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

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Submitted in part fulfilment of the requirements for the degree of
Doctor of Philosophy at the University of Southampton, April 13, 2020

Abstract

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 receortors (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 $\geq$ 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 α7 in the pharynx of *eat-2* mutant led to a cell-surface expression, as shown by staining with labelled α-bungarotoxin. However the feeding and pharmacological phenotypes of the mutant were not rescued. *C. elegans* strain in which human α7 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 phayngeal response to nAChR agonists cytisine, nicotine or acetylcholine were noted. The lack of apparent functionality of α7 receptor could be due to the incorrect cellular localisation of this protein. α-bgtx staining showed that α7 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.

Contents

List of Tables

List of Figures

List of Abbreviations

5-HT - 5-hydroxytryptamine

ACE - Acetylcholinesterase

AChBP - Acetylcholine Binding Protein

bgtx - bungarotoxin

Bovine Serum Albumin (BSA)

e. coli ER- endoplasmic reticulum polyvinylidene difluoride (PVDF) C. elegans GLIC - *Gloebacter* ligand gated ion channels

glutamine-gated chloride (GluCl) Green Fluorescent Protein (GFP)

Hermaphrodite Specific Neurons (HSNs)

BWM - Body Wall Muscle

Ch - choline

ChAt - Choline acetyltransferase

dimethyl sulfoxide (DMSO)

ECD - extracellular domain

E/R ratio ELIC - *Erwinia* ligand gated ion channels

GABA - Gamma aminobutyric acid

GPCR - G protein coupled receptor

nAChR : nicotinic acetylcholine receptor

NGM - nematode growth medium

NMJ - Neuromuscular junction

neurosecretory motor neurons (NSM

RIC-3 - Resistant to Inhibitors of Cholinesterase-3

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresi TM - transmembrane

RIC-3 - (resistant to inhibitors of cholinesterase-3

VAChT - Vesicular acetylcholine transferase

MBP - Maltose Binding Protein

Declaration

I, Monika Kudelska

declare that the thesis entitled:

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

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Signed:

Date:

Acknowledgements

Throughout the process of PhD study, research and writing this thesis, I have received a great deal of support and help. I would like to thank my supervisors: Dr Declan Doyle, Prof Vincent O'Connor and Prof Lindy Holden-Dye, whose expertise, knowledge, enthusiasm and motivation was invaluable. Without your guidance this PhD would have been unachievable.

I would like to acknowledge The Gerard Kerkut Charitable Trust for providing funding for this PhD project.

I am also very grateful to Prof Robert Walker, Prof Nullin Divecha and Dr James Dillon for their insightful comments and encouraging words.

My thanks go to Dr Alistair Bailey for his help with writing and formatting this thesis and for keeping me company throughout my studies.

Thank you to all members of the worm-lab and the lab of Dr Declan Doyle for all the fun we have experienced together. In particular, I would like to thank Dr Ben Yarnall, Dr Fernando Calahorro and Dr Anna Crisford for your support and technical help.

I am grateful to my all my family members who always believed in me.

Last but not least thank you to my partner, Damien Parsey for his continual emotional support, patience and understanding. I would not have made it without you.

Publications

Part of this thesis has been published in the following papers:

Kudelska, M. M., L. Holden-Dye, V. O'Connor, and D. A. Doyle. 2017. Concentration-Dependent Effects of Acute and Chronic Neonicotinoid Exposure on the Behaviour and Development of the Nematode *Caenorhabditis elegans*. Journal Article. Pest Manag Sci 73 (7): 134551. https://doi.org/10.1002/ps.4564.

Kudelska, M. M., A. Lewis, C. T. Ng, D. A. Doyle, L. Holden-Dye, V. M. O'Connor, and R. J. Walker. 2018. Investigation of Feeding Behaviour in *C. elegans* Reveals Distinct Pharmacological and Antibacterial Effects of Nicotine. Invert Neurosci 18 (4): 14. https://doi.org/10.1007/s10158-018-0219-1.

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 insectides (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 beatles, 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, bn 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 Commision 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).

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.

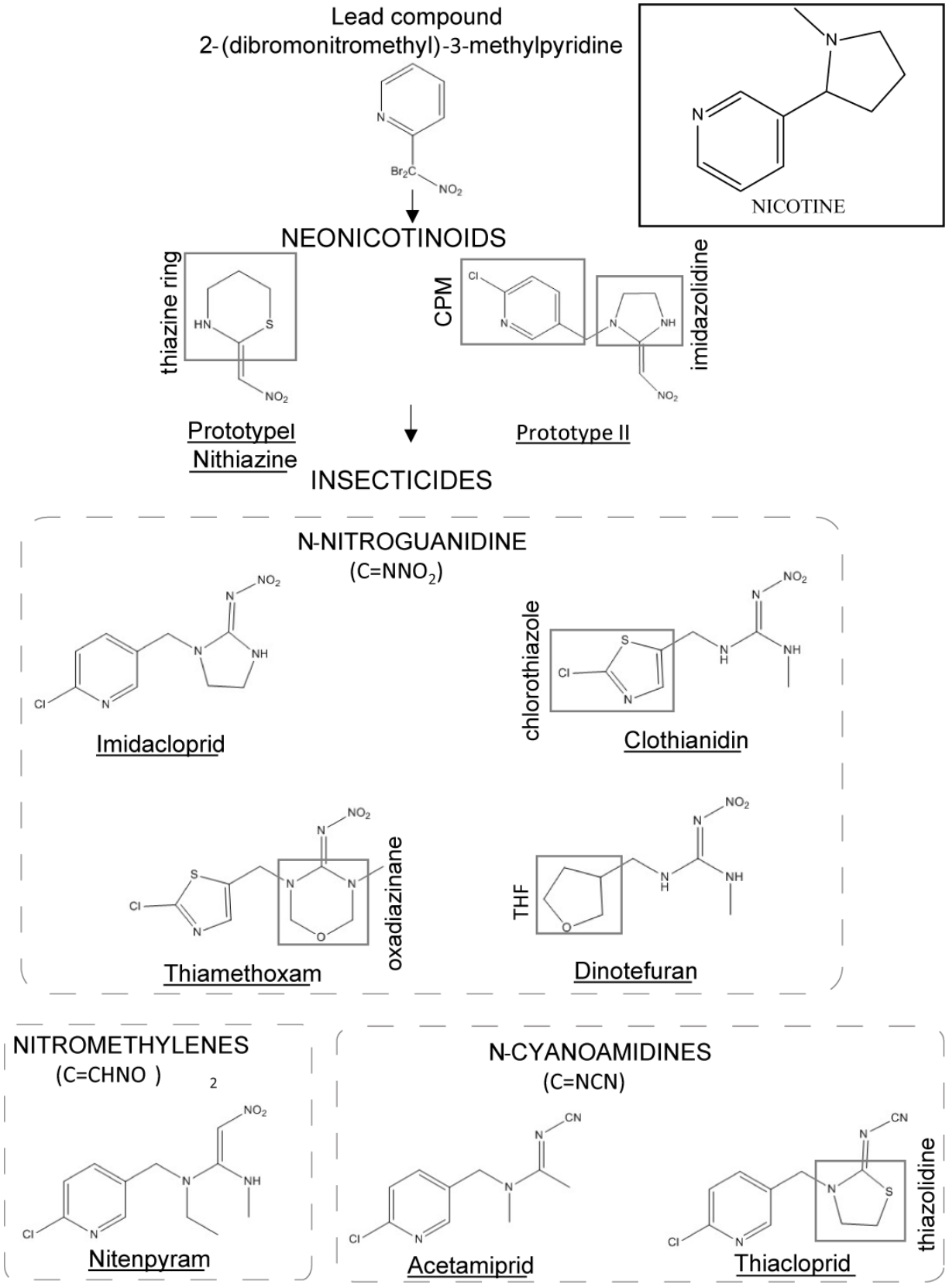


Figure 1.1: **Development and chemical structures of the synthetic insecticides, the neonicotinoids.** Systematic modification of the lead and prototype compounds led to the discovery of seven neonicotinoids currently used in agriculture and animal health. They are structurally related to nicotine (shown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

1.1.4 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 ~ 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-spam 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 Commision 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 **??**). 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 programmes. 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.

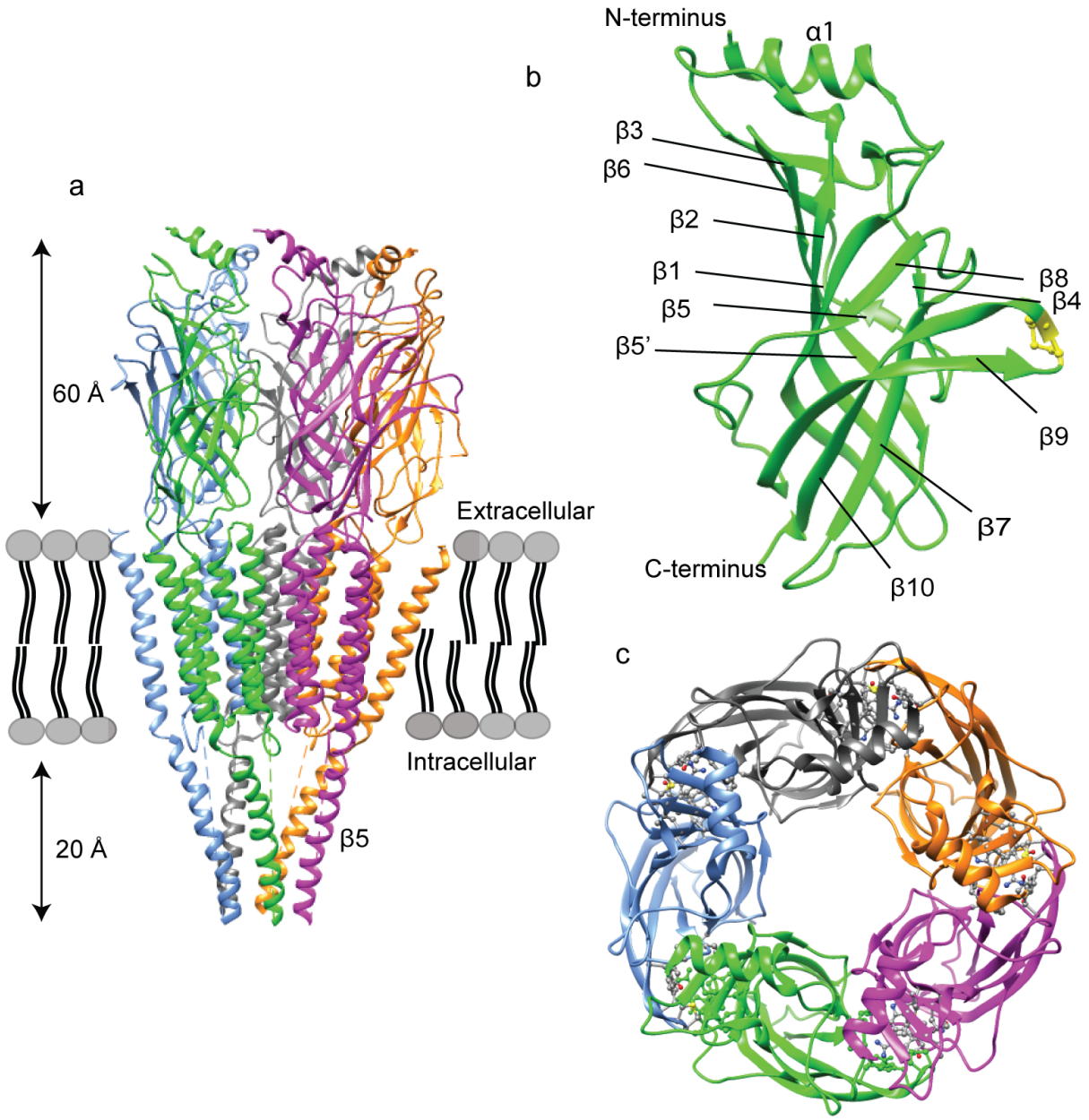


Figure 1.2: **Structural features of the nicotinic acetylcholine receptor.** Torpedo nAChR is a transmembrane protein, made up of 5 subunits (colour-coded), arranged around the ion conductivity pore. Each subunit consists of extracellular ligand-binding, transmembrane and intracellular domain (a) (PBD 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 a soluble 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 classical 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* (*stagnalis* are discussed.

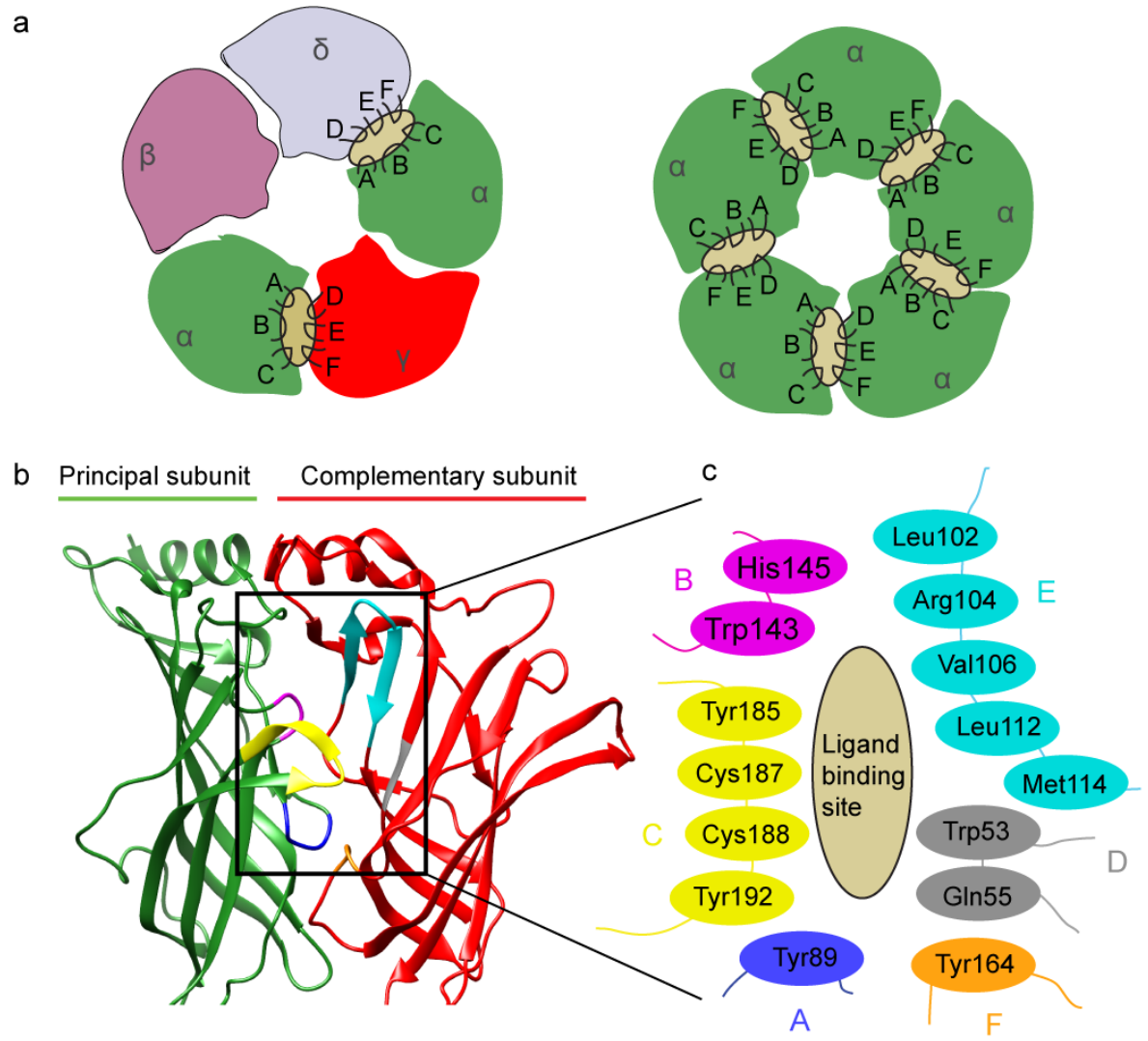


Figure 1.3: **The ligand binding domain of acetylcholine binding protein.** Agonist binds to the loops situated in the adjacent subunits of the nAChR. In muscle type receptor, there are 2 binding sites, and there are 5 in homopentameric receptor (a). The ligand binding pocket of the AChBP (PDB:1I9B) 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). 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). 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. 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

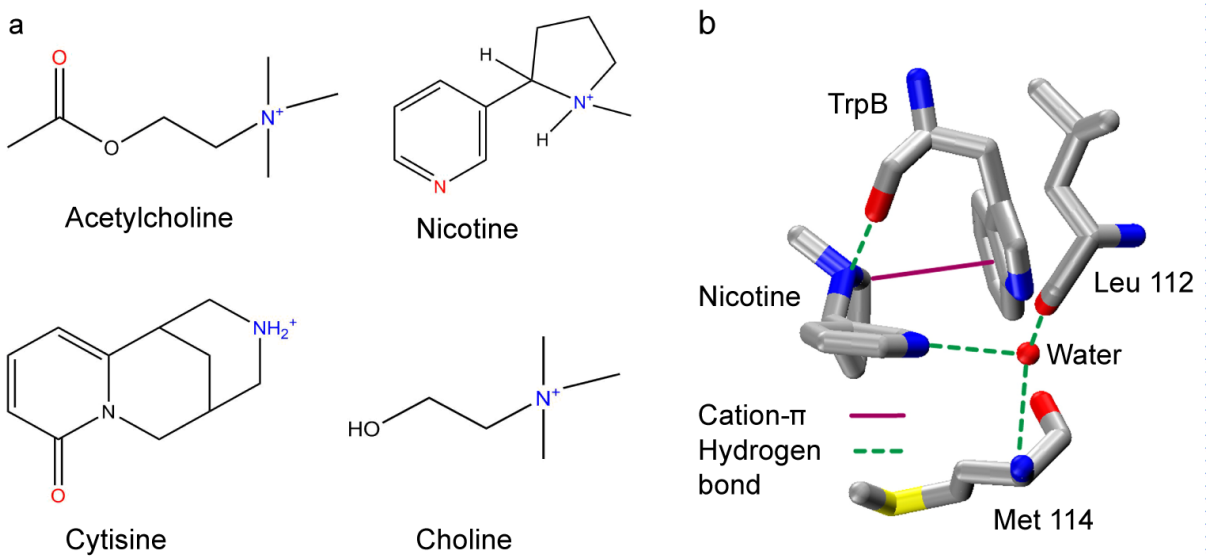


Figure 1.4: **Nicotinic acetylcholine receptor agonist pharmacophore.** Agonists of the nAChRs contain hydrogen bond acceptor (red) and cationic nitrogen (blue) (a). Interactions with the receptor based on the crystal structure of the AChBP and nicotine (PB:1UW6) (b). Image b) is taken from Blum, Lester,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

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 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 apibatidine 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

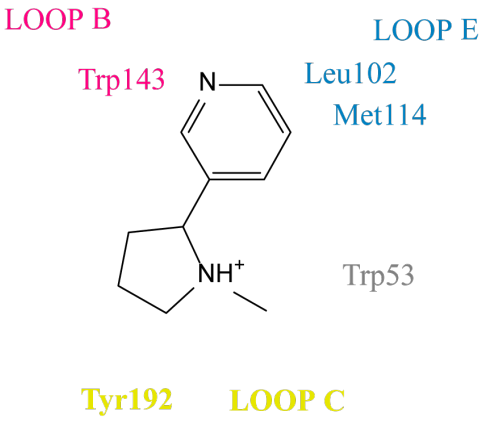
Structure of AChBP proved to be also valuable in determining structural elements which may account for neonicotinoids' selectivity. Ihara et al. (2008); Talley et al. (2008); Ihara et al. (2014) derived crystal structures of the great pond snail (*L. stagnalis*, Ls) and California sea slug (*Aplysia 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 **??** 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).

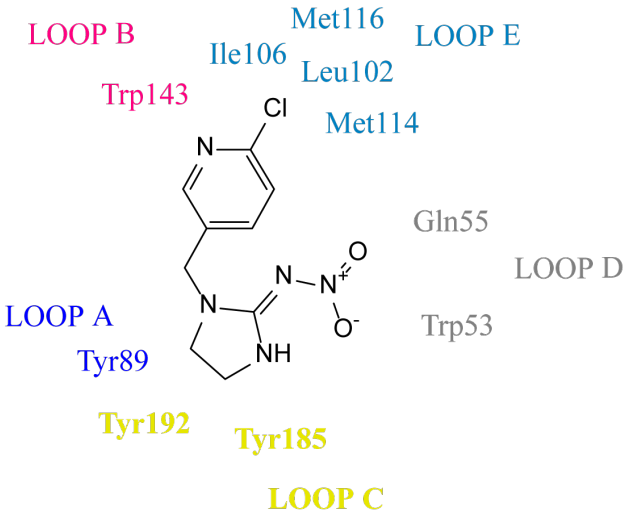
Regarding 5-membered ring interactions, in nicotine-bound structures, the cationic nitrogen forms 3 interactions when bound to AChBP: the cation-π with the ring of Trp143 (TrpB), the hydrogen bond with the backbone carbonyl of TrpB (Celie et al. 2004), as well as the cation-π interaction with Tyr192 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).



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.

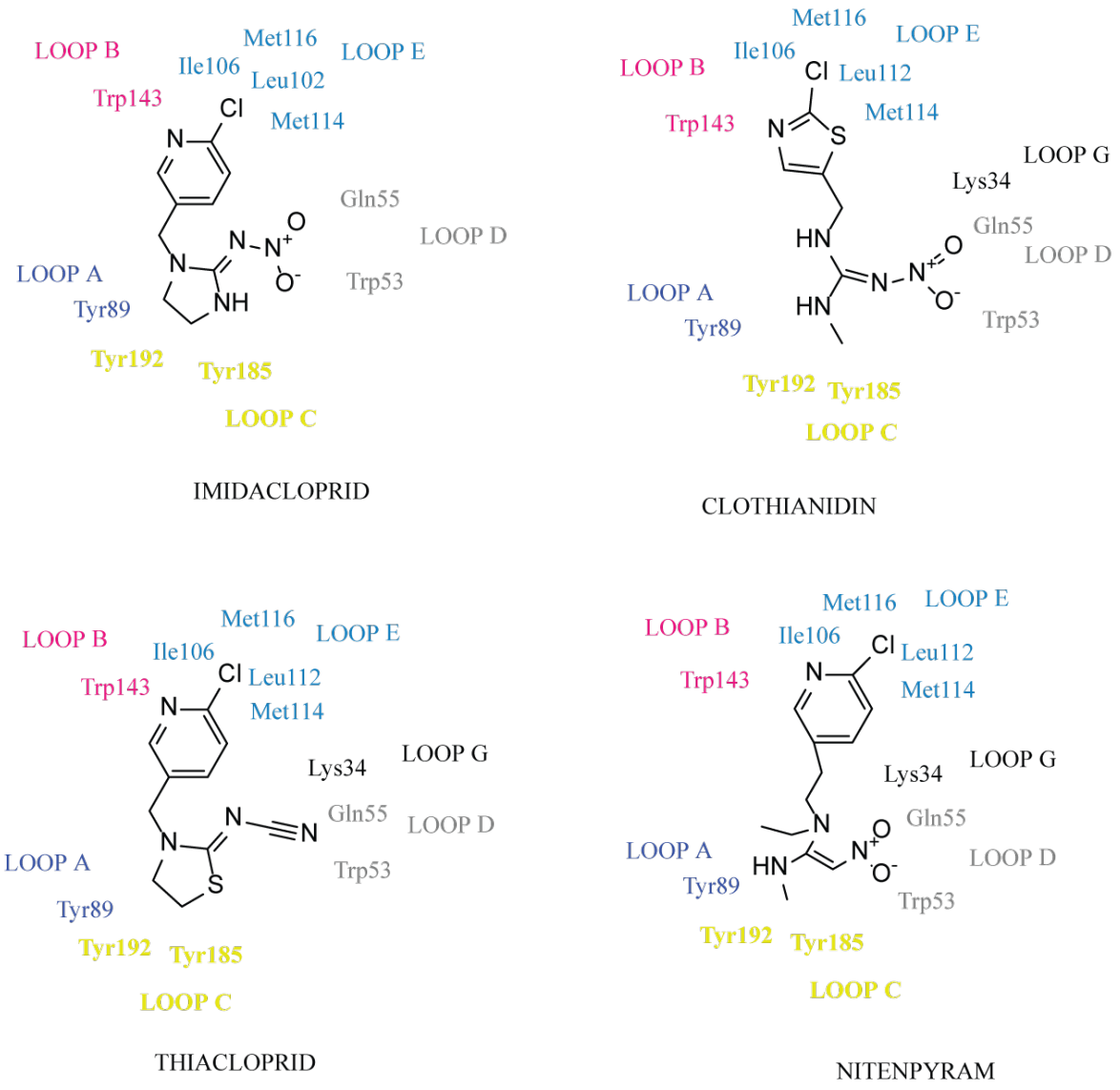


Figure 1.6: **Pharmacophore of neonicotinoids**. Schematic representation of the agonist binding site of acetylcholine binding protein, highlighting residues interacting with imidacloprid, thiacloprid, thiacloprid and nitenpyram. For nitenpyram, the interactions are predicted based on other structures.

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 $\alpha 4\beta 2$ 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

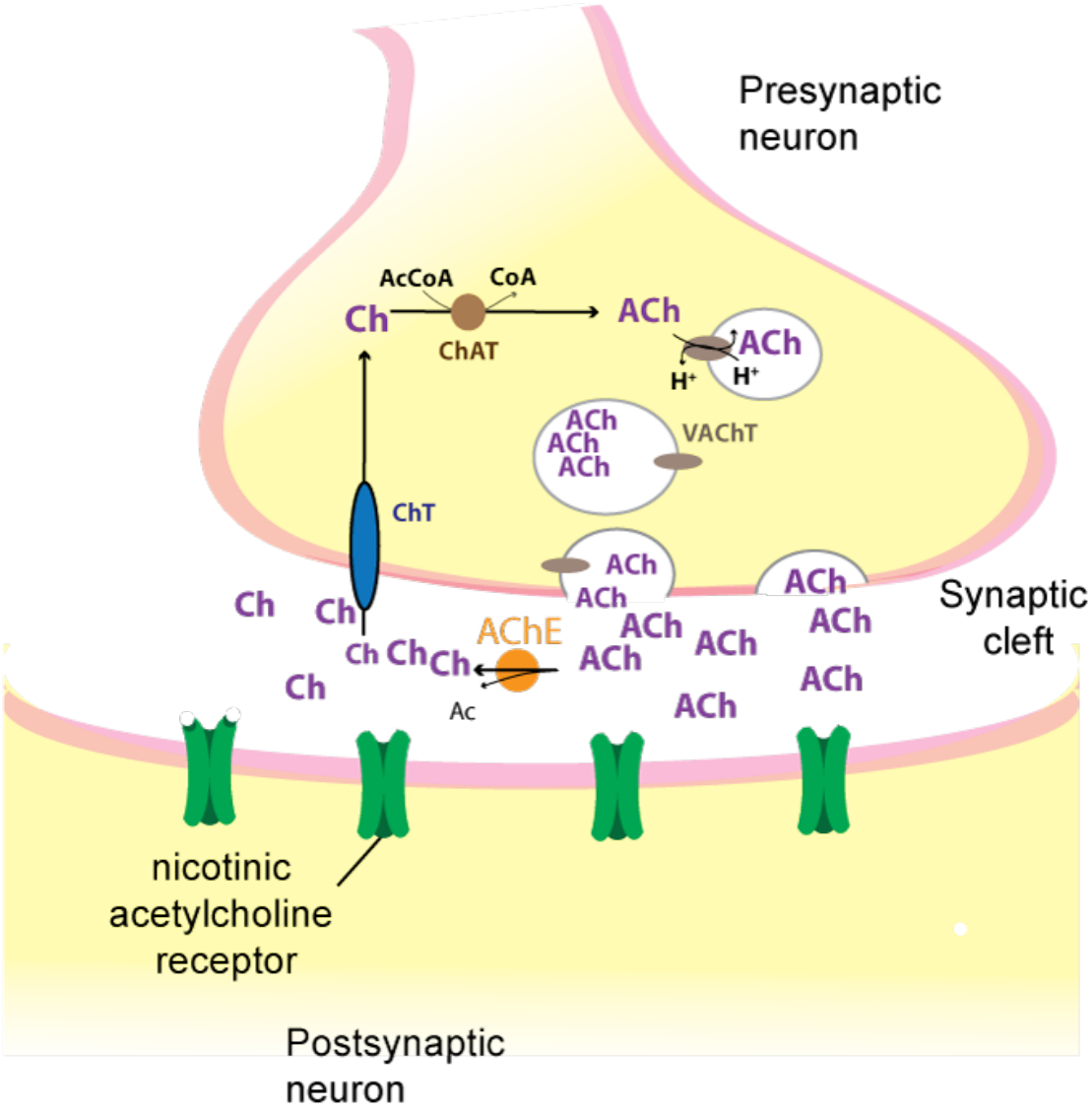


Figure 1.7: **Enzymes transporters and receptors at the cholinergic synapse.** Upon release into the synaptic cleft, acetylcholine binds to nicotinic acetylcholine receptors (nAChRs) for signal propagation. To prevent overstimulation of the post-synaptic neuron, acetylcholine is broken down to choline 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).

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 *cocroach* (Orr, Orr, and Hollingworth 1990). Incubation of α-bgtx with the ganglia of the american *cocroach* (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 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 *cocroach* 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 ~ 1.5 ms; this is interrupted by channel closing bursts of ~ 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 *cocroach* 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 *cocroach* thoracic ganglia (Tan, Galligan, and Hollingworth 2007; Thany 2009) potato beetle isolated neurons (Tan, Salgado, and Hollingworth 2008), and on cultured *cocroach* (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 neonicoinoids 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 (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.

In addition to the saturation studies, the competitive ligand binding studies were carried out. In the 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

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.

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 rectors varies, depending on the identity of the α subunit. Imidacloprid did not bind to Mp$\alpha 1$/rat$\beta 2$ receptor, whereas its Kd at Mp$\alpha 2$ and Mp$\alpha 3$-containing

receptor was 3 and 2.8 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 2.8 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
Rn$\alpha 4\beta 2$	>1000	Lansdell and Millar, 2000
Dm$\alpha 1$/Rn$\beta 2$	34.9	
Dm$\alpha 2$/Rn$\beta 2$	20	
Dm$\alpha 3$/Rn$\beta 2$	8.4	
Mp$\alpha 1$/Rn$\beta 2$	N/B	Huang et al., 1999
Mp$\alpha 2$/Rn$\beta 2$	3	
Mp$\alpha 3$/Rn$\beta 2$	2.8	
Mp$\alpha 4$/Rn$\beta 2$	N/B	
Nl$\alpha 1$/Rn$\beta 2$	24.3	Liu et al., 2005
Cf$\alpha 1$/Dm$\alpha 2$/Rn$\beta 2$	141	Bass et al. 2006
Cf$\alpha 3$/Dm$\alpha 2$/Rn$\beta 2$	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.

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
Nlα1/Rnβ2	Imidacloprid	61	Liu et al. 2009
Nlα2/Rnβ2	Imidacloprid	870	
Nlα3/Rnβ2	Imidaclorprid	350	
Nlα3α8/Rnβ2	Imidacloprid	3.2	Yixi et al. 2009
	Clothianidin	5.1	
	Thiacloprid	2.8	
	Nitenpyram	5.6	
Dmα1/Ggβ2	Imidacloprid	0.04	Dederer et al. 2011
	Clothianidin	0.34	
	Acetamiprid	0.23	
	Nitenpyram	0.4	
Dmα2/Ggβ2	Imidacloprid	0.84	
	Clothianidin	5.4	
	Acetamiprid	2	
	Nitenpyram	35.4	
Cfα1/Ggβ2	Imidacloprid	0.02	
	Clothianidin	0.15	
	Acetamiprid	0.11	
	Nitenpyram	0.63	
Cfα2/Ggβ2	Imidacloprid	1.31	
	Clothianidin	1.65	
	Acetamiprid	2.63	
	Nitenpyram	24.4	
Cfα4/Ggβ2	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 eletrophysiological 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

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

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).

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.

1.3.8.0.1 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 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 **??**). 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 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

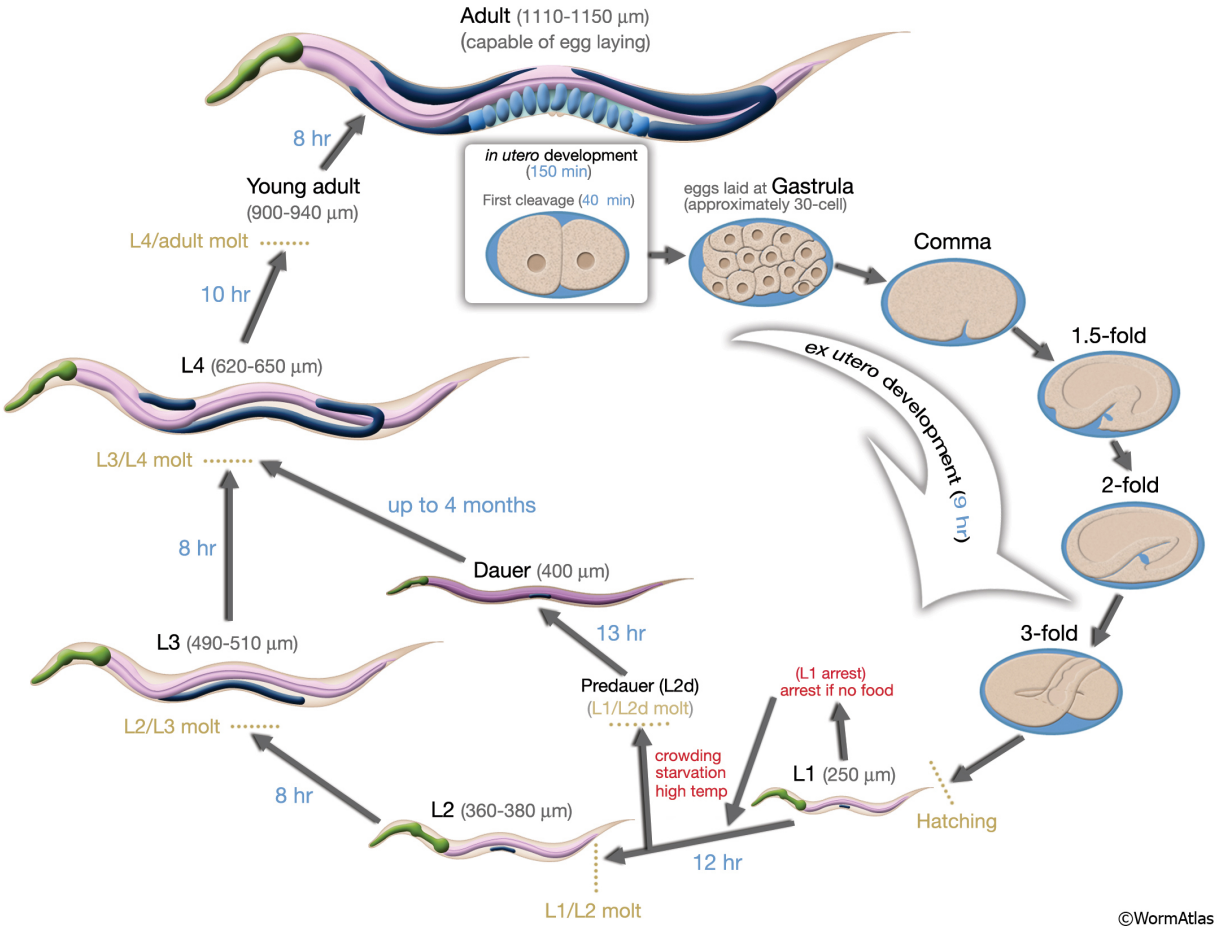


Figure 1.8: **The life cycle of *C. elegans*.** *C. elegans* develops into an adult through 4 larval stages L1 - L4. These stages are separated by molts associated with shedding of an old and exposure of a new cuticle. Adults can lay over a 1000 eggs a day which hatch within several hours. Dauer stage 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.

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 phayngeal muscle, namely the corpus, isthmus and the teminal 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 VC4/5 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, L. and H. R. Horvitz 1990; Sze et al. 2000) by acting on 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 **??**. 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 **??**) 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 **??**. 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 (Nguyen et al. 1995) 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. *C. elegans* contains 29 genes encoding for nAChR subunits (Jones et al. 2007). The receptor subunits are 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 (1.9), 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).

		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.

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).

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 **??**).

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) There are two receptor at the BWM differentiated based on their pharmacology into L-(levamisole) type and N-(nicotine) type (Richmond and Jorgensen 1999). EAT-2 is a predicted β nAChR subunit expressed in the pharyngeal muscle, believed to assemble with auxilary subunit EAT-18 (McKay et al. 2004).

1.4.8.0.1 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 body wall muscle (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.

1.4.8.0.2 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).

1.4.8.1 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 exhogenous 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.

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