticus* (Meyer and Reddy 1985) identified further regions where α-bgtx binds with high affinity: the abdominal ganglion in the region rich in interneurons which make synaptic connections with the sensory

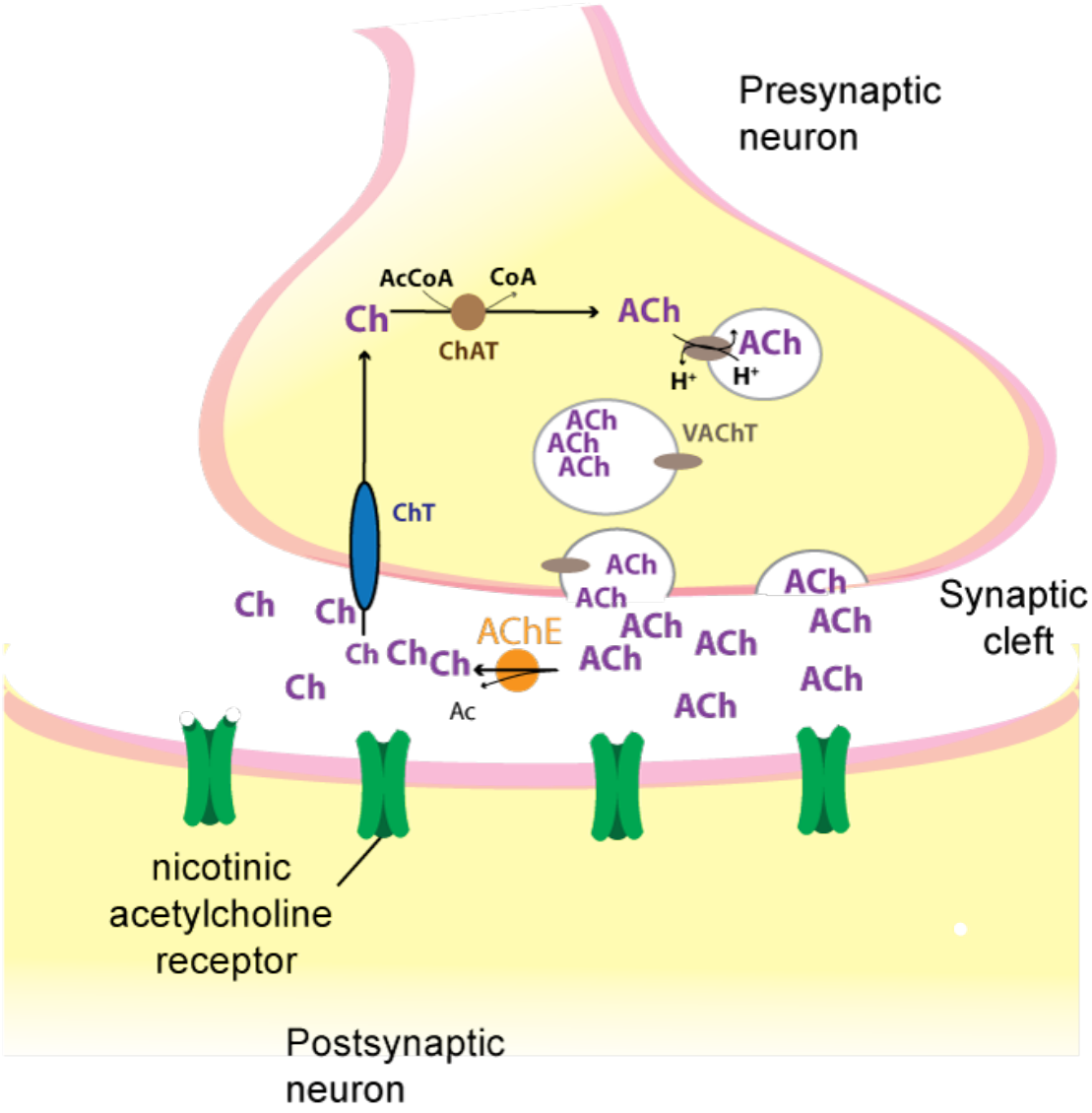


Figure 1.7: **Enzymes transporters and receptors at the cholinergic synapse.** Upon release into the synaptic cleft, acetylcholine binds to nicotinic acetylcholine receptors (nAChRs) for signal propagation. To prevent overstimulation of the post-synaptic neuron, acetylcholine is broken down to choline and acetate by acetylcholinesterase (AChE). Choline is then taken up to the pre-synapse by a choline transporter (ChT). The acetyl group in transferred onto choline to produce acetylcholine. This reaction is catalysed by choline acetyltransferase (ChAT). Generated acetylcholine is pumped into the synaptic vesicle by the vesicular acetylcholine transporter (VAChT).

afferent neurons (Daley and Camhi 1988), the abdominal ganglia and the thoracic ganglia (Sattelle 1981). Presence of nAChRs at the insect ganglia was confirmed using electrophysiological approaches (Sattelle 1981; Bai et al. 1992).

Based on the distribution of cholinergic-synapse markers in the insect nervous system and the quantitative analysis of acetylcholine in the insect brain (Florey 1963), it was concluded that acetylcholine is a major neurotransmitter in the nervous system of insects. In contrast to vertebrates (Brown, Dale, and Feldberg 1936; Bacq and Brown 1937; Chang and Lee 1963) and *C. elegans* (Richmond and Jorgensen 1999), acetylcholine in insects does not mediate muscle contraction at the NMJ, instead it is mainly involved in the sensory pathways and central information processing. The action of ACh in insects is exerted by nAChRs.

1.3.2 Role of nAChRs in insects

Biological role of nAChRs in insects was investigated in behavioural assays in response to nAChR agonists. Lethal doses of neonicotinoid imidacloprid induced complex symptoms in American *cockroach* and in honeybee (Sone et al. 1994; Elbart, Nauen, and Leicht 1997; Suchail, Guez, and Belzunces 2001). The following order of events was noted: hyperexcitation as evident by excessive pacing, collapse and diminishing uncoordinated leg and abdomen movement followed by paralysis and eventual death. Lethal dose of insecticide nicotine (David and Gardiner 1953), a naturally occurring alkaloid found in the *Solanaceae* family of plants (Steppuhn et al. 2004), induced similar effects on bees (McIndoo 1943). Distinct behavioural alterations can be induced by sub-lethal doses. Imidacloprid at < 4 nM inhibits feeding of *Myzus persicae*, which leads to their starvation (Nauen 1995; Elbart, Nauen, and Leicht 1997). In honeybees and bumblebees neonicotinoids impair on learning and memory, as well as reproduction (Section 1.1.7.1), highlighting an important role of nAChRs in insects biology.

1.3.3 Electrophysiological properties of insect nAChRs

The kinetic properties of insect nAChRs were investigated using neuronal preparations, where high density of nAChRs was found (Section 1.3.1.1). Acetylcholine and nicotine increased the rate of neuronal firing (Callec and Sattelle 1973; Sattelle et al. 1976; Meyer and Reddy 1985; Kerkut, Pitman, and Walker 1969; Sattelle 1981; Bai et al. 1992) by depolarising post-synaptic neurons (Callec and Sattelle 1973; Sattelle et al. 1976; Goldberg et al. 1999; Barbara et al. 2005; Brown et al. 2006; Palmer et al. 2013). These effects were inhibited by nAChR antagonist α-bgtx, suggesting effects of nicotine and acetylcholine were induced directly acting on nAChRs and that nAChRs are excitatory. Indeed, analysis of the agonist-evoked nAChR currents in the cultured honey bee neurons showed flux of mainly sodium and potassium but also calcium (Goldberg et al. 1999).

1.3.3.1 Single channel kinetics

Single channel recordings showed that insect nAChRs exhibit complex kinetics, resembling those found in vertebrates (Colquhoun and Sakmann 1985; Nagata et al. 1996, 1998). Using cholinergic neurons of the larva *Drosophila* CNS (Albert and Lingle 1993; Brown et al. 2006), and cultured neurons of *Musca domestica* (*M. domestica*) (Albert and Lingle 1993) it was shown that in response to nAChR agonists acetylcholine, nicotine, imidacloprid and clothianidin, the channel switches between an active and inactive form, with the active form interrupted by the short-lived channel closing bursts. Temporal characterisation of these events revealed that the frequency of channel opening and the duration of opening differs depending on the agonist applied and the neuronal preparation. However, typically the receptor remains opened for $\sim$ 1.5 ms; this is interrupted by channel closing bursts of $\sim$ 20 μs which occur at a frequency of 1-2 closures/opening burst (Albert and Lingle 1993).

Channel opening is not an all or nothing event. Instead, a channel typically exhibits two conductance states, one on which it is fully opened, named a full conductance state (i.e. the active form), and one in which the channel is partially opened, named the sub-conductance state. Although the conductance rates from various insect preparations are similar, the ratio between the two as well as their fine structure varies depending on the concentration, the agonist used and and the neuronal preparation (Albert and Lingle 1993; Brown et al. 2006).

1.3.3.2 Desensitisation of insect nAChRs

Exposure of insect neuronal preparations to high concentrations of agonists has a secondary effect. Following rapid depolarisation, the current slowly decreased until it it abolished completely due to nAChR desensitisation (Goldberg et al. 1999). Desensitisation is a period after agonist removal, whereby subsequent depolarisation cannot be elicited by agonist (Goldberg et al. 1999). The time taken for desensitisation varies between hundreds of mseconds (Goldberg et al. 1999) to tens of seconds (Salgado and Saar 2004) in insects. In vertebrates, there are receptors which desensitise in μseconds (Bouzat et al. 2008). Although the process of receptor desensitisation is typically reversible (Goldberg et al. 1999; Salgado and Saar 2004), full recovery may not occur or may be slower if the receptors are exposed to large doses of agonist for a prolonged time (Katz and Thesleff 1957).

1.3.4 Structural basis of major conformation states of nAChRs

Nicotinic acetylcholine receptors have three basic conformation states: the closed, the open and the desensitised state (Katz and Thesleff 1957; Monod, Wyman, and Changeux 1965). Structural features of the closed state channel are described in Section 1.2.1 and 1.2.2. Briefly, nAChR is a pentameric assembly of receptor subunits.

Each subunits contains 4 transmembrane helices (M1-M4) (Noda et al. 1982, 1983), an N-terminal helix and 10 βsheets (Brejc et al. 2001; Dellisanti et al. 2007; Li et al. 2011) and a large C-terminal domain (Unwin N. 1995; Dellisanti et al. 2007; Li et al. 2011). The N-terminal domain contains an agonist binding site formed by the loop contributions from the adjacent subunits (Brejc et al. 2001). One of the key features of the closed-channel is the presence of leucine residues originating from the pore-lining M2 helix, which project inwards (Unwin N. 1995). These residues form a gate which occludes the passage of ions of closed nAChR. High resolution structures of AChBP (Bourne et al. 2005; Hansen et al. 2005) and human $\alpha 7$-AChBP chimera (Li et al. 2011) highlighted the structural differences between the open (agonist-bound) and the closed states. In the agonist-bound structures, the aromatic residues in C loop form a cap above the agonist, suggesting that ligand binding leads to movement of the C-loop which folds over the agonist binding site, burying the ligand inside the protein and reducing the dissociation on/off rates. In addition, loop A moves towards the loop B, whereas loop F moves towards the agonist. These local changes propagate the rearrangement of the outer β sheet which rotates towards the centre of the pentamer and lead to structural changes at the level of the channel, leading to its opening.

Crystal structures of bacterial pentameric ligand gated ion channels shed light on the possible mechanism of channel opening. Although these channels are not members of the Cys-loop family due to the absence of N-terminal disulphide bond and a large cytoplasmic loop between M3-M4 transmembrane (TM) helices, they share common topology with nAChRs. Comparison of closed *Erwinia chrysanthemi* ligand gated ion channels (ELIC) (Hilf and Dutzler 2008) to opened *Gloeobacter violaceus* ligand gated ion channels (GLIC) (Hilf and Dutzler 2009), showed that in the open state pore-lining helices are tilted inwards, which leads to opening of the gate. An alternative hypothesis of channel opening was derived based on the cryo EM of the mammalian muscle nAChR in closed and open state (Unwin N. 1995). These structures suggest that binding of the agonist leads to rotation of 5 M2 helices. As they move, the distance between them increases, and so the ion conductivity pathway becomes wider, the gate opens, thus ions flow. More recently a higher resolution structure of muscle type nAChR has been derived (Unwin and Fujiyoshi 2012), suggesting that in the open state, TM helices not only rotate, but also bend towards the centre of the pore. Twisting and tilting of inner helices were also observed in the crystal structures of other representative of Cys-loop receptors, namely glycine receptors (Du et al. 2015) and glutamine-gated chloride (GluCl) channel (Althoff et al. 2014))

In 2016, the crystal structure of the human $\alpha 4/\beta 2$ receptor in desensitized state (Morales-Perez, Noviello, and Hibbs 2016) was derived. This was compared to the structures of open glycine (Du et al. 2015), closed GluCl (Althoff et al. 2014) and desensitized GABA (Miller and Aricescu 2014). Differences at the interface of the extracellular domain (ECD) and TM regions were noted, which arise as a result of the rotation motion at the level of the receptor. The structural rearrangements lead to the occlusion of the ion channel, reducing conduction (Monod, Wyman, and Changeux 1965) and tightening of the ligand binding site leading to an increase in ligand affinity to the desensitised receptor (Monod, Wyman, and Changeux 1965).

1.3.5 Neonicotinoids act as nAChR agonists

1.3.5.1 Mutations in nAChRs give rise to neonicotinoid-resistance

Several lines of evidence suggest that nAChR are the principal site of action of neonicotinoids. Genetic analysis of the neonicotinoid-resistant strains of insects showed that resistance arises as a consequence of mutations in nAChR subunits. Field isolates of peach aphid *M. persicae* (Bass et al. 2011), the cotton aphid *A. gossypii* (Hirata et al. 2015, 2017) and the Colorado potato beetle *L. decemlineata* (Szendrei et al. 2012), as well as lab-isolates of brown planthopper, *N. lugens* (Liu et al. 2005), fruit fly *D. melanogaster* (Perry et al. 2008) with decreased sensitivity to neonicotinoids have been identified. Behavioural analysis shows that their sensitivity is up to 1500-fold lower in comparison to the reference strains, as shown by the shift in the LD_{50}. Analysis of the DNA of the resistant strains identified mutations in nAChR subunit coding sequence (Bass et al. 2011; Perry et al. 2008; Hirata et al. 2015). However, mutations in other genes can also give rise to resistance (Zewen et al. 2003; Benting and Nauen 2004; Bass et al. 2015).

1.3.5.2 Neonicotinoids evoke nAChR-like current in insect neuronal preparations

Neonicotinoids induce nAChR-like current in insect neuronal preparations, which reassembles that induced by nAChR agonist nicotine (Section 1.3.3). Sone et al. (1994) investigated the effects of imidacloprid on the neuronal activity at the thoracic ganglia of male adult American cockroaches, *P. americana* using extracurricular recordings. This method allows for a record of changes in spontaneous neuronal activity in response to mechanical or pharmacological interventions. At a very low concentration of 1 nM, imidacloprid induced a sustained increase in the rate of neuronal firing for over 2 minutes. At concentrations ranging from 10 nM to 100 μM, the following sequence of events was noted: an increase of the rate of spontaneous action potentials of neurons followed by a gradual decline, leading to a complete block of neuronal activity (Sone et al. 1994). Imidacloprid had the same effect on various insect preparations including thoracic ganglion of the *L. decemlineata* (Tan, Salgado, and Hollingworth 2008) and on the abdominal ganglion of *P. americana* (Buckingham et al. 1997). The same observations were made for other neonicotinoids (Thany 2009; Schroeder and Flattum 1984).

Sattelle et al. (1989) used isolated *cockroach* neuronal preparation to record post-synaptic intracellular currents in response to neonicotinoid prototype 2(nitromethylene) tetrahydro-1, 3-thiazine (NMTHT). NMTHT depolarised the post-synaptic unpaired median neurons and the cell body of motor neurons of the abdominal ganglion. Agriculturally relevant neonicotinoids had the same effect on the post-synaptic membrane of the isolated *cockroach* thoracic ganglia (Tan, Galligan, and Hollingworth 2007; Thany 2009) potato beetle isolated neurons (Tan, Salgado, and Hollingworth 2008), and on cultured *cockroach* (Ihara et al. 2006), honeybee (Palmer et al. 2013) and fruit fly (Brown et al. 2006) neurons.

Pharmacological characterisation of neonicotinoids-induced currents provided further evidence for their mode of action. The inward current elicited by neonicotinoids were dose-dependent, whereby the higher the concentration, the grater the depolarisation. EC_{50} values (concentrations at which the half of the maximum current was observed) were in the region of 1 - 5 μM (Thany 2009; Tan, Galligan, and Hollingworth 2007). Such low values indicate highly potent action of neonicotinoids on insects, in agreement with toxicological data (Section 1.1.5). Neonicotinoid-induced currents were reminiscent of those induced by acetylcholine and nicotine, and were prevented by the application of nAChRs antagonists (α-bungarotoxin, methyllycaconitine, mecamylamine or d-tubocurarine) not by muscarinic receptor antagonists (atropine, pirenzepine), suggesting neonicotinoid-induced currents are due to the activation of nicotinic receptors.

1.3.6 Mode of action of neonicotinoids

Although neonicotinoids typically acts as agonists, they can have diverse mode of action. The currents produced by neonicotinoids and ACh on cultured or isolated insect neuronal preparation were compared. Neonicotinoids evoking current lower than that evoked by ACh were classed as partial agonists, those eliciting similar response were classed as true agonists, whereas those more efficacious than ACh, super-agonists. Thiacloprid and imidacloprid were shown to be partial agonists, nitenpyram, clothianidin, acetamiprid and dinotefuran were true agonists, whereas thiamethoxam had no effect on the isolated American cockroach thoracic ganglion neurons (Tan, Galligan, and Hollingworth 2007). This differs from the mode of action of neonicotinoids on cultured terminal abdominal ganglion neurons of this insect. Currents produced by all neonicotinoids tested was lower than that evoked by ACh (Ihara et al. 2006), suggesting they are all partial agonists on these cells. The mode of action of neonicotinoids on the fruit fly (Brown et al. 2006) and honey bee neurons (Palmer et al. 2013) differs still, implying the presence of distinct nAChRs in different insect species and neuronal preparations.

1.3.6.1 Neonicotinoids bind with high affinity to insect nAChRs

Neonicotinoids bind to insect nAChRs with high affinity, as shown in the saturation ligand binding studies. In the saturation binding experiment, various concentration of the labelled ligand is added to the preparation and the concentration of the ligand at the equilibrium is determined. This is then used to derive dissociation constant, Kd, to define the binding strength of neonicotinoids to insect nAChRs. In the whole membrane preparations of the housefly and aphid, the Kd of imidacloprid and thiamethoxam were in the low nM range (Table 4; Liu and Casida 1993; Wellmann et al. 2004; Liu et al. 2005) suggesting high affinity interaction. Interestingly, two binding affinities have been derived from the imidacloprid study in the brown planthopper and pea aphid (Wellmann et al. 2004; Taillebois et al. 2014) suggesting the presence of at least two imidacloprid binding sites in these animals.

Table 1.4: Binding affinity of neonicotinoids

Compound	Species	Common name	Kd (nM)	References
Imidacloprid	*Musca domestica*	housefly	1.2	Liu et al. 1993
Imidacloprid	*Aphis craccivora*	cowpea aphid	12.3	Wellmann et al. 2004
Imidacloprid	*Myzus persicae*	green peach aphid	4.1	
Imidacloprid	*Nilaparvata lugens*	brown plantohopper	<0.01 1.5	Liu et al. 2005
Imidacloprid	*Acyrthosiphon pisum*	pea aphid	0.008 0.002	Taillebois et al. 2014
Thiamethoxam	*Myzus persicae*	green peach aphid	15.4	Wellmann et al. 2004

Binding affinity measured in the whole membrane preparation, with the exception of housefly experiment where head membranes were used.

In addition to the saturation studies, the competitive ligand binding studies were carried out. In the competitive ligand binding studies, biological preparation is incubated with radiolabelled ligand. The ability of various concentrations of unlabeled ligand is measured to define its equilibrium inhibition constant (Ki). This method informs both on the affinity and on the interactions between ligands. Various concentrations of neonicotinoid prototype isothiaocynate were incubated with the homogenate of *Drosophila* and a homogenate of the abdominal nerve cords of *P. americana* before the exposure to radiolabelled nAChR antagonist α-bgtx (Gepner, Hall, and Sattelle 1978). Isothiaocynate inhibited binding of α-bgtx in the concentration dependent manner (Gepner, Hall, and Sattelle 1978), suggesting the two compounds share the binding site. Similarly, imidacloprid has been shown to displace α-bgtx from brain membrane preparations from honey bee *A. mellifera* (Tomizawa, M. and I. Yamamoto 1992; Tomizawa, M. and I. Yamamoto 1993), *Drosophila* (Zhang, Tomizawa, and Casida 2004), house fly *M. domestica* and isolated *cockroach* nerve cords (Bai et al. 1991). The binding affinity of neonicotinoid-related compounds was compared to the insecticidal activity; the correlative relationship between the two was found (Kagabu et al. 2002; Liu et al. 2005), providing further evidence that neonicotinoids act by targeting nAChRs.

1.3.6.2 High affinity of neonicotinoids to heterologously expressed insect-chimera receptors

Due to the difficulties in the heterologous expression of native insect receptors (Section 1.3.7), the binding affinity of neonicotinoids to isolated, native receptors is largely unknown. However, binding studies on hybrid receptors consisting of insect α-subunit and vertebrate β subunit, were carried out.

Mammalian $\alpha 4/\beta 2$ receptor expresses well in *Xenopus* oocytes (Cooper, Couturier, and Ballivet 1991) and cell lines (Lansdell and Millar 2000) and it has low affinity to imidacloprid (Kd >1000 μM) (Lansdell and Millar 2000). Chimera of rat $\beta 2$ and α subunits from the fruit fly (Lansdell and Millar 2000), aphid *M. Persicae* (Huang et al. 1999), planthopper *N. lugens* (Liu et al. 2009), cat flea *Ctenocephalides felis* (Bass et al. 2006) and sheep blowfly *Lucilia cuprina* (Dederer, Werr, and Ilg 2011) have been generated. It needs to be noted that the potency of neonicotinoids on these receptors is not reported, suggesting these receptors are not functional.

However, their pharmacological profiles have been determined using saturation ligand binding studies (Hulme and Trevethick 2010) (Table 1.5).

The affinity of neonicotinoids to insect-chimera receptors varies, depending on the identity of the α subunit. Imidacloprid did not bind to Mpα1/ratβ2 receptor, whereas its Kd at Mpα2 and Mpα3-containing receptor was 3 and 28 nM, respectively (Huang et al. 1999). Four to five fold-difference between the most and least susceptible fruit fly and cat flee receptor assemblies were also identified (Lansdell and Millar 2000; Bass et al. 2006).

Imidacloprid exhibits the highest affinity against target pest *M. Persicae* with the lowest reported Kd of 28 nM on $\alpha 3/\beta 2$ receptor (Huang et al. 1999). It binds less tightly to the non-target insect, the fruit fly nAChRs; the Kd values range from 8.4 to 34.9 nM (Lansdell and Millar 2000).

Table 1.5: Binding affinity of imidacloprid to recombinant insect-hybrid receptors

Receptor	Kd (nM)	Rerefence
Rnα4β2	>1000	Lansdell and Millar, 2000
Dmα1/Rnβ2	34.9	
Dmα2/Rnβ2	20	
Dmα3/Rnβ2	8.4	
Mpα1/Rnβ2	N/B	Huang et al., 1999
Mpα2/Rnβ2	3	
Mpα3/Rnβ2	2.8	
Mpα4/Rnβ2	N/B	
Nlα1/Rnβ2	24.3	Liu et al., 2005
Cfα1/Dmα2/Rnβ2	141	Bass et al. 2006
Cfα3/Dmα2/Rnβ2	28.7	

Receptors were expressed in insect S2 cell line
Rn = *Rattus norvegicus* (rat), Dm = *Drosophila melanogaster* (fruit fly), Mp = *Myzus persicae* (aphid), Nl = *Nilaparvata lugens* (planthopper), Cf = *Ctenocephalides felis* (cat flea)), N/B = no binding,

1.3.6.3 High potency of neonicotinoids on heterologously expressed insect-mammalian hybrid receptors

The potency of neonicotinoids on insect-mammal hybrid nAChRs have been also determined using cyanoamidines clothianidin and imidacloprid, nitroguanidines thiacloprid and acetamiprid and nitromethylene nitenpyram (Table 1.6).

Dose-dependent depolarising current was recorded from cells expressing insect-hybrid nAChRs in responses to all tested neonicotinoids. The potency of neonicotinoids varied, as indicated by the EC_{50} values between 0.04 and 45.8 μM, however it is generally in the region of 1 μM.

The rank order of potency of cyanoamidines, nitroguanidine and nitromethylene differs, depending on the

receptor identity. For example, in imidacloprid and clothianidin are the most potent on the fruit fly $\alpha 1$ containing receptors (Dederer, Werr, and Ilg 2011), whereas planthopper $\alpha 3\alpha 8$ hybrid, thiacloprid is the most potent (Yixi et al. 2009). Nitenpyram has consistently the highest EC_{50}.

Table 1.6: The potency of neonicotinoids on recombinantly expressed insect hybrid nAChRs.

Receptor	Compound	EC50 (μM)	Rerefence
Nl$\alpha 1$/Rn$\beta 2$	Imidacloprid	61	Liu et al. 2009
Nl$\alpha 2$/Rn$\beta 2$	Imidacloprid	870	
Nl$\alpha 3$/Rn$\beta 2$	Imidaclorprid	350	
Nl$\alpha 3\alpha 8$/Rn$\beta 2$	Imidacloprid	3.2	Yixi et al. 2009
	Clothianidin	5.1	
	Thiacloprid	2.8	
	Nitenpyram	5.6	
Dm$\alpha 1$/Gg$\beta 2$	Imidacloprid	0.04	Dederer et al. 2011
	Clothianidin	0.34	
	Acetamiprid	0.23	
	Nitenpyram	0.4	
Dm$\alpha 2$/Gg$\beta 2$	Imidacloprid	0.84	
	Clothianidin	5.4	
	Acetamiprid	2	
	Nitenpyram	35.4	
Cf$\alpha 1$/Gg$\beta 2$	Imidacloprid	0.02	
	Clothianidin	0.15	
	Acetamiprid	0.11	
	Nitenpyram	0.63	
Cf$\alpha 2$/Gg$\beta 2$	Imidacloprid	1.31	
	Clothianidin	1.65	
	Acetamiprid	2.63	
	Nitenpyram	24.4	
Cf$\alpha 4$/Gg$\beta 2$	Imidacloprid	13.8	
	Clothianidin	21.3	
	Acetamiprid	9.4	
	Nitenpyram	45.8	

Receptors were expressed in *Xenopus* oocytes
Rn = *Rattus norvegicus* (rat), Gg = *Gallus gallus* (chicken), Dm = **Drosophila melanogaster* (fruit fly), Nl = *Nilaparvata lugens* (planthopper), Cf = *Ctenocephalides felis* (cat flea)), Lc = *Lucilia cuprina* (sheep blowfly)

1.3.7 nAChR subunits in insects

The electrophysiological and ligand binding studies on neuronal preparations and hybrid receptors provides evidence that nAChR are molecular targets of neonicotinoids. nAChR are assemblies of 5 different or identical receptor subunits (Section 1.2.1). Each subunit is encoded by a separate gene and is classified as either α or non-α, depending on the primary amino acid sequence, whereby α subunits contain a disulphide bond formed between the adjacent cysteines in the ligand binding domain (Figure 1.2). Genome sequencing projects enabled identification of nAChR subunit families in several insect species. Fruit fly and model organism *D. melanogaster* has 10 subunits, 7 of which are α ($\alpha 1-7$) and 3 are β ($\beta 1-3$) (Adams et al. 2000; Sattelle et al. 2005). There are 11 subunits in the beneficial insect honeybee *A. mellifera* ($\alpha 1-9$, $\beta 1-2$) (Jones et al. 2006; Honeybee Genome Sequencing Consortium 2006), 12 subunits in the pest red flour beetle *Tribolium castaneum* ($\alpha 1-11$,, $\beta 1$) (*Tribolium* Genome Sequencing Consortium 2008) and 8 in the Pea Aphid, *Acyrthosiphon pisum* ($\alpha 1-6$, $\beta 1-2$) (Yi-peng et al. 2013; International Aphid Genomics Consortium and Others 2010). With the aid of molecular cloning techniques, equivalent subunits have been identified in many other insects, including cat flea *C. felis* (Bass et al. 2006) and green peach aphid *M. persicae* (Huang et al. 2000). Amino acid sequence alignment of equivalent subunits revealed that they are highly conserved, with sequence identity typically greater than 60% (Jones and Sattelle 2010).

Table 1.7: Nicotinic acetylcholine receptors in insects, nematodes, mammals and fish

Species	Localisation of nAChRs	Function of nAChRs	Major receptor types	Ref
Mouse	Nervous system	NT release modulation	$\alpha 4\beta 2$ and $\alpha 7$	1-3
	NMJ	Muscle contraction	$\alpha 1\beta 1\epsilon\delta$	4-6
D. melanogaster	Nervous system	Major NT	?	in-text
D. rerio	Nervous system	NT release modulator	$\alpha 4\beta 2$ and $\alpha 7$	7
	NMJ	Muscle contraction	$\alpha 1\beta 1\epsilon\delta$	8
C. elegans	Nervous system	Major NT	DES-2/DEG-3	9, 10
	NMJ	Muscle contraction	L-, N-type and EAT-2	11-14
A. mellifera	Nervous system	Major NT	?	in-text

NT = neurotransmitter, NMJ = neuromuscular junction
References: 1 = Chen and Patrick 1997, 2 = Araujo et al. 1988, 3 = Couturier et al. 1990; Cooper et al. 1991, 4 = Lee et al. 1967; 5 = Brown et al. 1936, 6 = Mishina et al. 1986, 7 = Zirger et al. 2003, 8 = Mongeon et al. 2011, 9 = Lewis et al. 1987, 10 = Treinin et al. 1998, 11 = Richmond and Jorgensen 1999; 12 = Boulin et al. 2008, 13 = Touroutine et al. 2005, 14 = McKay et al. 2004

Insect nAChR gene families are among the least diverse when compared to other animal phyla. Mammals express 17 subunits: $\alpha 1-10$, $\beta 1-4$, δ, γ and ϵ (Millar and Gotti 2009) and there are 29 subunits in the representative of the phylum *Nematoda, C. elegans* (Jones et al. 2007).

1.3.8 Difficulties in heterologous expression of insect nAChRs

To identify which subunits assemble to form functional receptors, recombinant expression techniques were used. Recombinant expression is a technique by which receptor stoichiometry and function can be studied in

a heterologous system. cDNA is injected into the *Xenopus* oocytes, or used to transfect insect or mammalian cell lines. Using internal cellular machinery, it is transcribed, translated and processed to the surface of the cell. Should a protein form, cell-surface expression can be detected using biochemical approaches (such as ligand binding studies), whereas function can be studied by means of electrical recordings. These approaches were utilised to identify the major receptor assemblies in mammals, nematode and fish (Table 1.7).

To determine which insect subunits form functional nAChRs, Lansdell et al. (2012) transfected cultured insect cells with over 70 *Drosophila melanogaster* nAChR subunit cDNAs either singularly or in combinations. No cell surface was achieved, as shown by the radiolabelled ligand binding studies. Difficulties in expression of *Drosophila* subunits were also encountered in *Xenopus* oocytes (Lansdell et al. 2012) and mammalian cell lines (Lansdell et al. 1997). The attempts to express receptors from other species were also largely unsuccessful. No ligand binding and/or agonist evoked currents were detected from cells transfected with genes en coding for the nAChR subunits of brown planthopper (Liu et al. 2005, 2009; Yixi et al. 2009), cat flea (Bass et al. 2006), aphid *M. persicae* (Huang et al. 2000) and brown dog tick *Rhipicephalus sanguineus* (Lees et al. 2014). Homomeric *Locust* $\alpha 1$ (Marshall et al. 1990), *M. Persicae* $\alpha 1$ and *M. Persicae* $\alpha 2$ (Sgard et al. 1998) produced receptors with nAChR-like pharmacological and electrophysiological characteristics, however the channel-generated currents were of low amplitude, and the expression was inconsistent.

1.3.8.1 Importance of chaperon proteins in heterologous expression of nAChRs

Difficulties in recombinant receptor expression highlight the complexity of receptor formation. Assembly and oligomerisation are critical steps in the receptor maturation (Brodsky and McCracken 1999) which are poorly understood in insects. Co-expression of mammalian and *C. elegans* nAChRs with chaperon proteins shed light on the critical role of endoplasmic reticulum (ER) and Golgi resident proteins in receptor maturation. Boulin et al. (2008) demonstrated that three chaperon proteins are necessary for the expression of *C. elegans* muscle-type receptors in *Xenopus* oocytes: UNC-50, UNC-74 and RIC-3 (described in more details in Sections 1.4.9.1; 1.4.9.2 and 1.4.9.3); ligand binding and agonist-evoked currents were abolished upon exclusion of any of the three proteins. Resistant to inhibitors of cholinesterase-3 (RIC-3) protein also improves the cell surface expression of the second type of the body wall muscle (BWM) *C. elegans* receptor (Ballivet et al. 1996) and the neuron-type *C elegans* receptor in *Xenopus* oocytes (Halevi et al. 2002). It also plays a role in the maturation of human receptor in *Xenopus* oocytes and cell lines (Section 6.1.2.4). More recently, RIC-3 has been shown to influence folding and maturation of insect nAChRs. Co-expression of Dm$\alpha 2$-containing and Dm$\alpha 5/\alpha 7$ receptors with RIC-3 improved (Lansdell et al. 2008), and in some instances enabled expression in otherwise non-permissible systems (Lansdell et al. 2012). Up to 3.5-fold increase in specific binding of radiolabelled antagonist was noted in insect cells co-transfected with RIC-3, suggesting the presence of greater number of folded receptors on the cell surface (Lansdell et al. 2008). Expressed receptors have been also shown to be functional: in *Xenopus* oocytes, ionic currents were detected in response to acetylcholine (Lansdell et al. 2012).

Inability to heterologous express insect nAChRs hinders their pharmacological characterisation and identification of subunits important in conferring the agricultural role of neonicotinoids. Development of a platform in which the heterologous nAChRs from pest-insects and non-target organisms could be expressed, would enable pharmacological characterisation of these proteins to better define the mode of action and selective toxicity of neonicotinoids. It would also open the door to screening of novel insecticides, to combat emerging and spreading neonicotinoid-resistance (Section 1.3.5.1 and Charaabi et al. 2018).

1.4 *C. elegans* as a model system for expression of nAChRs

As indicated, the expression of insect receptors is limited due to difficulties in heterologous expression in *Xenopus* oocytes or cell lines. This suggests that these systems do not offer appropriate cellular environment for receptor maturation. Model organism *C. elegans* is an alternative model in which heterologous receptor expression can be achieved (Crisford et al. 2011; Salom et al. 2012; Sloan et al. 2015).

C. elegans is a transparent non-parasitic nematode, inhabiting temperate soil environments. This worm was first described as a new species in 1900 (Maupas 1900) and named *Caenorhabditis elegans* Greek *caeno* meaning recent, *rhabditis* meaning rod-like and Latin *elegans* meaning elegant. The natural isolate of this species was extracted from the compost heap in Bristol by Sydney Brenner in 1960's and named N2. Since, *C. elegans* has become a valued lab tool and a model organism due to ethical, economical and biological reasons. In contrast to vertebral organisms, *C. elegans* is not protected under most animal research legislation. The cost of use is low, due to the cost of purchase (~$6/strain), maintenance, fast life cycle and high fertility of these animals. *C. elegans* is also the first multicellular organism to have the whole genome sequenced (The *C. elegans* Genome Consortium 1998). In addition, every single neuron of its nervous system was mapped (White et al. 1986). It has an advantage over other model organisms in that its nervous system is relatively simple and it is amenable to genetic manipulations.

1.4.1 General biology of *C. elegans*

C. elegans exists as a male and a hermaphrodite, with the latter sex being the more prevalent one. In the lab, 99.9% of worms are hermaphrodites, which self-fertilise their eggs. *C. elegans* has a fast life-cycle (www.wormbook.org), which is temperature-dependent. At 15°C, it takes 5.5 days from egg-fertilisation to the development of a worm into an adult. This process is shortened to 3.5 and 2.5 days at 20 and 25°C, respectively (Figure 1.8). At 20°C, hermaphrodite lay eggs 2.5 hours after the fertilisation. 8 hours later the embryo hatches as a larvae in the first stage of its development (L1). In the presence of food, larvae develops into an adult through three further developmental stages, namely L2, L3 and L4. The transition between each larval stage is marked by a process of molting, during which the old cuticle is shed and replaced by a new

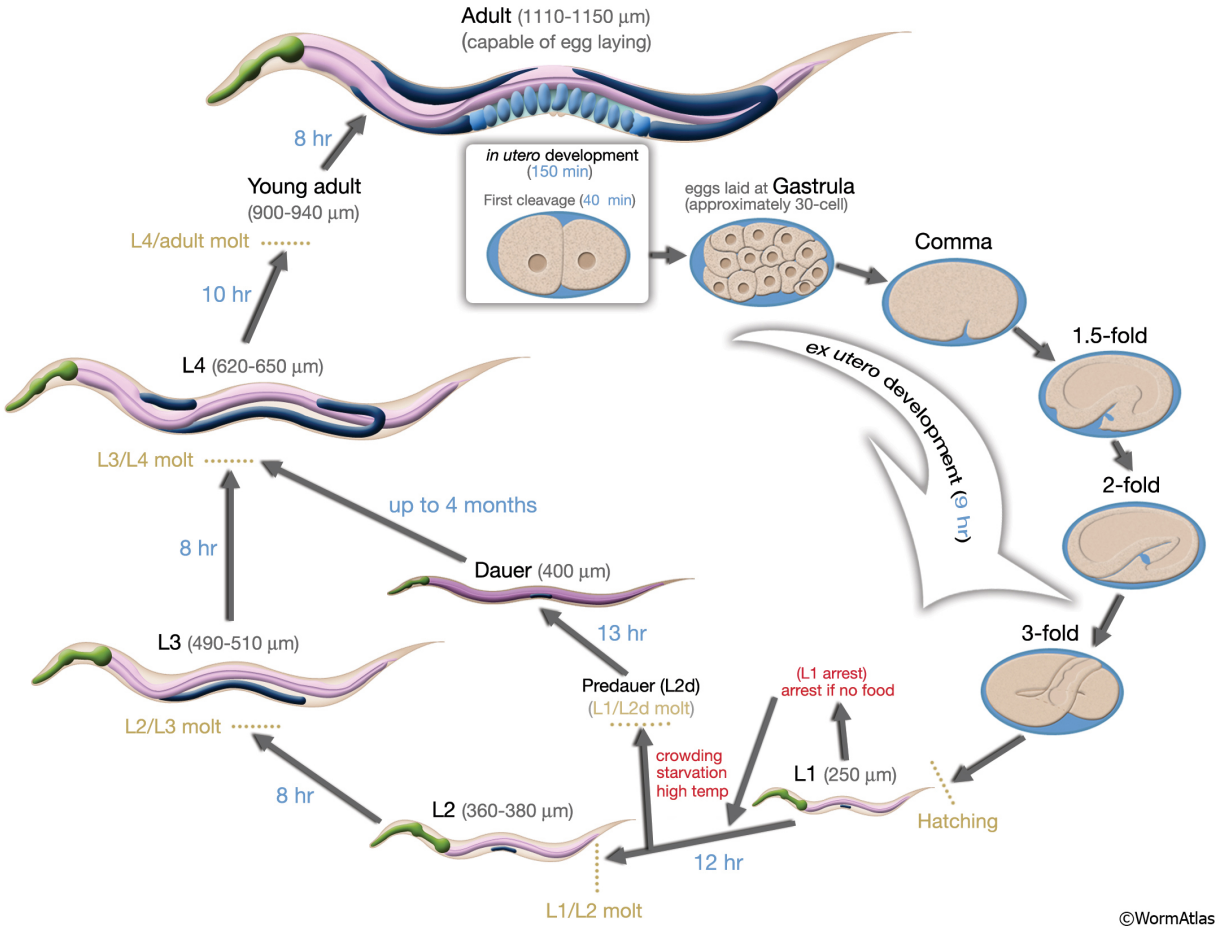


Figure 1.8: **The life cycle of *C. elegans*.** *C. elegans* develops into an adult through 4 larval stages L1 - L4. These stages are separated by molts associated with shedding of an old and exposure of a new cuticle. Adults can lay over a 1000 eggs a day which hatch within several hours. Dauer stage is a metabolically compromised worm stage entered in the absence of food. Upon re-appearance of food, worms develop into L4 and adults normally. Figure taken from www.wormatlas.org.

one. In the absence of food, developing L2 and L3 worms enter the dauer stage. The worms can remain arrested at this low metabolic activity state for up to several weeks, and will develop into adults, should the food re-appear. Hermaphrodites remain fertile for the first three days of their adulthood. Their eggs can be self-fertilised internally, or, if there are males available, by mating. Unmated worm can lay up to 350 eggs, whereas mated over a 1000 eggs. Figure 1.8 illustrates the full *C. elegans* life cycle.

1.4.2 Behaviour as an analytical tool

Over half of the century of *C. elegans* research developed a great depth of understanding of many of their simple and more sophisticated behaviours.

1.4.2.1 Pharyngeal pumping

Pharyngeal pumping is the feeding behaviour of the worm mediated by the pharynx. Successive and timed contraction-relaxation cycles of this muscular organ results in the capture, misceration and passage of the food particles down the alimentary track. Pharyngeal pumping can be easily scored by counting the number of pharyngeal pumps over time to determine the effects of compounds or genetic alteration on the function of the pharynx. In addition, pharyngeal cellular assays can be performed which offer not only a greater temporal resolution of the activity of the pharynx, but also allow for an analysis of the function of distinct anatomical features of the pharynx.

EPG (electropharyngeogram) is an extracellular electrical recording from the pharynx of the worm. It arises as a result of the flow of ions out of the worm's mouth, due to the changes in the membrane potential of the pharyngeal muscle. A single pharyngeal pump gives rise to a series of electrical transients collectively called an EPG. These electrical transients are temporally defined and represent activities of distinct anatomical features of the pharyngeal muscle, namely the corpus, isthmus and the terminal bulb (Raizen and Avery 1994; Franks et al. 2006).

1.4.2.2 Locomotion

C. elegans exhibits distinct locomotory behaviour in liquid and on solid medium. Whilst in liquid it flexes back and forth in the middle of the body. On solid medium, it performs S shaped, crawling movement. The direction of this movement is mostly forward and achieved due to the friction between the substrate and the body (Niebur and Erdos 1993). By counting the number of bends in the unit of time in the presence and absence of treatment, the effects on locomotory behaviour can be measured.

1.4.2.3 Egg-laying

C. elegans reproduces mainly by self-fertilisation of hermaphrodites or less frequently by mating with males. Hermaphrodite is sexually ready to be fertilised from young adult. The eggs are stored in the uterus and laid in defined spacio-temporal fashion. Typically, 5 eggs are expelled from the vulva at the time in approximately 20 minute intervals (Waggoner et al. 1998). The number of eggs laid in the unit of time can be counted and used to inform on the effects of treatment on the reproductive ability of the worm.

1.4.3 Nervous system and the neurotransmitters of *C. elegans*

One of the great advantages of *C. elegans* is that their entire nervous system has been mapped (White et al. 1986) and the neurotransmitters underpinning its function identified. Electron microscopy of serial worm cross sections (White et al. 1986) showed that a hermaphrodite has a total of 302 neurons present in the ventral nerve cord, the pharynx, the circumpharangeal ring and the tail. These neurons are assigned to 118 classes based on morphology and positioning. There are 39 sensory neurons, 27 motor neurons and 52 interneurons. Pharyngeal nervous system consists of 20 neurons belonging to 14 types.

The fast neurotransmission at the neuronal synapse and at the NMJ is mediated by a total of 6 neurotransmitters. Four of them are biogenic amines: octopamine, tyramine, dopamine and serotonin, which are involved in the modulation of many of the *C. elegans* behaviours.

Dopamine is synthesised in 8 mechanosensory neurons in hermaphrodites, whereas in males, it is present in those 8 neurons plus 6 tail mechanosensory neurons (Lints and Emmons 1999). It is involved in the modulation of the locomotion. Specifically, it mediates a reduction in movement velocity upon initial food encounter (Sawin, Ranganathan, and Horvitz 2000) and in the local exploratory behaviour in the vicinity of recently depleted food (Hills, Brockie, and Maricq 2004). It is also involved in learning. It has been shown to regulate the onset of the accelerated forward movement in response to nonlocalised mechanical stimulus such as plate tapping (Sanyal et al. 2004), and adaptation to the odourants (Bettinger and McIntire 2004).

Tyramine and octopamine are minor neurotransmitters, which are present in a small number of cells. Octopamine is synthesised from tyramine in the gonad sheet cells and a pair of RIC interneurons (Alkema et al. 2005). Its role is unclear, but it is potentially involved in the inhibition of egg-laying and pharyngeal pumping (Horvitz et al. 1982; Alkema et al. 2005). Tyramine is synthesised in the same cells as octopamine as well as in a single pair of interneurons and gonad sheath cells, RIM motor neurons and the 4 uterine UV1 cells (Alkema et al. 2005). It can act as an inhibitory neurotransmitter (Pirri et al. 2009) to prevent egg-laying and the head movement in response to anterior touch, as well as to modulate the reversal movement (Alkema et al. 2005).

5-HT (serotonin) functions in 8 types of neurons in *C. elegans* and has been shown to be involved in the regulation of locomotion, egg-laying and pharyngeal pumping. It is synthesised in a pair of Hermaphrodite Specific Neurons (HSNs) (Desai et al. 1988) and the Ventral C (VC) 4/5 neurons, which innervate vulval muscles (Duerr et al. 1999) and which upon release of 5-HT, stimulate egg-laying (Waggoner et al. 1998). In the pharynx, it is released from the neurosecretory motor neurons (NSM) and the I5 interneurons (Chase and Koelle 2007), where it stimulates pumping in the presence of food (Horvitz et al. 1982; Avery and Horvitz 1990; Sze et al. 2000) by acting on G-protein coupled receptors (GPCRs) SER1, SER4 and SER7 (Hobson et al. 2003; Song et al. 2013; Tsalik et al. 2003) and serotonin-gated chloride channel MOD-1 (Ranganathan, Cannon, and Horvitz 2000). More details on the role of 5-HT in the pharynx can be found in section 4.1.4.1.

Serotonin is also involved in the modulation of locomotory behaviour, specifically enhanced slowing upon food encounter in food deprived animals (Sawin, Ranganathan, and Horvitz 2000).

GABA acts primarily at the NMJ. It is present in a total of 26 neurons, which are motor neurons innervating the BWM, the head muscles and the enteric muscles, as well as interneurons (Schuske, Beg, and Jorgensen 2004). GABA serves both as excitatory and inhibitory neurotransmitter in *C. elegans*. It inhibits locomotion and foraging (McIntire et al. 1993) (Section 3.1.3.1) by activating $GABA_A$ ligand gated ion channels (Bamber et al. 1999) and induces enteric muscle contraction during defecation (McIntire, Jorgensen, and Horvitz 1993) by acting on cation selective GABA-gated ion channel EXP-1 (Beg and Jorgensen 2003).

Glutamate is the most rapid excitatory and the second most frequently used neurotransmitter in *C. elegans* (Serrano-Saiz et al. 2017). It is present in 79 cells in hermaphrodites, and 98 cells males. Found in the head, pharynx, ventral cord, body and tail neurons (Loer and Rand 2016). In the pharynx, it is released from 4 neurons and it shortens the duration of the pump (Raizen, Lee, and Avery 1995). More details on the function of glutamate in the pharynx can be found in section 4.1.4.2. Glutamate also plays a role in the backing response upon nose touch (Mellem et al. 2002), the response to hyperosmotic stimuli (Hilliard, Bargmann, and Bazzicalupo 2002) and repellent odours (Hart, Sims, and Kaplan 1995). It is also involved in the regulation of foraging behaviour (Hills, Brockie, and Maricq 2004) and long-term memory (Rose et al. 2003). These functions are induced by acting on ionotropic glutamate gated ion channel (iGluR). There are at least 10 iGluR subunits in *C. elegans* (Brockie and Maricq 2003), some of which are excitatory, whereas others are inhibitory (Cully et al. 1994).

Like in the insect brain (Florey 1963), ACh is a main excitatory neurotransmitter in the nervous system of *C. elegans*, where it is released from over a third of the *C. elegans* cells. In *C. elegans*, ACh is also a major neurotransmitter at the NMJ (Rand 2007).

1.4.4 Acetylcholine regulates feeding, locomotion and reproduction in *C. elegans*

Many of the *C. elegans* behaviours are regulated by acetylcholine, as evident from the behavioural analysis of mutant strains in which acetylcholine neurotransmission is affected. *C. elegans* cholinergic synapse expresses enzymes and transporters necessary for the cholinergic neurotransmission. Choline acetyltransferase (ChAT) encoded by the *cha-1* gene catalyses the formation of acetylcholine (Rand, J. B. and R. L. Russell 1985). Vesicular acetylcholine transferase (VAChT) encoded by *unc-17* loads acetylcholine into synaptic vesicles (Alfonso et al. 1993). Null mutations of these genes are lethal due to the inhibition of worm's locomotion and feeding and its eventual death due to starvation (Rand 1989; Alfonso et al. 1993). Polymorphic *ChAT* and *VAChT* mutants in which the expression is reduced, but not abolished, revealed somewhat opposite phenotypes. The pharyngeal pumping both in the presence and absence of food was reduced (Dalliere et al. 2015) the movement was highly uncoordinated and jerky (Rand and Russell 1984), whereas egg-laying

increased (Bany, Dong, and Koelle 2003).

The role of cholinergic transmission was also investigated using pharmacological studies, in which the effects of cholinergic agents on the behaviour of worms were observed. Aldicarb is a synthetic carbamate commonly used as a nematicide (Lue, Lewis, and Melchor 1984) in the pest management systems. Its mode of action is via inhibition of the acetylcholine esterase (AChE) - the enzyme that breakdowns acetylcholine released to the synaptic cleft (Johnson and Russell 1983). When applied on worms, aldicarb causes hypercontraction of the BWM, leading to paralysis (Nguyen et al. 1995; Mulcahy, Holden-Dye, and O'Connor 2013), hypercontraction of the pharyngeal muscle and inhibition of feeding as well as the inhibition of egg-laying (Nguyen et al. 1995). These observations in conjunction with the phenotypical analysis of *cha-1* and *unc-17* mutants, suggest acetylcholine stimulates feeding, coordinates locomotion and inhibits egg-laying in *C. elegans*.

1.4.5 *C. elegans* nAChRs

Acetylcholine exerts its action by activating nAChRs which are expressed at the post-synapse of the NMJ of the BWM and pharyngeal muscle and which mediate muscle contraction (Richmond and Jorgensen 1999; McKay 2004). *C. elegans* nAChRs are also expressed in non-synaptic regions, including the sensory endings of chemosensory neurons, which is in agreement with their role in chemosensation (Yassin et al. 2001). *C. elegans* contains at least 29 genes encoding for nAChR subunits (Jones et al. 2007) assigned to five groups based on the sequence homology: DEG-3, ACR-16, ACR-8, UNC-38, and UNC-26. The ECD domain sequence identity between members of these five groups and insect receptors is low (Figure 19), suggesting distinct pharmacophores.

1.4.6 Pharmacological evidence for the role of nAChRs in the regulation of *C. elegans* behaviour

Pharmacological experiments in which selective nAChR agonists, namely levamisole, nicotine and neonicotinoids, were tested against *C. elegans* behaviours provide evidence for the important role of these receptors in the regulation of locomotion, pharyngeal pumping and egg-laying.

Levamisole is a synthetic compound used in treatment of parasitic worm infestation in both humans and animals (Miller 1980). It is an agonist of a subset of receptors present at a BWM (Richmond and Jorgensen 1999). Levamisole causes spastic paralysis of worms (J. A. Lewis, Wu, Levine, et al. 1980) and stimulates egg-laying (Trent, Tsuing, and Horvitz 1983).

Nicotine is an alkaloid naturally occurring in the *Tobacco* plant (Steppuhn et al. 2004). It is an agonist of the second type receptor at a BWM, namely the N-type (Ballivet et al. 1996), but based on the nicotine-intoxication worm phenotype, it is also likely to target receptors regulating pharyngeal pumping and vulva muscle. Nicotine inhibits locomotion and egg-laying (Kudelska et al. 2017). It also inhibits pharyngeal pumping in the presence of food (Kudelska et al. 2018), whereas in the absence of food, it stimulates pharyngeal pumping (Raizen, Lee, and Avery 1995).

		honey bee receptor subunit										
Group	*C. elegans* subunit	alpha1	alpha-2	alpha-3	alpha-4	alpha-5	alpha-6	alpha-7	alpha-8	alpha-9	beta-1	beta-2
DEG-3	ACR-17	35%	33%	31%	32%	38%	34%	34%	30%	28%	31%	23%
	ACR-18	34%	32%	33%	33%	35%	33%	33%	31%	22%	28%	22%
	ACR-20	28%	30%	28%	29%	31%	29%	29%	30%	20%	23%	17%
	ACR-22	28%	27%	30%	29%	30%	25%	25%	26%	23%	28%	20%
	ACR-23	28%	32%	32%	30%	28%	28%	30%	29%	24%	25%	17%
	DES-2	34%	35%	34%	32%	31%	30%	31%	32%	22%	28%	21%
	DEG-3	29%	28%	30%	30%	31%	29%	31%	31%	23%	24%	19%
	ACR-24	31%	28%	31%	32%	34%	29%	31%	30%	23%	30%	19%
	ACR-5	31%	28%	30%	29%	28%	23%	26%	29%	20%	25%	17%
ACR-16	ACR-7	40%	38%	41%	41%	38%	42%	44%	37%	25%	35%	19%
	ACR-9	40%	39%	41%	41%	43%	49%	48%	40%	27%	39%	23%
	ACR-10	37%	40%	39%	38%	40%	43%	39%	35%	26%	37%	21%
	ACR-11	41%	40%	41%	40%	45%	47%	47%	35%	27%	40%	23%
	ACR-14	34%	34%	35%	35%	40%	41%	41%	34%	25%	37%	23%
	ACR-15	35%	40%	37%	39%	38%	46%	45%	36%	22%	36%	21%
	ACR-16	45%	45%	45%	45%	44%	52%	52%	42%	25%	42%	20%
	ACR-19	40%	43%	42%	40%	42%	45%	45%	39%	26%	39%	23%
	ACR-25	39%	38%	39%	40%	43%	49%	49%	37%	28%	39%	21%
	ACR-21	41%	43%	41%	40%	47%	43%	38%	36%	26%	35%	21%
	EAT-2	34%	37%	35%	35%	35%	41%	41%	34%	25%	34%	21%
ACR-8	ACR-8	46%	42%	47%	47%	39%	39%	41%	44%	24%	38%	18%
	ACR-12	52%	48%	50%	50%	38%	44%	44%	52%	29%	41%	20%
	LEV-8	47%	44%	46%	44%	38%	40%	41%	42%	26%	36%	19%
UNC-38	UNC-38	52%	53%	50%	48%	41%	39%	37%	50%	26%	40%	21%
	UNC-63	59%	59%	61%	58%	42%	42%	44%	58%	27%	46%	23%
	ACR-6	40%	44%	42%	40%	38%	35%	38%	40%	25%	34%	20%
UNC-29	ACR-2	46%	45%	43%	44%	39%	37%	39%	43%	24%	54%	24%
	ACR-3	43%	40%	40%	40%	42%	39%	37%	41%	25%	53%	25%
	UNC-29	45%	43%	44%	44%	41%	36%	38%	45%	24%	52%	23%
	LEV-1	43%	42%	44%	42%	40%	35%	36%	43%	26%	50%	24%

Figure 1.9: **Amino acid sequence identity between the insect and *C. elegans* nAChR subunits.** Sequences of the honeybee and *C. elegans* extracellular, ligand binding domains were aligned using the Multiple Sequence Comparison by Log- Expectation (MUSCLE). Sequence identities were derived with the HMMER alignment and color-coded using red-yellow-green scale. *C. elegans* subunits of the UNC-38 group are the most homologous to the insect subunits.

nAChR agonists neonicotinoids have been shown to affect motility (Hopewell et al. 2017; Mugova et al. 2018) and egg-laying (Gomez-Amaro et al. 2015; Ruan et al. 2009) (Section 3.1.4).

1.4.7 Genetic evidence for the role of nAChRs in the regulation of *C. elegans* behaviour

Behavioural analysis of *C. elegans* mutants supports the role of nAChRs in egg-laying, locomotion and pharyngeal pumping and reveals further functions of these receptors. Several mutant strains, including *unc-29*, *unc-38* and *unc-63* (J. A. Lewis, Wu, Levine, et al. 1980) as well as *unc-29; acr16* and *unc-63; acr-16* double mutants (Touroutine et al. 2005) exhibit disrupted, highly uncoordinated movement. *unc-29*, *unc-38* and *unc-63*, *lev-1*, *lev-8* mutants exhibits markedly inhibited egg-laying in the presence of levamisole (Waggoner et al. 2000). *C. elegans eat-2* mutant shows 70% reduction in the pumping rate in the presence of food (Raizen, Lee, and Avery 1995; McKay et al. 2004). Yassin et al. (2001) has shown that *deg-3* mutants are deficient in choline-chemotaxis, suggesting this subunit plays a role in sensory transduction. In the *unc-63* mutant, the action of nAChR agonist DMPP, which delays cell divisions and differentiation during the L2 developmental stage in hindered (Ruaud and Bessereau 2006) suggesting a role of this subunit in the development of *C. elegans*.

1.4.8 Stoichiometry of *C. elegans* nAChRs

C. elegans expresses at least 29 nAChR subunits (Section 1.4.5), which are expressed at the neuromuscular junction (NMJ) (Richmond and Jorgensen 1999) and in the nervous system (Lewis et al. 1987). To date, four receptor assemblies have been identified. (1) A single neuronal receptor composed of DES-2 and DEG-3 subunits (Treinin et al. 1998). (2, 3) There are two receptor at the BWM differentiated based on their pharmacology into L-(levamisole) type and N-(nicotine) type (Richmond and Jorgensen 1999). (4) EAT-2 is a predicted β nAChR subunit expressed in the pharyngeal muscle, believed to assemble with auxiliary subunit EAT-18 (McKay et al. 2004).

1.4.8.1 L-type receptors

L-type nAChR is one of the two *C elegans* nAChRs expressed at the post-synaptic membrane of the NMJ of the BWM (Richmond and Jorgensen 1999). Three of the five constituting subunits were identified in the behavioural analysis of *C elegans unc-29*, *unc-38* and *unc-63* mutants, which showed highly uncoordinated locomotion (J. A. Lewis, Wu, Levine, et al. 1980). *unc-29, unc-38* and *unc-63*, as well as *lev-1* and *lev-8* were also resistant to nAChR agonist levamisole (J. A. Lewis, Wu, Levine, et al. 1980). Expression of *lev-1*, *unc-29* and *unc-38* in *Xenopus* oocytes generated a protein with nAChR-like properties: in response to acetylcholine and levamisole, depolarising current was elicited (Fleming et al. 1997). Richmond and Jorgensen (1999) provided evidence that these receptors are expressed at the NMJ of the BWM. Intracellular recordings from the post-synaptic membrane at the NMJ of the BWM showed that in response to acetylcholine and levamisole inward current is elicited. That current was abolished in *unc-29* and *unc-38* mutants (Richmond and Jorgensen 1999). The identity of the levamisole sensitive nAChRs was revealed by Boulin et al. (2008), who showed that eight genes are required for the generation of fully functional receptor in *Xenopus* oocytes. Five genes encode for nAChR subunits UNC-29, UNC-38, UNC-63, LEV-1 and LEV-8, two of which, viz. UNC-29 and LEV-1 are non-α. In the absence of any one of the 5 subunits, agonist-evoked currents were abolished, suggesting all subunits are essential for the receptor function. The remaining 3 genes encode for the auxiliary subunits RIC-3, UNC-50, AND UNC-74. Their role is described in Section 1.4.9.

1.4.8.2 N-type receptors

Work of Richmond and Jorgensen (1999) identified the second type of nAChR at the NMJ of the BWM. This receptor showed high sensitivity to nicotine, thus was named N-type. N-type receptor is composed of ACR-16 subunits, which form homomeric receptors in *Xenopus* oocytes (Ballivet et al. 1996).

1.4.8.3 Receptors at the pharyngeal neuromuscular junction

A single receptor subunit, namely EAT-2 has been identified as essential in mediation of the feeding response (McKay et al. 2004). *C. elegans eat-2* mutant shows significantly reduced pumping in the presence of food (Raizen, Lee, and Avery 1995; McKay et al. 2004). A similar phenotype was noted in the *eat-18* mutants. EAT-18 however is not a nAChR subunit. Instead, it is predicted to be a single transmembrane protein. Based on the localisation and behavioural phenotype, EAT-18 and EAT-2 are believed to co-assembly to form a functional receptor (McKay et al. 2004).

ACR-7 is also expressed at the pharyngeal muscle (Saur et al. 2013), as was shown with a reporter construct, however its function in pharyngeal pumping is unclear as *acr-7* mutant pump normally in the presence of food (Saur et al. 2013).

1.4.9 *C. elegans* proteins important in nAChR maturation

The function of nAChRs in *C. elegans* depends on the successful receptor maturation, a process aided by a number of chaperon proteins.

1.4.9.1 RIC-3

RIC-3 is a evolutionary conserved, ER-residing (Roncarati et al. 2006; Alexander et al. 2010) TM protein (Wang et al. 2009). In *C. elegans*, it is ubiquitously expressed in most (if not all) neurons, and in the pharyngeal and BWMs (Halevi et al. 2002). The predicted topology of *C. elegans* RIC-3 has 2 transmembrane domains and 3 coiled-coils. The *C. elegans ric-3* mutant has impaired locomotor behaviour, resistance to levamisole (Miller et al. 1996) and impaired responses to cholinergic agents, as measured by electrophysiological recording from the BWM (Halevi et al. 2002). The *C. elegans ric-3* mutant has impaired cholinergic neurotransmission; there is a lack of cholinergic component of the EPG recording resulting in significantly retarded pharyngeal pumping and starved appearance (Halevi et al. 2002).

Heterologous expression of *C. elegans* nAChR in *Xenopus* oocytes provides evidence for their function in receptor maturation. Choline-evoked currents of neuronal DEG-3/DES-2 and muscle ACR-16 receptors increased upon RIC-3 co-expression (Halevi et al. 2002; Ballivet et al. 1996). The role of RIC-3 was also demonstrated *in-vivo*. Nicotine induced current at the BMW was markedly reduced in *ric-3* mutant, in comparison to wild-type (Halevi et al. 2002).

C. elegans RIC-3 can also promote maturation of mammalian $\alpha 7$ channels. Co-expression of this protein improved $\alpha 7$ function in *Xenopus* oocytes as shown by enhanced choline- and acetylcholine- evoked currents and cell-surface binding of radiolabelled α-bgtx (Lansdell et al. 2005; Williams et al. 2005). RIC-3 also enabled expression of $\alpha 7$ in otherwise non-permissive insect cell lines (Lansdell et al. 2008). It has been shown to not only promote the heterologous cell-surface expression of mammalian receptors, but it also increased the expression of insect chimera nAChRs (Lansdell et al. 2012).

1.4.9.2 UNC-50

UNC-50 is an ortholog of evolutionary conserved GMH1 protein. In *C. elegans* it was first identified in behavioural and pharmacological screens of *C. elegans* mutants. Several phenotypes have been described including: uncoordinated movement (J. A. Lewis, Wu, Berg, et al. 1980) reduced binding of radiolabelled levamisole to the membrane fractions (Lewis et al. 1987), resistance to levamisole in behavioural assays (Lewis et al. 1987; Abiusi et al. 2017) and no responses of L-type nAChRs at the BWM to levamisole (Eimer et al. 2007). The lack of cell-surface staining from antibodies against UNC-29 (Eimer et al. 2007) in *unc-50* mutant confirmed the role of UNC-50 in nAChR maturation. *unc-50* mutant is also characterised

by an increased lysozyme-dependent degradation of nAChRs, suggesting its preventative role in this process. UNC-50 is predicted to be expressed in the Golgi, as the expression of GFP::UNC-50 fusion protein resulted in fluorescence typical of the localisation to this organelle (Eimer et al. 2007).

1.4.9.3 UNC-74

UNC-74 is closely related to the human TMX3 protein which is thought to be ER-associated (Haugstetter, Blicher, and Ellgaard 2005). Reduced radiolabelled meta-aminolevamisole binding to membrane fraction of *C. elegans* mutant (Lewis et al. 1987) combined with its role in expression of L-type receptor in *Xenopus* oocytes (Boulin et al. 2008) confirms its role in receptor maturation.

1.4.9.4 EAT-18

EAT-18 is thought to be required for the function of pharyngeal nAChRs. It consists of a single transmembrane and an extracellular domain. Transgenic worms expressing EAT-18::GFP fusion protein reveal fluorescence in the pharynx with the strongest signal in the muscle, but also in the pharyngeal neuron M5 and unidentified 5 to 6 extrapharyngeal neurons (McKay et al. 2004). *eat-18* mutants are deficient in pumping and resistant to high concentration of nicotine, supporting the function of EAT-18 in cholinergic neurotransmission of the pharynx (Raizen, Lee, and Avery 1995). The association of *eat-18* with pharyngeal nAChR was indicated by comparison of the staining in the wild-type and *eat-18* mutant strains. Injection into the pseudocoelom of radiolabelled α-bgtx resulted in straining of the pharynx. This was however abolished in the mutant strain (McKay et al. 2004). In addition, the expression of EAT-2 in *eat-2* mutant was normal, suggesting EAT-18 is not involved in the trafficking of this receptor. It has been proposed that EAT-18 co-assembles with EAT-2 due to their common pharyngeal phenotypes in mutant strains and common cellular localisation in the pharyngeal muscle (McKay et al. 2004). Recently, successful expression of eat-2 co-assembled with eat-18 has been shown in *Xenopus* oocytes (personal communication).

1.4.10 Mode of action studies in *C. elegans*

The depth of *C. elegans* genetic, anatomical, biochemical and pharmacological knowledge combined with methods to generate mutant and transgenic lines, provides an opportunity to use this organism in the mode of action studies of cholinergic agents. Indeed, *C. elegans* has been utilised to investigate the molecular basis of biocides selectivity, including nAChR levamisole (Pinnock et al. 1988; Lewis et al. 1987; J. A. Lewis, Wu, Berg, et al. 1980). *C. elegans* is also a promising model in which functional and pharmacological properties of nAChRs can be studied.

1.4.10.1 Pharmaceutical characterisation of proteins in transgenic worms

A process of microinjection allows for generation of transgenic worms in which heterologous expression can be achieved. Microinjection is a process by which a plasmid containing cDNA encoding for a protein of interest is injected into the syncytium distal arm of the gonad(s) of the young adult hermaphrodite worm (Stinchcomb et al. 1985). The injected DNA in taken up by the the residing oocytes (Wolke, Jezuit, and Priess 2007), which become fertilised and develop into adult worms. Using cellular machinery, the DNA plasmid forms extrachromosomal arrays, from which the cDNA becomes transcribed, translated and expressed (Stinchcomb et al. 1985; Mello et al. 1991).

The expression of transgene can be driven in specific cells or tissues, including those reliant on cholinergic transmission. Conjugated monoclonal antibodies were used to show selective expression of myo-3 (heavy chain of myosin B) at the body-wall muscle and vulva muscle (Ardizzi and Epstein 1987) and myo-2 (myosin heavy chain C) in the pharyngeal muscle (Okkema et al. 1993) of the intact worm. Thus, by using myo-3 or myo-2 promoters upstream of the heterologous gene, expression at the body wall or pharyngeal muscle, respectively, can be achieved (Sloan et al. 2015; Crisford et al. 2011). There are also promoters, such as H2O, inducing expression in the nervous system (Yabe et al. 2005). Behavioural and cellular analysis of transgenic worms generated by microinjection, allows for pharmacological characterisation of native and exogenous proteins, including nAChRs (Sloan et al. 2015).

1.4.11 Biochemical methods to assess expression of nAChR in *C. elegans*

The cellular localisation of nAChRs expressed in *C. elegans* can be detected by an array of methods, such as using protein-specific pharmacological agents. α-bgtx is a high affinity antagonist of nAChRs (Blumenthal et al. 1997), widely used to label expression on native and heterologous channels. Audioradiography of tissues incubated with radiolabelled α-bgtx visualised mammalian nAChRs at the post-synaptic membrane of the end-plate (Barnard, Wieckowski, and Chiu 1971), in the peripheral (Clarke et al. 1985) and central nervous system (Carbonetto and Fambrough 1979). Fluorescently labelled α-bgtx was utilised to show successful expression of heterologous proteins such as mammalian $\alpha 7$ in HEK, P12 and SH SY5Y cell lines (Cooper and Millar 1997; Gu et al. 2016). In *C. elegans*, conjugated-α-bgtx injected into the pseudocoelom, labelled native nAChRs of the pharyngeal (McKay et al. 2004) and BWM (Jensen et al. 2012). It also allowed for the identification of heterologously expressed ACR-16 in the BWM of *C. elegans* (Jensen et al. 2012). α-Bgtx is used to demonstrate cell surface expression, because it binds to the extracellular domain of the nAChR (Dellisanti et al. 2007) and does not permeate membranes. There are methods used to label heterologous proteins intracellularly. For example, Salom et al. (2012) and Gu et al. (2016) used detergents to permeabilised membrane to allow protein-specific antibodies or α-bgtx to access protein sites inside the cell.

Taken together, *C. elegans* is an attractive biological system in which the mode of action and selective toxicity

of cholinergic agents can be studied. It is also a promising biological system for the heterologous expression of nAChRs, in particularly those that do not express well in other biological systems. Generally speaking, heterologous expression of proteins can have two consequences: (1) when introduced into the mutant strain, it can restore drug or cellular function (Crisford et al. 2011; Salom et al. 2012) and (2) heterologous expression in wild-type worm can lead to new or altered pharmacological sensitivity (Crisford et al. 2011; Salom et al. 2012). These changes can be scored using an array of behavioural and cellular methods. Therefore, heterologous expression combined with behavioural and pharmacological analysis of transgenic worms can also inform on functional and pharmacological properties of heterologously expressed proteins.

1.4.12 Aims

The overall aim of this project is to develop *C. elegans* as a platform for the heterologous expression of nAChRs, with the aim to gain insight into selective toxicity of neonicotinoids insecticides. This will be achieved in three steps:

1. Define sensitivity of *C. elegans* to these compounds. The representatives of three distinct chemical classes of neonicotinoids will be used: cyanoamidine clothianidin, nitroguanidine thiacloprid and nitromethylene nitenpyram. Their effects on *C. elegans* will by tested utilising behavioural and cellular assays to define their potency on distinct neuronal circuits.
2. Identify suitable *C. elegans* genetic background with defined cholinergic function for the expression of nAChRs.
3. Develop assays by which the functional nAChR expression and drug-sensitivity can be tested.

Chapter 2

Methods

2.1 General bacterial methods

2.1.1 Transformation of *E. coli* with DNA vectors

Briefly, 25 to 50 μL of chemically competent Mach1 or DH5α *Escherichia coli (E.coli)* cells were combined and gently mixed with ~10 pg of plasmid DNA. The mix was left on ice for 30 minutes. Cells were placed in 42°C water bath and after 45 seconds placed back on ice. Next, 250-500 μL of growth medium (Luria-Bertani (LB) broth) was added and cells placed in the 37°C shaking incubator for 45 minutes to 1 hour. The entire volume of cells was spread onto 10 cm LB-agar plate containing an appropriate antibiotic for selection. Antibiotics used for each vector selection are listed in Table 2.1. Cells were spread on plates and left in the 37°C incubator overnight to allow for growth of transformed cells into colonies.

Table 2.1: Selection pressure for DNA plasmids used in this study.

Plasmid	Antibiotic
pET26	Kanamycin
pBMH	Ampicillin
pET27b(+)	Kanamycin
pcDNA3.1	Ampicillin
PCR-8-TOPO	Spectinomycin
pDEST	Ampicillin

Note:
Ampicillin used at a final concentration of 0.1 mg/mL, whereas kanamycin and spectinomycin at 0.05 mg/mL.

2.1.2 Isolation of DNA plasmid from *E. coli*

Transformed *E. coli* colony was picked, placed in 5 mL of LB supplemented with appropriate antibiotic and incubated overnight at 37°C whilst shaking. DNA was extracted using the MiniPrep Kit (Thermo Scientific or Qiagen) following the manufacturers instructions. Centrifugation was carried out in table top centrifuge at 12 000 g. Isolated DNA was quantified in a Nanodrop UV-Vis spectrophotometer.

2.1.3 Analytic digestion of DNA plasmids

DNA plasmids were digested with restriction enzyme(s) (Promega). The reaction mix (Table 2.2) was incubated at 37°C for 2-8 hours and the DNA fragments were resolved on the agarose gel (Section 2.2.2).

Table 2.2: Components assembled to carry out restriction enzyme reaction.

10x Buffer	1 μL
BSA	0.1 μL
DNA	1-2 μg
Enzyme	5 units
dH_2O	up to 10 μL

2.2 General molecular biology methods

2.2.1 Amplification of DNA fragments by Polymerase Chain Reaction (PCR)

2.2.1.1 Primer design

To enable the amplification of the DNA of interest, appropriate PCR primers were designed applying the following criteria: primers were unique to the annealing site on the designated DNA, 18 to 25 nucleotides long, guanine-cytosine content from 40 to 60 %, melting temperature from 55 to 75°C. Where possible, primers rich in guanine and cytosine at 3' end were selected to facilitate high specificity of primer binding to the target. The primers were ordered from Eurofins Genomics, subsequently diluted in ddH_2O to the concentration of 100 pmol/μl and stored at -20°C. Sequences of primers used in this study can be found in Table 2.3.

Table 2.3: DNA primers used in this study.

DNA product	DNA primer	Size (bp)
NdeI-pelB-3C-SalI	Fw: gaaggagatatacatatgaaatacctg Rv: TAGCTTGTCGACgggcccctggaacagaacttc	1560
SalI-α7ECD-2GSC-NheI	Fw: AGCTCCGTCGACtttcagcgtaaactgtacaaag Rv: ACTAGCTAGCTTAaagcttagccgcaccacggcg	1051
CHRNA7	Fw: atgcgctgctcgccgggaggcg Rv: ttacgcaaagtctttggacacggc	1509
eat-2	Fw: atgaccttgaaaatcgca Rv: ttattcaatatcaacaatcgg	1425

Note:
Sequences are presented in 5' to 3' direction, non-complementary nucleotides are in capital letters whereas complementary in small letters. Fw stands for forward, whereas Rv for reverse primer.

2.2.1.2 PCR Protocol

PCR was performed using either Phusion High-Fidelity DNA Polymerase (Thermo Scientific) or Pfu DNA polymerase (Promega) as indicated. The components mixed and cycling conditions used are listed in Tables 2.4 and 2.5.

Table 2.4: Components assembled for Phusion polymerase-mediated polymerase chain reaction (PCR) reaction.

Component	Concentration
Buffer	1x
dNTP mix	200 μM
Reverse/ Forward primer	500 nM
DNA	10 ng / 50 μL reaction
Polymerase	0.5 U / 50 μL reaction
ddH_2O	up to 50 μL

Table 2.5: Components assembled for Pfu polymerase-mediated PCR reaction.

Component	Concentration
Buffer	1x
dNTP mix	200 μM
Reverse/ Forward primer	500 nM
DNA	25 ng / 50 μL reaction
Polymerase	0.25 U / 50 μL reaction
ddH_2O	up to 50 μL

Thermal cycling conditions used for amplification of *C. elegans* nAChR subunit *eat-2*, whole length and the extracellular domain of human $\alpha 7$ nAChR subunit as well as $pelB - HIS - MBP - 3C$ sequence (sequence for expression of genes and purification of proteins from *E. coli*) are shown in Tables 2.6 - 2.9.

Table 2.6: Thermal cycling conditions for amplification of *eat-2* from *TB207* plasmid with Pfu polymerase.

Step	Duration	Temperature °C	Number of cycles
Initial denaturation	2 mins	95	1
Denaturation	1 mins	95	
Annealing	30 secs	51.1	30
Extension	3 mins	73	
Final extension	5 mins	73	1

Table 2.7: Thermal cycling conditions for amplification of human $\alpha 7$ nAChR (*CHRNA7*) cDNA from *DNA3.1* plasmid with Phusion polymerase.

Step	Duration	Temperature °C	Number of cycles
Initial denaturation	30 secs	98	1
Denaturation	10 secs	98	
Annealing	30 secs	gradient	30
Extension	45 secs	72	
Final extension	7 mins	72	1

Note:
gradient annealing temperatures in degree Celsius were: 50, 51.1, 53.4, 57.2, 60.2

Table 2.8: Thermal cycling conditions for amplification of *pelB-HIS-MBP-3C* from *pET26-GLIC* plasmid with Pfu polymerase.

Step	Duration	Temperature °C	Number of cycles
Initial denaturation	3 mins	95	1
Denaturation	1 mins	95	
Annealing	30 secs	45	35
Extension	3 mins	73	
Final extension	5 mins	73	1

Table 2.9: Thermal cycling conditions for amplification of human α7 nAChR ligand binding domain from *pBMH* plasmid with Phusion HF polymerase.

Step	Duration	Temperature °C	Number of cycles
Initial denaturation	30 secs	98	1
Denaturation	10 secs	98	
Annealing	45 secs	51.7	35
Extension	45 secs	72	
Final extension	7 mins	72	1

2.2.2 DNA electrophoresis

To resolve the size of DNA samples, agarose gel electrophoresis was run using BioRad Wide horizontal electrophoresis system and PowerPac Basic Power Supply. The resolving gel was prepared by addition of agarose (0.6-1.2 % (w/v); Sigma Aldrich) to 1x TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer. This mix was heated in the microwave until agarose completely melted and left on the bench to cool down to $\sim 50^{\circ}$C. Subsequently, Nancy-520 DNA Gel Stain (Sigma-Aldrich) at 5 mg/mL was added in 1:1000 (v/v) dilution and the mixture was poured into the gel caster. Meanwhile, the DNA samples were prepared by mixing them with 1 % (v/v) loading dye (Blue/Orange Loading Dye, Promega). Once the gel set, the samples were loaded into wells alongside the indicated molecular weight marker. Markers variously used in this thesis include 1kb Hyperladder (Bioline), 1kb ladder (Promega) or 1 kb Plus DNA ladder (Thermo Scientific). Electrophoresis was run at 70 V until the samples were sufficiently resolved (typically 30 minutes to 2 hours) and gels imaged using Syngenta GBox.

2.2.3 DNA purification following PCR and electrophoresis

Following gel electrophoresis, the band of interest was visualised under the UV light and isolated by cutting with a surgical blade. DNA was subsequently purified using GeneJET Gel Extraction Kit (Thermo Scientific) or Gel Extraction Kit (Qiagen) following manufacturers protocols.

2.2.4 Ligation dependent cloning

The DNA vector for expression of human nAChR was generated by T4 dependent ligation. PCR-amplified, gel-excised and digested with *SalI* and *NdeI* *pelB-HIS-MBP-3C* gene was inserted into the digested *pET27* plasmid. The complementary sequences were ligated with T4 ligase (Table 2.10). Next, PCR-amplified, gel-excised and digested with *SalI* and *NheI* human $\alpha 7$ extracellular domain (ECD) was ligated into the digested *pET27-pelB-HIS-MBP-3C*. Both times reaction mixtures were incubated at room temperature for 3-4 hours and used to transform chemically competent Mach1 cells. 50-100 μL of cells were transformed with 2.5-8 μL ligation reaction mix.

Table 2.10: Components assembled to carry out ligation-dependent cloning reaction.

Ligase buffer	1 x
Backbone DNA	100 ng
Insert DNA	3:1 insert:backbone ratio
T4 DNA ligase	1 unit
ddH_2O	up to 10 μL

2.2.5 Gateway cloning

Vectors for generation of *C. elegans* transgenic genes were generated by recombinant Gateway Cloning.

2.2.5.1 Generation of the entry (TOPO) vector by TA recombination

3' adenine overhangs were added to the amplified and gel purified DNA fragment in the reaction using non-proofreading Extend Long Roche Polymerase (ThermoScientific) (Table 2.11).

Table 2.11: Addition of adenine overhangs to PCR product for entry clone generation.

Polymerase	5 U/20 μL reaction
10 x Buffer B	1x
DNA	up to 500 ng
dNTP	200 μM
ddH_2O	up to 10 μL

TA reaction was assembled (Table 2.12), incubated at room temperature for 1 hour and 2 μL of the reaction mix was used to transform 50 μL DH5α chemically competent cells. DNA was isolated from transformed colonies and sequenced to ensure the correct sequence and orientation of the insert.

Table 2.12: Components assembled for the generation of the entry clone for Gateway cloning.

PCR-8 TOPO vector	1 μL
Salt solution	1 μL
PCR product	up to 500 ng
ddH_2O	up to 6 μL

2.2.5.2 Generation of the expression vector by LR reaction

LR reaction was assembled using Gateway LR Clonase II Enzyme Mix (Invitrogen) (Table 2.13). Reaction was incubated at room temperature for 2 hours. To inactivate the enzyme, 2 μL of proteinase K was added and the reaction mix was incubated at 37°C for 10 minutes. 1 μL of reaction mix was used to transform 50 μL of One Shot OmniMAX 2 T1 phage resistant cells (Invitrogen). Transformed cells were plated and grew overnight. Following, plasmid was isolated from transformed cells and subjected to sequencing to ensure successful formation of the plasmid.

Table 2.13: Components assembled for the generation of recombinant vector by Gateway cloning.

Entry clone (PCR-8-TOPO-CHRNA7)	75 ng
Destination vector (pDEST-pmyo2)	75 ng
LR Clonase II	1 μL
TE buffer (pH=8)	up to 5 μL

2.3 Expression of human $\alpha 7$ nAChR in *E. coli*

Heterologous protein was expressed in and subsequently purified from *E. coli* (Figure 2.1).

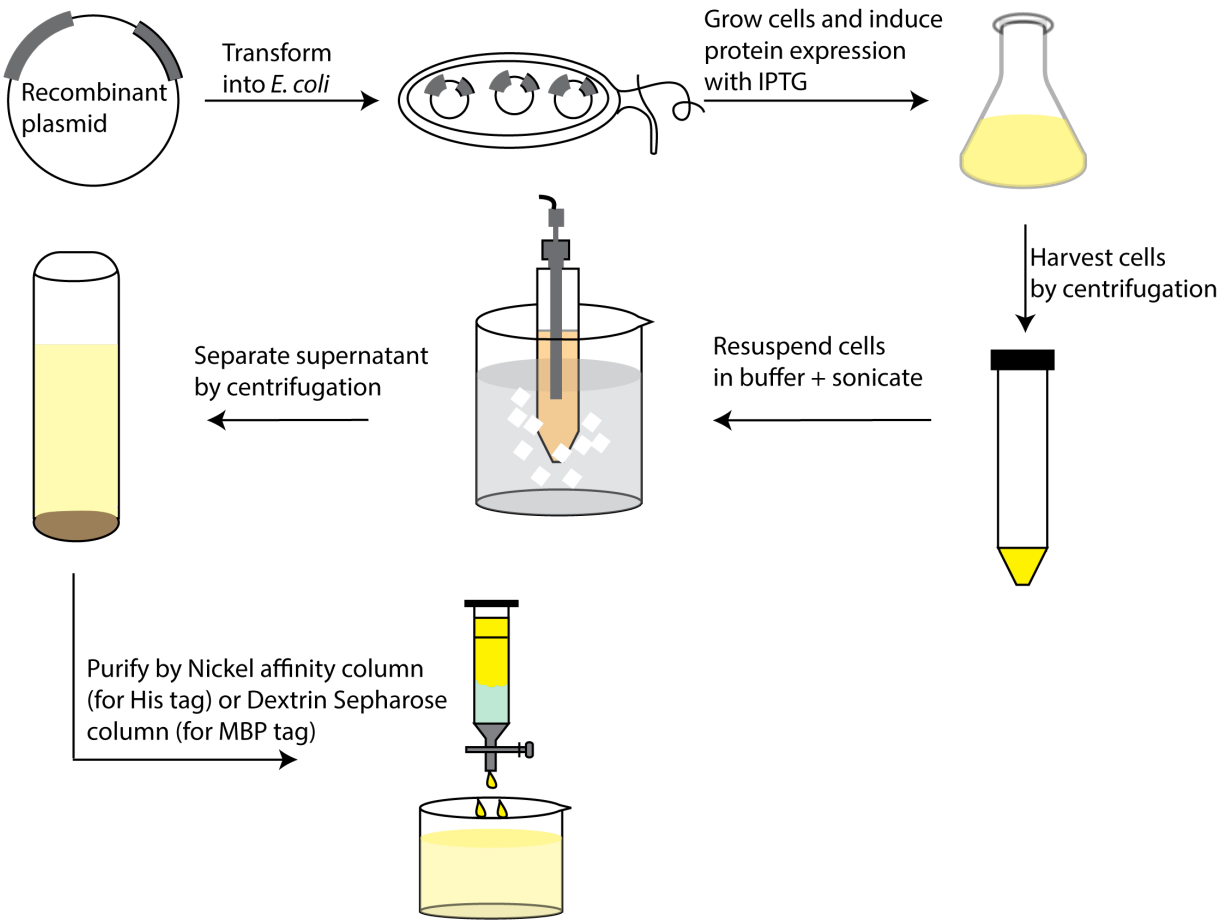


Figure 2.1: **The process of heterologous protein expression in *E. coli* and protein purification.** Plasmid containing gene of interest is transformed into *E. coli* cells. Transformed cells are grown in medium and the protein expression induced by addition of Isopropyl β-d-1-thiogalactopyranoside (IPTG). Cells are subsequently harvested, re-suspended in buffer and cellular content released by sonication. The supernatant containing soluble proteins is isolated from cellular debris by centrifugation and the heterologous protein isolated using metal affinity chromatography. His tagged protein bound to $Nickel^{2+}$ are eluted with imidazole, whereas Maltose Binding Protein (MBP) tagged proteins bound to Dextrin Sepharose beads are eluted with maltose.

2.3.1 Growth of transformed *E. coli* cells

Chemically competent bacterial cells (BL21(DE3)) engineered for high efficiency protein expression were transformed with the authenticated expression vector as described (Section 2.1.2). Transformed colonies were resuspended and placed in 5 mL of growth medium supplemented with the appropriate antibiotic. Seed culture was placed in the shaking incubator at 37°C and left to grow until OD_{600nm} of 1-2. This starter culture was used to inoculate growth medium supplemented with appropriate antibiotic in 2 L baffled flasks, to the final OD_{600nm} of 0.01-0.05. Inoculated flasks were placed in a shaking incubator at 37°C, 250 RPM. The following protocol was followed, unless otherwise stated: At $OD_{600nm} = 0.5$, the temperature was lowered to 18°C.

When the 18°C culture reached an OD_{600nm} 1, 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the growth continued overnight at 18°C.

2.3.2 Protein purification

E. coli were harvested by centrifuging the cell culture at 5000 g for 20 minutes at 4°C and either used immediately or stored at -20°C for further use. Harvested cells were kept on ice throughout the purification procedure. Two methods of purification were tested: HIS-tag purification using Ni-NTA resin and maltose binding protein (MBP) purification with Sepharose-Dextrin Beads (GE Healthcare Life Sciences).

2.3.3 HIS-tag purification

The composition of buffers used is as follows :

Re-suspension buffer: 0.1M TRIS (pH=8), 0.15 M NaCl. Wash buffer 1: as previous. Wash buffer 2: 0.1 M TRIS (pH=8), 1 M NaCl. Wash buffer 3: 0.1 M TRIS, (pH=8), 0.15 M NaCl. Elution buffer: 0.1 M TRIS, (pH=8), 0.15 M NaCl, 0.2 M imidazole (pH=7.5)

Cells harvested from 1 L of culture medium were re-suspended in 40 mL re-suspension buffer supplemented with 2 Pierce Protease Inhibitor Mini Tablets (Thermo Fisher Scientific) and sonicated on ice using the following settings: power 7, pulse on: 10 seconds, pulse off: 20 seconds, total time 6 minutes. Sonicated cells were subject to 16 000 g spin for 45 minutes at 4°C to sediment cellular debris. The supernatant was collected and spun again at 100 000 g for 1 hour at 4°C to separate non-soluble fraction (e.g. aggregated proteins) in the pellet from the supernatant containing soluble fraction. Supernatant was mixed with 0.5 mL of Ni-NTA resin (previously equilibrated in the resuspension buffer) and equilibrated for 1 hour or overnight at 4°C on the rotating tube rotator (speed 8-9). Following this incubation, the mix was decanted into a low pressure 5 mL chromatography column. Resin was washed with 10 mL of each one of the 3 washing buffers. Lastly, bound to Ni-NTA resin proteins were eluted off by addition of 5 x 0.5 mL of elution buffer. Eluted fractions were stored at 4°C. At each stage, samples consisting of the pre induction (pre-I; post induction (post-I) Homogenate (H) (Whole cells), high speed supernatant (LOAD), Flow through (FT0) wash (W) and eluate (E) fractions were collected for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Section 2.3.5.1).

2.3.4 Quantification of protein expression and purification

Protein content of the eluted samples was measured with NanoDrop 1000 Spectrophotometer V3.7 at 280 nM and the following parameters, as measured by Compute pI/Mw tool (http://web.expasy.org/compute_pi/): Mw (kDa) of pentameric full length protein = 420, extinction coefficient (/1000)= 132.95. Two μL of the

elution buffer/buffer C were used to blank the spectrophotometer, and 2 μL of the elution fractions was used to estimate the protein concentration of the sample.

2.3.5 Analysis of protein molecular weight using denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.5.1 Sample preparation

This section described how each sample collected during protein expression and purification was prepared. Pre- and post-induction whole cell samples: 1 mL of *E. coli* cell culture was taken, spun down in tabletop centrifuge at max speed for 5 minutes. Supernatant was discarded, pellet re-suspended in 150 μL dH_2O. Total protein content of 2 μL samples were measured with NanoDrop by measuring protein absorbance at 280 nm. Seventy mg/ml of protein was loaded onto a gel in each sample. Cell lysate: following sonication, 30 μL of cell lysate was taken. Cell-debris and supernatant: 30 μL of cell lysate sample taken, spun down in tabletop centrifuge for 10 mins at maximum speed, at 4°C. supernatant was pipetted into another micro centrifuge tube and labelled supernatant, whereas the pellet was re-suspended in 30 μL dH_2O and labelled whole-cell sample. Supernatant and Pellet samples: 50 μL of cell lysate spun down in tabletop centrifuge for 10 minutes at maximum speed, at 4°C. Thirty μL of the supernatant taken, spun down in ultracentrifuge at 100 000 g, at 4°C for 1 hour. Supernatant was pipetted into another microcentrifuge tube (ultra-supernatant) whereas debris was re-suspended in 30 μL of dH_20. The same volumes of cell-lysate, cell-debris, supernatant, ultra-supernatant, ultra-pellet and flow-through were loaded onto SDS-PAGE gels. Protein samples were mixed with sample buffer (2 % SDS, 2 mM DTT, 4 % glycerol, 0.04 M Tris pH = 6.8, 0.01 % bromophenol blue) and boiled for 5 – 10 minutes. Next, 4 – 10 μL samples were loaded onto 8 – 12 % acrylamide SDS-PAGE gel alongside 4 μL of Protein Marker PageRulerTM Prestained/Unstained Protein (Thermo Fisher Scientific).

2.3.5.2 Gel electrophoresis

2.3.5.2.1 Gel preparation

Protein samples were subject to denaturing SDS-PAGE and stained with the Coomassie stain to visualise and estimate the size of protein species. SDS-PAGE constituted from a stacking gel (5 % acrylamide/bisacrylamide, 0.72 M Tris pH = 8.4, 0.025 % ammonium persulfate, 0.4 % TEMED, 0.1 % SDS) casted over a resolving gel (12 % acrylamide/bisacrylamide, 1 M Tris pH = 8.4, 0.06 % ammonium persulfate, 0.13 % TEMED, 0.1 % SDS).

BioRad electrophoresis apparatus was used. Electrophoresis chamber was filled with the running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) and the electrophoresis proceeded at 80 V for 1 hour and then 120 V

for 2 hours. The gel was either stained to visualise all proteins present in samples, or used in Western blot to detect the presence of a specific protein.

2.3.5.3 Coomassie staining and imaging

Following SDS-PAGE electrophoresis, gels were incubated in fixing buffer (10 % acetic acid, 40 % ethanol, 50 % dH_2O) and placed on an oscillating platform for at least 1 hour to remove background staining. The fixed gel was then washed with dH_2O and incubated with Coomassie stain (14 mg of Coomassie Blue R-250 (ThermoScientific) /L of dH_2O) overnight. The gel was de-stained by incubation with dH_2O for at least 8 hours and imaged with Gel Doc^{TM} XR+ (Bio-Rad).

2.3.6 Analysis of protein molecular weight using one-dimensional non-denaturing polyacrylamide gel electrophoresis

2.3.6.1 Sample preparation.

Two protein eluate samples were collected from the immobilized metal affinity chromatography (IMAC) and mixed with sample buffer (4 % glycerol, 0.04 M Tris pH = 6.8, 0.01 % bromophenol blue). One sample was boiled for 5 minutes to denature proteins, whereas the other was not. Next, 4 – 10 μL prepared samples were loaded onto 12 % nondenaturing polyacrylamide gel, alongside 4 μL of 1 mg/mL of bovine serum albumin, which served as a marker.

2.3.6.1.1 Gel preparation

Nondenaturing gel (12 % acrylamide/bisacrylamide, 1 M Tris pH = 8.4, 0.06 % ammonium persulfate, 0.13 % TEMED) was used to resolve the size of proteins.

2.3.6.1.2 Gel electrophoresis

BioRad electrophoresis apparatus was used. Electrophoresis chamber was filled with the running buffer (25 mM Tris, 192 mM glycine) and the electrophoresis proceeded at 80 V for 1 hour and then 120 V for 2 hours. The gel was stained and imaged as described in Section 2.3.5.3

2.3.7 Western blots

2.3.7.1 Protein transfer

Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Membrane cut to the size of the resolving gel was equilibrated for 15 minutes to 1 hour in the transfer buffer (12.1 g Tris, 57.6 g glycine,

800 mL methanol in total volume of 4 L) then washed with dH_2O followed by methanol. Freshly run polyacrylamide gel was placed on top of the submerged in transfer buffer sponge, 2 x filter paper and PVDF membrane stack and covered with 2 x filter paper. Assembled transfer mount with the gel and PVDF membrane was placed in the BioRad Mini Trans-Blot Module which was in turn inserted into Mini-PROTEAN Tetra Cell tank. The tank was filled with transfer buffer. Proteins were transferred from the gel onto the membrane at 100 V constant voltage for for 1 - 3 hours at 4°C.

2.3.7.2 Antibody binding

Transfer of his-tagged human $\alpha 7$ nAChR ECD-chimera protein were detected using 1 mg/mL monoclonal mouse anti-Hexa-His primary antibodies (Thermo Fisher Scientific) and 1mg/mL IRDyeő 680RD Goat anti-Mouse IgG (Li-Cor) used at 1 in 1000 dilution. PVDF membrane was incubated for at least 1 hour in each blocking, primary and secondary antibody buffer (Table 2.14). To remove residual solution, three 10-minute-long washes were carried out in-between and after last incubations.

Table 2.14: Composition of buffers used for Western blotting.

Washing buffer	1 x phosphate buffered saline (PBS), 0.05 % TWEEN
Blocking buffer	1 x PBS, 0.05 % (v/v) TWEEN 20 (BioRad), 5 % BSA
Primary Antibody buffer	5 mL blocking buffer, 5 μL primary antibody
Secondary Antibody buffer	5 mL blocking buffer, 5 μL secondary antibody

2.3.7.3 Western blot imaging

Immunodecorated PVDF were imaged using Odyssey imaging system (Li-Cor Biosciences). Images in the 800 nm channel detects protein bands tagged by the IRDye 800CW secondary antibody. This was cross referenced to images scanned in the 700 nm to detect protein ladder bands and 800 nm channels.

2.3.7.4 Gel filtration

Protein purified and eluted with the Ni-NTA resin were subject to size exclusion chromatography with GE Healthcare Superdex^{TM} 200 10/300GL column with the separation range between 10 and 600 kDa. This methods allows for the molecular weight assessment and separation of proteins present in sample, based on their mobility through the resin-filled column.

2.3.7.5 Sample preparation

Sample was prepared with VIVASPIN20 column with the cut off point of 30 kDa (Sartorius) by spinning down in a centrifuge at 36 000 RPM at 4°C.

2.3.7.6 Buffers

Buffer used : 0.1M TRIS (pH=8), 0.15 M NaCl degassed and ddH_20 degassed.

2.3.7.7 Calibration of the column

To estimate size of proteins present in the sample, standard curve was generated. Four proteins were selected as protein standards: trypsin of 23.3 kDa, chicken serum albumin of 47.5 kDa, bovine serum albumin of 66.5 kDa and dextrin which forms large aggregates. These aggregates are larger than the column capacity, therefore dextrin served as void. Solutions of 3 mg/mL of trypsin, chicken and bovine serum albumin (BSA) were prepared and 1 mg/mL of dextrin. Protein solutions were injected into the column one at the time at a flow rate of 0.4 mL/ min. The eluted volume at which peak position as a function of volume eluted was noted for each protein. All peak positions were normalised to the position of the void (dextrin) (Figure 2.2). Linear regression line was plotted of the log protein size (kDa) as a function of normalised volume eluted (Figure D.11). The standard equation of the line was derived: y = - 0.1738 * peak position + 2. 776.

Protein	Mwt (kDa)	log Mwt	Peak position (mL)	Normalised peak position
Dextrin	NA	NA	9.48	0
Bovine Serum Albumin	66.45	1.82	14.73	5.25
Chicken Serum Albumin	47.29	1.68	16.38	6.90
Trypsin	23.30	1.37	17.23	7.75

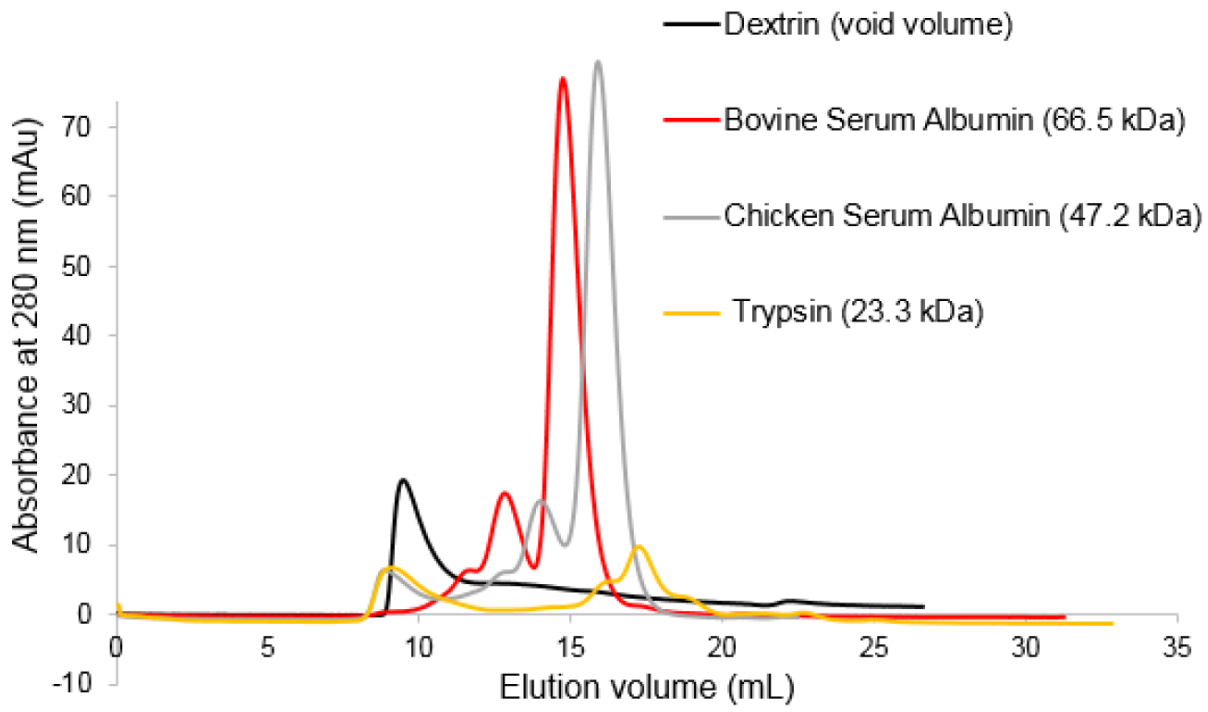


Figure 2.2: **Gel filtration of protein markers.** Data derived from the spectra and normalised to the dextran void.

2.4 *C. elegans* methods

2.4.1 *C. elegans* strains

Wild type strain:

N2 (Bristol)

Mutant strains:

CB7431 (genotype *bus-17, (allele br2)X.* ; outcrossed x4

AD465 (genotype *eat-2, (allele ad465)II.* ; outcrossed x0

FX863 (genotype *acr-7, (alleletm863)II.* ; outcrossed x0

Transgenic strains:

eat-2 (ad465) II Ex; [pDESTgcy32 (Pmyo-3::GFP)]

eat-2 (ad465) II Ex; [pDESTgcy32 (Pmyo-2::CHRNA7)]; [pDESTgcy32 (Pmyo-3::GFP)]]

eat-2 (ad465) II Ex; [[pDESTgcy32 (Pmyo-2::EAT-2); [pDESTgcy32 (Pmyo-3::GFP)]]

2.4.2 *C. elegans* culture

C. elegans strains were cultured at 20°C on the nematode growth medium (NGM) (Brenner 1974) and fed with OP50 strain of *E. coli*. Worms were picked with a platinum wire.

2.4.3 Preparation of *C. elegans* plates

NGM was prepared weekly in 4 or 8 L batches as described: 2 % agar (w/v), 0.25 % peptone (w/v), 50 mM NaCl (w/v) in dH_20. The components were autoclaved and cooled to 55°C, then 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM K_2HPO_4 and 0.1 % cholesterol were added. 10 mL NGM portions were poured into 5.5 cm Petri dishes with a peristaltic pump. Once solidified, NGM were seeded with 50 μL of OP50. OP50 was applied in the middle of the plate, creating a round food patch for *C. elegans* to feed on. Prepared plates were left overnight to allow bacteria growth.

2.4.4 Maintenance and preparation of *E. coli* OP50

Fresh stock of OP50 plates were prepared at monthly intervals. A single colony was picked from the OP50 stock plate and placed in 10 mL of LB. Following overnight growth, cells were streaked on a plate and allowed to form colonies by incubation at 37°C.

2.4.5 *E. coli* OP50 culture

To prepare OP50 culture, a single colony was picked from the OP50 stock plate and placed in 10 mL of LB. Bacterial culture was grown in a shaking incubator at 37°C until OD_{600nm} reached 0.6 to reach the exponentially growing phase. Cultures were stored at 4°C for up to 2 week and used to seed NGM plates.

2.4.6 General *C. elegans* methods

All experiments, with the exception of the development assay, were performed on young hermaphrodite adults (L4 + 1 day). Drugs and reagents were purchased from Sigma Aldrich, unless otherwise stated. Behavioural observations were made using a binocular microscope, unless otherwise stated. Results are expressed as mean ± SEM of 'N' determinations.

2.4.7 Dose-response curves

Dose-response curves and the measurement of EC_{50} or IC_{50} were performed in GraphPad (version 6.07). The curves were fitted into nonlinear regression sigmoidal dose-response (three parameter logistic) equation (Hill 1910). This model was chosen, because it allows for determination of the predicted EC/IC_{50} value even if the entire dose-response curve is not available, as it was the case in many behavioural assays involving neonicotinoids. A potential disadvantage of choosing this model is that it assumes Hill coefficient of 1, in other words, lack of cooperativity among binding sites in nAChRs, which is the predicted target of compounds used in this study. nAChRs have multiple binding sites: from two in the muscle-type receptor (Blount and Merlie 1989) to 5 in homomeric complexes (Celie et al. 2004; Li et al. 2011). Their opening is generally governed by positive cooperativity binding of ligands (Katz and Thesleff 1957; Edelstein et al. 1996), however some nAChRs do not seem to display this property. For example, a Hill coefficient of 1 was determined for acetylcholine binding to levamisole-type receptor (Boulin et al. 2008) as well as many agonists binding to AChBP (Kaczanowska et al. 2014) suggesting, a single agonist is sufficient to open the channel, despite the presence of three and five binding sites, respectively. Although the properties of L- and N- type *C. elegans* receptors have been described (Boulin et al. 2008; Touroutine et al. 2005), kinetics of most *C. elegans* nAChRs are unknown. Resultantly, it is uncertain whether the three parameter logistic model is appropriate for dose-response curves of the effects of nAChRs agonists on behaviour of *C. elegans*. Whilst IC_{50}/EC_{50} values derived using this model might not be accurate, they provide an estimation of the potency of agonist on *C. elegans* nAChRs.

2.4.8 Drug stocks

5-HT was used in form of serotonin creatinine sulfate monohydrate, ampicillin in form of sodium salt, whereas nicotine was in the form of hydrogen tartrate salt. Stock concentration of FITC-alpha-bungarotoxin

(FITC-α-bgtx) at 500 μg/ml was made in ddH_2O. Thiacloprid and clothianidin were dissolved in 100 % dimethyl sulfoxide (DMSO). Nitenpyram and nicotine stocks were prepared by dissolving drugs in dH_20 and diluted to the indicated final concentrations. Working concentration of 100 μg/mL FITC-α-bgtx was prepared and stored at 4°C for up to 2 weeks. The solution was span down briefly before use to pellet aggregates. Drugs were stored at -18°C for long term storage (>1 month). Once defrosted, they were used within 2 weeks or discarded. Nitenpyram stock was made immediately prior to the experiment and protected from light using foil to prevent photo-degradation. Buffers used for the behavioural assays in liquid were supplemented with 0.1 % (w/v) BSA, which prevents worms from sticking to the bottom of the experimental plate. Therefore, M9 and Dent's solution refer to buffers supplemented with BSA, unless otherwise stated.

2.4.9 Effects of drugs on intact *C. elegans* locomotion and feeding behaviour upon acute exposure

All assays were performed in M9 medium. M9 buffer composition is (g/litre): 6 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4.H_2O$. Worms were exposed to varying indicated concentrations of nicotine or neonicotinoids for a maximum period of 2 hours. The effects of these compounds on locomotion and feeding was scored.

10 x stock concentrations of nicotine, nitenpyram and 5-HT were added to the assay to give the indicated final concentration. To keep the concentration of DMSO below the concentration that have known effects (data not shown) the stocks of thiacloprid and clothianidin in 100 % DMSO were used in 1 in 200 (0.5 %) dilution and mixed vigorously with buffer.

2.4.9.1 Effects of drugs on intact *C. elegans* in liquid

Whilst in liquid, worms exhibit rhythmical swimming-like behaviour known as thrashing. A single thrash was defined as a complete bend in the mid-point of the body. Experiments were performed in a 24-well plate filled with 450 or 497 μL buffer. 50 μL nicotine/ nitenpyram/vehicle or 2.5 μL thiacloprid/clothianidin was added to the final volume of 500 μL to achieve final desired concentrations.

Worms were picked off the food and transferred to the experimental arena. After 5 minutes of acclimatisation to allow recovery from mechanical transfer, the first thrashing count was performed (time 0). This provided baseline thrashing for each worm. Only thrashing worms were included in the analysis. After estimation of the control thrashing wells were supplemented with drug or a vehicle. For experiments with 1.5 mM thiacloprid, worms were transferred in a small volume of liquid (~2 μL) from the control to the experimental well by pipetting. This method was adopted due to drug's limited solubility. It must be noted that after a period of about 40 minutes, 1.5 mM thiacloprid began to visibly precipitate. Measurements were taken for 30 seconds typically at

time points: 10, 30, 40, 60 and 120 post-addition of the drug/drug vehicle. At least three independent repeats for each condition were carried out. The number of worms in each experiment varied from 2 to 6.

2.4.9.2 Onset of paralysis

The trashing assay was performed as described above but the time interval between measurements was reduced to from 30 seconds to every 2 minutes for the first 10 minutes. Worms were exposed to drug concentrations which induced paralysis in the thrashing experiment. That is wild-type worms were submerged in 100 mM nicotine, whereas *bus-17* in 25 mM nicotine, 50 mM nitenpyram or 1.5 mM thiacloprid. These concentrations were achieved by addition of 100 μL nicotine stock or vehicle into 900 μL buffer, 5 μL of thiacloprid/clothianidin stock or vehicle into 995 μL buffer or 10 μL nitenpyram or vehicle into 90 μL buffer. Twelve well plates were used. The protocol was followed as described in Section 2.4.9.1, but after a period of acclimatisation, worms were transferred from control to the experimental well by pipetting.

2.4.9.3 Recovery from drug-induced paralysis

The recovery assay was designed to determine if and how quickly worms recovered from drug-induced paralysis. Twenty four-well plates were used and worms were assayed in a total volume of 500 μL (Section 2.4.9.1) with the exception of nitenpyram experiment in which 25 μL nitenpyram stock or vehicle was added to 225 μL buffer to give a final volume of 250 μL. Following the initial thrashing count (Section 2.4.9.1), worms were transferred to drug concentrations inducing paralysis. Worms were incubated in nicotine for 20 minutes, thiacloprid or nitenpyram for 1 hour - that is until a steady state inhibition or full paralysis was achieved. This was marked as time point 0. Subsequently, worms were transferred to a wash well to observe the recovery and thrashing was counted at 10, 30, 60, 90, 120 and 150 minutes. Alongside this test group, a positive and a negative control experiments were carried out. For the positive control, worms were transferred from buffer to drug containing medium. For a negative control, worms were transferred from buffer to buffer containing a drug solvent and back to the buffer.

2.4.9.4 Effects of drugs on pharmacologically induced pharyngeal pumping of intact *C. elegans*

Pharyngeal pumping assay was employed to determine the effects of compounds on the pharynx - a feeding organ of worms. Pharyngeal pumping is mediated by three main muscular anatomical structures (the corpus, anterior isthmus and the terminal bulb) which contract and relax to suck and push in the food. This activity is coupled with a movement of the grinder - a structure responsible for crushing the food into smaller particles so it can be passed down into the intestine. Therefore, to score this behaviour, the number of the grinder movements per minute was counted (where forward lateral movement of the grinder and its return to the resting place was counted as 1). In this experiment, worms were assayed in the presence of the pharyngeal stimulant

5-HT. In liquid 5-HT causes immobility and stimulates pharyngeal pumping. Experiments were performed in a 24-well plate in a total volume of 500 μL (or in 250 μL for nitenpyram). Worms were picked off the food and placed in a well containing buffer. To paralyse them and stimulate their pharyngeal pumping, 5-HT was added from stock to a final concentration of 10 mM. After 30 minutes, the 5-HT stimulated pump rate was measured. Following, the treatment/solvent was added and the effects on pumping were recorded 30 minutes later. Data was displayed in pumps per second (Hz). Data collection was carried out in collaboration with Amelia Lewis.

2.4.9.5 Effects of drugs on *C. elegans* size

To determine the effects of drug exposure on worms size, *bus-17* worms were submerged in 1 mL of buffer, 50 mM nicotine, 50 mM nitenpyram, 1.5 mM thiacloprid or 2.5 mM clothianidin or vehicle control (dilutions described in Section 2.4.9.2) in 12 well plates. Four hours later, worms were transferred by pipetting onto 2 % agarose pads and immobilised with 6 μL of 10 mM sodium azide. Images were taken immediately using Nikon Eclipse Microscope. Size of worms was determined in ImageJ. The scale was set using a graticule and length measured from the tip of the tail to the tip of the head with the freehand function. For improved accuracy, three measurements of each worm were taken and an average was derived.

2.4.10 Effects of drugs on intact *C. elegans* behaviour upon 24-hour exposure

On-plate assays were carried out to determine the effects of prolonged drug exposure on *C. elegans* behaviour. Worms were placed on NGM plates containing the indicated drug / drug vehicle and a food source in form of *E. coli* OP50 patch. All drugs were added to the NGM at 1 in 200 dilution.

2.4.11 Plate preparation

NGM was prepared as described in Section 2.4.3. Fifty μL of drug solution at appropriate concentration was added to 10 mL of molten NGM at approx 50°C and mixed by gentle inversion. Three mL of such mix was placed in each of the three successive wells of a 6-well plate (Figure 2.3). The medium was left overnight to solidify. One well was then seeded with 50 μL of OP50 culture, whereas the other two wells remained unseeded. This provided an experimental arena with the food on, the cleaning well and the experimental arena containing no food. In parallel, control plates containing drug solvent (water or 0.5 % DMSO) were prepared. Due to heat instability, nitenpyram plates were prepared by pipetting 50 μL of drug solution onto solidified 3 mL NGM. The plates were left overnight to enable diffusion of the compounds into the solid agar. The appropriate well was then seeded. Nitenpyram-containing plates were covered with aluminium foil at all times, to prevent photo-degradation.

2.4.11.1 Experimental protocol

Four L4 + 1 worms were picked off food from the culture plate and placed in the first well of the experimental plate containing treatment or solvent. Twenty four hours later, the following behaviours were scored:

1. Pharyngeal pumping on food (feeding behaviour): a pump was defined as described previously (Section 2.4.9.4). Only worms present on the food lawn were included in analysis.
2. Egg-laying: The number of eggs and larvae was counted to derive the total number of eggs laid over the period of 24-hours. The total value was divided by a number of worms present on a plate. Should a worm disappear from the experimental arena, the results were not included in the analysis.
3. Body bends is the measure of locomotory ability of worms on solid medium. A single body bend was defined as a bend of the below-the-head portion of the body and counted for a period of 1 minute in the absence of food.
4. Egg-hatching: After 24-hour exposure, adults were removed from the plate leaving the eggs and the progeny behind. 24-hours later, the number of unhatched eggs present on the plate was counted. This was expressed as a % of eggs hatched (formula used: 100-(number of unhatched eggs*100/total number of eggs laid).

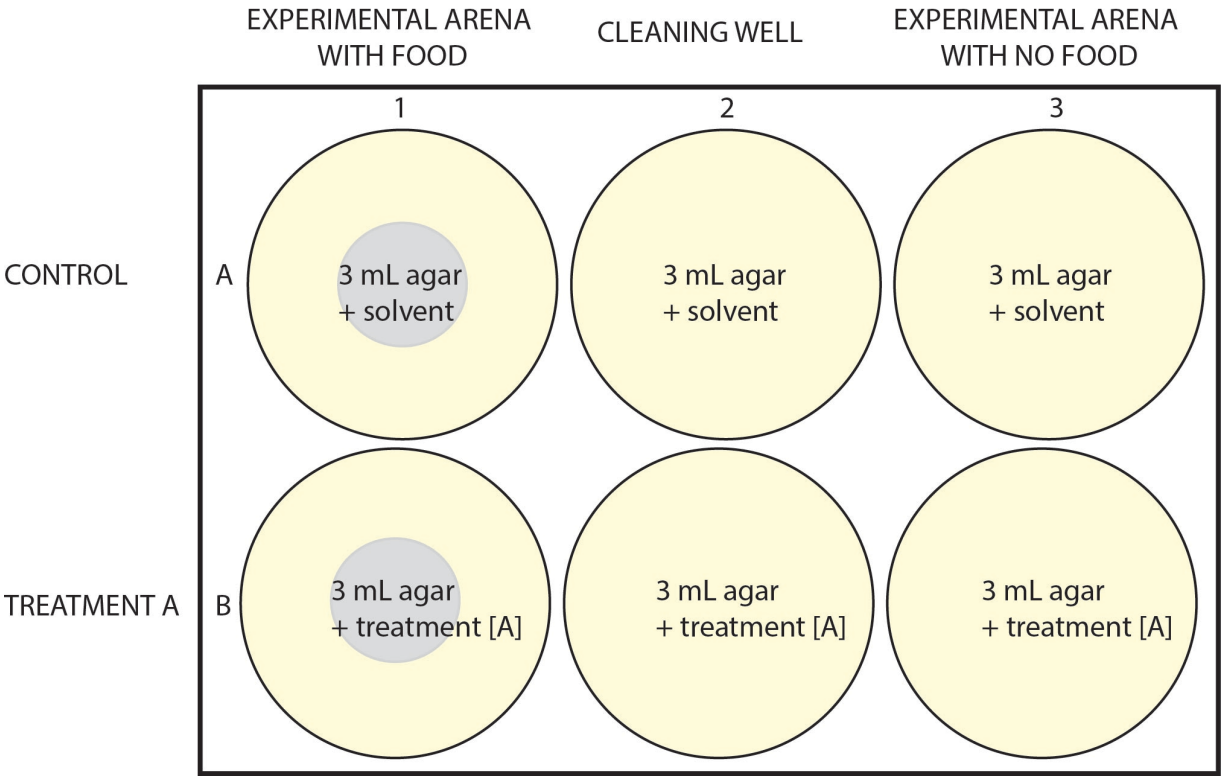


Figure 2.3: **Diagram of the 24-hour "on-plate" assay arena.** Drug or drug solvent was incorporated into the nematode growth medium (NGM) and poured into rows of a 6-well plate. Wells in the first column were seeded with the OP50. Two to four L4 + 1 worms were placed on the experimental arena containing food source. After 24 hours, pumping rate on food and the number of eggs laid per worm were counted. Following, worms were transferred to the cleaning well and left for 5-10 minutes to remove the residual food. Worms were then transferred to the experimental arena containing no food source. After period of acclimatisation (5-10 minutes), their locomotion on food was measured by counting body bends.

2.4.12 Effects of drugs on development of *C. elegans* upon long term (days) exposure

2.4.12.1 Development assay

Experiments were carried out in 12 well plates containing drug or solvent-incorporated and seeded-NGM (prepared as described previously). Six to twelve young adult hermaphrodites were placed on drug/solvent containing OP50 NGM plates. They were left on a plate for 1 hour to lay eggs, and removed from the plate, leaving the progeny behind. The number of worms in each developmental stage was counted at time points: 24, 30, 48, 54, 72, 80, 96, 120, 144, 168 and 192 hours. Larval stages were scored by following size/vulva/eggs present criteria, described by Karmacharya et al. (2009) and shown in Figure 2.4. If necessary, worms were viewed at higher magnification using Nikon Eclipse E800 microscope. Results were represented as mean % worms in each developmental stage.

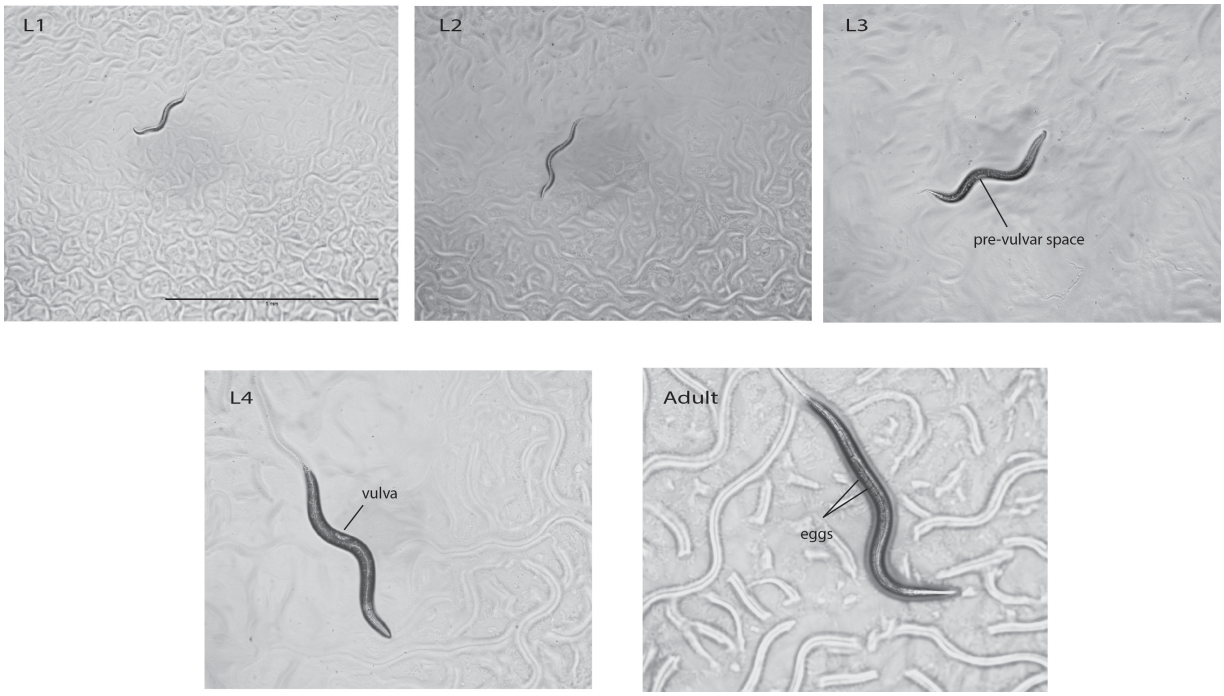


Figure 2.4: ***C. elegans* developmental stages.** Images showing all 4 larval stages of *C. elegans*. L1 are the smallest worms on the plate. L2 are slightly bigger, L3 are bigger still, more mobile and have a pre-vulvar space. L4 has a visible vulva, whereas adults have eggs present in their uterus. The same magnification was used to capture all images, thus the scale bar in the top left image applies to all images. Scale bar = 1mm.

2.4.13 Effects of drugs on *C. elegans* pharyngeal pumping in dissected head preparation

2.4.13.1 Dissection of worms to remove the cuticular barrier

L4 + 1 worms were picked off food and placed in 3.5 cm Petri dish filled with 3 mL Dent's saline. Heads containing pharyngeal musculature and nerves were separated from the rest of the body (Figure 2.5). By doing so, the cuticular barrier was removed and a portion of the pharynx was exposed to the external solution. The pharynx in cut-head preparation retained its function. It pumped at an average rate of 0.13 Hz over the period of 120 minutes (Figure 2.6). Pumping was defined as described previously (Section 2.4.9.4), counted for a period of 30 seconds and expressed in Hz. Only worms pumping at rate >0 were used in experiments.

2.4.13.2 Experimental arena

Cut heads were placed in a 12-well plate filled with 1 mL of Dent's saline (glucose 1.8 g, Hepes 1.2 g, NaCl 8.2 g, KCl 0.4 g, $CaCl_2$ 0.4 g, $MgCl_2$ - 1 mL at 1 M, pH adjusted to 7.4 with 10 M NaoH, 0.1 % BSA (w/v), made daily) with drug solution or vehicle. 100 μL or nicotine or 5-HT was added to 900 μL of buffer to achieve desired concentration of the drug. Clothianidin and thiacloprid were used at a 1 in 1000 dilution to keep the DMSO concentration at 0.1 % (v/v). Therefore, 1 μL of drug stock was added to 999 μL of buffer. Nitenpyram experiments were performed by addition of 10 μL of drug stock to 90 μL of buffer.

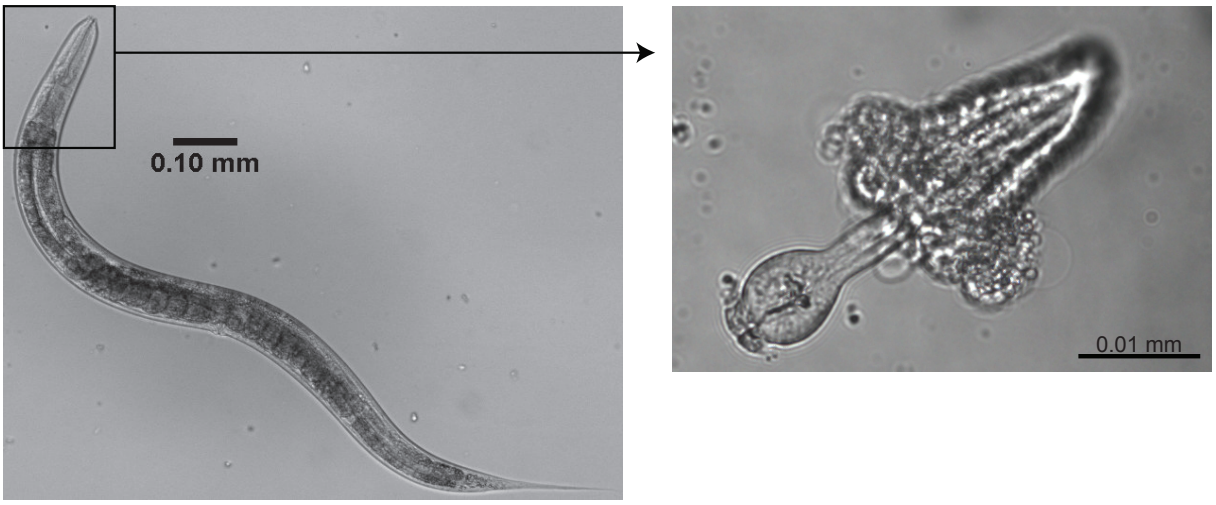


Figure 2.5: **Dissected worm preparation.** The pharynx was liberated from the rest of the body by cutting with a surgical blade just under the terminal bulb whilst viewing under the binocular microscope.

2.4.14 Stimulatory effects of drugs on pharyngeal pumping of dissected *C. elegans*

2.4.14.1 Effects of 5-HT

Following dissection, heads were placed in Dent's solution. After 5 minutes the initial count of pharyngeal pumping was made and heads were transferred to a drug containing well. Pharyngeal pumping was estimated at 10, 20, 30, 60 minutes after being placed in the drug. Heads were transferred to Dent's solution for recovery and pumping measured 30 minutes later (90 minutes after starting the measurements). As a negative control, worms were incubated in buffer throughout the duration of the experiment.

2.4.14.2 Effects of neonicotinoids and nicotine

Experiments were set up as described above (Section 2.4.14.1) but the time points were: 0 (Dent's), 2, 5, 10, 15, 20, 30, 45, 60 65, 75, 90 and 120 (treatment) and 130 (recovery). As a control, cut heads were incubated with a 5-HT concentration eliciting maximal response. That is 1 μM for wild-type N2 worms and 50 μM for *eat-2* mutant worms.

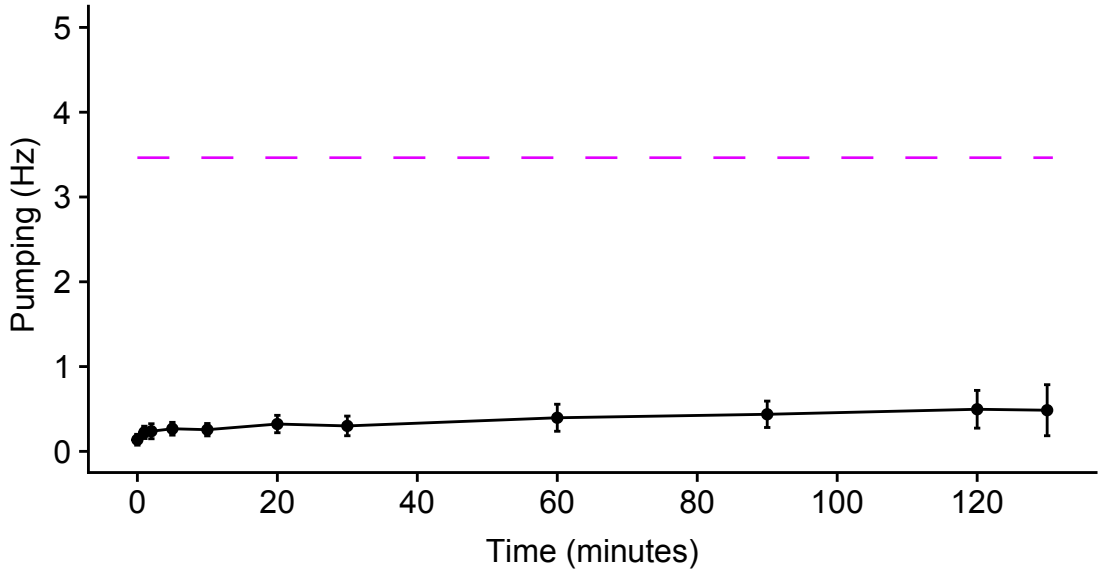


Figure 2.6: **Pharyngeal pumping of dissected *C. elegans* in liquid.** Cut heads were placed in Dent's saline and the pharyngeal pumping was counted over time. Measurements were made by visual observations, counted for 30 seconds and expressed in Hz. Data are $\pm$ SEM collected over $\geq$ 2 observations; number of replicates $\geq$ 4. For comparison, the average pharyngeal pumping in the presence of 1 μM 5-HT is shown is dashed purple line.

2.4.15 Inhibitory effects of drugs on pharmacologically induced pharyngeal pumping of dissected *C. elegans*

The effects of compounds on 5-HT stimulated pharyngeal pumping were tested. Cut heads were exposed to 1 μM 5-HT for 10 minutes to stimulate pumping. Following this, they were transferred to a well containing 5-HT and the indicated treatments. Pharyngeal pumping was measured before and 10, 20, 30, and 50 minutes after transfer into the 5-HT plus treatment incubation. To probe for recovery, heads were placed in 1 μM 5-HT and the pump rate 5, 10 and 30 minutes after being transferred into recovery (that is 55, 65 and 80 minutes after the start of the experiment) was recorded. As a control, heads were exposed to 5-HT plus solvent throughout the duration of the experiment.

2.4.16 Extracellular recording from the pharynx of cut head preparation of *C. elegans*

Cut heads were prepared (Section 2.4.13.1) and transferred to the experimental arena by pipetting. Extracellular recordings were made with an electropharyngeogram (EPG) technique (Figure 2.7).

2.4.16.1 Preparation of a microelectrode

Non-filamented borosilicate capillary tube (Harvard apparatus) with outer diameter (OD) of 1.5 mm and internal diameter (ID) of 0.1 mm was pulled with a Narishige puller (model PC:10). The puller was set at 98.2°C for step 1 and 72.8°C for step 2 to make a tip of $\sim$ 10 μm. The needle was back-filled with Dent's using a micropipette filler (250 μm ID, 350 μm OD, World Precision Instruments).

2.4.16.2 Experimental set-up

The microelectrode was inserted into a microelectrode holder containing a silver wire. The microelectrode was inserted into a headstage (HS-2A Asoclamp) and carefully lowered using a micromanipulator (Burleigh) into a recording chamber filled with Dent's saline and resting on a stage of Axoscope 2 (Zeiss) microscope. The reference electrode was made with a glass capillary filled with 2 % agar in 3 M KCl. The reference electrode was placed in the recording chamber and connected to the amplifier headstage via a dish filled with 3 M KCl solution and a silver wire electrode. The cut head was placed in a recording chamber and a tight seal between the tip of the nose and the microelectrode was made by applying suction. The extracellular electrical signals from the pharynx were amplified by an Axoclamp-2B Microelectrode Amplifier, digitized by Digidata 1322A and recorded with Axoscope 9.2.

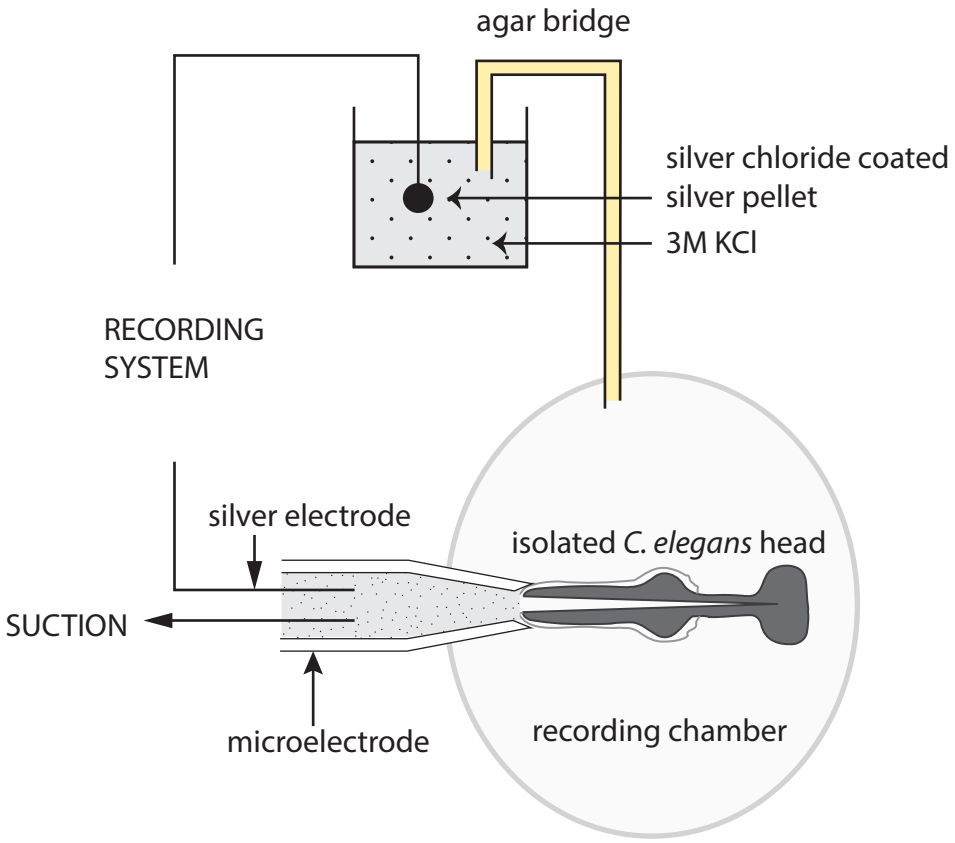


Figure 2.7: **Experimental preparation for extracellular recordings from the *C. elegans* pharynx.** A diagram showing the set-up used for electropharyngeogram (EPG) experimentation.

2.4.16.3 Experimental protocols

A cut head was placed in a recording chamber. The seal around the tip of the nose and the microelectrode was made and a worm was left for 5 minutes to acclimatise. Solutions changes were achieved by gravity perfusion with a flow rate of ~ 1 mL/min. The pharyngeal pumping was recorded during control perfusion, drug perfusion and recovery into buffer in 3 equal 5-minute blocks giving a total time of the recording of 15 minutes.

2.4.16.4 Data acquisition and analysis

A single EPG reflects a contraction-relaxation of the pharyngeal muscle. It consists of a series of peaks, including e and E or excitatory peaks, I or inhibitory peak as well as r and R, or repolarising peaks (Figure 5.1). The effects of exposure to drugs on three parameters were measured. (1) The pumping rate, which was derived by taking maximum pumping rate in a 10 second window. (2) The E/R ratio, which is the ratio between the amplitude of E and R spikes. This was measured by calculating the average of E/R ratios of all EPGs in the period of the maximum pumping. (3) The pump duration, which is the average duration of all EPGs in the period of the maximum pumping. If there were less then 10 EPGs, 10 consecutive peaks were taken to derive the final pump duration and E/R ratio value.

2.4.17 Microinjection to generate *C. elegans* transgenic lines

2.4.17.1 Preparation of a needle

Aluminosilicate capillaries SM100F-10 (1 mm external diameter, 0.5 mm internal diameter) needle was pulled with Narishige puller (model PC:10) using the following settings: step 1 at 99°C, step 2 at 79°C. The pulled needle was filled with 1 μL of injection mix and assembled into Transferman NK2 (Eppendorf) micromanipulator. Microinjection was performed with FemtoJet Microinjector (Eppendorf).

2.4.17.2 Generation of transgenic lines

2.4.17.2.1 Preparation of DNA microinjection mix

Test DNA plasmid was prepared with a Green Fluorescent Protein (GFP) co-injected marker to identify transgenic worms. Injection mix containing the test plasmid at (5 ng/L) and the co-injection marker (30 ng/L) were resuspended in ddH_2O and centrifuged at 15 000 rpm for 5 minutes to precipitate aggregates. One μL of the cleared DNA mix was back filled into the injection needle.

2.4.17.2.2 Injection

A single L4 + 1 was picked from NGM plate and immobilised by gently pressing it into a drop of Halocarbon oil 700 on 2 % agarose pad. The agarose pad was placed on a stage of Nikon Eclipse TE200 microscope and the syncytium of the anterior and/or posterior arm of the *C. elegans* gonad was injected with the DNA mix. Injected worms were gently liberated from the oil with a pick and placed on individual seeded NGM plates.

2.4.17.2.3 Screening of progeny of injected worms

The progeny of injected worms were viewed under the fluorescent microscope and GFP filter. Green F1 worms were picked individually onto separate seeded NGM plates and left to propagate. Plates containing green F2s were collected and kept as separate stable lines (Figure 2.8).

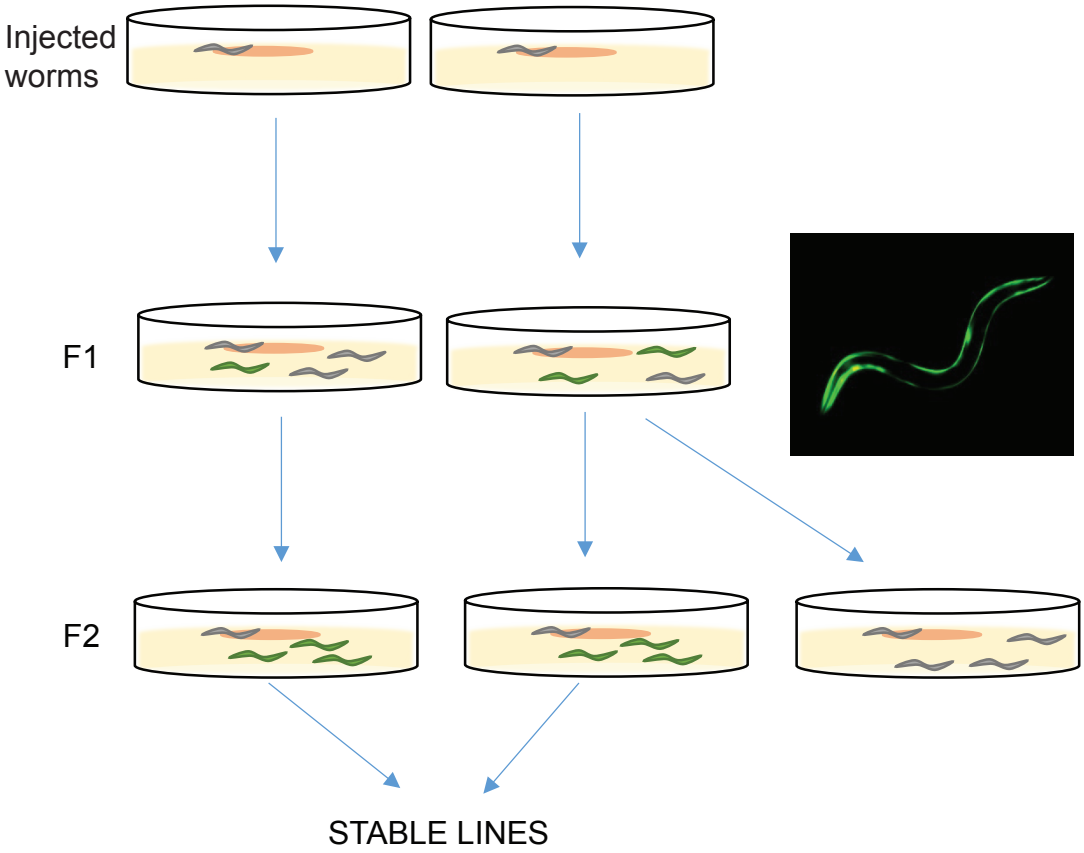


Figure 2.8: **Selection of transgenic worms.** L4 + 1 hermaphrodites are co-injected with selectivity marker (i.e. a vector containing gene encoding for GFP under the body wall muscle promoter). Green F1 worms were selected and kept separately; those that produce green worms were considered stable lines.

2.4.18 Determination of human nAChR expression in the *C. elegans* pharynx by staining with conjugated $\alpha 7$ selective antagonist FITC-α-bungarotoxin

2.4.18.1 Worm preparation

Worms were submerged in 3 mL of Dent's saline in 5 cm Petri dish. To ease dissection, they were paralysed by placing the dish at -20°C for 5 minutes. Following this, the tip of the nose was cut perpendicular to the head to allow the cuticle to roll back and expose the pharynx. Next, the cut just below the terminal bulb was made and liberated pharynxes were collected (Figure 2.9).

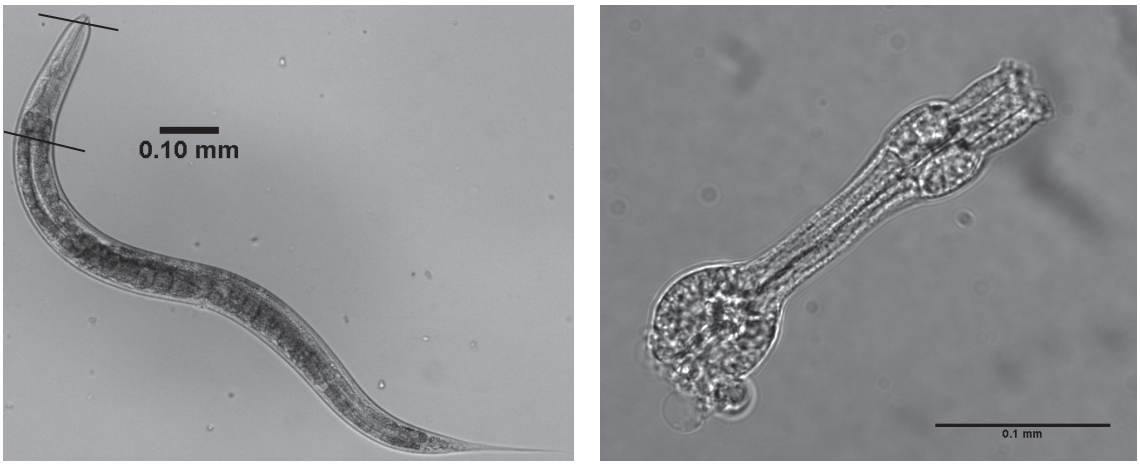


Figure 2.9: **Exposure of the *C. elegans* pharynx.** Using surgical blade, the cut was made at a tip of the nose and just below the terminal bulb (left image, black lines) to expose the pharynx (right).

2.4.18.2 Staining

Exposed pharynxes were placed in 1 mL of Dent's in a single well of a 12 well plate. FITC-αBgtx was added to the final concentration of 1 μg/mL. The plate was protected from light by covering with foil. The incubation proceeded for 1 hour at room temperature before being washed in 1 mL of Dent's.

2.4.18.3 Imaging

αBgtx treated pharynxes were transferred onto 2 % agarose pad and covered with a slip. Images were taken immediately at 10x magnification. The preparation was exposed for 0.1 s and FITC filter was applied on NIKON E800 fluorescence microscope. Staining was quantified in ImageJ by subtracting background fluorescence from the fluorescence in the terminal bulb.

Chapter 3

Effects of neonicotinoids on the behaviour and development of *C. elegans*

3.1 Introduction

Neonicotinoids are the most commonly used insecticides worldwide due to their high efficacy against pest insects (Section 1.1.5), selective toxicity to insect pests over mammals (Section 1.1.6) and advantageous physicochemical attributes (Section 1.1.4). The main disadvantage of these compounds is that they can be toxic to non-target species, including bees (Section 1.1.7). This undesired ecotoxicological effect spurred a debate over their environmental impact and revealed a necessity to further investigate their effects on other ecologically important organisms such as worms.

3.1.1 Ecological role of non-parasitic worms

Non-parasitic earth worms and nematodes, play an important biological role. They are the most abundant multicellular organisms on earth and are significant biomass contributors. In addition, they cycle nutrients contributing as much as 1/5 of all bioavailable nitrogen in soil (Neher 2001), promoting plant growth (Ingham et al. 1985) and soil fertility. They are also valuable bioindicators and have been used in the assessment of contaminated soil (Lecomte-Pradines et al. 2014).

3.1.2 Residues of neonicotinoids in soil

Neonicotinoids are commonly applied as seed dressing (Jeschke et al. 2011; Alford and Krupke 2017), due to a benefit of extended crops protection resulting in a reduction in the insecticide application frequency. However, on average, only 5 % of the active ingredient is taken up by and distributed throughout the developing plant (Sur and Stork 2003). The remainder enters the wider environment, including soils, where they can have a negative effect on inhabiting worm species.

The levels of neonicotinoids in terrestrial terrains vary depending on the composition and the physical properties of the soil (Mörtl et al. 2016; Selim, Jeong, and Elbana 2010; Zhang et al. 2018). Numerous studies investigated their levels in various soil types, following variable post planting period and generally report the sub μM concentrations (reviewed in Wood and Goulson (2017)). However, they persist in terrestrial terrains from few days to several years (reviewed in Goulson (2013)). Nitenpyram and thiacloprid typically remain there for several weeks, clothianidin for just over a year, whereas imidacloprid for several years. Long dissipation half-life and absorption capacity means that neonicotinoids may come in contact with soil- residing worms for prolonged time periods. Neonicotinoids can also enter the worm's interior by multiple routes. They may diffuse across the worm's cuticle, or be ingested with soil particles (Pisa et al. 2015). Exposure to residual neonicotinoids can have a negative impact on many aspects of worm's biology.

3.1.3 Cholinergic regulation of worm behaviour

The cholinergic system is the primary target of neonicotinoids in insects (Section 1.3.5). Acetylcholine is pivotal in regulation of worm behaviour (Section 1.4.4). Most of the current knowledge is derived from work on the soil nematode and model organism *C. elegans*.

3.1.3.1 Locomotion

C. elegans exhibits distinct locomotory behaviours in liquid and on solid medium (Section 1.4.2.2). In the liquid medium, it flexes back and forth, whereas on solid medium it crawls is a sinusoidal fashion. These behaviours are mediated by the body musculature composed of 95 muscle cells. The muscle cells are arranged into 4 muscle rows: a pair of longitudinal ventral rows and a pair of dorsal rows. Their function is under the control of the nervous system (Figure 3.1). There are 4 motor neuron classes synapsing onto the dorsal muscle (AS, DA, DB, and DD) and 4 innervating the ventral muscle (VA, VB, VC, and VD). Motor neurons belonging to class A, B and AS release ACh and are excitatory, whereas motor neurons of class D are inhibitory and release GABA (McIntire et al. 1993). Bending of the dorsal side is associated with excitation and contraction of the dorsal side and simultaneous inhibition and relaxation of the ventral side, whereas the reverse is true when the ventral side bends. A and B neurons not only innervate muscle, but also send out processes to the collateral

side, and synapse onto D, inhibitory neurons (White et al. 1986). By doing so, acetylcholine acts directly on the muscle to elicit contraction and indirectly to relax the opposite side. Taken together, this allows the bending of a particular portion of one and the relaxation of the opposing side of the body to enable the worm's locomotory activities. Whereas the propagation of the electrical signal down the axis of the muscle whilst on solid medium, results in forward movement.

3.1.3.1.1 Regulation of the direction of movement

The direction of worm's movement is controlled by so called command interneurons (Chalfie et al. 1985; White et al. 1986). There are 5 command interneurons, namely PVC, AVB, AVA, AVD and AVE. These make synaptic connections with appropriate motor neurons of the BWM. PVC and AVB innervate VB and DB neurons which regulate forward movement. AVA, AVD and AVE innervate VA and VB which regulate backward movement (Chalfie et al. 1985; White et al. 1986).

3.1.3.1.2 Sensory regulation of the locomotion

Locomotion can be regulated by the environmental cues detected by the sensory neurons which relay information into the locomotory circuitry. Locomotion on solid medium is greatly influenced by the presence of food (Dallière et al. 2017). Whilst on food, *C. elegans* exhibits two types of locomotory behaviour: dwelling and roaming. Dwelling is characterised by enhanced turning frequency but low movement speed rate, whereas roaming is associated with decreased turning frequency but higher movement speed. Upon transfer to the area with no food source, worms search for food evident by enhanced movement speed. *C. elegans* locomotion is also influenced by noxious stimuli and olfactory cues. For example, in response to a range of nociceptive stimuli ASH head neurons are activated (Hilliard et al. 2005). This leads to rapid and transient backward movement, followed by a change of direction in the forward movement (reviewed in Bono and Villu-Maricq 2005).

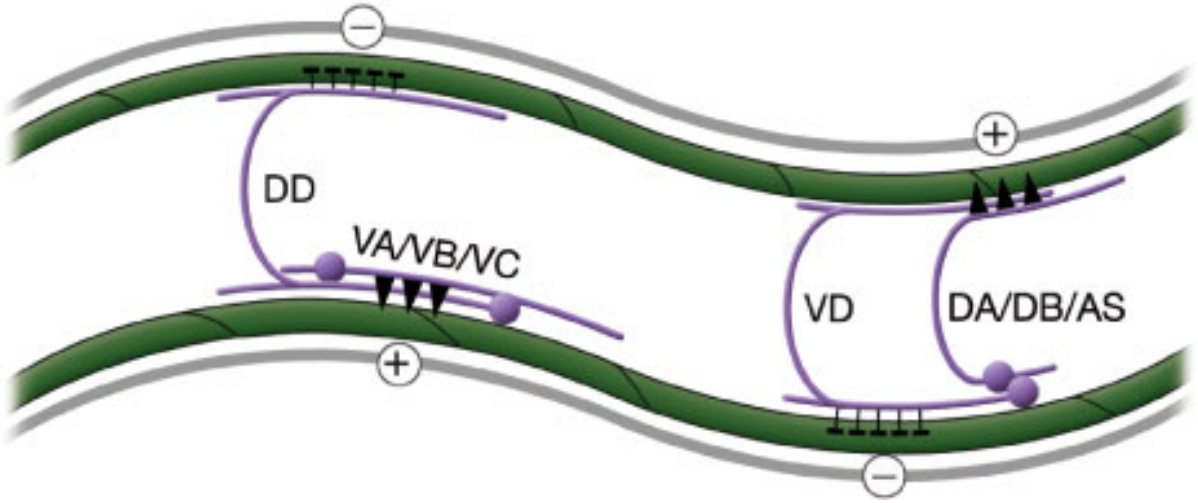


Figure 3.1: **Locomotory circuit in *C. elegans*.** Release of acetylcholine onto dorsal muscles (+) leads to their excitation and contraction. At the same time, acetylcholine activates GABAergic neurons contralaterally. Release of GABA leads to inhibition of ventral muscles. The signal propagates down the axis and leads to coordinated sinusoidal movement. Figure taken from www.wormatlas.org.

3.1.3.2 Egg laying

Egg-laying is controlled by the contraction of vulvar muscles under the influence of the nervous system (Figure 3.2). The main excitatory neurotransmitter is 5-HT (Waggoner et al. 1998) released from the HSNs. There are other neurotransmitters involved, such as excitatory acetylcholine (Trent, Tsuing, and Horvitz 1983) released from the VCs. In addition, there are four uv1 neuroendocrine cells linking uterus and vulva which release tyramine to inhibit egg laying (Alkema et al. 2005).

3.1.4 Effects of neonicotinoids on worms

There is limited literature regarding the effects of neonicotinoids on earthworms and nematodes (Section 1.1.7.2). In earth worms negative effects of neonicotinoid on the reproduction (Gomez-Eyles et al. 2009; Baylay et al. 2012; Kreutzweiser et al. 2008; Alves et al. 2013), avoidance (Alves et al. 2013) and locomotion (Dittbrenner et al. 2011) have been described. Studies by (Dong et al. 2014, 2017) revealed antiparasitic potential of these insecticides. Thiacloprid kills plant parasite *Meloidogyne incognita* with the LC_{50} of 24 μM (Dong et al. 2014), whereas thiacloprid inhibits its egg-hatching with the EC_{50} of 300 μM (Dong et al. 2014, 2017). They seem to have variable effects on *C. elegans*. Mugova et al. (2018) reports an inhibitory effect on motility of imidacloprid at concentration ranging from 120 μM to 2 mM. Thiacloprid seems to have an opposite effect. At concentrations from 2 to 40 μM it elevates locomotion in liquid of mixed developmental stage population of *C. elegans* (Hopewell et al. 2017). Variable effects of neonicotinoids on egg-laying are also reported. Low mM concentrations of clothianidin and thiacloprid inhibit egg-laying (Gomez-Amaro et al. 2015). In contrast, imidacloprid at a single concentration of 20 nM, elevates the number of egg-laid, but has no effect at 120 μM - 2 mM, suggesting this effect is not dose-dependent (Ruan et al. 2009).

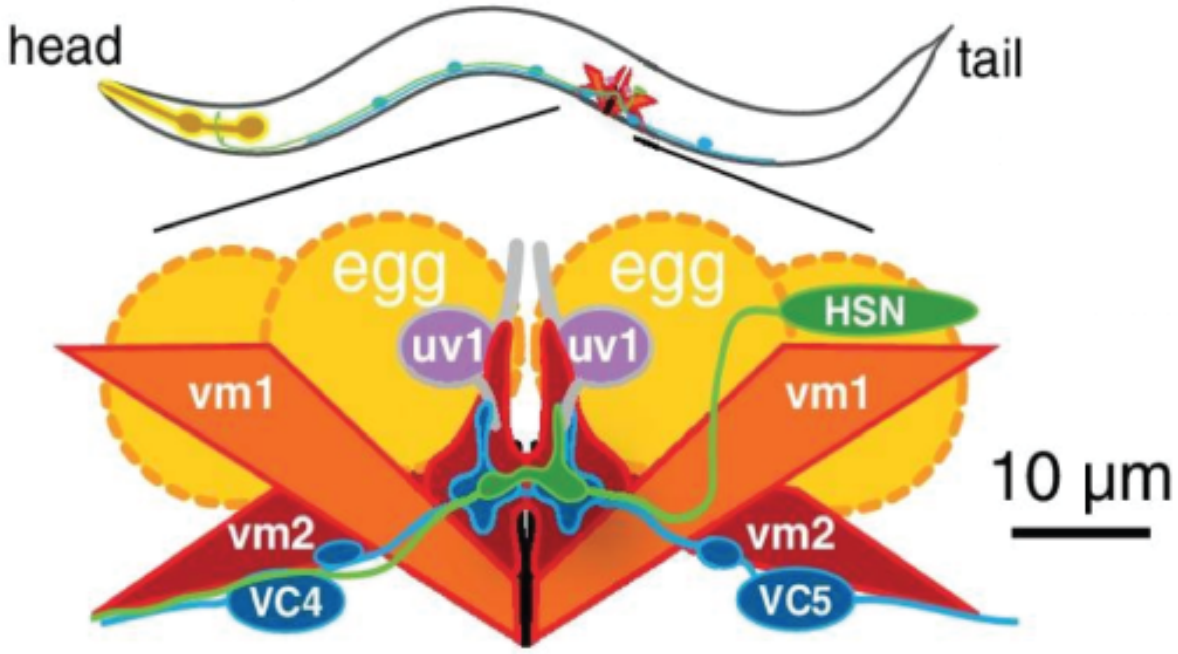


Figure 3.2: **Neuronal circuitry of *C. elegans* vulva.** Lateral image of the *C. elegans* hermaphrodite (top) and positioning of the vulva (bottom). Sixteen vulval muscles, out of which vm1 and vm2 are the most important, contract to expel eggs out. Hermaphrodite Specific Neurons (HSN) and Ventral C (VC) neurons synapse onto vm2 muscle. Uv1 neuroendocrine cells link the uterus and vulva and inhibit egg-laying. Image taken from Collins et al. (2016).

3.1.5 Chapter aims

The aim of this chapter is to better understand the effects of neonicotinoids on Nematoda representative *C. elegans*. Their impact on defined and well understood behaviours governed by the cholinergic transmission are tested and compared to the effects elicited by a classical nAChR agonist nicotine. This will be used to inform the potential risk of these environmental neurotoxins against Nematoda and on their mode of action.

3.2 Results

3.2.1 Effects of nicotine on thrashing

Upon immersion of *C. elegans* in liquid, it exhibits rhythmic swimming-like locomotory behaviour driven by the body wall musculature and inputs from the central nervous system (Section 3.1.3.1). This locomotory behaviour is known as thrashing. It can be easily scored and used as an assessment for the effects of acute exposure to nicotine and neonicotinoid on motility of *C. elegans*. Worms placed in untreated liquid medium retain a constant thrashing rate of ~40-45 repeats per 30s throughout the duration of the experiment (2 hours) (Figure 3.3). Addition of nicotine at concentrations ranging from 1 to 100 mM leads to dose-dependent inhibition of motility (Figure 3.3). The time course for the effects of nicotine varied dependent on the concentration. At doses eliciting partial paralysis (i.e. 10 and 25 mM) 2 "phases" of inhibition can be seen. The immediate one seen after 10 minutes which recovers slightly, and the second which reaches a steady state inhibition after 60 minutes. Nicotine at 100 mM led to a complete inhibition of thrashing. This effect was visible 10-minutes post exposure and sustained for 2 hours. The EC_{50} of nicotine on thrashing was 26.2 (Figure 3.3b). This low efficacy may be due to reduced bioavailability of nicotine in the worm.

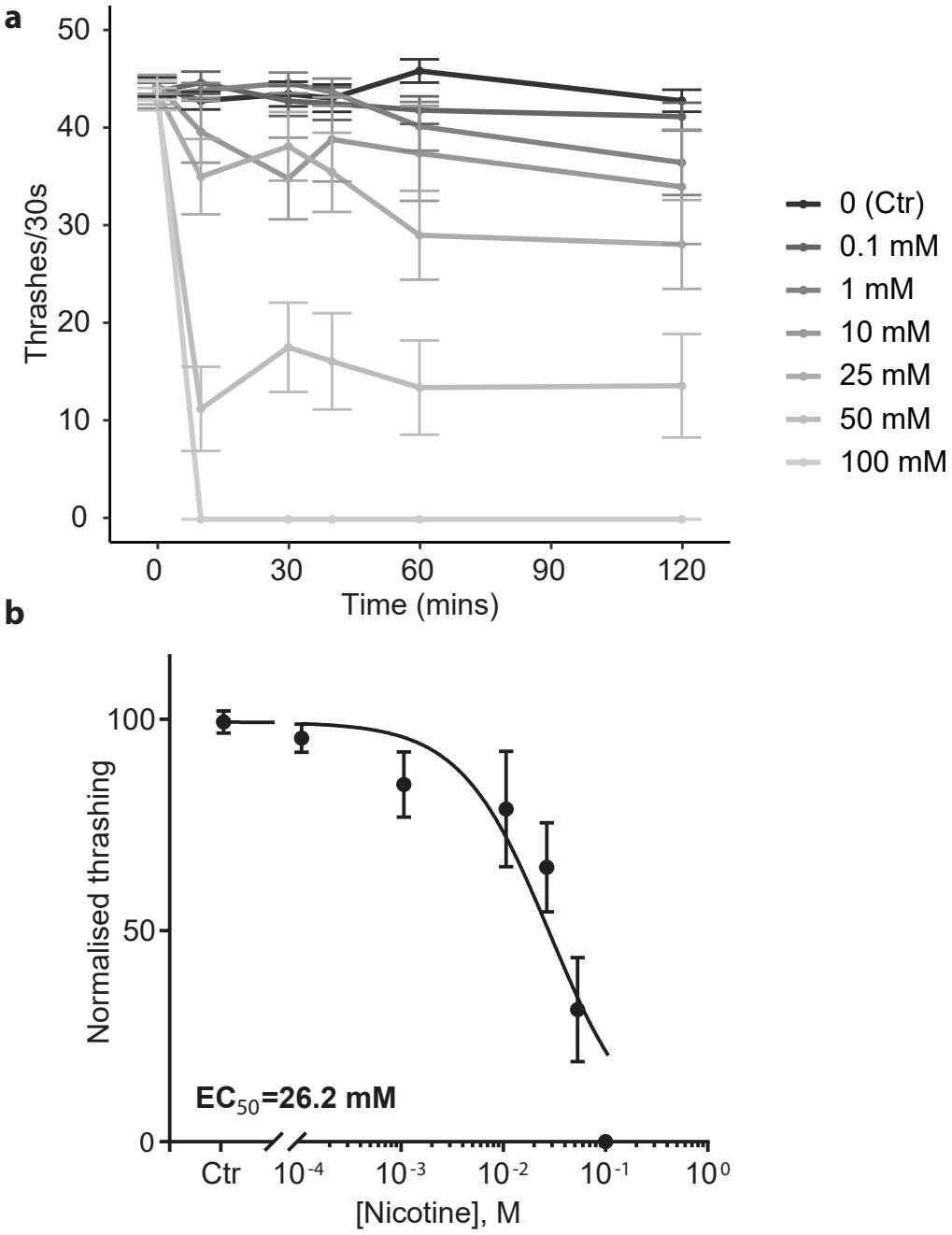


Figure 3.3: **The concentration and time dependence of the effects of nicotine on thrashing of *C. elegans*.** Wild type N2 worms were exposed to varying concentrations of nicotine. The number of thrashes were recorded for 30 seconds at the indicated time points (a). Concentration dependence of nicotine inhibition of thrashing on wild-type N2 worms (b). Dose-response curve were generated by taking the 60 minute time-points; that is when the steady-state inhibition of thrashing was reached, and expressed as % of control thrashing. EC_{50} values (dose at which thrashing was reduced by half) is shown. Data are mean $\pm$ SEM of $\geq$ 14 individual worms collected from experiments done on 3 days.

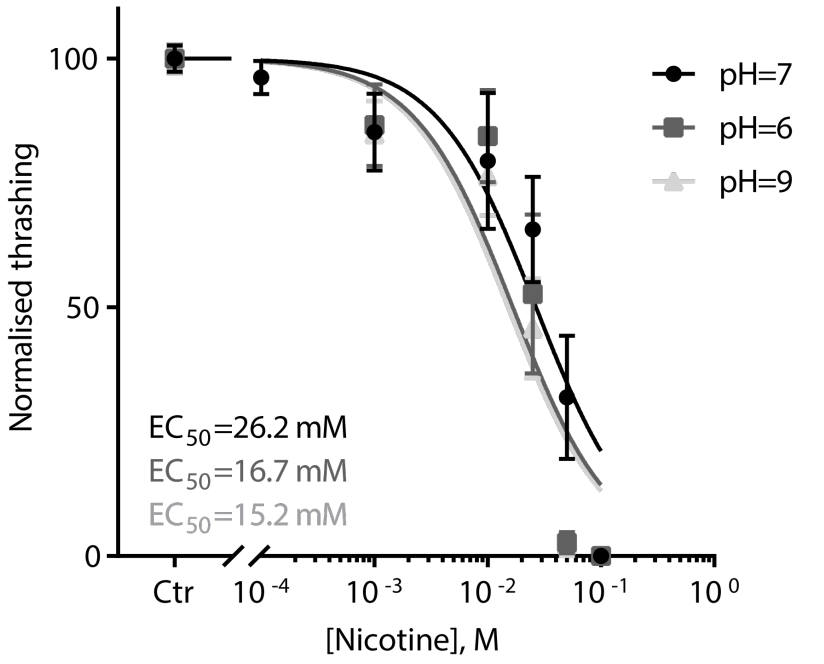


Figure 3.4: **Effects of pH on the concentration dependence for the effects of nicotine on *C. elegans* thrashing.** Dose-response curves for the effects of pH on efficacy of nicotine on thrashing. Wild-type worms were exposed to varying concentrations of nicotine whilst being suspended in a buffer with pH of 7, 6 and 9. The number of thrashes were scored for 30 seconds at 60 minute time-point. Data is expressed as % of control thrashing. EC_{50} values are shown. Data are mean $\pm$ SEM of $\geq$ 11 individual worms collected from paired experiments done on 3 separate days.

3.2.1.1 Effects of pH on nicotine induced inhibition of thrashing

Bioavailability of compounds might be impaired by the physicochemical properties of drugs, such as charge. Nicotine is a diprotic base, with pKa of pyridine ring of 3.12 and pKa of pyrrolidine ring of 8.02 (Ciolino et al. 1999). By altering the pH of liquid medium from 7 to 6 and 9, the equilibrium between charged and uncharged nicotine species shifts. One might predict this has an effect on the efficacy of nicotine. Indeed, The EC_{50} of nicotine at pH= 6 and 9 is slightly, but not significantly decreased in comparison to pH=7 16.7, 15.2 and 26.2 mM, respectively (Figure 3.4). Since pH of the external buffer does not have a marked effect on efficacy, all following experiments were performed at neutral pH.

3.2.2 Effects of the cuticle on nicotine induced inhibition of thrashing

The second factor limiting drugs' bioavailability is worm's cuticle. This idea is supported by the observation that the application of 0.1mM of nicotine on intact worm has no effects on thrashing (Figure 3.3), but when applied on the isolated body wall muscle or dissected worm, it induced large inward current and paralysis, respectively (Richmond and Jorgensen 1999; Matta et al. 2007). This suggests that the cuticle is a major physical barrier for drug entry. To provide a platform for investigation of the importance of the cuticle in drug permeability, *C. elegans bus-17* mutant strain was used. *Bus-17* is a GT13 glycosyltransferase mutant (Yook and Hodgkin 2007) exhibiting defective glycoprotein production resulting in abnormal surface coat and altered cuticular integrity. Mutation of *bus-17* genes have no significant effect on the thrashing frequency of *C. elegans*. Worms retained a constant thrashing rate of 40-45 thrashes per 30s over the duration of the experiment (Figure 3.5). Immersion in nicotine led to concentration dependent paralysis, but the potency of nicotine on the mutant vs the wild-type strain is almost 10-fold greater (Figure 3.5). Moreover, the inhibitory effects of nicotine at 10 and 25 mM on *bus-17* worms lacks 2 phases of inhibition seen previously. This might suggest that nicotine reaches the internal molecular targets more quickly.

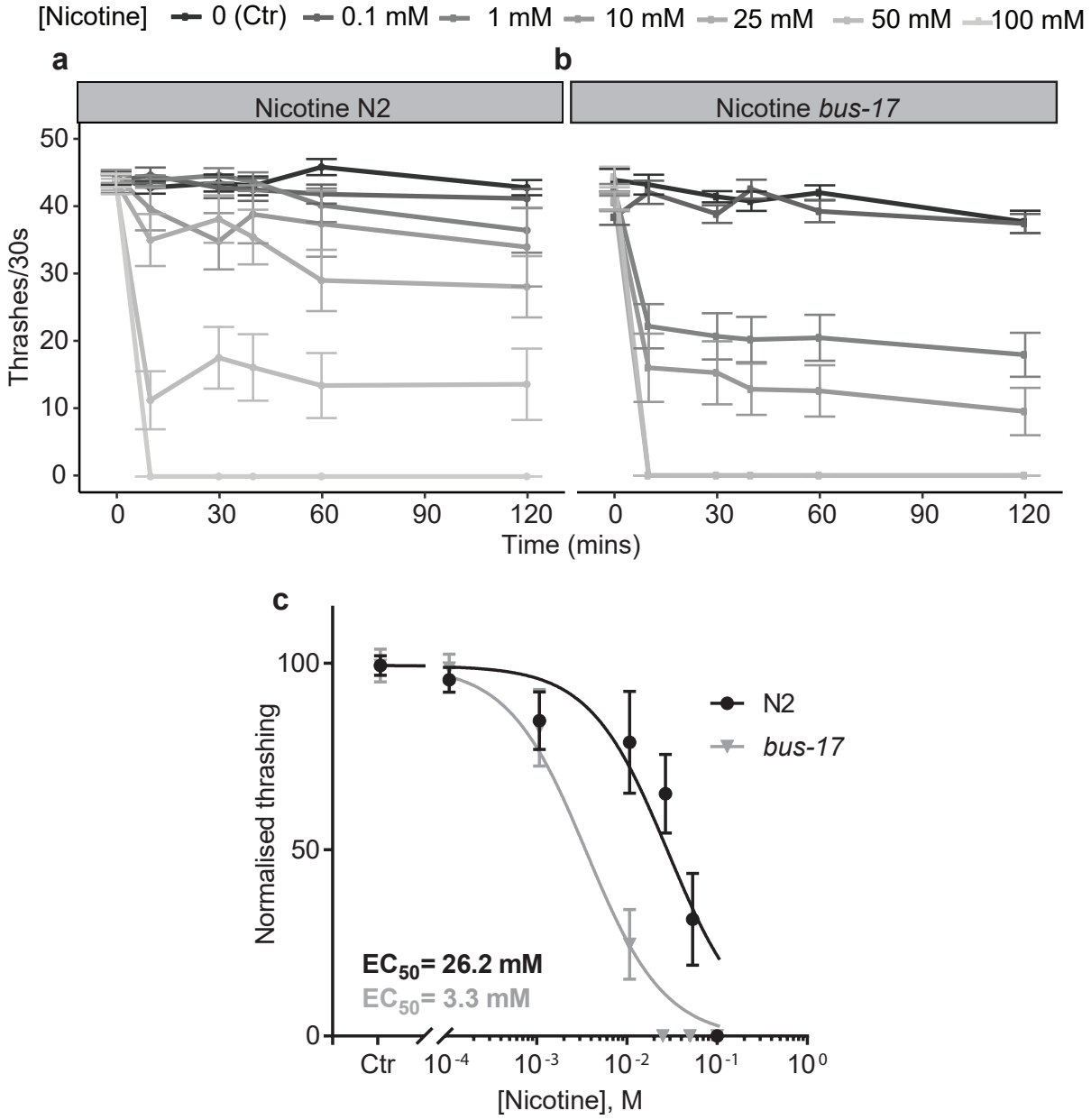


Figure 3.5: **The effects of the cuticle on the concentration and time dependence of nicotine inhibition of *C. elegans* thrashing.** N2 wild type (a) and *bus-17* mutant (b) worms were exposed to varying concentrations of nicotine. The number of thrashes were recorded for 30 seconds at the indicated time points. b) Concentration dependence of nicotine dependent inhibition of thrashing on N2 (black) and *bus-17* worms (grey). Dose-response curve were generated by taking the 120-minute time- points; that is when the steady-state of thrashing inhibition was reached, and expressed as % of control thrashing. EC_{50} values are shown on the graph. Data are mean $\pm$ SEM of $\geq$ 15 individual worms collected from paired experiments done on 3 days.

3.2.3 Effects of neonicotinoids on thrashing

To assess the effects of neonicotinoids on motility of worms in liquid, the thrashing experiment was repeated with nitenpyram, thiacloprid and clothianidin. Out of the three compounds tested, only nitenpyram at concentrations ranging from 1 to 100 mM induced concentration-dependent paralysis of N2 wild-type worms. Low water solubility of thiacloprid and clothianidin limited the maximum testable doses to 1.5 and 2.5 mM, respectively. Results in Figure 3.6, left panel show that at these doses neither of the two have an effect on thrashing of wild-type worm. To determine whether a cuticle also limits the bioavailability of neonicotinoids, experiments were repeated on *bus-17* mutant. Shift in potency of all compounds was noted (Figure 3.6 and 3.7). The EC_{50} of nitenpyram on wild-type increased by almost 12-fold on mutant worm (195.8 and 16.6 mM). Thiacloprid and clothianidin were with no effects on wild-type worms, but induced paralysis of the *bus-17* mutant with the EC_{50} of 377.6 μM and 3.5 mM, respectively. The time course for both clothianidin and thiacloprid have similar features: gradual increase in inhibition of thrashing with the maximal effect achieved after 1 hour followed by a slow retrieval. The gradual recovery might represent adaptation of the neuronal circuit for locomotion, desensitisation of receptors mediating the response, or precipitation of a drug (although no visual sigh of this were observed with exception of 1.5 mM thiacloprid after 45 minutes). The breakdown in liquid is unlikely, as both compounds have long half-live in water (Table 1.2) (Gilbert and Gill 2010).

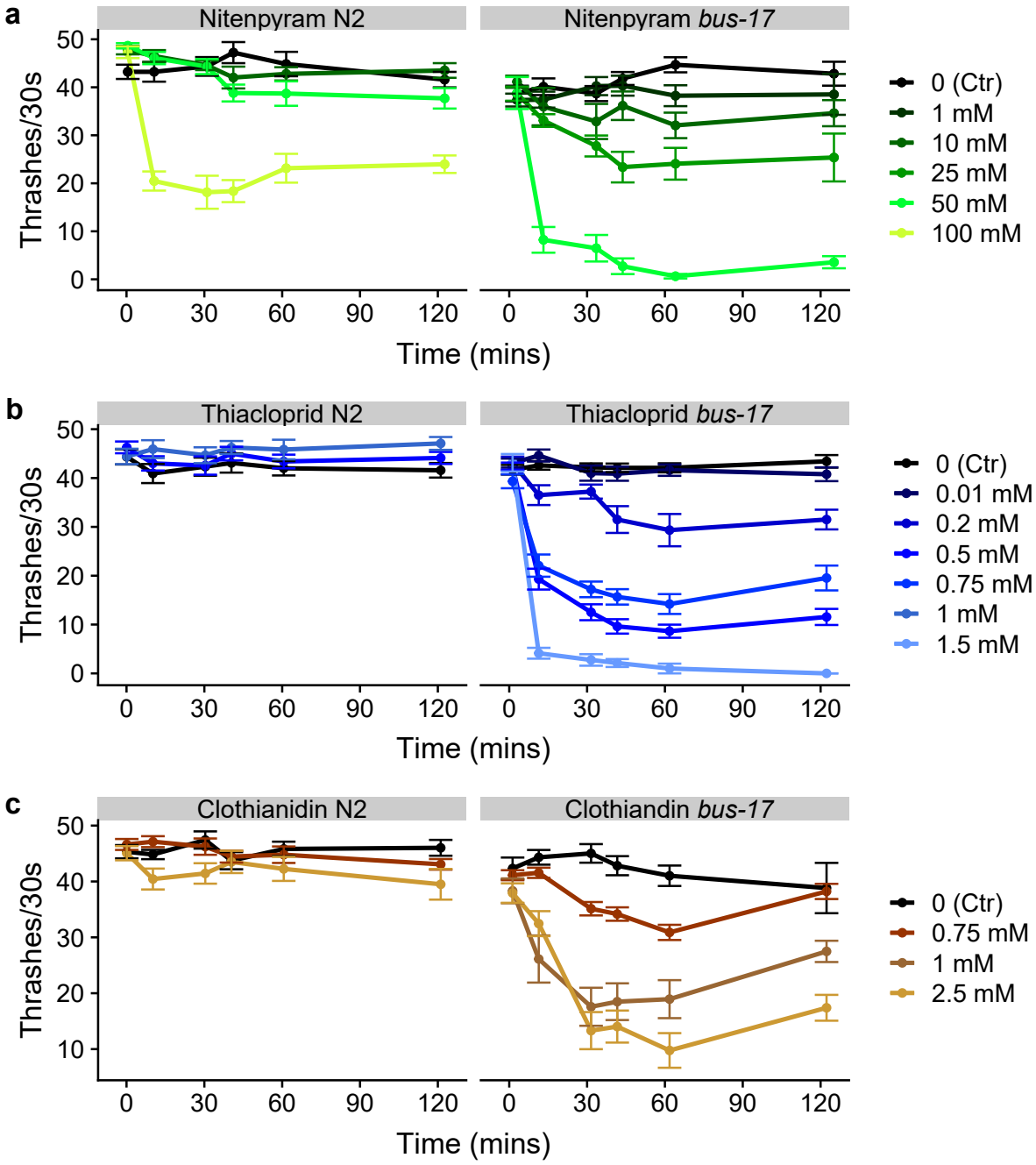


Figure 3.6: **The concentration and time dependence of neonicotinoids inhibition of *C. elegans* thrashing.** Wild type (left panel) and *bus-17* (right panel) worms were acutely exposed to varying concentrations of nitenpyram, thiacloprid, clothianidin or drug vehicle (0 (Ctr)). The number of thrashed over 30 seconds at indicated time points was scored. Data are mean ± SEM of ≥ 6 individual worms collected from paired experiments done on ≥ 2 days.

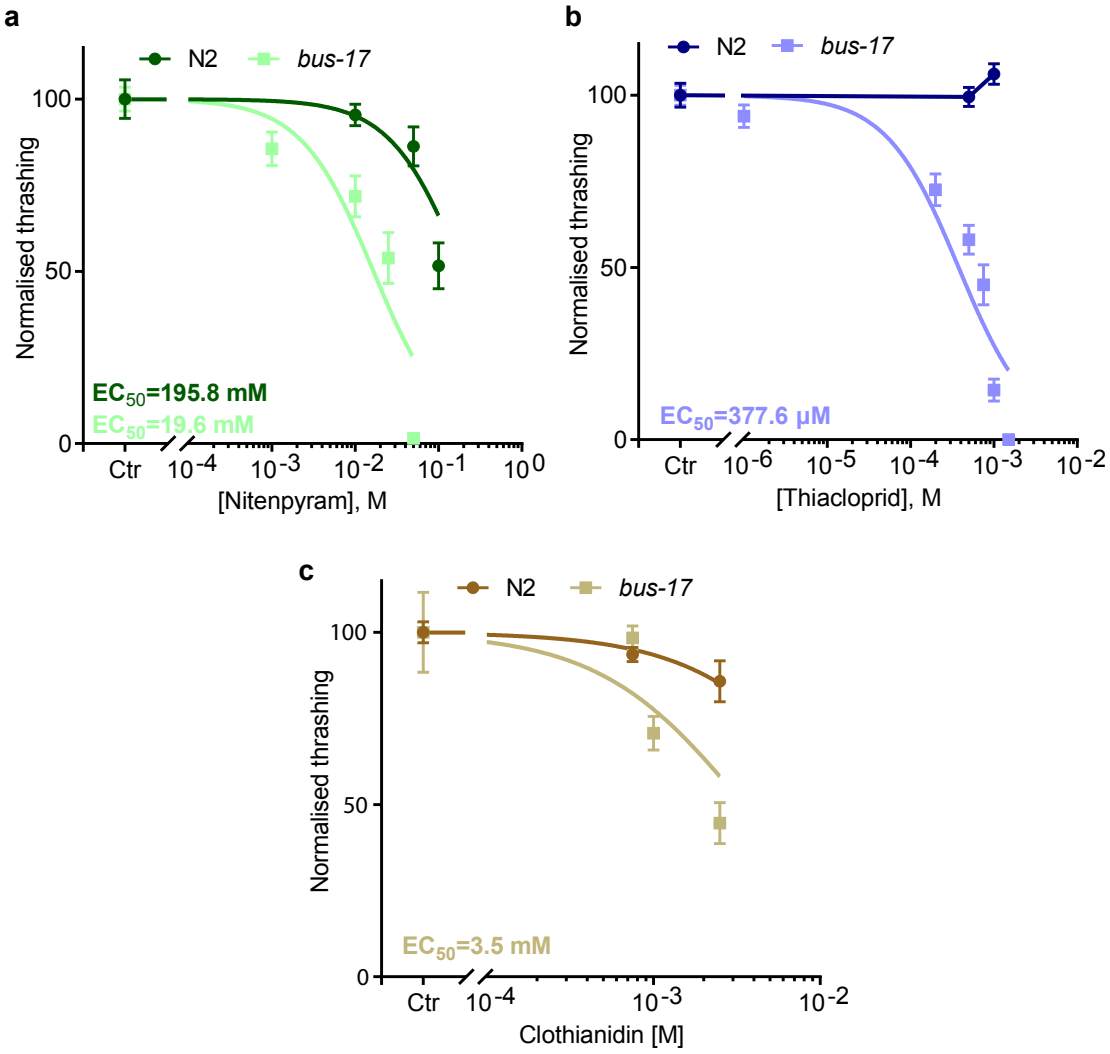


Figure 3.7: **Dose-response curves for the effects of neonicotinoids on *C. elegans* thrashing.** Concentration-response curves for the effects of nitenpyram (a), thiacloprid (b) and clothianidin (c) on thrashing of wild-type (black) and *bus-17* (grey) *C. elegans*. Dose-response curves were generated by taking 120-minute time-point for nitenpyram and 120-minute time points for thiacloprid and clothianidin; that is when the steady-state inhibition of thrashing was reached, and expressed as % control thrashing. Data and mean ± SEM. The EC_{50} for clothianidin are approximations, because the highest concentration tested (2.5 mM) inhibited thrashing by 55 % in *bus-17*.

3.2.4 Kinetic properties of nicotine- and neonicotinoid- induced inhibition of thrashing

To observe penetration properties of compounds, the thrashing experiment was repeated in the presence of drug doses inducing inhibition of thrashing. The observations at early time points were made to determine how quickly a maximum effect can be observed (Figure 3.8). High doses of nicotine paralysed all N2 wild-type and *bus-17* mutant worms within 6 minutes and time taken to paralyse 50 % of worms ($t_{1/2}$) of less than a minute. Since neonicotinoids did not induce full paralysis of wild-type worms, only the effects of thiacloprid and nitenpyram on *bus-17* mutant were investigated. The action of neonicotinoids was much slower when compared to nicotine. Worms became immobile after 1 hour of incubation, and the $t_{1/2}$ for both compounds was extended to 5 minutes.

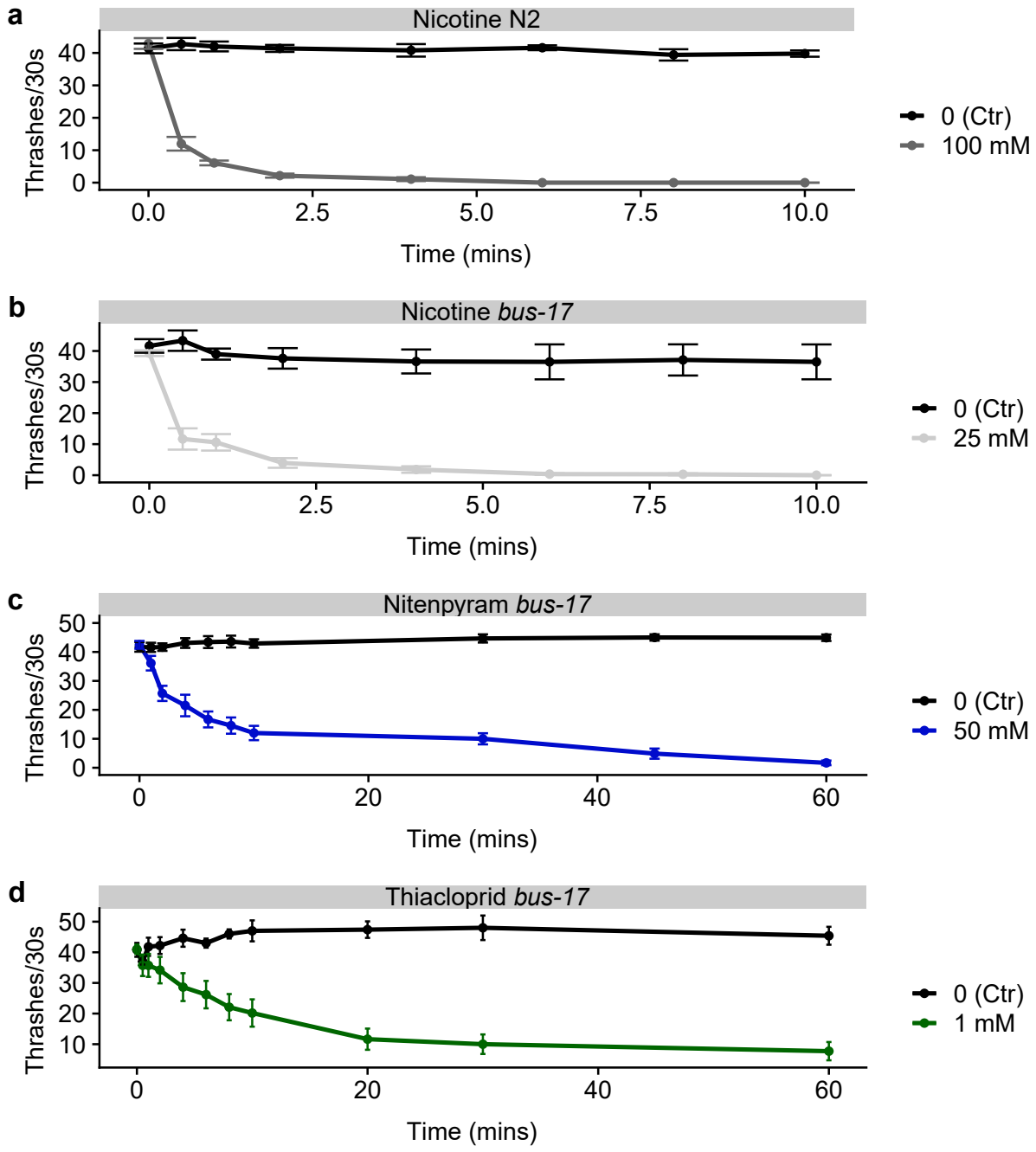


Figure 3.8: **The onset kinetics of nicotine and neonicotinoid induced inhibition of *C. elegans* thrashing.** Worms were submerged in drug concentrations at which complete paralysis was achieved; that is: a) N2 wild-type in 100mM nicotine, b) *bus-17* mutant in 25mM nicotine, c) *bus-17* mutant in 50 mM nitenpyram and d) *bus-17* mutant in 1mM thiacloprid. The number of thrashes over 30 seconds at the indicated time points were counted. Data are mean $\pm$ SEM of $\geq$ 4 individual worms collected from paired experiments done on 2 days. Note a different time scale in a, b compared to c and d.

Recovery assay gives an indication of how quickly the effects of compounds reverse. This reversal when drug inhibited worms are moved into a larger volume of drug free solution are thought to be due to a diffusion of a drug out of the worm or/and drug metabolism via various detoxifying systems (Lindblom and Dodd 2006). In this experiment, worms were placed in drug concentration that induced full paralysis for 20 minutes in the case of nicotine and 60 minutes in case of nitenpyram and thiacloprid. Once paralysis was achieved, worms were transferred to drug-free medium and the thrashing rates were monitored over time (Figure 3.9). Following the exposure to high concentration of nicotine and 2.5 hours of washing, a proportion of worms remained paralysed: 50% of wild-type and 25% of *bus-17* (data not shown). The remaining wild-type and *bus-17* worms recovered with $t_{1/2}$ of 1.5 hours and 50 minutes respectively. In contrast, all worms paralysed by nitenpyram or thiacloprid returned to normal basal thrashing within 2 hours of washing. The time taken for half recovery for both compounds was 1 hour.

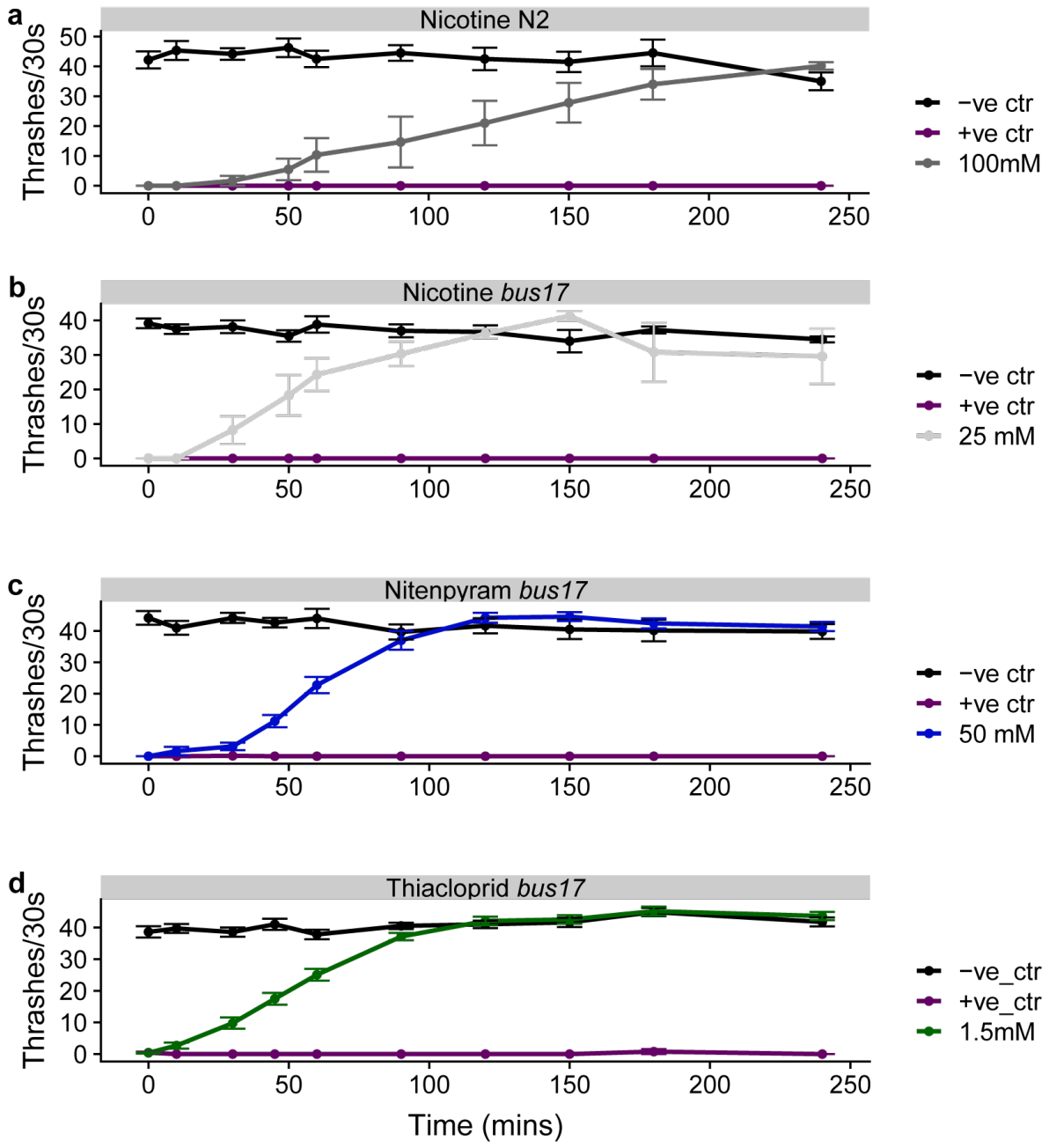


Figure 3.9: **Recovery kinetics of nicotine and neonicotinoid-paralysed *C. elegans*.** N2 wild-type and *bus-17* mutant worms were exposed to indicated concentrations of nicotine (a and b), nitenpyram (c) and thiacloprid (d). Paralysed worms were transformed to drug-free dish and recovery was scored by noting a number of thrashes / 30s. Only worms recovered are included in this analysis. Alongside these, worms were transferred from drug-containing to drug containing dish (+ve ctr) and from drug-free to drug-free dish (-ve ctr). Data are mean ± SEM of 8 individual worms collected from experiments done ≥ 2 days.

3.2.4.1 Effects of nicotine and neonicotinoids on *C. elegans* length

During acute and chronic exposure experiments, it was noted that a 4 hour incubation of worms with high nicotine concentrations had marked effect on morphology. Specifically, nicotine at concentrations inducing paralysis led to shrinking of the worm. To investigate this further and determine if neonicotinoids have the same effect, worms were exposed to either drug concentration inducing paralysis, or the highest possible testable concentrations. To maximise the concentration of drugs inside the worm, *bus-17* mutant was used in this experiment. The images of L4 + 1 incubated with nicotine or neonicotinoids for 4 hours were taken and the measurements of the length of the body were made. Exposure to 25 mM nicotine led to reduction in the body length. In contrast, neonicotinoids had no effect (Figure 3.10).

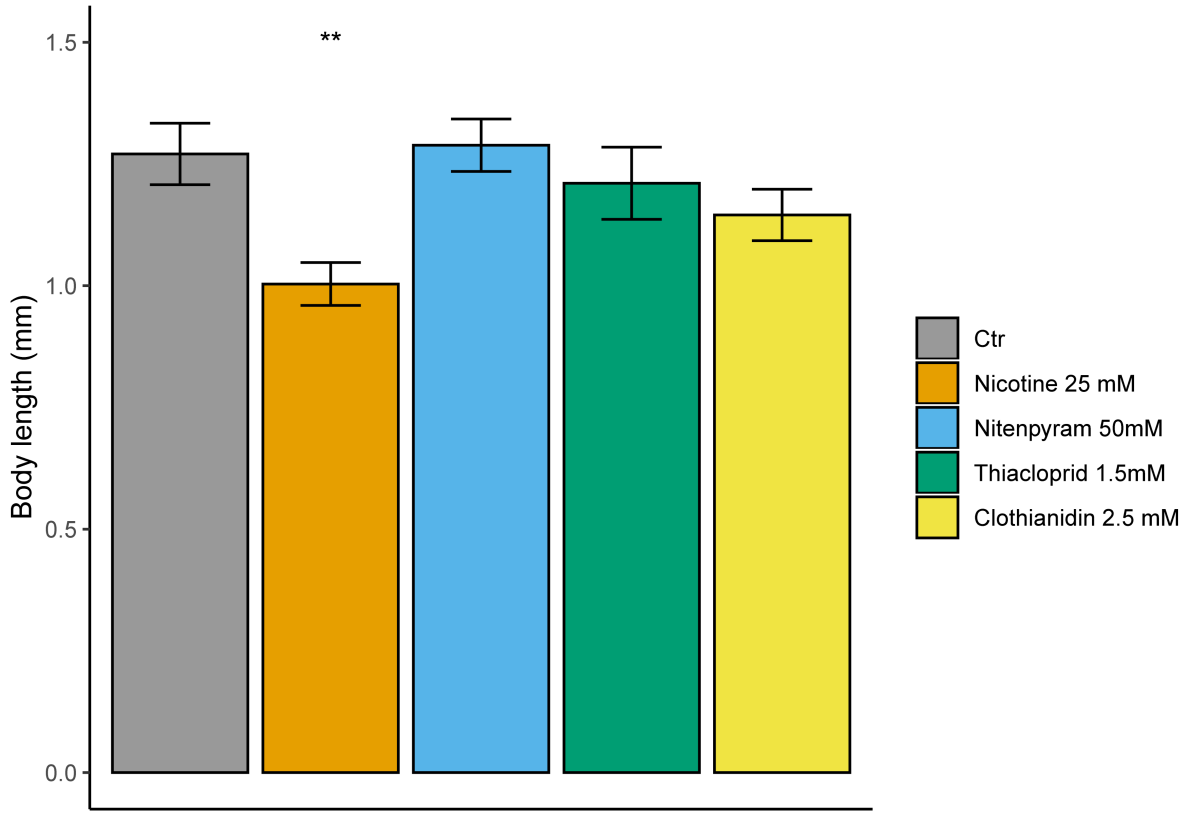


Figure 3.10: **Effects of nicotinic compounds on *C. elegans* body length.** *Bus-17* mutant was submerged for 4 hours in solution containing 25 mM nicotine, 50 mM nitenpyram, 1.5 mM thiacloprid or 2.5 mM clothianidin or vehicle control (Ctr). 4 hours later, the images of worms were taken and the body length measured. Data are mean $\pm$ SEM. Number of determinations $\geq$ 12 collected over 3 observations. One way ANOVA with Bonferonni corrections, $**$ P $\leq$ 0.01.

3.2.5 Effects of chronic exposure of *C. elegans* to nicotine and neonicotinoids on behaviour

Liquid assays allow for relatively short-term exposure. To test whether protracted exposure of worms provides a better paradigm for sensitivity on-plate assay was employed (Section 2.4.10). The concentrations used were ranged from 0.5 to 25 mM nicotine, 1 mM nitenpyram, 1 μM to 1.5 mM thiacloprid and 0.5 to 3.75 mM clothianidin. Worms were exposed to treatment for a period of 24-hours and their effects on locomotion and fertility were measured. This allows for prolonged drug exposure which may lead to accumulation of the drug inside the worm and increased efficacy of compounds on worm behaviour.

3.2.5.1 Effects of nicotine on avoidance

During the experimentation, an observation that the proportion of worms disappeared from nicotine containing plates was made. After 24 hour incubation with nicotine at concentrations $\geq$ 25 mM, the number of worms remaining on the plate was significantly reduced in comparison to the control (Figure 3.11). Closer observation revealed that in the presence of nicotine, worms escaped the experimental arena by crawling to the side of the plate. Neither of the three neonicotinoids had such effect (data not shown).

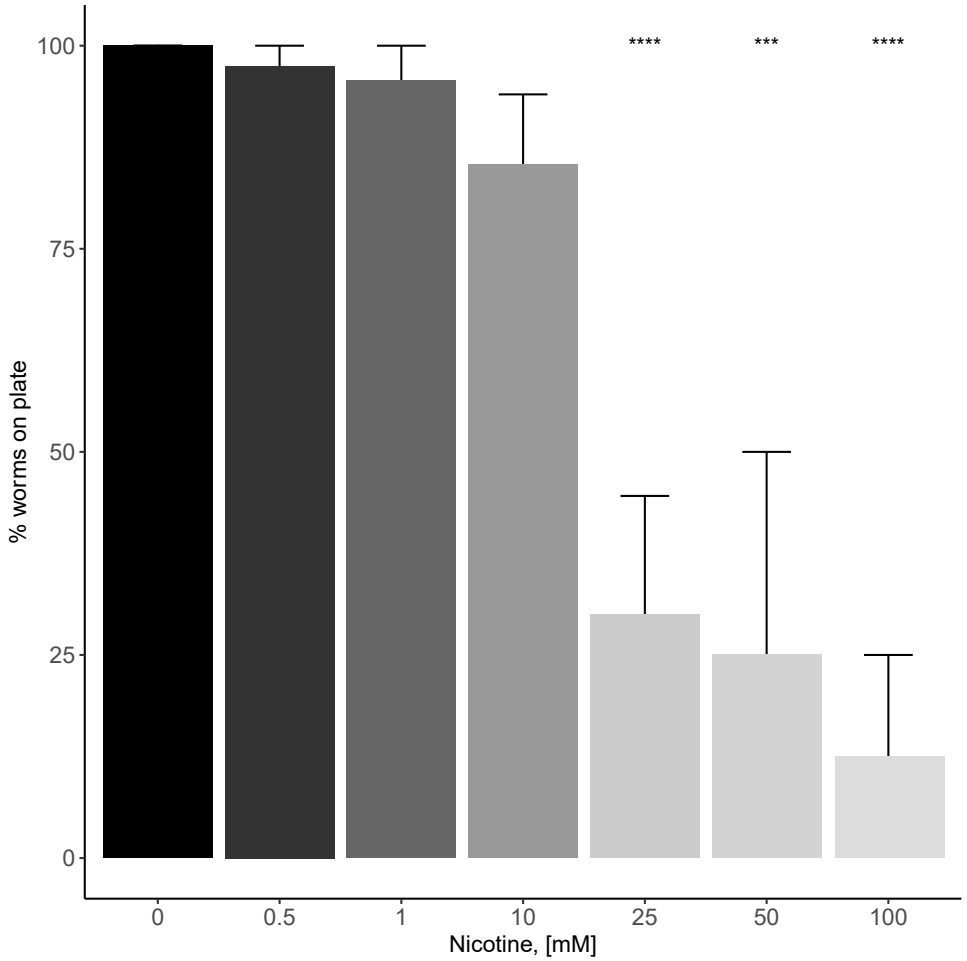


Figure 3.11: **The concentration dependence of the effects of nicotine on *C. elegans* avoidance.** 4-10 wild-type worms were placed on agar plate containing indicated nicotine concentrations or drug vehicle (0). 24 hours later, the % of worms remaining on the plate was scored. Data are mean $\pm$ SEM, collected from 2 - 4 individual experiments. One way ANOVA (Kruskal-Wallis test) with Sidak Corrections, $***P \leq 0.001$, $****P \leq 0.0001$.

3.2.5.2 Effects on body bends

Whilst on solid medium, *C. elegans* exhibits sinusoidal movement (Section 1.4.2.2). This can be quantified by counting a number of forward body bends per unit of time and is a measure of the motor function. The presence of food modifies this behaviour (Section 3.1.3.1.2), therefore the measurements were made on treatment - soaked solid medium containing no OP50 food patch (Section 2.4.10).

Untreated wild-type worms move at a rate of 39 body bends per minute (Figure 3.12, left panel). This is reduced to 33 bends per minute in *bus-17* mutant (Figure 3.12 right panel), due to a reduced traction of the body on agar medium (Yook and Hodgkin 2007). The body bends of wild-type *C. elegans* was altered by nicotine with with the EC_{50} of 3.6 mM, whereas nitenpyram, thiacloprid and clothianidin had no effect (Figure 3.12 and 3.13). In contrast, the body bends rate of *bus-17* mutant was reduced by all compounds, except for up to the 1 mM nitenpyram. The EC_{50} for the effects of nicotine and clothianidin was 1.6 and 3.3 mM, respectively. Thiacloprid was the most potent with the EC_{50} of 721.2 μM (Figure 3.13).

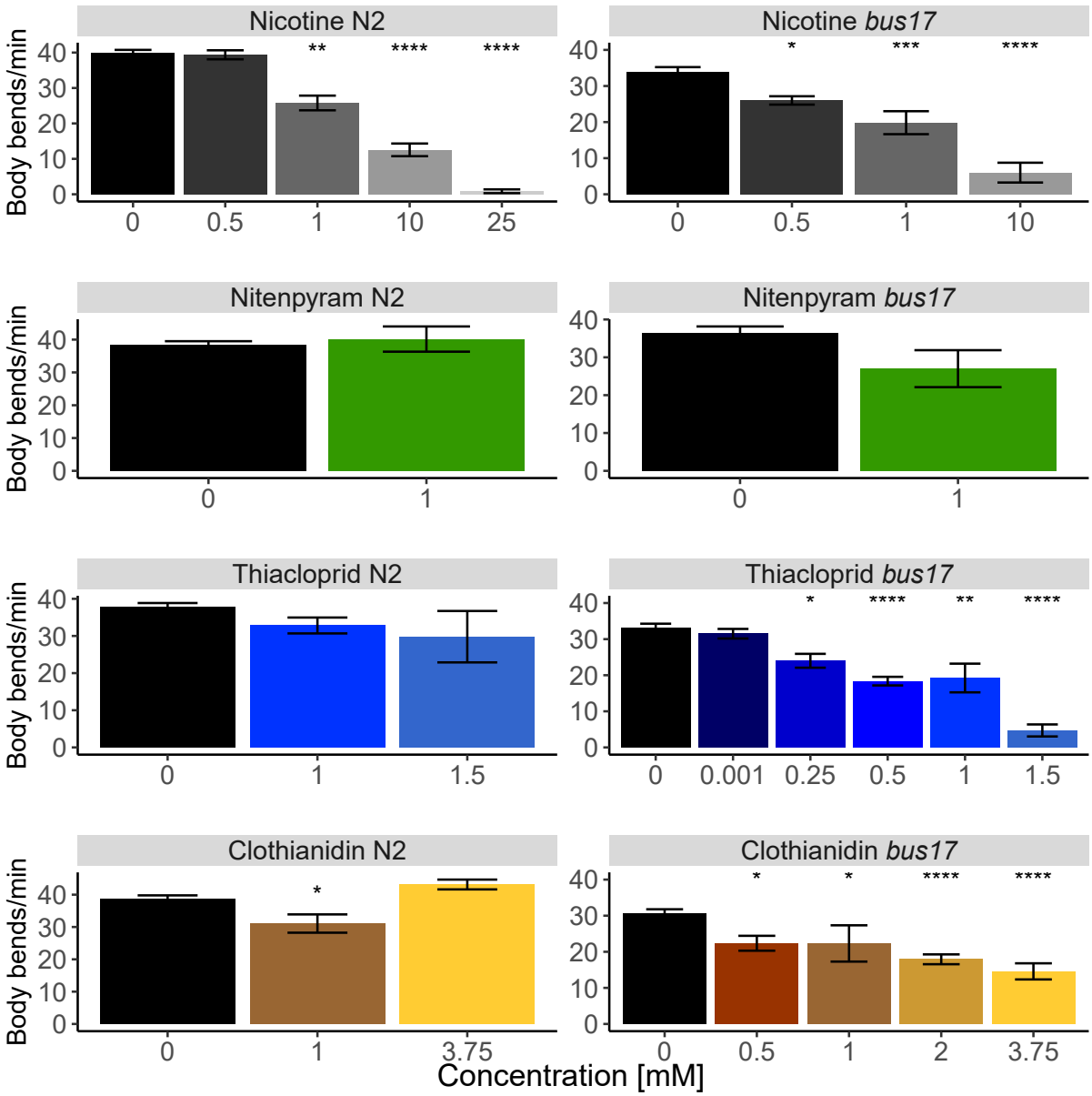


Figure 3.12: **The concentration dependence for the effects of nicotine and neonicotinoids on body bends of *C. elegans*.** N2 wild-type (left panel) and *bus-17* mutant (right panel) worms were exposed for 24 hours to varying concentrations of nicotine, nitenpyram, thiacloprid, clothianidin or drug vehicle (O), incorporated into solid medium. Body bends were counted by visual observation. Data are mean ± SEM of ≥ 5 individual worms collected from ≥ 3 paired experiments. One way ANOVA (Kruskal-Wallis test) with Dunnett's Corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

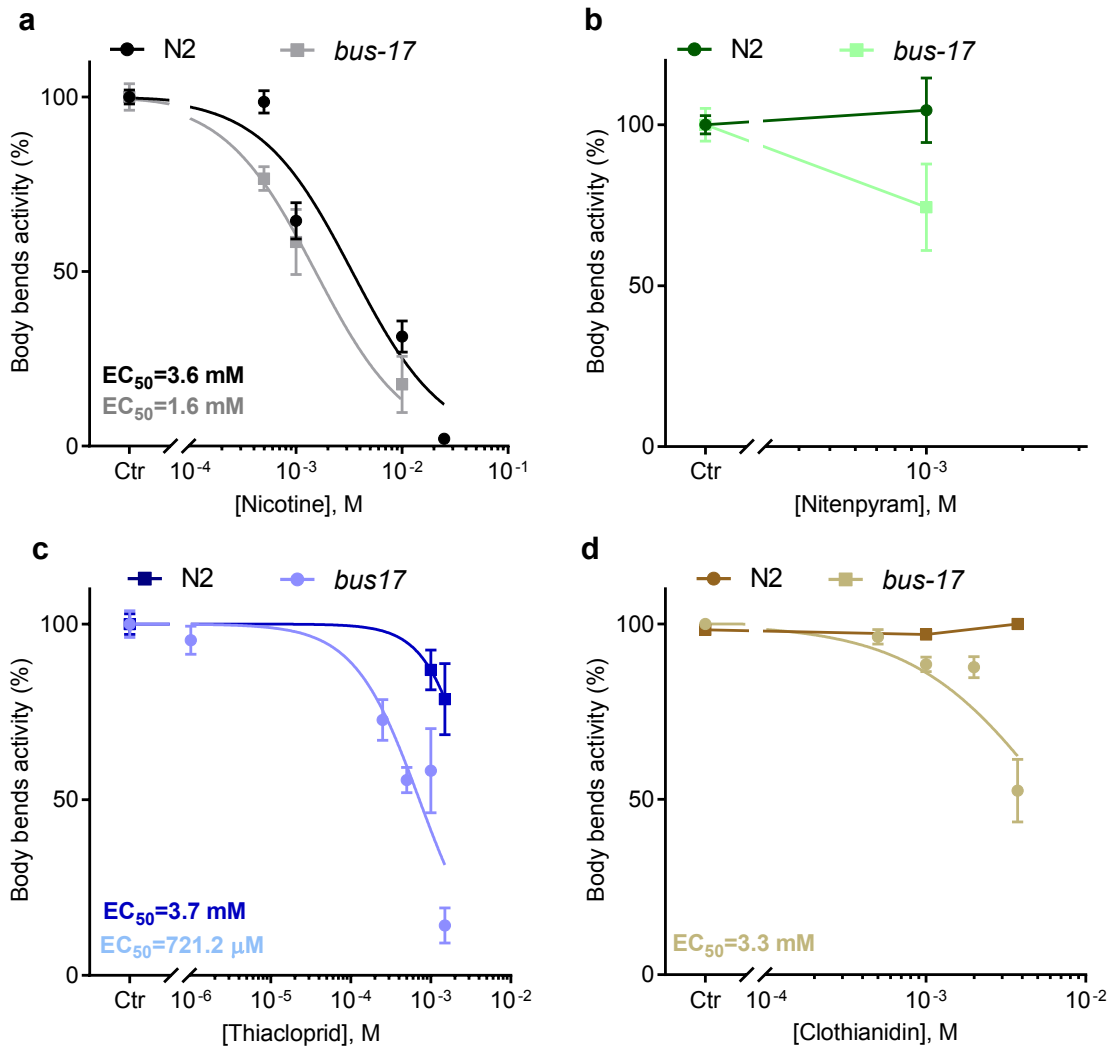


Figure 3.13: **The concentration dependence curves for the effects of nicotine and neonicotinoids on *C. elegans* body bends.** Concentration-response curves for the effects of nicotine (a), nitenpyram (b), thiacloprid (c) and clothianidin (d) on body-bend rates of wild-type N2 and *bus-17* mutant *C. elegans*. Body bend rates are expressed as a % of control activity. Data are mean ± SEM. The EC_{50} of thiacloprid on N2 and clothianidin on *bus-17* is an approximation, as at highest concentrations tested (1.5 mM thiacloprid and 3.75 mM clothianidin) the maximum inhibition observed was 21 and 42 %.

3.2.5.3 Effects on egg laying

On-plate experiments allow to assay for other aspects of *C. elegans* biology such as egg-laying (Section 1.4.2.3). The number of eggs laid per worm in the presence of nicotine and neonicotinoids over a period of 24 hours was counted and compared to the control (Figure 3.14 and 3.15). Both N2 wild-type and *bus-17* mutants lay $\sim$ 95 eggs/day/worm. No effects on wild-type worm of either compound was observed. However, egg-laying rate of *bus-17* mutant was reduced by low mM concentrations of thiacloprid and clothianidin.

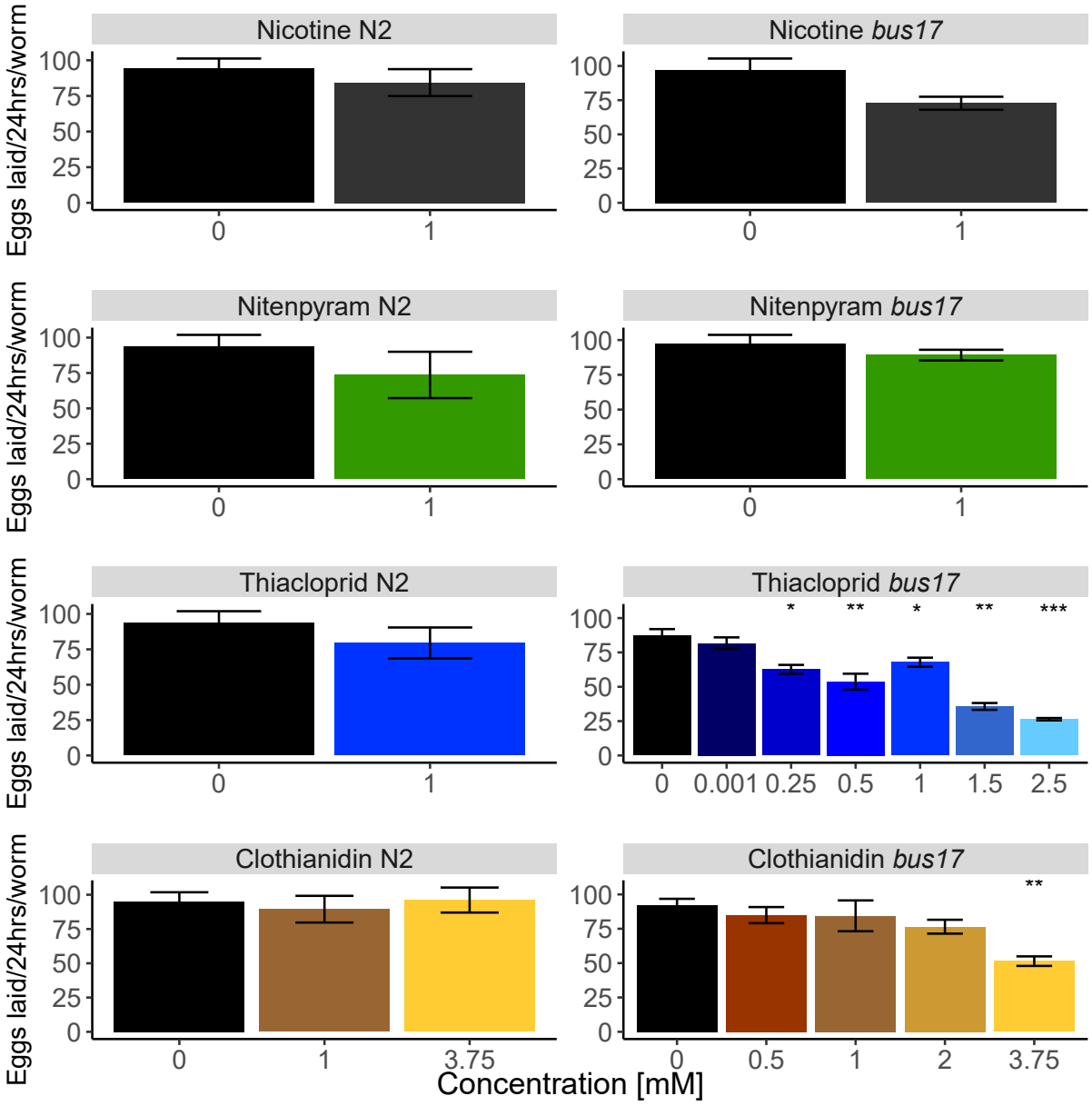


Figure 3.14: **The concentration dependence for the effects of nicotine and neonicotinoids on egg-laying of *C. elegans*.** Wild type (left panel) and *bus-17* (right panel) worms were exposed for 24 hours to varying concentrations of nicotine, thiacloprid, clothianidin or drug vehicle (0), incorporated into solid medium. Number of eggs laid in 24 hours were counted and expressed as eggs laid per worm. Data are mean ± SEM collected from ≥ 8 individual on ≥ 2 days. One way ANOVA (Kruskal-Wallis test) with Dunnett's Corrections, * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.

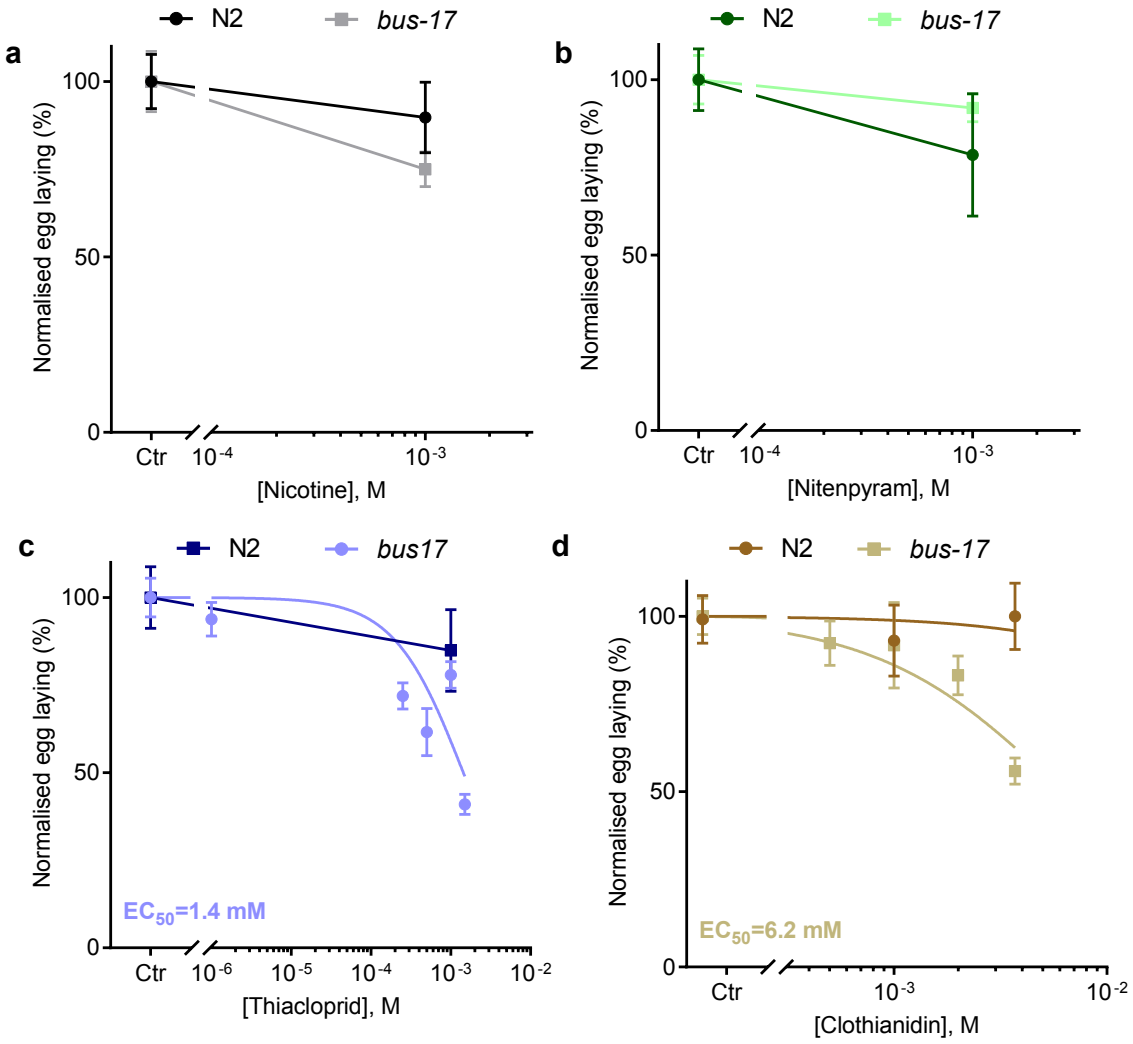


Figure 3.15: **Dose-response curves for the effects of nicotine and neonicotinoids on egg-laying of *C. elegans*.** Concentration-response curves for the effects of nicotine (a), nitenpyram (b), thiacloprid (c) and clothianidin (d) on egg-laying of N2 wild-type and *bus-17* mutant *C. elegans*. Egg laying is expressed as a % control activity. The EC_{50} for clothianidin is an approximation, as at the highest concentration tested (3.75 mM), the maximum response observed was 44 %. Data are mean ± SEM.

3.2.5.4 Effects on egg hatching

Eggs laid on the plate hatch after 9 hours of exo-utero development (Figure 1.8). To investigate the effects of nicotine and neonicotinoids on egg-hatching L4 + 1 worms were incubated with nicotine and neonicotinoids. After 24 hours of incubation, they were removed from the experimental plate, leaving the progeny and eggs behind. After further 24 hours, the number of unchanged eggs and larvae present were counted to derive the % hatching rate. Almost 100 % of eggs laid by N2 and *bus-17* hatched (Figure 3.16). Neither compound had an effect on hatching of N2 worms (Figure 3.16, left panel). However, thiacloprid at 1.5 mM and clothianidin at 2 mM reduced the proportion of hatched eggs of *bus-17* worms by 19 and 13 %, respectively (Figure 3.16, right panel).

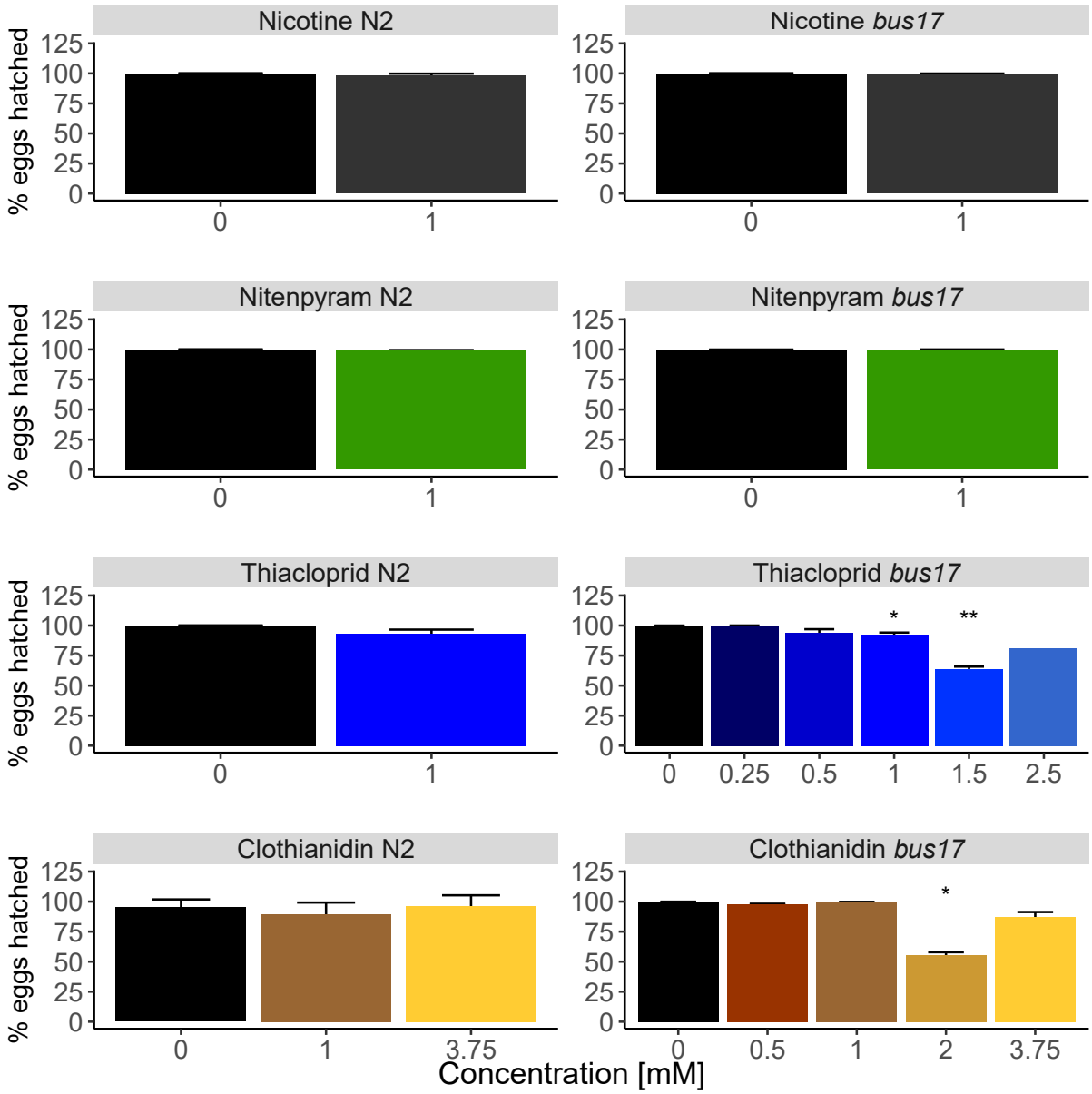


Figure 3.16: **The concentration dependence for the effects of nicotine and neonicotinoids on *C. elegans* egg-hatching.** N2 wild-type (a) and *bus-17* mutant (b) worms laid eggs in the presence of varying concentrations of nicotine, thiacloprid, clothianidin or drug vehicle (0). After 24 hours adult worms were removed and the eggs left behind. Number of unhatched eggs and larvae were counted 24 hours later. Data are mean $\pm$ SEM, of ≥ 2 paired experiments performed on $\geq$ days. One way ANOVA (Kruskal-Wallis test) with Dunnett's Corrections, $*$ P ≤ 0.05, $**$ P ≤ 0.01.

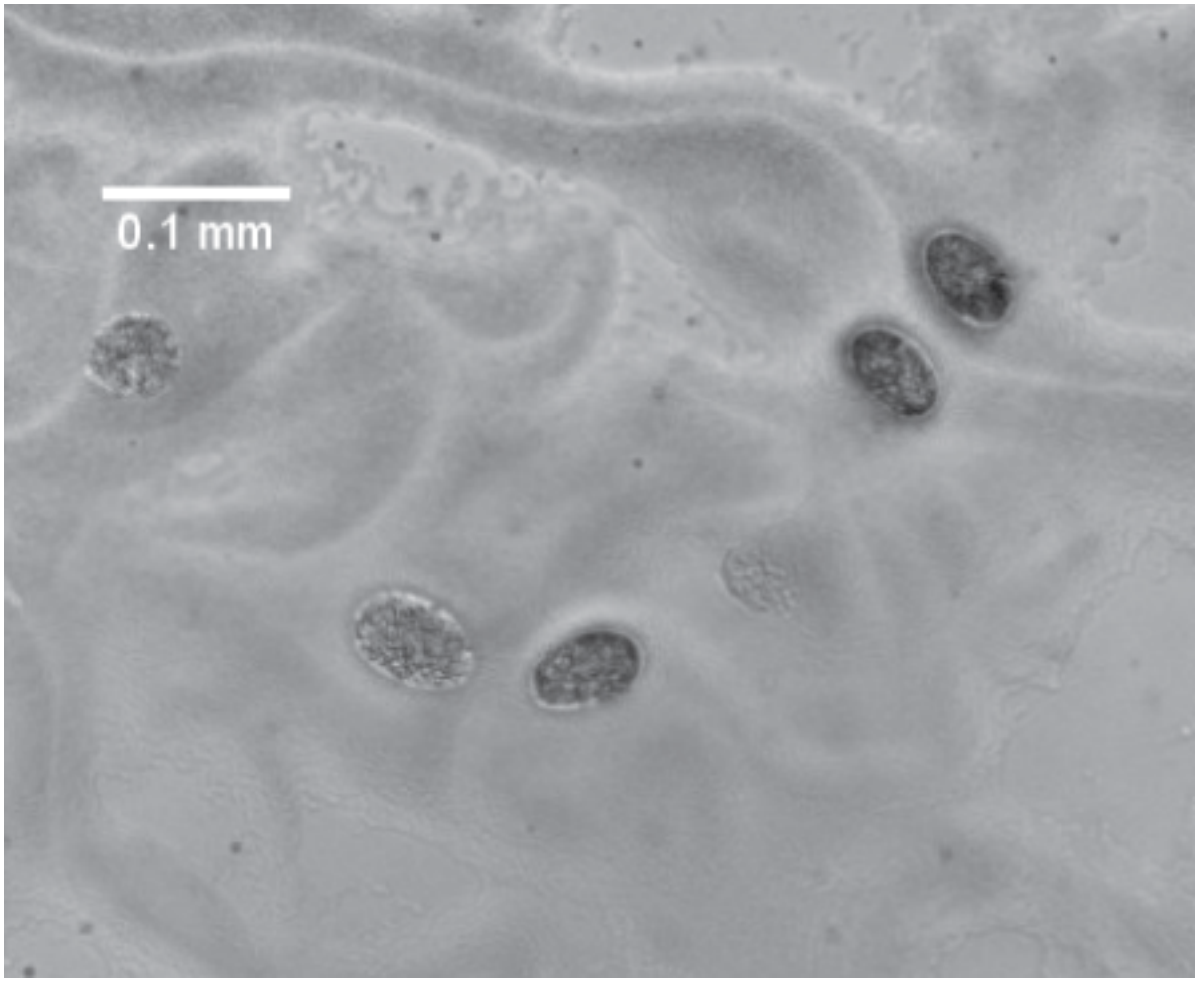


Figure 3.17: **Effects of thiacloprid and clothianidin on *C. elegans* egg-hatching.** The appearance of unhatched eggs laid by *bus-17 C. elegans* mutant in the presence of 1.5 mM thiacloprid.

To investigate whether compounds hinder the hatching of larvae, images of unhatched eggs were taken. As seen in (Figure 3.17), the eggs laid in the presence of thiacloprid and clothianidin are granular in appearance with no worm inside. This suggests thiacloprid and clothianidin interfere with the process of fertilisation or early developmental processes.

Figure 3.18: **Effects of nicotine and thiacloprid on larval development of *C. elegans*.** Eggs were laid by N2 wild-type worms on medium containing 1 mM nicotine or 1 mM thiacloprid. 72-hours later, the images of the progeny were taken. Worms developing in the presence of treatment are visibly smaller in comparison to the control.

3.2.5.5 Effects on development

Eggs that are laid on plate with a food source, hatch and develop into adults in three days. During the on-plate assay an observation was made that there were smaller worms present on the plate containing nicotine and thiacloprid (Figure 3.18). To investigate whether this was an effect of the drugs on the timing of development, larval development of age-synchronized progeny was made.

A synchronous population of L4 + 1 worms laid eggs on drug-treated plate. 2 hours later, the adults were removed. The development of the progeny was observed. The number of worms in each developmental stage, namely L1, L2, L3 and L4 were counted at days 1, 2, 3 and 6 days post egg laying. Clothianidin or nitenpyram at 1mM showed no effect as the proportion of each developmental stage shifted in parallel with N2 (data not shown). In contrast thiacloprid and nicotine slowed the larval development of worms (Figure 3.19). This difference was most clearly observed at day two. Almost all control worms reached L3 stage in control plate. 50 % of thiacloprid exposed worms were L3 and the rest were still at the L2 stage. Nicotine had a greater effect with almost all the worms being L2. This suggests L2/L3 transition was disturbed. All worms reached adulthood by day 6 of their life.

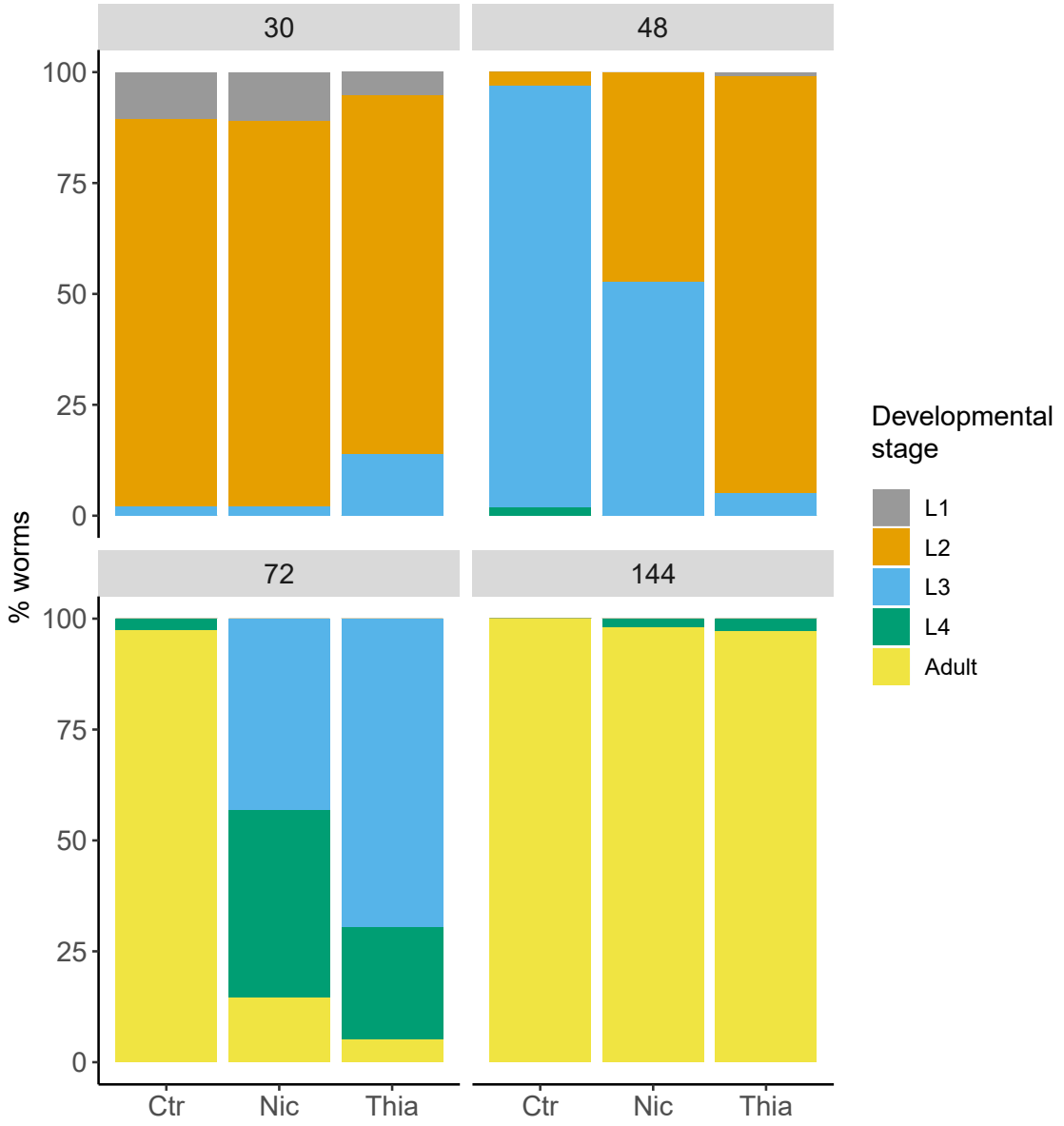


Figure 3.19: **Effects of nicotine and thiacloprid on the development of *C. elegans*.** N2 wild-type worms laid eggs on plates dosed with 1mM thiacloprid, 1mM nicotine or drug vehicle (Ctr). Larval development in the presence of drugs was monitored over time. Worms were assigned to each one of 5 life-stages, namely L1, L2, L3, L4 and gravid adults. The fraction of worms in each stage as a % of total population at time point: 30 hours (day 1), 48 hours (day 2), 72 hours (day 3), 144 hours (day 6) was measured. Data are shown as the mean of N $\geq$ 3.

3.3 Discussion

Investigation of the environmental safety profile of pest controlling compounds is essential to ensure safe use of these substances (Iyaniwura 1991). Neonicotinoids are the most commonly used insecticides worldwide, but their impact on many non-target invertebrates is poorly understood. To determine their potential environmental impact, the effects of neonicotinoids on the Nematoda representative *C. elegans* has been investigated.

Limited number of studies have investigated the effects of neonicotinoids on *C. elegans*. These investigations typically report disruption of behaviours governed by the cholinergic neurotransmission (Gomez-Eyles et al. 2009; Ruan et al. 2009; Mugova et al. 2018; Hopewell et al. 2017). In this chapter detailed description of the effects of neonicotinoids on various aspects of worm behaviour are described and compared to the effects exerted by a classical nAChR agonist, nicotine.

3.3.1 Nicotine and neonicotinoids affect locomotion of worms by differential mechanisms

Wild-type animals were exposed acutely to nicotine and neonicotinoids and their effects on thrashing a measure of motility was scored. Neither thiacloprid nor clothianidin impaired motility. In contrast, nitenpyram and nicotine paralysed worms when present at mM concentrations. Increase in exposure time from 2 to 24 hours resulted in increased efficacy of nicotine on wild-type worms in motility assay which now utilized body bends. This was reflected in the shift of EC_{50} from 31 to 3.6 mM. Thiacloprid had no effect on thrashing, but it inhibited body bends by 20 % at 1.5 mM. Clothianidin had no effect on thrashing or body bends.

Despite common effects of nicotine and neonicotinoids on locomotion, the mode of action is likely to be different. Exposure of worms to nicotine for 4 hours led to significant reduction in the size of the worm. This is due to the proposed mode of action of nicotine. Nicotine activates ACR-16 nicotinic acetylcholine receptors present at the body wall muscle (Touroutine et al. 2005; Richmond and Jorgensen 1999), which leads to BWM hypercontraction (Sobkowiak, Kowalski, and Lesicki 2011). The BWM is physically attached to the cuticle (Altun and Hall 2009a), therefore prolonged muscle stiffness and hypercontraction can lead to shrinkage of the cuticle (Petzold et al. 2011) and the reduction in the body size, as it was observed in this study. In contrast, neonicotinoids did not have the same effect. Thiacloprid and clothianidin treated worms showed uncoordinated, twitchy phenotype and no reduction in the body length. Taken together, this data suggest that nicotine and neonicotinoids can act to inhibit locomotion differently. It is possible that they all act on nAChRs in the worm, as these molecules are the primary targets for nicotine and neonicotinoids in other species, but they are likely to act on different types of *C. elegans* nAChRs. To provide an insight into the mode of action of these compounds, studies on wild-type and nAChR mutant worms should be performed. behavioural analysis of wild-type and mutant strains exposed to nicotine and neonicotinoids might allow for identification of strains resistant to these compounds and hence discover potential molecular targets.

3.3.2 Nicotine inhibition of thrashing

The time course for the effects of 10 and 25 mM nicotine on thrashing of wild-type worm revealed two "phases" of inhibition. First, seen after 10 minutes, followed by partial recovery and a second phase seen after 40 minutes. This could suggest nicotine targets multiple sites that alter thrashing. Previous research has shown that nicotine acts at a body wall muscle but also at sensory neurons. Nicotine is an agonist of TRP β receptors (Feng et al. 2006) which are expressed in nociceptive ASH and ADL neurons (Colbert, Smith, and Bargmann 1997). These neurons send out processes to the nerve ring where they make connections with a diversity of circuits, including those regulating movement (Rogers et al. 2006). A nicotine-related compound quinine has an effect on locomotion via nociceptive circuit (Hilliard et al. 2004). It is therefore possible that the first and rapid effect of nicotine on thrashing could be due to the action on sensory neurons. Sensory neurons have processes exposed to the external environment (Hall and Altun 2008), which are easily accessible to nicotine. Indeed, nicotine at high doses inhibits locomotion within 1 minute of incubation (Figure 3.8a). In addition, this effect is equally rapid on wild-type and *bus-17* mutant worms. This supports the notion that nicotine affects locomotion by targeting structures exposed to the surface and not buried within the worm.

The second phase of inhibition could be due to the effects of nicotine on the body wall muscle. It may take longer to reach muscular targets because worms ingest only a little material whilst in liquid (Gomez-Amaro et al. 2015) and so nicotine must cross the cuticle to reach the BWM. The complex structure and the thickness of the cuticle may slow the kinetics of absorption. This is supported by the lack of two phases of nicotine-induced paralysis in the leaky cuticle *bus-17* mutant. The effects of nicotine on thrashing of a mutant strain equilibrate after 10 minutes of incubation which may suggest improved permeability and hence reflect synergistic sensory and muscular effects of nicotine on locomotion.

3.3.3 The cuticle limits bioavailability of nicotine and neonicotinoids

This study reports low efficacy of neonicotinoids on the wild-type worm *C. elegans* (Table 3.1). This could be due to a low potency of compounds on target receptors, or due to the limited permeability. The increased efficacy of nicotine on wild-type worm motility in terms of body bend vs thrashing assay suggest that nicotine does not equilibrate across the cuticle readily. This suggest that low *C. elegans* sensitivity to nicotine (and potentially neonicotinoids) might be a result of hindered permeability of drugs.

Permeability of drugs across lipidic structures is dependent on the physicochemical properties of compounds (Avdeef 2004). The effects of the pH of the external buffer on the efficacy of nicotine on thrashing were investigated. The changes in pH shifts the ionization state of nicotine, which could affect absorption. A shift of pH from 7 to 6 and 9 moderately decrease EC_{50} from 26.2 mM to 16.7 mM and 15.2 mM, respectively. Therefore the pH of external solution does not markedly alter efficacy of nicotine. This may be due to worms' capacity to regulate their cuticular surface pH in tunnel-like and water-filled pores. These structure are on

the surface of the cuticle and keep constant pH microenvironment of ~5 (Sims et al. 1992, 1994). There are other physicochemical factors that could play a role in permeability of nicotine and neonicotinoids, such as lipophilicity. Nicotine and neonicotinoids have moderate lipophilicity (Blaxten 1993) which could limit the ability of drugs to enter and diffuse across lipidic structures (Liu, Testa, and Fahr 2011), limiting bioavailibity and efficacy of compounds. If the primary molecular targets are within and not on the surface of the worm, the low efficacy could be due to the limited diffusion through the cuticular structures.

To investigate the role of surface coat and cuticle in drug permeability *bus-17* mutant with fragile and more permeable cuticle was employed in acute and moderate exposure assays. Exposure of *bus-17* worms increased potency of all compounds. For example, in acute-exposure thrashing experiments, the EC_{50} for the effects of nicotine and nitenpyram increased by 8- and 5-fold, respectively. Moreover, thiacloprid and clothianidin had no effect on motility of the wild-type worm, but on *bus-17* they induced paralysis with the EC_{50} of 480 μM and 2.2 mM, respectively. Increased drug sensitivity was also observed in other cuticle-mutant strains for example *bus-8* (Partridge et al. 2008), *bus-5* and *bus-16* (Xiong, Pears, and Woollard 2017). This increased sensitivity is thought to be due to an increased cuticular permeability in mutant strains (Partridge et al. 2008; Xiong, Pears, and Woollard 2017). These data highlights the importance of the cuticle in protecting *C. elegans* against the outer environment. The cuticle is a common structural feature of all soil nematodes (Decraemer et al. 2003). It is likely that it limits bioavailability of residual insecticides in all soil nematodes, hindering their potential toxic effects.

3.3.3.1 Nicotine and thiacloprid have a neurodevelopmental effect on *C. elegans*.

Worms growing in the presence of 1 mM nicotine and thiacloprid developed into adults slower than the control worms. Neonicotinoids also disrupt larval development in bees (Souza Rosa et al. 2016). In would be interesting to investigate whether there is a common mechanism of neonicotinoid-induced neurodevelopmental defect in worms and bees. In worms, thiacloprid acts by disrupting L2/L3 transition. L2 stage is a stage at which multiple cell divisions and differentiation occurs (Hall and Altun 2008). Nicotinic acetylcholine receptors containing UNC-63 subunits seem to be involved in this process (Ruaud and Bessereau 2006). Developmental assays on *unc-63* and other nAChR mutants should be carried out to determine if the action of thiacloprid depends on this protein.

Table 3.1: Summary table of the effects of nicotine and neonicotinoids on *C. elegans*.

behavioural assay	Compound	Strain	EC_{50}
Thrashing	Nicotine	N2	26.2 mM
		bus-17	3.3 mM
	Nitenpyram	N2	195.8 mM
		bus-17	16.6 mM
	Thiacloprid	N2	$>$ 1.5 mM
		bus-17	377.6 μM
	Clothianidin	N2	$>$ 2.5 mM
		bus-17	3.3 mM
Body bends	Nicotine	N2	3.6 mM
		bus-17	1.6 mM
	Nitenpyram	N2	$>$ 1 mM
		bus-17	$>$ 1 mM
	Thiacloprid	N2	3.7 mM
		bus-17	721.2 μM
	Clothianidin	N2	$>$ 3.75 mM
		bus-17	3.3 mM
Egg-laying	Nicotine	N2	$>$ 1 mM
		bus-17	$>$ 1 mM
	Nitenpyram	N2	$>$ 1 mM
		bus-17	$>$ 1 mM
	Thiacloprid	N2	$>$ 1 mM
		bus-17	1.4 mM
	Clothianidin	N2	$>$ 3.75 mM
		bus-17	6.2 mM
Egg-hatching	Nicotine	N2	$>$ 1 mM
		bus-17	$>$ 1 mM
	Nitenpyram	N2	$>$ 1 mM
		bus-17	$>$ 1 mM
	Thiacloprid	N2	$>$ 1 mM
		bus-17	1.5 mM
	Clothianidin	N2	$>$ 3.75 mM
		bus-17	$>$ 3.75 mM

Chapter 4

The effects of nicotine and neonicotinoids on the *C. elegans* pharyngeal pumping

4.1 Introduction

Results from the previous results chapter show low susceptibility of *C. elegans* to neonicotinoids and that the cuticle is a limiting factor of the efficacy of these compounds on locomotion. This chapter aims to further characterise the concentration-dependent effects of neonicotinoids on worm and the role of the cuticle in their efficacy, by utilising the pharyngeal pumping assay.

The pharynx is responsible for feeding. It functions to capture the bacterial suspension, expel the fluid and trap bacteria inside the pharyngeal lumen (Song and Avery 2012). The bacteria is then smashed and passed to the gut for digestion (Avery and Horvitz 1987).

These complex pharyngeal functions are performed by two motions: pumping and peristalsis (Figure 4.1 (Avery and Horvitz 1989)). Pumping is the contraction and a subsequent relaxation of the corpus, anterior isthmus and the terminal bulb. Peristalsis is the motion of the posterior isthmus that pushes bacteria to the terminal bulb for grinding. On average, peristalsis occurs every 3.4 pumps (Song et al. 2013) and begins after the relaxation of the pharyngeal muscle. Grinding of the food particles is performed by the grinder. The grinder is positioned in the terminal bulb and composed of 3 pairs of muscle cells (Albertson and Thomson 1976). Contraction of these cells leads to their rotation, resulting in food maceration.

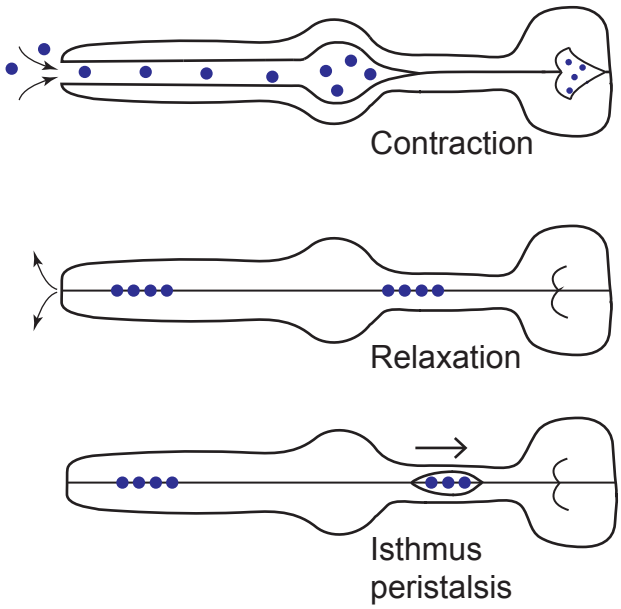


Figure 4.1: **Feeding of *C. elegans*.** As the corpus and anterior isthmus contract, bacterial suspension enters the pharynx through the mouth (top). Subsequent relaxation expels water out, trapping bacteria in the corpus-anterior isthmus lumen (middle). This pumping movement is synchronised with the terminal bulb contraction-relaxation cycle leading to movement of the grinder and crashing of food trapped between the muscle segments (not shown). This happens on average 3.4 times to allow food accumulation (Song and Avery 2012) and passage to the intestine. The peristalsis of the posterior isthmus opens the isthmus-terminal bulb lumen and pushes the next portion of food in (Avery and Horvitz 1987) for fragmentation (bottom).

4.1.1 Anatomy of the *C. elegans* pharynx

Electron micrograph analysis of the *C. elegans* pharyngeal sections (Albertson and Thomson 1976) provides detailed picture of its anatomy. The pharynx is a tubular feeding organ located in the head of the worm. It is 20 μM wide and 100 μM long, encapsulated by the basal membrane which separates the pharyngeal cells from the pseudocoelom. On the apical surface, the basal lamina lies directly below the cuticle. This is in contrast to the body wall muscle, where these two layers are also separated by the hypodermis. The pharynx can be divided into three anatomical features: most anterior corpus, middle isthmus and posterior terminal bulb. There are five different main cell types in the pharynx: muscles, neurons, epithelial, glands and marginal cells. The main constituents are the 20 muscle cells and 9 marginal cells which wrap around the pharyngeal lumen. Embedded within those cells are 4 glands and 20 neurons.

There are 8 layers of muscle cells encapsulating the pharynx (pm1-pm8, Figure 4.2). pm1 is the most anterior and constitutes a single cell surrounding the pharyngeal lumen and six processes running down the pro-corpus. Posterior to pm1 are the three cells of the pm2 muscle cells which also wrap around the lumen of the pharynx. Both pm1 and pm2 are, relative to other muscles of the pharynx, thin. pm3 together with pm1 and pm2 form pro-corpus, pm4 meta-corpus whereas pm5 the isthmus of the pharynx. These three sections are wedge-shaped and formed from three cells. There are three muscular layers forming the terminal bulb: pm6, pm7 and pm8.

pm6 and pm7 are composed of three cells, whereas pm is a single cell. pm8 is the most anterior and it is connected to the intestine by the toroidal valve composed of six cells.

There are nine marginal cells in the pharynx: mc1 - mc3 each with three fold symmetry. These cells run along the pharynx from the pm1 to pm8. They receive chemical synapses from M5 and form gap junctions with muscle cells.

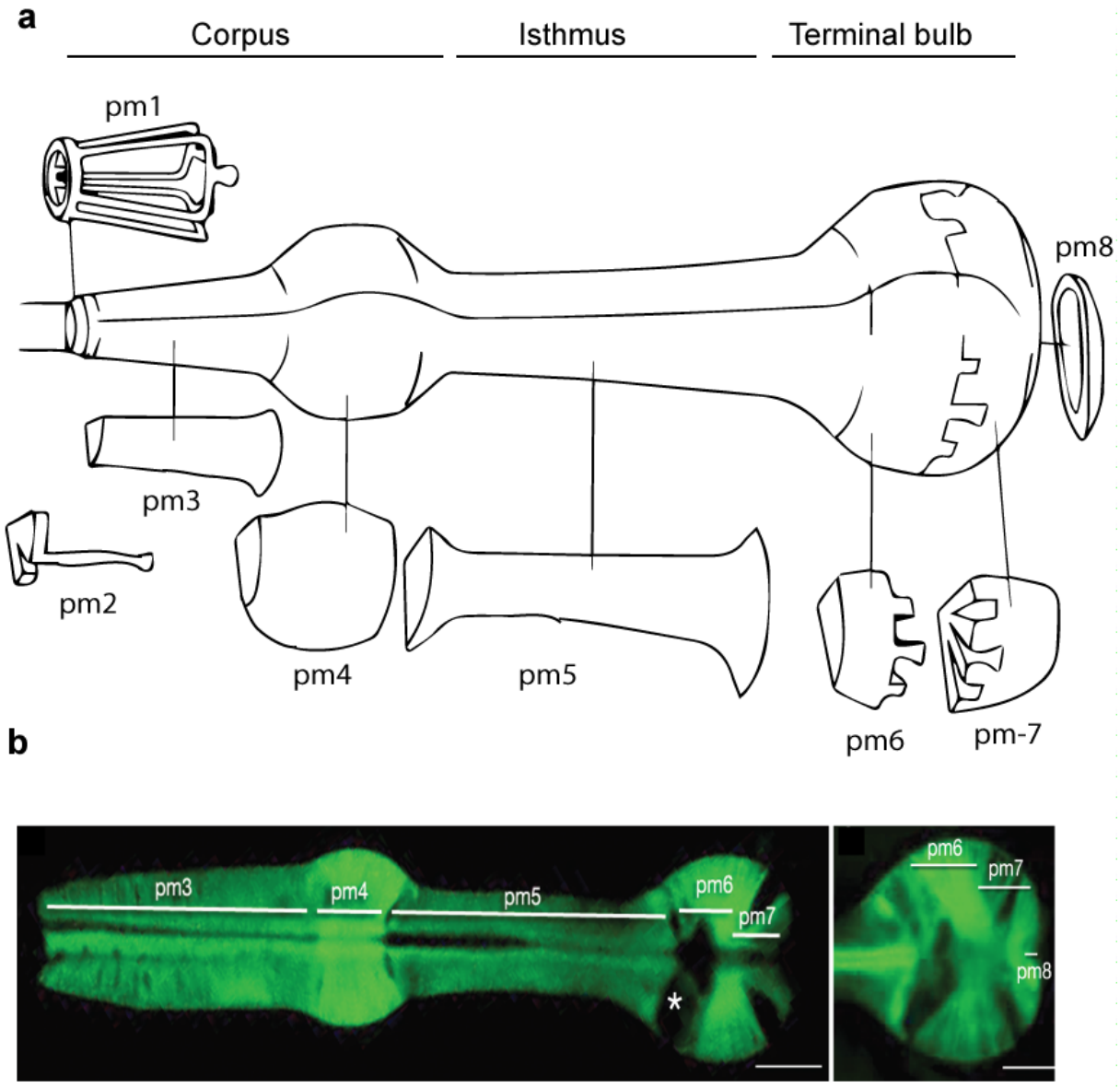


Figure 4.2: **The musculature of the *C. elegans* pharynx.** Cartoon representation of the muscle cells constituting each of the three anatomical features: the corpus, isthmus and the terminal bulb (a) A single cell of each layer is pictured. Epifluorescence image of pharyngeal muscle cells from the transgenic worm expressing myofilaments GFP reporter gene (b, left panel) and epifluorescence image of the transgenic worm expressing mitochondrial GFP reporter gene (b, right panel), to highlight the muscle cells of the terminal bulb. Images adapted from Albertson and Thomson (1976) (a) and Altun and Hall (2009b) (b).

4.1.2 Sensory regulation of pumping

There are 302 neurons in a *C. elegans* hermaphrodite, 20 of which are present in the pharynx (White et al. 1986). The pharyngeal nervous system is connected to the somatic nervous system a single point: a pair of gap junctions between the extrapharyngeal RIP and pharyngeal I1 neurons (Albertson and Thomson 1976; Figure 4.3). Laser ablation of RIP has no effect on pumping rates on or off food (Dalliere et al. 2015), suggesting that pharyngeal system is sufficient to drive pharyngeal responses. However, there is evidence that pharyngeal pumping can be influenced by the sensory cues. Animals with abolished sensory neurons and not RIP neurons have blunted pharyngeal response to light touch (Riddle, Blumenthal, and Meyer 1997), light (Bhatla et al. 2015), familial food, (Song et al. 2013), or attractive and repellent odours (Li et al. 2012). This evidence supports the sensory regulation of the pharyngeal activity. Numerous sensory cues associated with food are detected by the *C. elegans* to modulate pharyngeal pumping including odour (Li et al. 2012) and mechanical stimulation (Chalfie et al. 1985). These cues are detected by the sensory neurons. There are 60 sensory neurons in *C. elegans*. There are also pharyngeal neurons which are likely to serve sensory function. This includes NSM and all I neurons with the exception of I4. The activity of the pharynx is also under the influence of the humoral transmission. The pharynx expresses receptors activated by neurotransmitters not synthesised by the pharynx, such as dopamine (Sugiura et al. 2005). Mutation of genes not expressed in the pharynx has an effect on the pharyngeal response to food (Calahorro et al. 2018).

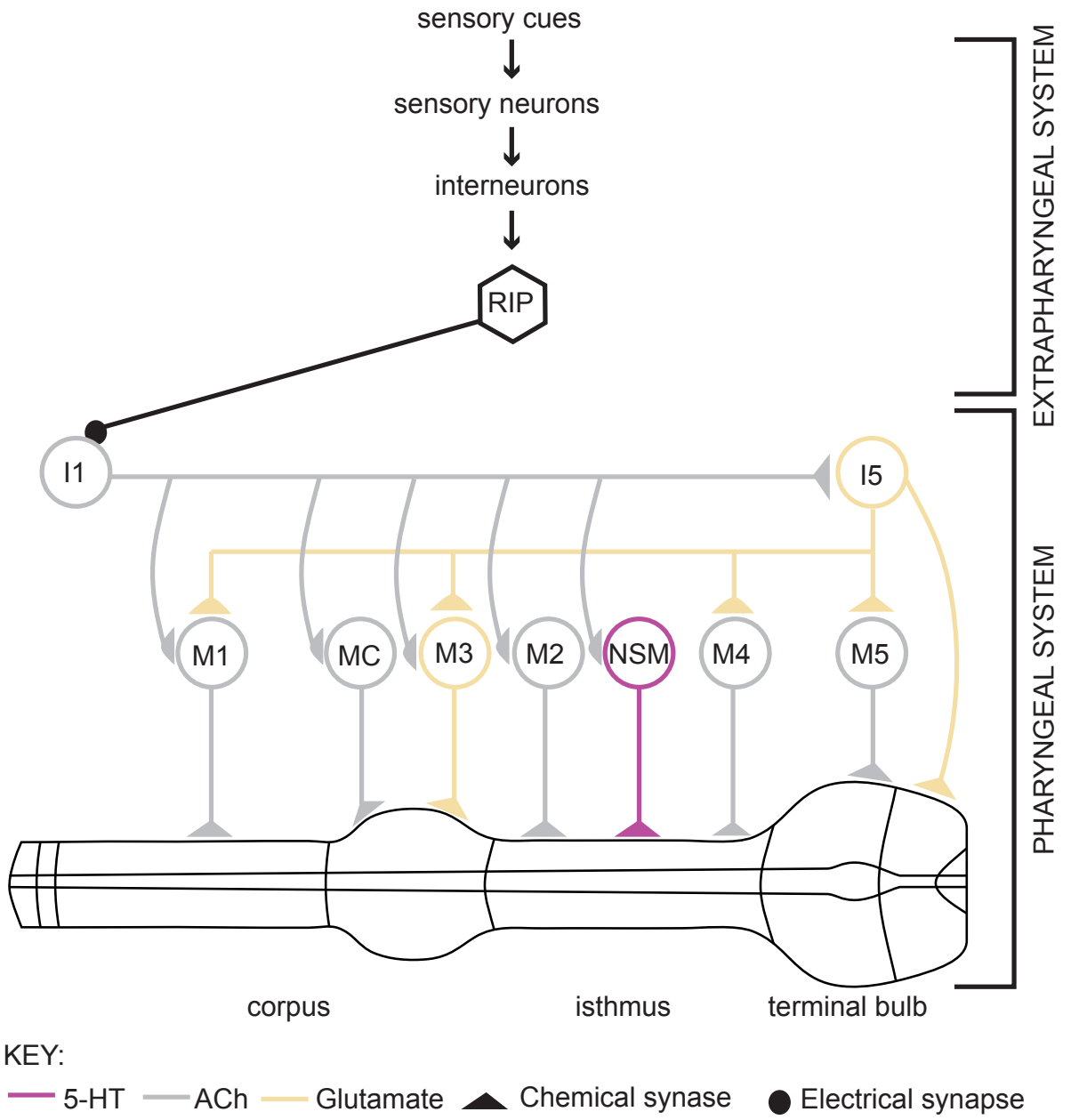


Figure 4.3: **Pharyngeal nervous system.** Simplified pharyngeal nervous system showing the major neurons, synaptic connection and neurotransmitters. Synapses between I1-RIP connect the extrapharyngeal and pharyngeal nervous system.

4.1.3 Pharyngeal nervous system

There are 20 neurons in the pharynx (Albertson and Thomson 1976 and Table 4.1). Laser ablation studies combined with behavioural analysis of generated worms provides an explanation of the role of the entire pharyngeal nervous system, and the role of individual neurons in the pharyngeal pumping.

The pharyngeal nervous system is not essential for the function of the pharynx, but it is crucial for the regulation and modulation of the pharyngeal function in response to the environment. In the absence of food, the wild-type worm pumps at a low rate. This increases markedly in the presence of food. The laser ablation of the pharyngeal nervous system does not abolish pumping entirely (Avery and Horvitz 1989). In the absence of food, wild-type worm makes 43 pumps per minute. This is reduced to 16 in worms lacking pharyngeal neurons. A stronger phenotype of neuron-ablated worms can be seen upon introduction of food. In the presence of food, pharyngeal pumping rate drops from 224 to 26 in laser ablated worms. In addition, there are abnormalities in the mechanism of pumping of worms lacking the pharyngeal nervous system: little food passes into the intestine resulting in retarded growth and compromised fertility (Avery and Horvitz 1989; Avery, L. 1993) showing the essential role of the pharyngeal nervous system in the feeding response.

Three out of 14 pharyngeal neuron types are sufficient to elicit feeding. These neurons are MC, M3 and M4. Laser ablation of MC neuron (MC^-strain) leads to reduction of pumping rate by 75% on food and 37% off food. Therefore, MC is important in initiation of pumping (Avery and Horvitz 1989; Raizen, Lee, and Avery 1995). Laser ablation of M3 or M4 has little effect on the pumping rate (Raizen, Lee, and Avery 1995), but it effects the mechanism of pumping. $M3^-$ animals have markedly extended latency of a single pump (Avery, L. 1993) suggesting M3 governs the timing of pumping. The sensory endings of M3 synapse onto the $M4^-$ animals accumulate bacteria in the corpus suggesting M4 initiates peristalsis (Avery and Horvitz 1989; Avery, L. 1993) by stimulating isthmus opening via mechanism that is not yet fully understood.

4.1.4 Neurotransmitters of the pharynx

Genetic studies of worm mutant strains provide evidence for a critical role of neurotransmission in the regulation of feeding. UNC-13 protein is involved in the regulation of neurotransmitter release at the synapse, as shown by the biochemical and behavioural analysis of the *unc-13 C. elegans* strain. Mutants deficient in UNC-13 show severe retention of vesicles in the pre-synapse (Richmond and Jorgensen 1999) and impaired synaptic transmission (Aravamudan et al. 1999). Their feeding is also affected: 70 % reduction of the pharyngeal pumping rate was noted (Richmond, Weimer, and Jorgensen 2001). These data highlight the key role of neurotransmitters in the regulation of pumping. The activity of the pharynx is influenced by acetylcholine, glutamate and 5-HT (Table 4.1 and Figure 4.3), and potential tyramine and octopamine (Alkema et al. 2005).

Table 4.1: Pharyngeal neurons and neurotransmitters they release.

Type	Pharyngeal neuron	Neurotransmitter released
Motorneuron	M1	Acetylcholine
	M2*	Acetylcholine
	M3*	Glutamate
	M4	Acetylcholine
	M5	Acetylcholine
Interneurons	I1*	Acetylcholine
	I2*	Glutamate
	I3	Acetylcholine
	I4	?
	I5	Glutamate
	I6	?
Other	MI	Glutamate
	NSM	5-HT
	MC*	Acetylcholine

Note:
Bilateral neurons are marked with *. Most neurons are either motor (M) or inter -neurons (I), but some have additional functions. NSM function as motor and secretory neuron, MI is a motor-interneurons, whereas MC is a motor and sensory neuron. Three neurons are sufficient for feeding: MC, M3 and M4.

4.1.4.1 5-HT

5-HT is synthesised in two pharyngeal neurons NSM and I5 (Chase and Koelle 2007). Mutant deficient in enzyme in the 5-HT biosynthetic pathway has blunted response to food (Sze et al. 2000). Exogenous application of 5-HT induces feeding response in the absence of food whereas competitive 5-HT antagonists inhibit pumping (Horvitz et al. 1982; Avery and Horvitz 1990). Collectively, this suggests that serotonergic neurotransmission induce food-evoked feeding response.

In the presence of food, 5-HT can be released from multiple sites, including NSM, ADF and HSN neurons. NSM are neurosecretory neurons in the pharyngeal nervous system. In the presence of food, they release 5-HT into the pseudocoelomic fluid (Horvitz et al. 1982) to inhibit locomotion, induce pumping and alter other behaviours (Horvitz et al. 1982). ADF are chemosensory neurons present on the head of the worm. Release of 5-HT selectively from ADF neurons is sufficient to drive the feeding response (Cunningham et al. 2012). Similarly, selective release of 5-HT from extrapharyngeal HSN neurons when NSM and ADF neurotransmission is defective, results in potent pumping response (Lee et al. 2017). Therefore, there are 3 possible routes by which elevated levels of 5-HT in the presence of food can be achieved. It is possible that these routes

co-function or operate at different conditions.

Detection of food leads to the elevation of pumping rate via 5-HT acting at multiple GPCRs (Avery and You 2012). 5-HT can regulate feeding response by binding to neuronal SER-4 receptors and SER-1 receptors expressed both in the pharyngeal muscle cells and neurons (Tsalik et al. 2003). It also binds to SER-5 receptors expressed on extrapharyngeal interneurons (Cunningham et al. 2012). However, the main driver of the 5-HT driven pharyngeal response it the activation of SER-7 receptors expressed on cholinergic MC and M4 neurons and on glutametergic M3 neurons (Hobson et al. 2003; Song et al. 2013). Acetylcholine released from MC and M4 neurons increases contraction frequency and induce isthmus peristalsis, respectively (Avery and Horvitz 1987; Raizen and Avery 1994). This leads to an increase in the activity of the pharynx to ~260 pumps/min. Glutamate released from M3 shortens pump duration to <200ms.

4.1.4.2 Glutamate

Glutamate is produced in at least 4 neurons (Table 4.1), but its function in M3 is the best studied. Laser ablated $M3^-$ animals have reduced pumping rate on food (Raizen, Lee, and Avery 1995). Similar phenotype is observed in animals deficient in glutamatergic neurotransmission. EAT-4 encodes for vesicular glutamate transporter (Lee et al. 1999). Mutation of this gene in *C. elegans*, leads to reduction of the pharyngeal pumping rate of food (Lee et al. 2008).

In response to food, released 5-HT activates M3 (Niacaris 2003), leading to release of glutamate which in turn contributes to the potent pharyngeal response. Specifically, it leads to shortening of the duration of the pump. M3-released glutamate acts via glutamate-gated chloride channel expressed on pm4 and pm5 pharyngeal muscles (Dent, Davis, and Avery 1997). Activation of these channels during depolarisation phase of the pharyngeal muscle action potential leads to the generation of post-synaptic inhibitory potentials, which bring about the repolarisation and hence relaxation of the muscle. This in turn shortens the pump duration (Avery, L. 1993), allowing another muscle depolarisation to occur.

4.1.4.3 Acetylcholine

The role of acetylcholine in the regulation of feeding was investigated in mutants deficient in proteins essential for the cholinergic neurotransmission (Section 1.4.4). Null mutants die soon after birth due to starvation (Rand 1989; Alfonso et al. 1993), whereas polymorphic mutants show reduced pharyngeal pumping both in the presence and absence of food (Dalliere et al. 2015). Hindered feeding was also observed in animals in which one of the 6 cholinergic neuron, namely MC neurons, were ablated (Avery and Horvitz 1989; Raizen, Lee, and Avery 1995).

Pharmacological and genetic studies suggest that acetylcholine stimulates pharynx by acting on nAChRs. Application of nAChR agonists, acetylcholine and nicotine, resulted in the stimulation of the pharyngeal pumping

in the absence of food (Raizen, Lee, and Avery 1995). In contrast, nAChR antagonist d-tubocurarine inhibited pharyngeal pumping in the presence of food (Raizen, Lee, and Avery 1995). Although there are at least 29 nAChR subunits expressed in *C. elegans* (Figure 1.9), only one, namely EAT-2 has been identified as essential in mediation of the feeding response (McKay et al. 2004) (section 1.4.8.1). EAT-2 is expressed in pm4 and pm5 muscle cells (McKay et al. 2004), which make synaptic connections with the MC (Albertson and Thomson 1976). *C. elegans eat-2* mutant shows significantly reduced pumping in the presence of food (Raizen, Lee, and Avery 1995; McKay et al. 2004). A similar phenotype was noted in the mutant of *eat-18* auxiliary protein (McKay et al. 2004).

Except from nAChRs, there are other acetylcholine sensitive receptors in the pharynx. GAR-3 is a ACh-sensitive GPCR (Hwang et al. 1999) expressed in metacorpus, isthmus, terminal bulb of the pharyngeal muscle and in the I3 pharyngeal neuron (Steger and Avery 2004). Its role may involve regulation of the membrane electrical potential (Steger and Avery 2004) and control of feeding during starvation (You et al. 2006). Whereas acetylcholine-gated chloride channels (ACC) are ionotropic channels and like nAChRs, members of Cys-loop receptor family. There are at least 8 members isoforms: ACC-1 to ACC-4, LGC-46 to LGC-49 (Takayanagi-Kiya, Zhou, and Jin 2016), which form homo- and hetero-pentamers (Putrenko, Zakikhani, and Dent 2005). They are generally expressed in a distinct subset of cholinergic and glutamatergic neurons (including acc-1 in pharyngeal M1 and M3) (Pereira et al. 2015; Takayanagi-Kiya, Zhou, and Jin 2016). Interestingly, *acc-4* is expressed in almost all cholinergic neurons. Electrophysiological data suggest ACC channels are inhibitory but they may be playing multiple functions. ACC-4 may be acting as autoinhibitory, as most neurons predicted to express it do not receive direct synaptic input from other neurons (Albertson and Thomson 1976). LGC-49 are expressed in presynaptic specialisations of cholinergic motor neurons where they regulate synaptic vesicle release (Takayanagi-Kiya, Zhou, and Jin 2016). Based on pharmacologically characterised ACC-1 and ACC-2 *in vivo*, ACC receptors are also pharmacologically distinct from other ACh receptors. Some of the nAChR and GPCR receptor compounds were potent at modulating the activity of ACC channels, but not others. For example, ACC channels are insensitive to nAChR compounds nicotine, cytisine and antagonist α-bgtx but are sensitivity to tubocurarine. This mixed pharmacological profile suggest ligand binding site is distinct from the binding site of nAChRs and cholinergic GPCRs.

4.1.5 Assays for scoring the effects of compounds on pharyngeal pumping

Pharyngeal pumping can be scored in two distinct animal preparations. One is the intact worm preparation, and the other is the cut-head preparation in which the cuticular barrier is removed by cutting the head away from the body, enabling easy drug access to pharyngeal binding sites. The pharyngeal pumping of the worm can be scored by visual observation. This is performed by counting the number of grinder movements in a period of time. Grinder activity is coupled to the contraction and relaxation cycles of the pharynx, therefore is a good indication of the pharyngeal function.

4.1.6 Chapter aims

This chapter describes the effects of 5-HT, nicotine and neonicotinoids on the pharyngeal pumping. The effects of compounds are scored on intact and cut-head worm in which access to the pharyngeal binding sites is increased. The aim of these investigations is to inform on the sensitivity of the pharyngeal system to 5-HT, nicotine and neonicotinoids and to provide an insight into the cholinergic regulation of the pharyngeal system. Additionally, effects of compounds on the pharyngeal pumping of mutants deficient in nAChR expression is investigated to elucidate the molecular basis of drug-induced pharyngeal alterations.

4.2 Results

4.2.1 Effects on pharyngeal pumping of intact worms on food

Pharyngeal pumping is a feeding behaviour of the intact worm mediated by the pharynx. In the presence of food, the pharynx pumps at an approximate rate of 4 pumps/s (4Hz) to ingest food particles (Figure 4.4). A typical way of assessing the effects of compounds on feeding is to place *C. elegans* on agar plate soaked with drug solution and laced with *E. coli* OP50 food patch. A drug present on the plate comes in direct contact with foraging worm, but also can enters the worm via ingestion and/or diffusion cross the cuticle. To assess the effects of nicotine and neonicotinoids, wild-type worms were exposed to nicotine, nitenpyram, thiacloprid and clothianidin for 24-hours before scoring their effects on pharyngeal pumping (Figure 4.4 left panel). Nicotine at concentrations $\geq$ 1 mM inhibited pumping in a dose - dependent manner. Nitenpyram and thiacloprid at 1 mM as well as clothianidin at 3.75 mM had no effect.

Analysis of responses to compounds in *bus-17* mutants show that the worm's cuticle can hinder the efficacy of drugs (Chapter 3). To determine whether the cuticle affects the efficacy of nicotine and neonicotinoids on the feeding behaviour, pharyngeal pumping experiments were repeated on the *bus-17*, cuticle-disrupted mutant of *C. elegans* (Figure 4.4 right panel)). As in the wild-type, the pharyngeal pumping of the *bus-17* mutant was inhibited by nicotine at 1 and 10mM, whereas nitenpyram had no effect. In contrast, the dose of thiacloprid and clothianidin was ineffective on wild-type, inhibited pumping of the mutant at low mM concentrations.

To compare the efficacy of compounds on wild-type versus mutant strain, dose-response curves for the effects of treatment on pharyngeal pumping were generated (Figure 4.5). Almost one fold difference in the potency of nicotine was observed. The EC_{50} on wild-type worm was 4.3 mM. This decreased to 2.3 mM in a mutant strain. Nicotine was also the most efficacious out of all compounds tested at inhibiting pumping. The estimated EC_{50} of thiacloprid and clothianidin on a mutant strain were 2.6 and 6.2 mM, respectively. Since they had no effect on wild-type it is not possible to estimate the fold-potency change between the wild-type and mutant strain.

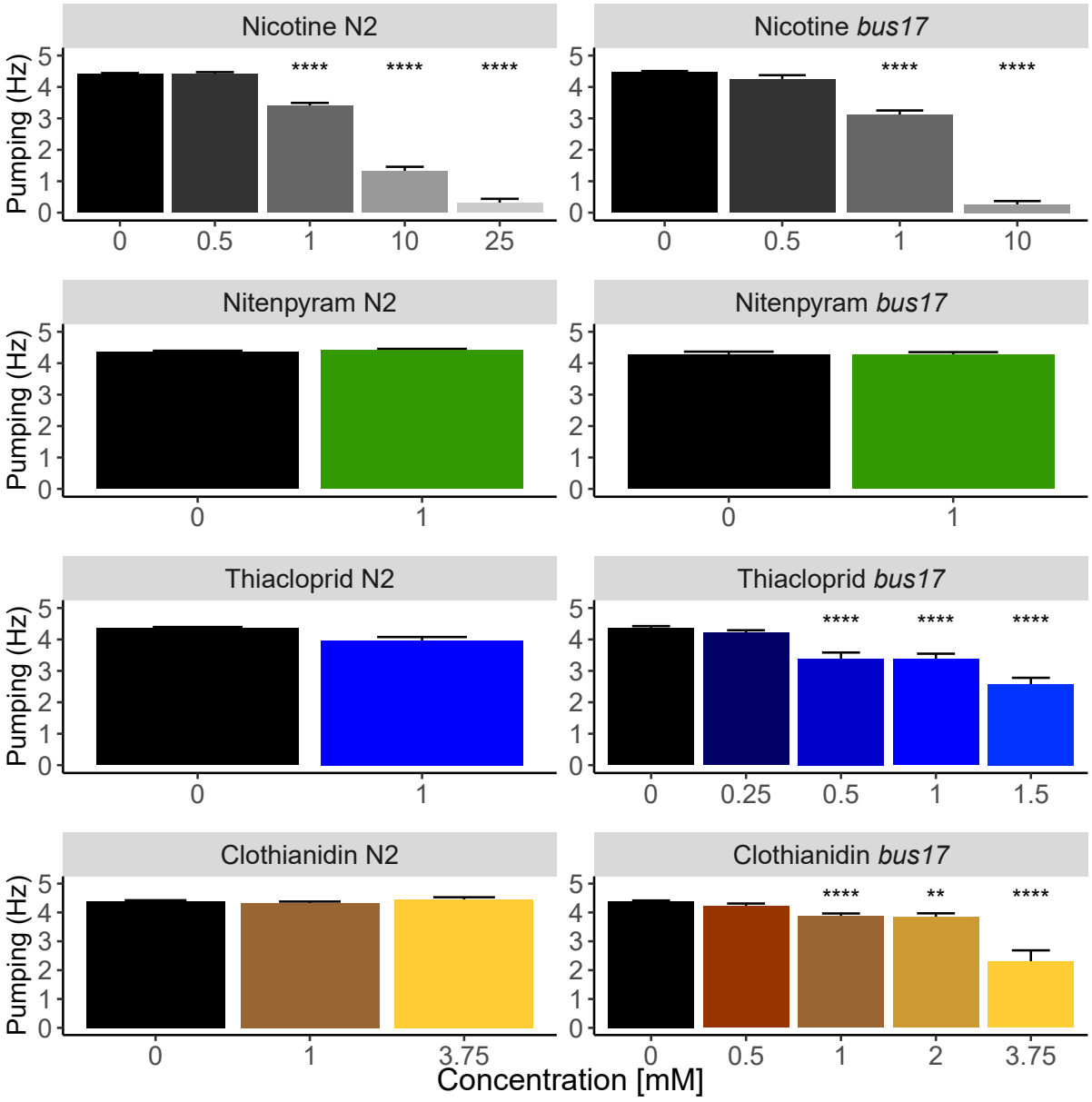


Figure 4.4: **The concentration-dependence for the effects of nicotine and neonicotinoids on feeding of *C. elegans*.** Wild-type (left panel) and *bus-17* (right panel) worms were exposed for 24 hours to nicotine, nitenpyram, thiacloprid, clothianidin or vehicle control (O), incorporated into solid medium. Pharyngeal pumps of worms present on food were counted by visual observation for 1 minute and expressed in Hz. Data are mean $\pm$ SEM, collected from 8 - 32 individual worms on $\geq$ 3 days. One way ANOVA (Kruskal-Wallis test) with Dunnett's Corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

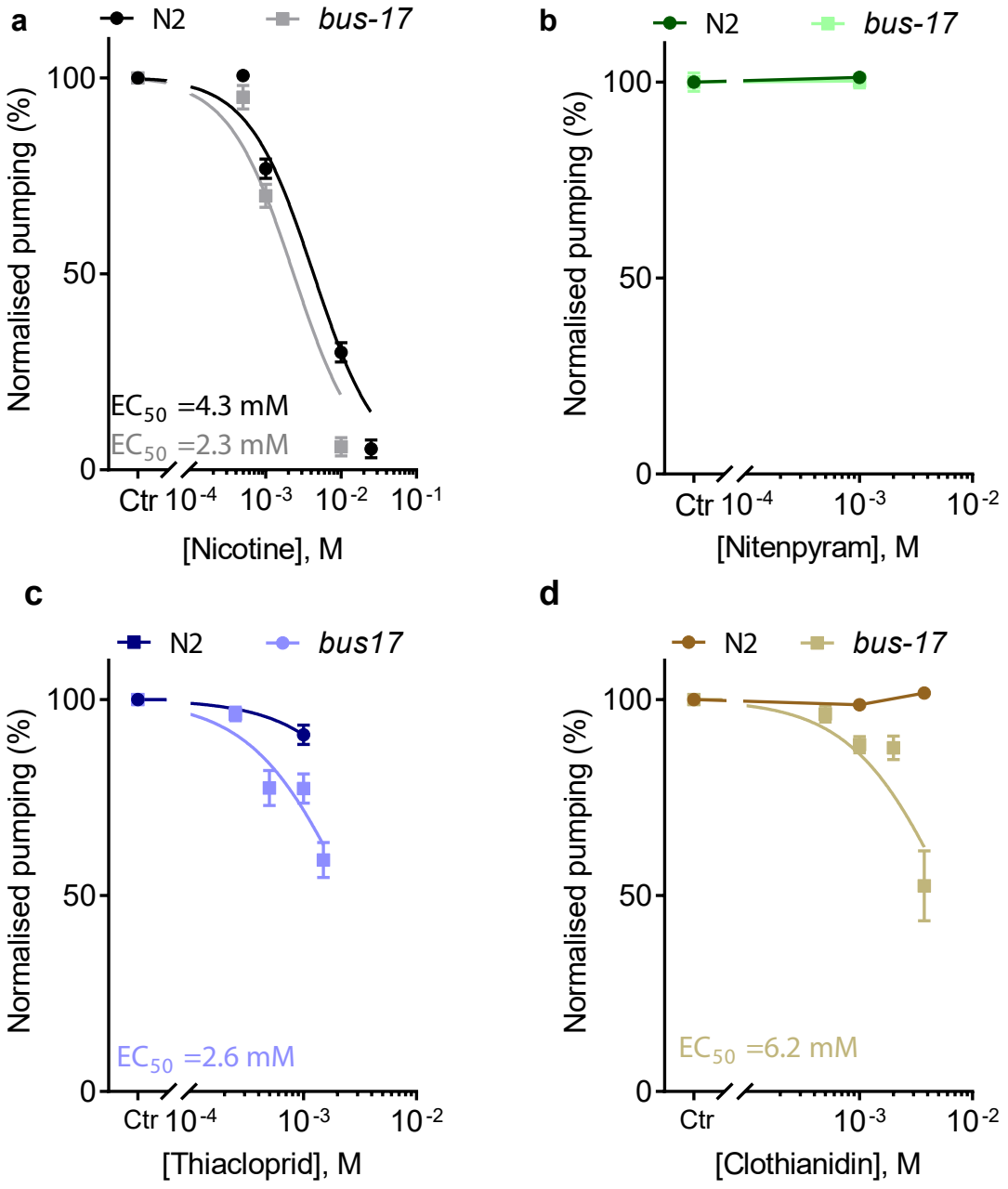


Figure 4.5: **Dose-response curves for the effects of nicotine and neonicotinoids on feeding of *C. elegans*.** Concentration-dependence curves for the effects of nicotine (a), nitenpyram (b), thiacloprid (c) and clothianidin (d) on feeding of wild-type and *bus-17 C. elegans*. Dose-response curves were generated by taking data points from Figure 4.4, and expressed as % control pumping. Data and mean ± SEM. The EC_{50} for thiacloprid and clothianidin are approximations, because at the highest concentration tested (1.5 and 3.75 mM) they inhibited *bus-17* pumping by 45 and 50 %, respectively.

4.2.1.1 Effects on pharyngeal pumping in liquid

During the course of the investigation, it was noted that high concentrations of nicotine had an antimicrobial effect on *C. elegans* food source - the OP50 *E. coli*. This raised a concern that the observed effects of nicotine on feeding could be partially due to the effects of this compound on the density of bacteria. To circumvent this issue, an alternative assay was developed in which the need for bacteria is removed. This alternative assay is performed in liquid, in the presence of 10 mM 5-HT. 5-HT has dual effect: it makes the worms immobile and stimulates their pumping (Figure 4.6). After a 5 minute incubation with 10 mM 5-HT, 45 % of worms were paralysed. This increased to 65 % after 1 hour of incubation. The paralysing effect of 5-HT enabled measurements of the pharyngeal pumping activity of worms. Whilst in liquid, worms display negligible food intake (Gomez-Amaro et al. 2015). 10 mM 5-HT stimulated pumping to 4 Hz - an effect seen after 5-minute exposure and sustained for 1 hour.

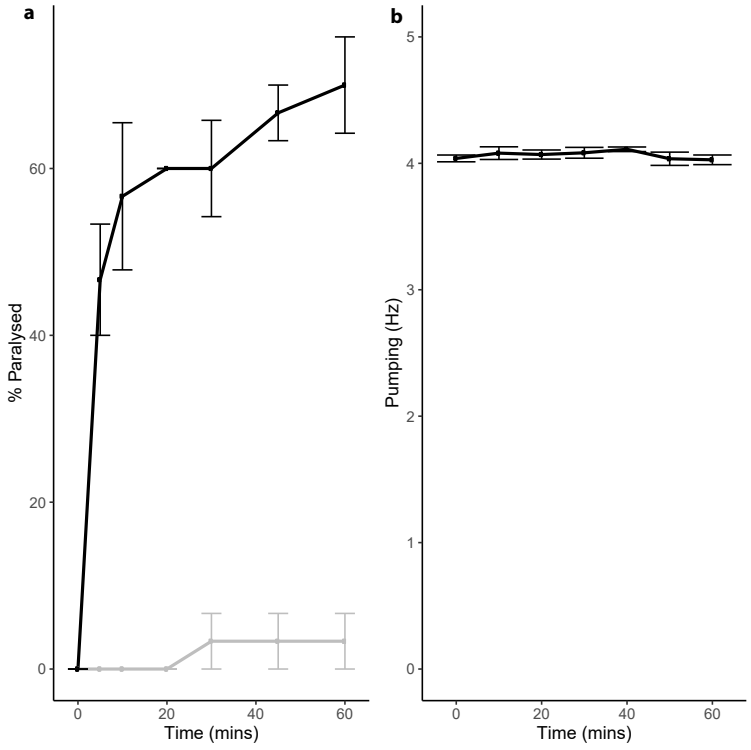


Figure 4.6: **The effects of 5-HT on *C. elegans* behaviour in liquid.** Worms were exposed to 10 mM 5-HT or vehicle control (0). The effects on locomotion over time was scored by counting the number of immobile worms pre- (time point zero) and post-exposure to treatment or vehicle control. Data are expressed as % of worms paralysed (a). Additionally, the effects of 5-HT on pumping of immobile worms was scored. The measurements were made immediately after addition of 5-HT or vehicle control (time point 0) and post-exposure at the indicated time points. Pumping was scored by visual observation by counting the number of pumps over the period of 30 seconds and expressed in Hz. Data are mean ± SEM of 3 independent repeats. Motility of 10 worms per condition was scored during each experiment.

To score the effects of compounds on 5-HT induced pumping, wild-type worms were exposed to 10 mM 5-HT. 30 minutes after, nicotine, nitenpyram, thiacloprid or clothianidin was added at the indicated concentrations, so that the worm was bathed in a solution containing both 5-HT and the drug of interest. The effect of nicotine and neonicotinoids on 5-HT stimulated pumping of paralysed worms was scored 30 minutes after the addition of nicotine or one of the neonicotinoids (Figure 4.7 and 4.8). Nicotine at concentrations ranging from 0.1 to 50 mM inhibited pumping with the EC_{50} of 1.7 mM. Concentration dependent inhibition was also caused by the incubation with nitenpyram. The estimated EC_{50} was 72.9 mM. Neither thiacloprid nor nitenpyram had an effect.

Incubation of *bus-17* mutant resulted in a decrease of EC_{50} of nicotine from 1.7 to 3.6 mM. A greater shift in potency of nitenpyram was noted. The EC_{50} increased from 72.9 in wild-type to 41.2 mM in a mutant strain. Estimated EC_{50} of thiacloprid was 4 mM whereas clothianidin at 2.5 mM did not significantly alter pumping.

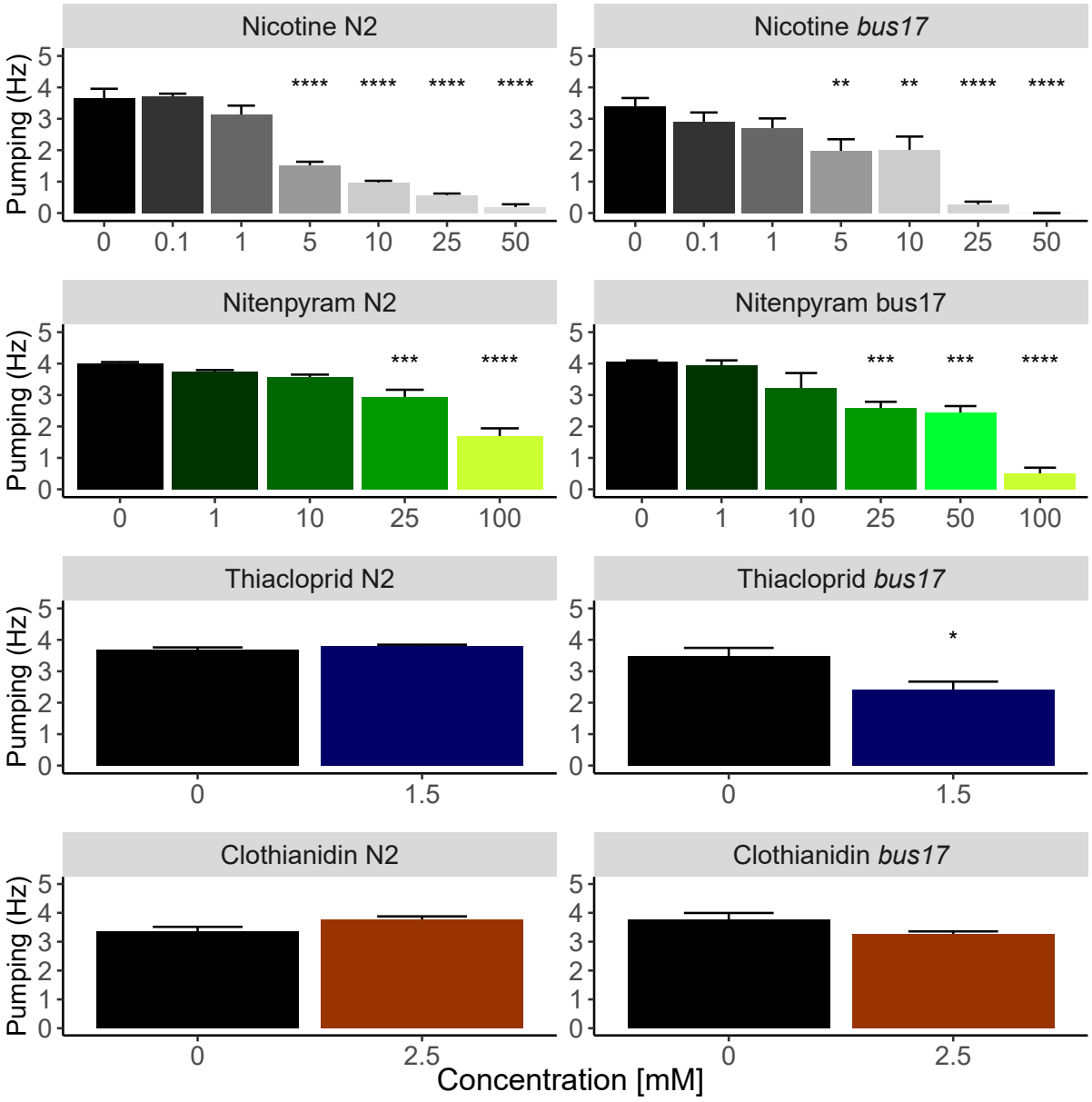


Figure 4.7: **The effects of nicotine and neonicotinoids on 5-HT stimulated pharyngeal pumping.** Pharyngeal pumping was stimulated by incubation of worms in 10 mM 5-HT for 30 minutes. Following, indicated concentrations of nicotine, nitenpyram, thiacloprid or clothianidin were added. The effect of nicotine and neonicotinoids on 5-HT induced pumping on N2 wild-type (left panel) and *bus-17* (right panel) was scored. Pharyngeal pumping was measured by visual observation for a period of 30 seconds and expressed in Hz. Data are mean ± SEM of 3-17 individual worms collected on ≥ 2 separate days. One way ANOVA (Kruskal-Wallis test) with Sidak Corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

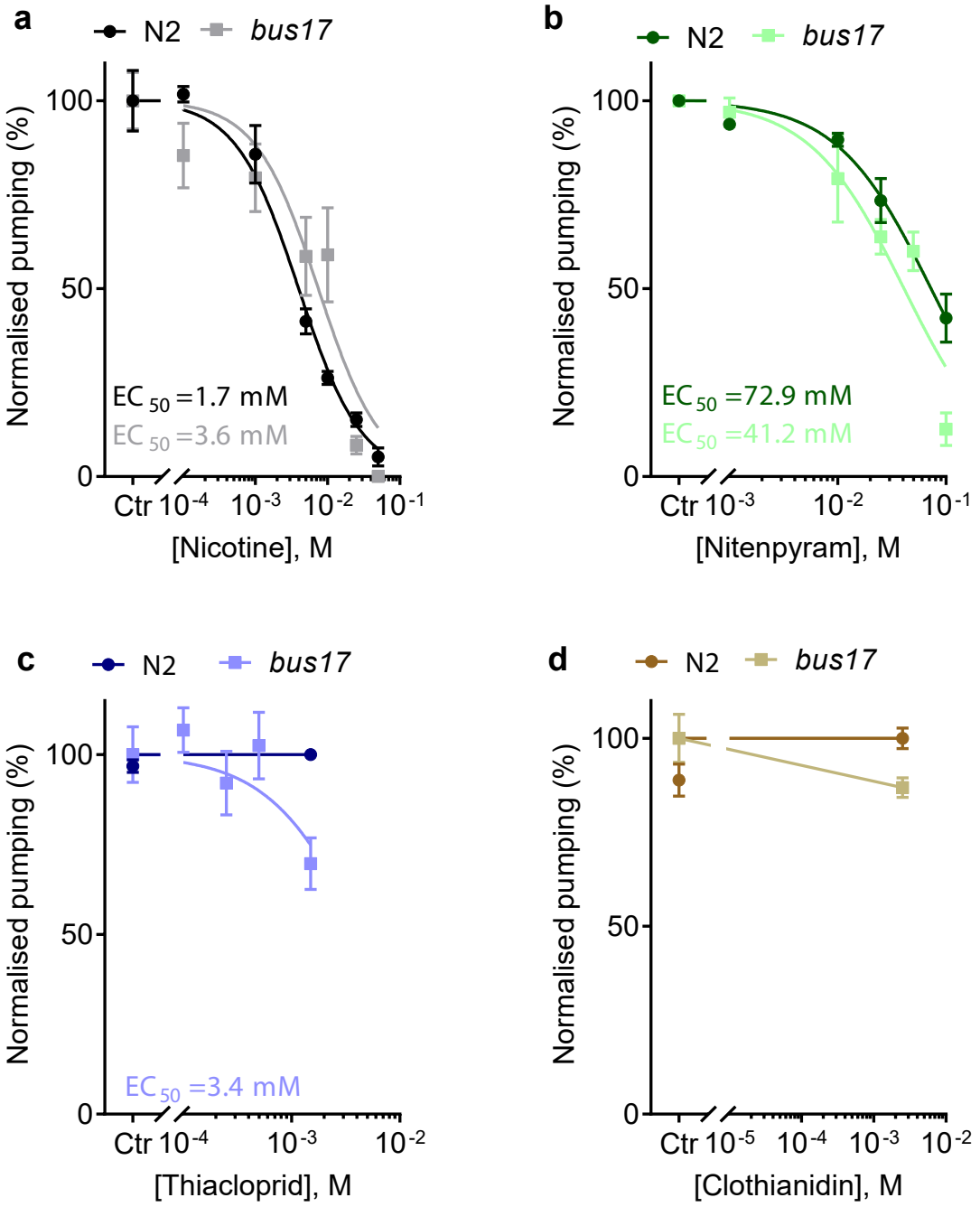


Figure 4.8: **The concentration dependence for the effects of nicotine and neonicotinoids on 5-HT stimulated pharyngeal pumping.** Concentration-dependence curves for the effects of nicotine (a), nitenpyram (b), thiacloprid (c) or clothianidin (d) on 5-HT stimulated pharyngeal pumping of N2 wild-type and *bus-17* mutant *C. elegans*. Data are expressed as % control pumping and are mean ± SEM. The EC_{50} for thiacloprid is an approximation, because at the highest concentration tested (2.5 mM) it inhibited pumping by 25 % in *bus-17*.

4.2.2 Effects on pharyngeal pumping of dissected animal

4.2.2.1 5-HT

These data show that nicotine and neonicotinoids have an effect on *C. elegans* feeding at mM concentrations and that the potency of compounds on pumping slightly increases in cuticle compromised *bus-17* mutant. This supports the idea that the cuticle presents a barrier for drug entry, limiting bioavailability and hindering the exerted effect. *Bus-17* mutant presents an attractive platform for studying the effects of the cuticle on drug's potency. An important caveat is that *bus-17* cuticle, although more permeable than the wild-type, could still hinder drug entry. To circumvent this, a liquid cut-head assay was performed. In this assay, the effects of nicotine and neonicotinoids on pharyngeal pumping of dissected, rather than the intact worm was performed.

4.2.2.2 Nicotine and neonicotinoids

To score the effects of nicotine and neonicotinoids on the pharynx, their effects on 5-HT stimulated pharyngeal pumping was determined. First, the effects of 5-HT were investigated (Figure 4.9 a and b). Exposure of cut-heads to 5-HT concentrations ranging from 10 nM to 100 μM resulted in dose-dependent stimulation of pharyngeal pumping. The maximal effect of effective doses was observed after 10 minutes of incubation and it typically gradually decreased for the next 50 minutes. 1 μM was the most effective and elicited a maximum average pumping response of 3.35 Hz. The EC_{50} for the effects of 5-HT on pharyngeal pumping of cut-heads was of 169 nM.

To determine whether the effects of 5-HT treatment were reversible, heads were washed for 15 minutes after indicated 5-HT treatment (Figure 4.9 a). Full recovery of all cut heads was noted, with the exception of those exposed to the highest concentration of 5-HT. Following treatment with 100 μM, only partial recovery of pumping was observed.

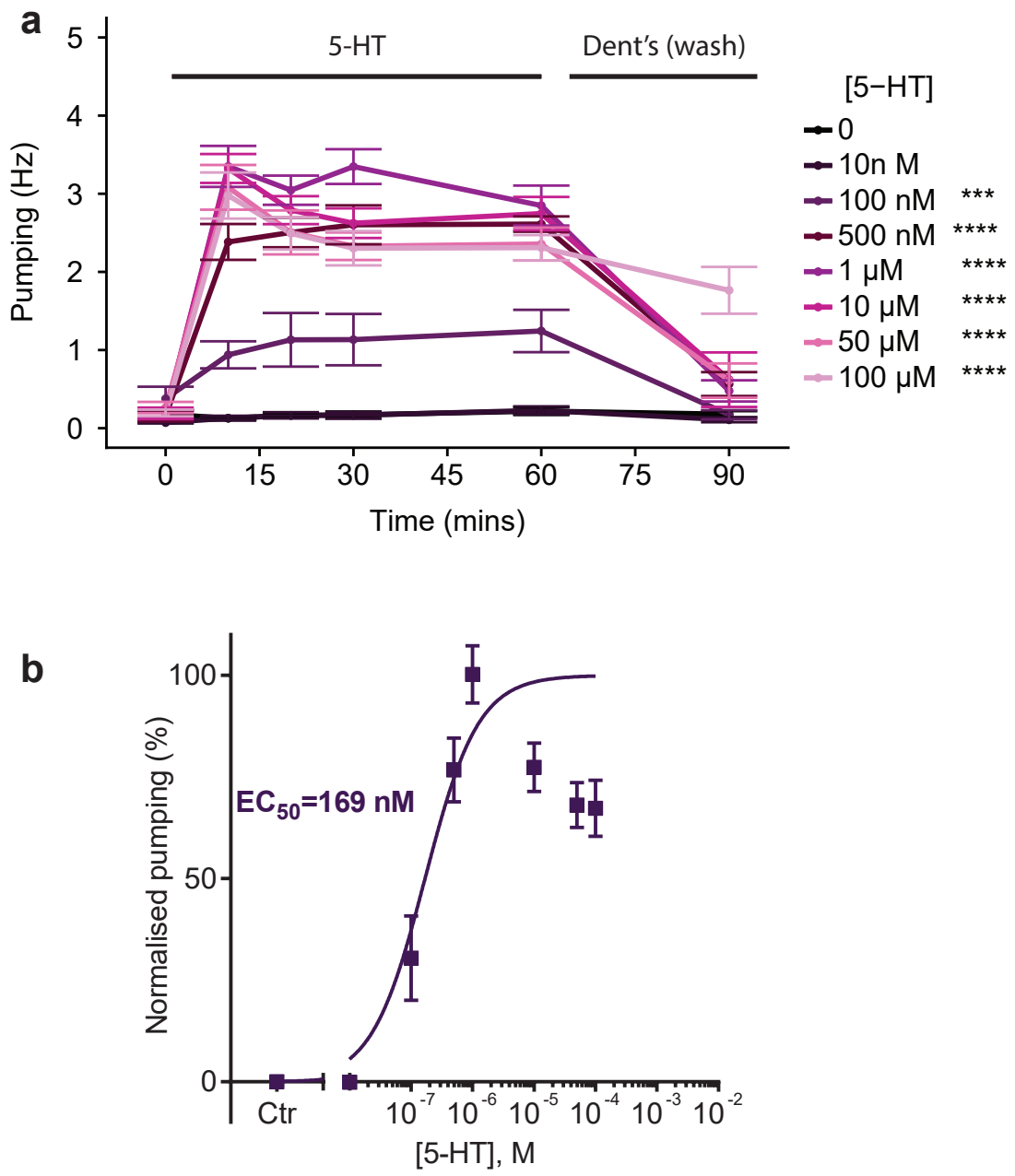


Figure 4.9: **The concentration and time dependence of the effects of 5-HT on pharyngeal pumping of dissected *C. elegans*.** a) Cut heads were exposed to varying concentrations of 5-HT, or vehicle control (0). The effects on pharyngeal pumping over time was scored by visual observation by counting the number of pharyngeal pumps in 30 seconds and the data was expressed in Hz. Data are mean ± SEM of 8 - 23 individual worms collected from experiments done on ≥ 3 days. Significance levels between the control and treatment are given in a figure legend and refer to 30 minute time points. One way ANOVA (Kruskal-Wallis test) with Sidak corrections, ∗P ≤ 0.05, ∗∗P ≤ 0.01, ∗ ∗ ∗P ≤ 0.001, ∗ ∗ ∗ ∗P ≤ 0.0001. b) Dose-response curve for the effects of 5-HT on pharyngeal pumping of dissected *C. elegans*. The graph was generated by taking 30-minute time points and fitted into nonlinear regression sigmoidal dose-response (three parameter logistic) equation. Data are mean ± SEM, normalised to control and a maximum response, and expressed as % maximum pumping.

To examine the effects of nicotine and neonicotinoids on pharyngeal pumping, cut heads were placed in 1μM 5-HT for 10 minutes. 1 μM concentration was chosen because it elicits maximal pharyngeal response, whereas 10 minutes is sufficient for the response to equilibrate. Following 5-HT incubation, cut heads were transferred to a dish containing 1 μM 5-HT a nd an indicated concentration of nicotine, nitenpyram, thiacloprid or clothianidin and the effects of nicotine or neonicotinoids on 5-HT induced pumping was measured at multiple time point over 50 minutes. To determine if the response was reversible, cut heads were then transferred to 1 μM 5-HT and recovery scored over the period of 15 minutes (Figure 4 .10).

Nicotine at concentration ranging from 1 - 20 μM inhibited 5-HT induced pumping partially, whereas 100 μM incubation resulted in acomplete termination of pumping activity (Figure 4.10 a). The maximal effects were typically observed after 10 minutes of incubation. For some concentrations i.e. 10, 20 and 50 μM, these effects weakened with time. Generally, the inhibitory effects of nicotine were reversible. Cut heads recovered from treatment with nicotine concentrations ranging from 1 - 50 μM after 15 minutes of washing. Cut heads previously incubated with 100 μM nicotine, pumped at half a rate of the control.

5-HT stimulated pharynxes were inhibited by mM c oncentrations of nitenpyram (Figure 4 .10 b). The effects were observed after 10 minutes and sustained throughout the course of the experimentation. Cut heads recovered well from nitenpyram-induced inhibition and returned to control pumping rate within 15 minutes of washing.

Thiacloprid at high μM concentrations had moderate, but not significant inhibitory effect, whereas dothianidin at asingle dose of 500 μM significantly inhibited 5-HT stimulated pumping (Figure 4.10 c and d).

To compare the relative potencies, dose-response curves for the effects of nicotine and the three neonicotinoids were plotted (Figure 4.11). The order of potency is: nicotine $>$ thiacloprid $>$ clothianidin $=$ nitenpyram. Nicotine is almost a magnitude more potent that neonicotinoids. It inhibits pumping with the EC_{50} of 10 μM, whereas neonicotinoids act at mM low range (2-3).

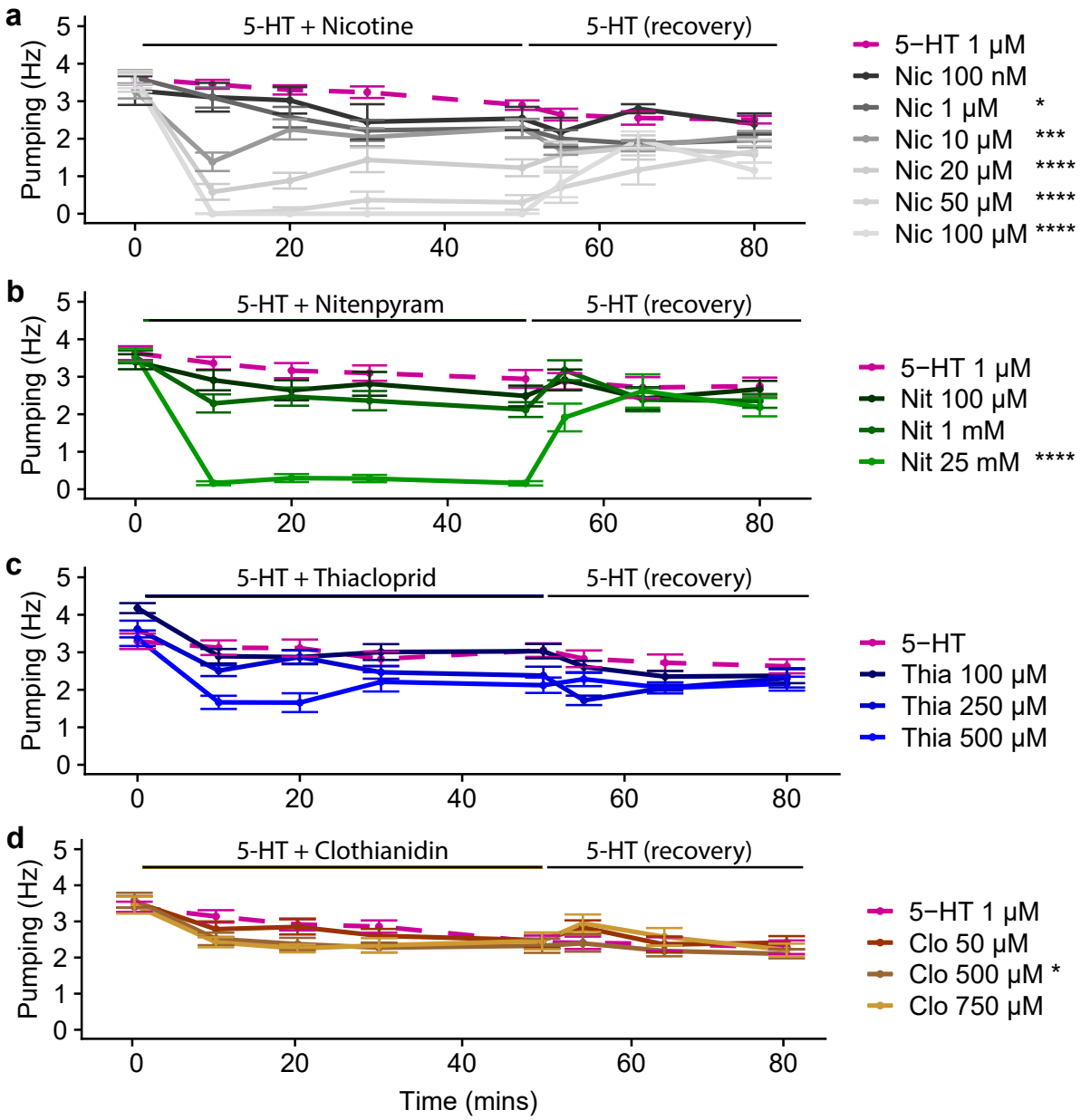


Figure 4.10: **The concentration and time dependence of the effects of nicotine and neonicotinoid on 5-HT stimulated pharyngeal pumping of dissected worm.** Pharyngeal pumping of cut heads pre- and post-exposed to 5-HT + nicotine (a), nitenpyram (b), thiacloprid (c) and clothianidin (d) or vehicle control (dashed purple line). Pharyngeal pumps were counted by visual observation for 30 seconds and expressed in Hz. Data are mean ± SEM from 3 - 34 individual worms collected on ≥ 3 days. Statistic analysis shown in legend refers to the 30 minute time points between 5-HT control and 5-HT + treatment. One way ANOVA (Kruskal-Wallis test) with Sidak corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

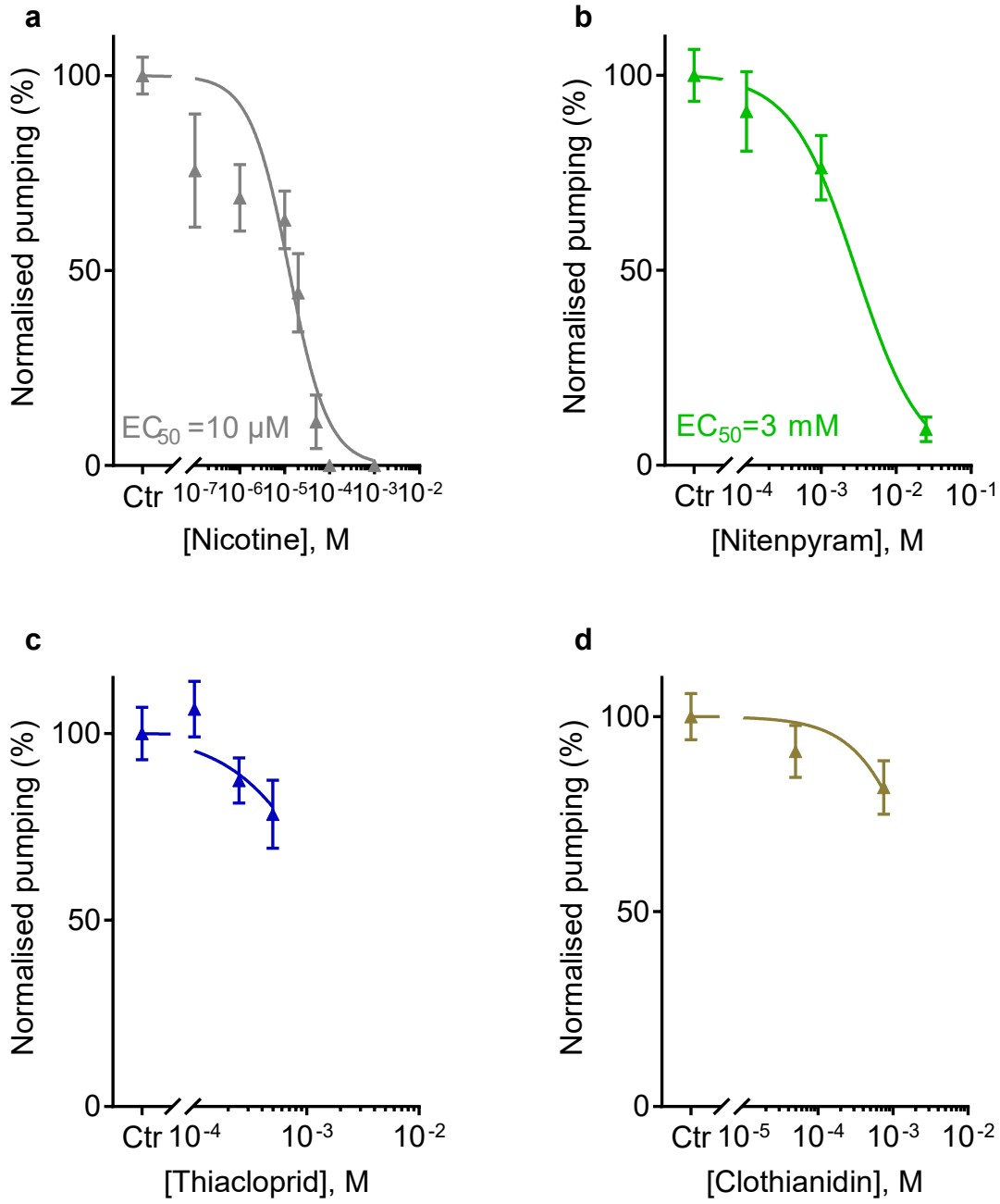


Figure 4.11: **Dose-response curves for the effects of nicotine and neonicotinoids on 5-HT stimulated pharyngeal pumping of dissected *C. elegans*.** Concentration-dependence curves for the effects of varying concentrations of nicotine, nitenpyram, thiacloprid or clothianidin on 5-HT stimulated pharyngeal pumping of cut heads. 30 minute time-points were taken (Figure 4.10) and expressed as % control (5-HT) pumping.

Cut head assays provide an opportunity to determine the effects of compounds on un-stimulated pharynx. Cut heads are not mobile, hence scoring of pharyngeal activity is possible. To score the effects of compounds on a pharynx of dissected animal, cut heads were incubated in buffer + solvent to record basal activity. Immediately after, cut heads were transferred to a dish containing an indicated concentration of nicotine, nitenpyram, thiacloprid, clothianidin or drug vehicle. The effects of treatment was scored for 1 hour and compared to the effects of 5-HT at 1 μM (Figure 4.12). Unstimulated pharynxes pump at an average rate of 0.1 Hz. Nicotine was tested at concentrations ranging from 100 nm to 100 μM (Figure 4.12 a). It had a dual action on the pharynx - stimulation at lower and inhibition at higher concentrations. At 10 μM it can be seen that a transient stimulation of pumping at 2 minute time point was seen. Incubation in 10 μM and 20 μM of nicotine led to weak but sustained stimulation of pumping - an effect seen at 20, 30 and 60 minute time points. 50 and 100 μM nicotine inhibited pumping completely. At these higher concentrations the muscle exhibited a visible twitching that can be described as fibrillation (data not shown). Neither nitenpyram nor thiacloprid had an effect on pharyngeal pumping of cut heads (Figure 4.12 b and c). In contrast, clothianidin stimulated pharynx (Figure 4.12 d). In response to 50 μM, an elevated pharyngeal pumping after 20 minutes was seen, whereas 500 and 750 μM of clothianidin elicited potent but short lived response. This response peaked after 2 minutes and returned to the basal rate after 10 minutes of incubation.

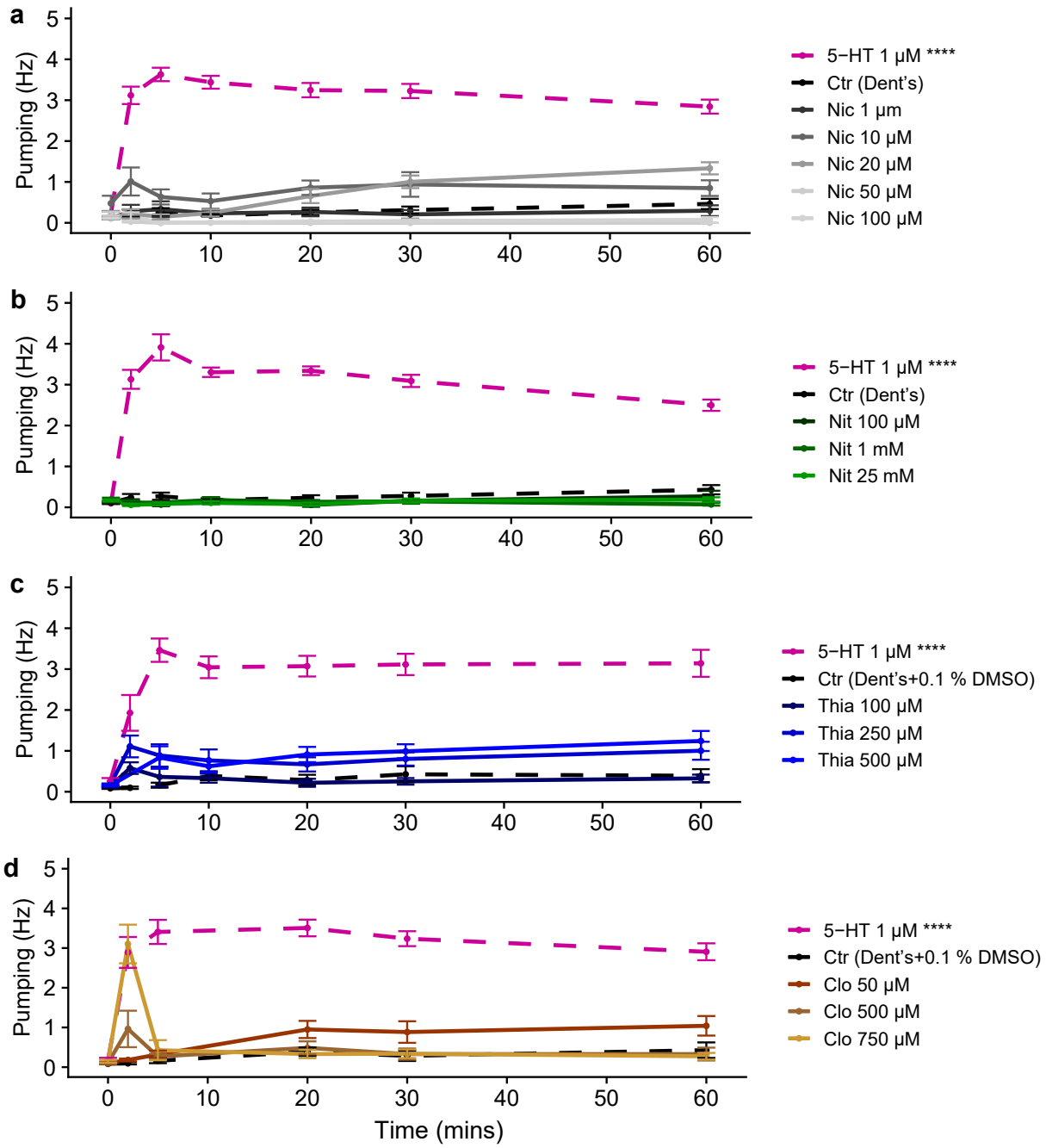


Figure 4.12: **The concentration and time dependence for the effects of nicotine and neonicotinoids on pharyngeal pumping of dissected *C. elegans*.** Cut heads were exposed to varying concentrations of (a) nicotine (Nic), (b) nitenpyram (Nit), (c) thiacloprid (Thia), (d) clothianidin (Clo), 1 μM 5-HT + vehicle control or vehicle control only (Ctr). The effects of treatment on pumping were scored over time by counting the number of pharyngeal pumps in 30 seconds time windows and expressed in Hz. Data are $\pm$ SEM of 5 - 24 worms collected on $\geq$ 3 days. Significance levels between the vehicle control and treatment are given in a figure legend and refer to 30 minute time points. One way ANOVA (Kruskal-Wallis test) with Sidak corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

To determine the onset of clothianidin induced stimulation of pumping, the experiment was repeated but the measurements were taken every minute for the first 5 minutes and at 10- minute time points (Figure 4.13). 500 and 750 μM clothianidin stimulated pumping with the onset of action at 1 minute. After 1 minute these effects began to gradually weaken.

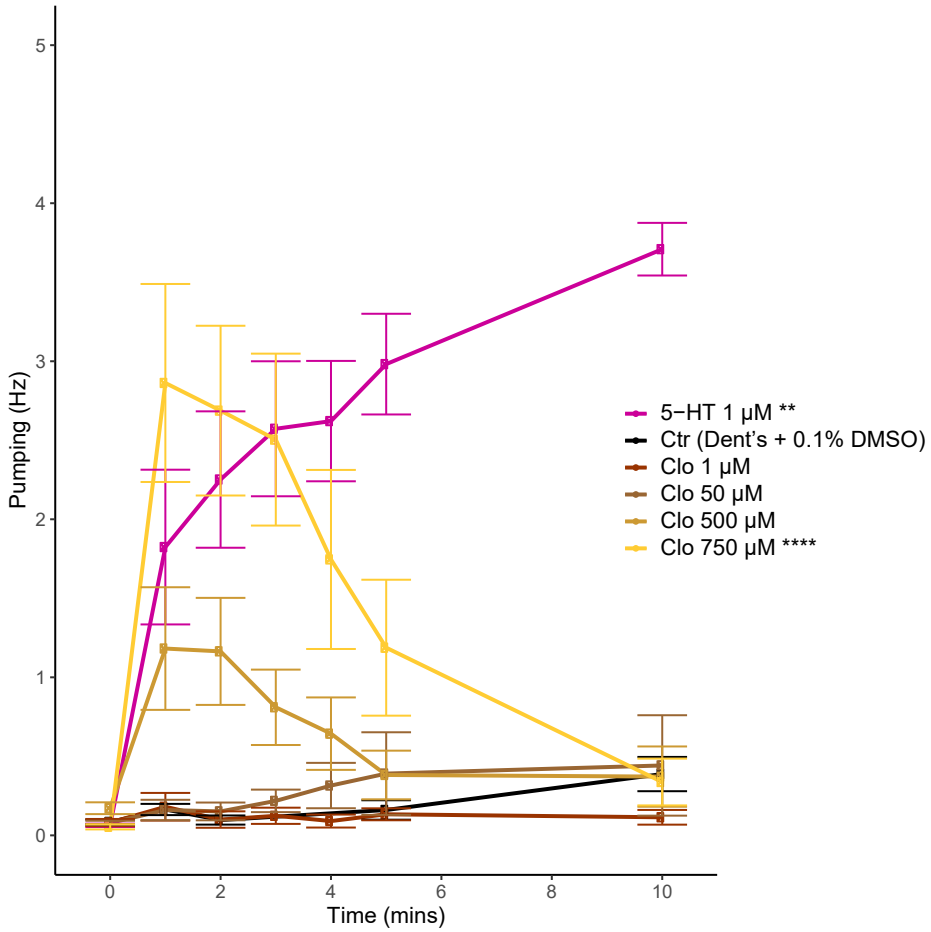


Figure 4.13: **The onset kinetics of clothianidin induced stimulation of pharyngeal pumping of dissected *C. elegans*.** Cut heads were exposed to varying concentrations of clothianidin (Clo), 1 μM 5-HT + solvent or solvent (Ctr). The effects on pumping were scored over time by counting the number of pharyngeal pumps over a 30 second time window. Data are expressed in Hz and are mean $\pm$ SEM of 7-13 individual worms collected on $\geq$ 3 days. Significance levels between the solvent control and treatment are given in a figure legend and refer to 2 minute time points. One way ANOVA (Kruskal-Wallis test) with Sidak corrections, $**P \leq 0.01$, $****P \leq 0.0001$.

4.2.3 Effects of 5-HT, nicotine and neonicotinoids on pharyngeal pumping in animals deficient in nAChR subunits

An investigation into the potential targets of action of 5-HT, nicotine and neonicotinoids on the pharynx were made. *C. elegans eat-2* and *acr-7* nAChR mutants were investigated. Both genes are expressed in the pharynx and have been implicated in the pharyngeal function (McKay et al. 2004; Saur et al. 2013) (Section 1.4.8.1). Wild-type and mutant worms were placed on agar plate containing food patch and the pharyngeal pumping rate of those present on food was scored (Figure 4.14). Pumping of the *acr-7* mutant was comparable to that of the wild-type strain. In contrast, pumping of the *eat-2* mutant was 70% lower than that of the wild-type.

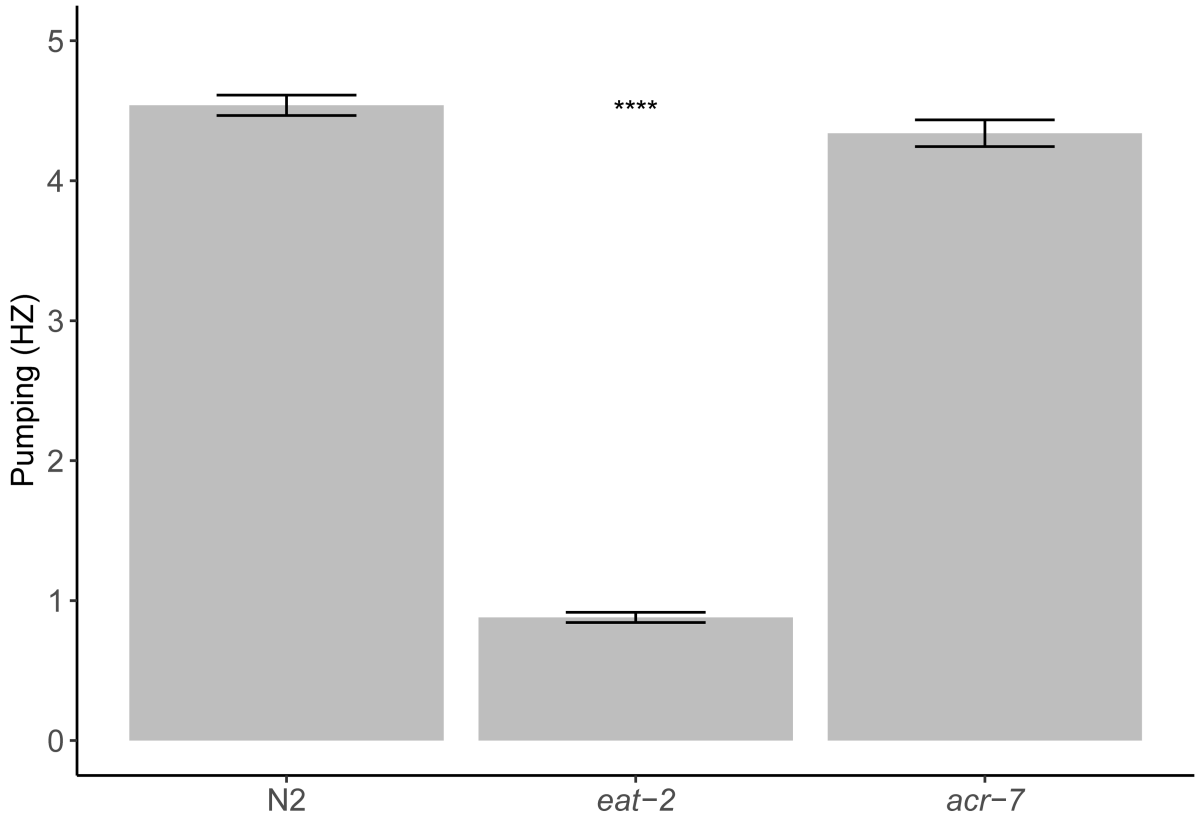


Figure 4.14: **Pharyngeal pumping of *C. elegans* nicotinic acetylcholine receptor mutants.** Pharyngeal pumping on food of N2 wild-type, *eat-2* and *acr-7* mutants. Pharyngeal pumps of worms present on food were counted by visual observation by counting the number of pharyngeal pumps in 30 seconds and expressed in Hz. Data are mean $\pm$ SEM, collected from $\geq$ 11 individual worms on $\geq$ 3 days. One way ANOVA (Kruskal-Wallis test) with Sidak Corrections, $***$ P $\leq$ 0.0001.

Further experiments were carried out to determine whether pharyngeal responses induced by 5-HT, nicotine and neonicotinoids are dependent on the expression of the EAT-2 nAChR. Cut heads of wild-type and *eat-2* mutant worms were exposed to 5-HT, nicotine, nitenpyram, thiacloprid and clothianidin. The effects of treatment on pharyngeal pumping of mutant strain was scored and compared to the wild-type. The effects of 5-HT concentrations ranging from 10 nM to 100 μM elicited dose-dependent stimulatory response in wild-type worm (Figure 4.15 a). The maximum rate of 3.5 Hz was achieved by 1 μM 5-HT after 10 minutes of incubation. 5-HT also stimulated pumping of *eat-2* mutant, but the responses were much weaker (Figure 4.15 b). The maximum rate of 0.78 Hz was achieved by 50 μM 5-HT after 60 minutes of incubation. To compare the effects of 5-HT on mutant to the effects elicited on the wild-type cut heads, data were plotted on dose-response curve (Figure 4.16). The efficacy of 5-HT on the pharyngeal pumping of *eat-2* mutant was markedly reduced. The maximum response achieved by 5-HT was 55 % lower in comparison to the wild-type. The potency was also reduced as reflected in the shift of the EC_{50} from 155 nM in wild-type to 104 μM in a mutant strain.

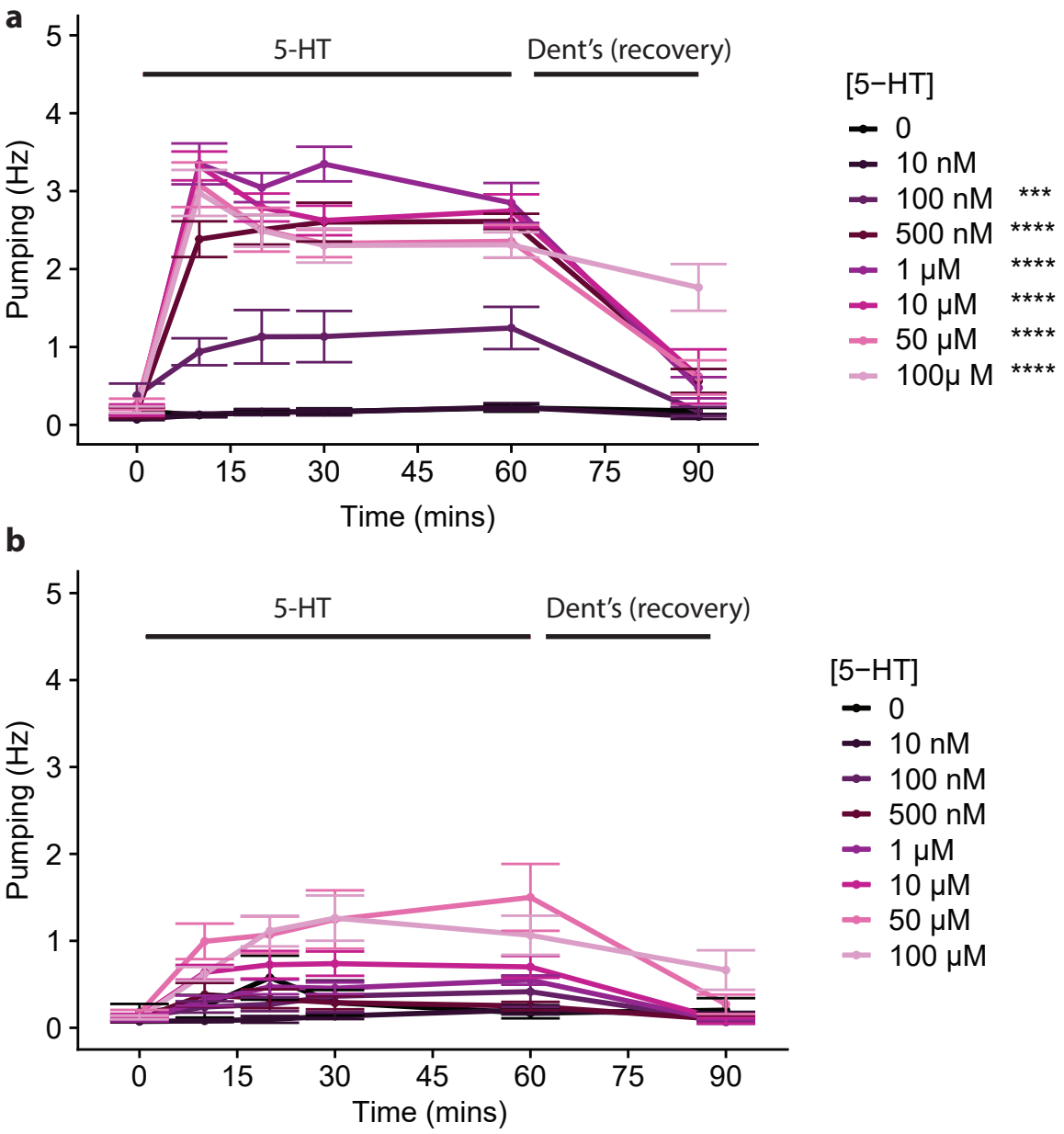


Figure 4.15: **The concentration and time dependence of the effects of 5-HT on pharyngeal pumping of dissected wild-type and *eat-2 C. elegans*.** a) Wild-type (N2) and mutant *eat-2* cut heads were exposed to varying concentrations of 5-HT, or vehicle control (0). The effects on pharyngeal pumping over time were scored by visual observation by counting the number of pharyngeal pumps in 30 seconds and expressed in Hz. Data are mean ± SEM of 8 - 23 individual worms collected from paired experiments done on ≥ 3 days. Significance levels between the control and treatment are given in a figure legend and refer to 30 minute time points. One way ANOVA (Kruskal-Wallis test) with Sidak corrections, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

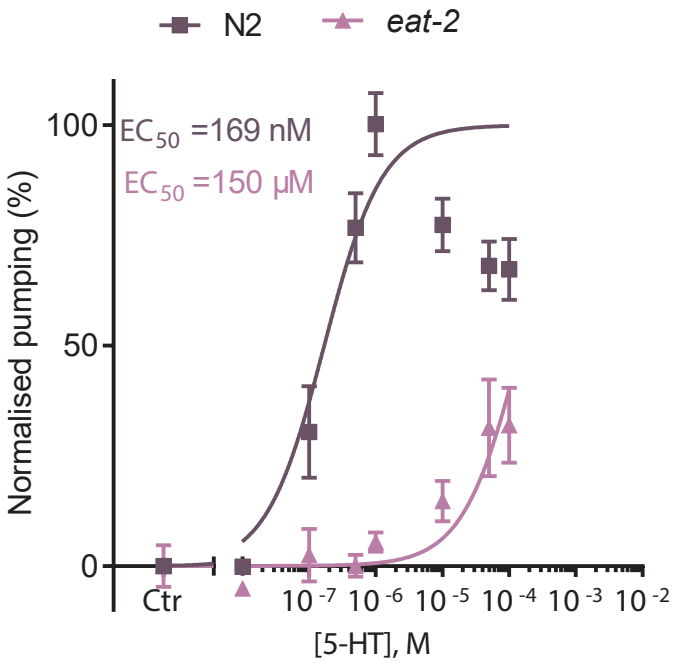


Figure 4.16: **Dose-response curves for the effects of 5-HT on pharyngeal pumping of N2 and *eat-2* cut heads.** The graphs were generated by taking 30-minute time points (Figure 4.15). Data are mean ± SEM, normalised to control and a maximum response elicited on N2 strain, expressed as a % maximum pumping.

The investigations into the direct effects of nicotine, nitenpyram, thiacloprid and clothianidin on *eat-2* cut-head pumping were carried out in visual observation experiments. These data were compared to the effects induced on the cut heads of wild-type animals (Figure 4.17). Nicotine stimulated wild-type pharynx at 10 and 20 μM. At 50, 100 μM and 1 mM it inhibited pumping. Nicotine stimulated pumping of *eat-2* mutant at concentrations ranging from 10 - 100 μM. A dose of 1 mM was required to observe pumping inhibition. Nitenpyram at 100 μM to 100 mM was with no effect on either of the strains (Figure 4.17 b). Thiacloprid at 250 and 500 μM stimulated pumping of both wild-type and *eat-2* strains (Figure 4.17 c). However, a greater stimulation of *eat-2* by thiacloprid at 250 μM was observed in comparison to the wild-type. Clothianidin had a stimulatory effects on the wild-type worm. This was also observed in the mutant (Figure 4.17 c).

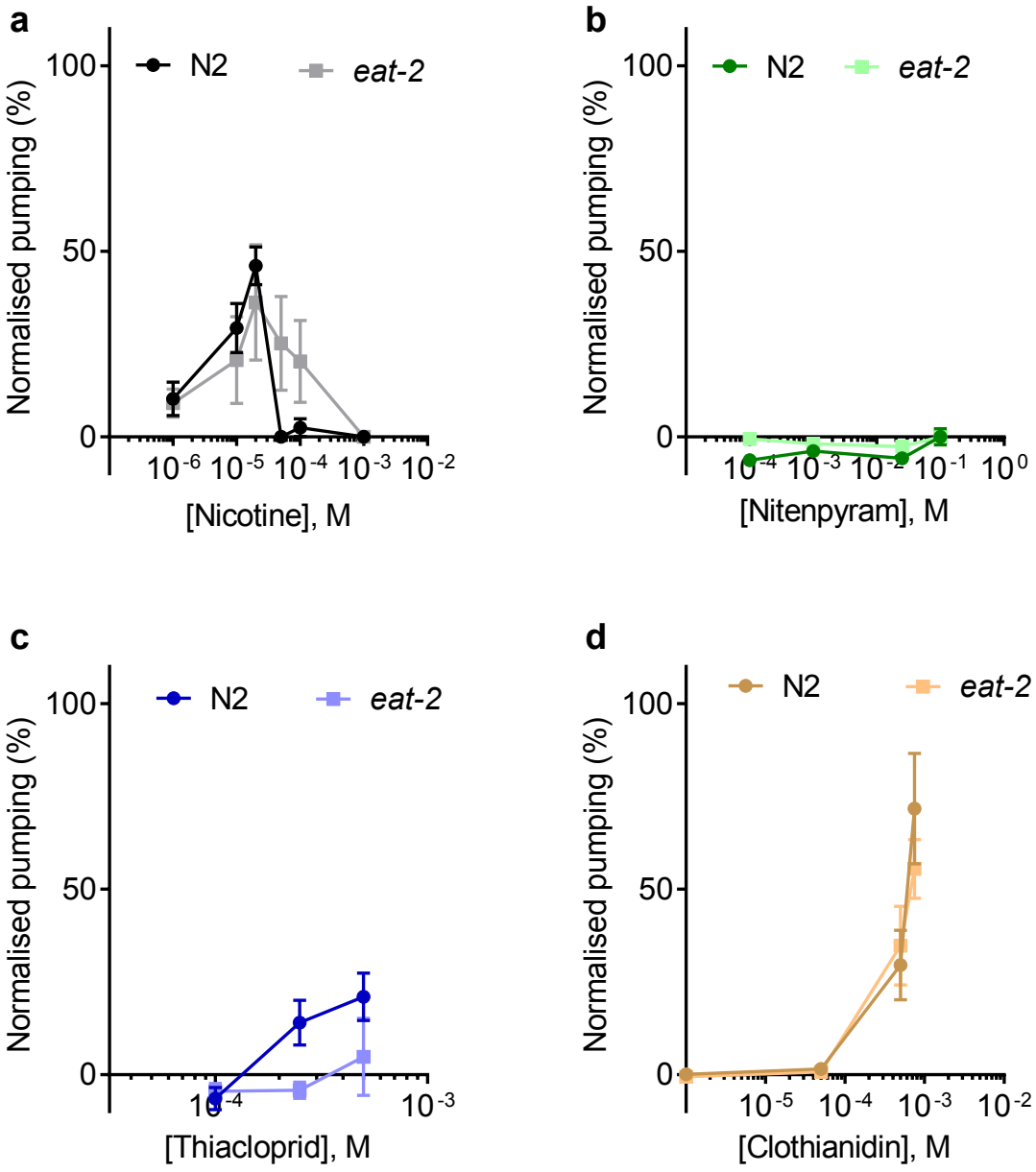


Figure 4.17: **The effects of *eat-2* mutation on the concentration dependence of nicotine and neonicotinoid-induced pharyngeal pumping responses.** N2 wild-type and *eat-2* mutant cut heads were exposed to varying concentrations of a) nicotine, b) nitenpyram, c) thiacloprid and d) clothianidin. The number of pharyngeal pumps over 30 s at 60 minute for nicotine, nitenpyram and thiacloprid or 2 minute time-points for clothianidin were taken (Figure 4.10) and expressed in Hz. Data are mean ± SEM of 5-25 individual worms collected from paired experiments done on ≥ 3 days. For comparison, the maximum pumping achieved by 5-HT is shown in dashed line.

4.3 Discussion

In this chapter, the effects of nicotine and neonicotinoids on pharyngeal pumping of intact and dissected, cut-head *C. elegans* are described. Pharyngeal pumping was assayed by visually scoring the frequency of pharyngeal pumps. These investigations were carried out to further investigate the toxicity of nicotine and neonicotinoids on worms, and to explore the role of the worm's cuticle on drug susceptibility.

4.3.1 5-HT induces fast pumping in *C. elegans* by the activation of EAT-2 containing nAChRs

Application of 5-HT on cut-head preparation elevated pumping from 0.17 Hz to 3.34 Hz with the EC_{50} of 169 nM (Table 4.2). These data suggest that 5-HT drives a feeding response in worms. The maximum pumping frequency achieved by the exogenous 5-HT is comparable to the pharyngeal pumping rate of the worm in the presence of food (3.34 Hz and 4.33 Hz, respectively). Pumping rate on food of a mutant with defective 5-HT biosynthesis pathway is markedly reduced (Sze et al. 2000). Endogenous 5-HT is released from NSM and ADF neurons in response to the presence of food. It then acts on MC and M4 neurons (Raizen, Lee, and Avery 1995; Niacaris 2003) to increase pumping frequency and on M3 neurons (Song et al. 2013; Niacaris 2003) to reduce the pumping latency. Application of 5-HT bypasses the sensory pathway to activate MC, M4 and M3 neurons directly by acting on GPCRs (Hobson et al. 2003; Song et al. 2013).

To provide an insight into the mechanism of 5-HT induced *C. elegans* responses, the effects of 5-HT on pharyngeal pumping of cut head *eat-2* nAChR mutant were performed and compared to the wild-type. The maximum response achieved by a 5-HT was reduced by 70 %, whereas the EC_{50} increased from 169 nM in wild-type to 150 μM in a mutant (Table 4.2). This relative 5-HT insensitivity of *eat-2* strain suggests 5-HT induces pumping by eliciting cholinergic neurotransmission via EAT-2 containing nAChRs. 5-HT acts on cholinergic MC and M4 motorneurons to stimulate ACh release. ACh binds to EAT-2 nAChRs expressed at the MC NMJ to stimulate pumping (McKay et al. 2004). Hence 5-HT evokes pharyngeal activity by indirectly activating EAT-2 containing nAChRs.

4.3.2 Cuticle limits efficacy of nicotine and neonicotinoids on *C. elegans* pharynx

To investigate the role of the cuticle in susceptibility of worms to neonicotinoids, experiments were carried out on intact worm of two strains: wild-type and *bus-17* mutant. *Bus-17* strain has altered surface coat (Gravato-Nobre et al. 2005) and reduced cuticle integrity (Yook and Hodgkin 2007). The pharynx was stimulated with 5-HT and the effects of nicotine and neonicotinoids on 5-HT induced pumping were examined (Figure 4.18). Nicotine and nitenpyram inhibited 5-HT evoked pharyngeal pumping at mM concentrations. The efficacy of nicotine as measured by EC_{50} was comparable on both strains: 2 and 3 mM on wild-type and

mutant strain, respectively. In contrast, the EC_{50} of nitenpyram increased from 73 in wild-type to 42 mM in a mutant strain. Further experiments were performed in which the cuticular barrier was removed and the effects of compounds on 5-HT stimulated pumping of dissected, cut-head wild-type *C. elegans* were investigated (Figure 4.18). Nicotine inhibited pumping with the EC_{50} of 10 μM, whereas the EC_{50} of nitenpyram was 3 mM. By comparing this to the intact worm data, it can be seen that the removal of the cuticle resulted in a 300 and 24-fold increase in efficacy of nicotine and nitenpyram on the 5-HT evoked pumping of wild-type worm, respectively. Due to poor solubility, testable concentrations of clothianidin and thiacloprid were limited. In the presence of high μM - low mM concentrations of thiacloprid or clothianidin, the 5-HT evoked pumping of intact and cut head worm was slightly reduced. No full dose-response curves were obtained, however, there is a trend towards left shift of partial dose-response curves (Figure 4.18) for both compounds. Taken together this suggested that the cuticular structures are limiting the access of nicotine and nitenpyram to the receptors of the pharyngeal system of *C. elegans*. The greater decrease of EC_{50} for nicotine versus nitenpyram might suggest better permeability of the latter, possibly due to its higher hydrophobicity (Table 1.2).

4.3.3 Differential effects of nicotine and neonicotinoids on the pharynx

To further characterise the effects of compounds on the pharyngeal pumping, nicotine and neonicotinoids were applied on unstimulated wild-type *C. elegans* cut-head (Table 4.2). Differential effects of nicotine and neonicotinoids are noted.

Nicotine had a concentration dependent effect: a moderate stimulation by 10 and 20 μM and an inhibition of pumping activity by 50 and 100 μM.

Scoring the effects of clothianidin on pharyngeal pumping revealed potent but transient effect at high μM concentrations. The maximum stimulatory effect was observed after 2 minutes of incubation and declined progressively to the basal pumping rate within 10 minutes.

Thiacloprid at high μM concentrations moderately stimulated pumping. This effect was sustained throughout the duration of the assay (1 hour). Nitenpyram at doses up to 100 mM had no effect on pharyngeal activity.

The differences in the pharyngeal effect achieved by nicotine and neonicotinoids could be due to targeting different receptors proteins. To investigate this further, the effects of nicotine and neonicotinoids on pharyngeal pumping of nAChR subunit mutant were investigated. Compounds were applied on cut heads of *eat-2 C. elegans* mutant and wild-type. Pharyngeal pumping was scored visually after 30 minutes of exposure (Table 4.2). Different responses at 50 and 100 μM nicotine were noted. A stimulation of pumping occurred in the mutant, whereas pumping was inhibited in wild-type worms. Clothianidin at 250, 500 and 750 μM stimulated pumping of both strains. A difference at a single concentrations of thiacloprid was noted. At 250 μM thiacloprid moderately elevated pumping of *eat-2*, but it had no effect on the wild-type. However, there was no difference at 100 and 500 μM. Nitenpyram was with no effect on either strain. Taken together, these data suggests that

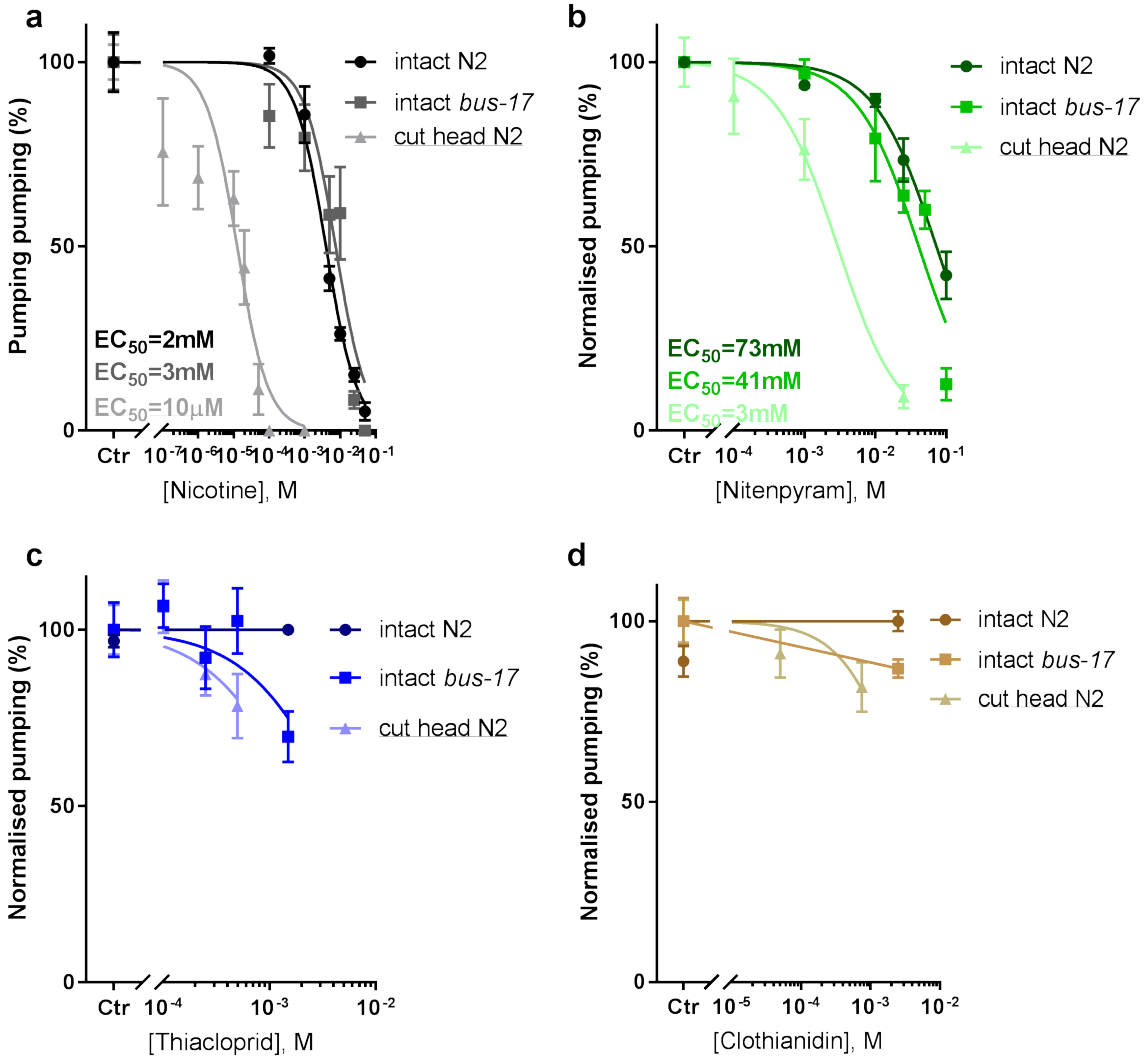


Figure 4.18: **The effects of the cuticle on nicotine and neonicotinoid - induced inhibition of 5-HT induced pumping.**

EAT-2 may be involved in the pharyngeal responses to nicotine, but there are likely other receptors involved too, whereas neonicotinoids act on receptors other than EAT-2.

Differential pharyngeal responses to nicotine and neonicotinoids suggest they have different mode of action in the *C. elegans* pharynx. This suggests the existence of multiple types of nAChRs with distinct pharmacology with regards to neonicotinoids.

4.3.4 Neonicotinoids may target different receptor protein in *C. elegans* pharynx

Neonicotinoids may have a differential mode of action on *C. elegans* pharynx. Clothianidin and thiacloprid have distinct stimulatory effects on pumping. Clothianidin induced transient, whereas thiacloprid induced sustained stimulation. In contrast, nitenpyram has no effect. Differential effects of neonicotinoids on animal behaviour was also observed in bumblebee (Moffat et al. 2016), honey and wild-bee (Woodcock et al. 2017) and could be underpinned by the differences in targeted receptors. Indeed, imidacloprid and clothianidin target distinct nAChRs in the honey bee mushroom body (Moffat et al. 2016) and in *Cockroach* neuronal preparation (Thany 2009).

4.3.5 Alternative sites for the action of nicotine and neonicotinoids

What are the alternative molecular sites for nicotine and neonicotinoids in worms? The response to nicotine does not depend on pharyngeal neurons (Raizen, Lee, and Avery 1995) suggesting nicotine acts directly on the muscle and/or on somatic nervous system. Behavioural and genetic analysis suggests nicotine can act on nAChRs other than EAT-2. The response to food in *lev-8* (Towers et al. 2005) mutant is reduced, making LEV-8 a potential target. Nicotine could also act on somatic nervous system, for example on IL1 and/or IL2 labial sensory neurons. IL2 are cholinergic (Pereira et al. 2015) and express nAChR DES-2 subunit (Treinin et al. 1998). IL1 neurons are involved in mechanosensation, they express ACR-2 nAChR subunits (Nurrish, Ségalat, and Kaplan 1999; Hallam et al. 2000). Both IL1 and IL2 output onto pharyngeal RIP neurons (Albertson and Thomson 1976; Serrano-Saiz et al. 2013) which are a point where extrapharyngeal and pharyngeal nervous system connect (Albertson and Thomson 1976).

Nicotine can also bind to receptors other than nAChRs, such as TRPV channels (Liu et al. 2004; Talavera et al. 2009; Feng et al. 2006). TRPV channels are expressed on IL1 neurons (Kindt et al. 2007), however effective nicotine doses are higher than those used in this study (i.e. typically $\geq$ 100 μM (Liu et al. 2004; Talavera et al. 2009).

4.3.6 Relative insensitivity of *C. elegans* to neonicotinoids

Concentrations of neonicotinoids effective against *C. elegans* feeding, are at least several fold higher than those effective against the feeding of insects. Clothianidin and thiacloprid stimulated pharyngeal pumping at high μM concentrations. In insects, they impair on feeding at sub μM concentrations. Imidacloprid inhibits feeding of mayflies (Alexander et al. 2007), thiamethoxam impairs on the feeding of bumble bees and some species of wild-bees (Baron, Raine, and Brown 2017). This supports results from the previous chapter that at field realistic concentrations, neonicotinoids have no impact on tested *C. elegans* behaviours.

4.3.7 Pharyngeal nAChRs have low sensitivity to neonicotinoids

The low potency of neonicotinoids on the pharynx in cut-head preparation suggest pharyngeal *C. elegans* nAChRs have low sensitivity to neonicotinoids relative to insects. Electrophysiological recordings from insect neuronal preparations show the effects of neonicotinoids at sub μM doses (Thany 2009; Moffat et al. 2016; Tan, Salgado, and Hollingworth 2008; Buckingham et al. 1997). In this study, a dose of at least 250 μM was required to observe an effect. Therefore, there is at least several fold difference in the susceptibility to neonicotinoids between the worm and insects. This suggests *C. elegans* nAChRs are pharmacologically distinct from those found in insects.

Table 4.2: Summary of the effects of compound on the pharyngeal pumping of *C. elegans* wild-type (N2) and nAChR mutant *eat-2*.

	N2	*eat-2*
5-HT	1. Dose dependent and sustained stimulation 2. Maximum pumping 3.34 Hz 3. EC_{50} = 169 nM	1. Dose dependent and sustained stimulation 2. Maximum pumping 0.87 Hz 3. EC_{50}=150 μM
Nicotine	1. Sustained stimulation by 10 and 20 μM 2. Inhibition by concentrations from 50 μM to 1 mM	1. Sustained stimulation by 10 100 μM 2. Inhibition by 1 mM
Nitenpyram	1. No effects at 0.1-100 mM	As N2
Thiacloprid	1. Weak stimulation by 500 μM	1. Weak stimulation by 250 and 500 μM
Clothianidin	1. Weak and sustained stimulation by 50 μM 2. Potent, dose-dependent and transient stimulation by 500 and 750 μM 3. Onset at 2 minutes	As N2

Chapter 5

Pharmacological characterisation of the *C. elegans* pharynx

5.1 Introduction

The previous chapter (Chapter 4) describes the effects of nicotine, 5-HT and neonicotinoids on pharyngeal pumping of the "cut-head worm". An alternative pharyngeal assay is an extracellular recording of the electrical action of the pharynx. This is an electropharyngeogram (EPG). Unlike scoring pharyngeal pumping by visual observation, EPG recordings allow for more detailed temporal resolution. Additionally, investigations into drug-induced changes of the EPG waveform may inform on the potential mode of action of compounds.

In an EPG assay, a worm's head is placed in a recording chamber. A tight seal between the electrode and the tip of the *C. elegans* nose is made. Contracting pharyngeal muscle produces currents which flows out of the worm's mouth; this is detected by the electrode. Each cycle of contraction and relaxation gives rise to a characteristic waveform. There are several different phases constituting an individual EPG (Figure 5.1). The beginning of the EPG signal marks excitatory phase. This phase mirrors depolarisation and contraction of the corpus and the terminal bulb and constitutes from 2 spikes. Excitatory e spike arises due to the release of ACh from MC neurons and an activation of nAChRs (Raizen, Lee, and Avery 1995). Excitatory E peak is due to a subsequent calcium channels activation (Lee et al. 1997; Shtonda and Avery 2005). e is often unseen on the EPG trace due to merging with the larger E. I or inhibitory spikes are diverse in number and amplitude and arise as a result of the inhibitory currents. These currents are produced by ligand-gated chloride channels in response to glutamate release from M3 neurons (Dent, Davis, and Avery 1997; Li et al. 1997). Lastly, R and r reflect relaxation of the corpus and the terminal bulb, due to a repolarisation of the terminal bulb muscle cells caused by the flow of potassium through the potassium ion channel (Shtonda and Avery 2005). r spikes are frequently merged with larger in amplitude R.

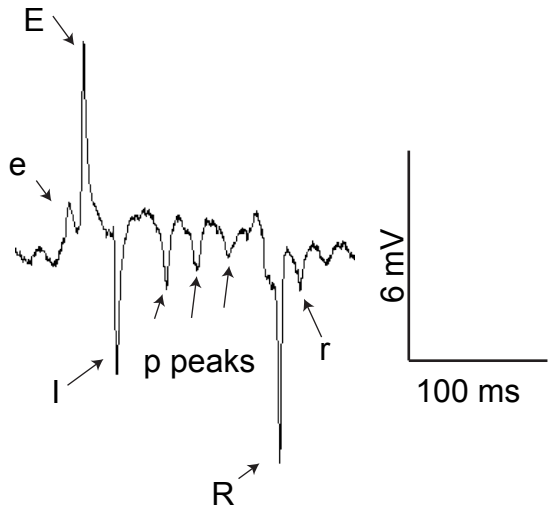


Figure 5.1: **Electropharyngeogram (EPG) of *C. elegans* pharynx.** A single pharyngeal activity recorded extracellularly from the wild-type cut head of *C. elegans*. E peaks arise due to contraction, whereas I and R peaks due to relaxation of the pharynx (Dillon et al. 2009).

The shape and amplitude of a single EPG varies between worms even of the same genetic make-up. Therefore caution should be taken when the effects of drugs on EPG characteristics are made. Typically, three parameters are quantified: the frequency of EPGs (in Hz), the duration of a single pump (measured by a time distance between E and R spike) and E/R ratio. E and R spikes are present on each EPG waveform, therefore they can be used to quantify mentioned parameters (Dillon et al. 2009).

5.1.1 Chapter aims

In this chapter, the effects of 5-HT and the cholinergic compounds on EPG are investigated. These results are compared to the effects elicited by nicotine and neonicotinoids to further inform on what is their mode of action.

5.2 Results

To assess the effects of exogenous drug application on the pharynx an EPG - an extracellular recordings - from the *C. elegans* pharynx in cut head preparation were made. The pharynx was perfused for 5 minutes in Dent's saline to record basal pumping rate, for 5 minutes in the drug treatment to record changes in the spontaneous pharyngeal activity due to drug application and again for 5 minutes in Dent's saline to determine if the pharynx recovers from drug-induced changes in pumping.

5.2.1 Effects of 5-HT on electropharyngeogram

Application of 5-HT concentrations ranging from 1 nM to 10 μM led to dose dependent stimulation of the pharyngeal pumping, sustained throughout the 5-minute perfusion (Figure 5.2). The maximum response achieved was 4.4 Hz by 10 μM. The EC_{50} was 255 nM. Washing the pharynx for 5 minutes was sufficient to observe recovery from the 5-HT induced stimulation of pumping.

To determine whether 5-HT had an effect on the shape of an EPG, individual EPG waveforms were examined closely (Figure 5.3a). A visible decrease in duration of the pump in response to 5-HT was noted. To quantify this, the pump duration of EPGs during the basal and treatment recording as well as after 5 minute wash were measured (Figure 5.3). The direct measurements were made by quantifying the time taken from E to R peak. 1 μM 5-HT reduced the latency by 27 %. This effect was reversible and returned to basal duration after 5 minutes of washing.

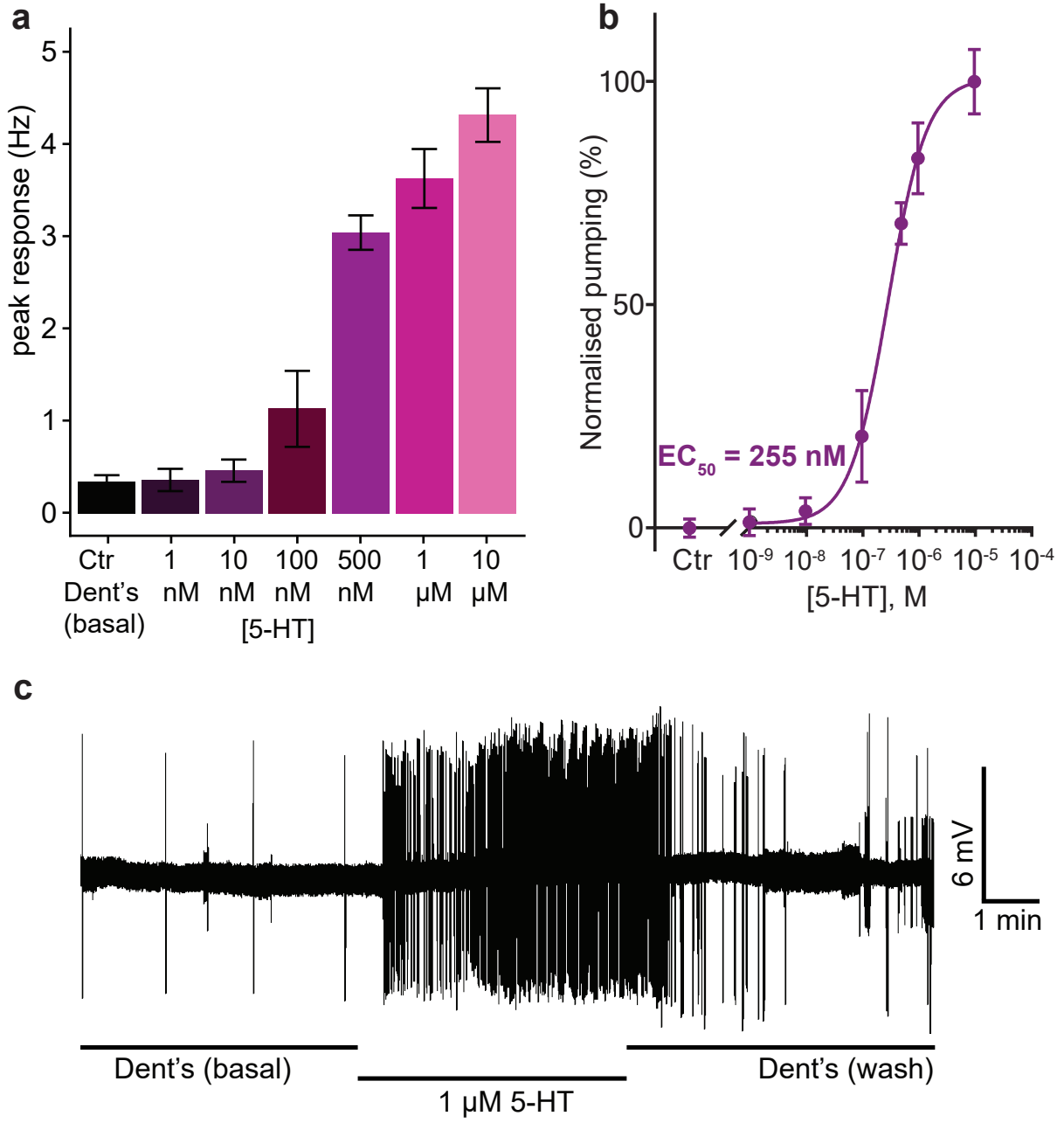


Figure 5.2: **The concentration dependence for the effects of 5-HT on the EPG frequency.** *C. elegans* cut heads were perfused with Dent's saline and 5-HT at indicated concentrations. The effects of varying concentrations of 5-HT on pharyngeal pumping was scored by extracting peak response in a 10 second window. Data are mean $\pm$ SEM from 3-6 individual worms collected on $\geq$ 2 days. b) Dose-response curve for the effects of 5-HT on the pharynx. Responses are normalised to basal pumping rate and expressed as a % maximum response. c) Example EPG recording showing basal activity, stimulated activity upon perfusion with 1 μM of 5-HT and recovery post 5-HT exposure. The response to 1 μM 5-HT represents the sustained excitatory effects of 5-HT on the pharynx.

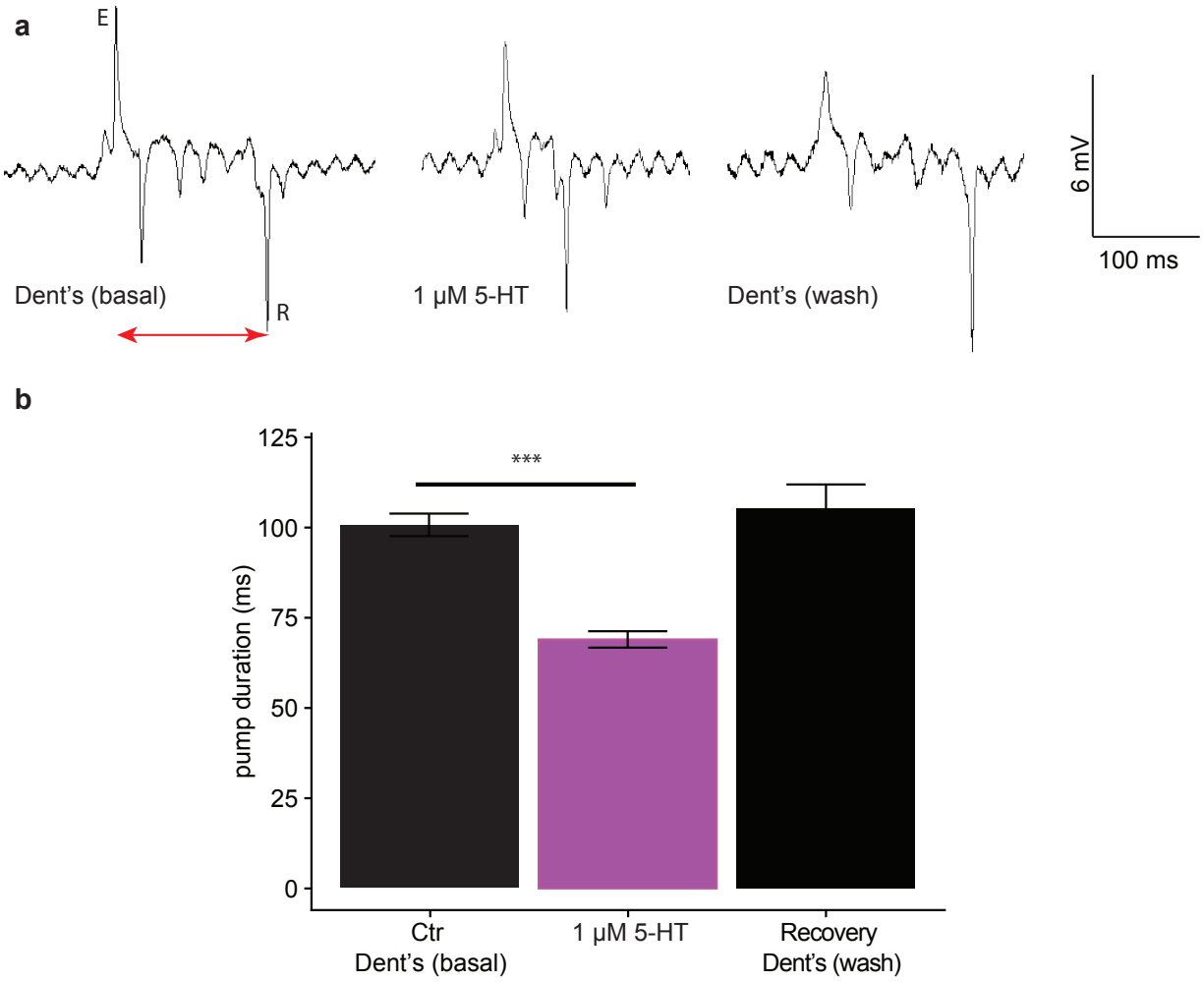


Figure 5.3: **Effects of 5-HT on the pump duration of *C. elegans*.** Basal and 5-HT exposed EPG waveforms were taken from the time window where maximum pumping activity was noted, whereas the wash EPG waveform was taken from the last minute of wash. Representative EPGs are shown in (a). Pump duration was derived by measuring the average time from E to R peaks (red arrow in a) of all EPGs over the peak response period (if there were less than 10 EPG in 10s window, 10 consecutive peaks were taken) (b). Data are mean ± SEM from 4 individual worms collected on 3 days. Two-tailed paired t-test with Welch correction. $***P \leq 0.001$.

5.2.2 Effects of acetylcholine on electropharyngeogram

Acetylcholine was applied at concentrations ranging from 1 to 100 μM. This led to concentration and time dependent effects on pumping (Figure 5.4). 10 μM acetylcholine stimulated pumping. This stimulation was sustained throughout the 5 minute perfusion and reversed to basal activity upon washing (Figure 5.4 a). Exposure of the pharynx to 15, 25, 50 or 100 μM acetylcholine led to potent stimulation of the pharyngeal activity before blocking its activity completely. Following, two types of activities were recorded: the pharynxes remained blocked even after 5 minutes of washing (Figure 5.4 b), or began pumping again whilst being perfused with acetylcholine (Figure 5.4 c). The ratio of pharynxes exhibiting the first or the second type of response to acetylcholine concentrations $\geq$ 15 μM was 1 : 1 and this was not concentration dependent (data not shown).

A closer look at the primary response of the pharynx to acetylcholine at 25 μM was taken (Figure 5.5). The observed stimulation was characterised by a train of EPG spikes, progressively increasing in frequency and deceasing in amplitude (Figure 5.5 b and c) until the pharyngeal activity was completely inhibited. To determine the potency of acetylcholine on the spike frequency, the stimulatory effect of acetylcholine was scored. The EC_{50} was 22 μM (Figure 5.6).

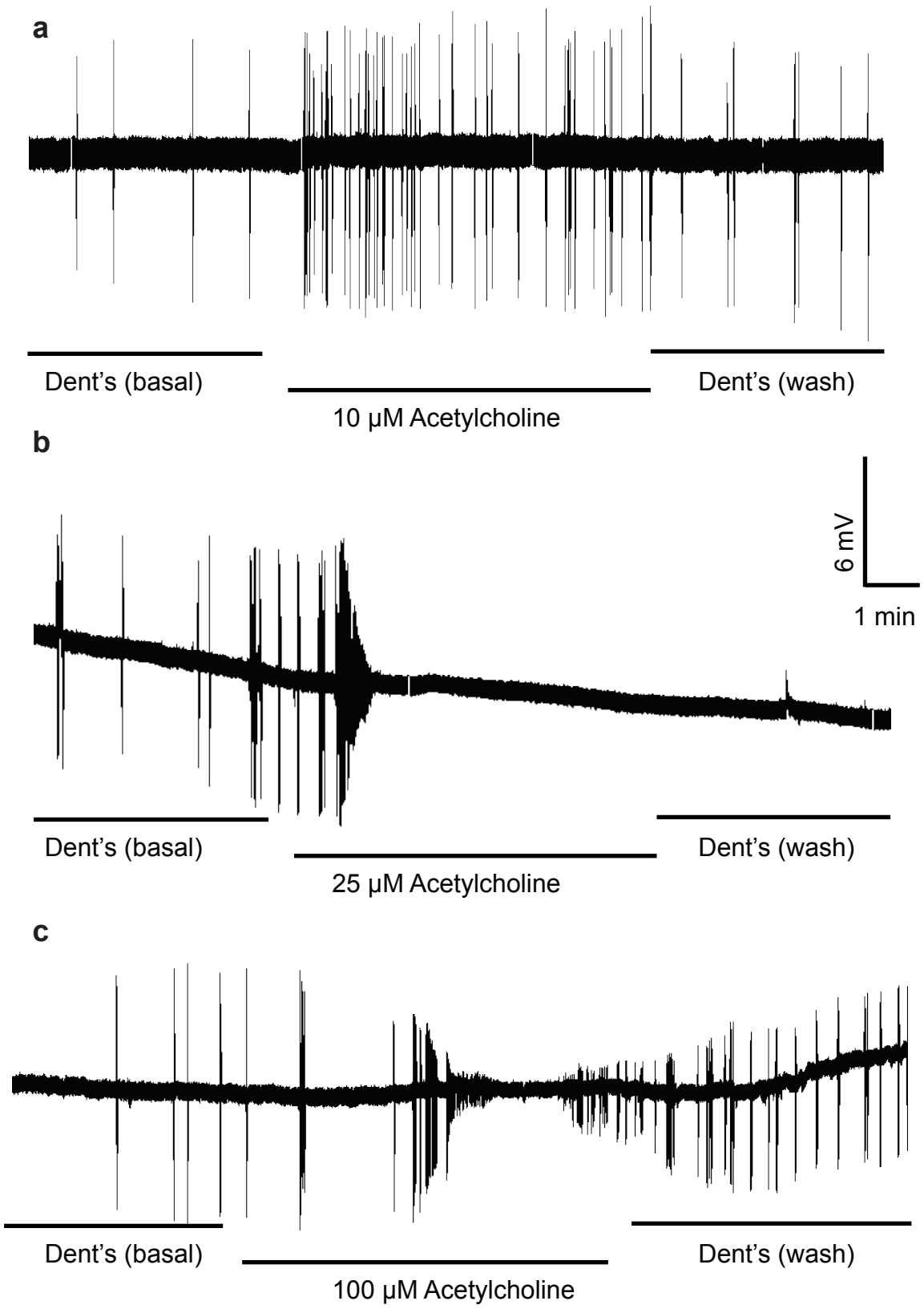


Figure 5.4: **Effects of acetylcholine on EPG.** Cut heads of *C. elegans* were perfused for 5 minutes in each Dent's saline (basal), acetylcholine and again in Dent's saline for recovery. Example EPG traces from the pharynx exposed to 10 μM (a), 25 (b), and 100 μM acetylcholine (c). Traces from 25 and 100 μM exposure represent variable responses of the pharynx to acetylcholine concentrations ranging from 15 to 100 μM. Each vertical line represents a single EPG.

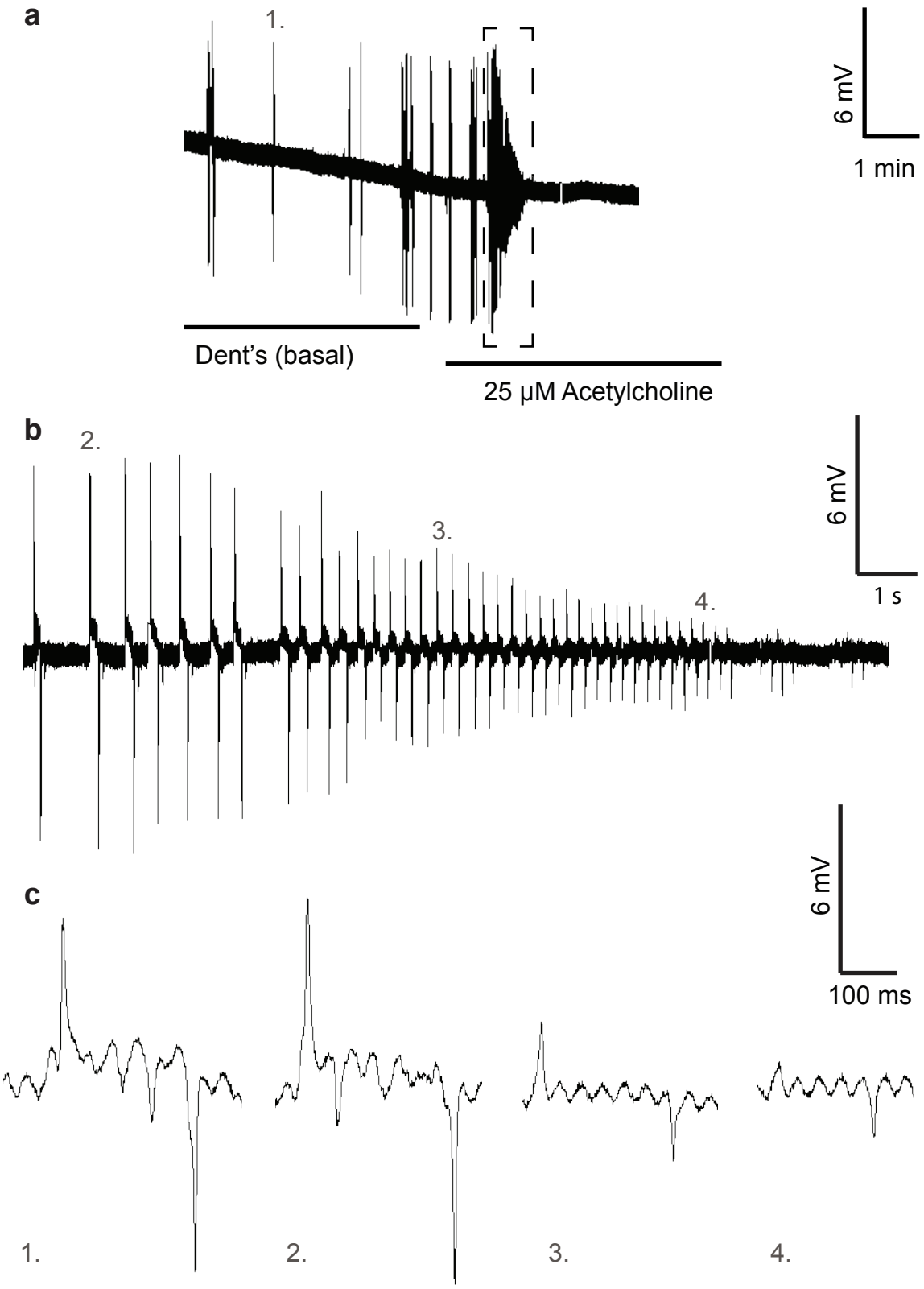


Figure 5.5: **Effects of acetylcholine on EPG frequency and waveform.** Example EPG recordings from the pharynx exposed to 25 μM acetylcholine representing effects of acetylcholine at $\geq$ 25 μM. Exposure of the pharynx to acetylcholine results in stimulation of pharyngeal activity (a) characterised by a train of spikes, (b), of decreasing amplitude (c). The stimulatory period shown in figure (b) represents trace boxed in figure (a). Example EPG waveforms from the basal pharyngeal activity (1), and activity at the beginning (2), in the middle (3) and at the end (1) of train of EPG spikes elicited by acetylcholine.

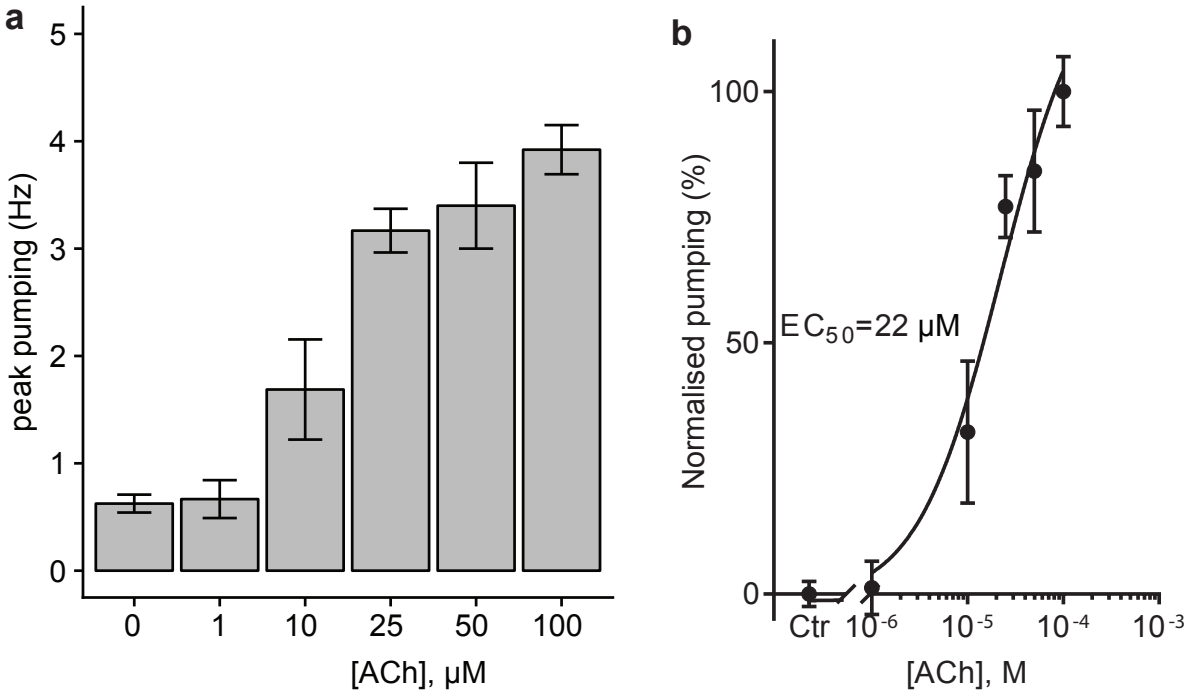


Figure 5.6: **The concentration dependence for the effects of acetylcholine on EPG frequency.** Cut-heads were perfused for 5 minutes in each Dent's saline and indicated acetylcholine concentrations. Pumping rates were derived by taking maximum pumping rate in each condition over the 10s time window, (or the entire stimulatory period, if the response to nicotine was under 10s). Data are mean $\geq$ SEM for 2-8 individual worms done on 2 days. b) Dose-response curve for the effects of nicotine on the pharynx. Data are mean $\geq$ SEM, normalised to the basal activity and expressed as a % maximum response.

5.2.3 Effects of nicotine on electropharyngeogram

Similarly to ACh, nicotine elicited concentration and time dependent changes to the EPG. At 1 μM it led to moderate but sustained stimulation of the pharyngeal activity. At higher doses (i.e. 5, 10, 25 and 50 μM) it caused a potent but transient elevation of pumping frequency followed by an inhibition of the pharyngeal activity (Figure 5.7). The stimulation by nicotine concentrations ranging from 5 to 50 μM consisted of a train of EPG spikes which decreased in amplitude with time (Figure 5.8).

To score for the stimulatory effects of nicotine on pumping, the peak pumping rates pre and post-application of nicotine were derived and the dose-response curve was plotted (Figure 5.9). The EC_{50} for the effects of nicotine on the pharyngeal activity was 2.7 μM.

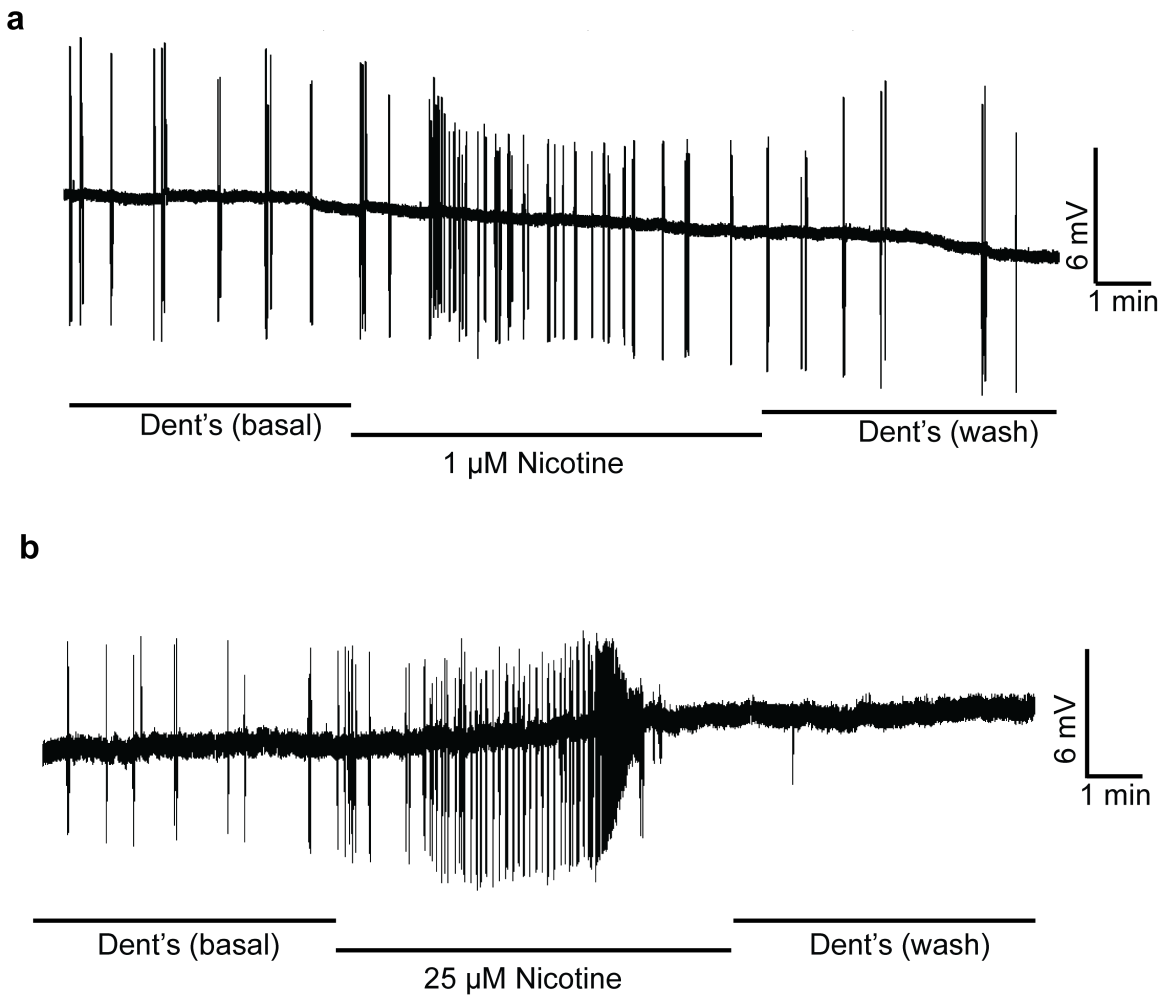


Figure 5.7: **The concentration dependent effects of nicotine on EPG frequency.** Cut heads were perfused for 5 minutes in each Dent's saline (basal), nicotine and again in Dent's saline for recovery. Example EPG traces from pharynxes exposed to 1 μM (a) and 25 μM nicotine (b). Response to 25 μM represent responses to nicotine concentrations ranging from 5 to 50 μM.

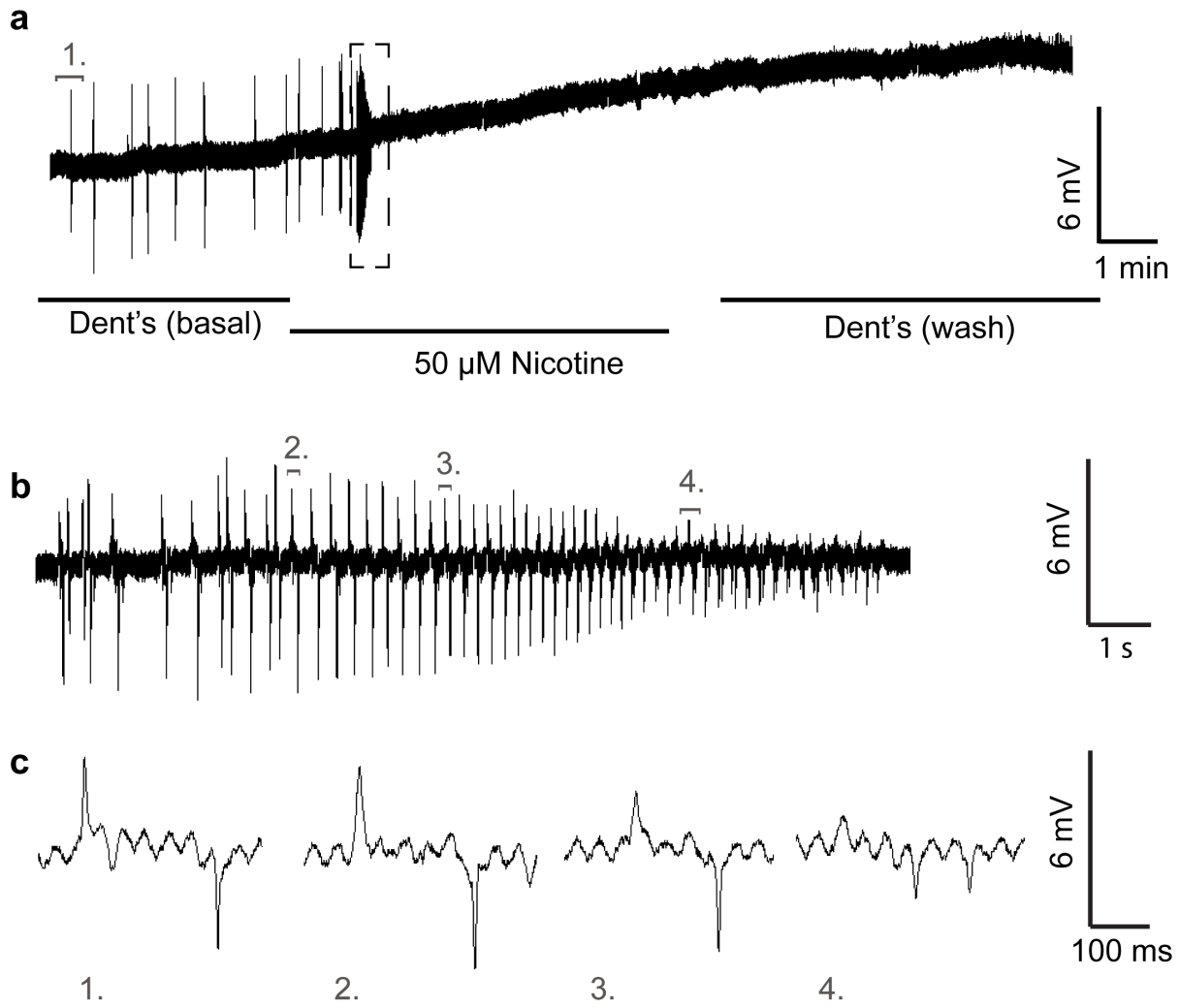


Figure 5.8: **Effects of nicotine on EPG frequency and waveform.** Example EPG recordings from the pharynx exposed to 50 μM nicotine representing effects of nicotine at $\geq$ 5 μM. Exposure of the pharynx to nicotine results in stimulation of pharyngeal activity (a) characterised by a train of spikes, (b), of decreasing amplitude (c). The stimulatory period shown in (b) represents trace boxed in (a). Example EPG waveforms from the basal pharyngeal activity (1), and activity at the beginning (2), in the middle (3) and at the end (1) of train of EPG spikes elicited by nicotine.

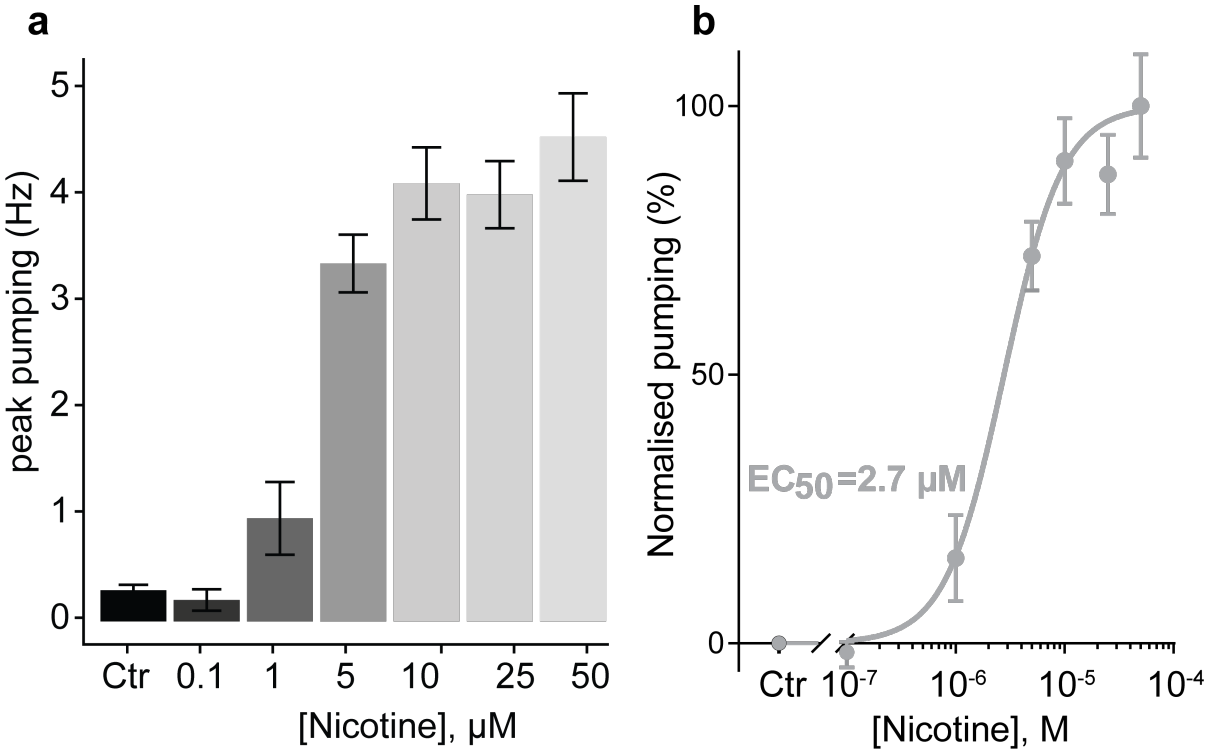


Figure 5.9: **The concentration dependence for the effects of nicotine on EPG frequency.** Cut-heads were perfused for 5 minutes in each Dent's saline and indicated nicotine concentrations. Pumping rates were derived by taking maximum pumping rate in each condition over the 10s time window, (or the entire stimulatory period, if the response to nicotine was under 10s). Data are mean ± SEM for 2-13 individual worms done on 2 days. b) Dose-response curve for the effects of nicotine on the pharynx. Data are mean ± SEM, normalised to the basal activity and expressed as a % maximum response.

To better understand the nicotine-induced pharyngeal events, photos (Figure 5.10) and video recordings (not shown) of pharynxes perfused with nicotine at 10 μM were taken. In agreement with the EPG data, nicotine induced short-lived train of pharyngeal contractions. Cycles of simultaneous contraction-relaxation of the corpus and the grinder in the terminal bulb could be seen. This suddenly ceased. Following, asynchronous contraction of the isthmus and the grinder began which led to sustained contraction and opening of the grinder and isthmus. In addition, twitching of the terminal bulb muscle could be observed throughout.

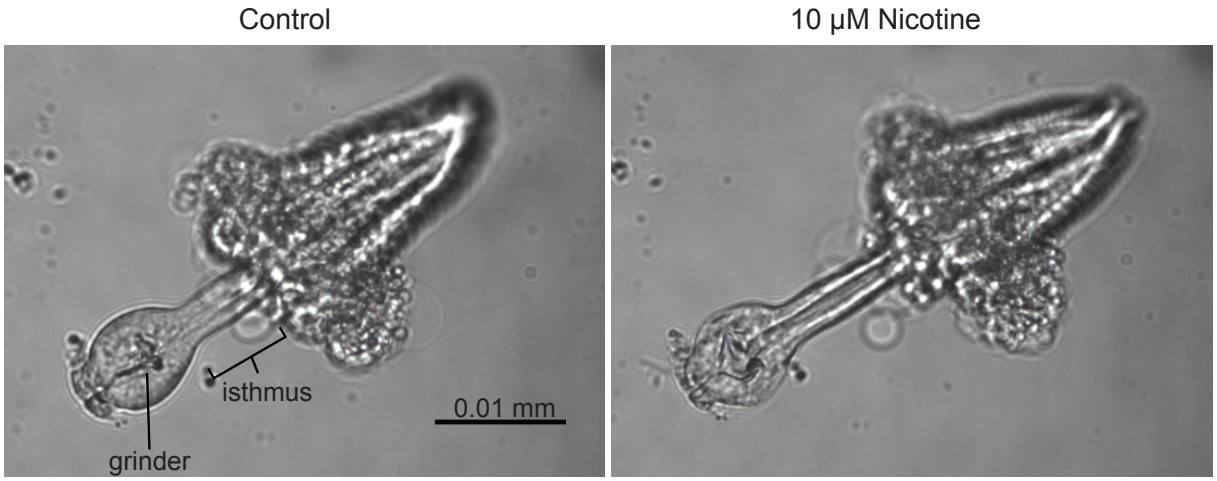


Figure 5.10: **The effects of nicotine on the morphology of the pharynx.** Appearance of the pharynx pre- and post exposure to nicotine at 10 μM. Images were taken immediately prior to nicotine exposure and post nicotine-induced inhibition of pumping.

Investigations into the recovery from nicotine-induced pharynx stimulation were made. Following stimulation by 1 μM nicotine, pharynxes returned to the basal pumping rate within 5 minute wash, those exposed to higher nicotine concentrations remained inhibited (Figure 5.7). To see if pharynxes begin to pump after longer wash, experiment were repeated. Cut heads were exposed to nicotine for 5 minutes, but after this time the washing period was extended until the pharyngeal activity was restored. To score for recovery, the time taken from the beginning of wash to the first EPG spike was taken (Figure 5.11). It took 8 minutes or longer to recover from the effects induced by 5 and 10 μM nicotine. The time needed to recover from 50 μM perfusion was 18 minutes, suggesting longer washing was required to remove residual nicotine after exposure to higher drug concentrations.

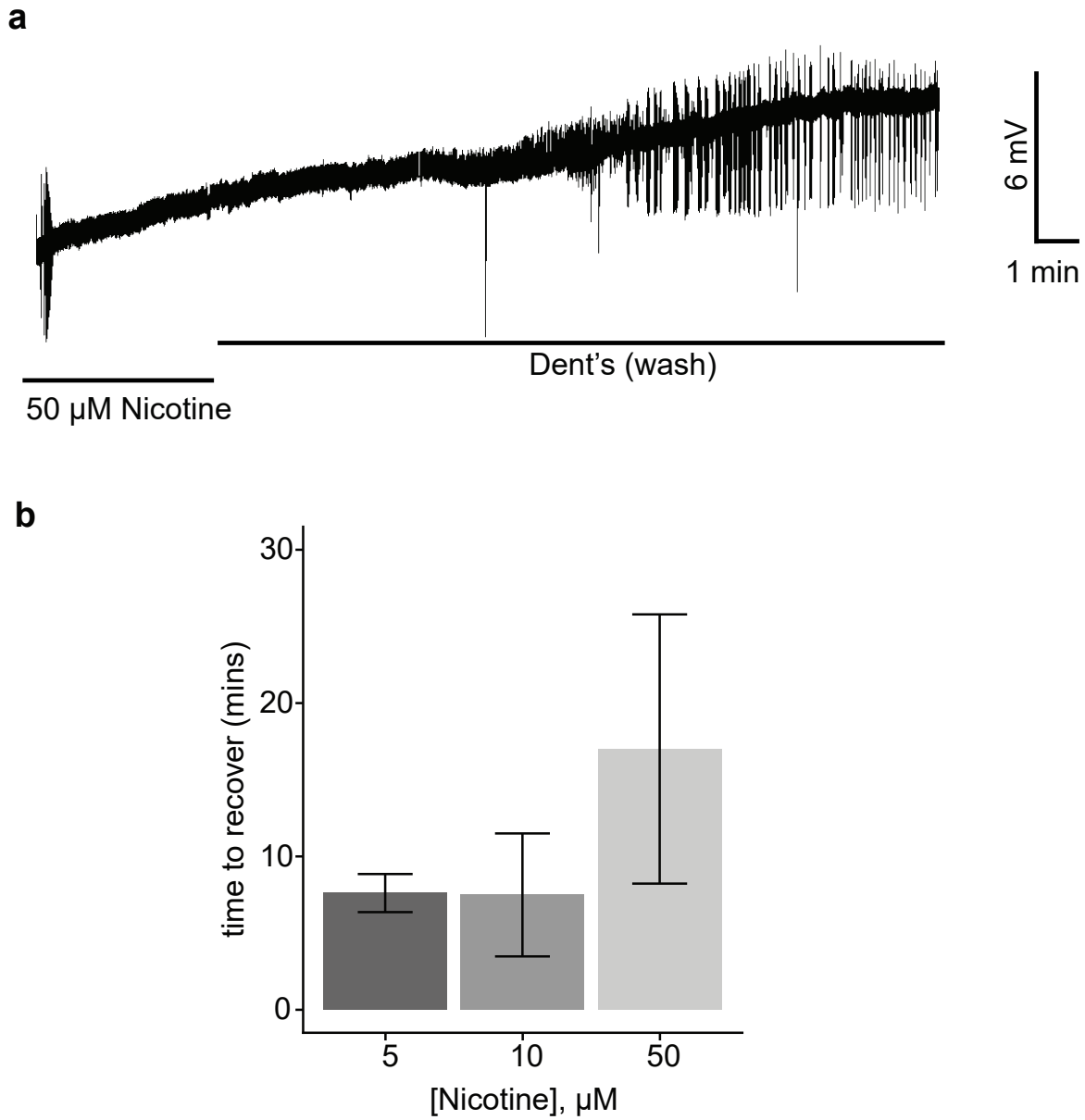


Figure 5.11: **Recovery from nicotine-induced inhibition of EPG of *C. elegans*.** Example trace showing recovery from the effects of 50 μM nicotine on EPG of cut head (a). Pharynxes were perfused with nicotine for 5 minutes before being flushed with Dent's saline. The time taken to recovery from 5, 10 and 50 μM nicotine induced inhibition of pumping was derived (b) by measuring the time period from the beginning of wash to the first EPG. Data are mean $\pm$ SEM from 2 - 3 individual worms collected from 2 paired experiments.

Comparing the effects of nicotine on the pharynx as revealed by EPG and by visual scoring revealed a discrepancy in the effects seen when pharynxes were exposed to 10 and 20 μM. In EPGs, perfusion of the pharynx for 5 minutes with either 10 or 20 μM elicited potent stimulation followed by a blockage of the pharyngeal activity (Figure 5.7 b). In contrast, no potent stimulation of the pharynx by these doses was observed when the pharyngeal pumping scored visually. Instead, a moderate stimulation of the pharynx was seen after ~20 minutes of incubation which increased in frequency over time (Figure 4.12). To determine if this effect can be replicated in the EPG experiments, pharynxes were perfused with 10 μM nicotine for 1 hour (Figure 5.12). After 10 minutes of perfusion, little EPG spikes began to emerge which increased in amplitude over time.

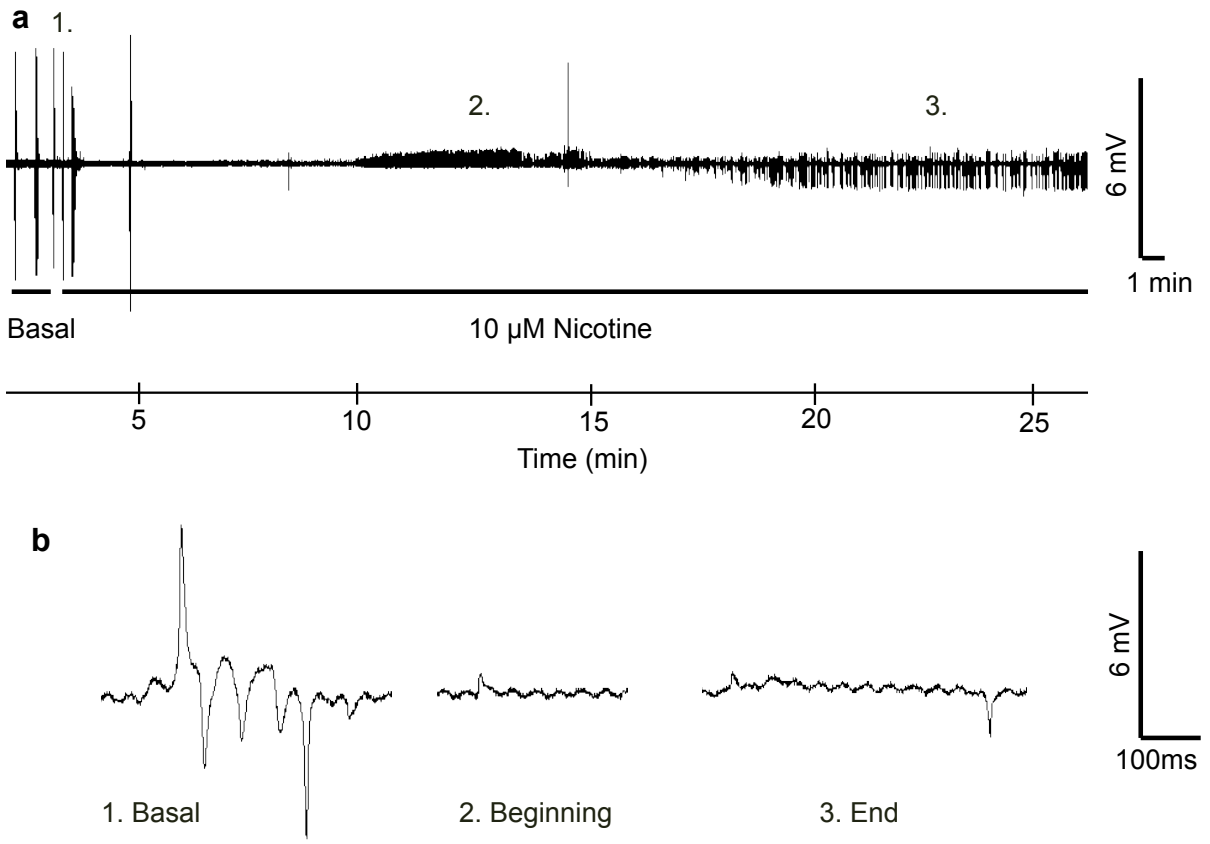


Figure 5.12: **Effects of prolonged nicotine exposure on EPG.** a) Example EPG recording from the *C. elegans* cut head showing the period of basal activity (Dent's), and the activity in response to 25 μM nicotine over the 1 hour perfusion period. The effects of nicotine on EPG waveform (b) showing the differences between the basal EPG (1), EPG at the beginning (1) and after 10 (2) and 20 minute (3) perfusion with nicotine.

5.2.4 Effects of cytisine on electropharyngeogram

The effects of cytisine, an agonist of nAChR was tested. Cytisine was applied at concentrations ranging from 1 to 100 μM. As in case of acetylcholine and nicotine, two types of responses were observed. Moderate but sustained stimulation of the pharyngeal activity was elicited by 5 μM, whereas at concentrations $\geq$ 10 μM, the pharynx was stimulated and subsequently inhibited (Figure 5.13). The EC_{50} of cytisine on EPG was 3 μM (Figure 5.14).

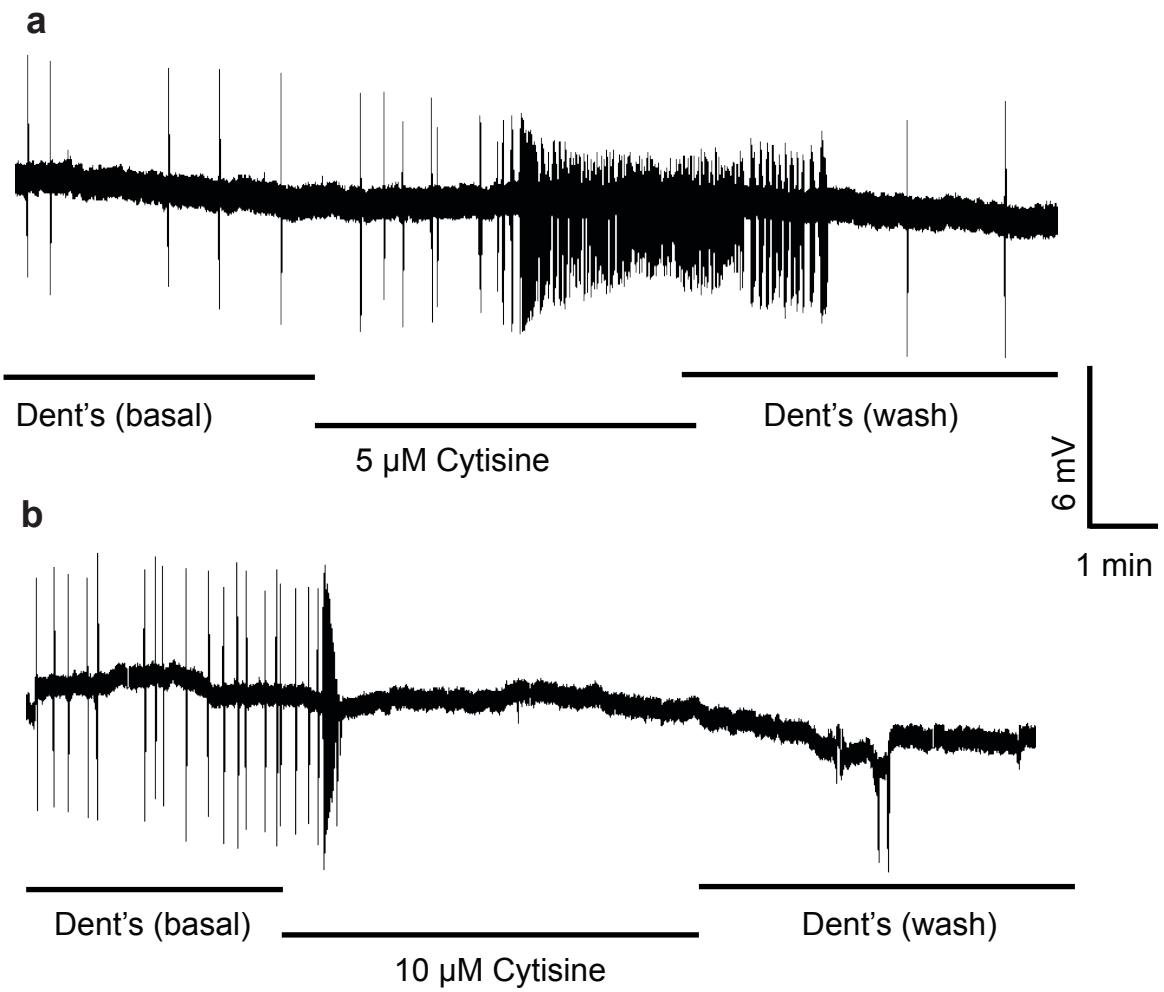


Figure 5.13: **Effects of cytisine on EPG.** Cut heads of *C. elegans* were perfused for 5 minutes in each Dent's saline (basal), cytisine and again in Dent's saline for recovery. Example EPG traces from the pharynx exposed to 5 μM (a) and 10 μM cytisine (b). Trace from 10 μM exposure represent response of the pharynx to cytisine concentrations ranging from 10 to 100 μM.

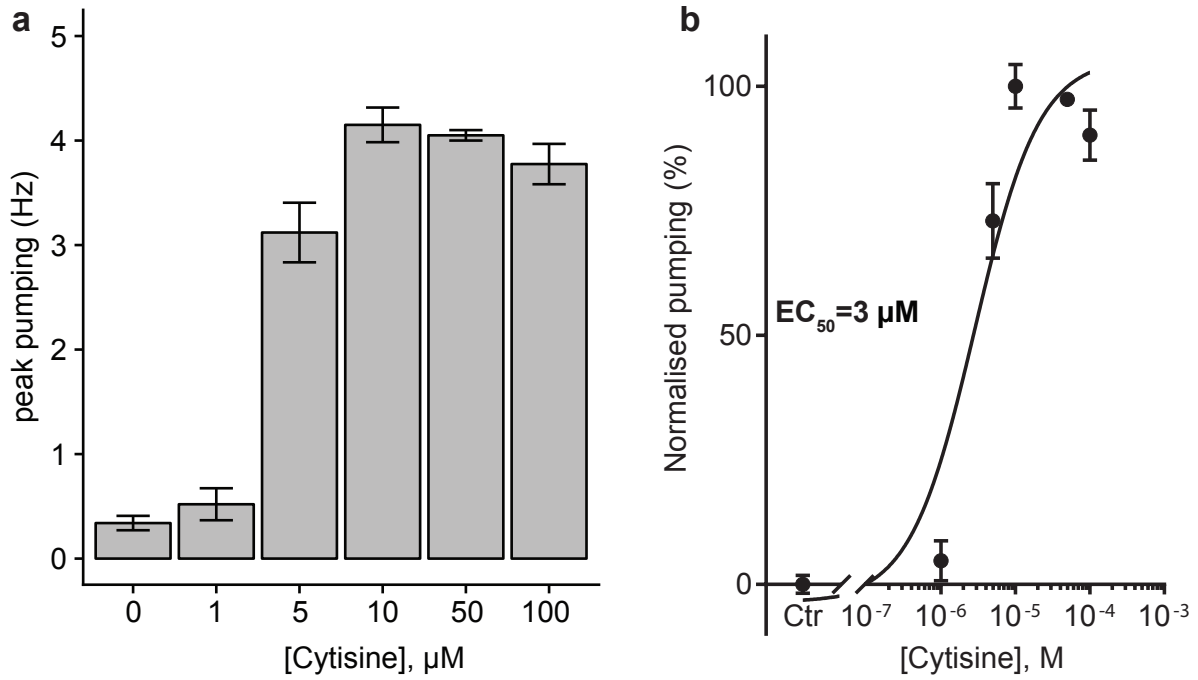


Figure 5.14: **The concentration dependence for the effects of cytisine on EPG frequency.** Cut-heads were perfused for 5 minutes in each Dent's saline and indicated cytisine concentrations. Pumping rates were derived by taking maximum pumping rate in each condition over the 10s time window, (or the entire stimulatory period, if the response to nicotine was under 10s). Data are mean $\pm$ SEM for 2-5 individual worms, from experiments done on $\geq$ 2 days. b) Dose-response curve for the effects of cytisine on the pharynx. Data are mean $\pm$ SEM, normalised to the basal activity and expressed as a % maximum response.

5.2.5 Effects of neonicotinoids on electropharyngeogram

The effects of neonicotinoids on EPG were also examined. Pharynxes were exposed to 100 μM nitenpyram, 50 μM thiacloprid and 75 μM clothianidin. Neither nitenpyram (Figure 5.15), nor thiacloprid had an effect on the frequency of pharyngeal activity (Figure 5.16). In contract, clothianidin stimulated pharyngeal activity. The frequency increased from 0.6 to 1.1 Hz and returned to basal following a 5 minute wash (Figure 5.17). EPGs from clothianidin-perfused pharynxes were examined and a reduction of R peak in relation to E peak was noted. Clothianidin significantly increased the E/R ratio from 1.3 to 1.8 (Figure 5.18 a and b). 5-minute wash did not reverse this effect. A change in duration of pumping activity was also observed. The latency of EPG decreased from 130 ms to 110 ms when pharynxes were exposed to clothianidin (Figure 5.18 a and c). This effect was not reversed upon 5 minute wash.

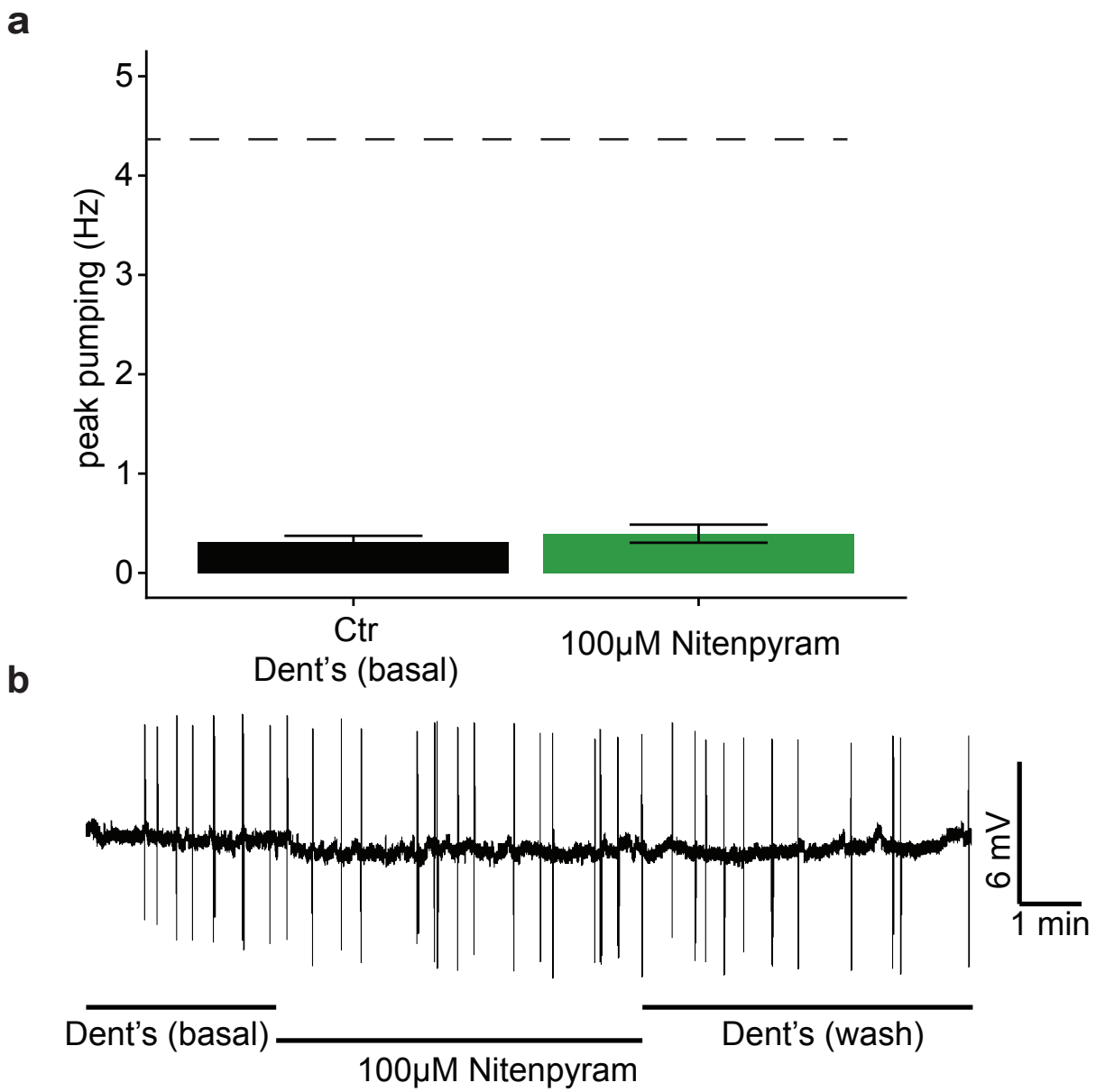


Figure 5.15: **The effects of nitenpyram on EPG.** Cut heads were perfused for 5 minutes with solvent (Dent's) and 100 μM nitenpyram. Peak pharyngeal response over 10-second window pre- and post exposure period were derived. Data are mean $\pm$ SEM from eight individual worms collected from 3 experiments. For comparison, the maximum pumping achieved by 5-HT is shown in dashed line. b) Example EPG recording showing basal, treatment with 100 μM nitenpyram and wash periods.

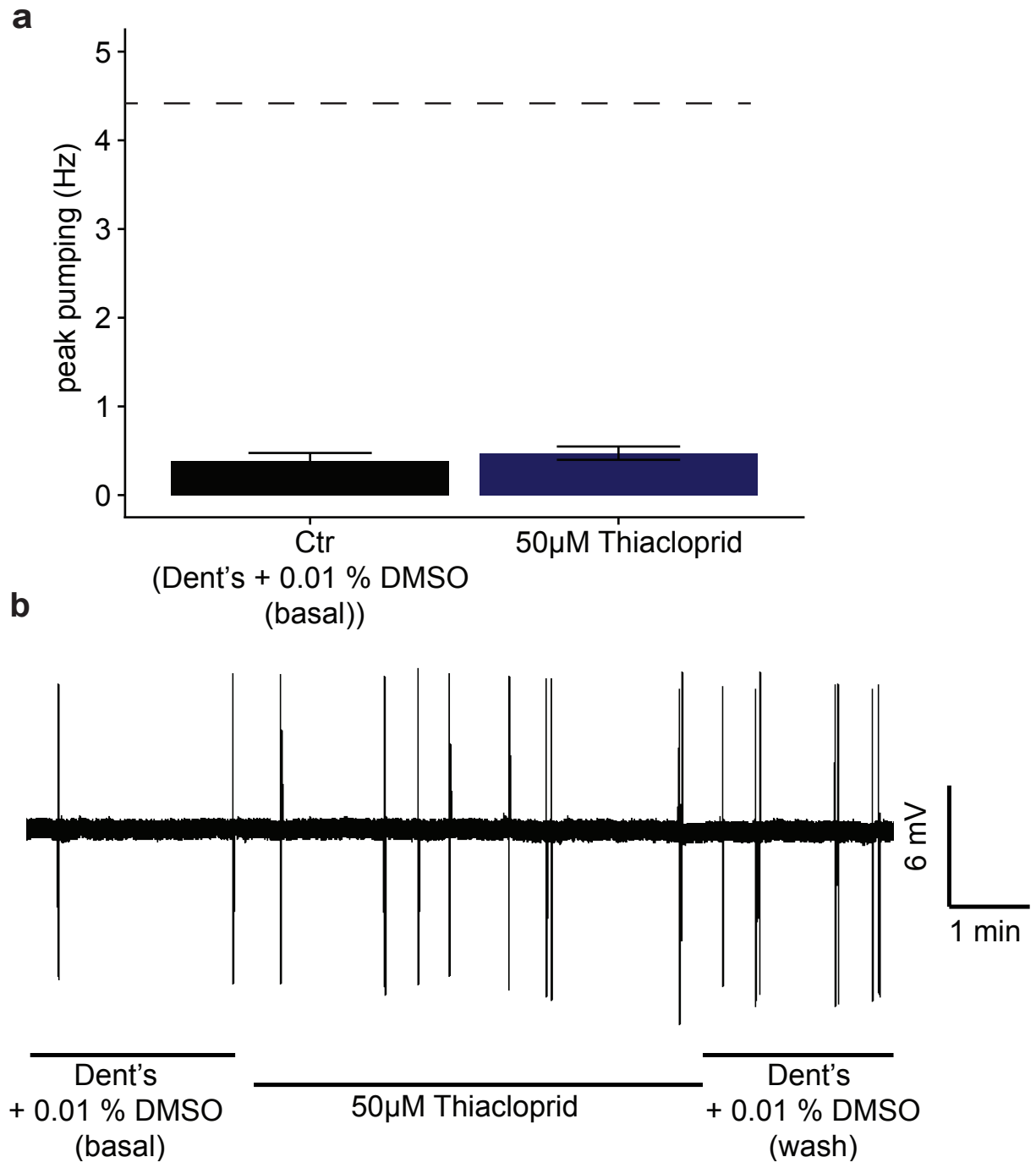


Figure 5.16: **The effects of thiacloprid on EPG.** Cut heads were perfused for 5 minutes with solvent (Dent's + 0.01% DMSO) and 50 μM thiacloprid. Peak pharyngeal response over 10-second window pre- and post drug treatment were derived. Data are mean $\pm$ SEM from 8 individual worms collected from 3 experiments. For comparison, the maximum pumping achieved by 5-HT is shown in dashed line. (b) Example EPG recording showing basal, treatment with 50 μM thiacloprid and wash periods.

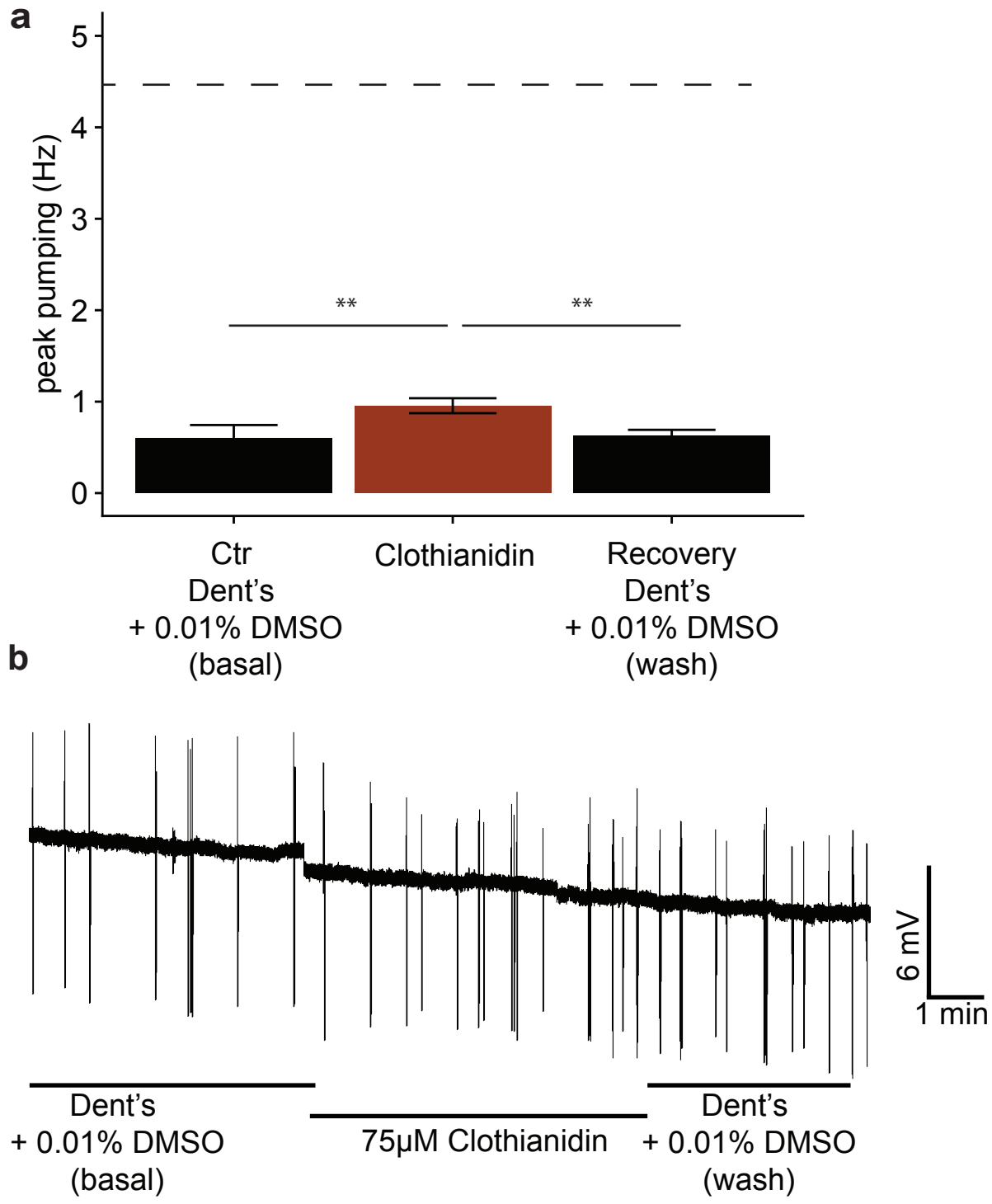


Figure 5.17: **The effects of clothianidin on EPG.** Cut heads were perfused for 5 minutes with solvent (Dent's + 0.01% DMSO) and 75 μM clothianidin (a). Peak pharyngeal response over a 10-second pre- and post drug treatment were derived. Data are mean $\pm$ SEM from 9 individual worms collected from 3 experiments. For comparison, the maximum pumping achieved by 5-HT is shown in dashed line. (b) Example EPG recording showing basal, treatment with 75 μM clothianidin and wash periods.

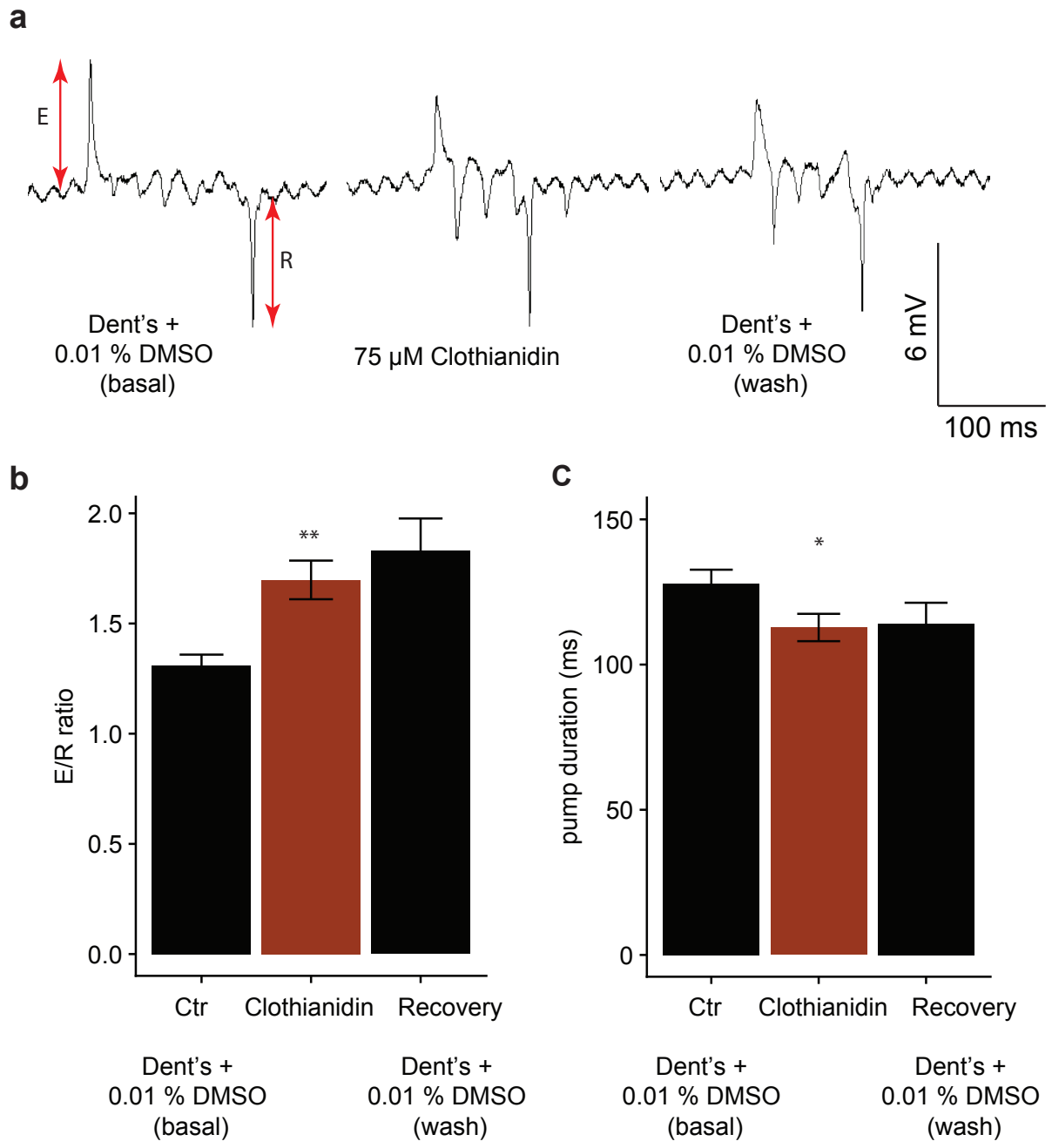


Figure 5.18: **The effects of clothianidin on EPG waveform.** Example individual basal (left), clothianidin stimulated (middle) and recovery EPG (right) (a). The effects of 75 μM clothianidin on E/R ratio (b) and pump duration (c). The amplitude of E relative to R was measured to derive E/R ratio, and the time taken from E to R to derive pump latency. The values are the average pump duration and the average E/R ratios from all EPGs in the period of the maximum pumping. If there were less then 10 EPGs, 10 consecutive peaks were taken. Data are mean $\pm$ SEM of 9 individual worms collected from 3 experiments. Two-tailed t-test, $*P \leq 0.05$. $**P \leq 0.01$.

5.2.6 Effects of acetylcholine in the presence of nitenpyram and thiacloprid on electropharyngeogram

Neither nitenpyram at 100 μM nor thiacloprid at 50 μM impaired on EPG frequency. To determine whether they inhibit the stimulatory effect of acetylcholine on EPG frequency, pharynxes were pre-incubated with nitenpyram or thiacloprid and then exposed to acetylcholine in the continual presence of thiacloprid or nitenpyram (Figure 5.19 and Figure 5.20). Acetylcholine at 10 μM was tested, because this dose is close to the EC_{50} on EPG frequency. The response of the pharynx to acetylcholine in the presence or absence of neonicotinoids was compared. Pre-exposure of the pharynx to either neonicotinoid did not influence the EPG spike frequency elicited by acetylcholine. In both cases, upon application of acetylcholine, the pumping frequency increased from 0.2 to $\sim$ 1 Hz.

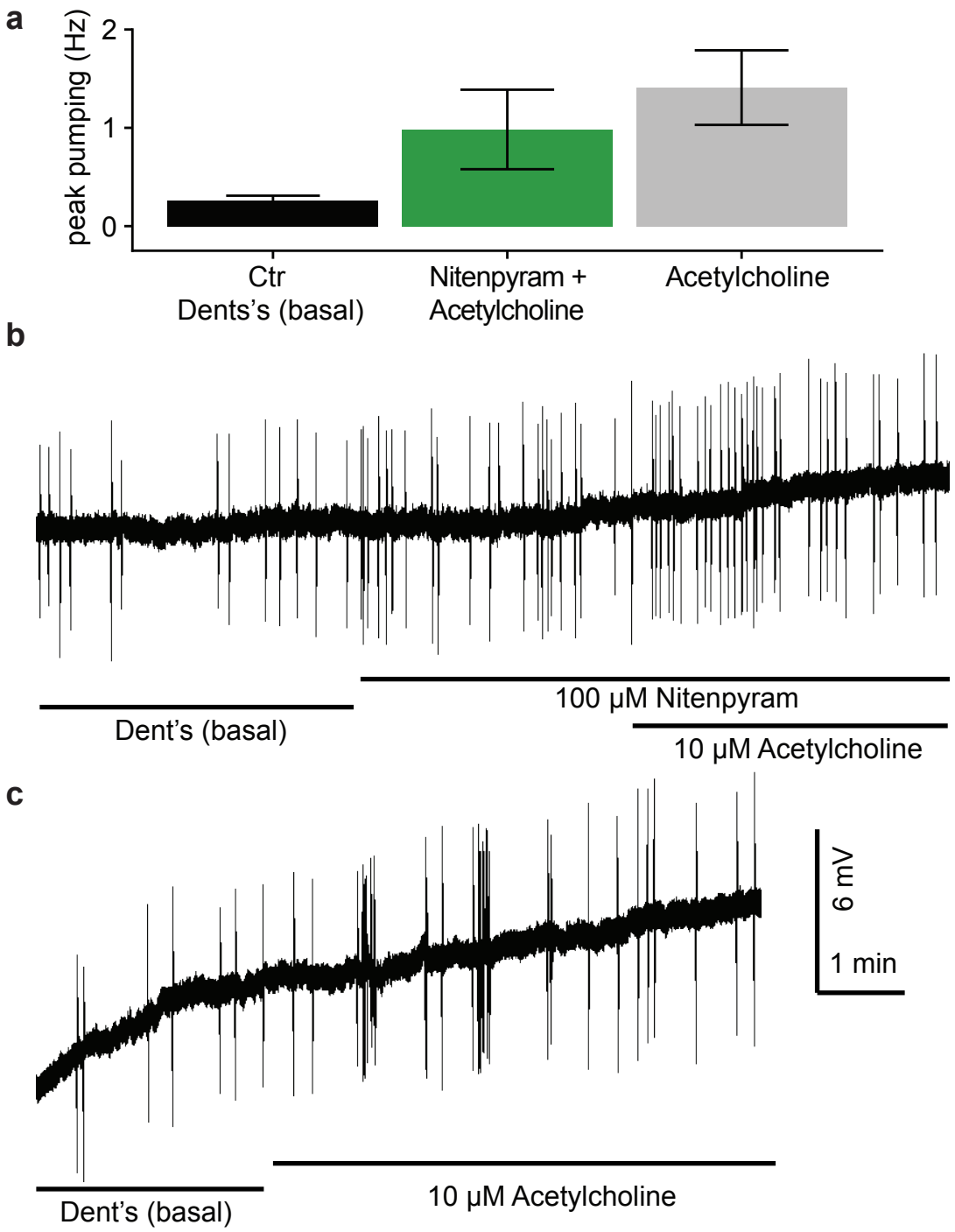


Figure 5.19: **Effects of acetylcholine on the EPG frequency in the presence nitenpyram.** Pharynxes were pre- exposed to 100 μM nitenpyram. 5 minutes later, 100 μM nitenpyram and 10μM acetylcholine were applied. Responses to acetylcholine in the presence of nitenpyram were compared to responses elicited by acetylcholine. Pharyngeal pumping rates were derived by extracting peak response in a 10 second window. Data are mean $\pm$ SEM from 3-8 individual worms collected from paired experiments done on $\geq$ 2 days. Example EPG traces of the pharyngeal responses in the presence or absence of nitenpyram (b and c respectively).

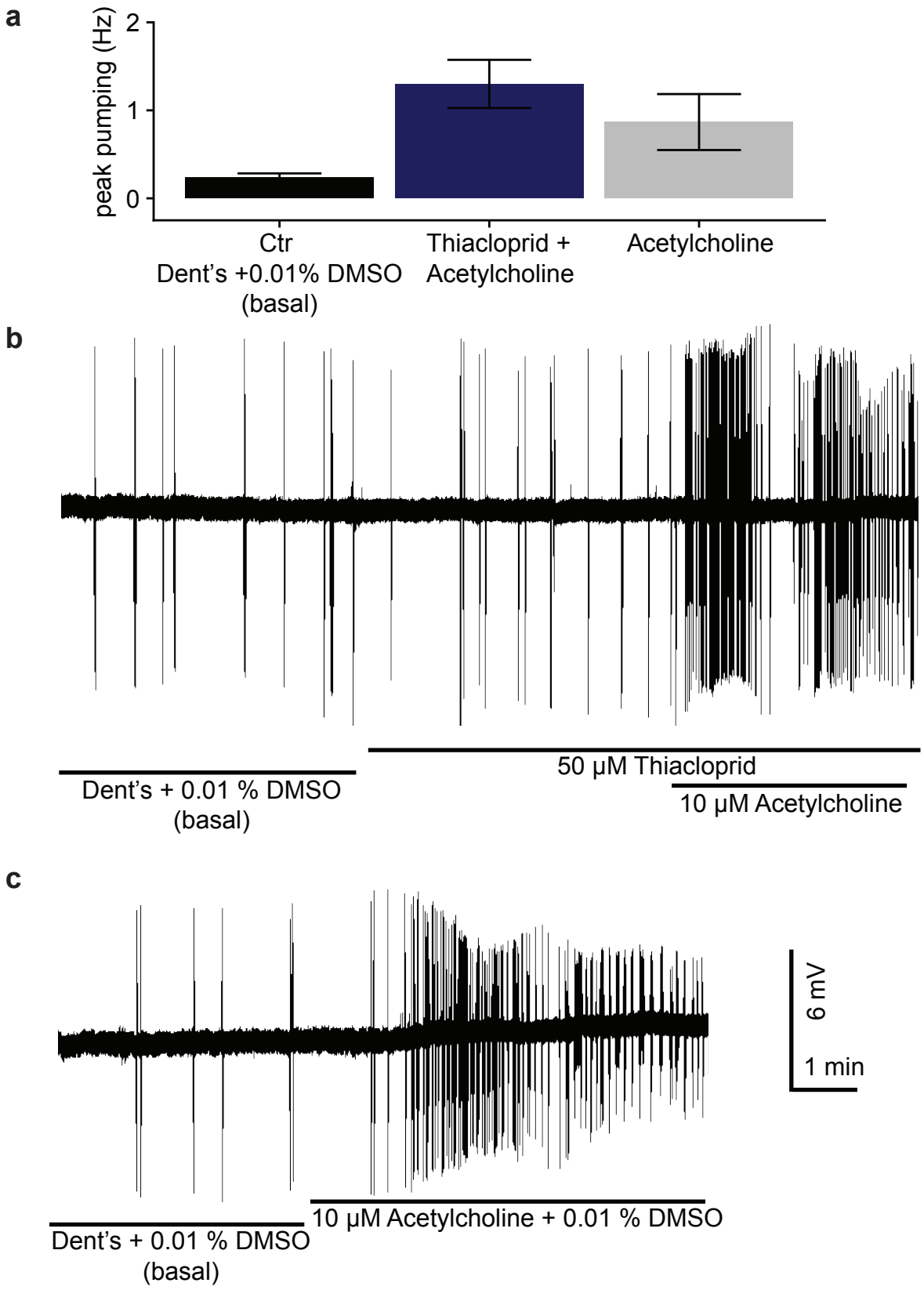


Figure 5.20: **Effects of acetylcholine on the EPG frequency in the presence of thiacloprid.** Pharynxes were pre- exposed to 75 μM thiacloprid. 5 minutes later, 75 μM thiacloprid and 10μM acetylcholine were applied. Responses to acetylcholine in the presence of thiacloprid were compared to responses elicited by acetylcholine. Pharyngeal pumping rates were derived by extracting peak response in a 10 second window. Data are mean $\pm$ SEM from 3-6 individual worms collected from paired experiments done on $\geq$ 2 days. Example EPG traces of the pharyngeal responses in the presence or absence of thiacloprid (b and c respectively).

5.2.7 Effects of nicotine on electropharyngeogram of worms deficient in nAChR

EPG analysis of the effects of nicotine on the pharynx revealed a nicotinic effect not seen in the visual observation experiments. To determine whether the effects of nicotine on EPG on nAChR *eat-2* mutant differs from wild-type, EPG recordings from both strains were obtained. The stimulatory effect of nicotine concentrations ranging from 100 nM to 50 μM was scored (Figure 5.21). No marked differences in nicotine-sensitivity of *eat-2* mutant vs wild-type worms were noted. The EC_{50} on wild-type was 3 μM, in comparison to 5 μM in *eat-2* mutant.

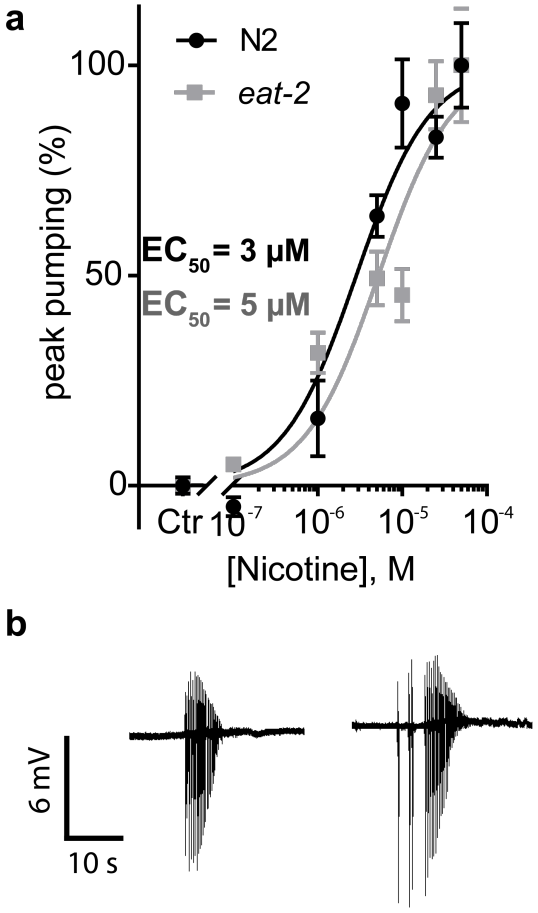


Figure 5.21: **Effects of nicotine on EPG frequency of *eat-2* nAChR *C. elegans* mutant.** Cut heads were perfused for 5 minutes in Dent's and nicotine at indicated concentrations. Peak response in 10s window was derived and normalised to the basal pumping (a). Data are mean $\pm$ SEM of paired experiments done on ≥ 2 worms on a single day. Example EPG recording showing response of the wild-type and *eat-2* mutant to 25 μM nicotine (b).

5.3 Discussion

C. elegans expresses at least 29 different nAChRs. Only a few of these subunits have been linked to the function of the pharynx (McKay et al. 2004). Cholinergic compounds were applied to the cut-head *C. elegans* and EPG recording were made to characterise the pharmacological profile of the pharynx, and in order to determine the expressed nAChRs. Results are summarised in Table 5.1.

5.3.1 Nicotine inhibits pumping by contracting pharyngeal muscle

EPG analysis revealed complex responses of the pharynx to nicotine. 1 and 10 μM induced sustained stimulatory effect. At concentrations ranging from 25 to 100 μM a dual response was observed: a potent stimulation characterised by a train of EPGs. The amplitude of spikes progressively decreased with time until a complete inhibition of pharyngeal activity occurred (Figure 5.7). This inhibition of pharyngeal activity coincided with a sustained contraction of grinder and isthmus of the pharynx. This suggests that nicotine induces potent contraction of the pharyngeal muscle and that a sustained stimulation leads to pumping inhibition.

5.3.2 *Eat-2* is not involved in the nicotine-induced pharyngeal responses

The EPG responses elicited by nicotine are reminiscent of the responses achieved by the cholinergic compounds such as acetylcholine, cytisine and choline. This suggests nicotine acts at the pharynx in a similar way to classical nAChR agonist and exogenously applied neurotransmitter targeting nAChRs. In the presence of food, acetylcholine acts on *eat-2* containing nAChRs to induce pumping (McKay et al. 2004). EPG recordings from wild-type and *eat-2* nAChR mutant worms show no difference in nicotine-induced responses, suggesting nicotine does not act on *eat-2* containing nAChRs.

5.3.3 Distinct effects of neonicotinoids on the pharyngeal system

The electrophysiological effects achieved by neonicotinoids on insect neuronal preparation and *C. elegans* pharynx differ. In *C. elegans*, clothianidin transiently stimulated pharynx for 5 minutes. EPG analysis revealed the effects of clothianidin on the shape of an EPG waveform. In the presence of 50 μM clothianidin, the amplitude of E spikes relative to R spike decreased leading to an increase of E/R ratio. E spikes arise due to simultaneous contraction of the pharyngeal muscle syncytium (Franks et al. 2006). Therefore, a reduction in E spike amplitude could be due to reduced synchronisation of the muscle syncytium depolarisation. 50 μM clothianidin also reduced a latency of pump duration. In contrast, thiacloprid and nitenpyram had no effect. In insects, neonicotinoids typically achieve biphasic effects on post-synaptic neurons: excitation followed by an inhibition (Section 1.3.5.2). Such effects was not observed in *C. elegans*. This supports the divergent nature of insect and pharyngeal *C. elegans* nAChR families.

5.3.4 *C. elegans* pharyngeal nAChRs are more closely related to human than insect nAChRs.

Investigation of the effects of nicotine and neonicotinoids on *C. elegans* pharyngeal system revealed high efficacy of nicotine, and low efficacy of neonicotinoids, which is also seen in mammals (Tomizawa, M. and J.E. Casida 2003). In contrast, neonicotinoids are more efficacious on insects in comparison to nicotine (Tomizawa, M. and J.E. Casida 2003). This suggests that mammalian and *C. elegans* nAChRs are more closely related than insect and *C. elegans* receptors. This is supported by the similarities in the effective doses of cholinergic compounds on the *C. elegans* pharynx and on the mammalian nAChRs. Acetylcholine, nicotine and cytisine all stimulated the pharynx with the EC_{50} of low μM. These concentrations are also effective against mammalian neuronal preparations and isolated nAChRs. Based on the $\alpha 7$ receptors expressed in *Xenopus* oocytes, the EC_{50} of acetylcholine, nicotine and cytisine is 21 μM, 12.6 μM and 5.6 μM, respectively (Ballivet et al. 1996; Papke and Porter-Papke 2002). This suggests there are similarities in the pharmacophore of pharyngeal nAChR and mammalian $\alpha 7$ receptors.

Table 5.1: Summary of the effects of compounds on the pharyngeal activity of *C. elegans*.

	Pump frequency	EPG waveform
5-HT	1. Dose-dependent, sustained stimulation 2. $EC_{50} = 255$ μM	1. Reduced pump duration
Acetylcholine	1. Sustained stimulation by 10 μM 2. Potent stimulation followed by inhibition by ≥ 25 μM 3. $EC_{50} = 22$ μM	1. The train of spikes elicited by $\geq 10\mu$M characterised by EPG spikes decreasing in amplitude
Cytisine	1. Sustained stimulation by 5 μM 2. Potent stimulation followed by inhibition by ≥ 10 μM 3. $EC_{50} = 3$ μM	1. The train of spikes elicited by ≥ 10 μM characterised by EPG spikes decreasing in amplitude

	Pump frequency	EPG waveform
Nicotine	1. Sustained stimulation by 1 μM 2. Potent stimulation followed by inhibition by $\geq$ 10 μM 3. $EC_{50} = 2.7$ μM	1. The train of spikes elicited by ≥ 10 μM characterised by EPG spikes decreasing in amplitude
Nitenpyram	1. No effect at 0.1 mM	1. No effect at 0.1 mM
Thiacloprid	1. No effect at 50 μM	1. No effects at 50 μM
Clothianidin	1. Weak and sustained stimulation by 75 μM	1. Increased E/R ratio 2. Decrease in pump duration
Clothianidin	1. No effects on ACh-evoked pharyngeal response	NA
Thiacloprid	1. No effects on ACh-evoked pharyngeal response	NA

Chapter 6

C. elegans pharynx as a platform for heterologous nAChR expression

6.1 Introduction

nAChRs are the major site of action of neonicotinoids. Several lines of evidence suggest that even within the same species, different neonicotinoid-compounds target distinct nAChRs. They also have adistinct modes of action; some neonicotinoids are true-, partial- or super- agonist whilst other are antagonists of nAChRs (Section 13.6). The pharmacological characterisation of insect nAChRs is needed to better understand the interactions between the nAChRs and neonicotinoids and to identify subunits sensitive to different members of this dass of insecticides.

6.1.1 Biological systems for heterologous protein expression

To pharmacologically characterise the receptor ion channel, a recombinant protein can be heterologously expressed in a number of different systems (reviewed in Millar 2009). The two most commonly used are mammalian cells, insect cells or *Xenopus* oocytes. Each presents advantages but also disadvantages (summarised in Table 6.1). These systems have been extensively used to characterise mammalian and *C. elegans* nAChRs (Millar 2009).

6.1.2 Properties of vertebrate $\alpha 7$ nAChR

One of the well-studied nAChR subunit is the vertebrate homomeric $\alpha 7$. Heterologous expression of these receptors in *Xenopus* oocytes and mammalian cell lines.

Table 6.1: Advantages and disadvantages of heterologous expression systems.

System	Advantages	Disadvantages
Xenopus oocytes	Cheap and easy to maintain Easy to inject Express a low number of endogenous membrane proteins Can be transfected with multiple mRNA species simultaneously Amendable to electrophysiological techniques	Functional properties may be altered Preparation short lived Single-cell technique
Cell lines	Temporal control of expression Favorable cellular environment for many proteins Amendable to electrophysiological and biochemical techniques	High cost

6.1.2.1 $\alpha 7$ nAChR is rapidly desensitising

Acetylcholine and nicotine are classical nAChR agonists that activate vertebrate $\alpha 7$ and many other nAChR types. In addition, there are selective compounds that bind to $\alpha 7$ receptors, such as cytisine and choline. The rank order of potency of these agonists is: cytisine $>$ nicotine $>$ ACh $>$ choline (Papke et al. 2000). The EC_{50} values vary depending on the method of measure and the expression system (Papke and Porter-Papke 2002). Nicotine is generally at least 5 times more potent than ACh (Couturier et al. 1990), whereas the potency of choline is at least 10 times lower than that of ACh (Papke and Porter-Papke 2002). The EC_{50} of the most potent compound cytisine is between 5.6 and 7.1 μM (Wonnacott and Baric 2007).

The kinetics of agonist-evoked nAChR responses are reminiscent to those of other nAChR types. Briefly, in the presence of agonist, receptor channels open rapidly allowing flux of ions, which gradually declines (Corrie et al. 2011) until a full depolarisation and desensitization occurs.

Extremely rapid desensitising kinetics is the unique feature of the $\alpha 7$ receptor. In 1990, Couturier et al. (1990) heterologously expressed $\alpha 7$ in *Xenopus* oocytes and recorded the macroscopic current in response to acetylcholine. In the presence of acetylcholine, receptors desensitised in under a millisecond (Couturier et al. 1990; Papke and Porter-Papke 2002). A more precise temporal characterisation of this response was obtained in 2008. Using patch-clamp, a single channel recording from transfected human cell lines was obtained (Bouzat et al. 2008). In the presence of 1 μM acetylcholine, $\alpha 7$ receptor opens and desensitises in 0.4 ms. This is much faster than other receptor channels. For example, $\alpha 8$ typically desensitises in hundreds of millisecond (Gerzanich, Anand, and Lindstrom 1994), whereas *C. elegans* L-type and N-type nAChRs in tens of seconds (Boulin et al. 2008; Touroutine et al. 2005).

Single channel recordings revealed another striking difference between $\alpha 7$ and other nAChRs. In response to agonist, nAChR channel typically display several bursts of channel opening flanked by periods of inactivity

(Mishina et al. 1986; Weltzin et al. 2019). In contrast, $\alpha 7$ typically opens once before entering the inactive form (Bouzat et al. 2008). This feature combined with rapid desensitising kinetics suggest that in physiological conditions, $\alpha 7$ receptors are primarily involved in the response to ACh released in a phasic and not tonic fashion.

Heterologous expression of $\alpha 7$ also allowed for a detailed analysis of the recovery kinetics of this receptor channel. Following desensitisation and removal of the agonist by washing, receptor returns to the resting state, allowing for the subsequent activation upon agonist application. Although the activation and desensitisation kinetics of $\alpha 7$ evoked by many agonists are almost identical, the recovery kinetics are compound, time and concentration- dependent (Mike, Castro, and Albuquerque 2000). After acetylcholine-evoked desensitisation and a 5-minute wash, the subsequent response of $\alpha 7$ receptors to ACh seemed unaffected (Briggs and McKenna 1998). In contrast, subsequent response to ACh following nicotine desensitisation was reduced (Briggs and McKenna 1998). Thus, recovery kinetics following acetylcholine application are faster. Recovery time is also more rapid for choline than for acetylcholine (Mike, Castro, and Albuquerque 2000).

6.1.2.2 Sensitive to α-bungarotoxin (α-bgtx)

A distinct pharmacological feature of $\alpha 7$ receptors is their high sensitivity to α-bgtx. Interaction between α-bgtx and $\alpha 7$ receptors was first revealed by biochemical techniques. $\alpha 7$ receptor was isolated from membrane fraction of transformed bacterial cells with α-bgtx-affinity chromatography (Schoepfer et al. 1990). Whereas, radiolabelled α-bgtx bound to $\alpha 7$ receptors immunoprecipitated from the chick retina (Keyser et al. 1993). Binding to mammalian brain receptors was also revealed by radiography of mammalian brain slices incubated with labelled α-bgtx (Clarke et al. 1985; Segal, Dudai, and Amsterdam 1978). The signal was consistent with the expression profile of $\alpha 7$ receptor, as shown by immunocytochemistry (Toro et al. 1994). In contrast, there was no α-bgtx binding in mice deficient in $\alpha 7$ expression (Orr-Urtreger et al. 1997). Finally, the crystal structure of $\alpha 7$ receptor showed binding of α-bgtx to the extracellular domain of the receptor (Dellisanti et al. 2007).

Electrophysiological evidence provided mechanistic details of the interaction between α-bgtx and $\alpha 7$ receptor. Incubation with nM concentrations of α-bgtx prevented responses to classical nAChR agonists of heterologous receptors in *Xenopus* oocytes (Couturier et al. 1990) as well as native receptors in PC12 cells (Blumenthal et al. 1997) and hippocampal neurons (Alkondon and Albuquerque 1991). Upon removal and wash, the receptor responds to ACh normally (Couturier et al. 1990), thus the interaction between α-bgtx and $\alpha 7$ are of high affinity, competitive and reversible.

6.1.2.3 Highly permeable to calcium ions

$\alpha 7$ receptors are highly permeable to calcium ions (Séguéla et al. 1993; Bertrand et al. 1993). Historically, calcium ion permeability of nAChRs was measured by establishing the reversal potential of the agonist-evoked

current by changing the concentration of calcium ions in the buffer and representing calcium ion flux as a function of sodium ion flux. Using this methods, is was established that there is up to 20-fold difference between calcium and sodium permeability of heterologously expressed $\alpha 7$ receptors (Séguéla et al. 1993). More recently fluorescent calcium indicators (Neher 1995) were used to measure calcium ion flux as a function of the entire transmembrane current. Calcium ions account for 11 % of the entire ionic conductance of heterologously expressed $\alpha 7$ receptors (Fucile et al. 2000). In comparison to other nAChRs, $\alpha 7$ has up to a 200-fold difference in calcium ion permeability when the channels are expressed heterologously (reviewed in Fucile 2004).

6.1.2.4 Matured with the aid of NACHO and RIC-3

$\alpha 7$ receptors processing is aided by several auxiliary proteins. Mammalian RIC-3 is a small protein with 2 transmembrane domains and a single coiled-coil domain (Wang et al. 2009). It is expressed in most brain regions, enriched in the areas common to the $\alpha 7$ expression, namely the hippocampus and the cerebellum, as shown by *in-situ* hybridisation (Halevi et al. 2003). Fluorescently tagged-RIC-3 localised to the ER and not the surface when expressed heterologously (Roncarati et al. 2006), whereas immunostaining of native RIC-3 in PC12 and hippocampal neurons showed co-localisation with neuronal and ER markers (Alexander et al. 2010), providing evidence that RIC-3 is an ER residing protein.

RIC-3 has a role in receptor maturation. Co-expression of RIC-3 protein with heterologously expressed mammalian $\alpha 7$ resulted in increased functional expression of this receptor, as measured by ACh-evoked current (Williams et al. 2005) and radiolabelled ligand binding (Lansdell et al. 2008). Additionally, RIC-3 promotes association of nAChRs with proteins involved in post-translational modification, receptor trafficking and transport (Mulcahy et al. 2015). Thus, RIC-3 promotes cell-surface expression of nAChRs.

NACHO is an 18-kDa multi-pass protein ER protein expressed in neurons of hippocampus, cerebral cortex and the olfactory bulb (Gu et al. 2016). ACh-evoked current and cell-surface labelling of heterologously expressed $\alpha 7$ receptor were elicited upon co-transfection of cells with NACHO (Gu et al. 2016). Absence of $\alpha 7$ mediated current in the hippocampus of NACHO-knock-out mice, the lack of binding of classical antagonists as well as behavioural phenotype, consistent with the disruption of cholinergic neurotransmission (Matta et al. 2017) supports the role of NACHO in maturation of $\alpha 7$ and other nAChRs including $\alpha 4\beta 2$, $\alpha 3\beta 2$ and $\alpha 3\beta 4$ (Matta et al. 2017).

Further experiments by Gu et al. (2016) and Matta et al. (2017), provided details of the interactions between the receptor, RIC-3 and NACHO. Transfection of HEK cells with $\alpha 7$ and RIC-3 resulted in no surface expression, based on the lack of α-bgtx or epibatidine binding. Surface expression was achieved when cells were co-transfected with $\alpha 7$ and NACHO, and augmented by RIC-3. Based on these observations it was proposed that NACHO promotes early events in the receptor assembly, whereas RIC-3 in synergy with NACHO aids receptor maturation. (Matta et al. 2017).

RIC-3 may also aid interactions with many other proteins in the cells, as shown by the enhanced interactome of nAChR $\alpha 7$ and other proteins in the cell upon co-expression of RIC-3 (Mulcahy et al. 2015).

RIC-3 and NACHO are the two must studied proteins involved in the maturation of $\alpha 7$ nAChRs, but there are also many other proteins of less defined role involved in the biogenesis of nAChRs (reviewed by Crespi, Colombo, and Gotti (2018)). For example, evolutionary conserved CRELD and EMC-6, which are ubiquitously expressed and ER membrane-bound (D'Alessandro et al. 2018; Richard et al. 2013), as well as NRA-2/nicalin (nicastrin-like protein) and NRA-4/nodal modulator (NOMO) involved in the regulation of receptor stoichiometry (Almedom et al. 2009).

Taken together, *Xenopus* oocytes and eukaryotic cell lines can be used as an heterologous expression platform for vertebrate nAChRs. They have been used to describe the receptor maturation, stoichiometry, pharmacological and kinetic properties of nAChRs, such as $\alpha 7$. However, the expression of many invertebrate receptors including insect ones in these systems has failed (Huang et al. 1999; Liu et al. 2005, 2009; Yixi et al. 2009; Bass et al. 2006), hindering their functional characterisation. Thus, other approaches need to be considered.

6.1.3 *C. elegans* pharynx as a suitable biological system for the expression of nAChRs

C. elegans is considered an attractive biological system for heterologous nAChR expression for several reasons. Firstly, it is amendable to genetic manipulation (Section 1.4.10.1). Using a process of microinjection, transgenic strains expressing heterologous nAChRs in chosen tissues or cells can be generated. Secondly, it expresses a number of chaperon proteins important for maturation of nAChRs (Section 1.4.9), including RIC-3 which plays a role in insect (Lansdell et al. 2008, 2012) and mammalian receptor cell-surface expression (Williams et al. 2005; Lansdell et al. 2008) (Section 6.1.2.4). In addition, acetylcholine and nAChRs are involved in control of many of the key *C. elegans* behaviours, including pharyngeal pumping. Acetylcholine acting on EAT-2 containing nAChRs in the pharynx is a main driver of fast pumping (Chapter 3 and McKay et al. (2004)). EAT-2 is expressed in pm4 and pm5 muscle cells (McKay et al. 2004), which make synaptic connections with the MC (Albertson and Thomson 1976). The feeding response and 5-HT sensitivity is markedly hindered in *eat-2 C. elegans* mutant (Raizen, Lee, and Avery 1995; McKay et al. 2004). Thus, selective expression of nAChRs at the MC synapse of the pharyngeal muscle in *eat-2 C. elegans* mutant is expected to have behavioural, cellular and pharmacological consequences. The difference in the function of the pharynx can be easily scored using behavioural or cellular assays (Section 1.4.2), whereas receptor expression can be detected using biochemical approaches (Section 1.4.11).

6.1.4 Chapter aim

This chapter describes efforts into development of the *C. elegans'* pharynx as a biological system for heterologous expression of nAChRs, with the aim to use it as a platform to characterise their interactions with neonicotinoids. Expression of endogenous and exogenous proteins in the wild-type and mutant *C. elegans* pharynx was carried out. The endogenous EAT-2 receptor was chosen because it is key in the function of the pharynx. The ultimate goal is to express insect receptors, however their stoichiometry is unknown (Section 1.3.8), therefore to demonstrate that the expression of exogenous proteins in the *C. elegans* pharynx can be achieved, a human $\alpha 7$ was selected. Human $\alpha 7$ is considered a good candidate because its stoichiometry, pharmacology, kinetic properties and molecular chaperons are known (Section 6.1.2). Behavioural and biochemical assays were utilised to detect cell surface expression and functionality of heterologous expression.

6.2 Results

This chapter describes the development of the method for the heterologous expression of nAChRs in the *C. elegans* pharynx.

As a positive control, EAT-2 rescue experiment was carried out. The literature suggests EAT-2 is a single molecular determinant of the fast pharyngeal pumping. The *eat-2* knock-out strain has been shown to have reduced pharyngeal response to food and 5-HT (McKay et al. 2004), which can be rescued by the expression of EAT-2 in the pharyngeal muscle, however no data was provided to support this claim. Additionally, the rescue strains are no longer available (personal communication). Therefore, the first step was to generate these strains and to confirm the function of EAT-2.

Transgenic lines were generated by the process of microinjection (Section 2.4.17). *eat-2 C. elegans* worms were injected with DNA construct containing *nAChR* cDNA downstream of the *myo-2* promoter, which drives expression in all muscle cells of the pharyngeal musculature (Altun and Hall 2009b).

6.2.1 Heterologous expression of native EAT-2 nAChRs in the *C. elegans* pharyngeal muscle

6.2.1.1 Generation of the expression vector

The expression vector was cloned using Gateway cloning method (Section 2.2.5). Briefly, *eat-2* coding DNA was PCR-amplified from the *pTB207* vector (a gift from Dr. Cedric Neveu; Table 2.6; Figure 6.1). Adenosine overhangs were added to the PCR-product (Table 2.11), which was subsequently cloned into the *TOPO* vector (Table 2.12, Figure 6.2). Cloning success was tested by performing PCR with one gene specific and one insert specific primers (Figure 6.3). *Eat-2* was then inserted into the expression vector by recombination cloning (Section 2.2.5.2 and Figure 6.4). This was authenticated by the analytical digestion (Figure 6.4) and sequencing (Appendix B).

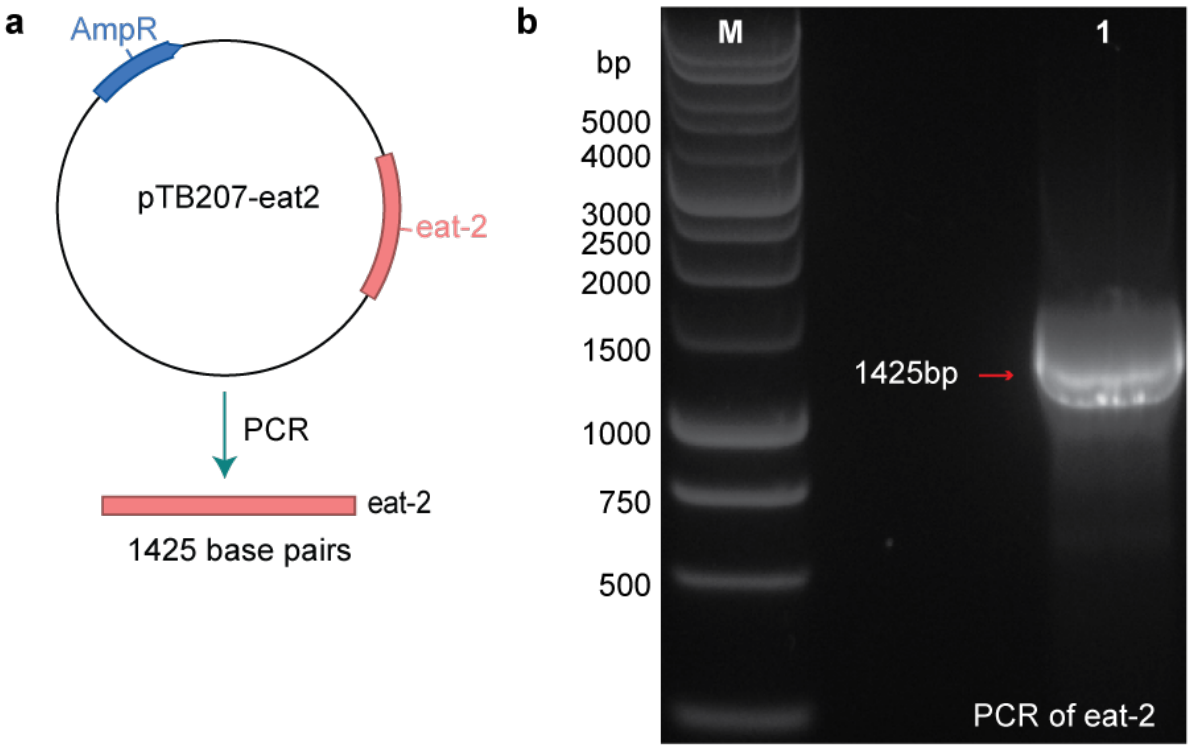


Figure 6.1: **Amplification of *eat-2* gene.** *Eat-2* cDNA was amplified from *pTB207* vector, gel excised and purified for downstream cloning. (a) Cartoon representation of the process of amplification of the gene by PCR including the expected PCR product size (b). Agarose gel of the PCR product with the corresponding size, against DNA ladder (M).

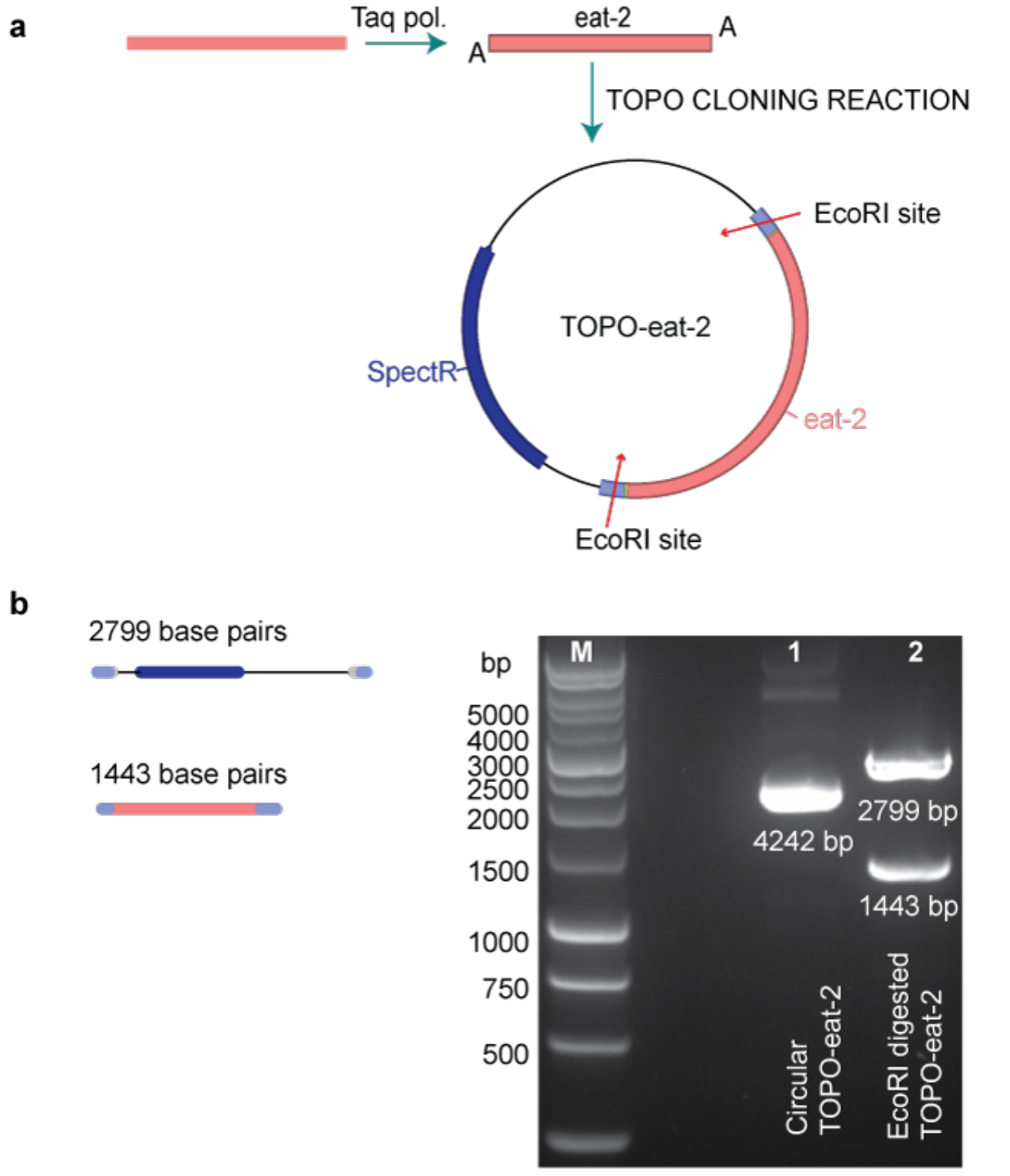


Figure 6.2: **Insertion of *eat-2* into the *TOPO* vector.** (a) Cartoon representation of the process of generation of *TOPO-eat-2* vector. 3' adenine overhangs were added to the purified *eat-2* cDNA to enable TOPO cloning. *Eat-2* containing *TOPO* vector was digested with *EcoRI* which cuts at sites flanking the inserted *eat-2* gene yielding 2 linear DNA fragments (b). (c) Agarose gel of non-digested *TOPO-eat-2* plasmid and the two bands following *EcoRI* digestion.

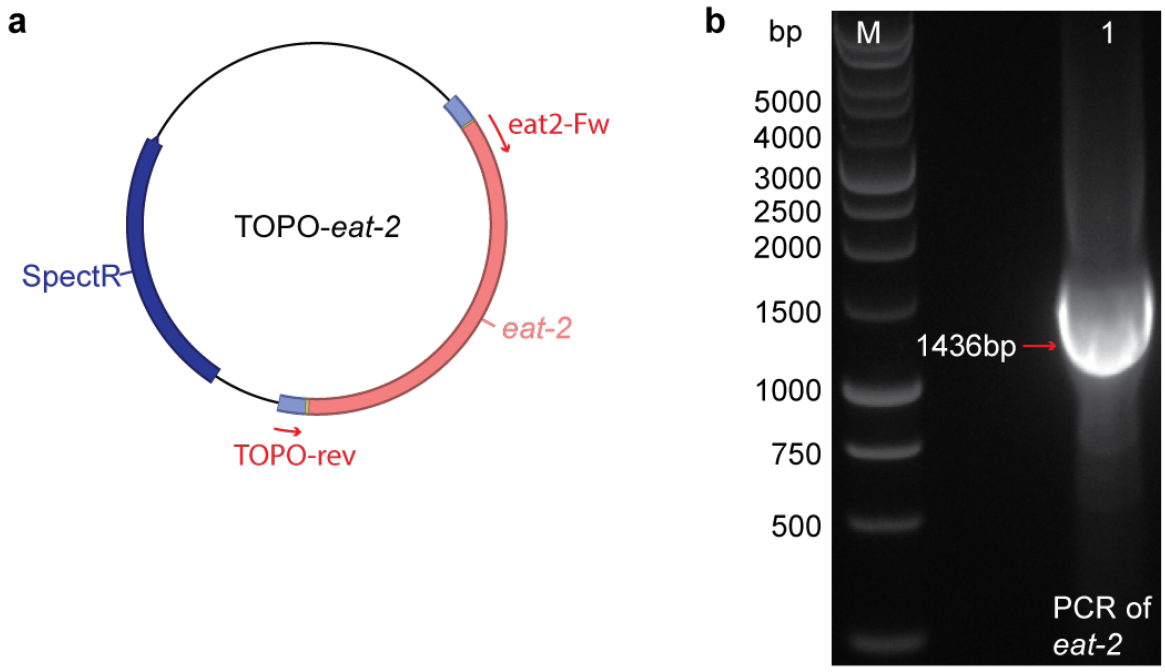


Figure 6.3: **PCR of *eat-2* gene from the *TOPO* vector.** (a) Cartoon representation showing the positioning of primers used to PCR amplify *eat-2* from *TOPO* vector using insert specific (*eat2-Fw*) and vector specific (*TOPO-Rev*) primers. Only clone containing *eat-2* inserted in the right direction should produce a PCR product of 1436 bp. (b) Picture of agarose gel of PCR product against DNA ladder (M).

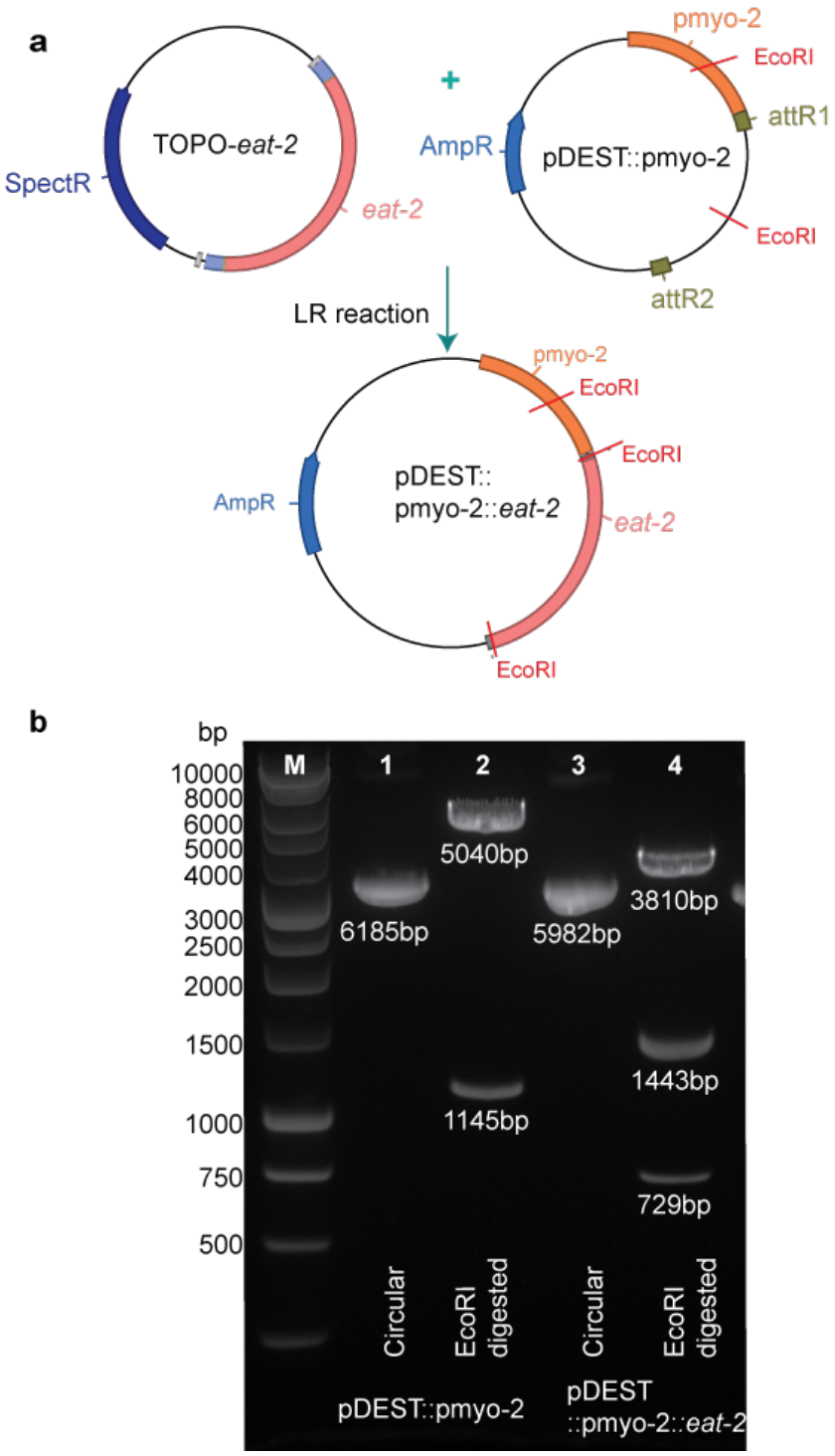


Figure 6.4: **The generation of the vector for the expression of EAT-2 nAChR in the *C. elegans* pharynx.** (a) Cartoon representation of the cloning process and location of *EcoRI* restriction sites within plasmids. *Eat-2* was cloned into the expression backbone vector downstream of the *myo-2* promoter. To identify successful clones, expression vector with and without the cloned *eat-2* were analytically digested with *EcoRI*. *EcoRI* cuts the backbone plasmid in two places producing 2 DNA fragments. In contrast, digestion of the plasmid containing the *eat-2* sequence yields 3 DNA fragments. (b) Picture of the agarose gel containing the digested plasmids against DNA ladder (M). Plasmid names and DNA fragment sizes are given on the gel.

6.2.1.2 Generation of the transgenic strain

Transgenic strains were generated by the process of microinjection (Section 2.4.17). A DNA mix of vector containing *eat-2* gene and vector containing the selectivity marker was prepared. Selectivity marker was a vector containing GFP under the *myo-3* promoter (*pmyo-3*). Endogenous *myo-3* promoter drives the expression of myosin in the body wall muscle. Therefore by driving the expression of GFP with *pmyo-3*, worms will appear green under the fluorescence microscope. This enabled identification of successfully transfected worms. Worms expressing GFP are also predicted to express another injected gene, in this case EAT-2.11

64 adult *eat-2* worms were injected. 6 of those generated 33 green progeny. Only 2 of the 6 F1 produced green progeny- these were kept as stable lines. The genotype of these lines is *eat-2::pmyo3::GFP;pmyo2::eat-2*, but for simplicity, they will be referred to as *eat-2::eat-2* or *eat-2* rescue.

Alongside, a control line was generated in which GFP was expressed at the body wall muscle of *eat-2* mutant. Worms were injected with a plasmid DNA containing *GFP* gene. 32 worms were injected. There were 5 green progeny present on a single plate, one of which produced green offspring. This transgenic line was kept and used as a control. The genotype of this line is *eat-2::pmyo3::GFP*, but for simplicity, they will be referred to as *eat-2::GFP* or *eat-2* transgenic control line.

6.2.1.3 Feeding phenotype of EAT-2 expressing transgenic lines

Eat-2 mutant has an overt feeding phenotype (Figure 4.14, McKay et al. (2004)). To determine whether the expression of *eat-2* under *myo-2* promoter rescues the feeding retardation of *eat-2* mutant, behavioural assays were carried out. The feeding phenotype of generated strains, *eat-2* mutant and wild-type *C. elegans* were assayed (Figure 6.5). Worms were placed on an agar plate containing an OP50 food patch and pharyngeal pumping on food of adult worms was scored. Wild-type worms pumped at a rate of 4.65 Hz. This dropped to 0.94 and 0.89 Hz in *eat-2* mutant and *eat-2* transgenic control strain, respectively. The feeding phenotype of two *eat-2* rescue lines was assayed. Their feeding phenotypes did not differ (data not shown), therefore results were pooled. Expression of *eat-2* in *eat-2* mutant restored feeding rate to 3.12 Hz.

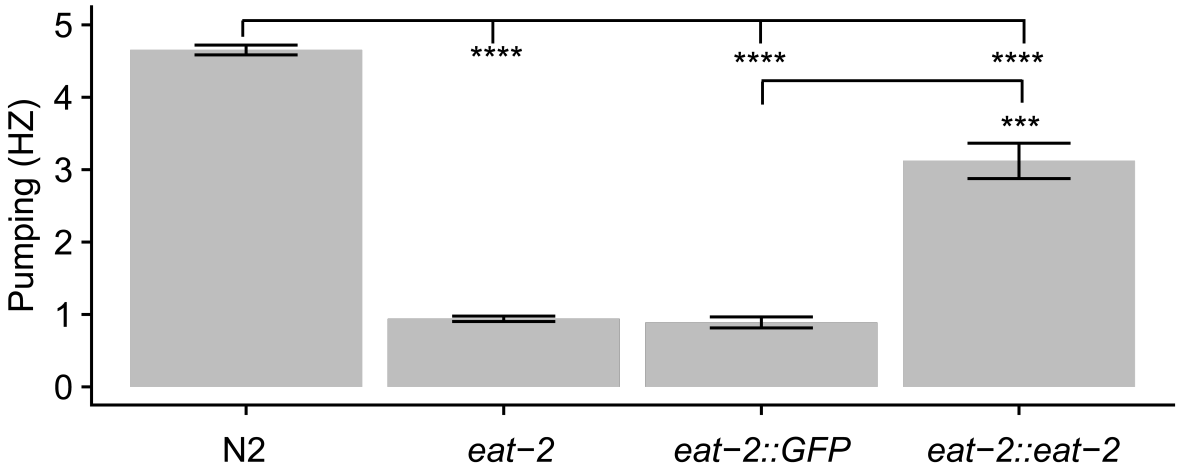


Figure 6.5: **Pharyngeal pumping of *C. elegans* nicotinic acetylcholine receptor mutant and rescue strains.** Pharyngeal pumping on food of N2 wild-type, *eat-2* mutant, *eat-2* transgenic control strain (*eat-2::GFP*) and *eat-2* rescue (*eat-2::eat-2*) strains. Pharyngeal pumps of worms present on food were counted by visual observation for 30 seconds and expressed in Hz. Data are mean ± SEM, collected from 10-46 individual worms on 3 days. One way ANOVA (Kruskal-Wallis test) with Sidak Corrections, $***\mathrm{P} \leq 0.0001$, $***\mathrm{P} \leq 0.001$.

6.2.1.4 Effects of 5-HT on pharyngeal pumping

The effects of 5-HT on pharyngeal pumping was scored to determine if expression of eat-2 in *eat-2* mutant worms rescues their 5-HT insensitivity (Figure 4.15). Cut-heads were used in this experiment and worms were exposed to 1 μM 5-HT because this dose elicits maximum response in the wild-type strain (Figure 4.9). After 30 minutes of incubation, the effects of 5-HT on pumping was scored (Figure 6.6). Wild-type pharynxes pumped at a rate of 3.00 Hz. This was reduced to 0.89 and 0.91 Hz in *eat-2* mutant and *eat-2* mutant expressing GFP. Transgenic lines were scored separately. The pharyngeal pumping rate induced by 5-HT did not differ between lines, hence the results were pooled. Rescue *eat-2* worms pumped at an average rate of 2.97 Hz.

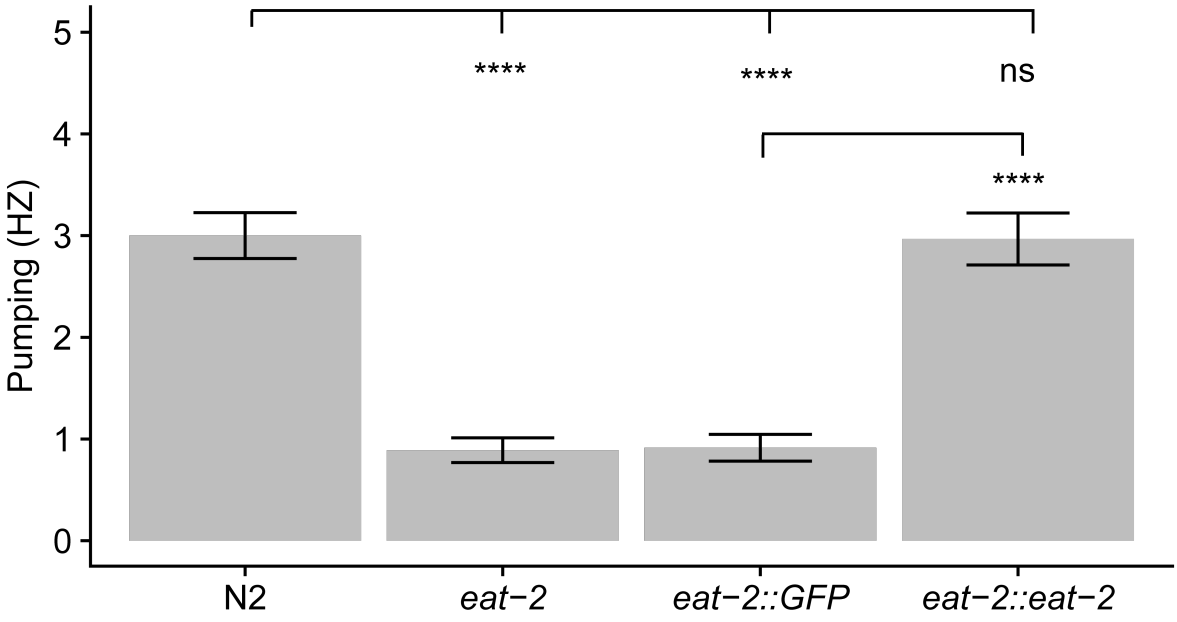


Figure 6.6: **The effects of 5-HT on wild-type, *eat-2* mutant and *eat-2* rescue strains.** Cut heads of wild-type, *eat-2* mutant, transgenic control and rescue strains were incubated with 1 μM 5-HT or control vehicle. 30 minutes later, the effects of 5-HT on pumping was scored. Pharyngeal pumping was counted by visual observation for 30 seconds and expressed in Hz. Data are mean $\pm$ SEM of 7 - 30 worms collected from paired experiments done on 2 days. $***$P $\leq$ 0.0001.

6.2.2 Heterologous expression of human $\alpha 7$ nAChRs in the *C. elegans* pharyngeal muscle

To test whether successful expression of non-native nAChRs can be achieved, human $\alpha 7$ receptor was introduced into the *C. elegans* pharynx. This receptor was chosen because it is homopentameric (Couturier et al. 1990; Cooper and Millar 1997; Gu et al. 2016), therefore does not need to interact with other subunits to form a functional receptor. Additionally, its pharmacology has been thoroughly studied (Papke and Porter-Papke 2002).

Two approaches were taken as they provide alternative ways of assessing the functionality of the introduced receptor.

First, human $\alpha 7$ encoding gene (*CHRNA7*) was introduced into the *eat-2* background to determine whether the *eat-2* mutant behavioural and pharmacological phenotype can be reversed. Alongside, *CHRNA7* was introduced into the wild-type background of *C. elegans* to determine whether the pharmacology of $\alpha 7$ receptor can be imposed on the pharynx.

6.2.2.1 Generation of the expression vector

A cDNA sequence was inserted downstream of *myo-2* promoter by LR recombination. Briefly, *CHRNA7* was PCR amplified from *pDNA3.1* vector (AddGene plasmid #62276) using flanking primers (Table 2.3, Table 2.7

and Figure 6.7). Amplified PCR product was gel-purified and incubated with non-proofreading PCR polymerase to add 3' adenine-overhangs. This enabled cloning into the *TOPO* vector (Figure 6.8 a). Produced plasmid was analytically digested with *EcoRI* (Figure 6.8 b). *CHRNA7* was then cloned into the *pDEST* expression vector downstream of the *myo-2* promoter by LT recombination (Figure 6.9). Four clones were analysed by digestion. A single positive clone (clone number 4 in lane 5 of the agarose gel in Figure 6.9 b) was selected, sequenced and used in downstream experiments. The entire *pmyo-2::CHRNA7* sequence can be found in the Appendix C.

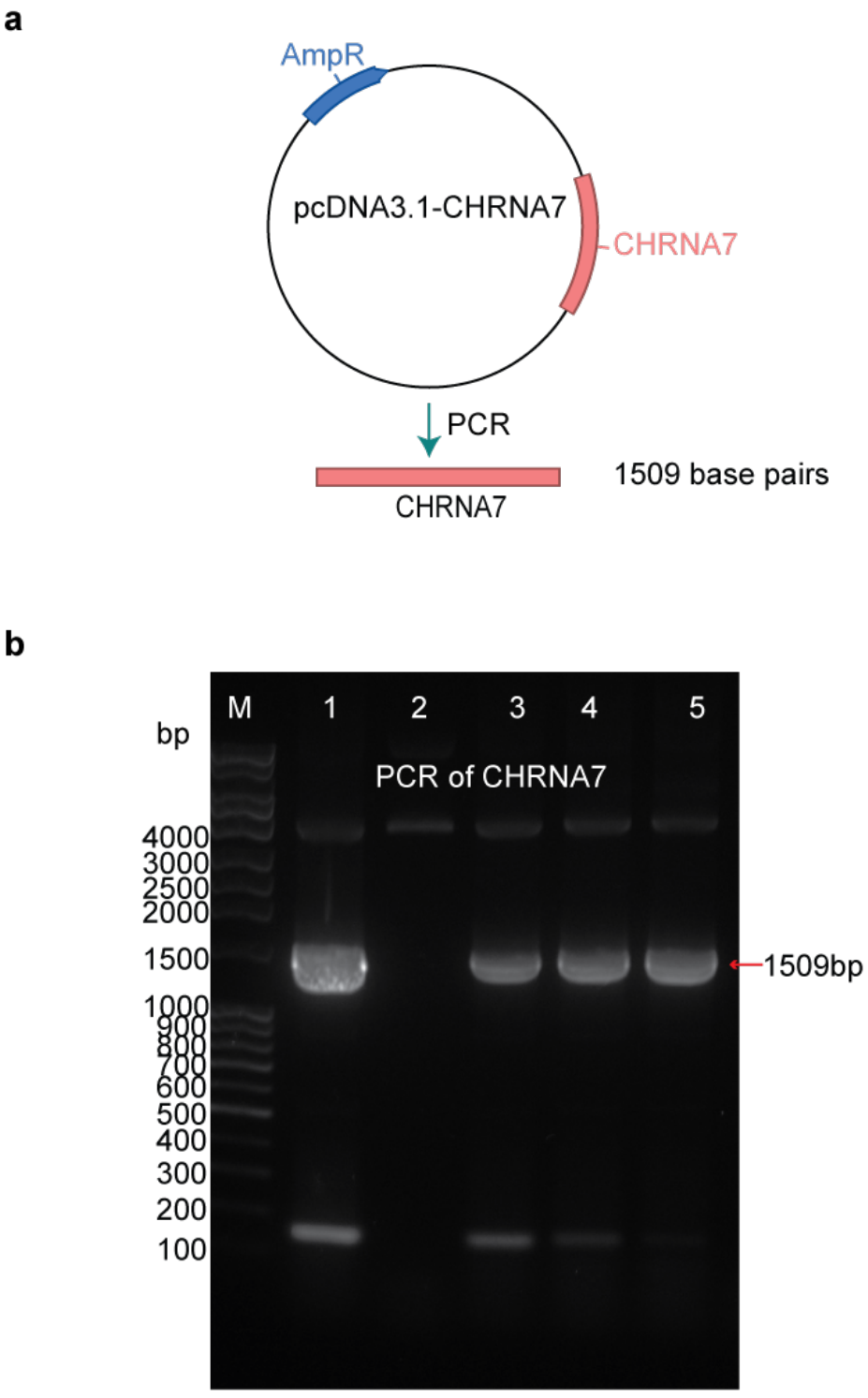


Figure 6.7: **Amplification of the gene encoding for human $\alpha 7$ subunit of nAChR.** (a) Cartoon representation of the process of amplification of *CHRNA7* by PCR. *CHRNA7* was amplified from pcDNA3.1 vector, gel excised and purified for downstream cloning. (b) Picture of the agarose gel of the PCR products of 1509 bp against DNA ladder (M).

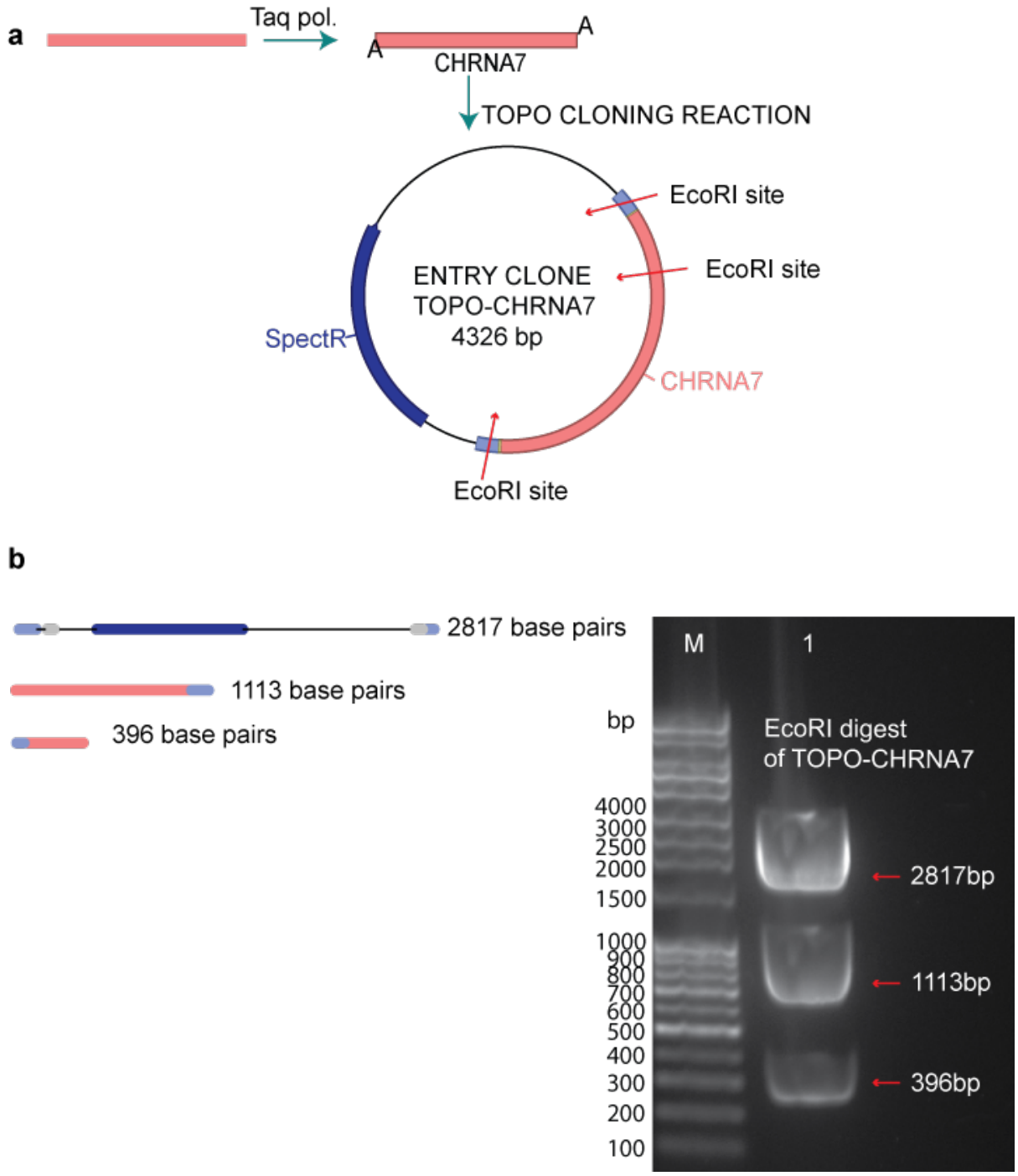


Figure 6.8: **Insertion of *CHRNA7* into the *TOPO* vector.** (a) Cartoon representation of generation of *TOPO-CHRNA7* vector. 3' A overhangs were added to the purified PCR product to enable TOPO cloning. *CHRNA7* containing *TOPO* vector was digested with *EcoRI* which cuts at sites flanking and within the inserted gene, yielding 3 linear DNA fragments. (b) Schematic representation (left) and an agarose gel (right) of DNA fragments generated upon *EcoRI* digestion against DNA ladder (M).

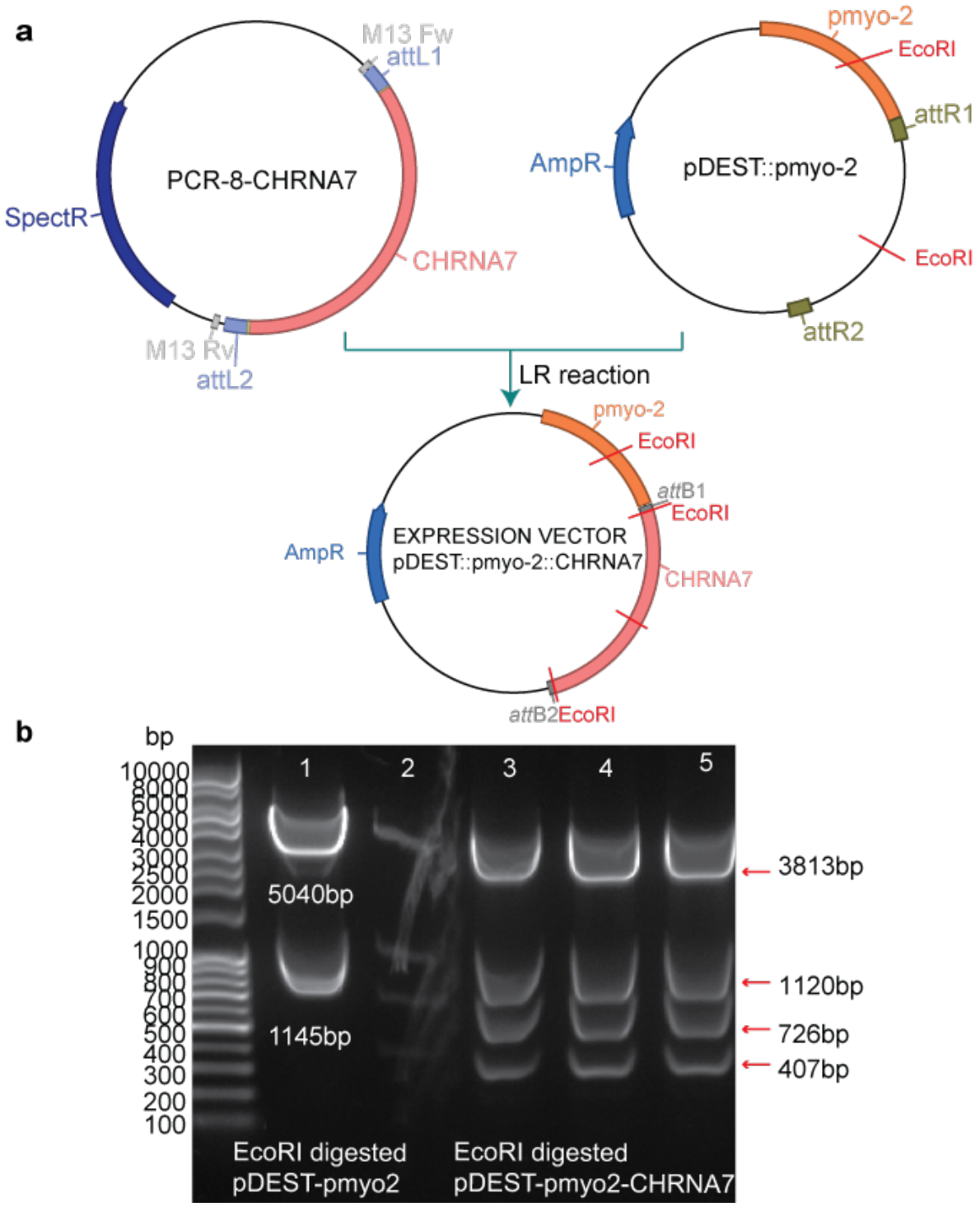


Figure 6.9: **Generation of the vector for the expression of $\alpha 7$ nAChR in the *C. elegans* pharynx.** (a) *CHRNA7* gene was cloned into the *pDEST* expression vector downstream of the *myo-2* promoter. Four clones of the generated plasmid and the backbone *pDEST* plasmid were digested with *EcoRI*. The *EcoRI* restriction sites within the backbone plasmid and the cloned plasmid are shown in a. (b) The resulting DNA fragments were run on the agarose gel. Digestion of *pDEST* backbone results in the generation of 2 fragments (Line 1 on the gel), where digestion of *CHRNA7*-containing plasmid yields 4 fragments (Lines 2-5).

6.2.2.2 Generation of transgenic strain

Transgenic worms were generated by microinjection. DNA mix containing two plasmids: *CHRNA7* downstream of *myo-2* promoter and *GFP* downstream of *myo-3* promoter was prepared. 64 adult *eat-2* worms were injected. 3 plates contained a total of 23 green progeny. These 23 worms were separated and allowed to propagate. Green offsprings were found on three plates. These plates were kept separately and treated as individual worm lines. The genotype of these lines is *eat-2::pmyo3::GFP;pmyo2::α7*, but for simplicity, they will be referred to as *eat- 2:: α7*.

The three *eat-2:: α7* lines were assayed separately. The behavioural output did not differ (data not shown), therefore the results were pooled.

A control strain in which GFP is expressed at the body wall muscle was generated previously (Section 6.2.1.2).

6.2.2.3 Generation of N2 transgenic strain

59 adult N2 worms were injected with the DNA mix used previously (Section 6.2.2.2). 12 plates contained a total of 74 green progeny. These 74 worms were separated and allowed to propagate. Green offspring were found on two plates. These plates were kept separately and treated as individual worm lines. The genotype of these lines is *N2::pmyo3::GFP;pmyo2::α7*, but for simplicity, they will be referred to as *N2::α7*, or N2 transgenic. Their behavioural output did not differ (data not shown), therefore the results were pooled.

A control strain in which GFP is expressed at the body wall muscle was also generated. 8 worms were injected with the *GFP* containing vector. 2 generated 8 green offspring. 1line was stable. The genotype of this line is *N2::pmyo3::GFP*, simply *N2::GFP* or N2 transgenic control line.

6.2.2.4 Feeding phenotype of transgenic lines

The feeding phenotype of wild-type, *eat-2* mutant, *α7*-expressing and control lines were assayed. Worms were placed on agar plate containing a food patch and the pharyngeal pumping was scored (Figure 6.10).

The feeding phenotype of worms did not change upon introduction of human *α7* in the pharynx. N2 wild-type, and transgenic *C. elegans* pumped at an average rate of 4.64 - 4.68 Hz. Pharyngeal pumping of *eat-2* mutant and *eat-2* transgenic worms varied between 0.89 and 0.98 Hz.

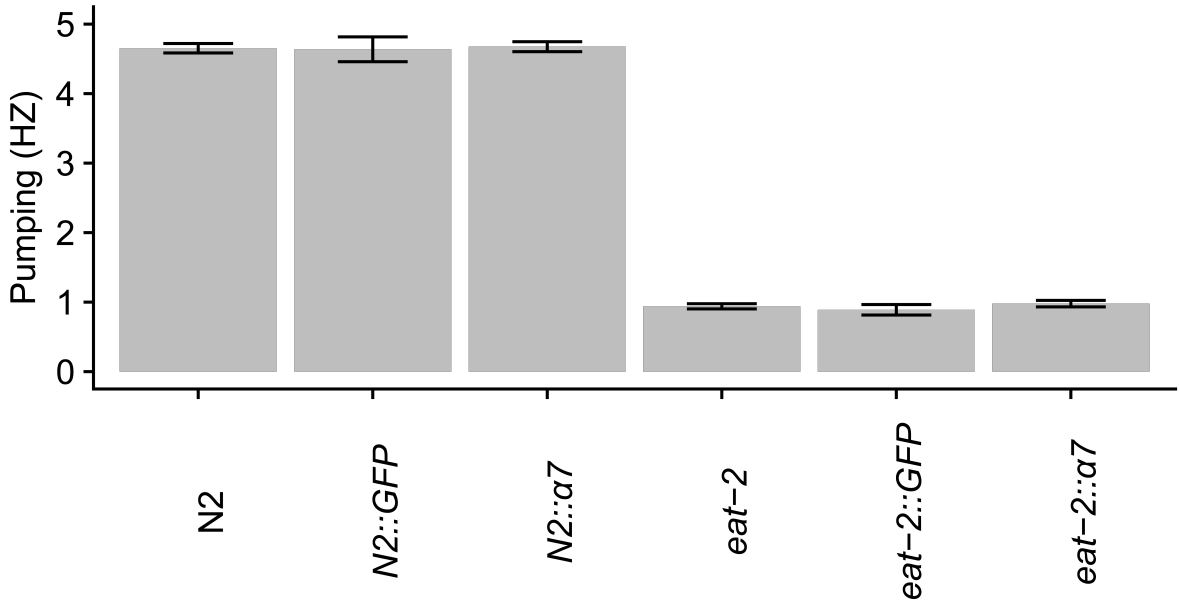


Figure 6.10: **Effects of human $\alpha 7$ nAChR expression on the feeding phenotype of *C. elegans*.** Pharyngeal pumping of N2 wild-type, *eat-2* mutant, transgenic strains expressing human $\alpha 7$ nAChR in the pharyngeal muscle (*N2::α7* and *eat-2::α7*) and transgenic control worms (*N2::GFP* and *eat-2::GFP*). Pharyngeal pumps of worms present on food were counted by visual observation for 30 seconds and expressed in Hz. Data are mean $\pm$ SEM, collected from 7-30 individual worms on 3 days. One way ANOVA (Kruskal-Wallis test) with Sidak Corrections.

6.2.2.5 Effects of 5-HT on pharyngeal pumping

The effects of 5-HT on the pharyngeal pumping of wild-type, *eat-* mutant, transgenic and transgenic control worms expressing human receptor was assayed. (Figure 6.11). Cut-heads were bathed in 1 μM 5-HT. 30 minutes later, the effects on pharyngeal pumping were scored.

Introduction of human $\alpha 7$ receptor into the pharynx had no effect on the pharyngeal responses to 5-HT. After 30 minutes of incubation, the pharyngeal pumping of transgenic and control *C. elegans* did not differ.

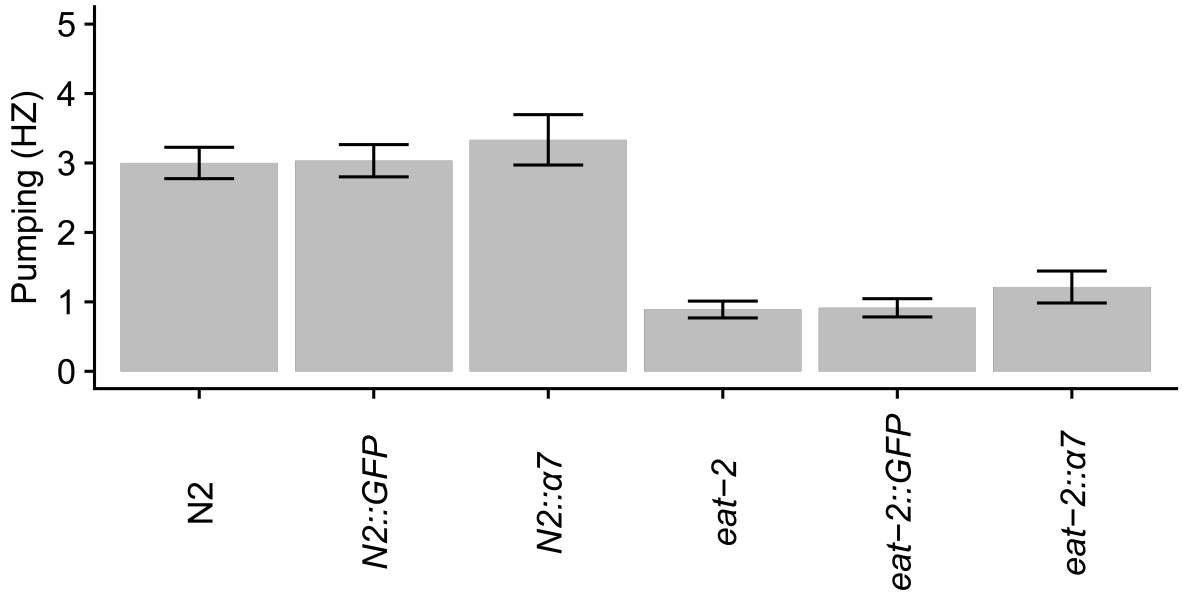


Figure 6.11: **Effects of human $\alpha 7$ nAChR expression on the 5-HT induced pharyngeal pumping of *C. elegans*.** The effects of 5-HT on N2 wild-type, *eat-2* mutant, transgenic strains expressing human $\alpha 7$ nAChR in the pharyngeal muscle (*N2::α7* and *eat-2-α7*) and transgenic control worms (*N2::GFP* and *eat-2-GFP*). Cut heads were incubated with 1 μM 5-HT or control vehicle. 30 minutes later, the effects of 5-HT on pumping was scored. Pharyngeal pumping was measured by counting the number of pharyngeal pumps/30s and expressed in Hz. Data are mean $\pm$ SEM of 7 - 30 worms collected from paired experiments done on 3 days.

6.2.3 Pharmacological characterisation of $\alpha 7$ expressing worms

The experiments utilizing the pharyngeal pump phenotype on food and in the presence of 5-HT suggest lack of functionality of the introduced receptor. However, the experiments that we used may not be sensitive enough to detect functional expression of the $\alpha 7$. Thus, we extended the analysis to investigate the pharmacological sensitivity with the aim of determining if the transgenic lines heterologously expressing $\alpha 7$ exhibit known $\alpha 7$ pharmacology.

To asses whether the introduction of receptor into the pharynx of wild-type worms imposes $\alpha 7$ pharmacology on the pharyngeal system, a series of pharyngeal assays were performed. nAChR agonists were tested to determine if there is a differential sensitivity between the N2 wild-type and transgenic worms. Compounds tested were acetylcholine, nicotine, choline and cytisine.

Effects of nAChR agonist on 5-HT induced pharyngeal pumping on cut-heads were tested. Cut heads were exposed to 1 μM 5-HT for 10 minutes. Following this the activated pharynxes were transferred to a dish containing 5-HT and an indicated nAChR agonist. The effects of agonist on 5-HT induced pumping was scored for 50 minutes (Figure 6.12, 6.13, 6.14).

Exposure to 5-HT results in dose and time dependent elevation of the pharyngeal pumping that is indifferent in the wild-type and the transgenic lines. Exposure to nAChR agonists led to dose-dependent inhibition of this response.

Exposure of cut heads to nicotine from 1 to 50 μM, resulted in concentration-dependent inhibition of pumping in both wild-type and transgenic strains (Figure 6.12). The IC_{50} values were comparable: 13 and 11 μM, respectively indicating no shift in the sensitivity to nicotine upon introduction of human receptor in the *C. elegans* pharynx.

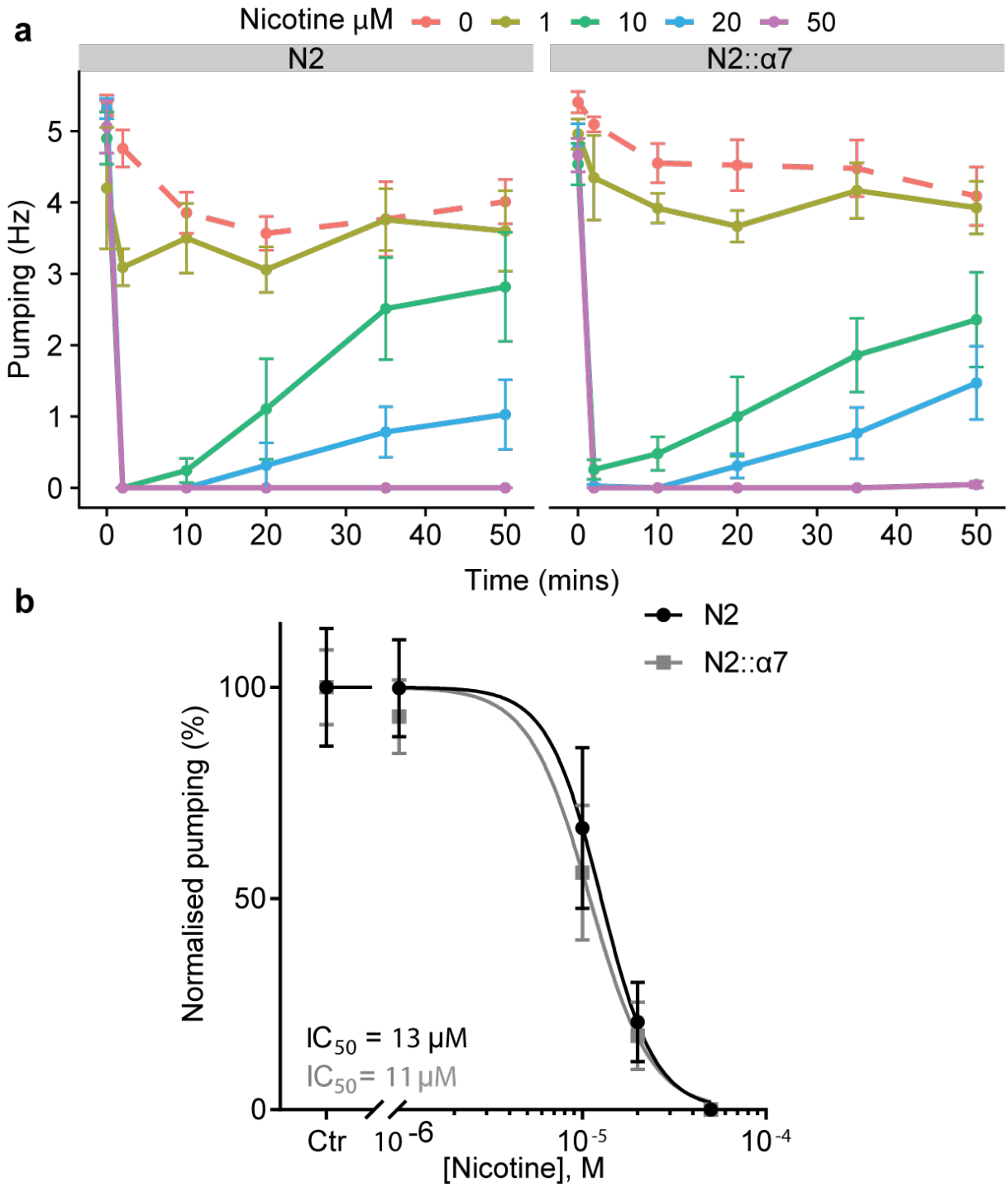


Figure 6.12: **The effects of human $\alpha 7$ nAChR expression on the nicotine-induced inhibition of 5-HT evoked pumping.** Cut heads of wild-type (N2) and transgenic worms expressing human $\alpha 7$ in the pharynx (*N2::α7*) were exposed for 10 minutes to 1 μM 5-HT to stimulate pumping. They were then transferred to 5-HT + indicated concentration of nicotine or vehicle control. a) The effects on pharyngeal pumping pre- (time point 0) and post- nicotine exposure were scored by visual observation for 30 seconds and expressed in Hz. b) 35-minute time points were taken, and normalised to the maximal (5-HT induced) and minimal response. Data are mean $\pm$ SEM from 5 - 10 individual worms collected from paired experiments done on 2 days.

The responses of both strains to choline at 1 μM to 1 mM were also indiscernible (Figure 6.13). Choline inhibited 5-HT evoked pumping of the wild-type worms with the IC_{50} of 22 μM. The IC_{50} of choline on transgenic line was 15 μM.

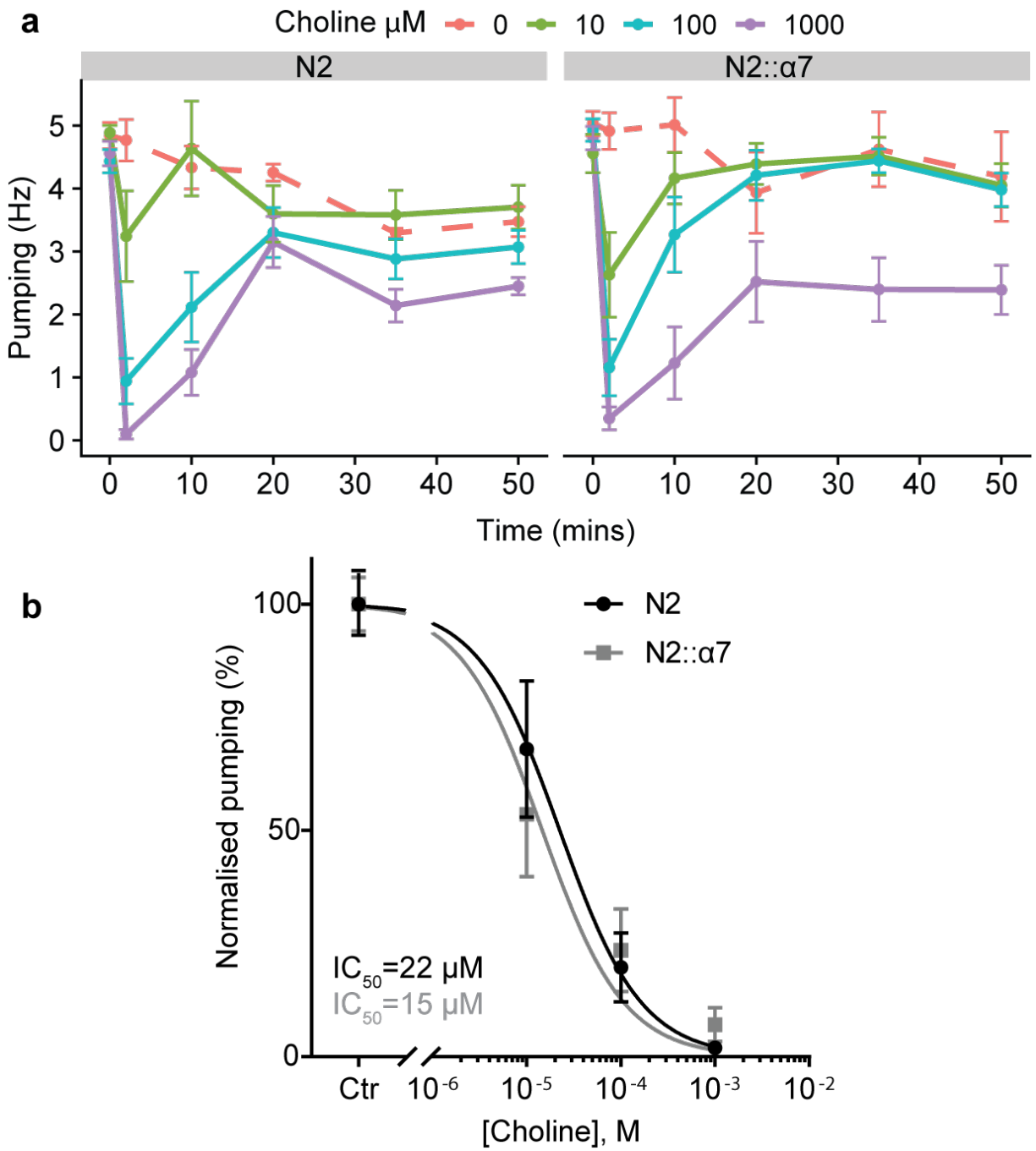


Figure 6.13: **The effects of human $\alpha 7$ nAChR expression on the choline-induced inhibition of 5-HT evoked pumping.** Cut heads of wild-type (N2) and transgenic worms expressing human $\alpha 7$ in the pharynx (*N2::α7*) were exposed for 10 minutes to 1 μM 5-HT to stimulate pumping. They were then transferred to 5-HT + indicated concentration of choline or vehicle control. a) The effects on pharyngeal pumping pre- (time point 0) and post- choline exposure were scored by visual observation for 30 seconds and expressed in Hz. b) 2-minute time points were taken, and normalised to the maximal (5-HT induced) and minimal response. Data are mean ± SEM from 3 - 12 individual worms collected from paired experiments done on 2 days.

Wild-type cut-head were also exposed to cytisine at 100 nM, 1, 10 and 50 μM (Figure 6.14 a left panel). The two lowest doses had no effect on 5-HT induced pumping. 10 μM inhibited pumping almost completely and transiently after 2 minutes of incubation. After 10 minutes, the pumping rate was comparable to the control. Comparing the effects of cytisine on 5-HT evoked pumping of N2 wild-type to the effects on transgenic worms revealed a difference at a single dose of 50 μM (Figure 6.14 a and b). In wild-type, cytisine rapidly inhibited pumping. Pharynxes remained paralysed for 10 minutes. They began to progressively recover, however the rate of the control was not reached. Transgenic pharynxes also remained paralysed for 10 minutes, however, pumping returned to a rate comparable to control after 20 minutes.

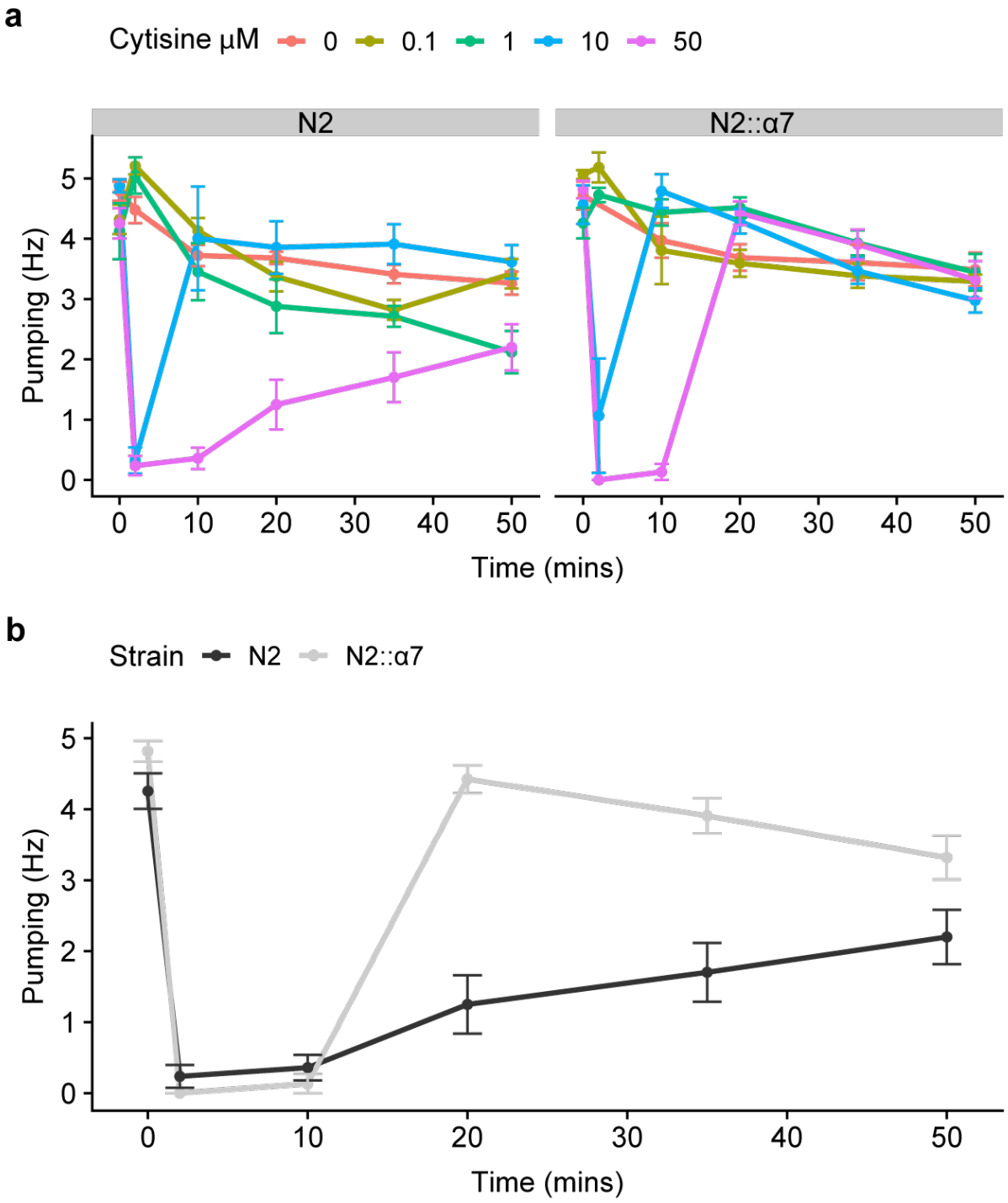


Figure 6.14: **The effects of human $\alpha 7$ nAChR expression on the cytisine-induced inhibition of 5-HT evoked pumping.** (a) Cut heads of wild-type (N2) and transgenic worms expressing human $\alpha 7$ in the pharynx (*N2::α7*) were exposed for 10 minutes to 1 μM 5-HT to stimulate pumping. They were then transferred to 5-HT + indicated concentration of cytisine or vehicle control. The effects on pharyngeal pumping pre- (time point 0) and post- cytisine exposure were scored by visual observation for 30 seconds and expressed in Hz. b) Comparison of the 50 μM cytisine to show the difference in pharyngeal response between N2 and N2 transgenic worms. Data are mean $\pm$ SEM from 5 - 14 individual worms collected from paired experiments done on 2 days.

6.2.3.1 α-Bgtx staining

To determine if FITC-α-bgtx binds to native nAChRs, an isolated pharynx was used (Section 2.4.18 and Figure 2.9). In this preparation, there is no cuticular barrier, therefore, α-bgtx should access to the extracellular nAChR binding sites in the cell membrane of the pharyngeal muscle. Pharynxes were incubated with α-bgtx for 1 hour before being washed to remove unbound toxin.

Images of N2 wild-type and transgenic as well as *eat-2* mutant and transgenic pharynxes were taken (Figure 6.15). There was either no fluorescence (12 out or 21 preparations) or very weak fluorescence in the corpus and/or terminal bulb of the wild-type and mutant strains. In comparison, exposure of transgenic pharynxes in which human α7 was expressed in both *C. elegans* N2 wild-type and *eat-2* mutant strains led to strong fluorescence in the pharynx. Comparison of the fluorescence localisation to the localisation of muscle cells in the pharynx (Figure 4.2) showed that the fluorescence was selectively present in the terminal bulb and largely localised to pm7 and pm8 muscle cells. Weak fluorescence was also observed in the isthmus and corpus, however this was inconsistent among preparations (6 out of 14).

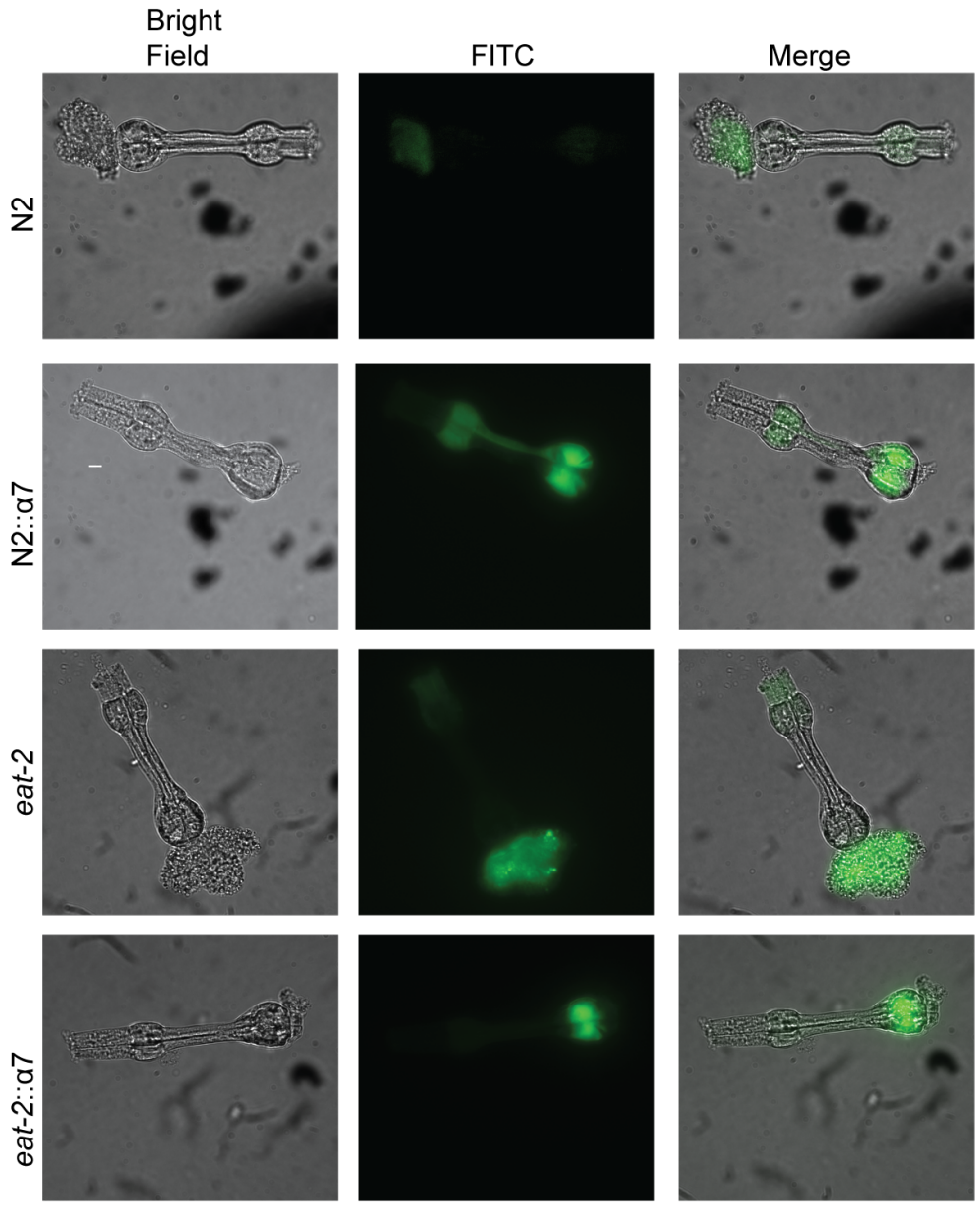


Figure 6.15: **The staining of *C. elegans* pharynxes with FITC-αbungarotoxin (FITC-αbgtx).** Representative images of the isolated pharynxes of N2 wild-type, *eat-2* mutant, and human $\alpha 7$ nAChR expressing transgenic worms (*N2::α7* and *eat-2::α7*). Isolated pharynxes were incubated with FITC-α-bgtx. 1 hour later, they were washed for 1 hour and imaged immediately after. Bright field (left column) and fluorescent images (middle column) were taken and superimposed (right column). Note that fluorescence posterior to the terminal bulb may represent staining of the extrapharyngeal structures (McKay et al. 2004). Scale bar (5 μM) is in the second top image on the left.

6.3 Discussion

Understanding the molecular basis of the mode of action of insecticides is the first step towards development of more selective compounds. Neonicotinoids act by targeting nAChRs, but the receptor subunit specificity remains to be elucidated. Several other heterologous systems have been used for the expression of exogenous nAChR (Millar 2009). However, to enable pharmacological characterisation of nAChRs, we focused on using the *C. elegans* system.

C. elegans' pharynx is an attractive platform firstly because it has low relative sensitivity to neonicotinoids (Chapter 4 and 5). Secondly, it expresses chaperone proteins including RIC-3 and UNC-50 that are known to be important in aiding the maturation of nAChRs (Millar 2008; Eimer et al. 2007). Additionally, *C. elegans* has been previously used for the recombinant expression of ion channels, including potassium-activated calcium channels (Crisford et al. 2011) and nAChRs (Sloan et al. 2015). Lastly, it allows for scoring of the functionality of introduced receptors in behavioural assays.

6.3.1 *Eat-2* as a genetic background for functional nAChR expression

EAT-2 nAChR subunit is the single determinant of the fast pharyngeal function in the pharynx. Literature suggest that the *eat-2 C. elegans* mutant has profoundly altered feeding and disrupted cholinergic neurotransmission in the pharynx (Raizen, Lee, and Avery 1995). These can be rescued by the re-introduction of the EAT-2 receptor into the pharyngeal muscle (McKay et al. 2004). Thus, the selective expression of the nAChR at the muscle should be a platform for the functional expression of the introduced receptor. However, *eat-2* transgenic strain used in McKay et al. (2004) is no longer available and data supporting rescue was not provided in the publication. Therefore, the first step in this study was to generate *eat-2* rescue of *C. elegans*. Worms were injected with a DNA construct containing *eat-2* cDNA downstream of *myo-2* promoter. As a result, the expression of the nAChR was driven in the pharyngeal muscle of *C. elegans*.

The *eat-2* mutant and rescue strains were analysed behaviourally and pharmacologically. The intact mutant worms pump on food 70 % less than the wild-type. *Eat-2* worms are also relatively insensitive to the application of 5-HT in the cut-head pharyngeal pumping assays. Introduction of *eat-2* partially rescued the feeding phenotype of the mutant as measured by counting the number of pharyngeal pumps of the intact worms on the food patch (Figure 6.5). The insensitivity of the pharynx to 5-HT was reversed (Figure 6.6).

These results support the argument from Chapter 2, that EAT-2 nAChR is a major driver of the feeding response and the 5-HT induced pharyngeal response in worms. Whilst the 5-HT sensitivity was restored fully (5-HT induced pumping rate in wild-type was 3.00 Hz in comparison to 2.97 in transgenic lines), the pumping rate on food was not. Rescue lines pumped at rate slightly lower in comparison to the wild-type (3.12 vs 4.65 Hz). This lack of full rescue in feeding assay may suggest there are some differences in which pharyngeal pumping

is elevated in response to food and 5-HT. Indeed, exogenous application of 5-HT acts directly onto pharyngeal nervous system to stimulate pumping. In contrast, food stimulates MC and M4 indirectly. Initially it was thought that food evoked response was mediated by 5-HT released from NSM neurons (Horvitz et al. 1982). More recently it was shown that the release of 5-HT selectively from ADF neurons is sufficient to drive the feeding response (Cunningham et al. 2012). 5-HT released from ADF activates ADJ neurons and MC and M3 neurons. MC and M3 release ACh leading to pharyngeal response. Therefore the 5-HT elevation caused by exogenous 5-HT application is likely to be more diffused in comparison to the 5-HT levels in response to food. 5-HT application in the presence of food causes a small increase in the pumping rate, indicative of the presence of circuits regulating feeding in response to 5-HT but not activated in the presence of food.

Although the reversal of the feeding deficiency and 5-HT insensitivity in rescue lines suggests that the *myo-2* promoter is suitable for the heterologous nAChR expression in the *C. elegans* pharynx, this promoter may also have some limitations. Transgenic strain carrying *myo2-GFP* reporter gene, expresses GFP in all muscles of the pharynx (Altun and Hall 2009b and (Figure 4.2). In contrast, the EAT-2 receptor in native worms is expressed at the NMJ of the MC and pm4 muscle (McKay et al. 2004). Therefore, cellular EAT-2 expression driven by the native promoter, may be much more restricted in comparison to the expression driven by *myo-2* promoter.

6.3.2 No apparent functionality of human $\alpha 7$ receptors in the *eat-2* mutant pharynx

To determine whether exogenous nAChR can be successful expressed in *C. elegans*, human $\alpha 7$ receptor was introduced into the pharynx. This receptor protein was chosen because it is known to function as a homopentamer, its pharmacology has been well studies, and there are a number of $\alpha 7$ selective compounds which could be used to detect their functionality (Mazurov, Hauser, and Miller 2006).

A DNA construct containing $\alpha 7$ nAChR cDNA was cloned and used to generate transgenic lines that should drive expression in the pharyngeal muscle of N2 and *eat-2* strains. Introduction of human receptor into the *eat-2* pharynx did not rescue the feeding phenotype, nor did it reverse the 5-HT insensitivity. This could suggest that $\alpha 7$ may not be capable of performing the function of EAT-2 possibly due to the biological and molecular distinct nature of the pharynx.

One of the unique feature of the pharynx is the presence of acetylcholine-gated chloride channels, expressed in cholinergic and glutamatergic neurons (Pereira et al. 2015; Takayanagi-Kiya, Zhou, and Jin 2016), which are involved in the regulation of the synaptic release and inhibition of the neurons they are expressed in.

The properties of EAT-2 channel differ from those of $\alpha 7$. In contrast to $\alpha 7$, EAT-2 is classified as a non-α subunit, due to the lack of vicinal cysteine in the extracellular domain. Typically, non- α subunit must assembly with α subunit to form a functional receptor, however, EAT-2 may function as a homooligomer; a current was elicited in response to application of nAChR agonist on the *Xenopus* oocyte injected with *eat-2* (Lindy Holden-Dye, personal communication).

The function of the EAT-2 is dependent on EAT-18 (McKay et al. 2004). EAT-18 is a single pass transmembrane protein of unclear function, but it seems critical in eliciting feeding response (McKay et al. 2004). The native EAT-2 channels are localised to the pm4 muscle of the pharynx (McKay et al. 2004) in the juxtaposition to cholinergic MC neuron (Albertson and Thomson 1976). Expressed $\alpha 7$ is mainly localised to pm7 and pm8 which make synaptic connections with cholinergic motor neuron M5 (Albertson and Thomson 1976).

The EC_{50} for ACh acting on EAT-18-containing receptors is not known, but these receptors are likely activated by the phasically and synaptically released agonist. The EC_{50} of ACh on heterologously expressed $\alpha 7$ is 173 μM (Papke and Porter-Papke 2002). The concentration of ACh in the pharynx may not be adequate to activate human receptors in response to 5-HT or food.

Taken together, the cellular environment, the differences in structure and functional properties as well as the likely different localisation of EAT-2 and $\alpha 7$ receptors in the pharynx, may account for inability of the human receptor to perform the function of EAT-2 in the pharynx. If so, the feeding and 5-HT assays are not appropriate for the detection of $\alpha 7$ functionality.

6.3.3 Distinct response of the N2 transgenic worms to cytisine.

An alternative approach was taken, in which human receptor was introduced into the pharynx of the wild-type worm. $\alpha 7$ is a Ca^{2+} specific channel, so one might expect its ability to couple to muscle upon ACh activation. In addition, wide expression of $\alpha 7$ driven by the *myo-2* promoter might result in the wide activation of ACh receptor, resulting in feeding behaviour that could result in abnormal rate of growth (Halevi et al. 2002). However, development of transgenic lines was normal and there was no subtle feeding phenotype observed.

Further experiments were carried out to determine whether pharmacology of $\alpha 7$ receptor can be imposed on the *C. elegans* pharynx. In the presence of exogenous receptor that is selectively disrupted upon the application of the pharmacological agent, a change in pharyngeal response is expected. The function of the pharynx was scored in the presence of a series of nAChR agonist: acetylcholine, nicotine, cytisine and choline. The latter are selective $\alpha 7$ agonists with the EC_{50} on the heterologously expressed $\alpha 7$ receptors at 14.3 and 565 μM, respectively (Chavez-Noriega et al. 1997; Briggs and McKenna 1996). The ability of these compounds to inhibit 5-HT induced pharyngeal responses of cut heads were examined.

No differences between the wild-type and transgenic lines were noted in response to acetylcholine, nicotine and choline (Figure 6.12, 6.13, 6.14). The intrinsic sensitivity of the pharynx to these compounds precludes these experiment from being diagnostic for human $\alpha 7$ expression.

Investigating the effects of cytisine on wild-type and transgenic lines shows an interesting difference in the kinetics of the response at a single dose of 50 μM. Following rapid inhibition of pumping, a pumping recovery was observed in the continual presence of cytisine in both genetic backgrounds. In wild-type, the recovery was

slow. In contrast, transgenic lines recovered rapidly. However, it is unclear whether the recovery is due to cytisine acting on $\alpha 7$, since these receptors desensitise rapidly in the presence of agonist and do not recover until the drug is washed off (Briggs and McKenna 1998).

Overall the intrinsic sensitivity of the pharynx to nAChR agonists confounds this approach to determine expression of endogenous receptor but it hints at distinct signature in terms of dynamic of the receptor activation in response to cytisine. Further experiments should be carried out to characterise the responses of the wild-type and transgenic lines in experiments emitting the 5-HT stimulation. The EPG approach should be taken to observe the early time points effects, prior to receptor desensitisation. The effects of choline and other selective nAChRs should be investigated on the wild-type and transgenic worms.

6.3.4 $\alpha 7$ receptors are expressed on the surface of the pharyngeal muscle.

The lack of significant behavioural and pharmacological differential between the transgenic and control strains suggests lack of functionality of these receptors in the *C. elegans* pharynx, or the inability to resolve the functional human receptor. To independently assess whether $\alpha 7$ is expressed, staining of isolated pharynxes with conjugated $\alpha 7$ nAChR selective antagonist FITC-α-bgtx was carried out (Figure 6.15).

The lack of intense staining in wild-type suggest that pharynx does not endogenously express $\alpha 7$-bgtx sensitivity. This is in contrast with the body wall muscle where ACR-16 is readily detected by α-bgtx (Jensen et al. 2012). This reinforces the distinct nature of pharyngeal cholinergic receptors. In contrast, the robust detection of α-bgtx-binding in excised transgenic heads was observed. α-bgtx can bind to partially assembled receptors. X-ray crystal structure of human $\alpha 1$ shows α-bgtx binding to the extracellular domain of a single subunit, suggesting, a fully assembled receptor is not necessary for the binding of this antagonist (Dellisanti et al. 2007). This may suggest that the expressed $\alpha 7$ subunits are monomeric. However, nAChR assembly is strictly regulated in the cell (Crespi, Colombo, and Gotti 2018). Incorrectly assembled receptors are degraded (Brodsky and McCracken 1999) or accumulate inside the cell (Han, Yang, and Phillips 2000). In this study, there was no detergent permeabilisation, thus, the robust α-bgtx staining is likely concentrated to extracellular cell surface. This favors the idea that staining is to the cell surface homopentamers.

The α-bgtx staining of $\alpha 7$ receptors is consistently localised to pm7 and pm8 muscle cells, and inconsistently in other muscle cells of the pharynx. The receptor expression is driven by the *myo-2* promoter, which should result in protein production in all muscle cells of the pharynx (Altun and Hall 2009b), however selectivity has been observed previously (Anna Crisford, personal communication). In the endogenous system, EAT-2 functions at pm4, suggesting eat-2 promoter should be used to avoid restriction of endogenous promoter expression.

Chapter 7

General discussion

7.1 Environmental levels of neonicotinoids do not impact on the behaviour or development of *C. elegans*

Increased use of insecticides requires a better understanding of their environmental impact. Therefore, the initial aim of this project was to investigate the effects of neonicotinoid-insecticides on the Nematoda representative *C. elegans*. Neonicotinoids have been introduced to the market in the 1990s and since then have become the most commonly used insecticides worldwide (Jeschke et al. 2011). They have many advantages, including a high potency against a wide range of pest insects (Section 1.1.5) and low mammalian toxicity. However, neonicotinoids can have significant field effects on non-target species. The adverse effects of environmental neonicotinoids on bees have been studied for decades (Section 1.1.7.1), whereas the effects on other ecologically important species is less understood.

There are several forms in which neonicotinoids are delivered onto fields including spray and granules, but seed-coating is the most common method (Jeschke et al. 2011). This focused application ensures the presence of drug inside the plant at effective concentrations (Stamm et al. 2016), nevertheless neonicotinoids have a long half-life and leeching potential, therefore can reside in soil for prolonged time periods, coming in contact with inhabiting organisms. This leads to potential exposure to soil nematodes and earth worms, two important cultivars of the biomass and nutrient cyclers, that contribute to the soil fertility (Ingham et al. 1985; Neher 2001). The lethal dose of neonicotinoids on earths worms and nematodes varies between 2.74 and 62.08 μM (Table 1.3), however neonicotinoids at lower doses can induce sublethal effects (Section 1.1.7.2). The investigations into their effects on the nematode representative and model organism *C. elegans* were carried out using thiacloprid, clothianidin and nitenpyram.

The effects of these three compounds on locomotion in liquid and solid media, egg-laying and egg-hatching of wild-type worm were investigated (Chapter 3 and 4). This study reports low potency of neonicotinoids against

wild-type *C. elegans* (Kudelska et al. 2017). Neonicotinoids were either not effective, or effective at mM concentrations.

These data suggests that neonicotinoids have minimal, if any, effect on locomotion, reproduction or feeding of *C. elegans*, however it is possible that they affect more intricate aspects of worm's physiology. They impact learning and plasticity in honeybees (Williamson and Wright 2013), therefore efforts into determining their effects on the ability to perceive and process information could be made. For example, decision making could be tested utilising food leaving (Shtonda and Avery 2006) or chemotaxis assays (Law, Nuttley, and Kooy 2004), whereas learning and memory, using olfactory associative assays (Kauffman et al. 2010).

Exposure of *C. elegans* mutant with increased cuticle permeability (Xiong, Pears, and Woollard 2017) resulted in increased sensitivity of *C. elegans* to tested compounds. Ten-fold increase in the potency of nitenpyram was noted, as indicated by the shift in the EC_{50} in the thrashing assay. Thiacloprid and clothianidin did not affect a wild-type strain, but inhibited locomotion with an EC_{50} of 337.6 μM and 3.5 mM, respectively (Section 3.2.3). The nematode cuticle is the major route of entry for many drugs, therefore the ability of compounds to cross the cuticular barrier often defines their potency (Alvarez, Mottier, and Lanusse 2007). The cuticle limits bioavailability of many compound used in agriculture (Xiong, Pears, and Woollard 2017) and by doing so it protects the worm against their potentially harmful effects.

The cuticle encapsulates the body of nematodes as well as earthworms. Their structures have several similarities, as revealed by the electron microscopy of redworm *L. terrestris*, greenworm *Allolobophora chlorotica* (Reed and Rudall 1948; Coggeshall 1966; Knapp and Mill 1971) and *C. elegans* preparations (Cox, Kusch, and Edgar 1981). The main components of the cuticle are the collagen fibrils embedded within the matrix. This relatively thick layer is covered by a much thinner epicuticle, which consists mainly of lipids. In earthworms, the epicuticle is perforated by a coat of outward projections, shorter ellipsoidal bodies and longer microvilli. In *C. elegans* the surface coat consists of the glycoprotein-mesh. Based on the architectural and chemical similarities, it is likely that the cuticle of earthworms may also play a role in drug permeability. This is however poorly investigated.

Generally, the concentrations at which neonicotinoids impair on *C. elegans* behaviour are several folds higher than those found in the field (Sanchez-Bayo, Goka, and Hayasaka 2016) and concentrations effective against insect (Goulson 2013). These data suggests *C. elegans* is not impacted by neonicotinoids in the field, however no field-studies are available to confirm this.

The sensitivity of *C. elegans* to neonicotinoids differs from that of parasitic nematodes and earth worms. In *C. elegans* neonicotinoids impact on locomotion at low mM concentrations (Figure 3.6 and 3.12), but have no effects on reproduction (Figure 3.14 and 3.15). In the plant parasitic nematode, neonicotinoids are effective at μM concentrations. The LD_{50} of thiacloprid on the root-knot nematode *M. incognita* is 143.23 μM (Dong et al. 2014), whereas the IC_{50} from the *C. elegans* egg-hatching experiments is 300 μM (Dong et al. 2014, 2017). Earthworms seem to be the most susceptible, with reported toxic lethal doses greater or equal to

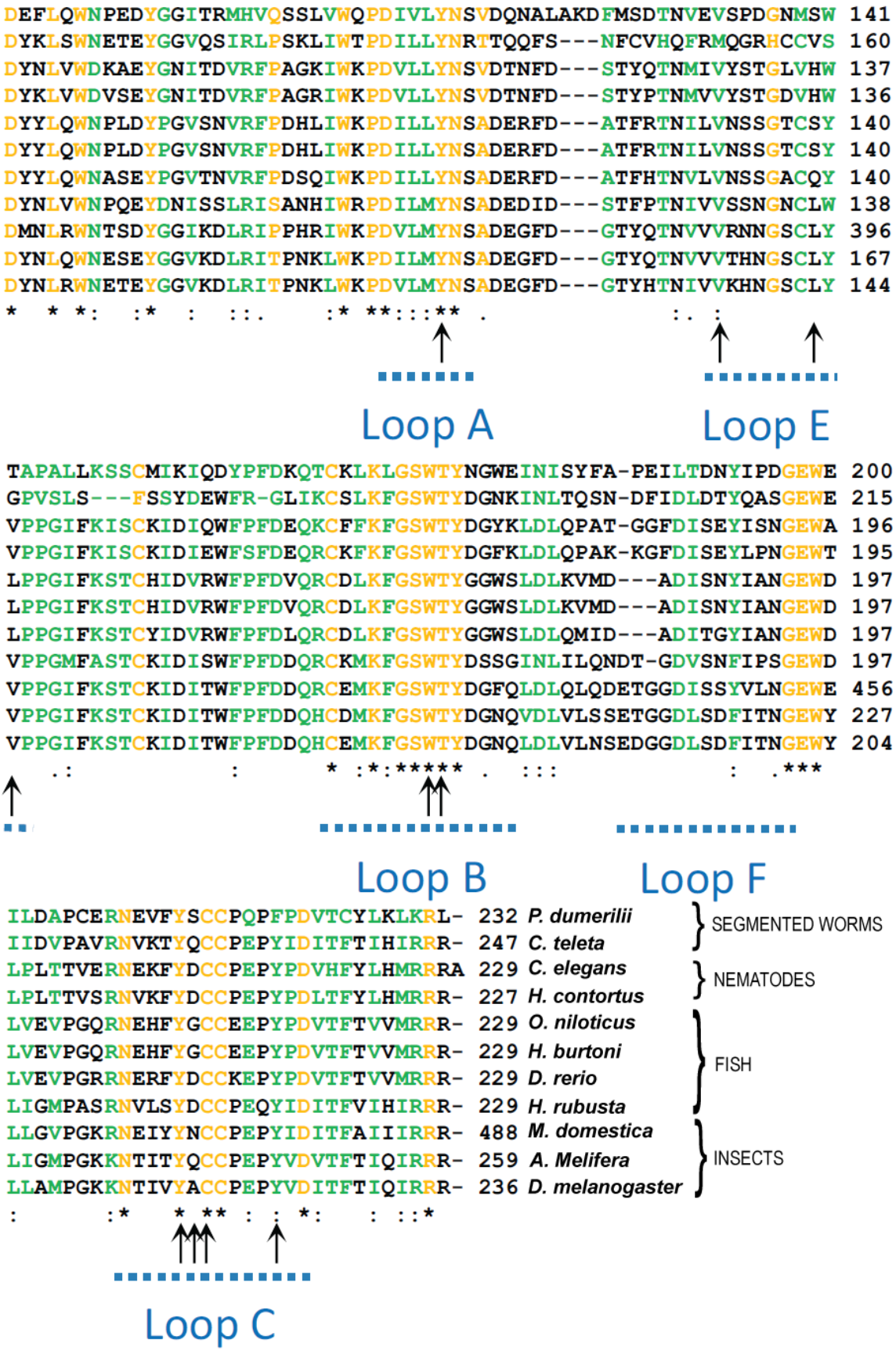


Figure 7.1: **Amino acid sequence alignment of the nAChR ligand binding domains from *C. elegans* and thiacloprid-susceptible species.** Amino acid sequences of nAChRs from representatives species of segmented worms, nematodes, fish and insects. Residues important for thiacloprid binding are indicated with a blue arrow. Figure taken from Hopewell et al. 2017.

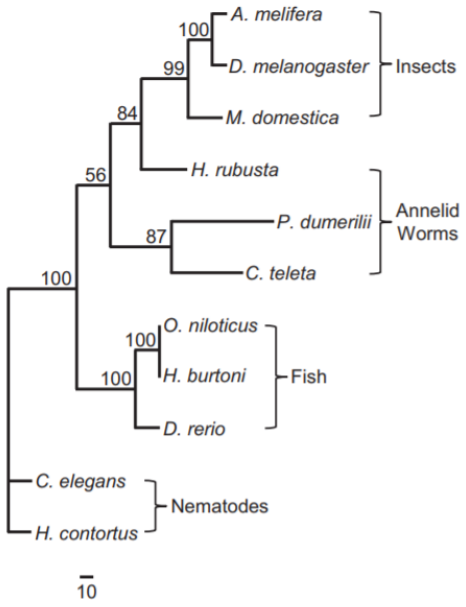


Figure 7.2: **Neighbour joining phylogenetic tree analysis of the amino acid sequences of nAChR ligand binding domains from *C. elegans* and thiacloprid-susceptible species.** To generate the tree, the ligand binding domain of *C. elegans* ACR-16 subunits and homologous sequences from parasitic nematode and representative species of insects, annelid (segmented) worms and fish were selected. Bootstrap values are shown as percentages after 1000 replicates. The scale bar indicates the fraction of substitutions per amino acid. Figure taken from Hopewell et al. 2017.

2.74 μM (Table 13) and doses effective against their reproduction and mobility at 488.85 nM, or higher (Section 1.1.7.2). Thus, there is a differential neonicotinoid-susceptibility between *C. elegans* and parasitic nematodes and between *C. elegans* and earth worm species. The same is true among the insects, where some species are much more susceptible than others (Section 13). Therefore, the sensitivity of each species to neonicotinoids should be considered separately when evaluating the environmental impact of these insecticides. This highlights the complexity and difficulty of the neonicotinoids risk characterisation and usage management.

Can the differential susceptibility to neonicotinoids be predicted based on nAChR sequence analysis and phylogenetic relationships? Comparison of the amino acid sequence of *C. elegans* muscle-type receptor with homologous subunits from neonicotinoid-susceptible species of insects, fish and annelid (segmented) worms revealed amino acids important in thiacloprid binding are highly conserved (Figure 7.1). However, phylogenetically, *C elegans* receptors are more distantly related to insects that receptors from segmented worms and fish (Figure 7.2), which could explain lower susceptibility of *C. elegans* to clothianidin, thiacloprid and nitenpyram in comparison to earthworms. Therefore, phylogenetic relationships between nAChRs from insects and other species might be a useful tool for prediction of neonicotinoid-sensitivity.

7.2 *C. elegans* as a model to study the mode of action of neonicotinoids

The adverse effect of neonicotinoids on biological pollinators and the emerging resistance (Section 1.3.5.1) creates a threat to the food safety and highlights the need for the synthesis of novel and more selective insecticides. One of the first steps towards the synthesis of new insecticides is the understanding of their mode of action (Metcalf 1971). Neonicotinoids act by targeting insect nAChRs (Section 1.3.5), however their mode of action and receptor specificity differs. They can have agonistic, antagonistic and super-agonistic action, depending on the animal preparation upon which they act on, and on the neonicotinoid itself (Section 1.3.6). In addition, even neonicotinoids sharing the same pharmacophore, target distinct receptors (Thany 2009; Moffat et al. 2016). Difficulties in heterologous expression of insect's receptors hiders their pharmacological characterisation and description of neonicotinoid-receptor specificity (Section 1.3.8.0.1).

The model organism *C. elegans* is an alternative system in which the mode of action of chemical agents can be studied *in-vivo* (Lewis et al. 1987; J. A. Lewis, Wu, Berg, et al. 1980). *C. elegans* expresses at least 29 nAChR subunits (Figure 1.9) (Jones et al. 2007) which form receptors at the neuromuscular junction and in the nervous system. Muscle-type receptors are involved in the regulation of locomotion, reproduction and feeding (Section 1.4.7 4.2.3). Neuronal type receptors are expressed in circuits involved in the sensory processing and chemosensation (Yassin et al. 2001). The suitability of the *C. elegans* system for the mode of action studies was investigated by scoring the sensitivity of native pharyngeal nAChRs receptors to neonicotinoids.

The pharynx is a neuromuscular system in which feeding is carried out by a musculature under the influence of the pharyngeal nervous system. In the presence of food, 5-HT is released, which stimulates MC neuron. MC releases acetylcholine which acts directly on EAT-2 containing nAChRs to drive fast pumping. Thus, EAT-2 is a molecular determinant of the fast pharyngeal response. Although EAT-2 is the only nAChR subunit with clearly characterised function, ACR-7 is also expressed in the pharyngeal muscle (Saur et al. 2013).

To determine the effects of neonicotinoids on the pharyngeal nAChRs, dissected head preparations were exposed to clothianidin, thiacloprid and nitenpyram. Their effects on 5-HT stimulated pharyngeal pumping were investigated. Nitenpyram and clothianidin inhibited 5-HT stimulated pumping at 25 mM and 500 μM, respectively (Section 4.2.2.2). The impact of neonicotinoids on unstimulated pharynx was also investigated and revealed no effects of thiacloprid and nitenpyram. In contrast clothianidin stimulated pumping. The lowest dose of clothianidin effective against this *C. elegans* behaviour was 75 μM. These data suggest nAChR expressed in the pharynx have low affinity to neonicotinoids. In contrast, neonicotinoids are effective at nM concentrations on target insect receptors (section 1.3.5.2 and 1.3.6.1) suggesting nAChR pharmacophore present in pest and beneficial insects are distinct from those found in the *C. elegans*.

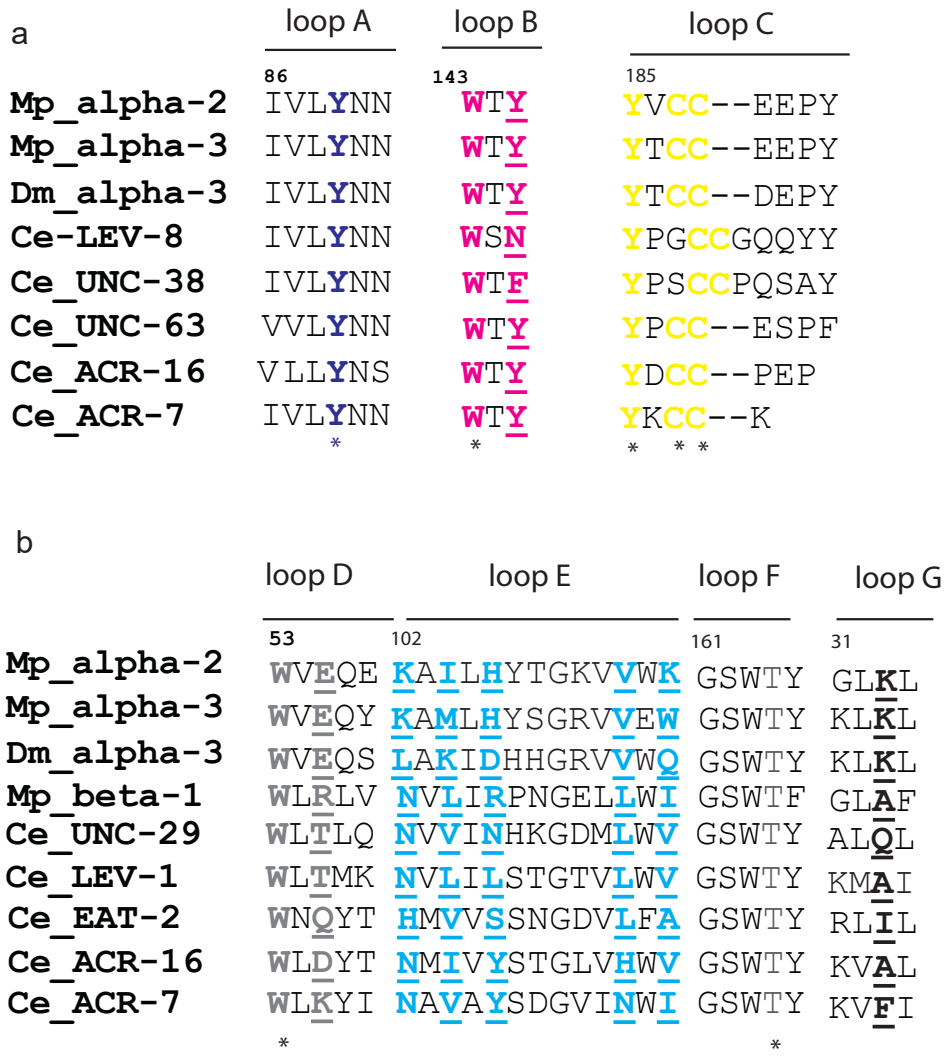


Figure 7.3: **Sequence alignment of the pharmacophore of insect and *C. elegans* nAChR subunits.** Ligand binding pocket is formed from the loops originating from the principal (a) and complementary (b) receptor subunits. Amino acids important in forming drug-receptor interactions are color-coded as in Figure 1.3. Non-conserved residues are underlined. Numbering is according to the AChBP of *Lymnaea stagnalis* (great pond snail) sequence. Mp = *M. persicae* (peach aphid), Dm = *D. melanogaster* (fruit fly), Ce = *C. elegans*. Sequence alignment generated by MUSCLE.

The nAChR pharmacophore consists of the contributions from the α (principal) subunit and non-α (complementary) subunit (Section 1.2.3 and 1.2.4). Residues important in binding of agonists have been identified in mollusc AChBP and nAChR bound to agonists, including nicotine and acetylcholine, thiacloprid, imidacloprid and clothianidin (Celie et al. 2004; Hansen et al. 2005; Ihara et al. 2008, 2014; Talley et al. 2008; Zouridakis et al. 2014; Morales-Perez, Noviello, and Hibbs 2016). The residues identified as those important in binding of agonists were compared between worm and insect nAChRs, by aligning their sequences (Figure 7.3).

The chosen insect subunits are those that form receptor chimeras in the recombinant system and confer high binding affinity to neonicotinoids (i.e Kd below 10 nM, Section 1.3.6.2 and Table 1.5) and β1 subunit, identified as a molecular determinant of neonicotinoid-resistance in *M. persicae* (Bass et al. 2011). Amino acid sequences of these subunits were aligned against nAChR subunits forming functional receptors at the body wall muscle, namely ACR-16 (Ballivet et al. 1996; Boulin et al. 2008 and Section 1.4.8), as well as EAT-2 and ACR-7 which are two subunits identified in the pharyngeal system (McKay et al. 2004; Saur et al. 2013 and Section 1.4.8.1).

A comparison of nAChR binding pocket residues in *C. elegans* and insect receptors identified several differences, in particular in the loops from the complementary site of the binding pocket, loops D, E and G. This might suggest that contributions from the complementary site determine neonicotinoids-selectivity (Marotta et al. 2014, 2014; Hansen et al. 2004; Harpsøe et al. 2013). However, there are receptor subunits, such as ACR-16, in which almost all residues are conserved, implying that susceptibility to neonicotinoids cannot be predicted based on the sequence of nAChR binding pocket, or that residues important in neonicotinoid binding have not been identified correctly and that crystal structures of insect receptors are needed to better understand the interactions between these insecticides and their target.

7.3 *C. elegans* pharynx as a platform for the pharmacological characterisation of nAChRs

Low sensitivity of *C. elegans* to nitenpyram, clothianidin and thiacloprid in behavioural and cellular assays precludes its use as a model to study the mode of action of neonicotinoids per se, but highlights its potential use as a suitable background for the heterologous expression of insect nAChRs. New insecticides are needed to prevent the negative environmental impact of neonicotinoids and combat emerging resistance. The first step towards the synthesis of neonicotinoids with improved selective toxicity profile is the heterologous expression of nAChRs from pests and non-target species in a suitable host, but no heterologous or native insect nAChR complexes have been expressed yet.

Cell lines and *Xenopus* oocytes are routinely used as biological systems for heterologous nAChR expression (Section 6.1.1). Model organism *C. elegans* is an alternative system in which recombinant proteins can be expressed by generation of transgenic worms (Crisford et al. 2011; Sloan et al. 2015). In comparison to other

systems, *C. elegans* is cheap and easy to maintain, whereas transgenic worms can be preserved for years at -80 °C. The function of recombinant nAChRs can be studied *in-vivo* by evaluating their impact on behaviours underpinned by the cholinergic neurotransmission.

Acetylcholine is a major neurotransmitter of the pharynx, released by at least 7 out of 14 neurons (Table 4.1). It is key and necessary for the induction of fast feeding in response to food (Section 4.1.4.3). Its action on the pharynx is mediated by nAChRs, most notably EAT-2 expressed at the NMJ (Section 1.4.8.1). The *C. elegans* pharynx does not possess an innate susceptibility to low concentrations of neonicotinoids, creating an appropriate genetic background in which effects of these compounds on recombinantly expressed receptor can be studied. In addition, it expresses an array of chaperon proteins, creating a favorable environment for the maturation and function of these ion channels (Section 1.4.9). *C. elegans* pharynx has been successfully used for the heterologous expression of non-native proteins (Crisford et al. 2011), including nAChRs (Sloan et al. 2015).

EAT-2 is a single nAChR subunit that confers pharyngeal 5-HT sensitivity and feeding response in *C. elegans* (McKay et al. 2004). We show that *eat-2* mutant is a suitable genetic background, in which the expression of heterologous nAChRs could be scored in behavioural assays. Transgenic line in which *eat-2* is heterologously expressed, rescued the blunted pharyngeal response to food and restored 5-HT resistance of the *eat-2* mutant (Section 6.2.1.3). However, the expression of human $\alpha 7$ nAChR in the *C. elegans* mutant pharynx had no observable phenotypical consequences (Section 6.2.2.4 and Section 6.2.2.5). Similarly, expression of this receptor in the wild-type worm revealed no differences in pharyngeal responses to nAChR agonists nicotine or choline (Section 6.2.3). Staining with fluorescently labelled human $\alpha 7$ nAChR antagonist α-bgtx, revealed increased fluorescence in the pharyngeal muscle of transgenic, when compared to control worms (Section 6.2.3.1). α-bgtx binds to the extracellular domain of the receptor (Dellisanti et al. 2007). This suggests that $\alpha 7$ receptor is expressed on the cell surface of the pharyngeal muscle, however due to the lack of phenotype its function is unclear. Further pharmacological experiments of transgenic strains (as described in the Discussion of Chapter 6) should be carried out to determine whether $\alpha 7$ retains its function upon expression in the pharynx of *C. elegans*.

The expression of $\alpha 7$ was driven by a *myo-2* promoter, which should drive expression in all cells of the pharyngeal musculature (Altun and Hall 2009b). However, α-bgtx staining of transgenic worms was concentrated in pm7 and pm8 muscle cells, suggesting $\alpha 7$ is expressed in the terminal bulb, which does not overlap with the localisation of native EAT-2. Thus, native EAT-2 promoter should be used to ensure correct localisation of the expressed protein.

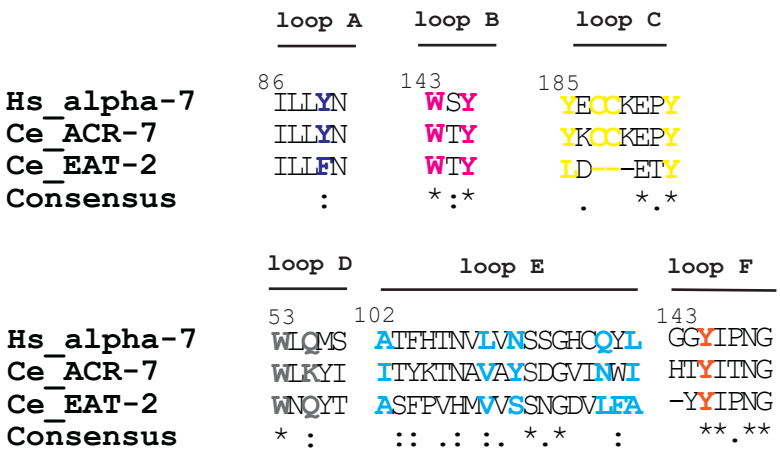


Figure 7.4: **Amino acid sequence alignment of human $\alpha 7$ and two of the pharyngeal *C. elegans* nAChRs ligand binding pockets.** Amino acid sequences forming nAChR binding pocket were aligned. Amino acids important in agonist binding are highlighted, as in Figure 1.3. Ce = *C. elegans*, Hs = human. Sequence alignment generated with MUSCLE.

7.4 *C. elegans* as a model for mammalian toxicity studies

To better understand the pharmacological profile of *C. elegans* nAChRs, the effects of endogenous neurotransmitter acetylcholine as well as agonist nicotine and cytisine were tested on the pharynx. Acetylcholine, nicotine and cytisine were applied on cut-head and the effects on EPG was assayed. All three induced potent and transient stimulation of pumping leading to muscle tetanus (Figure 5.8). Concentrations effective were in the low μM range. The order of potency as measured by the EC_{50} was: nicotine > cytisine > acetylcholine. In comparison to human $\alpha 7$, effective concentrations were in a similar range (Papke and Porter-Papke 2002). In addition, like pharyngeal nAChRs, human $\alpha 7$ receptors are insensitive to low doses of neonicotinoids with the EC_{50} values of 0.74 mM and 0.73 mM, respectively for clothianidin and imidacloprid, on the heterologously expressed channel (Cartereau, Martin, and Thany 2018). This suggests the pharmacophore of human $\alpha 7$ and pharyngeal nAChRs is conserved. This was confirmed by aligning the sequences of key amino acids forming the nAChR binding pocket in $\alpha 7$ and two of the pharyngeal nAChRs (Figure 7.4).

Comparison of human $\alpha 7$ and pharyngeal nAChR subunits EAT-2 and ACR-7 revealed high sequence similarity. Almost all residues forming ligand binding pocket are conserved between these subunits, suggesting pharyngeal nAChR subunits are homologous to human $\alpha 7$. Besides pharyngeal nAChR subunits, homologs of over two thirds of human proteins can be found in *C. elegans* (Sonnhammer and Durbin 1997; Lai et al. 2000). The similarities between mammals and *C. elegans* extends beyond genetics. *C. elegans* has conserved synaptic function, due to the presence of almost all vertebrate neurotransmitters and conserved neuronal signaling pathways (Bargmann 1998; Kaletta and Hengartner 2006). There are however some differences. *C. elegans* expresses inhibitory glutamate-gated chloride channel which is absent in mammals (Cully et al. 1994), but lacks voltage-gated sodium channels which is present in humans (Bargmann 1998). Despite these limitations,

C. elegans emerges as an attractive model for toxicity studies (Hunt 2017).

Methods to use *C. elegans* as a model to study acute toxicity as well as developmental and reproductive toxicology have been developed (Boyd et al. 2010; Xiong, Pears, and Woollard 2017). A good correlation between the rank order of toxicity of many compounds on *C. elegans* and mammals for acute toxicity, growth and reproduction endpoints have been found (Williams and Dusenbery 1988; Boyd et al. 2010). This includes organophosphates, which act by disrupting cholinergic neurotransmission at the synapse (Chadwick and Hill 1947). The rank order of acute toxicity, as measured by LC_{50}, correlated well with the LD_{50} ranking of these agents in rats and mice (Cole, Anderson, and Williams 2004). This highlight the potential suitability of *C. elegans* as a model for toxicity testing of cholinergic and other neurotoxins. Introduction of *C. elegans* into toxicity testing has a potential to reduce the use of conventional mammalian models, resulting in the reduction of cost and duration of such studies (Van Norman 2019).

Appendices

Appendix A

Pharmacophore of the nicotinic acetylcholine receptor

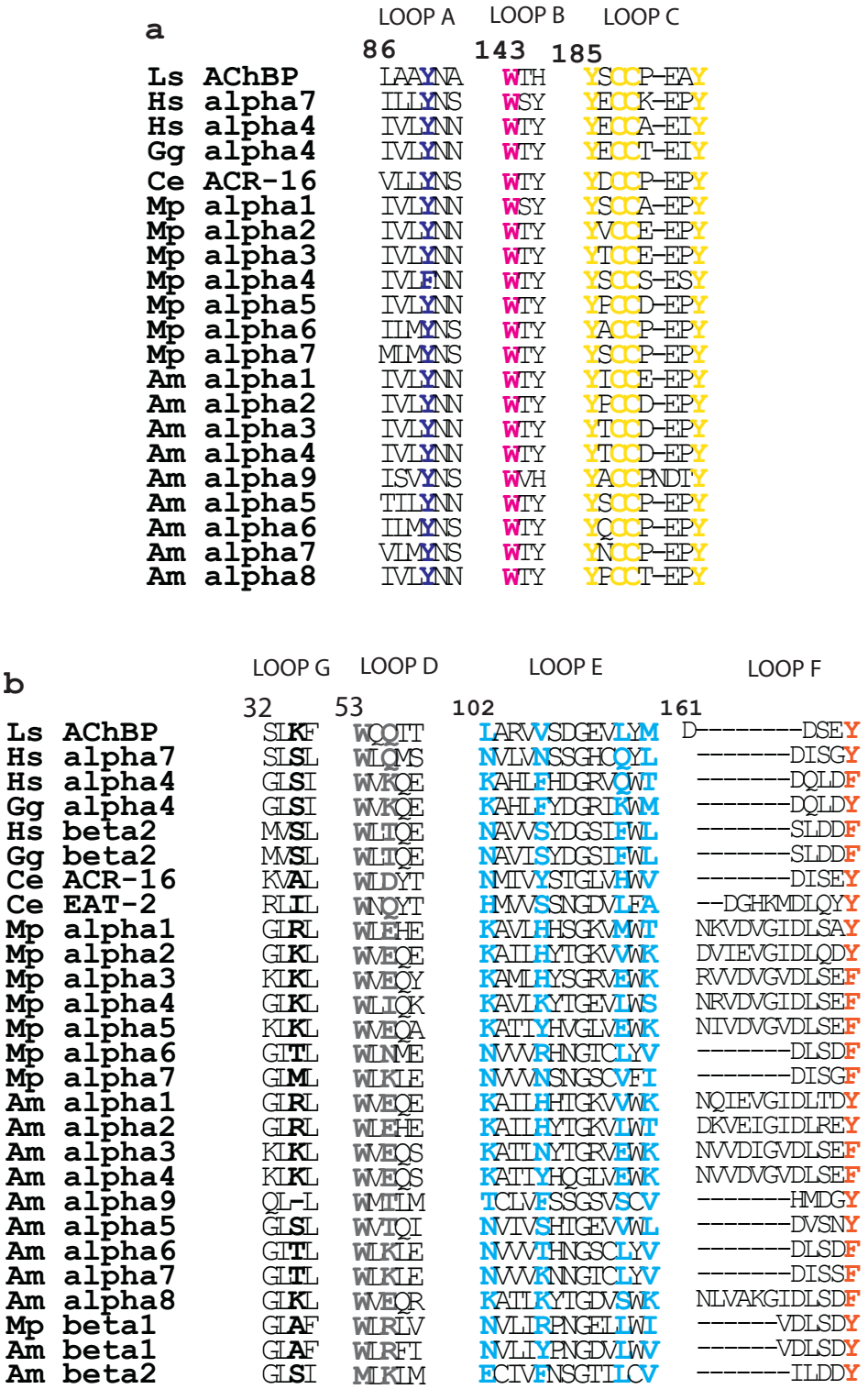
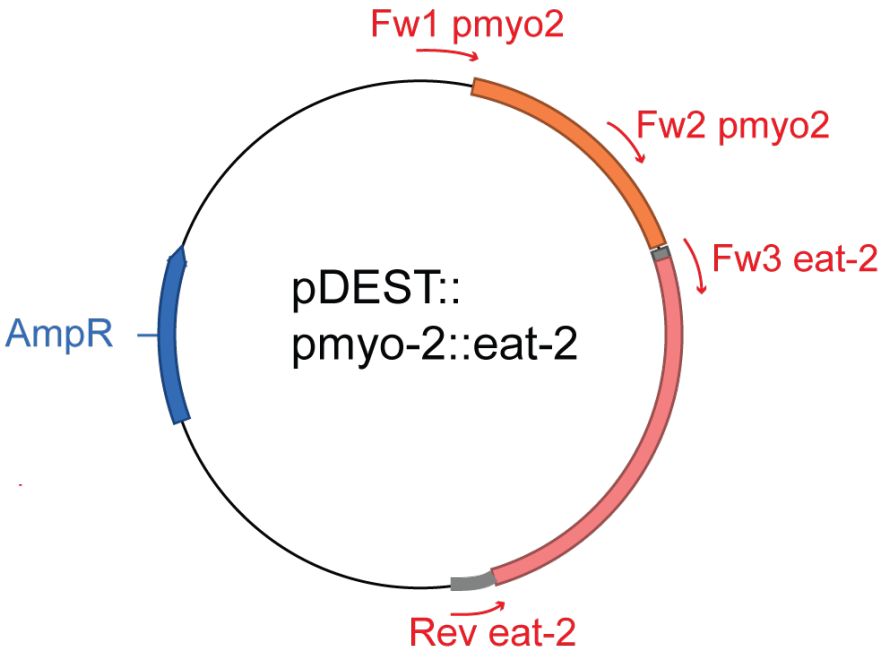


Figure A.1: **Sequence alignment of the binding pocket of the ligand binding protein and nicotinic acetylcholine receptors.** Amino acid sequences from the principal (a) and complementary (b) binding site loops, which form the ligand binding pocket. Residues important for agonist binding are highlighted and color coded as in Figure 1.3. Numbering corresponds to the sequence of the great pond snail acetylcholine binding protein (AChBP). Alignment was generated with MUSCLE (Edgar 2004). Abbreviations used: Ls - *Lymnaela stagnalis* (great pond snail), Am- *Apis mellifera* (honeybee), Mz- *Myzus persicae* (peach aphid), Hs- *Homo sapiens* (human), Gg- *Gallus gallus* (chicken), Ce - *C. elegans*.

Appendix B

DNA sequence used for the expression of *eat-2* in the pharyngeal muscle of *C. elegans*.

a)



b)

Fw1 pmyo2	GGCTTTACACTTTATGCTTCCGG
Fw2 pmyo2	GGCAGGAAGAGCACTTTGCGCGC
Fw3 eat-2	CCCACCGAGCGGTTGACTTCTCTCC
Rev eat-2	CGAGATGGCGATCTGATGACAGCGGC

c)

ataacaatttcacacaggaaacagctatgaccatgattacgccaagcttgcatgcctgcaggtcgaggcatttga < 75

attgggggtggtggacagtaactgtctgtaataataattactcctgaccaggttgcaattcgagttttgataagc < 150

ataattataccttgtacattgtgggttttgtgctgtggacgttttattgtgacatccccataagctacaagaaca < 225

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aaactaattttataatcaataaaacaacgtttgtaaatcaactgagtttacaagtagagacattgagggatactt < 375

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>eat-2

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>AttB2

tattgaataaaagggcgaattcgacccagctttcttgtacaaagtggtgata < 2752

d

```
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YKLRWSPEEYGNITTLQIPHGTLWKPDILLFNSANEHFDASFPVHMVVSSNGDVLFAPPGIVSFSCSLSMTWFPYDQQVCYLK
FGSWTYGKKLDLQIDDSDLPDGHKMDLQYYIPNGEFDLLATPAFRKSTTFLDETYVELYFHMHLKRRTMYYGLNWIVPSILIS
LSNILGFTMPPECGEKITLQITNFLSVMVFLAMVSEVAPPTSESIPIIAAFFSLSIVILGLSICASLIIVNIFFRHPKTHRMG
DWTRYVFLEWLPWFLLMSRPEHTFCRPRREEEKNDEEAGGDGTKLLENQQHQPRPRLLVNSQLVMDSTVPYLEEIIGYLKVFK
AKLDDDEEEEEEILNWRFMAMVIDRLSLFLFTGLIFGTTALIFAFCPNLFTDSPIVDIE*
```

Figure B.1: **Sequencing of *myo-2-eat-2* from the *pDEST* vector.** *myo-2::eat-2* nucleotide fragment from the expression vector used to generate *C. elegans* transgenic strains was sequenced following cloning. 3 forward (Fw) and a reverse (Rev) primers were used to generate overlapping sequencing fragments spanning the entire sequence of interest (a). Sequencing results authenticated the identity of the construct (b) and confirmed the amino acid sequence of the *eat-2* gene.

Appendix C

DNA sequence used for the expression of human $\alpha 7$ in the pharyngeal muscle of *C. elegans*.

ataacaatttcacacaggaaacagctatgaccatgattacgccaagcttgcatgcctgcaggtcgaggcatttga < 75

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ataattataccttgtacattgtgggttttgtgctgtggacgttttattgtgacatccccataagctacaagaaca < 225

aaaatgaaatgtaaaagtattgaaaacgtcgtacattttatatctgagtagtatcctttgctttaaatgtccata < 300

aaactaatttttataatcaataaaacaacgtttgtaaatcaactgagtttacaagtagagacattgagggatactt < 375

tcactatgctaaagtgataatcgaccaaataataacttcactttggtatttattcctgtcttataaatgttatgt < 450

atgaattaaattcatatgcatatggctcactctgacaatttttaataatcttccagatcaatattgactaccgat < 525

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>pmyo-3
|
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aataaagataagtctcaagataggttggtaatcgctaaagtggttgtgtggataagagtagcaaaatggcaggaa < 750

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gtccaaagactttgcgtaaaagggcgaattcgacccagctttcttgtacaaagtggtgata < 2836

b

MRCSPGGVWLALAASLLHVSLQGEFQRKLYKELVKNYNPLERPVANDSQPLTVYFSLSLLQI
MDVDEKNQVLTTNIWLQMSWTDHYLQWNVSEYPGVKTVRFPDGQIWKPDILLYNSADERFDA
TFHTNVLVNSSGHCQYLPPGIFKSSCYIDVRWFPFDVQHCKLKFGSWSYGGWSLDLQMQEAD
ISGYIPNGEWDLVGIPGKRSERFYECCKEPYPDVTFTVTMRRRTLYYGLNLLIPCVLISALA
LLVFLLPADSGEKISLGITVLLSLTVFMLLVAEIMPATSDSVPLIAQYFASTMIIVGLSVVV
TVIVLQYHHHDPDGGKMPKWTRVILLNWCAWFLRMKRPGEDKVRPACQHKQRRCSLASVEMS
AVAPPPASNGNLLYIGFRGLDGVHCVPTPDSGVVCGRMACSPTHDEHLLHGGQPPEGDPDLA
KILEEVRYIANRFRCQDESEAVCSEWKFAACVVDRLCLMAFSVFTIICTIGILMSAPNFVEA
VSKDFA*

Figure C.1: **Sequencing of *pmyo2-CHRNA7* from the *pDEST* expression vector.** *myo-2::CHRNA7* nucleotide fragment from the expression vector used to generate *C. elegans* transgenic strains was sequenced following cloning. Primers were used to generate overlapping sequencing fragments spanning the entire sequence of interest (a). Sequencing results authenticated the identity of the construct and confirmed the amino acid sequence of the *CHRNA7* gene (b).

Appendix D

Expression of the nicotinic acetylcholine receptor extracellular domain in *Escherichia coli*

D.1 Introduction

nAChRs are the molecular targets of neonicotinoid insecticides. The adverse effects of neonicotinoids on non-target insects led to the restricted use and an eventual ban of these chemicals in the EU (The European Commission 2013), which highlights the need for the synthesis of more selective compounds. The first step towards the generation of compounds effective on pest and not beneficial insects, is the understanding of the interactions between the neonicotinoid and their targets based on the knowledge of the agonist binding site structure.

D.1.1 Structural basis of acetylcholine and agonist binding

The neonicotinoid-pharmacophore was studies from the crystal structure of the homologue of nAChR extracellular domain (ECD) mollusc AChBP bound to imidacloprid (Ihara et al. 2008; Talley et al. 2008) and genetic analysis of the insect resistant strains (Liu et al. 2005). The key determinants for acetylcholine-binding are also well known (Section 1.2.4). Neonicotinoids and acetylcholine forms similar interactions with the conserved residues in the binding pocket (Section 1.2.5): cation-π interactions with the aromatic amino acids, hydrogen bonds via water molecule and Van der Waals interactions with loop E residues - these are common to interactions made with other agonists of nAChRs. However, there are also some proposed unique interactions between imidacloprid and AChBP, such as a hydrogen bond with Gln 55 of loop D. This Gln is not conserved and corresponds to basic residue in many insect nAChR subunits, thus the unique interaction may be present in some, but not all insect receptors. The importance of this residue was highlighted in the genetic analysis of naturally occurring resistant strains of *Myzus Persicae* (Bass et al. 2011).

Although AChBP is a useful model to study nAChR-drug interactions, as indicated above, there are differences in the amino acid sequence in the ligand binding pocket of AChBP and nAChR (Appendix A), including those that appear as key determinants of drug sensitivity. Therefore, a high resolution structure of nAChRs are needed to fully understand the underpinnings of selectivity of neonicotinoids and the chemical space in which improved selectivity might be achieved. Structures of $\alpha4\beta2$ (Morales-Perez, Noviello, and Hibbs 2016) and nAChR extracellular domain (ECD) of $\alpha2$ (Kouvatsos et al. 2016), mouse $\alpha1$ (Dellisanti et al. 2007) human $\alpha9$ (Zouridakis et al. 2014) are known. Structures of nAChR extracellular domain bacterial homolog GLIC (Hilf and Dutzler 2009) and ELIC (Hilf and Dutzler 2008) as well as pentameric ion channels GABA (Miller and Aricescu 2014) and glycine (Huang et al. 2015) have also been described. However, the crystal structure of insect receptors is not available. This may be caused by the difficulties in expression and purification of folded and soluble recombinant proteins (Rosano and Ceccarelli 2014), which are essential and first steps towards the structural characterisation.

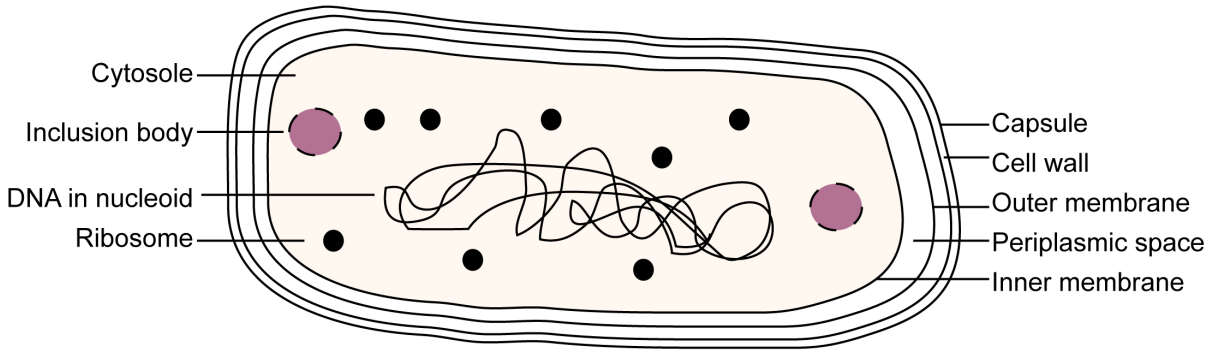


Figure D.1: **The *E. coli* cell.** *E. coli* is a Gram negative bacteria shielded by a capsule and encapsulated by 2 layers of the membrane separated by the periplasmic space. Main organelles in the cytosol are nucleoid containing the genetic material, inclusion bodies typically containing protein aggregates and ribosomes for protein translation.

D.1.2 Biological systems for recombinant protein expression

The requirements for the structural analysis of any protein is the high level expression of stable and correctly folded product (Rosano and Ceccarelli 2014). There is an array of biological systems used for the recombinant protein expression, however there is no single system that subserves as the single route to the best production of all proteins. This often means a trial and error approach, until the right system is identified. Some of the commonly used systems are: isolated cell lines of mammals or insects, and whole organisms such as yeast, fungi or algae. *E. coli* is one of the most commonly used host organisms for heterologous soluble protein expression (Berman et al. 2000) due to its simplicity, low cost, relatively good knowledge of transcription, translation and protein folding processes as well as ease of manipulation. According to Protein Data Bank, almost 75 % of all structures have been derived from proteins expressed in *E. coli* (Berman et al. 2000).

D.1.3 Strategies used to express pentameric ligand gated ion channels in *Escherichia coli (E. coli)*.

Despite the intrinsic difficulties of expressing multimeric and membrane proteins in *E. coli*, they have been used to express and purify high quantity of folded nAChRs and related proteins. This includes AChBP (Abraham et al. 2016), ELIC (Nys et al. 2016; Hilf and Dutzler 2008), full length human $\alpha 7$ nAChRs (Tillman et al. 2016), and the ECD of the rat $\alpha 7$ (Fischer et al. 2001). Strategies employed in these studies highlight ways of overcoming major difficulties faced when trying to express recombinant proteins in *E. coli*.

Eukaryotic secretory and membrane proteins are targeted to ER and Golgi where they undergo a process of maturation, before being sent to their correct localisations. As part of the maturation process, vast number of these proteins undergo a process of post-transcriptional modification (Khoury, Baliban, and Floudas 2011), such as addition of carbohydrates (glycosylation) or formation of disulphide bonds. These modifications can contribute to the stability of the protein (Xu and Ng 2015). The *E. coli* secretory system is much simpler,

therefore the nature, the frequency and the mechanism of post-translational modifications differ from those in eukaryotic cells (Dell et al. 2010). There are a number of strategies employed to overcome this problem. For example, proteins requiring formation of disulphide bonds can be targeted to the periplasmic space of the *E. coli*, where this process can take place, due to the presence of active alkaline phosphatases. To enable formation of disulphide bonds of recombinant human nAChR, $\alpha 7$ was targeted to the periplasmic space of *E. coli* with the pelB sequence (Tillman et al. 2016).

Targeting to the periplasmic space may also have other advantages. Periplasmic environment is less crowded due to reduced number of secreted proteins, in comparison to the cytoplasm, therefore the likelihood of proteolysis of the recombinant protein is reduced (Makrides 1996). This may lead to potential increased stability of proteins. Expression in the periplasm may be advantageous for some proteins, even those that do not require formation of disulphide bonds, such as ELIC (Hilf and Dutzler 2008; Nys et al. 2016).

One of the major obstacles when producing a recombinant protein in *E. coli* is it that many proteins tend to form insoluble aggregates (Peternel and Komel 2011). This issue can be addressed by a number of approaches. First, recombinant protein can be tagged by another protein, native to the biological host. Maltose binding protein (MBP) is an *E. coli* periplasmic protein (Bedouelle 1983) which can increase the solubility and stability of recombinant proteins expressed in both cytoplasmic and periplasmic space (Raran-Kurussi, Keefe, and Waugh 2015) and including those rich in disulphide bonds (Planson et al. 2003). MBP fusion strategy was employed by Fischer et al. (2001) to express $\alpha 7$ ECD and Hilf and Dutzler (2008) to express ELIC. Another way to increase solubility is to to produce a fragment and not a full length of the protein. This is a common approach employed when studying the soluble domains of transmembrane proteins, such as ECD containing the agonist-binding pocket of nAChRs. ECD is soluble, therefore it may be easier to successfully express and purify than the full-lent protein containing hydrophobic sections. Importantly, ECDs can form pentameric assemblies and folded binding sites (Kouvatsos et al. 2016; Dellisanti et al. 2007). Another approach is to modify the genetic code, to introduce amino acid mutations, some of which may favour expression. Amino acid mutations can increase solubility and improve expression by as as much as 264-fold (Dale et al. 1994). This strategy was successfully employed for the expression of $\alpha 7$ ECD (Tsetlin et al. 2002). There is also another version of the $\alpha 7$ ECD containing greater alterations generated by Zouridakis et al. (2009) which is also characterised by improved solubility in *E. coli* (Figure D.2).

Summarising, there is no universal protocol for the expression of folded and stable recombinant proteins particularly in the challenging subdomain of the membrane protein that derives from proteins with complex quaternary (pentameric) structure. Therefore, it is a common practice to try several approaches, optimising the *E. coli* growth and expression conditions along the process. Years of research developed the use of solubility enhancers, targeting signalling sequences and other approaches to allow for the expression of complex molecules, such as nAChRs and bacterial structurally related proteins. For example, to express and purified ELIC from *E. coli*, Hilf and Dutzler (2008) expressed ELIC tagged by MBP and targeted it to the

```
Aplysia AChBP   AHSQANLMRLKSDLFN--RSPMYPGPTKDDPLTVTLGFTLQDIV        58
WT Hu-a7        GEF---QRKLYKELVKNYNPLERPVANDSQPLTVYFSLSLLQIM        41
mut10           GEY---QRKLYKELVKNYNPLERPVANDSQPLTVYFSLSLLQIM        41
Hu-a7*          --F---QRKLYKELVKNYNPLERPVANDSQPLTVYFSLSLLQIM        39
Apis-a5*        --G---EYRLTKYLLDGYDAGVRPAENSSQPLAVVFGLSLHHII        39
Cel-ACR21*      ------GERLYRDLLYDYNNEVRPSVHSKEPINVTFVFSLTQII        38
                       :*   *.        *   ..:*: * : ::* .*:

Aplysia AChBP   KADSSTNEVDLVYYEQQRWKLNSLMWDPNEYGNITDFRTSAADIWTPDITAYSSTRPVQV 118
WT Hu-a7        DVDEKNQVLTTNIWLQMSWTDHYLQWNVSEYPGVKTVRFPDGQIWKPDILLYNSADERFD 101
mut10           DVDEKNQVLTTNIWLQMSWTDHYLQWNTSEYPGVKTVRFPDGQIWKPDILLYNSADERFD 101
Hu-a7*          DVDEKNQVLTTNIWLQMSWTDHYLQWNVSEYPGVKTVRFPDGQIWKPDILLYNSADERFD 99
Apis-a5*        DVDEKNQILTTNCWVTQIWTDHHLKWNASEFAGIRVIRVPYNRVWRPDTILYNNADPQYS 99
Cel-ACR21*      DVDERNQILTTNSWIRLHWVDYKLVWDPRLYQNVTRIHIPSDKIWKPDIILYNNADAQYM 98
                ..*. .: :    :    *    * *:   : .:  .:     :* **   *..:

Aplysia AChBP   --LSPQIAVVTHDGSVMFIPAQRLSFMCDPTG-VDSEEGATCAVKFGSWVYSGFEIDLKT 175
WT Hu-a7        -ATFHTNVLVNSSGHCQYLPPGIFKSSCYIDVRWFPFDVQHCKLKFGSWSYGGWSLDLQM 160
mut10           -ATFHTNVLVNSSGHSQYLPPGIFKSSCDVSC-VDTESGATCKLKFGSWSYGGWSLDLQM 159
Hu-a7*          -ATFHTNVLVNSSGHCQYLPPGIFKSSCDPTG-VDSEEGATCKLKFGSWSYGGWSLDLQM 157
Apis-a5*        SAVINTNVIVSHTGEVVWLSHGIFRSSCDPTG-VDSEEGATCVLKWASWTYDGYQLELEK 158
Cel-ACR21*      KSVMSTDVIVDYLGNIHWPLSAIFTSSCDPTG-VDSEEGATCILKYASWAYDGTKIDLLL 157
                       .:*   *   :    :   *         .   * :*:.** *.* .::*

Aplysia AChBP   DTDQVDLSSYYASSKYEILSATQTRQVQHYSCCPEPYIDVNLVVKFRER   235
WT Hu-a7        --QEADISGYIPNGEWDLVGIPGKRSERFYECCKEPYPDVTFTVTMRRR   209
mut10           --QEADTSGYIPNGEWDLTGIPGKRSERYYECCKEPYPDVTFTVTMRRR   208
Hu-a7*          --QEADISGYIPNGEWDLVGIPGKRSERFYECCKEPYPDVTFTVTMRRR   204
Apis-a5*        QSEQGDTSNYQANGEFDLTNFSARRNVEYYSCCPEPYPDITYEIRLRRR   207
Cel-ACR21*      KSEQGDTTNYITNTEWSTIGIRAEKNQVYYSCCPEPYPFIDVHVTIERR   206
                  :: * :.*  . ::.  .    :.  .*.** ***  :   : :..*
```

Figure D.2: **Sequences of the extracellular domain (ECD) nAChR variant with increased solubility.** Sequences of ECD used in this study: human $\alpha 7$ (hu a7), honey bee $\alpha 5$ (Ap a5) and *C. elegans* ACR-21 (Cel ACR-21) have been mutated (residues in red) based on the mutant version of $\alpha 7$ (mut-10) (Zouridakis et al. (2009)). In addition, Cys-loop of the honey bee and *C. elegans* subunits have been replaced for the more soluble Cys-loop sequence of *Aplysia* AChBP.

periplasmic space by pelB. pelB is native to *Erwinia carotovora* (Lei et al. 1987). It is a 22-amino acid sequence: *MKY*LLPTAAAGLLLLAAQ**PAMA**. As many other signal sequences, it contains 3 basic residues at the N-terminus (in italic), a string of hydrophobic amino acids, and a cleavage site (in bold) (Perlman and Halvorson 1983). The cleavage site is recognisable by the membrane bound signal peptidase (Pugsley 1993), releasing the downstream peptide into the periplasmic space. Although not native to *E. coli*, pelB directs tagged proteins to the periplasm of this bacterium (Yoon, Kim, and Kim 2010), via the Sec translocation pathway. Despite all these advances, the successful expression of nAChRs in *E. coli* has been achieved only in a handful of cases. The inability to produce high quantity and quality of nAChRs, hinders their structural analysis and understanding of the molecular basis of selectivity of important agricultural compounds, such as neonicotinoids.

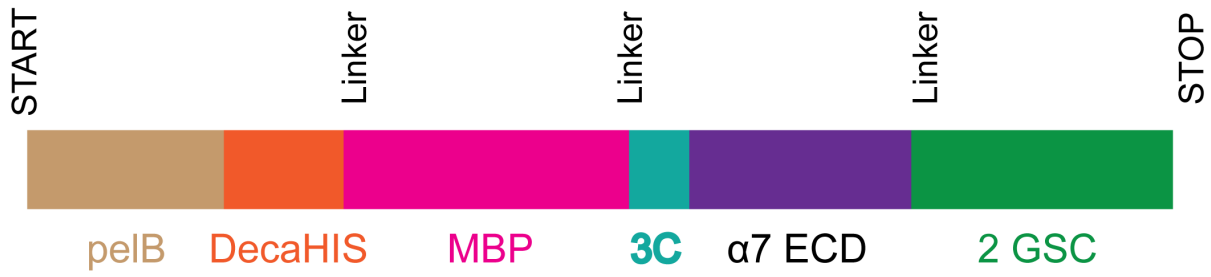


Figure D.3: **Schematic diagram of the DNA construct used for the expression of $\alpha 7$ ECD in *E. coli*.** This is more text that belongs here.

D.1.4 Chapter aims

The aim of this chapter is to develop an *E. coli* based expression platform for insect nAChRs to enable characterisation of the ligand binding site and determination of structural features underpinning their interactions with neonicotinoids.

First, the expression and purification of human $\alpha 7$ was initiated as a test bed. This receptor was chosen because it forms homopentameric receptors in which the recombinant expression of a single subunit can potentially drive functional expression of the ECD.

To enable expression of proteins in *E. coli* cells, necessary DNA elements were cloned into the expression vector (Figure D.3). These elements are the START codon, pelB, sequence encoding for a string of 10 histidines (DecaHIS), maltose binding protein (MBP) and cleavage site 3C.

START codon initiates protein translation, pelB targets protein to the periplasm, DecaHIS and/or MBP enable purification with a nickel or dextrin chromatography column, respectively. MBP also acts as a solubility enhancer, whereas 3C is cleavage site enabling removal of pelB-HIS-MBP-3C from downstream peptide upon treatment with an appropriate protease. Downstream of these elements sequence encoding for the $\alpha 7$ extracellular domain was cloned. There are several reasons why the expression of the ligand binding domain without the transmembrane domain was carried out. First, this study is concerned with the structure of the ligand binding site, which is contained in the ECD domain of the receptor. ECD is potentially soluble, therefore easier to successfully express and purify than the full-length protein containing hydrophobic sections. Although ECDs can form pentameric channels, the sequences within the TM region of the receptor may be also important in the process of receptor assembly (Wang et al. 1996). To account for this, the ECD was flanked by sequence encoding for 2GSC - a single subunit of a pentameric protein.

2GSC is a cytosolic protein endogenous to Gram-negative bacteria *Xanthomonas campestris*. Its structure was derived by X-ray crystallography, following the expression in and purification from *E. coli* (Figure D.4) (Lin et al. 2006). 2GSC is a four-helical protein assembling into pentameric bundles. The overall architecture is similar to that of the nAChR membrane spanning domain (Figure D.4), however, 2 GSC is soluble. It is therefore

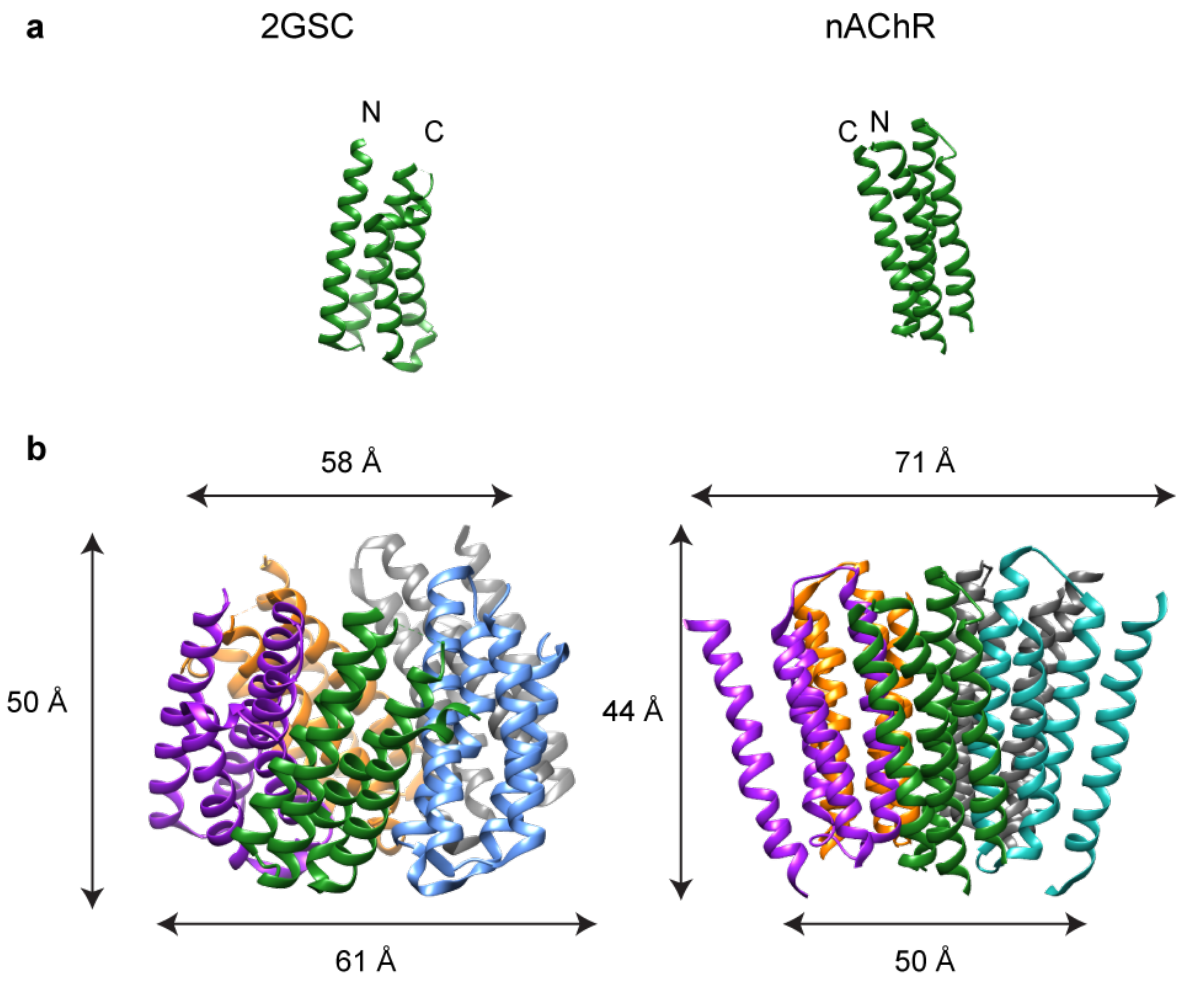


Figure D.4: **Comparison of the pentameric soluble bacterial protein with transmembrane domain of the nicotinic acetylcholine receptor.** 2GSC is a 4-helical protein, assembling into a pentameric bundle (a). The general architecture and dimensions closely reassemble those of the nAChR transmembrane domain (b). Images and dimensions were derived in UCSA Chimera (PDB codes:2GSC and 2BG9 for muscle nAChR). Distances were derived by calculating distances from the most distal atoms on the polar ends of the structures.

hypothesised that the oligomerisation of the 2GSC could aid assemble of pentameric ECD of nAChRs.

It needs to be noticed that although this chapter describes the expression of human $\alpha 7$ receptor, the expression of several other genes have been tested.

Two further ECDs, namely honey bee $\alpha 5$ and *C. elegans* ACR-21 subunit were cloned. Their expression was driven from plasmids containing 2GSC, as well as two other proteins: 1VR4 and 2GUV (Appendix E). 1VR4 and 2GUV are bacterial proteins of unknown function, shown to form pentamers in *E. coli*. Out of 9 constructs tested, the results from the $\alpha 7$ were the most promising.

D.2 Results

D.2.1 Generation of the vector for the expression of human $\alpha 7$ nAChR in *E. coli* periplasm.

The expression vector was generated in a two-step reaction. First, *pelB-HIS-MBP-3C* was cloned, followed by the *α7-2GSC*. For simplicity, *pelB-HIS-MBP-3C* sequence will be refereed to as MBP-3C.

MBP-3C was PCR amplified (Figure D.5, Table 2.8) with *SalI*, *NdeI* flanking primers (Table 2.3) from the vector used for expression of ELIC (Hilf and Dutzler 2008). Purified PCR product and the destination *pET27* vectors were sequentially digested with *SalI* and *NdeI* restriction enzymes to enable ligation (Figure D.5). Ligation and colony selections were performed to generate *pET27-pelB-HIS-3C* (*pET27-MBP-3C* for short), suitable for expression of proteins in the periplasm. The success of cloning was provisionally indicated by *NdeI* and *SalI* digestion to produce the backbone and insert DNA fragments of purified plasmid. The positive clones were amplified and sequenced using primers flanking the insert. A single nucleotide mutation (C substituted by A) occurred between the *pelB* and *DecaHIS*, but this conservative mutation (GCC to GCA codon; both encode for alanine (Appendix F).

α7-ECD-2GSC was cloned into the *pET27-MBP-3C* expression vector (Figure D.6). *pBMH* plasmid containing coding *α7-ECD-2GSC* sequence was synthesised. The *Huα7-2GSC* gene was PCR amplified with primers containing non-complementary sequences containing *SalI* and *NheI* restriction sites (Table 2.3 and Table 2.9). PCR product and *pET27-MBP-3C* were sequentially digested with *SalI* and *NheI* restriction enzymes. Purified DNA fragments were ligated, colonies selected and DNA purified. DNA was then analytically digested with *SalI* and *NheI* as well as send for sequencing. Cloned DNA sequence was error free (Appendix G). The generated *pET27-MBP-3C-α7-ECD-2GSC* vector was used for the expression of $\alpha 7$-ECD.

D.2.2 Expression of $\alpha 7$ chimera in *E. coli*

The *pET27-pelB-3C-α7-ECD-2GSC* was used to express the chimera protein in *E. coli* cells. To enable protein expression, *E. coli* cells were transformed with the expression plasmid and grown in the presence of antibiotic kanamycin. Bacteria were grown in LB growth media, which contains nutrient to support bacterial growth. The conditions at which bacteria are grown can be modified to optimize protein expression (PhD thesis of Ben Yarnall, data not shown). The factors were investigated with respect to the induced expression of $\alpha 7$ ECD chimera. Transformed *E. coli* culture was grown at 37°C until $OD_{600nm} = 1.0$ before addition of 0.5 mM IPTG for 6 hours. This method allowed for high levels of protein expression over a short period of time.

In parallel, culture was grown at 37°C until it reached the exponentially growing phase ($OD_{600nm} = 0.6$). At that point, the temperature was lowered to 18°C, and the growth allowed to proceeded until $OD_{600nm} = 1.0$. At this point, 0.2 mM IPTG was added and the cultures incubated overnight at 18°C.

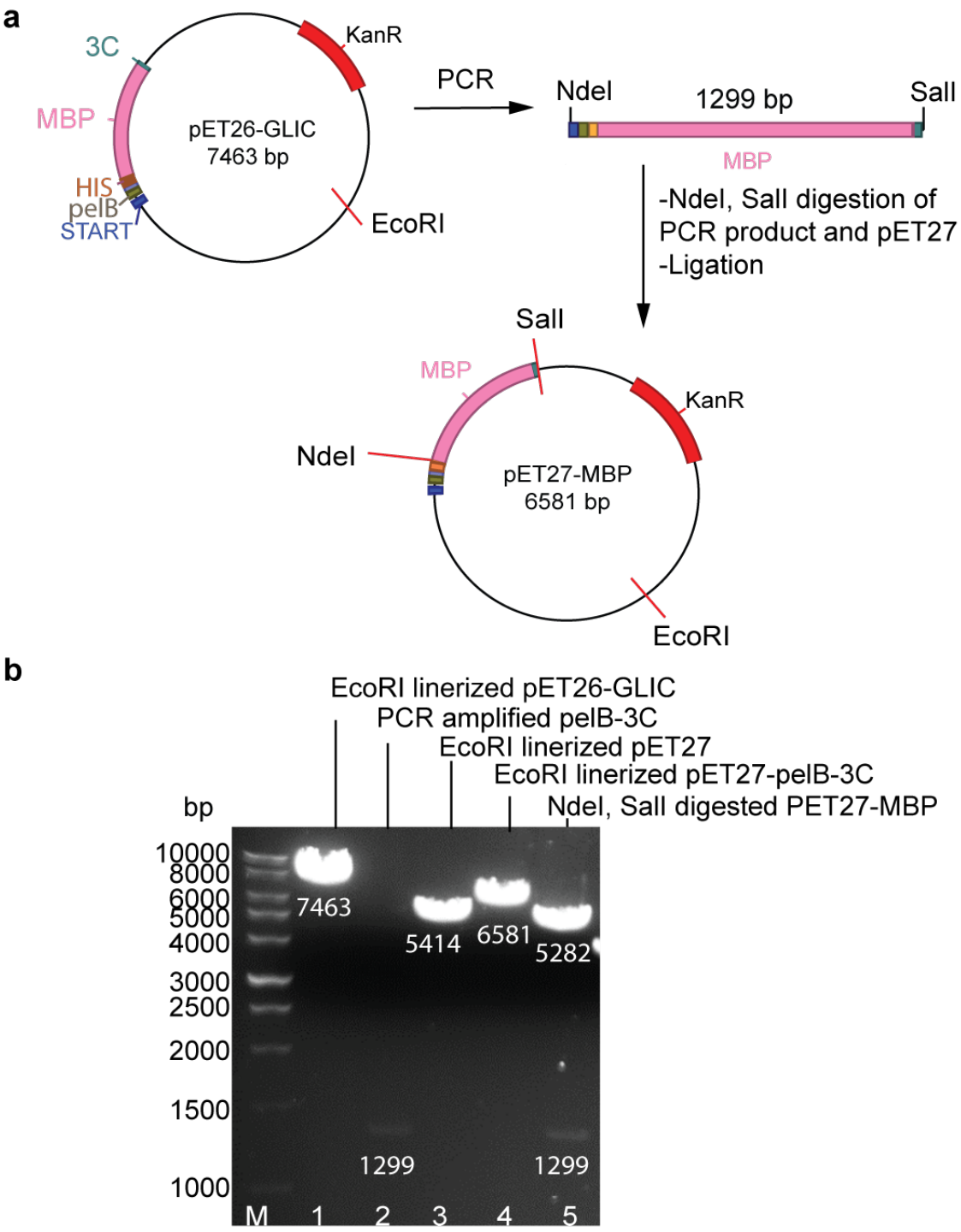


Figure D.5: **Generation of the vector for the expression of proteins in the periplasm of *E. coli*.** (a) Cartoon representation of the process of amplification of the gene by PCR (a) indicating the restriction sites of enzymes used for DNA digestion. *pelB-HIS-MBP-3C* sequence was amplified from *pET26-GLIC* vector, gel excised and purified. Digested with *SalI* and *NdeI* PCR fragment was cloned into digested *pET27*. (b) Agarose gel of digested PCR template (*pET26-GLIC*), PCR products (2), *pET27* vector backbone (3) and cloned expression *pET27-pelB-HIS-MBP-3C* vector (4 and 5) against DNA ladder (M). The sizes of generated DNA fragments in bp are given under the DNA bands. The localisation of restriction sites within the DNA fragments are indicated in a.

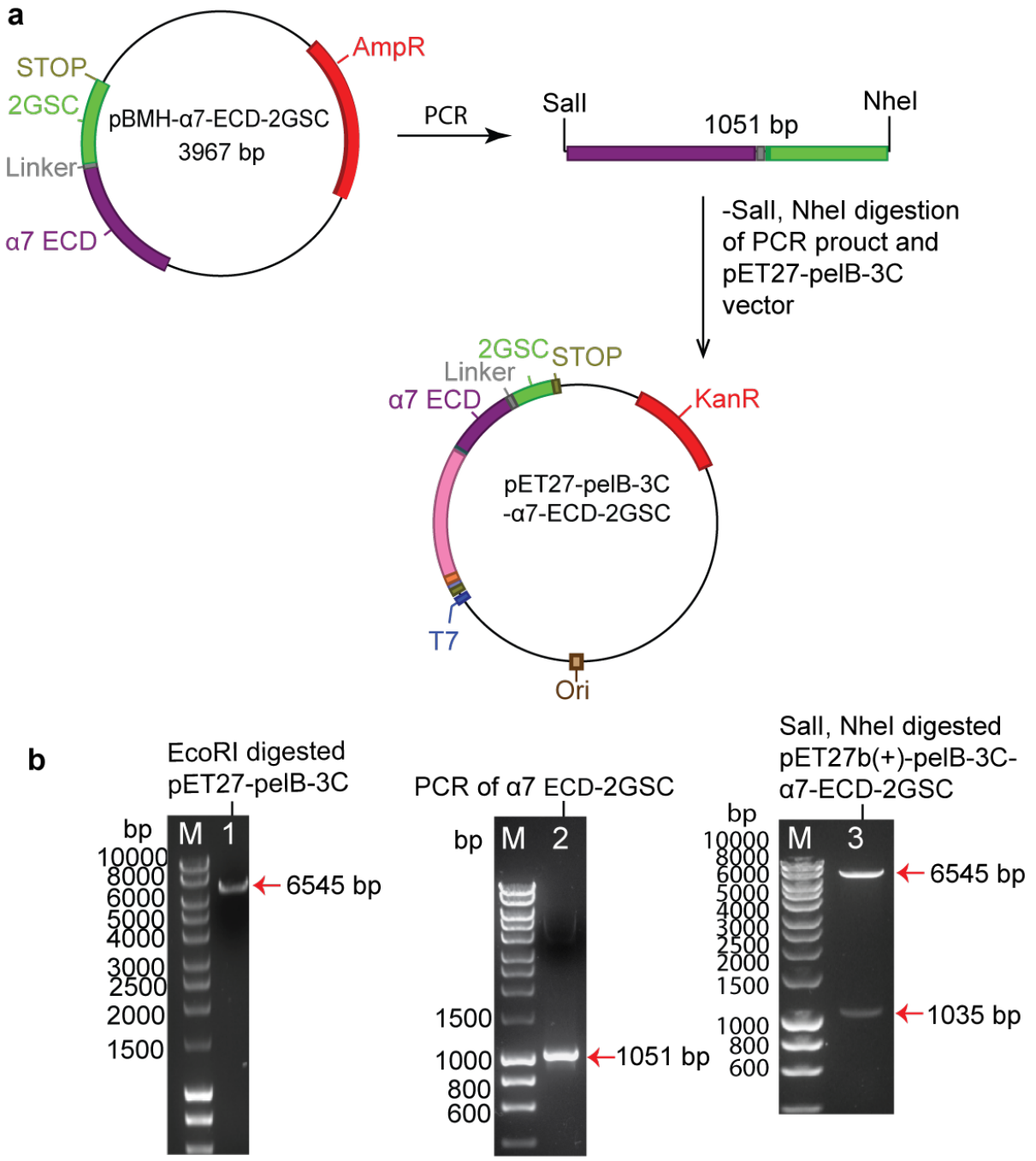


Figure D.6: **Generation of the vector for the expression of ligand binding domain of human $\alpha 7$ nAChR.** Schematic representation (a) and DNA agarose gel (b) of generation of expression vector. $\alpha 7$ was PCR amplified using primers flanked with restriction enzyme recognition sites, digested and cloned into digested *pET27-pelB-3C* vector.

Pre- and post-induction samples were collected from both growth and IPTG induction conditions (Section 2.3.5.1) and mixed with denaturing sample buffer. Proteins present in the SDS-bacterial cell extracts before and after IPTG induction were resolved with SDS-PAGE and visualised with the Coomassie staining (Figure D.7).

Addition of IPTG should lead to the expression of the $\alpha 7$ construct. Indeed, a distinct band of 84 kDa can be seen on the gel. The size of this band corresponds to the predicted size of expressed coding sequence from the $\alpha 7$ construct.

To authenticate the band as the protein of interest Western blot of the samples run on the SDS-PAGE was performed (Section 2.3.7). Western blot uses antibodies with high affinity for the N-terminal DecaHIS tag (Section 2.3.7.2). As seen in Figure D.7, no protein was detected in the pre-induction sample. Whereas addition of IPTG inducer resulted in production of HIS-tagged protein. This protein was of expected size of $\sim$ 84 kDa.

Western blot was examined closely to establish which condition resulted in the production the highest amount of the recombinant protein. Samples run on the SDS-PAGE gel were volume-normalised, thus the intensity of bands on Western blot were be compared. The expression of $\alpha 7$ chimera at 37°C and induction with 0.5 mM IPTG was the highest after 2 hours and decreased over time. Interestingly, multiple bands were detected in sample collected after the overnight protein expression at lower temperature and lower IPTG concentration. This suggests multiple sized HIS-tagged proteins are present in the sample, including truncated or proteolysed ones. Based on band intensities, the highest level of expression was achieved after overnight expression with low IPTG concentration and at low temperature.

Following overnight protein expression at 18°C, induction by 0.2 mM IPTG, and 6-hour expression at 37°C induction with 0.5 mM IPTG, the protein was purified. (Section 2.3.2). At each stage of purification the samples were collected to run them on the SDS-PAGE gel. Briefly, purification was done is a three-step process. The cells were precipitated and broken down by sonication to release their content. Homogenised cells were then spun down (low speed spin) to remove the unbroken cells, nucleic acids, organelles and large insoluble cellular particles, such as inclusion bodies (precipitant was collected as a whole cell sample). The supernatant from this low speed spin was then spun at 100 000g to precipitate cellular organelles. $\alpha 7$ ECD in the 100 000g soluble fraction was subsequently resolved using solid phase Ni^{2+}-NTA IMAC purification (Section 2.3.3). Briefly, the soluble fraction was incubated with Ni^{2+}-NTA resin for 2 hours at 4°C to allow binding of the expressed HIS-tagged $\alpha 7$ ECD chimera to beads. The mixture was decanted into the chromatography column. The unbound proteins were collected in the flow-through, before the beads were washed three times. The wash fractions were pooled and run as "Wash" on the gel. The resin-bound protein was then eluted by washing the column with 5 mL of 0.2 mM imidazole, to displace the HIS-tagged protein from the immobilized Nickel by competition. The eluted proteins were collected in two eluate fractions (Eluate 1 and 2).

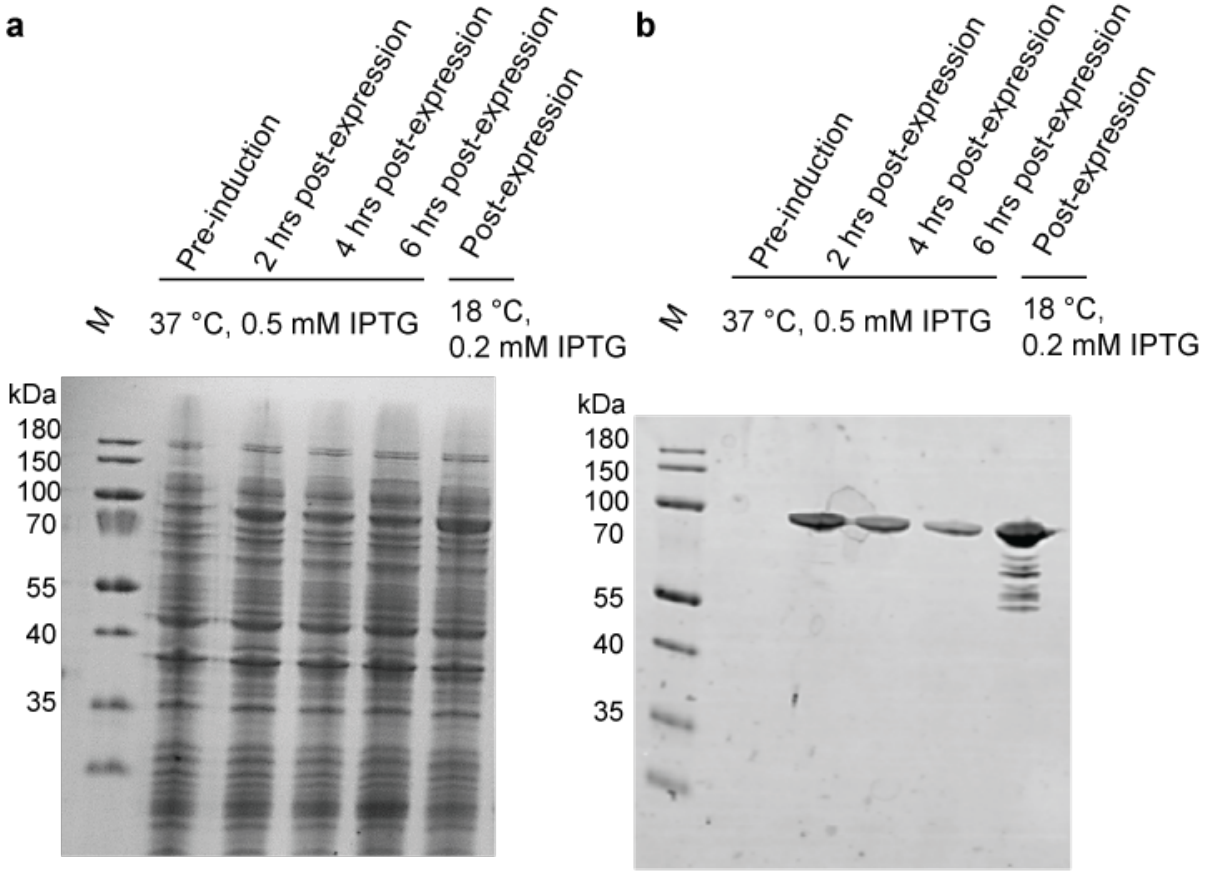


Figure D.7: **Expression of the $\alpha 7$ ECD chimera protein in *E. coli*.** Coomassie stained SDS-PAGE gels (a) and corresponding Western blot (b) of proteins obtained from the lysed whole cell samples. Transformed with $\alpha 7$ ECD containing plasmid *E . coli* were grown in 1 L of LB. Protein expression was induced with either 0.5 mM IPTG and proceeded to be grown at 37°C or with 0.2 mM IPTG and proceeded to be raised at 18°C. Samples of the cellular suspension prior to induction (pre-induction) and at 2, 4, 6 hours after the induction with high IPTG concentration were taken. Alongside, a sample after overnight (16-hour expression) driven by low concentrations of IPTG (0.2 mM) were taken. Samples were prepared as described in Section 2.3.5.1.

Representative samples from each of the fractions indicated above were resolved on the SDS-PAGE gel (Figure D.8) and the presence of recombinant protein was detected by a Western blot with anti-HIS-tag antibodies (Figure D.8 b). Since most of the purification features are common following expression at 18 and 37°C, some general comments are be made first. Then the comparison between the total purified protein will be made between the two.

The whole cell sample is the precipitate collected after the low-speed spin of the sonicated cells. As expected, a large number of proteins of various sizes were present in this sample, as visible on the Coomassie stained SDS-PAGE. There is a high intensity band of 84 kDa (size corresponding to the expression product of $\alpha 7$ ECD chimera) on both the Coomassie stained SDS-PAGE and the Western blot. This suggests that following harvest, the cells were either not broken up entirely and the recombinant protein retained intra-cellularly, or the protein was present in the inclusion bodies. To account for this, the sonication steps were extended from 6 to 8 minutes in the future experiments.

"Flow through" and "Wash" were samples collected during the first two steps of IMAC, and are expected to contain proteins with no- or weak - affinity to Ni^{2+}-NTA resin. Indeed, a large number of proteins of various sizes can be seen on the Coommassie stained gel, particularly in the "Flow through". Additionally, there are also immunoreactive proteins of the expected $\alpha 7$ ECD chimera protein size present in both the Flow Through and Wash, suggesting it failed to bind to the Ni^{2+}-NTA resin with high affinity. This could indicate that the insufficient amount of resin was present, therefore not all HIS-tagged protein managed to bind. Alternatively, the HIS-tag was buried in the tertiary and/or quaternary structure of the protein and was thus not accessible for interactions. Therefore, the amount of resin was increased for future experiments from 0.5 mL to 1 mL used for purification of the protein from 1 L of culture. Additionally the incubation time of the incubation of the soluble fraction with resin was increased from 2 hours to overnight.

Eluate samples are expected to contain proteins with high affinity to Ni^{2+}-NTA resin. However, no immunoreactive protein was detected in the eluate following expression induced at 37°C and 0.5 mM IPTG. In contrast, there is a band on the Western blot in the eluate collected after expression at 18°C and 0.2 mM IPTG, corresponding to the $\alpha 7$ ECD, based on its size of 84 kDa. Thus, more protein is being successfully purified following extended expression at lower temperature.

D.2.3 Purification of the $\alpha 7$ chimera protein

The expression and purification process was repeated with the modified conditions. That is, 1. lower IPTG concentration and low temperature during the expression, 2. extended sonication time, 3. overnight equilibration of the soluble fraction with resin and 4. increased amount of resin used.

To determine whether modified conditions have an effect on the protein purification efficiency, samples were collected during the purification procedure. Following expression of the protein at 37°C induced with 0.2 mM IPTG, cells harvested from 1 L of culture were sonicated and centrifuged at 16000g. The centrifugation

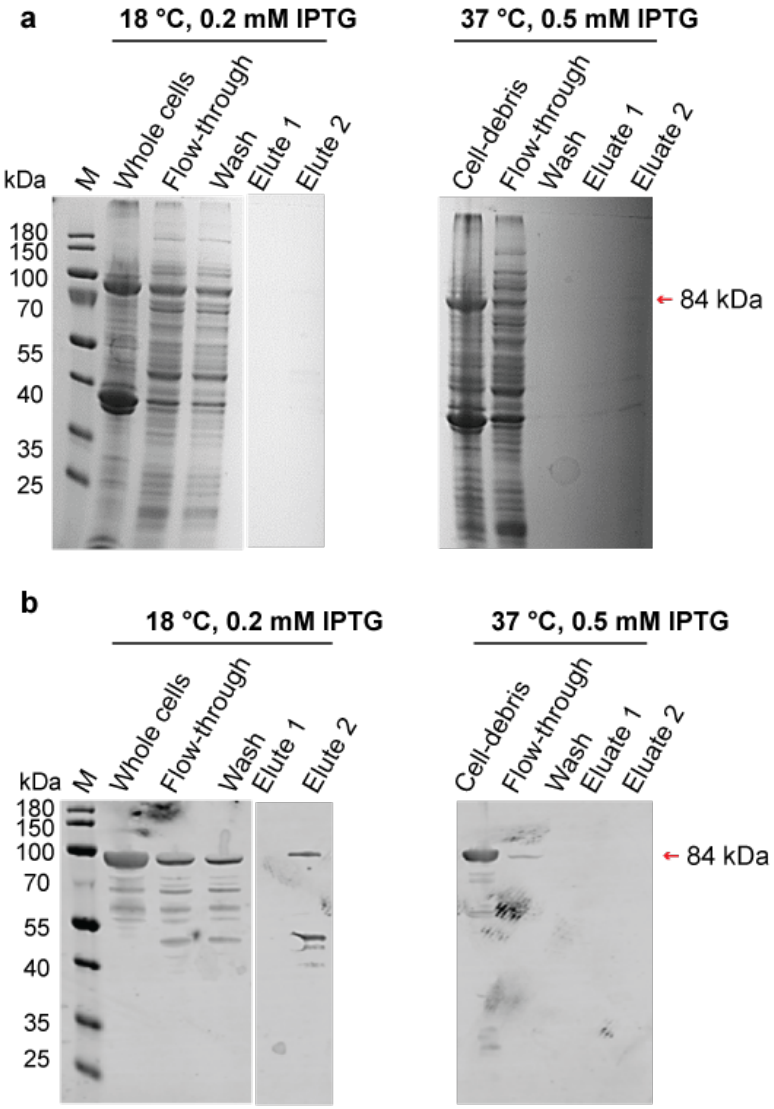


Figure D.8: **The effects of the temperature and inducer concentration on the expression of $\alpha 7$ nAChR chimera in *E. coli*.** SDS-PAGE gel (a) and corresponding Western blot (b) of samples collected during the purification of $\alpha 7$ nAChR chimera following the side by side expression of the protein at two different conditions. The expression was proceeded as explained in Figure D.7. Following expression, cells were harvested and homogenised. Protein purification proceeded as described in Section 2.3). The expected size of monomeric $\alpha 7$ nAChR chimera is 84 kDa.

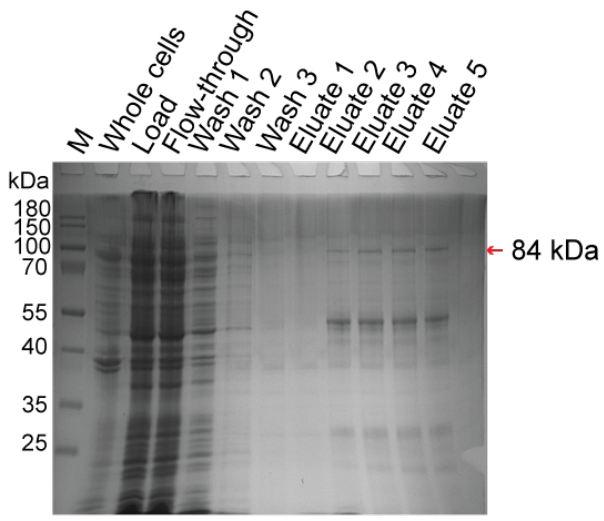


Figure D.9: **Coomassie stained SDS-PAGE gel of samples collected during purification of $\alpha 7$ ECD chimera protein.** *E. coli* cells were grown in 1L of TB. Protein expression was induced with 0.2 mM IPTG and proceeded overnight.

precipitate sample was run on the SDS-PAGE (Whole cells). The supernatant was spun down again at 100 000g to collected a soluble fraction (Load) which was subsequently incubated with 1 mL of Ni^{2+}-NTA resin (binding capacity of up to 40 mg) overnight. The mix was decanted onto the chromatography column. This was followed by 3 washes in 10 mL of buffer and 1 mL of 0.2 mM imidazole-containing buffer to generate 5 distinct eluate fractions obtained from the Ni^{2+}-NTA IMAC samples. Collected samples were prepared and run on the SDS-PAGE gel (Figure D.9). A band of the expected size of 84 kDa was present in eluate samples suggesting successful purification of $\alpha 7$-ECD chimera together with few other contaminants, the 50 kDa one being the most prominent, as judged by the staining intensity. Intensely stained band corresponding to the size of the $\alpha 7$-ECD chimera is also seen in the whole cell and the load fractions, suggesting that the significant proportion of the induced protein had been lost during centrifugation steps.

During preparation of samples for the SDS-PAGE, samples are heated resulting in protein denaturation and disintegration of individual subunits in multimeric complexes. Therefore, based on obtained SDS-PAGE results, it is impossible to state whether the expressed $\alpha 7$ ECD chimera is monomeric or pentameric. It is crucial for the recombinant protein to form multimeric complexes, because the nAChR ligand binding sites are on the interface of two neighbouring subunits. A blue native PAGE gel of non-denatured and non-reduced samples was run to allows for separation of proteins based on their mass and charge. The bands were visualised using a Coomassie stain.

The collected eluate (Figure D.9) was pooled. Two samples were prepared, one of which was boiled for $\sim$ 5 minutes to denature any multimeric proteins into their constituent subunits. The boiled sample should contain proteins only in the monomeric form, whereas non-boiled sample should contain proteins in their native state. The boiled sample was cooled and together with the un-boiled one, run on the native gel with the aim

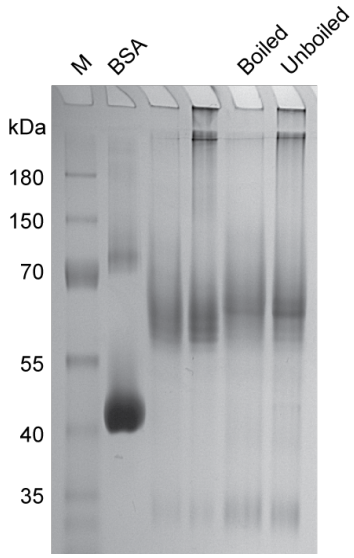


Figure D.10: **Coomassie stained Native Blue PAGE gel of $\alpha 7$ ECD chimera eluates.** Boiled and unboiled eluate samples of $\alpha 7$ ECD chimera protein (Figure D.9) were run on native non-denaturing gel alongside molecular weight markers (M) and un-boiled Bovine Serum Albumin (BSA) sample of 66.5 kDa.

to determine whether there are any high molecular weight bands selectively present in the un-boiled sample (Figure D.10).

Boiled sample contains a single band of $\sim$ 55kDa. There is also a corresponding band in the unboiled sample. A clear and strong staining present at the top of the gel produced from the un-boiled sample is also evident. This could represent a multimeric form of the $\alpha 7$ ECD chimera. The size of a pentamer is 420 kDa, therefore it is possible that the electrophoresis was run not for long enough to allow the protein to enter the gel. Alternatively, the staining could represent protein aggregates.

Size-exclusion chromatography, also known as gel filtration, is a complementary method for accessing protein sizes. The advantage of this method over PAGE is that the size estimation is much more accurate and the separation range is much greater (in this case, 10 - 600 kDa). This procedure uses a matrix filled column, containing pores of defined size. Loaded proteins can travel through the column at a defined speed, depending on their molecular weight. There is a reversal relationship between the molecular weight and the motility rate. That is, smaller molecules travel slower and are eluted later from the column, in comparison to the larger ones. Proteins are detected by spectroscopy because their amide bonds absorb at 280 nM. The result is a spectra of the absorbance against the eluted volume (Figure D.11).

To estimate the size of proteins present in a sample, the standard curve was generated (Section 2.3.7.7). The homogeneous solutions of proteins of known sizes were run to derive their spectra. The proteins used were: trypsin of 23.3 kDa, chicken serum albumin of 47.5 kDa, BSA of 66.5 kDa and Dextrin which forms large aggregates. The peak position as a function of volume eluted was derived and normalised to the peak position of the void (aka protein which does not enter the column pores, but passes straight through). The normalised peak positions for blue dextran, BSA, Chicken Serum Albumin and Trypsin were 0, 5.25, 6.90 and

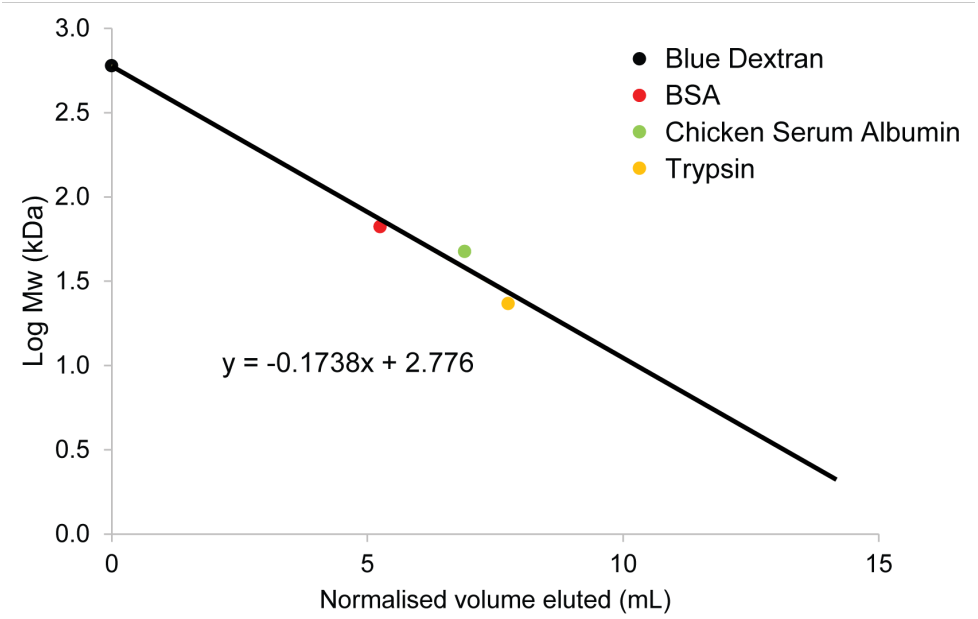


Figure D.11: **Calibration curve for molecular weight determination by gel filtration.** 1 mL of standard proteins were applied to the column. Blue dextran was used to determine the void volume.

7.75, respectively (Figure 2.2). These values were plotted on a logMw against normalised peak position graph to produce an equation of a straight line of y = -0.17 x + 2.78, where y is the log molecular weight of the protein and x is the normalised peak position. This equation was then used to calculate the size of the proteins present in $\alpha 7$ ECD chimera eluate.

To prepare samples, protein was expressed in 1 L of the growth medium and purified using optimised protocol. Eluate samples collected following Ni^{2+}-NTA IMAC were pooled and concentrated to 500 μL. The final concentration of the sample was 3 mg / mL, as measured by spectroscopy. This sample was run on the SDS-PAGE gel (Figure D.13 a).

A protein of desired size of 84 kDa was present, as well 50 and 25 kDa bands. The sample was run through the filtration column and the peak spectra was derived (Figure D.13 b). The highest peak was eluted at 16.20 mL. Normalised to void, this is 6.72 mL. This equates to 42.6 kDa. There are also small peaks: one eluted at 22 mL, and the other at 26.70 mL. These proteins are below 10 kDa. A small peak can be also seen at $\sim$ 10 mL which overlaps with the void peak and may represent aggregated proteins.

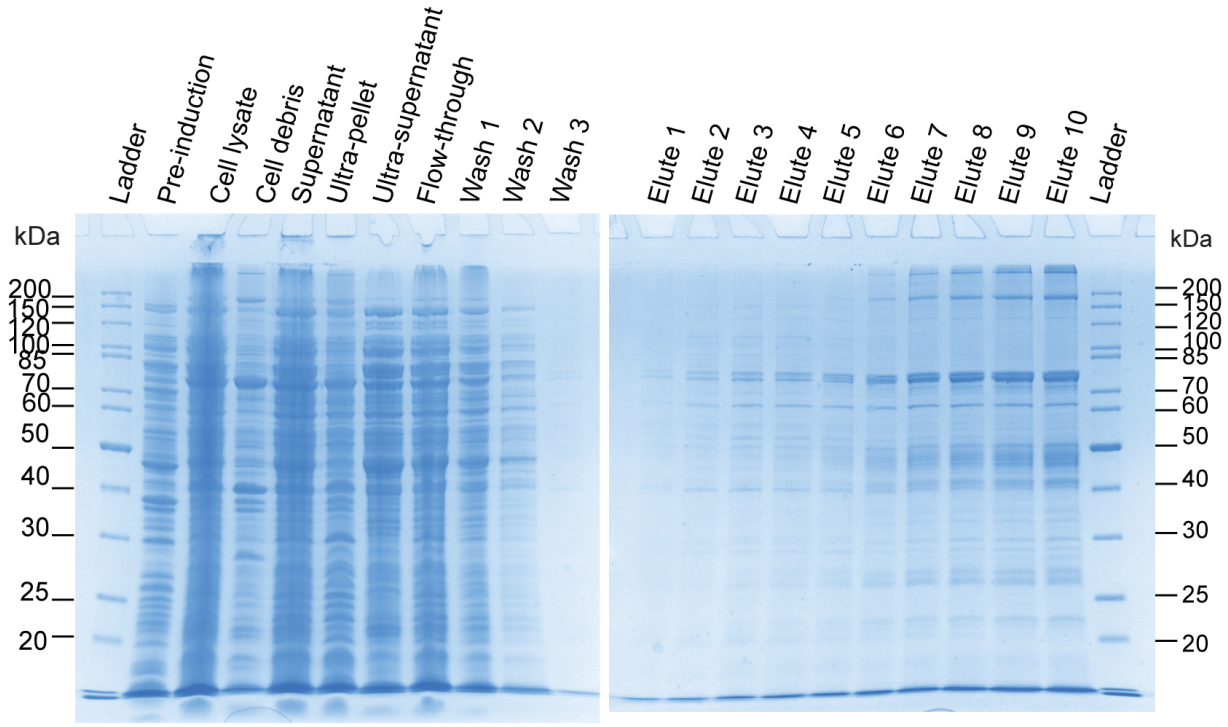


Figure D.12: **Expression and purification of $\alpha 7$ ECD chimera for size-exclusion chromatography**. SDS-PAGE gel of samples collected during protein expression and purification.

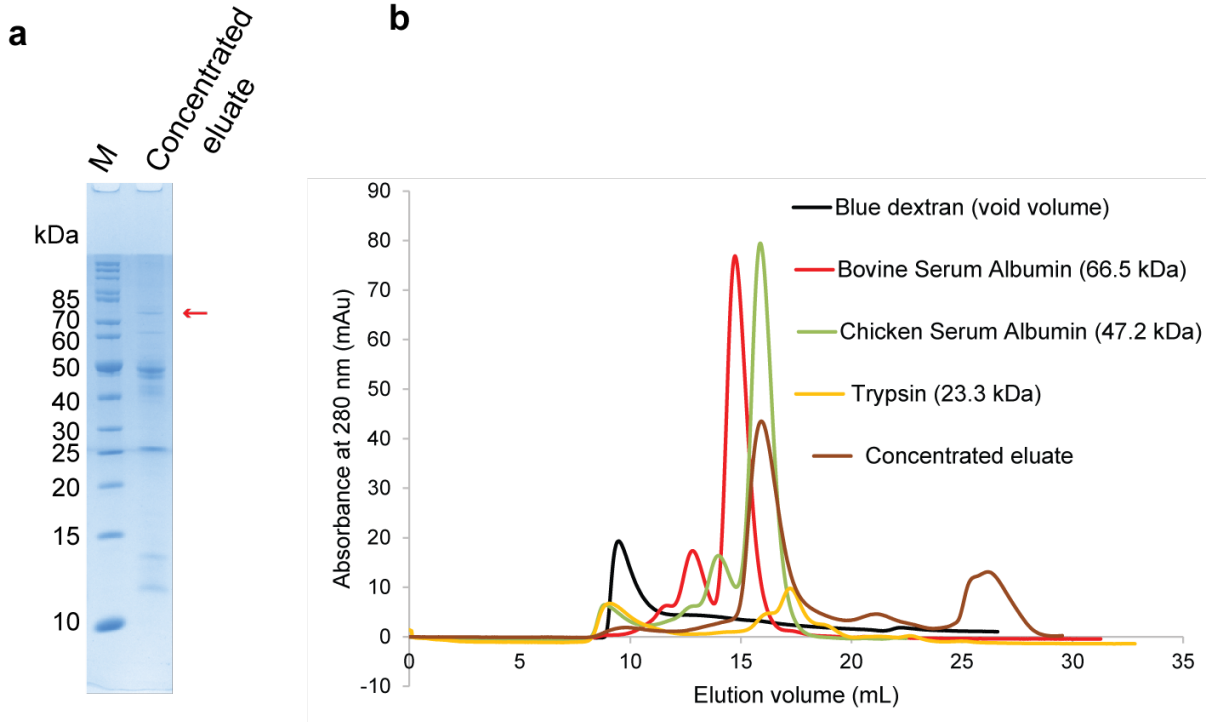


Figure D.13: **Estimation of protein sizes following $\alpha 7$ chimera expression and purification.** SDS-PAGE gel (a) and gel filtration spectra of concentrated $\alpha 7$ ECD chimera eluate.

D.3 Discussion

This chapter aims to determine whether *E. coli* BL21(DE3) cells are appropriate for the expression of ECD of nAChRs. This was done with a view to characterize candidate neonicotinoid binding sites. The difficulties in expression and purification of recombinant proteins, hinders their structural analysis and hence identification of molecular interactions between the target and the ligand.

There are several host systems available for the production of nAChRs and related proteins. For example, yeast cells have been successfully used to express assembled and folded mammalian $\alpha 2$ (Kouvatsos et al. 2016), $\alpha 1$ (Dellisanti et al. 2007) and $\alpha 9$ (Zouridakis et al. 2014) ECD of nAChRs and nAChR ECD structural surrogate mollusc AChBP (Hilf and Dutzler 2008, 2009). Functional mammalian $\alpha 4\beta 2$ receptors were successfully expressed and subsequently purified from both mammalian (Morales-Perez, Noviello, and Hibbs 2016) and insect cell lines (Kouvatsos et al. 2014). *E. coli* is an attractive alternative due to the relative low cost of use and ease of manipulation. The successful expression of folded AChBPs (Abraham et al. 2016) and full length human $\alpha 7$ (Tillman et al. 2016) was achieved in *E. coli* cells.

The suitability of *E. coli* as an expression system for nAChR ECD was tested by expressing and purifying human $\alpha 7$ ECD - chimera protein.

D.3.1 Expression and purification of $\alpha 7$ extracellular domain chimera yields product of the correct size

The initial experiments were carried out to determine whether and under what conditions can the expression of $\alpha 7$ ECD - chimera can be achieved. Two conditions were tested: rapid expression at 37°C and 0.5 mM IPTG and slower expression at 18°C and 0.2 mM IPTG. The cells expressing $\alpha 7$ ECD - chimera were collected from both conditions. Pre- and post-induction samples were run on the SDS-PAGE gel and Coomassie stained to resolve and visualise proteins (Figure D.7). $\alpha 7$ ECD-chimera was authenticated by Western blot using anti-HIS antibodies. Clear band of 84 kDa was present in the induced samples, confirming successful expression. Greater intensity of the band from 18°C and 0.2 mM IPTG suggest this is a favorable condition for the expression of $\alpha 7$ ECD-chimera. Lower temperature and IPTG concentrations were also beneficial for the expression of other $\alpha 7$ ECD construct (Abraham et al. 2016). In addition, to the band representing $\alpha 7$ ECD - chimera there was also an induction of the 50 kDa protein, which is likely a proteolytic fragment with the HIS-tag, based on immunoreactivity.

Western blot with anti-HIS antibodies detected the presence of HIS-tagged proteins with immunoreactivity consistent with the expressed size of $\alpha 7$ ECD - chimera. However, purification results showed that the proportion of induced protein available for binding was disappointing. The expressed protein was lost during the purification procedure, some was precipitated following centrifugation of sonicated cells (Figure D.8 Whole Cell sample),

suggesting the formation of inclusion bodies. In addition, some expressed protein failed to bind to the nickel resin, potentially due to misfolding or aggregation. The expression of remaining construct was even more challenging, with a smaller proportion of the ECD - chimera purified.

D.3.2 Analysis of the quaternary structure.

To determine whether the protein was purified as a pentamer, a native-PAGE gel was run, which enables separation of folded and assembled proteins on the gel. Two samples were prepared: one containing denatured by boiling proteins and the other containing non-denatured, un-boiled proteins. A clear staining of high molecular weight proteins was observed in the un-boiled sample, but not in the boiled sample, suggesting purification of multimeric proteins. The presence of high molecular weight protein was not confirmed by gel filtration. Therefore further experiments are needed to investigate whether expressed $\alpha 7$ ECD chimera forms pentameric structures. This could include binding of radiolabelled ligands, such as α-bgtx (Barnard, Wieckowski, and Chiu 1971; Carbonetto and Fambrough 1979; Clarke et al. 1985).

In summary, this chapter validates the use of *E. coli* as a system for the expression of $\alpha 7$ ECD and highlights the need for further optimisation to improve stability and purification efficiency of the recombinant protein.

Appendix E

Bacterial homopentameric soluble domains.

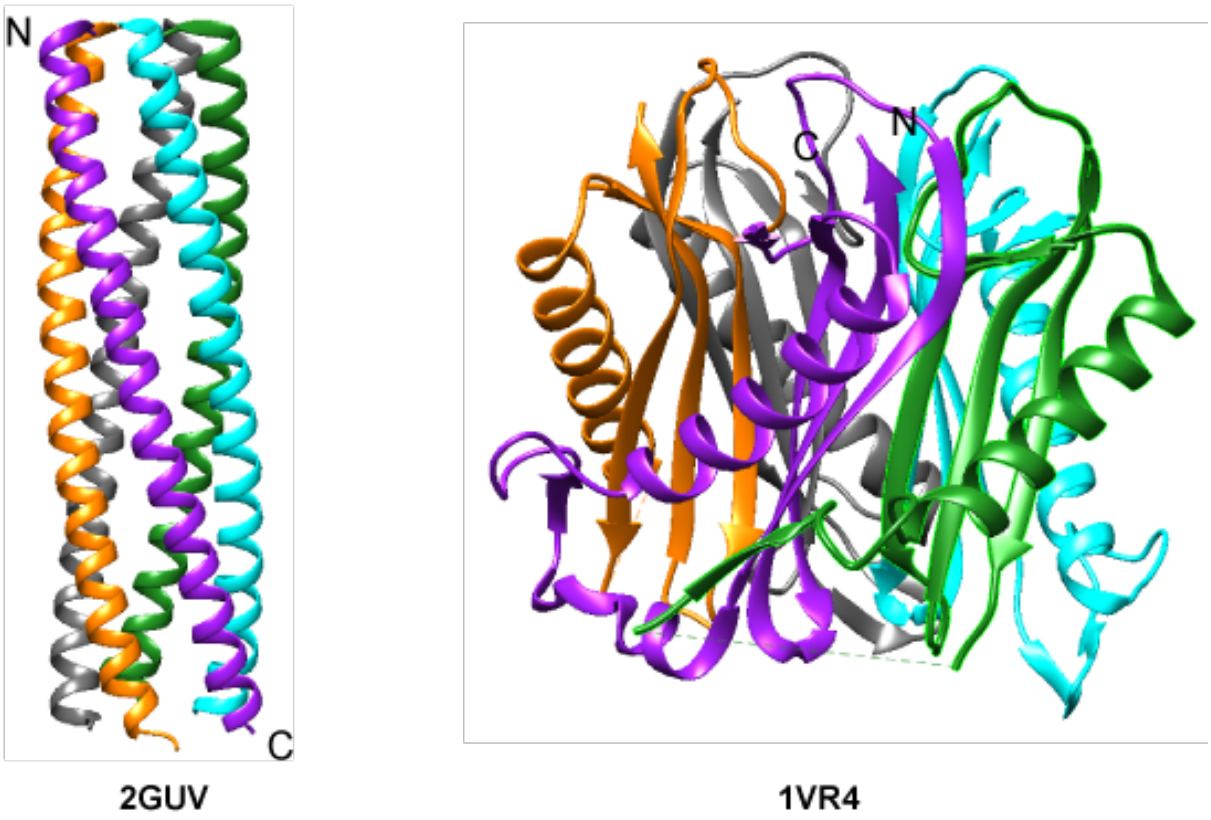
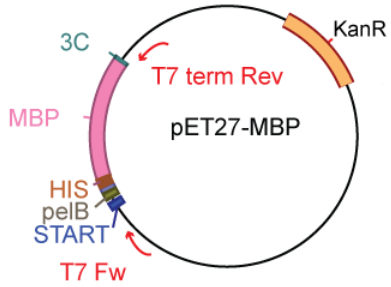


Figure E.1: **Structures of soluble homopentameric soluble domains**. Single subunits are color coded and the termini of a single subunits (in purple) are shown. 2GUV and 1VR4 subunits were used as C-terminal tags of nAChR expressed in *E. coli* to promote pentamerisation. Images generated in USCF Chimera (PDB codes: 2GUV and 1VR4).

Appendix F

DNA sequence used for the expression of human $\alpha 7$ extracellular domain in *E. coli*

a



T7 Fw : TAA TAC GAC TCA CTA TAG GG
T7 term Rev: CTA GTT ATT GCT CAG CGG T

b

START pelB

```
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1    CATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCG
```

ERROR decaHIS

```
Query   61   GCGATGGCAATGGATATCGGAATTAATTCGGATCCGAATTCGAGCTCACATCACCATCAC
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
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```

MBP

```
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             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query   361  TCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCGTTCCAGGACAAGCTGTATCCGTTT
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Sbjct   421  ACCTGGGATGCCGTACGTTACAACGGCAAGCTGATTGCTTACCCGATCGCTGTTGAAGCG

Query   481  TTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   481  TTATCGCTGATTTATAACAAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATC

Query   541  CCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   541  CCGGCGCTGGATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAA

Query   601  GAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   601  GAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTCAAGTATGAA
```

```
Query  661   AACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661   AACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCTGGCGCGAAAGCGGGTCTG

Query  721   ACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   ACCTTCCTGGTTGACCTGATTAAAAACAAACACATGAATGCAGACACCGATTACTCCATC

Query  781   GCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   GCAGAAGCTGCCTTTAATAAAGGCGAAACAGCGATGACCATCAACGGCCCGTGGGCATGG

Query  841   TCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   TCCAACATCGACACCAGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGT

Query  901   CAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   CAACCATCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCGAAC

Query  961   AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   AAAGAGCTGGCAAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAAGGTCTGGAAGCG

Query  1021  GTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  GTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAGTCTTACGAGGAAGAGTTGGCG

Query  1081  AAAGATCCACGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAAC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  AAAGATCCACGTATTGCCGCCACTATGGAAAACGCCCAGAAAGGTGAAATCATGCCGAAC

Query  1141  ATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  ATCCCGCAGATGTCCGCTTTCTGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGC
                                                                    3C
Query  1201  GGTCGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTCCGGGTAGCCTGGAAGTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  GGTCGTCAGACTGTCGATGAAGCCCTGAAAGACGCGCAGACTCCGGGTAGCCTGGAAGTT

Query  1261  CTGTTCCAGGGGCCC  1275
              |||||||||||||||
Sbjct  1261  CTGTTCCAGGGGCCC  1275
```

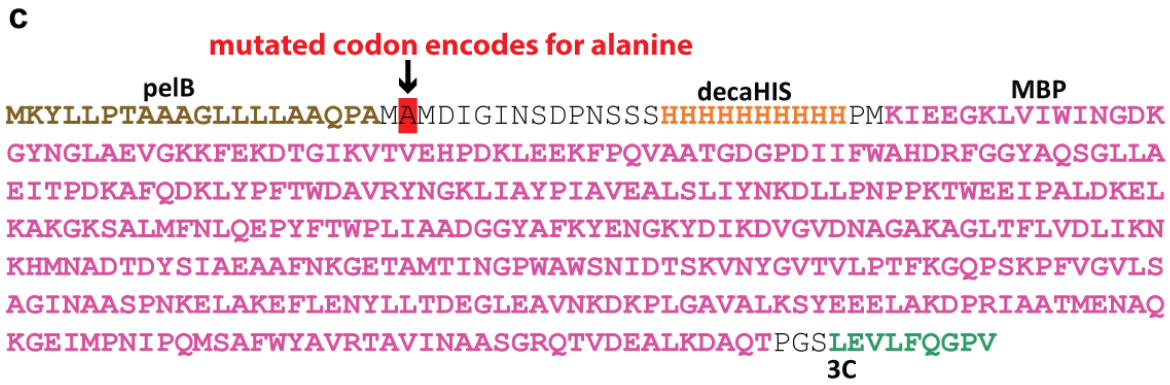
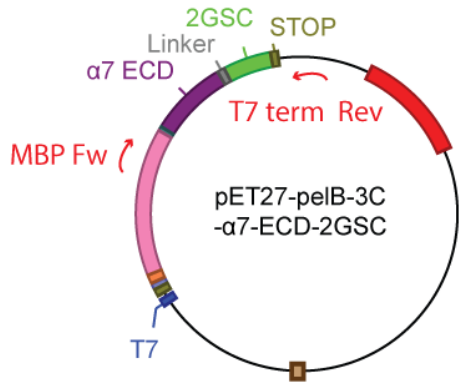


Figure F.1: **Sequencing of *pelB-3C* cloned into *pET27* expression vector.** Inserted into *pET27 pelB-3C* sequence was sequenced using universal T7 forward and T7 terminator primers (a). The cloned sequence (Query) was compared to the expected sequence (Subject) (b). Single nucleotide mutation from A to C occurred, highlighted in red, changing the codon from GCC to GCA, both of which encode for alanine. The cloned nucleotide sequence was translated (c) and major functional domains highlighted.

Appendix G

Sequencing of the DNA used for the expression of of the $\alpha 7$ chimera protein

a



MBP Fw : CCCGCAGATGTCCGC
T7 Term Rev: CTA GTT ATT GCT CAG CGG T

b

3C α7 ECD

```
Query   GTTCTGTTCCAGGGGCCCGTCGACTTTCAGCGTAAACTGTACAAAGAGCTGGTGAAGAAC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   GTTCTGTTCCAGGGGCCCGTCGACTTTCAGCGTAAACTGTACAAAGAGCTGGTGAAGAAC

Query   TACAACCCGCTGGAACGCCCGGTTGCTAACGATTCCCAGCCGCTGACTGTTTACTTCTCT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   TACAACCCGCTGGAACGCCCGGTTGCTAACGATTCCCAGCCGCTGACTGTTTACTTCTCT

Query   CTGTCCCTGCTGCAGATCATGGATGTTGACGAAAAAAACCAGGTGCTGACCACCAACATC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   CTGTCCCTGCTGCAGATCATGGATGTTGACGAAAAAAACCAGGTGCTGACCACCAACATC

Query   TGGCTGCAGATGTCCTGGACCGACCACTACCTGCAGTGGAACGTGTCTGAGTATCCGGGT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   TGGCTGCAGATGTCCTGGACCGACCACTACCTGCAGTGGAACGTGTCTGAGTATCCGGGT

Query   GTGAAGACTGTTCGTTTCCCGGATGGTCAGATTTGGAAACCGGATATTCTGCTGTATAAC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   GTGAAGACTGTTCGTTTCCCGGATGGTCAGATTTGGAAACCGGATATTCTGCTGTATAAC

Query   TCTGCGGATGAGCGTTTTGACGCGACCTTCCACACCAACGTGCTGGTTAACTCTAGCGGT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   TCTGCGGATGAGCGTTTTGACGCGACCTTCCACACCAACGTGCTGGTTAACTCTAGCGGT

Query   CACTGCCAGTATCTGCCGCCGGGTATCTTCAAATCTTCTTGCGACCCGACCGGTGTTGAC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   CACTGCCAGTATCTGCCGCCGGGTATCTTCAAATCTTCTTGCGACCCGACCGGTGTTGAC

Query   TCTGAGGAAGGCGCGACTTGCAAACTGAAGTTCGGTTCTTGGTCTTACGGCGGTTGGTCT
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   TCTGAGGAAGGCGCGACTTGCAAACTGAAGTTCGGTTCTTGGTCTTACGGCGGTTGGTCT

Query   CTGGACCTGCAGATGCAGGAAGCAGATATTTCTGGTTACATTCCGAACGGCGAGTGGGAC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   CTGGACCTGCAGATGCAGGAAGCAGATATTTCTGGTTACATTCCGAACGGCGAGTGGGAC

Query   CTGGTGGGTATCCCGGGCAAGCGTTCCGAGCGCTTTTACGAATGCTGCAAGGAACCGTAC
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   CTGGTGGGTATCCCGGGCAAGCGTTCCGAGCGCTTTTACGAATGCTGCAAGGAACCGTAC
```

```
Query    CCGGACGTGACCTTTACCGTTACCATGCGTCGTCGTGGTGAGGTTGAACAGCCGCTCGAG
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    CCGGACGTGACCTTTACCGTTACCATGCGTCGTCGTGGTGAGGTTGAACAGCCGCTCGAG
```

2GSC

```
Query    ATGGAAAACCGTGAATCTGCTCAGCGTCCGCATGAACGTCTGGATGCTTGGCGTGACTCT
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    ATGGAAAACCGTGAATCTGCTCAGCGTCCGCATGAACGTCTGGATGCTTGGCGTGACTCT

Query    ATGGAACTGGTTGAGATGATCTACCGTCTGACCGAAGTTTTTCCGGATCAGGAGCGTTAC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    ATGGAACTGGTTGAGATGATCTACCGTCTGACCGAAGTTTTTCCGGATCAGGAGCGTTAC

Query    GGCCTGACTGCACAGCTGCGCCGTGCTGCTGTTTCTATCCCGTCTAACATCGCGGAAGGT
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    GGCCTGACTGCACAGCTGCGCCGTGCTGCTGTTTCTATCCCGTCTAACATCGCGGAAGGT

Query    GCAGCTCGCCGCTCTACTCCGGACTATTCTCGTTTTCTGTCCATCGCGCGTGGTAGCCTG
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    GCAGCTCGCCGCTCTACTCCGGACTATTCTCGTTTTCTGTCCATCGCGCGTGGTAGCCTG

Query    TCTGAACTGGACACCCAGGTTCAGATCGCGGCACGCCTGGGTTATTCCCGTTCTGAAGAC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    TCTGAACTGGACACCCAGGTTCAGATCGCGGCACGCCTGGGTTATTCCCGTTCTGAAGAC

Query    GACCAGTCTGTTCGCCGTCAGGTGGATCTGGTGTTCGCTAAGCTGACCGCGCTGATGAAC
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    GACCAGTCTGTTCGCCGTCAGGTGGATCTGGTGTTCGCTAAGCTGACCGCGCTGATGAAC

Query    GCGCTGCGTCGCCGTGGTGCGGCTAAGCTTTAAGCTAGCCAGCCAGAACTCGCCCCGGAA
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct    GCGCTGCGTCGCCGTGGTGCGGCTAAGCTTTAAGCTAGCCAGCCAGAACTCGCCCCGGAA
```

STOP

```
Query    GACCCCGAGGATGTCGAGCACCACCACCACCACCATAA
         |||||||||||||||||||||||||||||||||||||
Sbjct    GACCCCGAGGATGTCGAGCACCACCACCACCACCATAA
```

c

pelB decaHIS MBP

MKYLLPTAAAGLLLLAAQPAMAMDIGINSDPNSSSHHHHHHHHHHPMKIEEGKLVIWINGDK

GYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLA

EITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKEL

KAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKN

KHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLS

AGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQ

3C α7 ECD

KGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTPGSLEVLFQGPVGEFQRKLYK

ELVKNYNPLERPVANDSQPLTVYFSLSLLQIMDVDEKNQVLTTNIWLQMSWTDHYLQWNVSE

YPGVKTVRFPDGQIWKPDILLYNSADERFDATFHTNVLVNSSGHCQYLPPGIFKSSCDPTGV

DSEEGATCKLKFGSWSYGGWSLDLQMQEADISGYIPNGEWDLVGIPGKRSERFYECCKEPYP

2GSC

DVTFTVTMRRRGEVEQPLEMENRESAQRPHERLDAWRDSMELVEMIYRLTEVFPDQERYGLT

AQLRRAAVSIPSNIAEGAARRSTPDYSRFLSIARGSLSELDTQVQIAARLGYSRSEDDQSVR

RQVDLVFAKLTALMNALRRRGAAKL*

Figure G.1: **Sequencing of $\alpha 7$ *ECD-2GSC* cloned into *pET27-pelB-3C* expression vector.** Inserted into *pET27-pelB-3C* vector $\alpha 7$ *ECD-2GSC* was sequenced using universal T7 Terminator reverse primer and primer within the 3' region of *MBP* (a). The cloned sequence (Query) was compared to the expected sequence (Subject) (b). The sequence was translated and merged with the *pel-3C* to depict the entire protein expressed (c). The major domains are highlighted, as well as the mutated Cys-loop (in red).

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