

Investigating the pharmacology of ladybird alkaloids on ligand gated ion channels



**The University of
Nottingham**

By Rohit Narendra Patel, BSc (Hons), MRes

School of Life Sciences

**Thesis submitted to the University of Nottingham for the Degree of
Doctor of Philosophy**

October 2017

Abstract

The WHO has forecasted the global population to reach 9 billion by 2050 and increased productivity of crops and livestock is crucial in order to meet the heightened demand for food. Pest organisms are a major factor in limiting this productivity and pesticides are a key tool in their control. Environmental concerns and the spread of pesticide resistance have hastened the need for new pesticides. Natural toxins targeting the nicotinic acetylcholine receptor (nAChR) have proven a valuable source of lead compounds for the development of new pesticides.

Alkaloids from multiple species of ladybird beetles have been shown to target the nAChR. Recently, studies have shown that harmonine from the Harlequin ladybird *Harmonia axyridis* and (-)-adaline from the 2-spot ladybird *Adalia bipunctata* show promise as leads for new pesticides as they have demonstrated to be selective for the neuronal-type nAChRs expressed in locust neurons over mammalian muscle-type nAChRs expressed in human TE671 cells. However, mammalian neuronal-type nAChRs need to be investigated in order to confirm this selectivity.

The *Xenopus* oocyte expression system was used to investigate the effects of (-)-adaline and whole alkaloid extract from the harlequin ladybird (HAE) on multiple mammalian neuronal-type nAChRs and the hybrid $D\alpha 2/\beta 2$ *Drosophila*/chick receptor using the two electrode voltage-clamp technique. This technique also allowed the expression and investigation of α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors. HAE was found to be selective for the insect hybrid $D\alpha 2/\beta 2$ over the other mammalian receptors investigated with an IC_{50} (95% CI) of 0.44 μ g/ml (0.211 – 0.91). Analysis of the current-

voltage (IV) relationship of HAE inhibition showed voltage-dependent block of rat $\alpha 4\beta 2$ and voltage-independent block of rat $\alpha 3\beta 4$ and human $\alpha 7$. Examination of the effect of HAE on the ACh EC_{50} revealed competitive block for rat $\alpha 4\beta 2$ and potential non-competitive block of human $\alpha 7$ and rat $\alpha 3\beta 4$.

Selectivity for the hybrid insect/chick receptor was not demonstrated with (-)-adaline instead showing selectivity for rat $\alpha 3\beta 4$ with an IC_{50} (95% CI) of 0.66 μM (0.24 – 1.67). (-)-adaline was found to competitively block rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ with potential non-competitive block of human $\alpha 7$. (-)-adaline was also found to block in all nAChRs examined in a voltage-independent manner.

As HAE showed selectivity for the hybrid $D\alpha 2/\beta 2$ receptor there is promise for component(s) of HAE to serve as lead structures for the development of new pesticides. However, HAE is a mixture of various alkaloids that could have differing modes of action. This study revealed a competitive mode of action consistent with binding to the ACh binding site and voltage-dependent block consistent with open channel blocking on rat $\alpha 4\beta 2$. Separation of HAE into individual components needs to be carried out for further investigation. HAE was also found to inhibit the rat GluN1-1a/2A NMDA receptor with an IC_{50} (95% CI) of 0.179 $\mu g/ml$ (0.116 – 0.278) at $V_h = -75$ mV indicating a potential therapeutic use in neurodegenerative diseases associated with NMDA receptor excitotoxicity as well as pesticidal use.

Acknowledgements

This thesis would not have been possible without the amazing support of my supervisors Dr Ian Duce and Dr Ian Mellor at the University of Nottingham. Their patience and guidance throughout the years is something I can never thank them enough for.

I would like to thank Dr Mike Birkett and Prof John Pickett at Rothamsted Research for providing the help and facilities needed to produce the material required for experiments. I would also like to give a big thanks to Dr David Richards, Dr Mark Burton, Dr Hamid Kachel, Dr Saleh Alhewairini, Dr Abu Izuddin, Dr Mahmoud Sherif, Dr Alix Blockley, John Grzeskowiak, Neville Ngum, Maria Koromina and everyone else who has been a part of the A70/A74 Electrophysiology team. Their friendship and dedication to their work was an inspiration and kept me going throughout the project.

I would also like to express my deep gratitude to Prof David Sattelle at the University of Manchester/University College London who gave me my start in scientific research. His teaching and supervision was essential in compelling me to go into further education/research. Many thanks also go to Dr Kristin Lees, Dr Behrooz Esmaeili, Dr Steve Buckingham and Dr Frederick Partridge at the University of Manchester/University College London for their help, generosity and for providing me with material essential for current and future work.

Last but not least I would like to express my eternal gratitude to my family; especially my parents for their encouragement and backing throughout my education. A special mention should also go out to Yogesh Bhai and family for providing me a

place to stay during my visit to Rothamsted Research which allowed the production of material essential for this project.

Contents

Abstract	2
Acknowledgements.....	4
Contents	6
Abbreviations	9
CHAPTER 1: Introduction	11
1.1 The need for investigating natural products to find promising new leads ..	11
1.2 Natural products as pesticides	13
1.3 The Nicotinic acetylcholine receptor.....	18
1.4 The insect nicotinic acetylcholine receptor.....	26
1.5 Ladybird beetles	30
1.6 Chemical defence of ladybird beetles	33
1.7 Ladybird beetle alkaloids.....	36
1.8 Aims	39
CHAPTER 2: MATERIALS & METHODS	41
2.1 Extraction and Purification of Ladybird Alkaloids.....	41
2.1.1 Collection of ladybird beetles	41
2.1.2 Solvent Extraction	41
2.1.3 Acid-Base Extraction.....	42
2.1.4 Liquid column and thin layer chromatography	43
2.2 Molecular Biology	44
2.2.1 Transformation of <i>E. coli</i> with plasmid DNA.....	44
2.2.2 Isolation of plasmid DNA from transformed <i>E. coli</i>	46
2.2.3 Linearization of plasmid DNA	47
2.2.4 cRNA Production.....	48
2.3 Electrophysiological recordings of <i>Xenopus laevis</i> oocytes	49
2.3.1 The Two electrode voltage-clamp technique.....	49
2.3.2 Preparation and injection of oocytes	52
2.3.2 Two-electrode voltage clamp procedure	53
2.4 Experimental procedure and data analysis	55

2.4.1 Measuring peak, late current and area under graph	55
2.4.2 Determining the EC ₅₀ value	57
2.4.3 Determining the IC ₅₀ value	58
2.4.4 Current-Voltage Experiments	59
2.4.5 Effect of ladybird alkaloid on EC ₅₀	60
CHAPTER 3: Characterising the effects of ladybird alkaloids on LGICs expressed in <i>Xenopus</i> oocytes	62
3.1 Characterising the responses of nAChRs expressed in <i>Xenopus</i> oocytes to ACh	62
3.1.1 Introduction	62
3.1.2 ACh EC ₅₀ of nAChRs expressed in <i>Xenopus</i> oocytes	62
3.1.3 Current-voltage relationship of ACh responses	68
3.1.4 Response of nAChRs to repeated applications of ACh	70
3.2 Analysis of alkaloid fractions from <i>Harmonia axyridis</i> and <i>Adalia bipunctata</i>	72
3.3 Concentration-inhibition relationship of HAE and (-)-adoline on mammalian and insect/mammalian hybrid neuronal nAChRs	75
3.4 Current-voltage relationship of HAE and (-)-adoline on mammalian neuronal nAChRs	89
3.5 Effect of HAE and (-)-adoline on ACh EC ₅₀ of mammalian neuronal nAChRs	98
3.6 Effect of HAE and (-)-adoline on glutamate LGICs	101
CHAPTER 4 - Discussion	107
4.1 Effects of ACh on mammalian and hybrid mammalian nAChRs	107
4.2 <i>Harmonia axyridis</i> alkaloid extract inhibits neuronal-type nicotinic acetylcholine receptors expressed in <i>Xenopus</i> oocytes	108
4.3 Mechanism of inhibition of <i>Harmonia axyridis</i> extract on nicotinic acetylcholine receptors differs depending on the receptor subtype	111
4.4 Voltage-dependent inhibition of NMDA glutamate receptors by <i>Harmonia axyridis</i> extract	113
4.5 (-)-adoline inhibits neuronal-type nicotinic acetylcholine receptors expressed in <i>Xenopus</i> oocytes	115
4.6 Mechanism of inhibition of (-)-adoline on neuronal-type nicotinic acetylcholine receptors	117

4.7 Some selectivity was observed for insect nAChRs over mammalian nAChRs for <i>Harmonia axyridis</i> alkaloid extract but not (-)-adoline	118
4.8 Bioinformatic analysis of nAChR subunits	120
4.9 Investigation of potential therapeutic applications of HAE is promising as inhibition of NMDARs is exhibited.....	123
4.10 Alkaloids contribute to the chemical defence of <i>Harmonia axyridis</i> and <i>Adalia bipunctata</i>	123
4.11 Conclusions	126
References	128
Appendix.....	156

Abbreviations

2-spot ladybird	<i>Adalia bipunctata</i>
7-spot ladybird	<i>Coccinella septempunctata</i>
ACh	Acetylcholine
AChBP	Acetylcholine binding protein
AChR	Acetylcholine receptor
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AUG	Area under graph
CI	Confidence interval
CNS	Central nervous system
Da2/ β 2	<i>Drosophila</i> SADa2/chick β 2 nicotinic acetylcholine receptor
EC ₅₀	Concentration at which half maximal effect of agonist is observed
ECD	Extracellular domain
GC-MS	Gas chromatography-mass spectrometry
HAE	<i>Harmonia axyridis</i> whole alkaloid extract
Harlequin ladybird	<i>Harmonia axyridis</i>
I	Current
IV	Current-voltage
IC ₅₀	Concentration at which half maximal effect of inhibitor is observed
KA	Kainic Acid
LGIC	Ligand-gated ion channel
m-nAChR	Muscle-type nicotinic acetylcholine receptor
mAChR	Muscarinic acetylcholine receptor
n-nAChR	Neuronal-type nicotinic acetylcholine receptor
nAChR	Nicotinic acetylcholine receptor

NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
PNS	Peripheral nervous system
SEM	Standard error of the mean
TEVC	Two-electrode voltage clamp
TM	Transmembrane
V	Voltage
V _h	Holding potential

CHAPTER 1: Introduction

1.1 The need for investigating natural products to find promising new leads

The global population is rapidly increasing and expected to reach 9 billion by 2050. The Food and Agriculture Organisation of the United Nations has projected that a 70% increase in food production is needed to meet the increasing demands of this population growth. The current rate at which food production is increasing for various key crops is forecasted to be insufficient to meet this demand (Ray *et al.* 2013). In order to increase this, the productivity of the land currently used needs to be maximised. A major limiting factor in the productivity of crops worldwide is insect pests that feed on crops. Insecticides have been used as a form of control dating back to the 19th century (Casida & Quistad 1998). Natural compounds were mainly used until 1945 when synthetic, organic insecticides were introduced. These allowed further improvements in agricultural productivity. By 2005, worldwide sales of insecticides reached US\$ 7.798 billion, accounting for 25% of global pesticide sales (Zhang *et al.* 2011).

A new class of organic, synthetic insecticides with a new target named the neonicotinoids was developed with the first compound, Imidacloprid, made commercially available in 1991 (Bai *et al.* 1991). In 2006, sales of neonicotinoids reached US\$ 1.56 billion representing a 17% share of the global insecticide market (Jeschke & Nauen 2008). Their success (Fig. 1.1.1) was helped by their initial ability to be effective in populations resistant to other insecticides and without resistant populations arising. However resistance has been reported due to overuse which will

limit their effectiveness in areas where resistance is present (Nauen & Denholm 2005; Bass *et al.* 2015). Resistance to synthetic pyrethroids, another main group of insecticides representing a large market share, has also been reported (Højland *et al.* 2015). Considerable economic losses are predicted if insecticide usage is stopped (Webster *et al.* 1999) and so alternatives are required in order to combat this resistance.

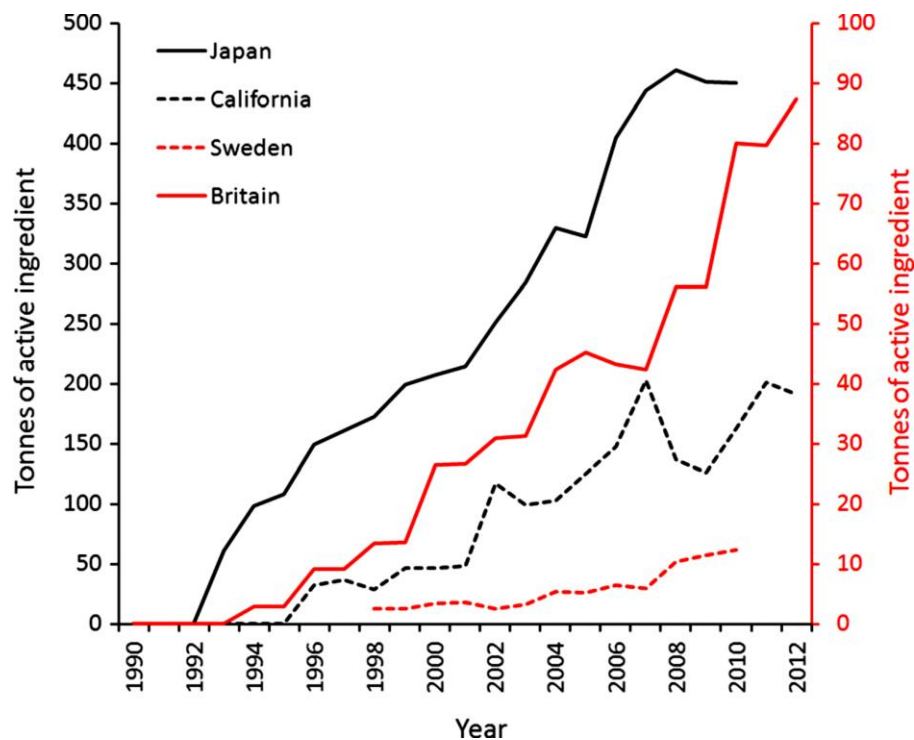


Fig. 1.1.1: Combined sales and usage of neonicotinoids and fipronil, an insecticide from a different class to the neonicotinoids. Japan shows domestic shipments, California shows use, Sweden shows sales and Britain shows agricultural use. The y-axis for Japan/California and Sweden/Britain are separate. Image from (Simon-Delso *et al.* 2015).

Another area of concern for neonicotinoids is the implied link between neonicotinoids and colony collapse disorder of honeybee populations. Exposure to neonicotinoids has been implicated as the cause of many unhealthy behaviours in honeybee populations that have in turn been linked to colony collapse disorder, the sudden disappearance of large populations of honeybees. Sub lethal doses of

neonicotinoids have been linked to adversely affecting the reproductive system of hive queens (Williams *et al.* 2015) and interfering with motor and sensory functions (Blacqui re *et al.* 2012). Infection of honeybee colonies by parasitic organisms is also a major concern and neonicotinoids are thought to play a role in compromising the immune system of honeybees making colonies more susceptible to infections (Alaux *et al.* 2010; Di Prisco *et al.* 2013). Concerns about the decline in populations of pollinators in general and their importance to the pollination of both crop and wild plants (Blacqui re *et al.* 2012) has led to a temporary ban of the use of neonicotinoids in the EU (Gross 2013).

1.2 Natural products as pesticides

Natural products created by organisms as a defence mechanism against pests and predators have proved invaluable resources in the search for leads for new pesticides and for other potential commercial uses. The nicotinoid compound nicotine found in the leaves of tobacco plants of the genus *Nicotiana* is present as a defence mechanism. Nicotine exhibits insecticidal activity and has proved to be of great commercial value (Steppuhn *et al.* 2004). Naturally occurring nicotinoid compounds such as nicotine and epibatidine, isolated from the skin of the Ecuadoran frog *Epipedobates anthonyi*, served as lead compounds for the development of neonicotinoids. Structural components of these nicotinoids were identified and used to create new compounds with improved activity (Tomizawa *et al.* 2005). Plants from the genus *Chrysanthemum* naturally contain pyrethrins that, like nicotine, are present as a defence mechanism for the plant. Natural pyrethrins are unstable in direct

sunlight and so pyrethroids, synthetic analogues of natural pyrethrins, were created (Elliott 1976). Natural compounds from animals have proven useful too. Investigation of nereistoxin from the annelid worm *Lumbriconereis heteropoda* showed insecticidal activity and synthetic analogues were also produced which has led to the commercial release of the insecticide Cartap, a synthetic analogue of nereistoxin (Sattelle *et al.* 1985). Spinosyns produced by the actinomycete, *Saccharopolyspora spinosa*, were also found to have insecticidal activity and a mixture of spinosyn A and D is commercially available as the insecticide Spinosad (Millar & Denholm 2007).

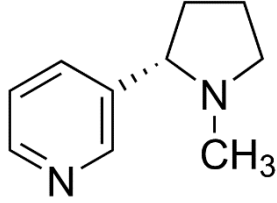
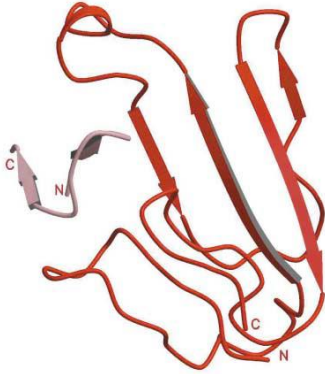
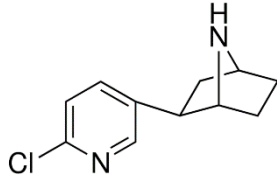
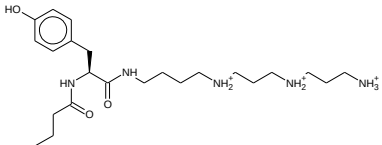
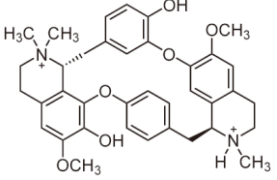
Nicotine, neonicotinoids, Spinosad and Cartap all target the nicotinic acetylcholine receptor (nAChR), a ligand-gated ion channel located in the central nervous system (CNS) of insects (Millar & Denholm 2007; Bai *et al.* 1991; Sattelle *et al.* 1985). Acetylcholine (ACh) is the main neurotransmitter in the insect CNS (Breer & Sattelle 1987) and so disruption of ACh transmission can be an effective mechanism for insecticidal activity which is proven by the number of insecticides targeting the nAChR on the market. Among the first natural toxins found to target the nAChR were α -bungarotoxin (α -BgTX) from the krait snake *Bungarus multicinctus* and d-tubocurarine from the vine plant *Chondrodendron tomentosum*. These toxins were not developed into commercial insecticides but have proved crucial as tools for characterising the nAChR (Changeux *et al.* 1970).

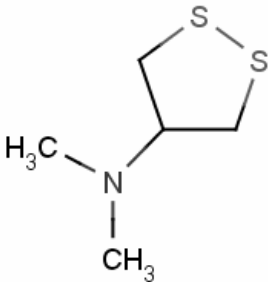
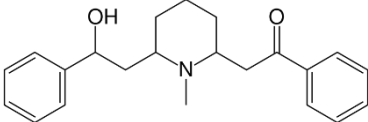
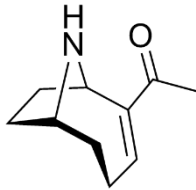
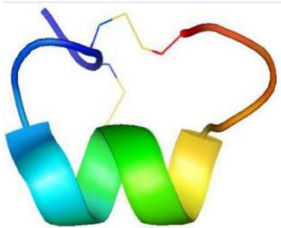
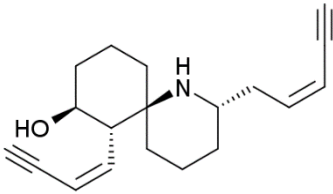
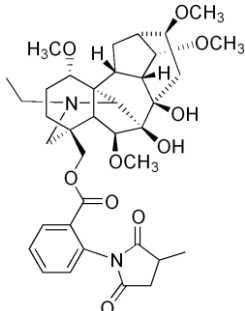
Many other venoms and toxins from the natural world have exhibited activity on the nAChR indicating a frequent mode of action for a naturally occurring chemical defence system (Table 1.2.1). The cone snail, *Conus purpurascens*, contains a family of peptides collectively known as α A-conotoxins that target the nAChR (Hopkins *et al.* 1995; Jacobsen *et al.* 1997). A variety of different venoms from species belonging to

the order Hymenoptera including wasps and ants were found to target the nAChR (Zalat *et al.* 2005). The solitary digger wasp, *Philanthus Triangulum*, was found to contain philanthotoxin (PhTX) which targeted NMDA and AMPA glutamate receptors as well as nAChRs (Rozental *et al.* 1989; McClymont *et al.* 2012; Franzyk *et al.* 2014a). Any nAChR targeting venom/toxin discovered has the potential of targeting other receptors as well. PhTX has served as a lead structure for a number of synthetic analogues that also target nAChRs (Mellor *et al.* 2003; Brier *et al.* 2003; Olsen *et al.* 2006). Potential useful biological activity has encouraged chemical syntheses of different natural toxins and subsequent screening for activity. This has led to the identification of the alkaloids *cis*-223B and xenovenine (Barthelme *et al.* 2013) and 5-*epi-cis*-275B (Procopiou *et al.* 2014).

As a variety of different species contain nAChR targeting venoms/toxins as a natural defence mechanism and given that targeting the nAChR has proved an effective method for producing insecticidal activity, investigation of venoms/toxins from organisms mainly predated upon by other insects would have a good probability of revealing further nAChR targeting venoms/toxins. Such candidates could then serve as lead compounds for the development of a novel insecticide.

Table 1.2.1 – Naturally occurring nAChR-targeting venoms/toxins

Species	Venom/Toxin Name	Mode of Action	Chemical Structure
Tobacco Plants (<i>Nicotiana</i>)	Nicotine	Agonist (Papke <i>et al.</i> 2007)	
Taiwanese many-banded krait snake (<i>Bungarus multicinctus</i>)	α -Bungarotoxin	Irreversible antagonist (Harel <i>et al.</i> 2001)	
Ecuadorian poison-arrow frog (<i>Epibatodes tricolor</i>)	Epibatidine	Agonist (Marks <i>et al.</i> 2010)	
Egyptian solitary wasp (<i>Philanthus triangulum</i>)	Philanthotoxin-433	Non-competitive antagonist (Kachel <i>et al.</i> 2016)	
South American vine (<i>Chondrodendron tomentosum</i>)	d-Tubocurarine	Competitive antagonist (Bowman 2006)	

Marine annelid worm (<i>Lumbrinereis heteropoda</i>)	Nereistoxin	Antagonist (Sattelle <i>et al.</i> 1985)	
Plants of the genus <i>Lobelia</i>	Lobeline	Non-competitive antagonist (Hillmer <i>et al.</i> 2013)	
Cyanobacteria	Anatoxin-a	Agonist (Wonnacott & Gallagher 2006)	
Cone snails of the genus <i>Conus</i>	α -conotoxin Vc1.1	Competitive antagonist (Lebbe <i>et al.</i> 2014)	 <chem>GCCSDPRCNYDHPEIC</chem>
Harlequin poison-dart frog (<i>Oophaga histrionica</i>)	Histrionicotoxin 283A	Non-competitive antagonist (Betz 1982)	
Larkspur plants of the genus <i>Delphinium</i>	Methyllycaconitine	Competitive antagonist (Panagis <i>et al.</i> 2000)	

1.3 The Nicotinic acetylcholine receptor

The nAChR was first theorised in 1906 when experiments on the effects of nicotine on the muscle contraction of fowl birds led to the conclusion that a “receptive substance” was responsible for the binding of nicotine and the generation of muscle contraction (Changeux *et al.* 1984). This theory was given further credence when a protein “which presents (in solution) several characteristic properties of the cholinergic receptor macromolecule” was isolated from the electric organ of the electric eel, *Electrophorus electricus*. Binding of α -BgTX to the electric organ was found to irreversibly block the actions of cholinergic agonists, stop depolarisation of the organ membrane, prevent binding of the radiolabelled cholinergic agonist decamethonium and prevent binding to the theorised receptor macromolecule in solution (Changeux *et al.* 1970).

The nAChR is a transmembrane pore activated by the binding of the ligand ACh and due to this it is referred to as a ligand-gated ion channel (LGIC). LGICs are broadly split into Cys-loop and ionotropic glutamate receptor families with ACh receptors belonging to the Cys-loop family. Cys-loop receptors are characterised by a disulphide bond linking two highly conserved cysteine residues on the extracellular $\beta 6$ and $\beta 7$ sheets (Morales-Perez *et al.* 2016). nAChRs are distinct from other ACh receptors in that they are selectively sensitive to nicotine and are ionotropic as opposed to metabotropic like muscarinic ACh receptors (Changeux & Edelstein 2006).

The nAChRs of the electric ray genus *Torpedo* have been studied extensively and have contributed greatly to the understanding of the structure and function of nAChRs. The *Torpedo* nAChR was found to consist of 5 transmembrane subunits (2α ,

β , γ and δ subunits) (Reynolds & Karlin 1978). Investigations into the arrangement of the subunits showed that the subunits formed a barrel around a central pore and that the subunits had to be arranged in a specific order (Fig. 1.3.1). If the β subunit was sandwiched between 2 α subunits then binding sites for ACh could not form (Karlin *et al.* 1983). The DNA/mRNA sequence of each subunit was cloned with all 4 resultant protein subunits proposed to have 4 transmembrane domains due to the identification of 4 regions containing approximately 20 uncharged, mainly hydrophobic amino acid residues surrounded by charged residues on α subunits (Devillers-Thiery *et al.* 1983) (Fig. 1.3.2). Similar regions were found in β , γ and δ subunits indicating that the 4 transmembrane domains could be a common feature between different subunits (Claudio *et al.* 1983; Noda *et al.* 1982; Noda *et al.* 1983). Further analysis of the subunit structure showed that all 4 *Torpedo* subunits were similar in size and formed rod shapes arranged in a ring perpendicular to the cell membrane. This ring structure is 160 Å in length and 80 Å in diameter (Unwin 2013).

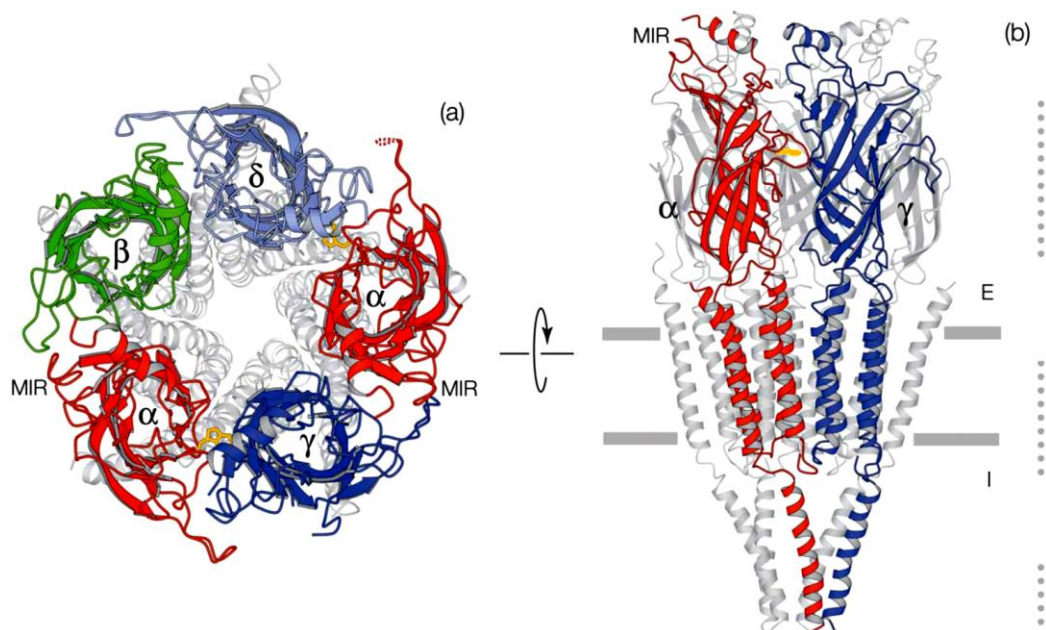


Fig. 1.3.1: Ribbon diagram showing the pentameric subunit arrangement for the *Torpedo* 2 α β γ δ nAChR as viewed from (a) the extracellular side of the membrane and (b) perpendicular to the cell membrane. Image from (Unwin 2005).

Xenopus oocytes can be used to express non-native proteins where injection of RNA into unfertilised oocyte can allow the expression of the encoded functional protein. Initially this was used to express haemoglobin (Gurdon *et al.* 1971). Injection of mRNA extracted from *Torpedo* electric organ caused the formation of functional ACh receptors giving the potential for the *Xenopus* oocyte expression system to be used as tool for investigating nAChRs (Barnard *et al.* 1982). Later, injection of different nAChR subunits and subsequent measuring of the expression levels of the *Torpedo* nAChR was used to investigate which combinations of subunits were required to form functional receptors. All four *Torpedo* subunits were found to be required to form nAChRs with normal sensitivity to ACh and reversible block by d-tubocurarine. Functional receptors without δ subunits can form with a lowered sensitivity to ACh. The reversal potential (membrane potential at which there is no net flow of ions) of the expressed receptors was also not changed by the removal of Cl^- ions indicating the expressed receptors were cation selective (Takahashi *et al.* 1985).

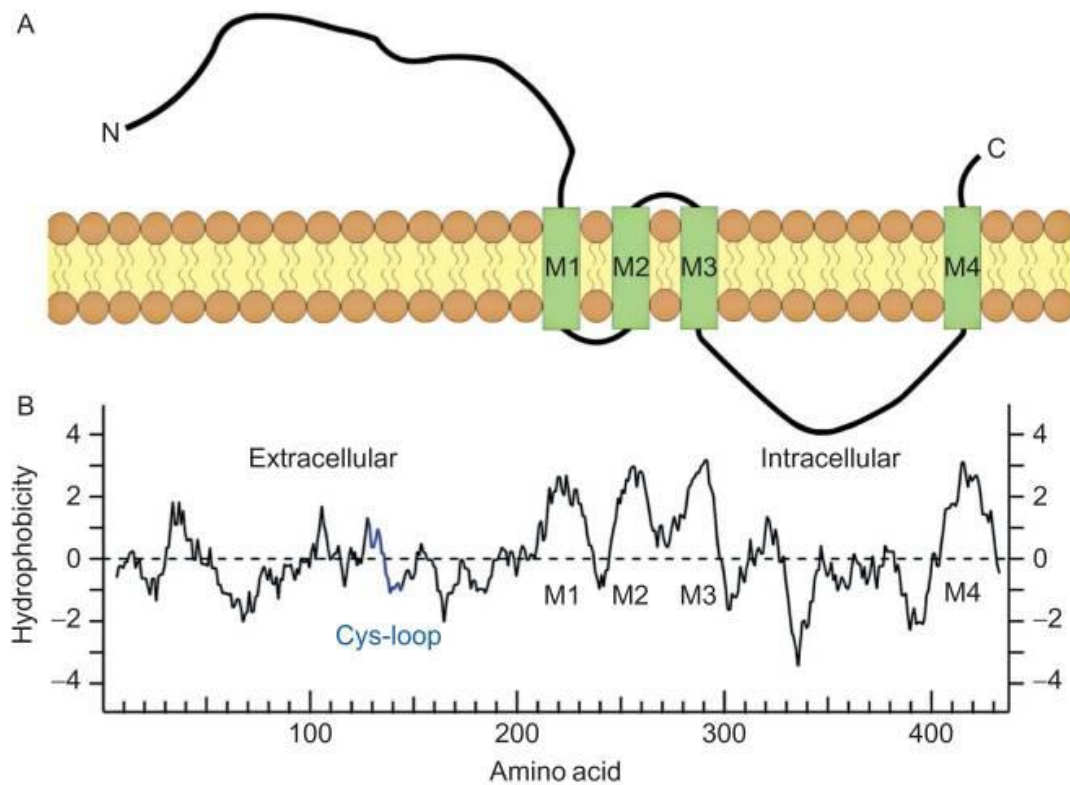


Fig. 1.3.2: (A) A diagram showing the arrangement of the amino acid sequence of a nAChR subunit with 4 transmembrane domains (M1-M4) through the cell membrane from N terminus to the C terminus. (B) Hydrophobicity profile of the human $\alpha 1$ subunit aligned with (A) to show regions of high hydrophobicity corresponding to the transmembrane domains. Image from (Dani 2015).

Examination of the rat central nervous system resulted in the identification of multiple α and β subunits that were insensitive to α -BgTX, a blocker of the *Torpedo* nAChR, indicating a pharmacologically distinct set of nAChRs classified as neuronal-type nAChRs (n-nAChRs) (Boulter *et al.* 1987a; Boulter *et al.* 1987b). $\alpha 2$ - $\alpha 9$ and $\beta 2$ - $\beta 4$ subunits were identified (Changeux & Edelstein 2006) and later $\alpha 10$ was identified (Lustig *et al.* 2001). Injection of cRNA into *Xenopus* oocytes revealed that $\alpha 7$ subunits could form functional receptors when injected alone (Thany *et al.* 2007) but $\alpha 3$ and $\alpha 4$ required co-injection of $\beta 2$ suggesting that homomeric and heteromeric n-nAChRs existed (Boulter *et al.* 1987a). Analysis of chick neuronal $\alpha 4$ and non- α nAChR subunits revealed that a stoichiometry of 2α and 3 non- α existed in functional heteromeric n-

nAChRs similar to muscle-type (Cooper *et al.* 1991). N-nAChRs in the chick retina were discovered to bind α -BgTX indicating the existence of different subtypes of n-nAChRs (Keyser *et al.* 1993). The $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs were found to have 2 variants consisting of $2\alpha-3\beta$ and $3\alpha-2\beta$ forms with the latter form having an increased sensitivity to ACh (Briggs *et al.* 2006). Analysis of the X-ray crystallographic structure of the $2\alpha-3\beta$ form of $\alpha 4\beta 2$ confirmed a subunit arrangement of $\alpha-\beta-\beta-\alpha-\beta$ in a pentameric ring. The presence of a disulphide bond formed between adjacent cysteines at the tip of Loop C in the $\alpha 4$ subunits characteristic of nAChR α subunits was also confirmed (Morales-Perez *et al.* 2016). LGICs were found to be conserved in prokaryotic organisms, a ligand-gated channel from *Gloeobacter* (GLIC), a homologue of the *Torpedo* nAChR was discovered as well as a channel from *Erwinia chrysanthemi* (ELIC). These are easily expressed in bacterial systems and share very similar structures so have been used as models of nAChRs from eukaryotic organisms (Hilf & Dutzler 2009). Structural analysis of GLIC showed hydrophobic residues dominating the extracellular domain, polar residues dominating the intracellular domain that make the domain hydrophilic and both ends of the channel being lined with acidic residues providing a negatively charged environment which contributes to the selectivity of the channel for positively charged cations. This structure was found to be conserved in nAChRs (Hilf & Dutzler 2009). GLIC has also been used to investigate the interaction between LGICs and membrane lipids. A study using GLIC has proposed that lipids influence the coupling of agonist binding and opening/closing of the channel via interaction with the M4 transmembrane domain. The M4 domain interacts with the Cys-loop which has a role in agonist binding and so lipids could modulate agonist binding via interaction with the M4 domain (Labriola *et al.* 2013).

The binding of ACh elicits a number of conformational changes that results in the transient opening of the channel allowing the cell membrane to be permeable to cations. A disulphide bond on the N-terminal of α subunits is implicated in the binding of ACh and is created by the interaction of two adjacent cysteine residues (Cys192 & Cys193) that are conserved in α subunits (Kao & Karlin 1986). The bond is readily reducible causing conformational changes in the receptor which allows affinity labelling agents to bind. Use of the affinity label [^3H]-4-(N-maleimidobenzyl) trimethylammonium revealed the two cysteine residues to be extracellular and close to the binding site of ACh. Cys192 and Cys193 were also found to be conserved across species boundaries (Karlin 2002) (Fig. 1.3.3). Conversion of Cys128 and Cys142 in α subunits to serine residues abolished both the ACh response and α -BgTX binding indicating these residues also play a key role in ACh binding (Mishina *et al.* 1985). Cys128 and Cys142 also form disulphide bonds and are conserved in beta, gamma and delta subunits in an extracellular region (Kao and Karlin, 1986).

Acetylcholine binding protein (AChBP) is a soluble protein found in the snail *Lymnaea stagnalis* most similar to the homomeric $\alpha 7$ subtype of nAChR (Olsen *et al.* 2014; Smit *et al.* 2001) consisting of N-terminal sequences the same length as those of pentameric LGICs (approx. 210 amino acids), has 15-28% sequence similarity to all LGICs (Celie *et al.* 2004), contains almost all residues conserved in the nAChR family, binds known nAChR ligands and forms cavities lined by residues known to be involved in ligand binding (Brejc *et al.* 2001). Studies have revealed that the AChBP ACh binding site is formed by a series of loops (A, B and C) from the principal face of one subunit and another series of loops (D, E and F) from the complementary face of an adjacent subunit (Fig. 1.3.4). This binding site contains a double cysteine motif conserved in α

subunits of nAChRs. Also, a Cys123-Cys136 disulphide bond exists which is important for the packing of β -sheets and explains the importance of Cys128-Cys142 in *Torpedo* nAChRs (Brejc *et al.*, 2001). An analysis of AChBP binding to nicotine, HEPES and carbamylcholine confirmed the binding of ligands to the interface between subunits by making contact with loops A, B and C on the principle face of the AChBP subunit. When HEPES is bound, loop C was found to make significant backbone movements and all side chains involved in binding were rearranged to accommodate binding. The Lys139 residue moved and formed a hydrogen bond with Tyr185 confirming previous indications that Tyr190 (the equivalent of Tyr185) in α subunits is critical for ACh binding (Celie *et al.*, 2004). The movements caused by ACh binding to the *Torpedo* nAChR was also investigated. Binding caused outward displacement of the β subunit transmembrane helices which caused the pore lining helices of the other subunits to straighten when they are normally bent inwards. The inward bend is thought to contribute to forming the channel gate and so this movement allows the opening of the gate (Unwin & Fujiyoshi 2012). The $\alpha 4\beta 2$ nAChR contains 2 different binding sites, one made from the interaction between an $\alpha 4$ and $\beta 2$ subunit and one made from the interaction between an $\alpha 4$ and $\alpha 4$ subunit. The main differences between the 2 binding sites were residues on the complementary sides of the $\alpha 4$ and $\beta 2$ subunits. Those being His142, Gln150 and Thr152 on the $\alpha 4$ subunit and Val136, Phe144 and Leu146 on the $\beta 2$ subunit (Indurthi *et al.* 2016). Channel opening has also been demonstrated to occur through binding at a transmembrane cavity rather than in the extracellular domain showing that channel opening is not exclusive to binding at the ACh binding site on the extracellular domain (Gill *et al.* 2011).

nAChR α Subunit Sequences that Bind α BGT		181	185	190	195	200
Torpedo α 1	YREFWKHWVYITCCPOTPWLD					
Chick Muscle α 1	YREFWKHWVYVACCPOTPWLD					
Chick α BGTBP α 1	IPGKRTESEFYECCKE-PYPD					
Chick α BGTBP α 2	UPGKRNELYVECCKE-PYPD					
Drosophila ALS	UPAURNEKFVSCCEE-PYLD					
Drosophila SAD	UPPERHEKYVPECAE-PYLD					

nAChR α Subunit Sequences that Do Not Bind α BGT		181	185	190	195	200
Cobra Muscle α 1	YREFWHSUNYSCCLITPWLD					
Chick Brain α 2	AIGRYNSKKVDCCTE-TPD					
Chick Brain α 3	APGYKHDIKVNCCEE-TPD					
Chick Brain α 4	AVGNYSKKVECCTE-TPD					
Rat Brain α 2	ATGTYSKKVDCCAE-TPD					
Rat Brain α 3	APGYKHEIKVNCCEE-TPD					
Rat Brain α 4	AVGTYNTRVVECCAE-TPD					

Fig. 1.3.3: Sequence alignment of α subunits from different species showing conservation of Cys192 and Cys193 across different species. Image from (McLane *et al.* 1991).

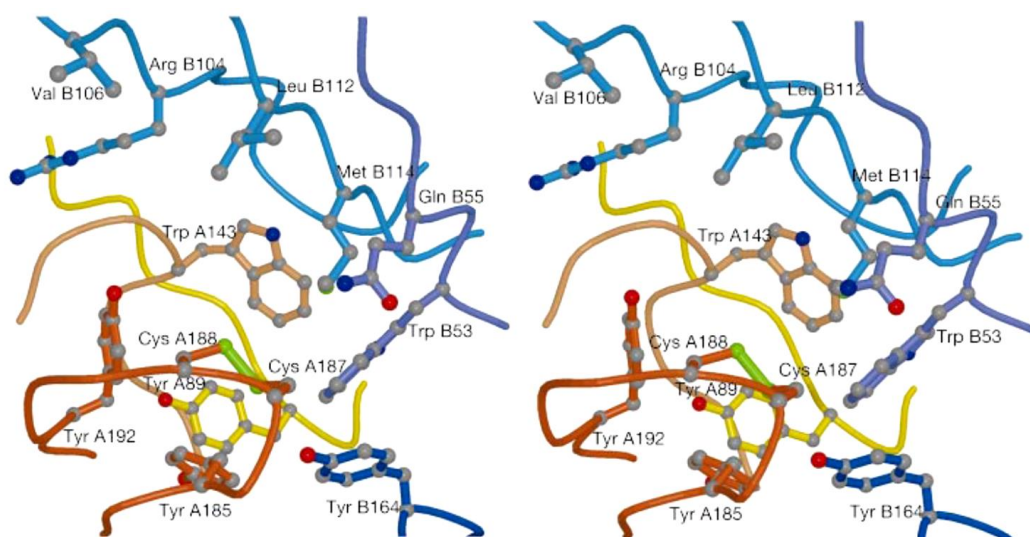


Fig. 1.3.4: Diagram of the ACh binding site of *Lymnea stagnalis* AChBP showing the residues found on the loops from the principal and complementary faces of the AChBP subunit that form the ACh binding site. Image from (Brejc *et al.*, 2001).

Continued exposure to agonist cause LGICs to enter a desensitisation state that closes the channel. This is thought to be protective in order to prevent constant stimulation of neurons or muscle fibres. Investigations have shown that the shifting of the channel from the open state to the desensitisation state could be due to movements in the transmembrane domains closing an intracellular desensitisation gate (Nemecz et al. 2016).

1.4 The insect nicotinic acetylcholine receptor

ACh is the main excitatory neurotransmitter in insects and nAChRs are abundant in the insect nervous system (Breer & Sattelle 1987). The nAChR families of various insects are known and appear to express fewer subunits (Table 1.4.1) compared to humans (16) and nematodes (29) (Jones & Sattelle 2010).

Table 1.4.1 – Currently known insect nAChR families

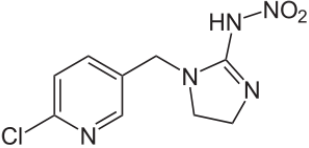
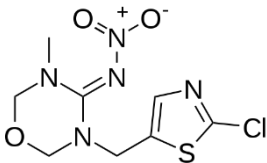
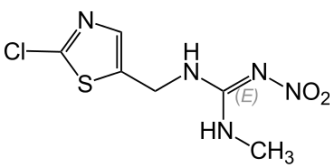
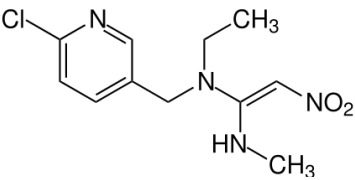
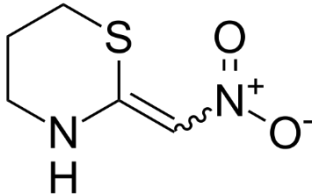
Common Name	Species Name	No. of nAChR Subunits	Reference
House fly	<i>Musca domestica</i>	10	(Scott et al. 2014)
Pea Aphid	<i>Acyrtosiphon pisum</i>	11	(Dale et al. 2010)
Parasitoid wasp	<i>Nasonia vitripennis</i>	16	(Jones et al. 2010)
Mosquito	<i>Anopheles gambiae</i>	10	(Jones & Sattelle 2010)
Honeybee	<i>Apis mellifera</i>	11	(Jones & Sattelle 2010)
Silkworm	<i>Bombyx mori</i>	12	(Jones & Sattelle 2010)
Fruitfly	<i>Drosophila melanogaster</i>	10	(Jones & Sattelle 2010)
Red Flour Beetle	<i>Tribolium castaneum</i>	12	(Jones & Sattelle 2010)

Direct recordings of insect neurons have confirmed the presence of nAChRs (H. Thany & Tricoire-Leignel 2011) and allowed the investigation of the mode of action of different neonicotinoid insecticides (Bai *et al.* 1991; Brown *et al.* 2006). However, insect nAChRs have proved difficult to express in heterologous expression systems such as the *Xenopus* oocyte expression system and therefore are not as well characterised as their nematode and mammalian counterparts. Locust (*Schistocerca gregaria*) alpha L1 subunits have expressed in *Xenopus* oocytes at low levels but the subunit composition of the receptor remains unknown (Amar *et al.* 1995). The *S. gregaria* β 1 subunit can also form functional nAChRs when co-expressed with the α 1 subunit (Jones *et al.* 2005). More recently, *Drosophila* α 5 and α 7 subunit cRNA expressed homopentameric nAChRs (Lansdell *et al.* 2012) and α 6, α 5 and *ric-3* expressed functional nAChRs (Watson *et al.* 2010) in *Xenopus* oocytes but expression of the other 7 subunits found in *Drosophila* remains elusive. This inability to form functional receptors in expression systems indicates there could be an additional component to insect nAChRs that are yet unknown. It has been proposed that in *Drosophila*, three potential complexes form: one containing at least D β 1 and D β 2, one containing at least D β 1 and D α 3 and one containing at least D α 1, D α 2 and D β 2 (Jones & Sattelle 2010).

A combination of *Drosophila* α subunits and chick β 2 subunits in *Xenopus* oocytes gives robust expression of hybrid nAChRs sensitive to α -BgTX (Bertrand *et al.* 1994) and this provides a viable alternative to expressing pure insect nAChRs. The hybrid *Drosophila* α 2/chicken β 2 (D α 2/ β 2) is more sensitive to neonicotinoids than the α 4 β 2 receptor (Ihara *et al.* 2003). This approach has successfully allowed investigation of the neonicotinoid interaction with nAChRs (Matsuda *et al.* 2009) and gives the

potential for its use in discovering novel insecticidal compounds. *Drosophila* $\alpha 1$ can also form functional hybrid receptors when co-expressed in *Xenopus* oocytes with chick $\beta 2$ (Ihara et al. 2014). The neonicotinoids represent a diverse range of structures (Table 1.4.2) and show differing actions on the insect nAChR. Dinetofuran and Nitenpyram act as full agonists while Imidacloprid acts as a partial agonist of $D\alpha 2/\beta 2$ (Matsuda et al. 2009). Comparison of the effect of neonicotinoids on native receptors examined using patch-clamp electrophysiology showed that the rank order of potency observed on native receptors was similar to those observed in $D\alpha 2/\beta 2$ showing that $D\alpha 2/\beta 2$ has use as a model for the native receptor (Brown et al. 2006).

Table 1.4.2 – Structure of neonicotinoids and other nAChR-targeting insecticides

Name	Type	Chemical Structure
Imidacloprid	Neonicotinoid	
Thiamethoxam	Neonicotinoid	
Clothianidin	Neonicotinoid	
Nitenpyram	Neonicotinoid	
Nithiazine	Neonicotinoid	

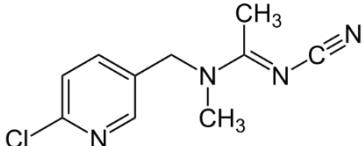
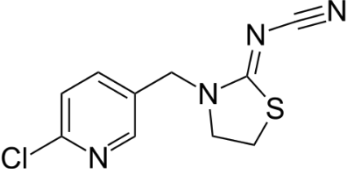
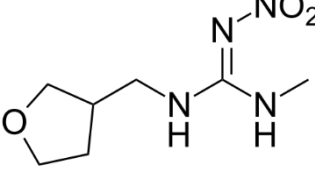
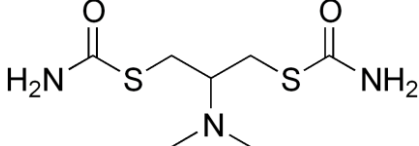
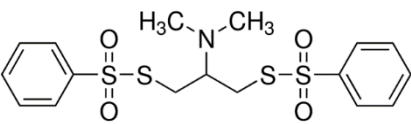
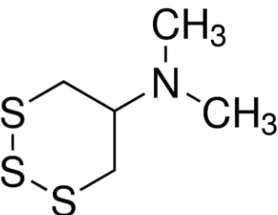
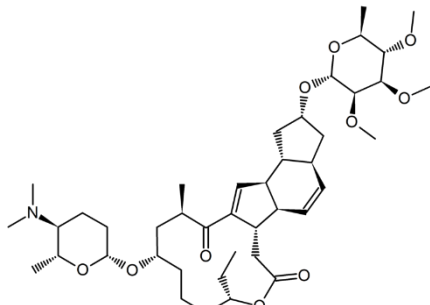
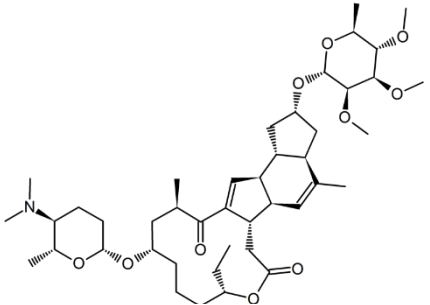
Acetamiprid	Neonicotinoid	
Thiacloprid	Neonicotinoid	
Dinotefuran	Neonicotinoid	
Cartap	Nereistoxin analogue	
Bensultap	Nereistoxin analogue	
Thiocyclam	Nereistoxin analogue	
Spinosyn A	Spinosad (when combined with Spinosyn D)	
Spinosyn D	Spinosad (when combined with Spinosyn A)	

Table adapted from (Simon-Delso et al. 2015).

1.5 Ladybird beetles

Ladybird beetles (Coleoptera: Coccinellidae) also known as coccinellids are beetles ranging from 1.3 – 10 mm in length belonging to the endopterygota group as they undergo complete metamorphosis, consisting of egg, larval, pupal and imaginal (adult) stages (Majerus 1994). Carl Linnaeus began the classification of coccinellids in the mid-1700s (Anon n.d.) and now over 4300 species have been identified (Majerus, 1994) and documented on all continents (Poutsma et al. 2008). They have an oval/round form with an exoskeleton (Fig. 1.5.2) and a hard wing casing called the elytra which can be brightly coloured with some species displaying a characteristic pattern and other species having great variation in their elytra pattern (Fig. 1.5.1) (Bezzarides et al. 2007). Eggs are laid in batches of 2-100 and also show variation in their colouring (Majerus, 1994).

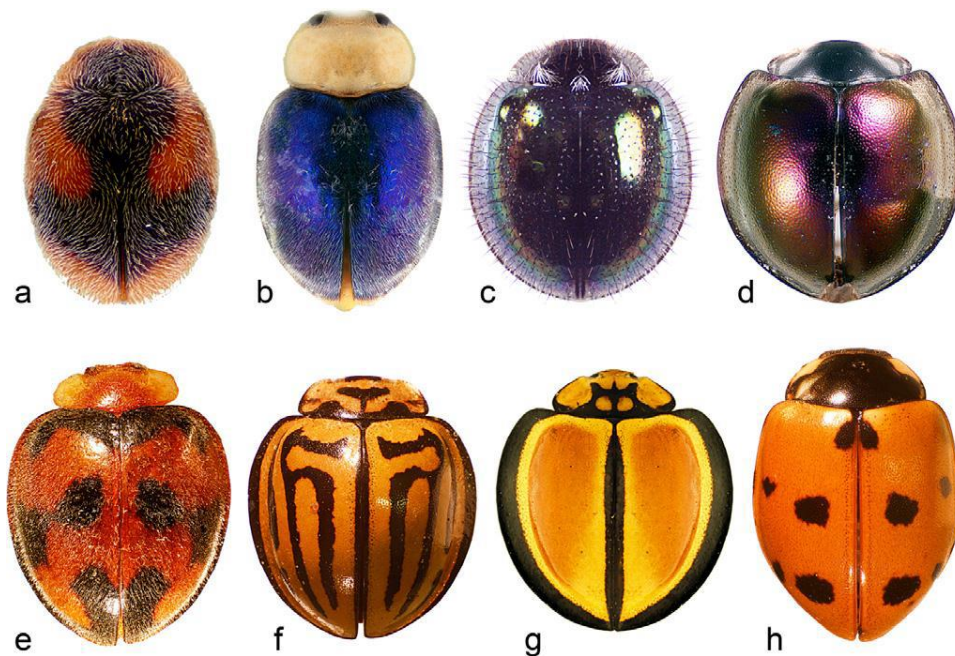


Fig. 1.5.1 - Elytra (hard wing casing) colours, textures and patterns can vary greatly between different species of coccinellids. Dorsal views of (a) *Scymnus mitior*; (b) *Rhyncortalia australis*, (c) *Chaetolotis amy*; (d) *Halmus cupripennis*; (e) *Epilachna mjoebergi*; (f) *Archegleis delta*; (g) *Australoneda bourgeoisi*; (h) *Coccinella undecimpunctata* are shown. Taken from (Seago et al. 2011).

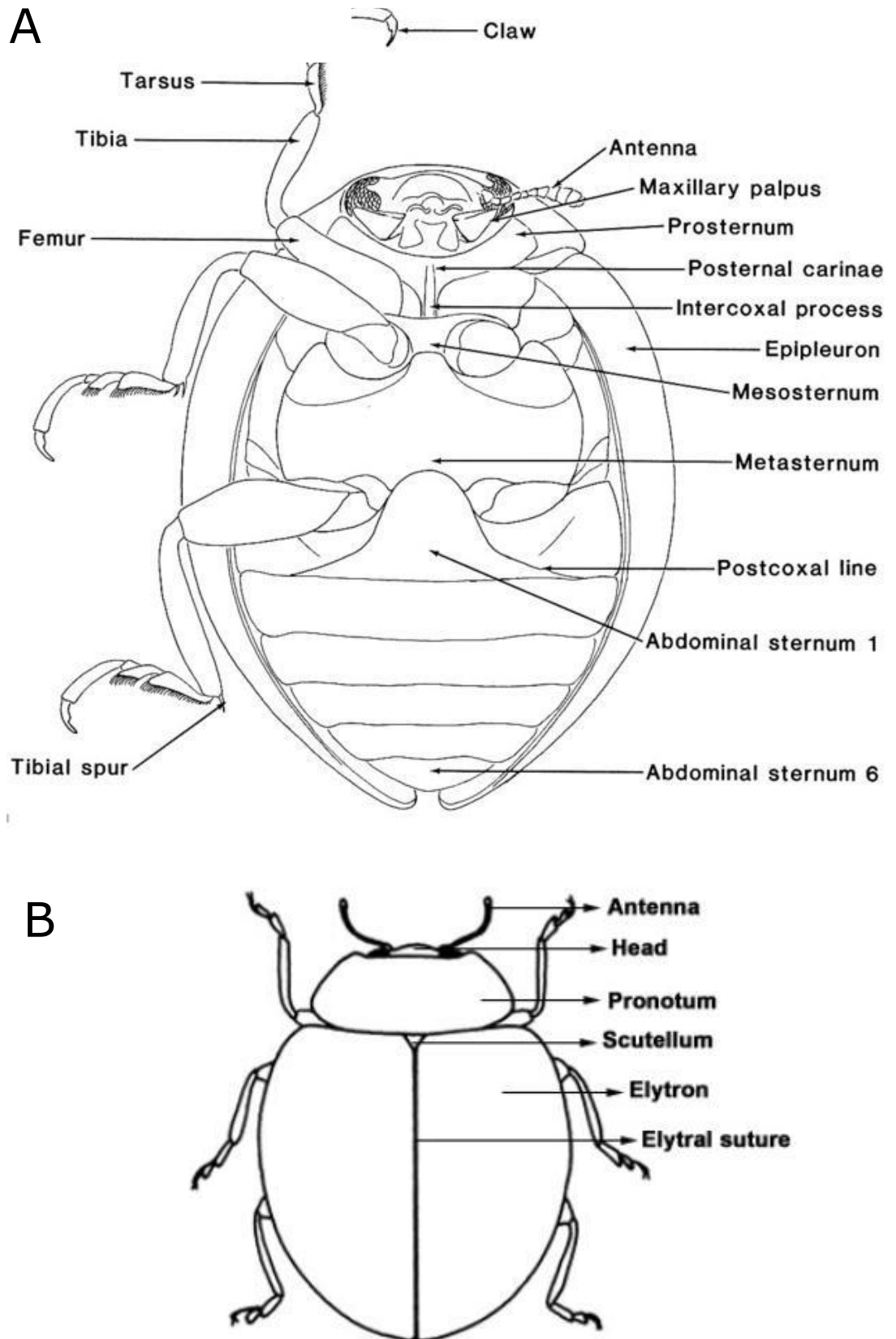


Fig. 1.5.2 - The key anatomical and morphological features of coccinellids from (A) a ventral and (B) a dorsal view. The ventral view is a schematic of *Coccinella novemnotata*. Ventral view taken from (Poorani 2008) & dorsal view. Image from (Gordon 1985).

Coccinellids can vary from monophagy to polyphagy but most species are polyphagous (Hagen 1962). Coccinellids can be prey on aphids, other coccinellids and consume plants. They are also known to feed on extra floral nectary secretions as well as pollen and fungi (Hagen, 1962). There is evidence that coccinellids that prey on aphids prefer different species of aphids (Finlayson et al. 2010) and so can be divided into groups depending on their preferred prey. When populations of their preferred prey are low they will feed on egg and larval stages of other insects and other coccinellid species. Egg cannibalism is common and the amount to which this occurs varies between species (Phoofolo & Obrycki 1998). Many coccinellids compete with other coccinellid species for their prey and so egg predation can occur on the eggs of other coccinellid species when prey is scarce. This is known as intraguild predation and is defined as when predators of the same prey (the same guild of predators) in the same area predate upon each other (Polis et al. 1989). It is seen to increase when aphid populations decrease so IGP is thought to be adaptive (Sato & Dixon 2004). Successful IGP would allow the elimination of competitors for the same prey and allows the prey population to recover so it is very beneficial for the species involved.

Some species of coccinellids are much more aggressive and successful intraguild predators (Michaud 2002) and this is thought to be a major reason for the decline of native populations of coccinellids when a foreign, more aggressive species invades or is introduced (Ware & Majerus 2008).

1.6 Chemical defence of ladybird beetles

Coccinellids usually have a bright coloration and are rarely predated upon by other species (Fig. 1.5.1) (King & Meinwald 1996). Coccinellids secrete a defensive fluid or reflex bleed from the tibio-femoral jointss in response to stress (Fig. 1.6.1).

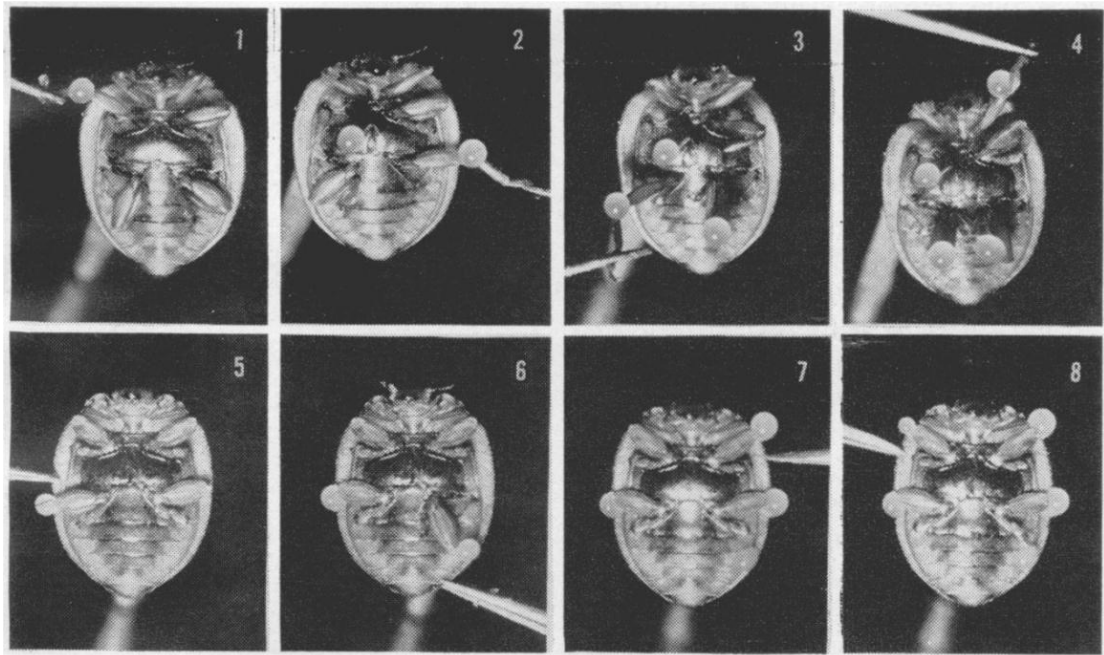


Fig. 1.6.1 – Photos 1-8 show an adult *Epilachna varivestis* reflex bleeding in response to pinching of the leg. Blleding occurred from legs closest to where the pinching occurred. Image from (Happ & Eisner 1961).

This is thought to be the haemolymph of the insect rather than a separately produced defensive fluid (Happ & Eisner 1961). This fluid was secreted in response to attacks by the ant *Formica exsectoides*. This successfully ceased the attacks when the ants came into contact with the secretion and the ants exhibited a characteristic dragging of the mouthparts behaviour that occurs when coming into contact with a defensive secretion (Happ & Eisner, 1961). Ants exhibited the same behaviour when exposed to pupae of the coccinellid *Epilachna varivestis* (Attygalle et al. 1993). Droplets of defensive secretory fluid were found on the glandular hairs on the ant's

surface. This behaviour could be attributed to the odour of the defensive fluid. Pyrazines are found in the secretions of some coccinellids and are known to be responsible for a warning odour (Hautier et al. 2008).

The confirmation of some form of chemical defence combined with the bright elytra colouring possessed by ladybird beetles indicates that this chemical defence could be an interesting source of useful natural products. Usually in the natural world the exhibition of bright coloration indicates that the organism possesses a strong chemical defence system and serves as a warning for potential predators. This is known as aposematism. Compounds such as epibatidine from the frog *Epipedobates anthonyi* (Fig. 1.6.2) have proven useful for the development of novel pesticides and therapeutics (Marks et al. 2010) and so ladybird beetle defensive chemistry could provide novel lead compounds for the same purposes.



Fig. 1.6.2 – The phantasmal poison frog *Epipedobates anthonyi*. Image from (Berec 2011).

Toxic effects have been observed when blue tits (*Parus caeruleus*) were fed adult *Coccinella septempunctata* beetles (Marples et al. 1989) and there are reports

of allergic reactions (rhinoconjunctivitis) in humans attributed to coccinellids (Yarbrough et al. 1999). There is evidence that some defensive secretions of certain coccinellid species may be more effective than others. *Myrica rubra*, showed less aggressive behaviour towards the Harlequin ladybird *Harmonia axyridis* when compared to the 7-spot ladybird *Coccinella septempunctata* (Finlayson et al., 2009). Crab spiders (*Misumenops tricuspidatus*) also reject *H. axyridis* in favour of other coccinellid species (Koch 2003). *H. axyridis* has proven a successful invasive species originating in the Far East and spreading across the continent to mainland Europe and the UK (Poutsma et al., 2008). In the previously mentioned studies *H. axyridis* was rejected by predators in favour of other coccinellid species so either their defensive chemistry is more effective, the appearance of the beetle is more effective at repelling than other species or a combination of both. *H. axyridis* has also shown to be a more aggressive predator of aphids when compared to 3 other coccinellids (Finlayson et al., 2010). A more effective chemical defence coupled with more aggressive predation is thought to contribute greatly to the invasive success of *H. axyridis* and success when introduced as a biological control method (Koch, 2003). Analysis of harmonine, an alkaloid produced by *H. axyridis* showed anti-microbial activity indicating the defensive chemistry serving as defence from pathogens as well as predators or competitors (Röhrich et al. 2012).

Coccinellid defensive chemicals are not limited to defensive secretions. The survival of the common green lacewing, *Chrysoperla carnea*, was limited when feeding on the eggs of the 2-spot ladybird, *Adalia bipunctata* with no individuals surviving when feeding on *H. axyridis* eggs (Santi & Maini 2006). Some form of chemical defence in coccinellid eggs that is varied between different coccinellid species must be present.

The bright elytra colouring of coccinellids is therefore an aposematic warning signal similar to brightly coloured poisonous reptiles and amphibians for the chemical defence the coccinellid possesses. The aposematic warning itself is seen as the first line of defence (Majerus, 1994) as rate of attack by avian predators increased after the bright colouring was altered which increased further after removal of the elytra (DolenskÁ et al. 2009).

1.7 Ladybird beetle alkaloids

The haemolymph and defensive secretions of coccinellids are known to contain a group of organic compounds called alkaloids. Alkaloid structure varies greatly between coccinellid species (Fig. 1.9.1) and are prime candidates for contributing to the toxic effects of the secreted defensive fluid (Pasteels 2007; Glisan King & Meinwald 1996). They may also contribute to repelling by foul taste and odour (Daloze et al. 1994). Alkaloids were detected throughout the body of *C. septempunctata* so could easily be a constituent of haemolymph and be secreted as a defensive response (Holloway et al. 1991). The first alkaloids to be identified were coccinelline and precoccinelline from *C. septempunctata* and coccinelline was found to repel ants (Daloze et al. 1994). More alkaloids were identified in *Hippodamia convergens* called hippodamine, convergine and myrrhine. These shared the same tricyclic tertiary amine structure as coccinelline and precoccinelline (Fig. 1.9.1) (Tursch et al. 2010). Defensive fluid from *E. varivestis* was found to contain 5 compounds, the major compound being an azamacrolide called epilachnene (Attygalle et al., 1993). Pupal defensive secretions of *Epilachna borealis* were found to contain a

combinatorial library of macrocyclic polyamines called polyazamacrolides with ring sizes containing up to 98 members (Schröder & Tolasch 1998). A different structure of alkaloids exist in the 22-pointed ladybird *Psyllobora vigintiduopunctata* called Psylloborine A, a dimeric alkaloid (Schroder and Tolasch, 1998). The alkaloids Harmonine and 3-hydroxypiperidin-2-one were isolated from *H. axyridis* and *Ailocaria hexaspilota* (Alam et al. 2002). Adaline and adalinine were found in *Adalia bipunctata* (G. Lognay et al. 1996). This is a small fraction of the 4000 plus species of coccinellid and shows a large diversity in the structure of alkaloid compounds they contain.

Feeding of radiolabelled acetate to *E. paenulata* (Camarano et al. 2009) and *A. bipunctata* (Laurent et al. 2002) followed by GC-MS analysis showed high incorporation of radiolabelled acetate into the alkaloids. Feeding of radiolabelled oleic acid and serine showed high incorporation into the alkaloid epilachnene (Attygalle et al., 1993). Feeding of radiolabelled sodium acetate showed high incorporation into coccinelline indicating coccinelline being formed from a β -polyketoacid and this was found to occur in the fat body (Laurent et al., 2002). This strongly points to the alkaloids being synthesised *de novo* from fatty acid precursors in the fat bodies across multiple species of coccinellids. The alkaloids of *H. axyridis* have also been implicated as having a similar method of creation (Haulotte et al. 2012).

Due to the bright colouration of coccinellids and their effective defensive chemistry, alkaloids from various species were assayed for their effects on the nAChR. The nAChR is a common target for natural toxins as acetylcholine is the main neurotransmitter in insects (Breer & Sattelle, 1987). As coccinellid beetles primarily compete with other insects, alkaloids could potentially be selective for insect over mammalian nAChRs and would be a promising lead compound for the development

of a novel insecticide. Analysis of coccinelline, precoccinelline and their analogues as well as exochomine showed binding to *Torpedo* muscle nAChRs. Subsequent electrophysiological analysis of precoccinelline and coccinelline showed inhibition of mammalian m-nAChRs expressed in TE671 cells and inhibition of human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes by precoccinelline (Leong et al. 2015). This was confirmed in a further study showing inhibitory activity of alkaloid extracts from multiple species of coccinellids when examined using patch-clamp electrophysiology of TE671 cells and locust neurons. Whole alkaloid extract of *Harmonia axyridis* as well as (-)-adaline and harmonine isolated from the alkaloid extracts showed selectivity for the insect nAChRs expressed in locust neurons over the mammalian m-nAChRs expressed in TE671 cells (Richards 2016).

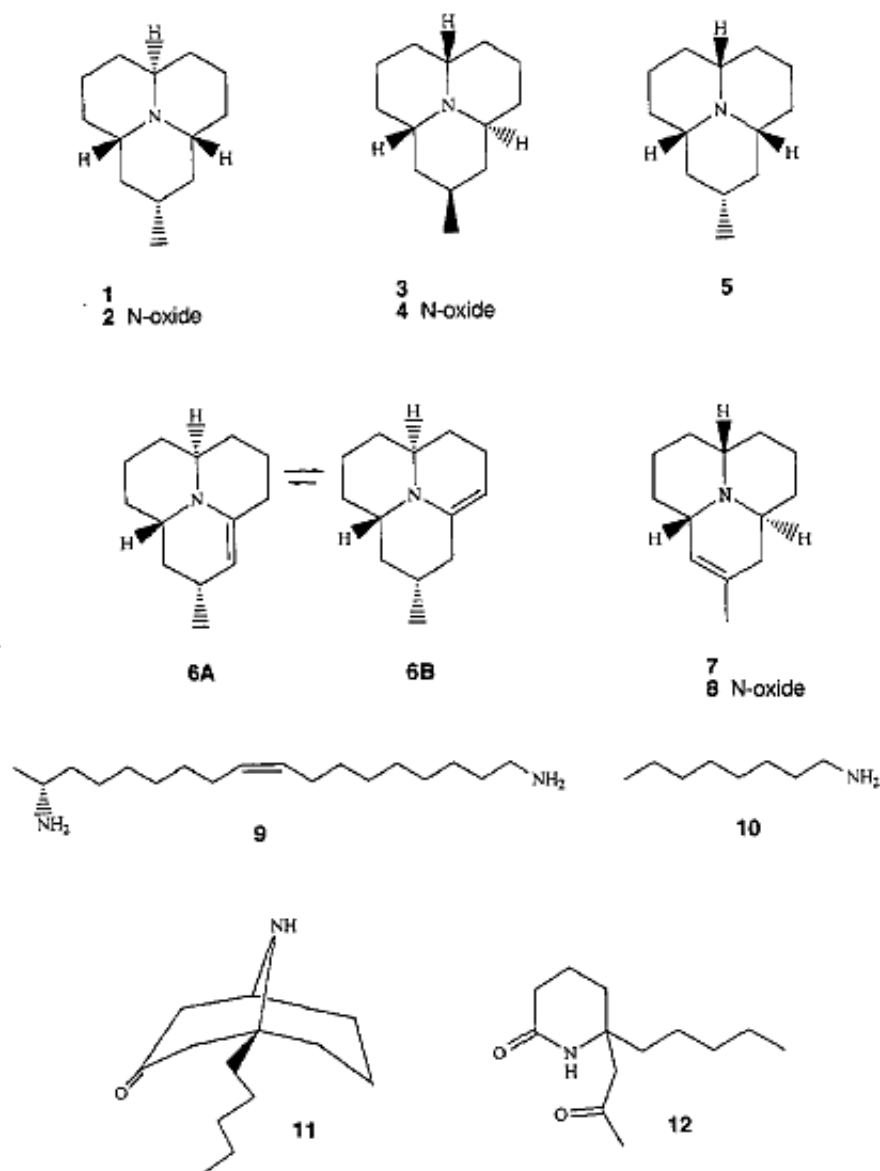


Fig. 1.9.1: Structure of different alkaloid compounds found in coccinellids: (1) coccinelline, (2) pre-coccinelline, (3) hippodamine, (4) convergine, (5) myrrhine, (6A&B) propyleine, (7) hippocasine, (8) hippocasine N-oxide, (9) harmonine, (10) n-octylamine, (11) adaline and (12) adalinine.

1.8 Aims

The aim of this study is to determine whether inhibition caused by *Harmonia axyridis* alkaloid extract (HAE) and (-)-adaline extends to mammalian n-nAChRs and the hybrid insect D α 2/ β 2. HAE will be obtained from adult *H. axyridis* beetles and (-)-adaline will be isolated from alkaloid extract from *Adalia bipunctata*. The

aforementioned receptors will be expressed in *Xenopus* oocytes and the effect of HAE and (-)-adoline will be investigated via the response to ACh of each receptor. Once inhibitory activity is established, any potential selectivity for a nAChR subtype will be examined. Further analysis of the mechanism of inhibitory action will be carried out in order to compare with the findings in previous studies. This involves investigating the concentration-inhibition relationship, current-voltage (IV) relationship of inhibition and carrying out a competition assay with ACh in order to determine whether HAE or (-)-adoline competes with ACh for the same binding site. Glutamate AMPA and NMDA receptors will also be investigated to ascertain whether inhibition is specific to nAChRs.

Selectivity for insect nAChRs would confirm (-)-adoline and HAE as prime candidates for a lead compound to develop insecticides. In a similar process to the development of neonicotinoids (Tomizawa et al., 2005), the molecular basis for selectivity can be examined and used to improve selectivity.

CHAPTER 2: MATERIALS & METHODS

2.1 Extraction and Purification of Ladybird Alkaloids

2.1.1 Collection of ladybird beetles

Adult *Harmonia axyridis* beetles were collected from the University Park campus of the University of Nottingham (UK) from 2013-2014 and stored in clear, universal tubes at -20 °C (Fig. 2.1.1.1). Adult *Adalia bipunctata* beetles (product name – Adaline B) were purchased from Syngenta Bioline and also stored at -20 °C.



Fig. 2.1.1.1: Universal tube containing adult *Harmonia axyridis* (Image from Richards et al., 2011).

2.1.2 Solvent Extraction

To extract alkaloids from the haemolymph and other tissues of the beetles, a solvent extraction using methanol was conducted. Collected beetles were weighed, counted and transferred to a large mortar and frozen by addition of liquid nitrogen. Beetles were ground up with a pestle and then transferred to a conical flask. A first extraction was carried out by adding approximately 50 ml of HPLC grade methanol per

100 beetles and left to stir overnight at room temperature. The methanol was decanted into a separate flask and the process was repeated with the same amount of fresh methanol. The methanol from the first extraction was stored at 4 °C and then combined with the methanol from the second extraction.

2.1.3 Acid-Base Extraction

An acid-base extraction was then used to separate alkaloid compounds from other compounds. The extractions were placed in a rotary evaporator until the methanol had completely evaporated. 2 ml 1M hydrochloric acid (HCl) was used to wash the remaining solid from the evaporator spherical flask and this was transferred to a separation funnel. Approximately 50 ml HPLC grade diethyl ether (Fisher Scientific, UK) was also used to wash the flask and added to the separation funnel. The contents inside the separation funnel were mixed and left to separate into an organic layer and an aqueous layer. The layers were decanted into separate beakers, the organic layer was discarded and the aqueous layer was returned to the separation funnel. This process was repeated twice and the aqueous layers were combined.

The collected aqueous layer was adjusted to pH > 10 with 2M sodium hydroxide (NaOH) and returned to the separation funnel. The beaker was washed with approximately 50 ml HPLC grade dichloromethane (DCM) (Fisher Scientific, UK) and this was also added to the separation funnel. The liquids were mixed, left to separate into organic and aqueous layers and decanted into separate beakers. The organic layer was kept, the aqueous layer was returned to the separation funnel and the process was repeated washing the beaker which had contained the aqueous layer with 50 ml

DCM. The organic layers were combined and a final wash with approximately 10 ml saturated sodium chloride (NaCl) solution and approximately 1 g magnesium sulphate (MgSO₄) powder was carried out. The mixture was then filtered (Whatman, Grade 4 filter paper) and the remaining DCM was evaporated using a rotary evaporator. A minimal volume of DCM was used to dissolve the remaining solid alkaloid and transferred to a glass vial. Ladybird beetle alkaloid extract was then evaporated by directing a pressurised stream of nitrogen gas onto the surface of the DCM. The remaining solid was weighed, dissolved in 0.5 – 1 ml DCM and aliquoted into glass ampoules sealed under nitrogen and stored at 4°C.

2.1.4 Liquid column and thin layer chromatography

Liquid column chromatography was carried out using a 30 cm glass column containing neutral alumina (Activity grade 1, Woelm Pharmaceuticals) mixed with DCM. After washing the column with DCM, alkaloid extract from *A. bipunctata* was added to the top. The column was then washed again with DCM and 10 approximately 1 ml fractions were collected in glass vials. This was repeated twice with DCM replaced by 4:1 DCM:methanol and 99:1 methanol:ammonium hydroxide with 10 fractions being collected for each solvent used. Approximately 1-2 µl of each fraction was blotted along a straight line onto an aluminium backed silica thin-layer chromatography plate (Sigma-Aldrich, UK) and placed standing up with the blotted fractions at the bottom end into a beaker containing 99:1 methanol:ammonium hydroxide. The volume contained in the beaker was enough to ensure the blotted alkaloid extract was just above the meniscus. After the 99:1 methanol:ammonium hydroxide had run to the top of the plate, the plate was stained with Dragendorff's

reagent to find out which fractions contained alkaloid. Fractions that tested positive were combined.

There are 2 alkaloids known to be produced by *A. bipunctata*, adaline and adalinine. A previously published method for separating these 2 alkaloids was used (G. Lognay et al. 1996). Liquid column chromatography was again used with a 30 cm column containing neutral alumina mixed with CHCl₃ – hexane 1:1. CHCl₃ – hexane 1:1 was then used to wash the column and 30 fractions were collected. Thin layer chromatography was used to determine which fractions contained adaline which is eluted from the column first and these fractions were combined. The remaining fractions were combined and stored at 4 °C.

The fractions containing adaline were combined into a glass vial and evaporated under a pressurised stream of nitrogen gas. The remaining solid was weighed, dissolved in 0.5 – 1 ml DCM and aliquoted into glass ampoules sealed under nitrogen and stored at 4°C.

All fractions were analysed using a gas chromatograph (Agilent 5890 GC, Agilent Technologies, Santa Clara, California, USA).

2.2 Molecular Biology

2.2.1 Transformation of *E. coli* with plasmid DNA

Chemically competent cells were created using *E. coli* strain XL10-Gold. An overnight culture of cells was prepared in 5 ml lysogeny broth (LB) (10 g NaCl, 10 g Tryptone & 5 g Yeast extract per 1 l H₂O) and incubated in an orbital shaker (Excella E24, New Brunswick Scientific) at 37 °C and 250 rpm. 50 ml LB was added and

incubated for a further 2 h. The culture was placed on ice for 15 min and then centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was removed and pellets re-suspended in 10 ml ice cold, filter sterilised 0.1 M CaCl_2 , left on ice for 15 min and then centrifuged at 2000 rpm for 10 min at room temperature. The supernatant was removed, the pellet re-suspended in 2 ml 0.1 M CaCl_2 and stored on ice for the subsequent bacterial transformation.

Plasmid DNA (pDNA) (Table 2.1) containing the LGIC subunit encoding gene was used to create capped RNA (cRNA). Human $\alpha 7$, chick $\beta 2$ and *Drosophila* SAD/ $\alpha 2$ pDNA was kindly provided by Prof David B. Sattelle (Wolfson Institute for Biomedical Research, University College London formerly Faculty of Life Sciences, University of Manchester). Rat $\alpha 3$, $\alpha 4$, $\beta 2$, $\beta 4$, GluN1-1a, GluN2A and GluA1 (flop variant) pDNA was acquired from the Salk Institute (La Jolla, California, US). Transformation was carried out using the heat shock method by adding 1 μl of plasmid DNA (pDNA) to 200 μl of chemically competent cells and leaving on ice for 20 min. The cells were then placed in a water bath at 42 °C for 1 min then immediately placed on ice for 2 min. 1 ml LB + 10 mM glucose was added and the cells were placed in a water bath at 37 °C for 30 min. 100 μl of cells were then spread over 95 mm Petri dishes filled with LB agar (10 g NaCl, 10 g tryptone, 5 g yeast extract & 17 g agar per 1 l H_2O) containing 50-100 $\mu\text{g/ml}$ Ampicillin and incubated overnight at 37 °C.

Table 2.1: Details of plasmids encoding LGIC subunits

LGIC	Species	LGIC subunit	Plasmid Vector	Linearization enzyme	Promoter
nAChR	Rat	$\alpha 3$	pSP64	<i>EcoRI</i>	SP6
	Rat	$\alpha 4$	pSP64	<i>EcoRI</i>	SP6
	Rat	$\beta 2$	pSP65	<i>HindIII</i>	SP6
	Rat	$\beta 4$	pBSSK(+)	<i>XhoI</i>	T3
	Human	$\alpha 7$	pcDNA3.1	<i>XbaI</i>	T7
	Chicken	$\beta 2$	pcDNA3.1	<i>XbaI</i>	T7
	Fruit Fly (<i>Drosophila melanogaster</i>)	SAD/ $\alpha 2$	pcDNA3.1	<i>SmaI</i>	T7
NMDAR	Rat	GluN1-1a	pRK7	<i>BamHI</i>	SP6
	Rat	GluN2A	pRK7	<i>EcoRI</i>	SP6
AMPA	Rat	GluA1 (flop variant)	pcDNA3.1	<i>XhoI</i>	T7

2.2.2 Isolation of plasmid DNA from transformed *E. coli*

A starter culture of *E. coli* was prepared by inoculating 5 ml LB + 50 $\mu\text{g/ml}$ Ampicillin using a sterile pipette tip to pick a single colony of the transformed *E. coli* from the LB agar plates. This was incubated in an orbital shaker (Excella E24, New Brunswick Scientific) overnight at 37 °C and 250 rpm. A GenElute Plasmid MiniPrep kit (Sigma-Aldrich) was used to isolate the plasmids from 3-4.5 ml of starter culture using the manufacturer's instructions with an Eppendorf 5417R centrifuge. All steps were carried out at room temperature.

A 1.5 ml Eppendorf tube was filled with 1.5 ml starter culture and centrifuged at 14,000 rpm for 1 min. The supernatant was removed; a further 1.5 ml culture was added and centrifuged again for 1 min. The supernatant was removed; the pellet was re-suspended in 200 μl resuspension solution and tubes were vortexed until the pellet was mixed thoroughly. 200 μl lysis buffer was added and mixed gently by inverting 4-6 times until the solution became clear. 350 μl neutralisation/binding solution was added, mixed thoroughly by inverting 4-6 times and centrifuged at 14,000 rpm for 10

min. The supernatant was added to a GenElute Miniprep Binding Column that had been previously washed by placing the column in a 1.5 ml Eppendorf tube, adding 0.5 ml column preparation solution, centrifuging for 1 min and discarding the flow-through. The column was then centrifuged at 14,000 rpm for 1 min and the flow-through was discarded. 0.5 ml optional wash solution was added and the column was centrifuged at 14,000 rpm for 1 min. The flow-through was discarded, 0.75 ml wash solution was added then centrifuged again at 14,000 rpm for 1 min and the flow-through discarded. The column was centrifuged again at 14,000 rpm for 3 min to ensure all the buffer had flowed through. The column was then placed in a fresh 1.5 ml Eppendorf tube and 50 μ l elution solution was added and left to stand for 1 min. The column was then centrifuged at 14,000 rpm for 1 min to elute the pDNA. The concentration of pDNA was then determined using a spectrophotometer (NanoDrop 1000, Thermo-Scientific).

2.2.3 Linearization of plasmid DNA

Linearization of plasmid DNA was carried out using the restriction enzymes listed in Table 2.1. 1 μ g pDNA was mixed with 5 μ l buffer, 5 μ l of restriction enzyme (New England Biolabs) and nuclease-free water up to 50 μ l for each reaction. High-fidelity enzymes (New England Biolabs) required the same protocol but with 1 μ l enzyme.

Linearization was checked after 1 h by running a 1 μ l sample on a 1.5% TAE gel with a 1kb ladder. This was made using 1xTAE (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA) and the gel was made by adding 0.75 g Agarose to 50 ml 1xTAE then

melting in a microwave for approximately 2 min followed by the addition of 1 µl of 10 µg/ml ethidium bromide. The gel was run for 1 h at 70 V. One clear band was visible for each subunit which ensures that no other cuts were made by the restriction enzyme.

The restriction digest was terminated by adding 2.5 µl 0.5 M EDTA, 5 µl 5M ammonium acetate and 100 µl 100% ethanol. This was left for 1 h at -20 °C and then centrifuged at 13,000 rpm/4 °C for 15 min. The supernatant was removed by pipetting and the tubes were left to dry in a sterile cabinet for 5 min. The pellet was then re-suspended in 6 µl nuclease-free water and the concentration was checked using a spectrophotometer (NanoDrop 1000, Thermo-Scientific).

2.2.4 cRNA Production

Production of cRNA from the linearized plasmids was carried out using the T7, SP6 or T3 mMESSAGE mMACHINE kits (Ambion) depending on the plasmid's promoter (Table 2.1). Up to 1 µg linearized pDNA was mixed with 15 µl 2xNTP/CAP, 3 µl 10x reaction buffer, 3 µl enzyme buffer and nuclease-free water up to 20 µl. The mixtures were incubated at 37 °C for 2 h followed by the addition of 30 µl lithium chloride precipitation solution and 30 µl nuclease-free water. The mixture was left to precipitate overnight at -20 °C. Following this the mixtures were centrifuged at 13,000 rpm/4 °C for 15 min. The supernatant was removed and the pellet washed with 1 ml 70% ethanol. This was centrifuged again at 13,000 rpm/4 °C for 15 min and the

supernatant was removed. The remaining pellet was re-suspended in 15 μ l nuclease-free water and the concentration of RNA was determined using a spectrophotometer (NanoDrop 1000, Thermo-Scientific).

2.3 Electrophysiological recordings of *Xenopus laevis* oocytes

2.3.1 The Two electrode voltage-clamp technique

Oocytes from the African clawed frog, *Xenopus laevis*, can be used to express non-native proteins by injecting foreign DNA into the nucleus or RNA into the cytoplasm. They are obtained by ovariectomy of adult females and are immature eggs not competent for fertilisation (Bianchi & Driscoll 2006). Oocytes go through six stages and stages IV-V are used for experiments. These are approximately 1mm in diameter and segmented into an animal pole (black region) and a vegetal pole (white region) (Bianchi and Driscoll, 2006) (Fig. 2.3.1.1). Expression of non-native proteins was first achieved by injecting haemoglobin mRNA (Gurdon et al. 1971) and the *Torpedo* acetylcholine receptor was the first transmembrane receptor to be investigated (Barnard et al. 1982). Use of this expression system allowed the examination of different subtypes of nAChRs (Takahashi et al. 1985; Boulter et al. 1987a) and the role of specific amino acids in receptor function (Mishina et al. 1985).

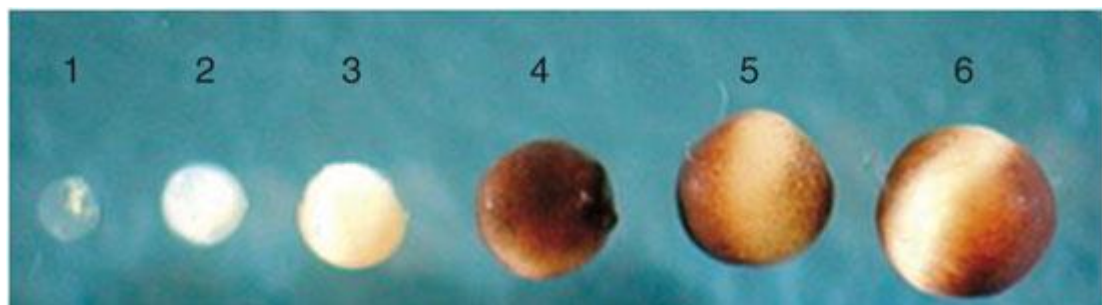
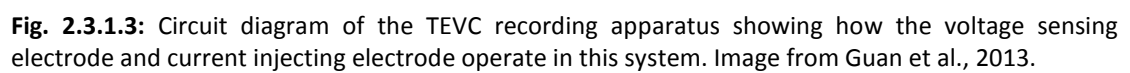
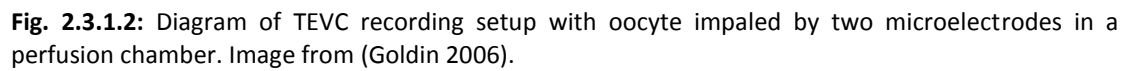


Fig. 2.3.1.1: *Xenopus* oocytes go through six stages with stages IV-V selected for experimentation. Image from (Allen et al. 2007).

Recording the electrical response of *Xenopus* oocytes injected with the mRNA of LGICs is achieved by using the two electrode voltage clamp (TEVC) technique. A voltage sensing microelectrode and a current injecting microelectrode are inserted into the oocyte cytoplasm in a perfusion chamber (Fig. 2.3.1.2). Saline is perfused and a command voltage is set. The voltage sensing microelectrode measures the membrane potential and an amplifier is used to level the membrane potential and a set command voltage by injection of current through the current microelectrode. The injected current is proportional to the change in current caused by the opening of expressed receptors and therefore is used to measure the total membrane current when ligands and drugs are perfused (Fig. 2.3.1.3) (Guan et al. 2013). This allows the analysis of the electrical response of a receptor in isolation and can allow the confirmation of the target site of different compounds. This has successfully been used to confirm the target sites of many anthelmintics and insecticides on the functioning of their target receptors. The oocyte expression system also allows the expression of hybrid insect/mammal nAChRs and this has proven useful in confirming the nAChR target of the neonicotinoids (Ihara et al. 2015; Matsuda et al. 2009) and nereistoxin (Raymond Delpech et al. 2003).



2.3.2 Preparation and injection of oocytes

Xenopus laevis ovaries extracted by ovariectomy of female *Xenopus laevis* were purchased from the European Xenopus Resource Centre (University of Portsmouth, UK). Ovaries were split into smaller pieces using scissors and incubated in 0.5-1 mg/ml collagenase (type 1A, Sigma) in Ca^{2+} free Barth's GTP solution (96mM NaCl, 2mM KCl, 5mM HEPES, 2.5mM pyruvic acid & 0.5mM theophylline, pH 7.5) for 1-2 h followed by thorough washing with Ca^{2+} free GTP. Oocytes were selected based on a clear divide between the light and dark poles and for their structural integrity then placed in fresh Ca^{2+} free GTP. Injection needles were pulled from 3.5 nl glass bores (World Precision Instruments, Inc.) using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co.) and a small part of the tip was broken off. The needle was then "back filled" with filtered paraffin and placed into a Nanoliter injector (World Precision Instruments, Inc.). 1 μl subunit cRNA at a concentration of 100 – 300 ng/ μl for mammalian subunits and 500 – 1500 ng/ μl for D α 2/ β 2 was pipetted onto the surface of a sterile petri dish. The glass needle was manoeuvred into the solution and the cRNA was taken up by the needle ensuring that no air bubbles were present. Viable oocytes were then selected and placed into a dish, lined with grooved plastic to hold the oocytes in place, containing GTP (96mM NaCl, 2mM KCl, 1.8mM CaCl_2 , 5mM HEPES, 2.5mM pyruvic acid & 0.5mM theophylline, pH 7.5 followed by the addition of 0.05 mg/ml gentamicin after autoclaving) (Fig 2.3.1.1). 50 nl cRNA was injected into the cytoplasm of each oocyte and then oocytes were transferred to a separate dish with fresh GTP. Damaged or discoloured oocytes were removed and those remaining were incubated at 18 °C for 2-4 days. GTP was changed daily and any damaged oocytes

were removed. For heteromeric receptors (rat $\alpha 4/\beta 2$, rat $\alpha 3/\beta 4$, GluN1-1a/2A, *Drosophila* $\alpha 2/\text{chick } \beta 2$) the cRNA was injected in a 1:1 ratio.

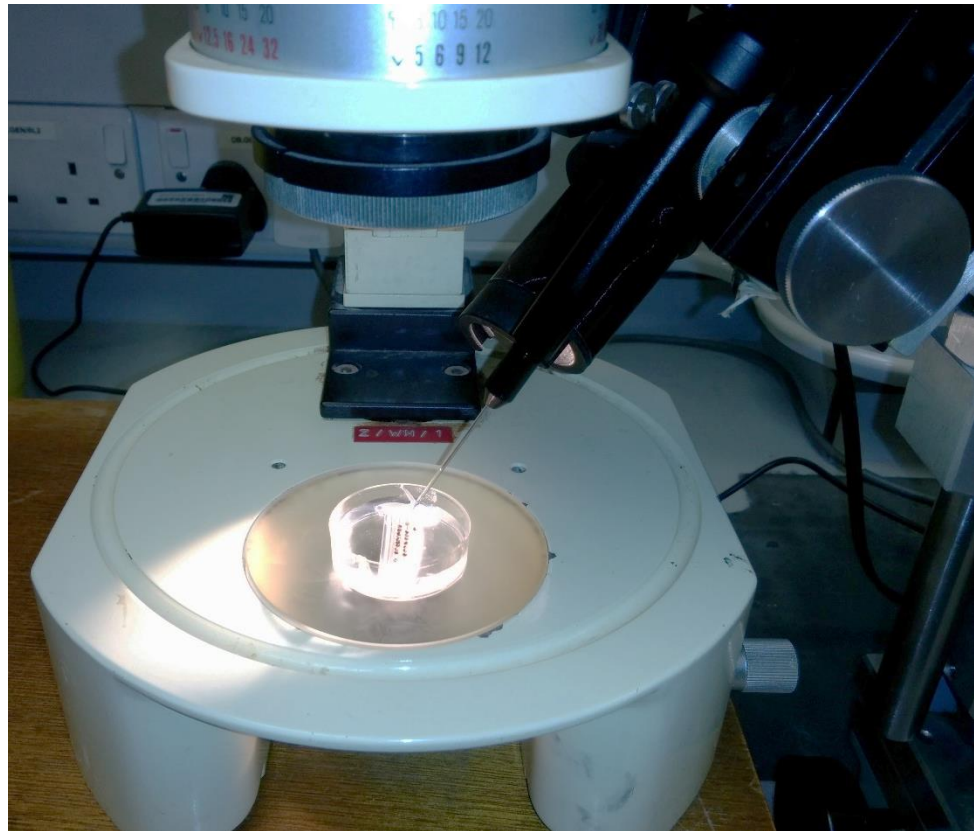


Fig. 2.3.1.1: Oocyte injection apparatus setup. Selected oocytes are placed into a dish containing GTP and the injection needle is moved into the solution in preparation to puncture the oocyte membrane and inject cRNA.

2.3.2 Two-electrode voltage clamp procedure

Microelectrodes were pulled from borosilicate glass capillaries (TW150F-4, World Precision Instruments Inc., US) using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co.) and used to impale oocytes placed in a perfusion bath. The microelectrodes were filled with 3M KCl and resistance was no higher than 0.5M Ω . Oocytes displaying a resting membrane potential of at least -20 mV were clamped at holding potentials (V_h) of -25, -50, -75, -100 or -125 mV using an Axoclamp 2A or

Geneclamp 500 amplifier (Axon Instruments Inc., USA). Output currents were transferred using a PCI-6221 A/D converter (National Instrument Corp., US) to a PC and WinEDR software (Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK) was used for recording and analysis (Fig. 2.3.2.1 & 2.3.2.2). Standard oocyte saline (SOS) (100mM NaCl, 2mM KCl, 1.8mM CaCl₂, 1mM MgCl₂, 5mM HEPES, pH 7.6) was applied using a MPS-2 multi-channel gravity fed perfusion system (World Precision Instruments Inc.). 0.5μM Atropine was added to the SOS to prevent any muscarinic response when testing nAChRs and Mg²⁺ free SOS was used when testing NMDA receptors.

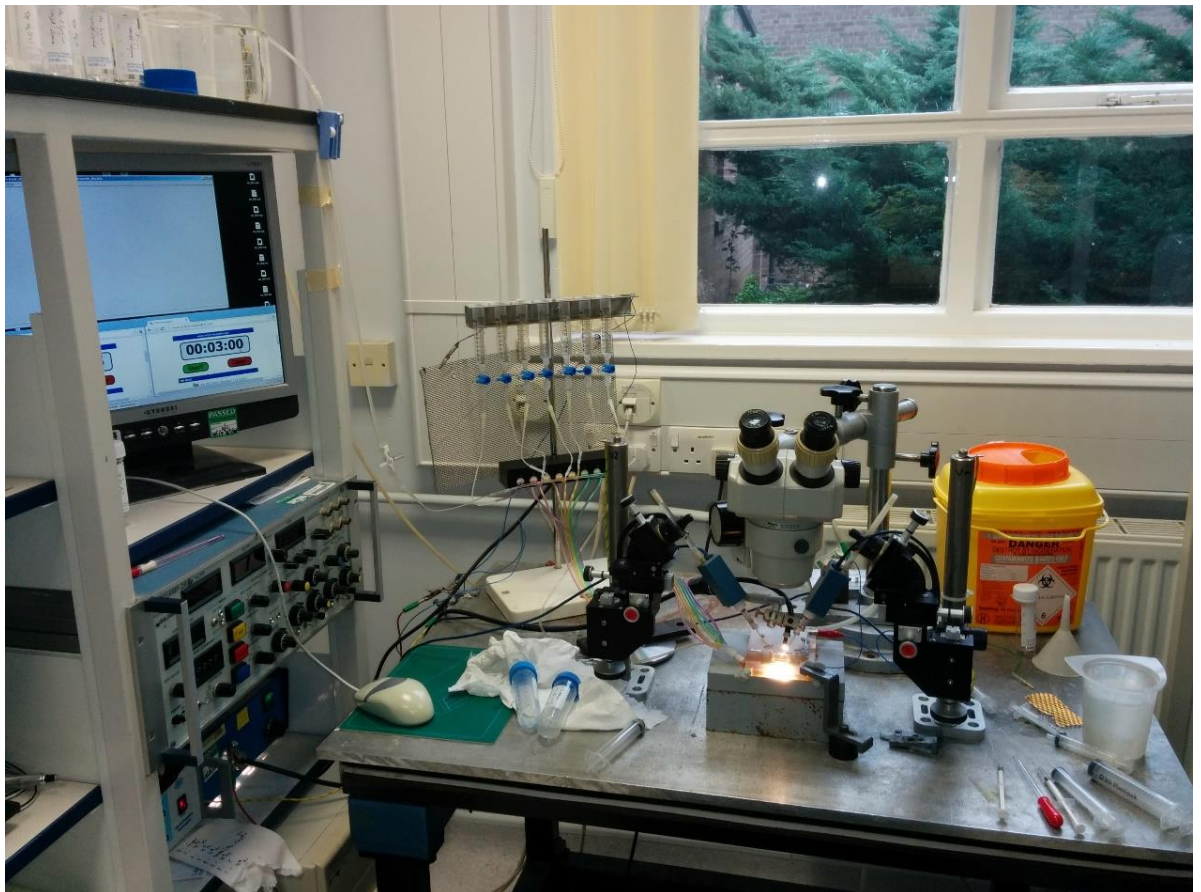


Fig. 2.3.2.1: Two electrode voltage-clamp setup. An injected oocyte is held in a perfusion chamber where a perfusion system is used to control the application of saline and drugs. Microelectrodes are connected to an amplifier and A/D converter and moved into the oocyte. These in turn are connected to a PC and analysed using the WinEDR program.

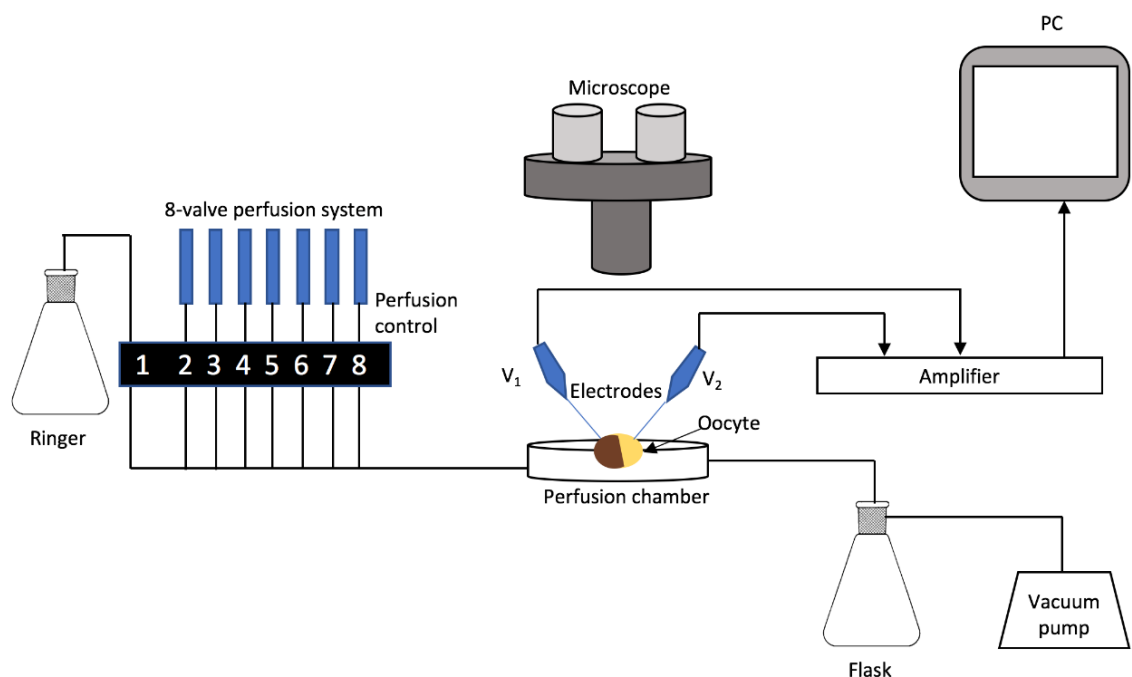


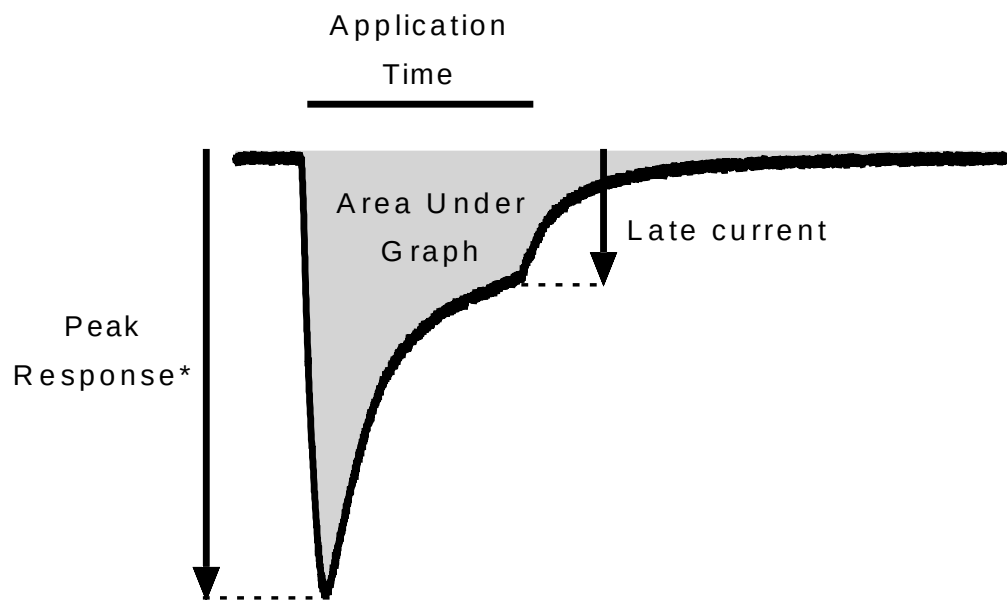
Fig. 2.3.2.2: Schematic diagram of two electrode voltage-clamp set up linked to a multi-channel perfusion system.

2.4 Experimental procedure and data analysis

2.4.1 Measuring peak, late current and area under graph

Each application of ACh caused a change in current represented by a downward deflection (at negative V_h) from a baseline in its absence. The peak response, late current response and area under graph was measured from each trace (Fig 2.4.1.1). It should be noted that for the rapidly desensitising human $\alpha 7$ receptor, the opening and closing of the receptor is so rapid that complete exchange

of solution surrounding the cell has not occurred. This means receptors exposed to agonist first open and close before the receptors exposed to agonist last and so the peak response observed on the trace is not the response of every receptor open in unison (Papke & Porter Papke 2002). Papke and Porter Papke also found that measuring the area under graph was a more accurate representation of the peak response of the cell and so the area under graph was also calculated.



*Not peak response of cell when human $\alpha 7$ is expressed

Fig. 2.4.1.1: Representative trace of the response of a *Xenopus* oocyte expressing rat $\alpha 3\beta 4$ nAChRs during application of 100 μM ACh. Marked on the trace are the parameters used to measure the response.

The late current measurement was used for rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs traces as the ACh response desensitised slowly. ACh and ACh co-applied with ladybird alkaloid was applied for 15 s and a 3 min washing interval was given between each application. Responses for human $\alpha 7$ and $D\alpha 2/\beta 2$ desensitised rapidly so measuring

the late current was unfeasible. Due to this only the peak response and area under graph was measured and the application time was reduced to 5 s and the washing interval remained 3 min. The area under graph was calculated using the trapezoid rule to approximate the integration of the line using the formula below where Δx is the interval between points on the x-axes.

$$\sum_{i=0}^n \frac{1}{2} (y_n + y_{n+1}) \Delta x$$

For Investigation of GluN1-1a/2A and GluA1 (flop), agonist (NMDA/glycine for GluN1-1a/2A and kainic acid (KA) for GluA1 (flop)) was applied until a steady current had been established. Once this occurred ladybird alkaloid was co-applied with agonist and perfusion was maintained until any changes in current were stabilised. Once stabilised the next concentration of ladybird alkaloid was applied until current changes were stabilised again. This process was repeated ensuring that all concentrations of ladybird alkaloid were applied in ascending order of concentration. Once the last concentration of ladybird alkaloid was applied and stabilisation was reached, SOS alone was applied until the current response reached the baseline established before application of agonist (McClymont et al. 2012; Franzyk et al. 2014b).

2.4.2 Determining the EC₅₀ value

Different concentrations of ACh were applied to oocytes clamped at $V_h = -75$ mV and normalised to the ACh concentration that gave the highest response. Prism 7 (GraphPad) was used to plot graphs of peak, late current and area under graph

responses against log(agonist) and calculate the EC₅₀ using the non-linear regression “log(agonist) vs. normalised response -- Variable slope (four parameters)” equation (shown below) with the top constrained to 100 and the bottom constrained to 0.

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{((LogEC50 - X) \times HillSlope)}}$$

2.4.3 Determining the IC₅₀ value

A control ACh concentration (10 µM for α4β2 and 100 µM for α7, α3β4 and Dα2/β2 nAChRs) was applied 2-3 times to ensure a stable ACh response, followed by co-application of the control ACh concentration with varying concentrations of ladybird alkaloid to oocytes clamped at V_h = -75 mV. Peak, late current and area under graph responses were normalised to the control ACh response. For GluN1-1a/2A and GluA1 (flop) experiments oocytes were also clamped at V_h = -75 mV and agonist concentrations of 100 µM kainic acid for GluA1 (Franzyk et al., 2014) and 100 µM NMDA/10 µM Glycine for GluN1-1a/2A (McClymont et al., 2012) were used. Stable current response in the presence of ladybird alkaloid was normalised to the stable current response to the agonist alone. Graphs were plotted in Prism 7 (GraphPad) using the non-linear regression “log(inhibitor) vs. normalised response (Variable slope)” equation (shown below).

$$Y = 100(1 + 10^{(LogIC50 - X) \times Hillslope})$$

IC₅₀s determined from these curve fits were compared in a pairwise manner using an extra sum of squares F-test; this is available in Graphpad Prism for this purpose and takes into account both the variability of the data and the quality of the curve fit.

2.4.4 Current-Voltage Experiments

A repeated voltage ramp was applied via Win EDR software (Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK) to nAChRs that slowly desensitise during continued ACh application (rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$). The V_h was repeatedly ramped up from an initial amplitude of -100 mV to a final amplitude of +50 mV over a duration of 1 s with a 0.5 s delay at -75 mV. Baseline recordings of the leakage current were measured for 5-10 s before application of ACh (10 μ M for $\alpha 4\beta 2$ and 100 μ M for $\alpha 3\beta 4$). The changes in current caused by ACh application were allowed to stabilise before co-application of ACh with ladybird alkaloid. The current was allowed to stabilise again and co-application was stopped. The current from 3 voltage ramps was averaged for the leakage current, stabilised ACh response and stabilised response of ACh co-applied with ladybird alkaloid for each cell. Multiple cells were tested and the mean was taken from combined cell data. The leakage current from the baseline recordings was subtracted from the stable current readings from both ACh alone and ACh co-applied with ladybird alkaloid. Current and voltage data was then plotted in Prism 7 (GraphPad). Data from the most negative 10 mV and the most positive 10 mV was used to calculate whether any significant difference had occurred in the level of inhibition using a paired t-test.

The voltage ramp protocol could not be used for receptors that rapidly desensitise after the onset of response to an agonist (human $\alpha 7$) as the receptor closes

before a stabilised current in response to the ramp can be achieved. A different protocol where control applications of 100 μ M ACh at holding potentials manually changed in 25 mV steps from -25 mV to -125 mV with 3 min wash intervals was used. This was then repeated with applications of the same concentration of 100 μ M ACh co-applied with ladybird alkaloid. The %inhibition of the ACh response at each holding potential was then used to determine whether any significant differences in the level of inhibition. After determining the normality of distribution using the Shapiro-Wilk test, a one-way ANOVA test followed by Tukey's multiple comparisons test was used.

For GluN1-1a/2A the V_h was manually changed and an IC_{50} value was obtained as described above for each V_h (-50, -75 and -100 mV). IC_{50} s were then compared using an extra sum-of-squares F-test.

In each case the approximate EC_{50} concentration of agonist was used to ensure that there was no saturation of the response to agonist due to excess agonist being available. Therefore the effect of ladybird alkaloids does not need to overcome the excess concentration of agonist and so the effect of ladybird alkaloids can be observed.

2.4.5 Effect of ladybird alkaloid on EC_{50}

EC_{50} curves were constructed in the presence and absence of ladybird alkaloid on the same oocyte. Due to the limited number of channels in the perfusion system used, a maximum of 3 concentrations could be examined on each oocyte. In order to construct a curve with a larger number of concentrations, a control concentration of 1 mM ACh was applied along with application of 1 mM ACh + ladybird alkaloid to each oocyte used. Data from each oocyte was normalised to the 1 mM ACh data point and

as this was present in recordings from each oocyte, this allowed the data sets from multiple oocytes to be combined. Each oocyte was clamped at $V_h = -75$ mV and three different concentrations of ACh were applied. Following this the same three ACh concentrations were co-applied with a constant concentration of ladybird alkaloid and peak responses were measured. This allowed the plotting of ACh concentration-response graphs in the absence and presence of alkaloid that could be fitted using the same non-linear regression equation to calculate the EC_{50} using Prism 7 (GraphPad) but with the Top parameter being set as less than 100 to allow a potential lower plateau to be plotted. The EC_{50} values were compared by an extra sum-of-squares F-test.

CHAPTER 3: Characterising the effects of ladybird alkaloids on LGICs expressed in *Xenopus* oocytes

3.1 Characterising the responses of nAChRs expressed in *Xenopus* oocytes to ACh

3.1.1 Introduction

In order to use nAChRs comprised of different subunits and expressed in *Xenopus* oocytes to examine the effects of ladybird alkaloids, the responses to ACh of each nAChR needed to be characterised. Determining the sensitivity to ACh is important to ensure appropriate ACh concentrations are used in further experiments to ensure that the oocyte's ACh response is not saturated. A range of ACh concentrations was applied to each oocyte to allow the construction of a concentration-response curve. The EC₅₀ values derived from the curves were then used to determine the appropriate ACh concentrations to use for each experiment. All experiments were carried out in the presence of 0.5 μ M atropine, a selective muscarinic ACh receptor antagonist, to ensure the ACh responses are solely due to activation of nAChRs.

3.1.2 ACh EC₅₀ of nAChRs expressed in *Xenopus* oocytes

The TEVC technique was used to examine the ACh responses of *Xenopus* oocytes expressing nAChR. Oocytes were clamped at $V_h = -75$ mV and a range of ACh concentrations was applied at 3 min intervals. Application of ACh generated an inward current represented by a negative change in current from the baseline (Fig 3.1.2.1-4 A-G). Concentration-response graphs were created by normalising the ACh responses

to the ACh concentration which gave the largest response (Figs 3.1.2.1, 3.1.2.2, 3.1.2.3 & 3.1.2.4).

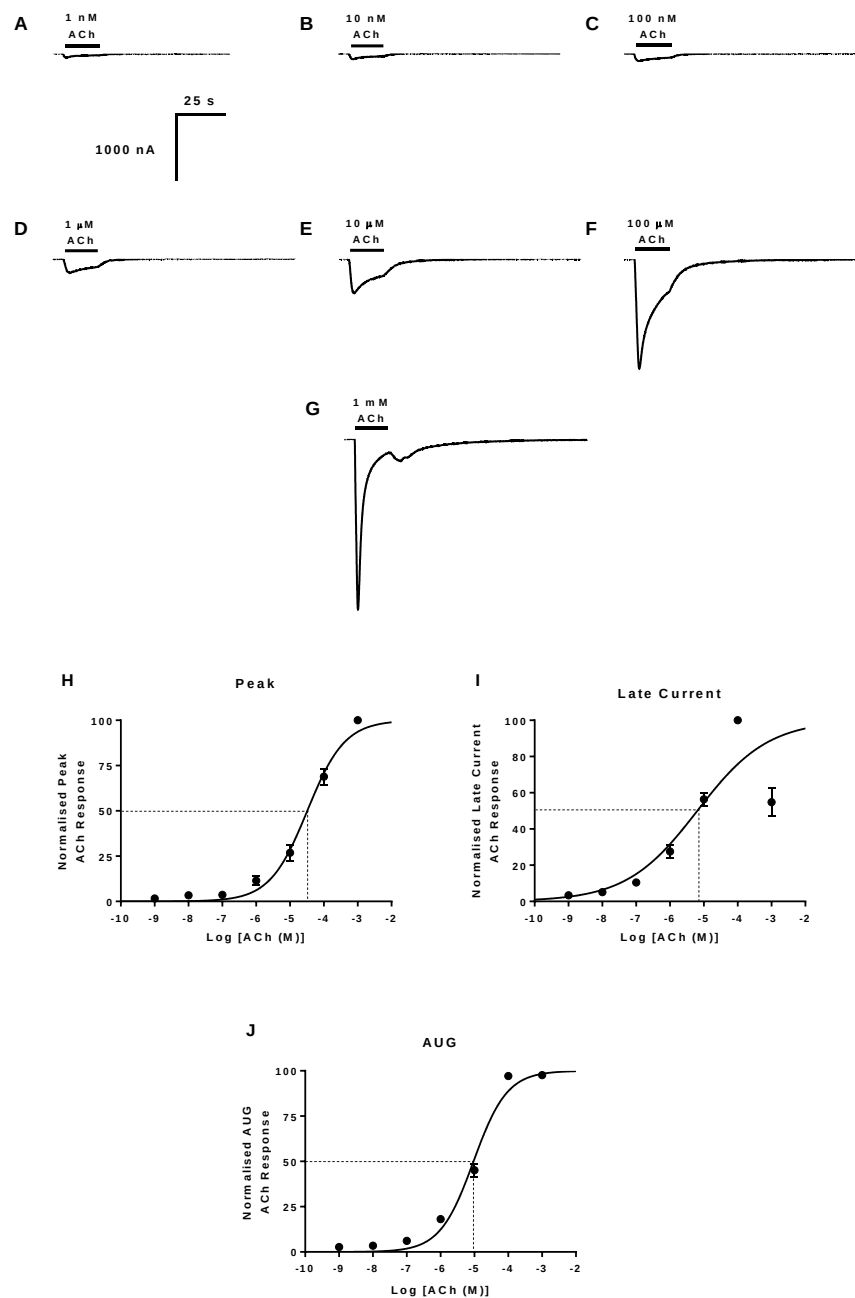


Fig. 3.1.2.1: Traces showing the standard response of a single *Xenopus* oocyte expressing rat $\alpha 4\beta 2$ nAChRs at $V_h = -75$ mV to (A) 1 nM ACh, (B) 10 nM ACh, (C) 100 nM ACh, (D) 1 μ M ACh, (E) 10 μ M ACh, (F) 100 μ M ACh and (G) 1 mM ACh applied for 15 s (black bars). Concentration-response curves fitted using the log(agonist) vs normalised response – Variable slope equation in GraphPad Prism 7 were used to calculate the EC₅₀ values of 32.4 μ M for the peak response (H), 6.41 μ M for the late current (I) and 9.42 μ M for the area under graph responses (J). All responses are normalised to the maximum ACh response and plotted as mean $\pm$ SEM (n=6).

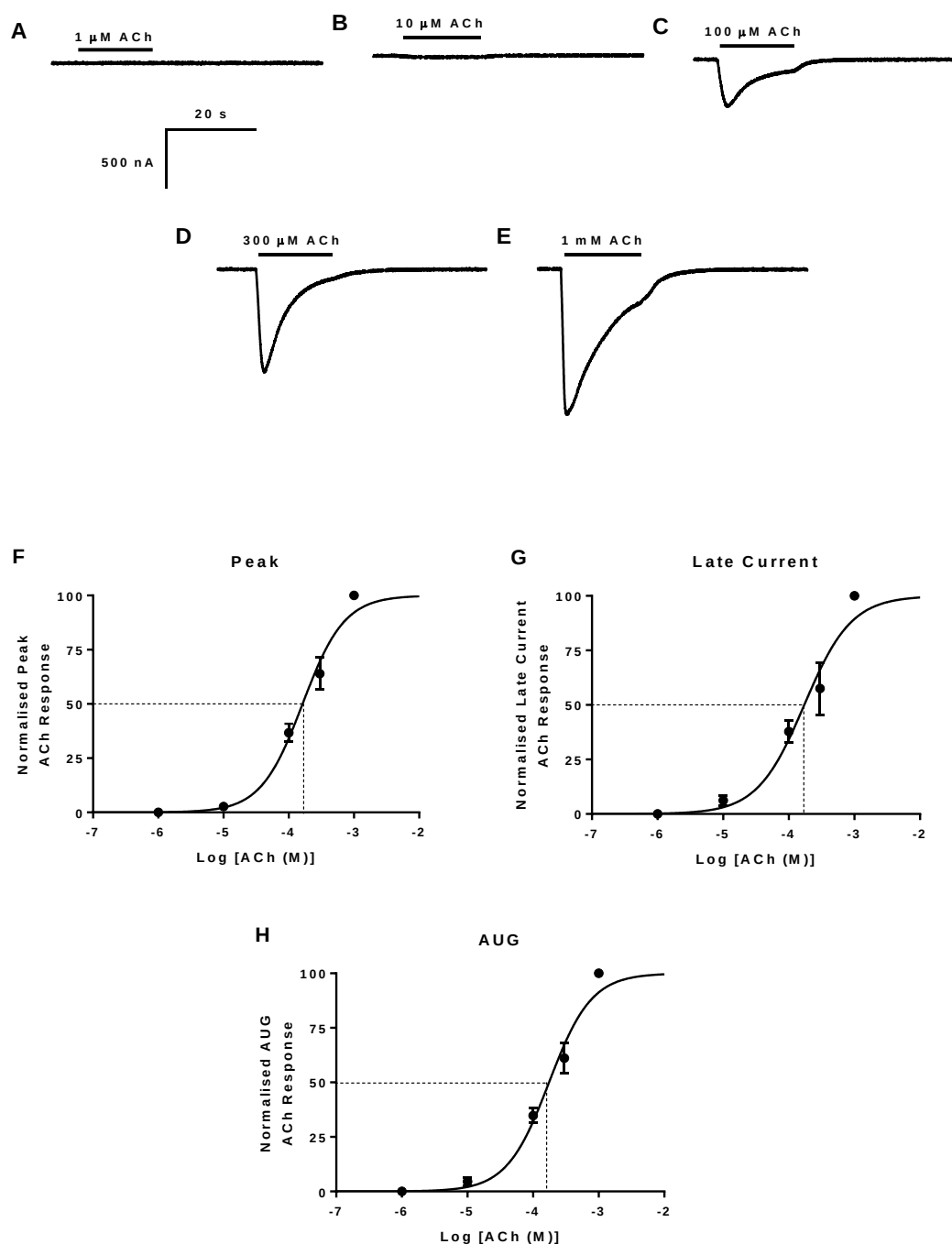


Fig. 3.1.2.2: Traces showing the standard response of a single *Xenopus* oocyte expressing rat $\alpha 3\beta 4$ nAChRs at $V_h = -75$ mV to (A) 1 μ M ACh, (B) 10 μ M ACh, (C) 100 μ M ACh, (D) 300 μ M ACh, and (E) 1 mM ACh applied for 15 s (black bars). Concentration-response curves fitted using the log(agonist) vs normalised response – Variable slope equation in GraphPad Prism 7 were used to calculate the EC₅₀ values of 163 μ M for the peak response (F), 172 μ M for the late current (G) and 175 μ M for the area under graph responses (H). All responses are normalised to the maximum ACh response and plotted as mean $\pm$ SEM (n=5).

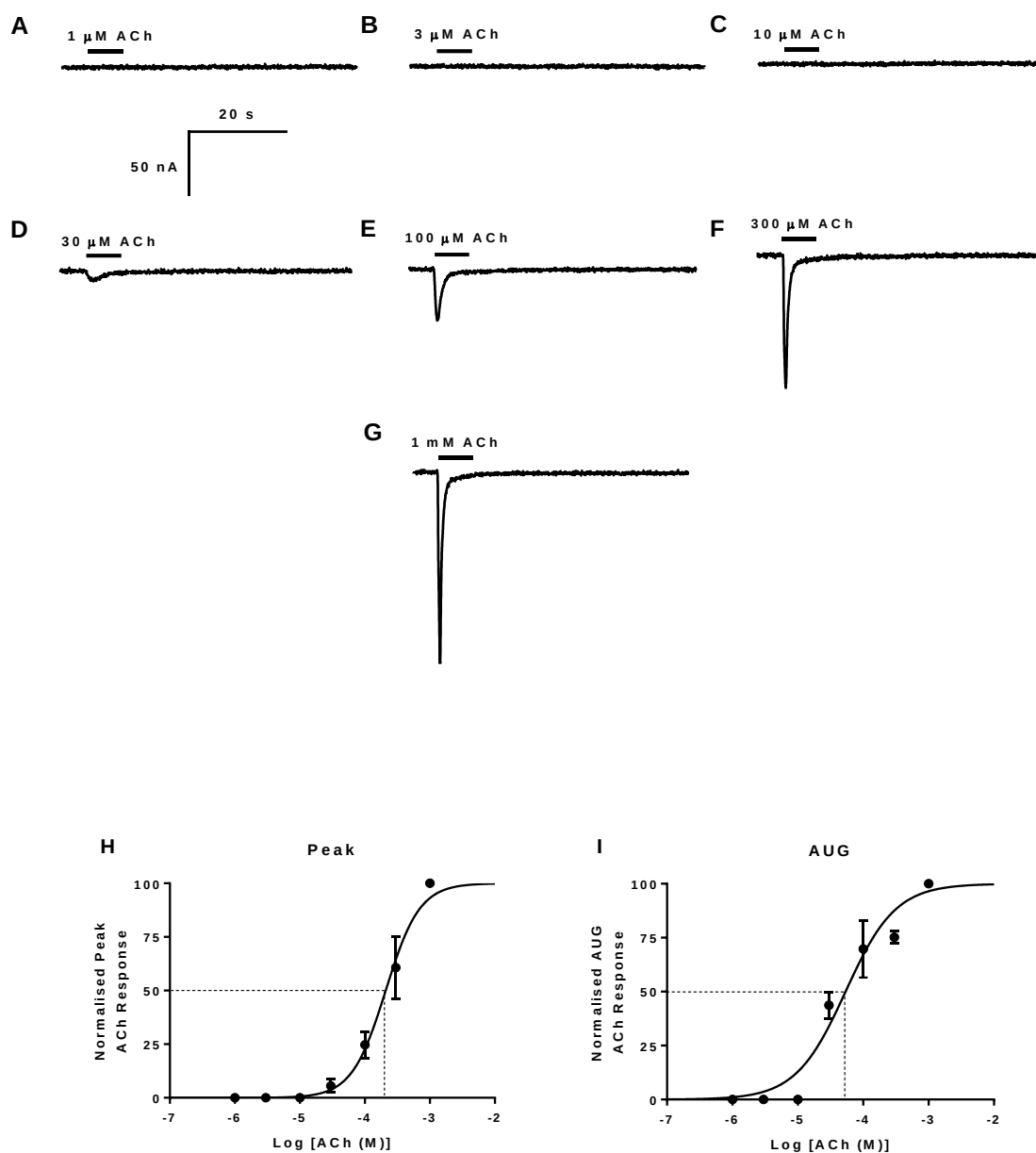


Fig. 3.1.2.3: Traces showing the standard responses of *Xenopus* oocytes expressing human $\alpha 7$ nAChRs at $V_h = -75$ mV to (A) 1 μ M ACh, (B) 3 μ M ACh, (C) 10 μ M ACh, (D) 30 μ M ACh, (E) 100 μ M ACh, (F) 300 μ M ACh and (G) 1 mM ACh applied for 5 s (black bars). Concentration-response curves fitted using the log(agonist) vs normalised response – Variable slope equation in GraphPad Prism 7 were used to calculate the EC₅₀ values of 209 μ M for the peak response (H) and 53.8 μ M for the area under graph responses (I). All responses are normalised to the maximum ACh response and plotted as mean $\pm$ SEM (n=6).

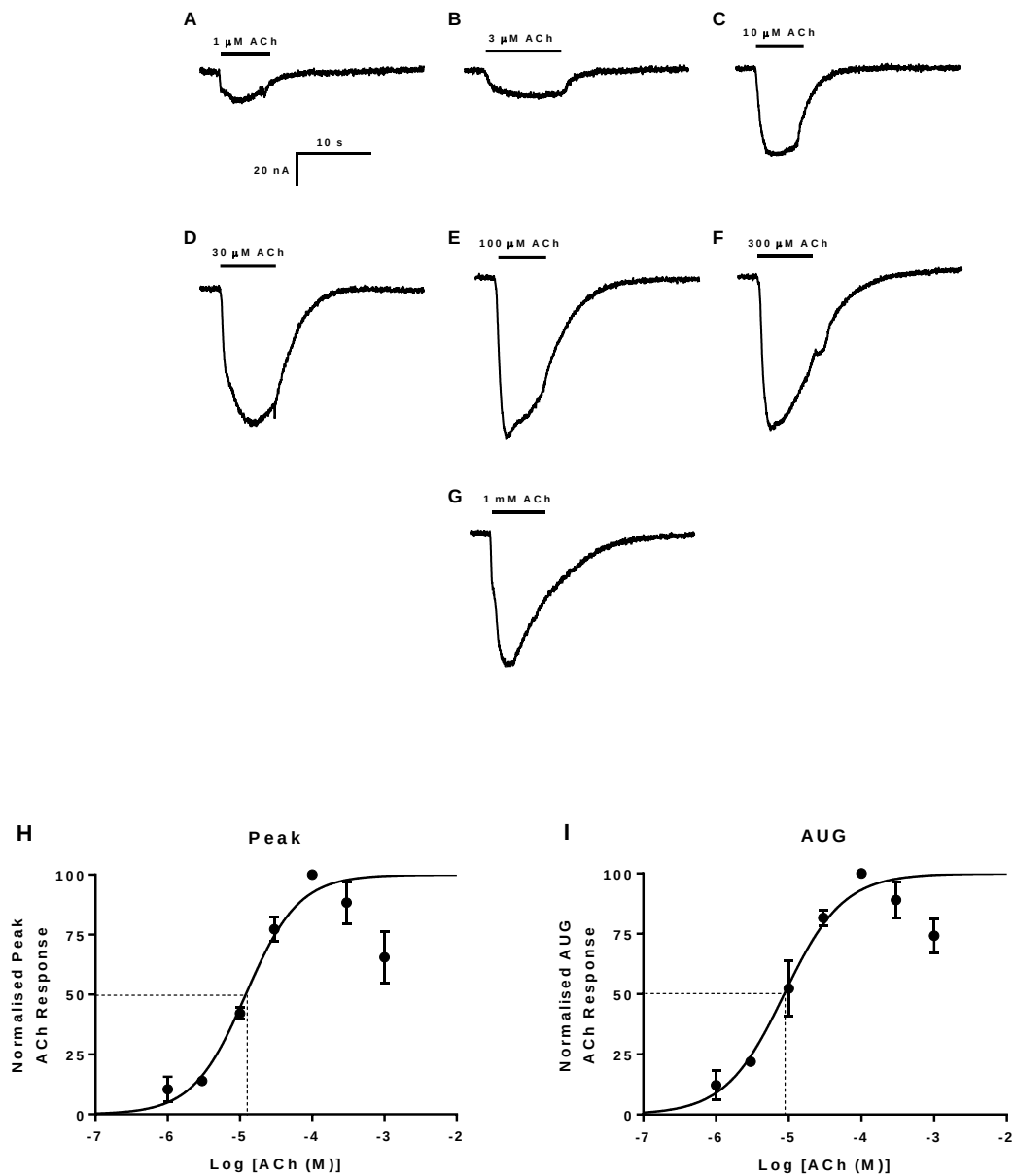


Fig. 3.1.2.4: Traces showing the standard responses of *Xenopus* oocytes expressing hybrid Da2/ β 2 nAChRs at $V_h = -75$ mV to (A) 1 μ M ACh, (B) 3 μ M ACh, (C) 10 μ M ACh, (D) 30 μ M ACh, (E) 100 μ M ACh, (F) 300 μ M ACh and (G) 1 mM ACh applied for 5 s (black bars). Concentration-response curves fitted using the log(agonist) vs normalised response – Variable slope equation in GraphPad Prism 7 were used to calculate the EC_{50} values of 12 μ M for the peak response (H) and 8.69 μ M for the area under graph responses (I). All responses are normalised to the maximum ACh response and plotted as mean $\pm$ SEM (n=3).

The EC₅₀ values for each receptor were compared (Table 3.2.1) and hybrid Dα2/β2 receptors were shown to have the lowest EC₅₀ for peak responses followed by rat α4β2 with the calculated EC₅₀ values for all parameters measured being closer to 10 μM than 100 μM. The EC₅₀ values for rat α3β4 and human α7 were found to be higher with the calculated EC₅₀ values being closer to 100 μM than 10 μM.

Table 3.1.2.1 – EC₅₀ values of peak current, late current and AUG responses of mammalian and hybrid insect/mammalian nAChRs to ACh

Receptor	EC ₅₀ (μM)(95% CI)		
	Peak current	Late Current	AUG
α4β2	32.4 (25.4 – 41.3)	6.41 (2.65 – 15.5)	9.42 (7.47 – 11.8)
α3β4	163 (131 – 199)	172 (118 – 239)	175 (141 – 214)
α7	209 (187 – 234)	-	53.8 (39.1 – 74.5)
Dα2/β2	12.0 (7.24 – 20)	-	8.69 (5.48 – 13.7)

Human α7 nAChRs showed rapid desensitisation after the peak ACh response had been reached (Fig 3.1.2.3). The late current could not be measured and so could not be used to examine the time dependent effects of ACh with human α7. A similar but slower desensitisation was exhibited by hybrid Dα2/β2 nAChRs (Fig 3.1.2.4). At lower concentrations of ACh this desensitisation did not occur but at ACh concentrations ranging from 30 μM to 1 mM desensitisation occurred within 5 s of the onset of the ACh response. As the higher concentrations would be used in further experiments the decision was made to continue all applications in further experiments for 5 s in the same manner as applications for human α7. Rat α4β2 and α3β4 ACh

responses showed a slower desensitisation and the current reached a steady state for both receptors (Fig 3.1.2.1 & Fig 3.1.2.2). An application time of 15 s allowed the current to begin to enter a steady state and so the time-dependent effects on the ACh response could be examined.

For rat $\alpha 3\beta 4$ and human $\alpha 7$, 100 μM ACh was chosen as the control concentration for further experiments with ladybird alkaloids as it is near the EC_{50} concentration and not a high enough concentration to saturate the response of the receptors. Because rat $\alpha 4\beta 2$ had a much lower EC_{50} , 10 μM ACh was chosen as the control concentration. Application of ACh to $\text{D}\alpha 2/\beta 2$ produced very small responses for the majority of oocytes tested and so 100 μM ACh was chosen as the control concentration to ensure that an easily measurable current could be produced.

3.1.3 Current-voltage relationship of ACh responses

Investigating the current-voltage (IV) relationship of ACh responses of human $\alpha 7$, rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ required two different methods, as the ACh responses of human $\alpha 7$ receptors do not enter a steady state current. The V_h was changed in 25 mV steps across a 100 mV range from -25 mV to -125 mV. The peak response to 100 μM ACh was measured at each V_h and as the V_h decreased, the response to ACh increased but not in a linear manner (Fig. 3.3.1).

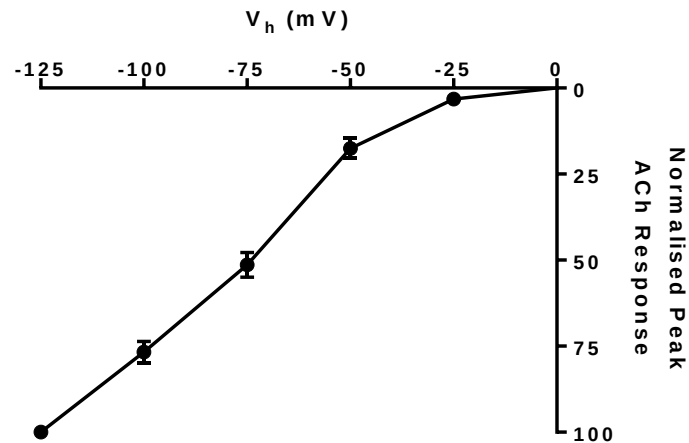


Fig. 3.1.3.1: Responses of human $\alpha 7$ receptors to 100 μ M ACh as the V_h is changed showing the IV relationship. All points are normalised to the -125 mV ACh response and plotted as mean $\pm$ SEM ($n = 6$) with some error bars not visible due to being smaller than the data point symbol.

As the ACh responses of rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ nAChRs reach a steady state current, a voltage ramp can be applied. This is preferable as it allows a wider range of V_h to be studied. V_h was repeatedly ramped from -100 mV to +50 mV over a 1 s duration with a 0.5 s delay between ramps. ACh was applied and current was measured once a stable response was achieved (Fig 3.3.2 & 3.3.3). As with human $\alpha 7$, rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ showed an increase in current generated by the ACh response at more negative V_h that was proportionally greater. At more positive V_h than -25 very little outward current was detected this is known as inward rectification.

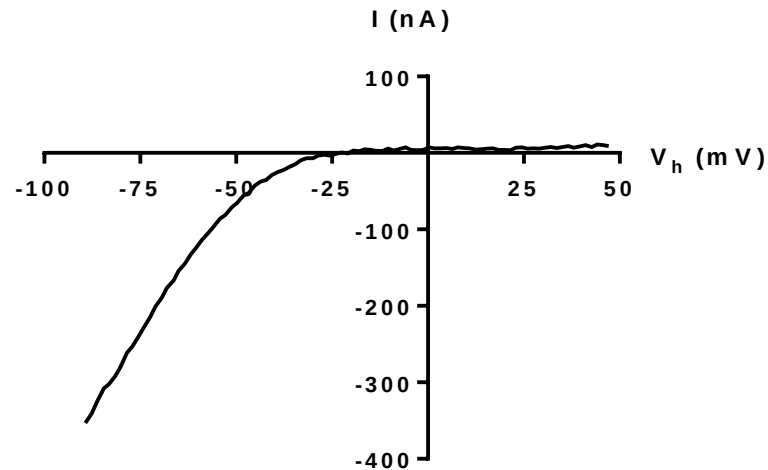


Fig. 3.1.3.2: IV relationship of rat $\alpha 3\beta 4$ in response to 100 μM ACh at a stable current. For each oocyte ($n=5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during the application of ACh. Leakage current was subtracted from stabilised ACh current.

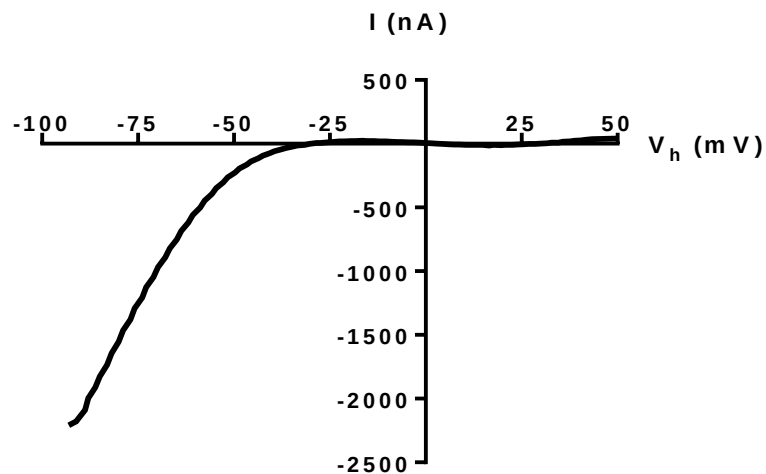


Fig. 3.1.3.3: IV relationship of rat $\alpha 4\beta 2$ in response to 10 μM ACh at a stable current. For each oocyte ($n = 5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during the application of ACh. Leakage current was subtracted from stabilised ACh current.

3.1.4 Response of nAChRs to repeated applications of ACh

The time interval between applications of ACh is critical to ensure that the ACh response is stable thereby ensuring that any reduction in current observed is due to the effect of ladybird alkaloid and not due to the receptors not recovering after desensitisation has occurred. A 3 min interval between each application was chosen as

the ACh response remained stable (defined as within 10% of the previous responses) and so enough time had passed so the receptors had recovered from desensitisation (Fig 3.1.4.1).

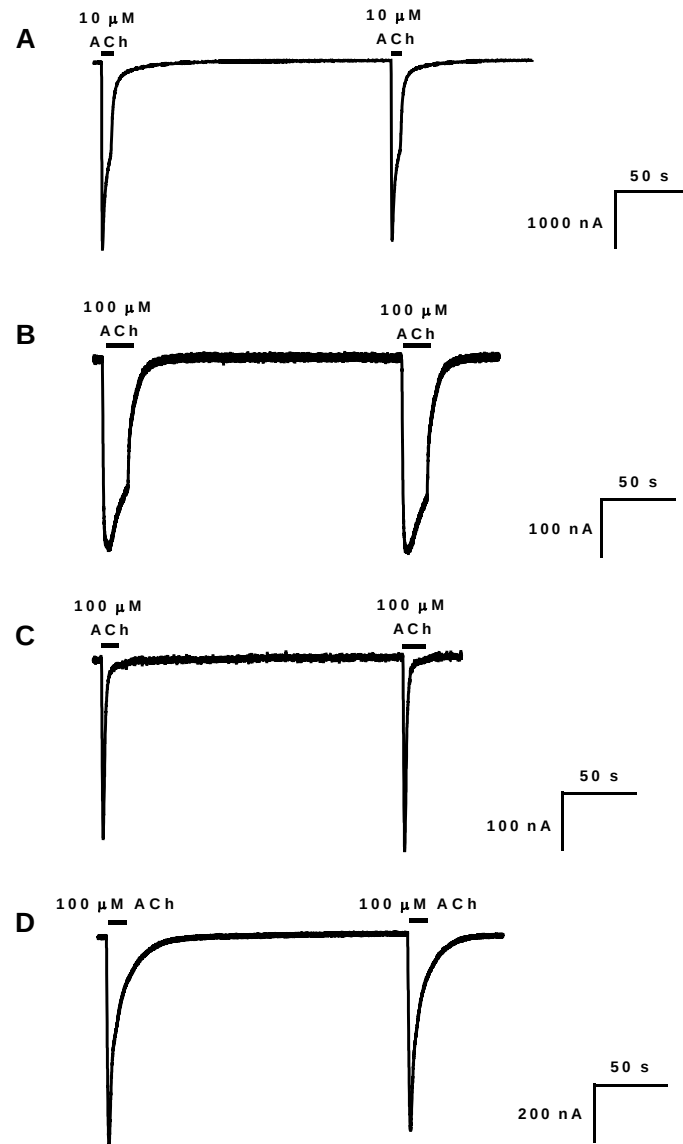


Fig 3.1.4.1: The response to consecutive applications of ACh after a 3 min wash period for (A) rat $\alpha 4 \beta 2$, (B) rat $\alpha 3 \beta 4$, (C) human $\alpha 7$ and (D) D $\alpha 2/\beta 2$ nAChRs.

3.2 Analysis of alkaloid fractions from *Harmonia axyridis* and *Adalia bipunctata*

After creating fractions of extracted alkaloids from *H. axyridis* beetles using liquid column chromatography, thin layer chromatography was then used to determine the fractions that contained alkaloid by testing with Dragendorff's reagent. Fractions that tested positive were combined and analysed using gas chromatography – mass spectrometry (Fig 3.2.1). The chromatograph showed the presence of multiple alkaloid compounds with a large peak indicating one alkaloid compound possibly accounting for the majority of HAE. Fractions of alkaloid extract were also obtained from *A. bipunctata* and a previously published protocol was used to separate the alkaloids (-)-adaline and adalinine from the fraction (Lognay et al., 1996). The presence of (-)-adaline was then confirmed in the separation using gas chromatography – mass spectrometry (Fig 3.2.2). Fractions that contained solely (-)-adaline were then combined and used in the experiments in this study

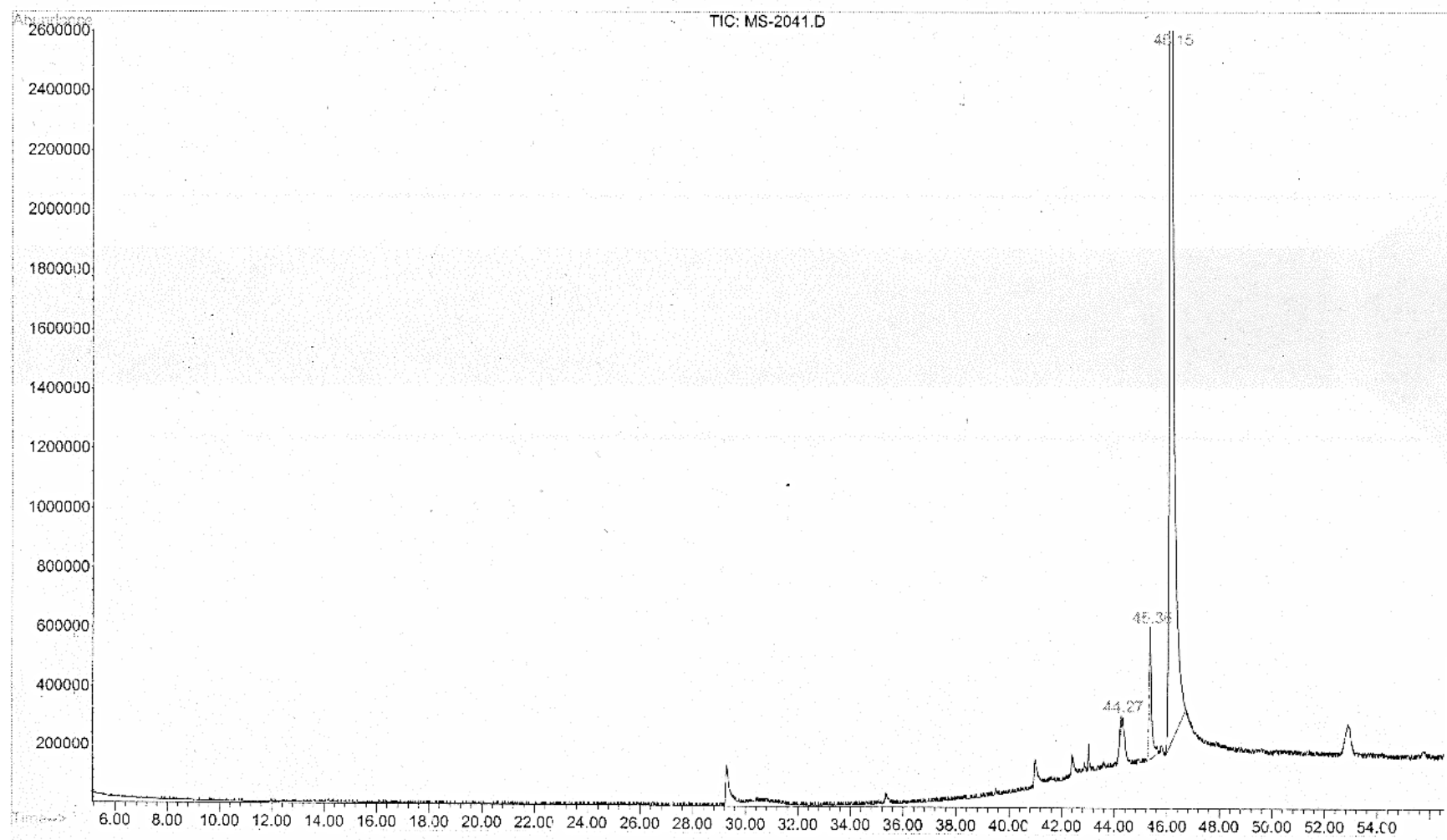


Fig 3.2.1: Chromatogram of HAE showing multiple alkaloid compounds present in HAE with one peak possibly representing an alkaloid compound present in a larger quantity than all others.

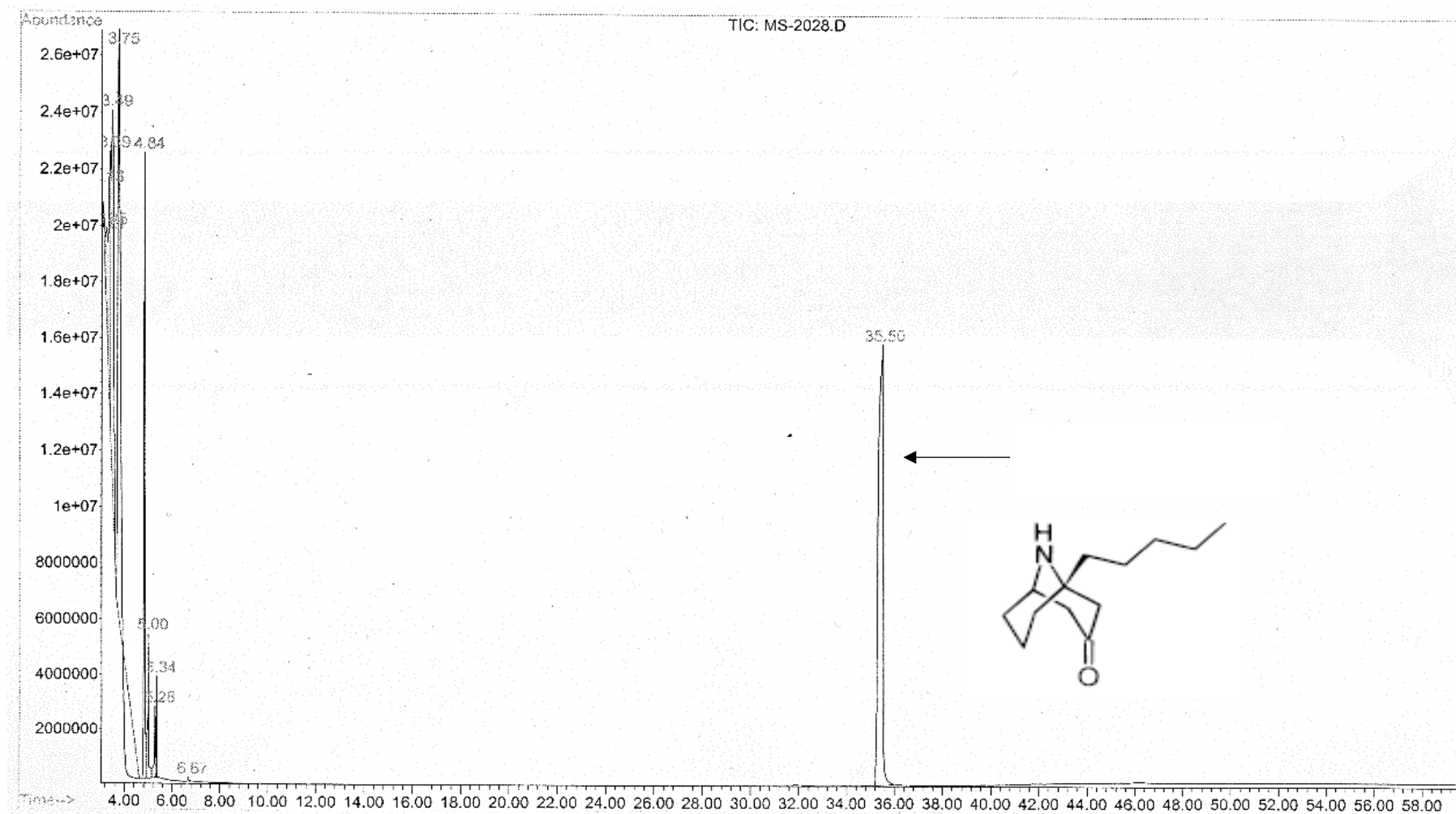


Fig. 3.2.2: Chromatogram of *A. bipunctata* alkaloid fraction showing major peak of (-)-adaline present in the sample. (-)-adaline structure taken from (Glisan King & Meinwald 1996).

3.3 Concentration-inhibition relationship of HAE and (-)-adaline on mammalian and insect/mammalian hybrid neuronal nAChRs

The whole alkaloid extract of *Harmonia axyridis* (HAE) and the alkaloid (-)-adaline has been shown to inhibit the ACh responses of m-nAChRs expressed in human TE671 cell lines and n-nAChRs expressed in neurons of the mushroom bodies of the desert locust *Schistocerca gregaria* (Richards, 2015). Recently, alkaloids from a variety of different coccinellid species have been shown to have non-competitive inhibitory activity on m-nAChRs expressed in TE671 cells and human $\alpha 7$ n-nAChRs expressed in *Xenopus* oocytes (Leong et al., 2014). However alkaloids from *H. axyridis* and (-)-adaline were not included in this study and therefore the activity on the homomeric human $\alpha 7$ as well as the heteromeric rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ n-nAChRs was investigated. As many natural toxins target multiple nAChRs, investigating the effects on more nAChR subtypes would show whether this inhibitory activity extended to other nAChR subtypes. The hybrid D $\alpha 2/\beta 2$ was also investigated in order to ascertain whether any selectivity existed between insect and mammalian n-nAChRs. Pure insect receptors have proven difficult to express in *Xenopus* oocytes and studies using D $\alpha 2/\beta 2$ have shown successful expression. However, due to the low levels of expression of D $\alpha 2/\beta 2$ (responses less than 50 nA) a higher concentration of 100 μ M ACh was used as an agonist. This concentration gave the largest response without causing desensitisation of the receptor and provided more observable current for analysis. HAE and (-)-adaline was co-applied at varying concentrations with ACh at the approximate EC₅₀ on oocytes expressing n-nAChRs clamped at $V_h = -75$ mV. The effect on the peak response, late current and AUG was measured. As the peak response of oocytes expressing the rapidly desensitising human $\alpha 7$ are not representative of the

whole cell response the AUG was measured across all receptors to provide a more accurate representation of the whole cell response (Papke). The effect on the late current shows the effect of ladybird alkaloids over time as differences in the decay of the response can give insight into mode of action. No effect was observed when HAE or (-)-adaline was applied alone (data not shown) but concentration-dependant inhibition of the ACh response was observed when HAE and (-)-adaline was co-applied with ACh on all nAChRs tested (Figs 3.3.1 – 3.3.4 & 3.3.6 – 3.3.9).

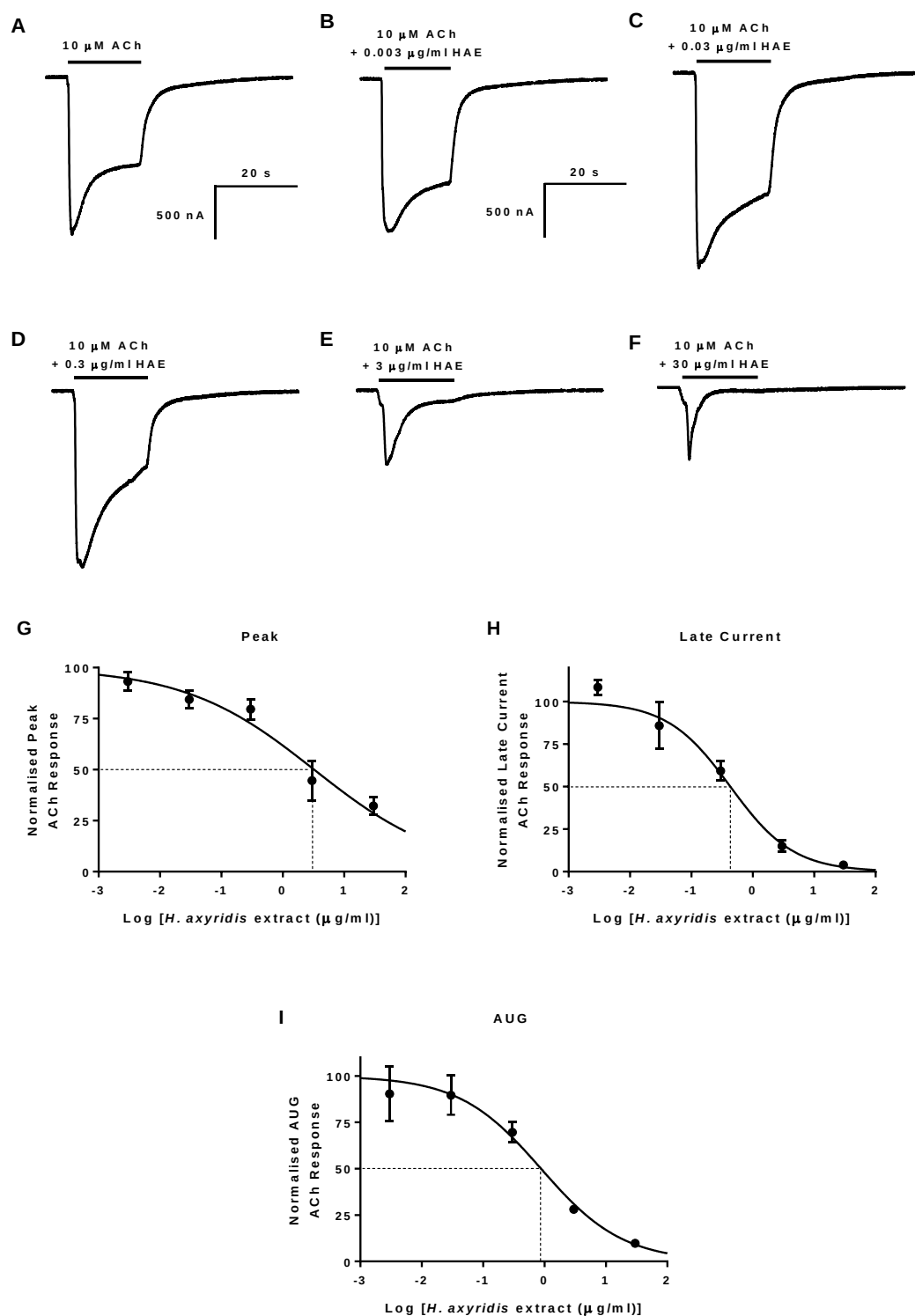


Fig. 3.3.1: Traces of the response to 10 μM ACh of rat $\alpha 4 \beta 2$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 0.003 $\mu\text{g/ml}$ HAE, (C) 0.03 $\mu\text{g/ml}$ HAE, (D) 0.3 $\mu\text{g/ml}$ HAE, (E) 3 $\mu\text{g/ml}$ HAE and (F) 30 $\mu\text{g/ml}$ HAE. Concentration-inhibition curves of the peak current, late current and area under graph responses show IC_{50} values of 3.24 $\mu\text{g/ml}$ for peak response (G), 0.4314 $\mu\text{g/ml}$ for late current (H) and 0.8672 $\mu\text{g/ml}$ for AUG (I). All responses were normalised to the control 10 μM ACh response and plotted as mean $\pm$ SEM (n = 4).

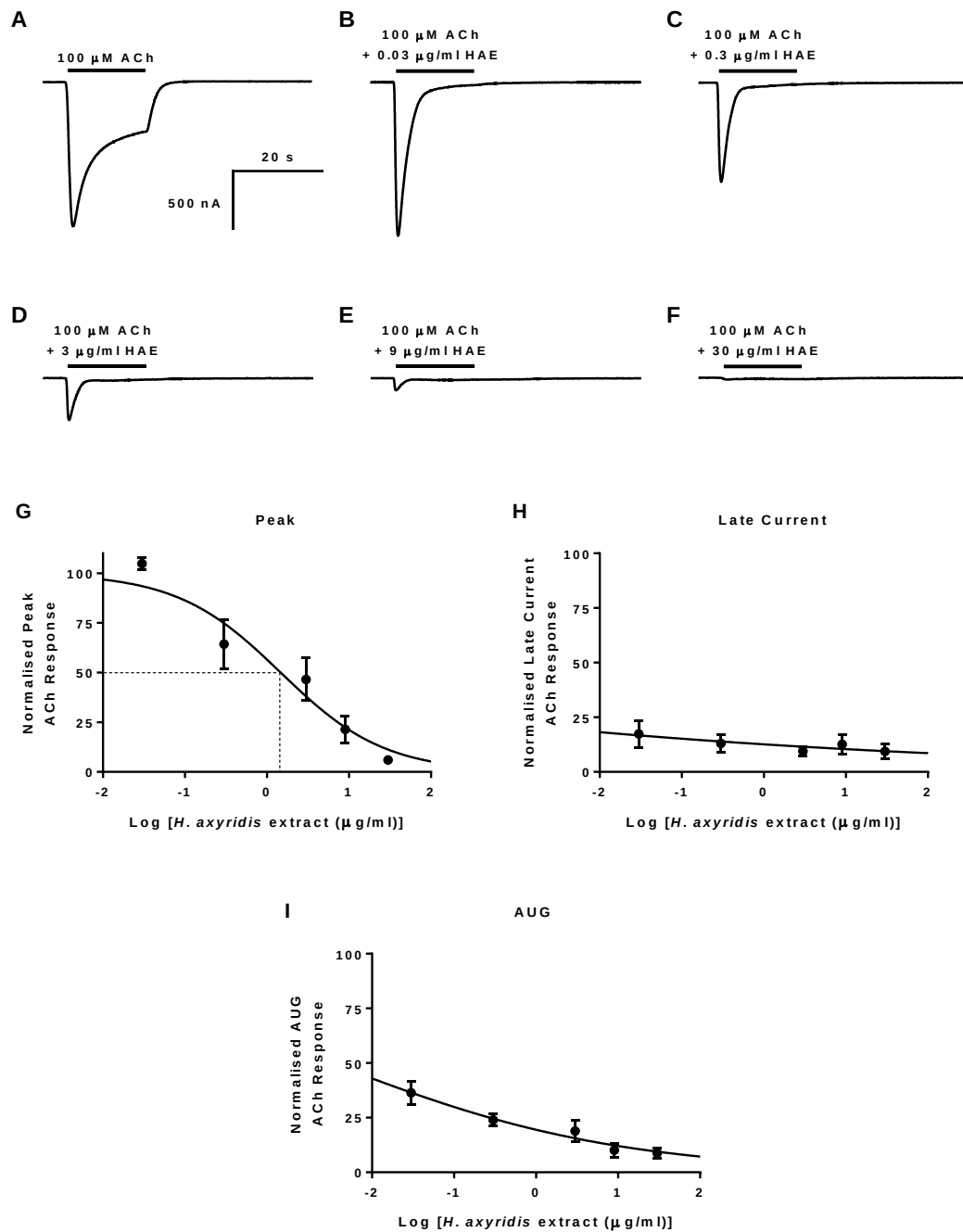


Fig. 3.3.2: Traces of the response to 100 μM ACh of rat $\alpha 3\beta 4$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 0.03 $\mu\text{g/ml}$ HAE, (C) 0.3 $\mu\text{g/ml}$ HAE, (D) 3 $\mu\text{g/ml}$ HAE, (E) 9 $\mu\text{g/ml}$ HAE and (F) 30 $\mu\text{g/ml}$ HAE. Concentration-inhibition curves of the peak current, late current and AUG responses show IC_{50} values of 1.469 $\mu\text{g/ml}$ for peak response (G), 1.07×10^{-9} $\mu\text{g/ml}$ for late current (H) and 0.00317 $\mu\text{g/ml}$ for AUG (I). All responses were normalised to the control 100 μM ACh response and plotted as mean $\pm$ SEM ($n = 7-8$).

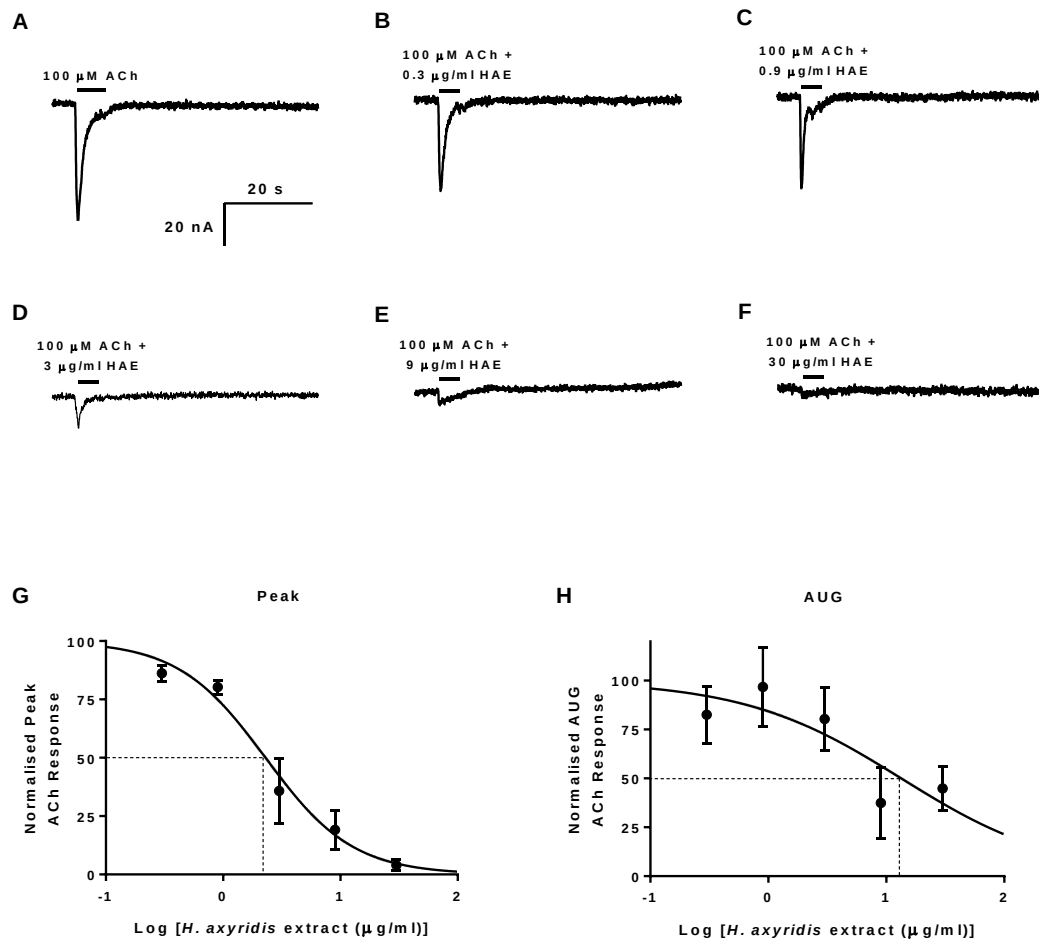


Fig. 3.3.3: Traces of the response to 100 μM ACh of human $\alpha 7$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 0.3 $\mu\text{g/ml}$ HAE, (C) 0.9 $\mu\text{g/ml}$ HAE, (D) 3 $\mu\text{g/ml}$ HAE, (E) 9 $\mu\text{g/ml}$ HAE and (F) 30 $\mu\text{g/ml}$ HAE. Concentration-inhibition curves of the peak current and AUG responses show IC_{50} values of 1.47 $\mu\text{g/ml}$ for peak response (G) and 13.4 $\mu\text{g/ml}$ for AUG (H). All responses were normalised to the control 100 μM ACh response and plotted as mean $\pm$ SEM ($n = 6-9$).

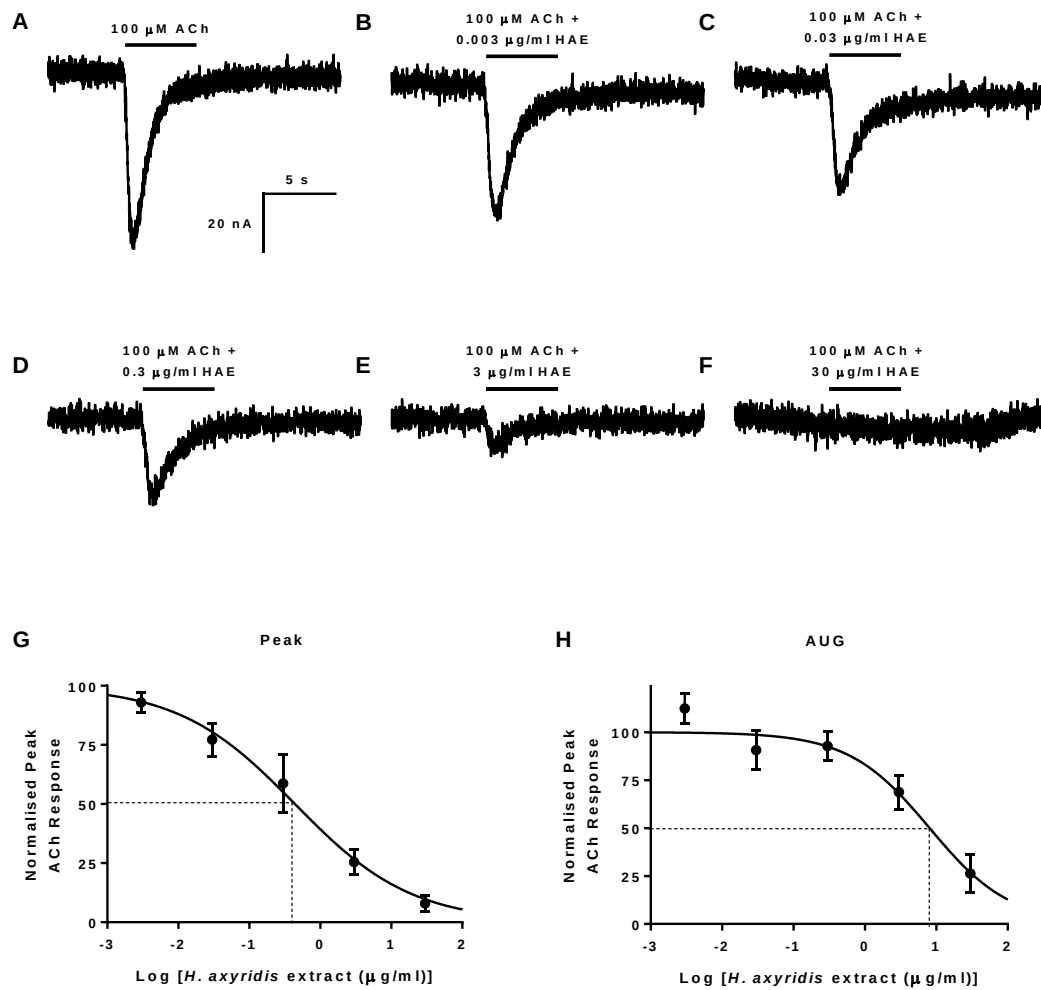


Fig. 3.3.4: Traces of the response to 100 μ M ACh of hybrid insect/mammalian D α 2/ β 2 nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 0.003 μ g/ml HAE, (C) 0.03 μ g/ml HAE, (D) 0.3 μ g/ml HAE, (E) 3 μ g/ml HAE and (F) 30 μ g/ml HAE. Concentration-inhibition curves of the peak current and AUG responses show IC_{50} values of 0.4397 μ g/ml for peak response (G) and 8.063 μ g/ml for AUG (H). All responses were normalised to the control 100 μ M ACh response and plotted as mean $\pm$ SEM ($n = 5$).

The peak current IC_{50} of HAE was measured for each nAChR and compared to the late current and AUG IC_{50} . Statistically significant differences were observed when analysed using an extra sum-of-squares F-test for all receptors tested. Late current and AUG IC_{50} were significantly different from peak current IC_{50} for rat α 3 β 4 ($P < 0.0001$ for AUG and late current could not be calculated), rat α 4 β 2 ($P = 0.0002$ for

late current and $P=0.0334$ for AUG), human $\alpha 7$ ($P=0.0005$ for AUG) and $D\alpha 2/\beta 2$ ($P<0.0001$ for AUG). The late current/AUG IC_{50} for rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ nAChRs that hold a steady state response were much lower than the IC_{50} for the peak response indicating a larger inhibitory effect on the decay of the response than on the peak of the response itself. Human $\alpha 7$ and hybrid $D\alpha 2/\beta 2$ do not hold a steady state current and also larger inhibitory effect on the peak IC_{50} over the AUG IC_{50} was observed indicating that there was a lesser effect on the decay of the response in these receptors (Table 3.3.1).

Table 3.3.1 – IC_{50} values on peak, late current and AUG responses of mammalian and hybrid insect/mammalian nAChRs to HAE

Receptor	IC_{50} ($\mu g/ml$) (95% CI)		
	Peak Current	Late Current	AUG
$\alpha 7$	2.30 (1.67 – 3.17)	-	13.4 (4.217 – 118)
$\alpha 4\beta 2$	3.24 (1.47 – 8.65)	0.431 (0.233 – 0.804)	0.88 (0.365 – 2.23)
$\alpha 3\beta 4$	1.47 (0.708 – 2.88)	1.07×10^{-9} *	0.00317 (0.000173 – 0.00131)
$D\alpha 2/\beta 2$	0.440 (0.211 – 0.910)	-	8.06 (3.62 – 19.6)

* - 95% CI could not be calculated

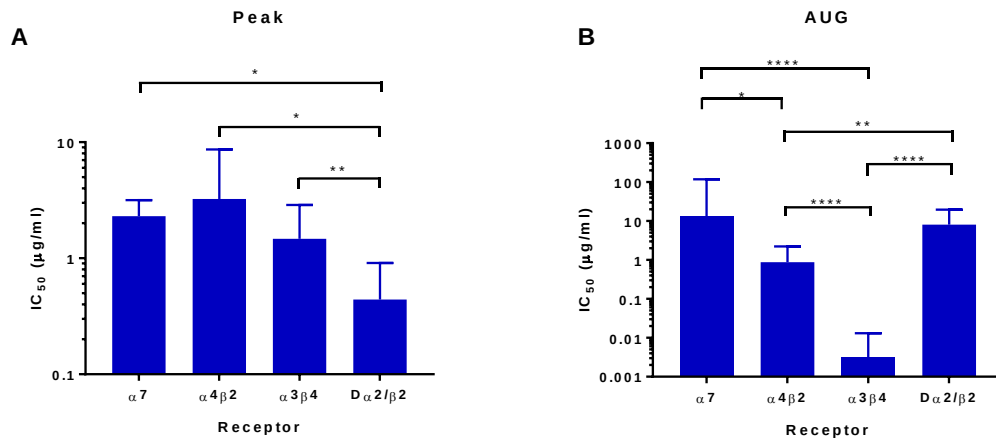


Fig. 3.3.5: IC₅₀ values for (A) peak current and (B) AUG of HAE were plotted as mean $\pm$ 95% CI and compared using an extra sum-of-squares F-test. Significant differences were observed in the peak current IC₅₀ for Dα2/β2 vs. α3β4 ($P=0.0217$), Dα2/β2 vs. α4β2 ($P=0.0016$) and Dα2/β2 vs. α7 ($P=0.0102$). Significant differences were observed in the AUG IC₅₀ for α3β4 vs. α4β2 ($P<0.0001$), α3β4 vs. α7 ($P<0.0001$) and α3β4 vs. Dα2/β2 ($P=0.0069$), α4β2 vs. α7 ($P=0.0178$) and Dα2/β2 vs. α4β2 ($P=0.0012$). (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, **** = $P<0.0001$).

HAE peak current IC₅₀ values across the different receptors (Fig 3.3.5) showed significantly lower IC₅₀'s for Dα2/β2 when compared to rat α3β4 ($P=0.0217$), rat α4β2 ($P=0.0016$) and human α7 ($P=0.0102$). There were no significant differences between the peak current IC₅₀s for the mammalian receptors indicating a selectivity for the hybrid Dα2/β2 nAChR (Fig. 3.3.5A). This selectivity did not extend to the AUG IC₅₀s with rat α3β4 having a significantly lower AUG IC₅₀ than rat α4β2 ($P<0.0001$), human α7 ($P<0.0001$) and Dα2/β2 ($P=0.0069$). This is also the case when comparing the late current IC₅₀ for rat α3β4 vs. rat α4β2 ($P<0.0001$). The AUG IC₅₀ for Dα2/β2 was also significantly larger than rat α4β2 ($P=0.0012$). There was more variability between the AUG IC₅₀s of mammalian receptors with significantly lower IC₅₀ for rat α4β2 when compared to human α7 ($P=0.0178$). Therefore while it appears that there is selectivity for Dα2/β2 as it did show the lowest peak IC₅₀, the AUG IC₅₀ value for rat α3β4 was even lower.

The concentration dependent effects of (-)-adaline were also investigated on *Xenopus* oocytes expressing human $\alpha 7$, rat $\alpha 3\beta 4$, rat $\alpha 4\beta 2$ and D $\alpha 2/\beta 2$ nAChRs clamped at $V_h = -75$ mV with (-)-adaline co-applied with ACh at the approximate EC_{50} . A range of (-)-adaline concentrations were applied and responses were normalised to a control ACh response in order to generate a concentration-inhibition curve and therefore an IC_{50} value (Fig 3.3.6, 3.3.7, 3.3.8 & 3.3.9).

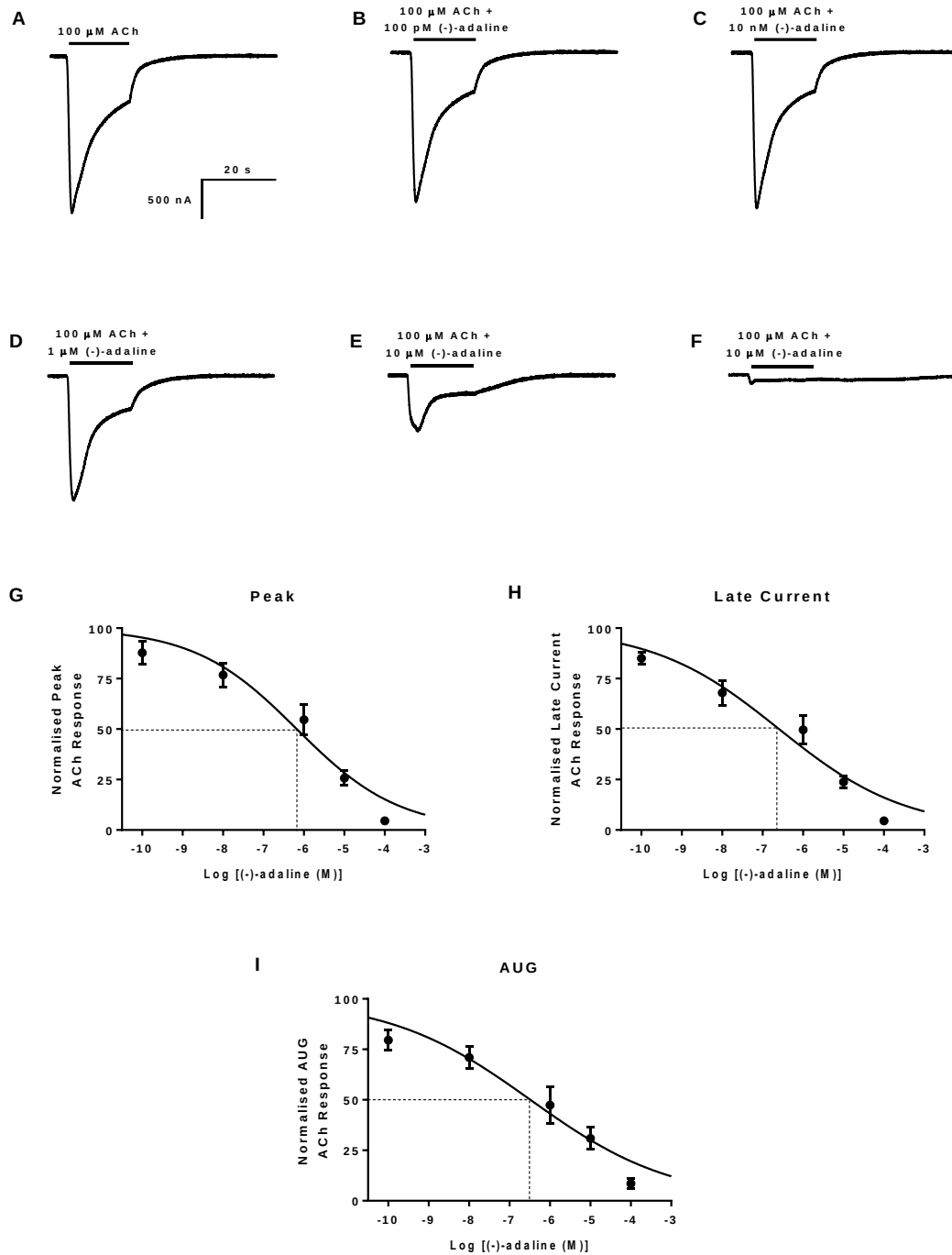


Fig. 3.3.6: Traces of the response to 100 μ M ACh of rat $\alpha 3 \beta 4$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 100 pM (-)-adaline, (C) 10 nM (-)-adaline, (D) 1 μ M (-)-adaline, (E) 10 μ M (-)-adaline and (F) 100 μ M (-)-adaline. Concentration-inhibition curves of the peak, late current and AUG responses gave IC_{50} values of 0.66 μ M for peak response (G) , 0.25 μ M for late current (H) and 0.33 μ M for AUG (I). All responses were normalised to the control 100 μ M ACh response and plotted as mean $\pm$ SEM ($n = 8$).

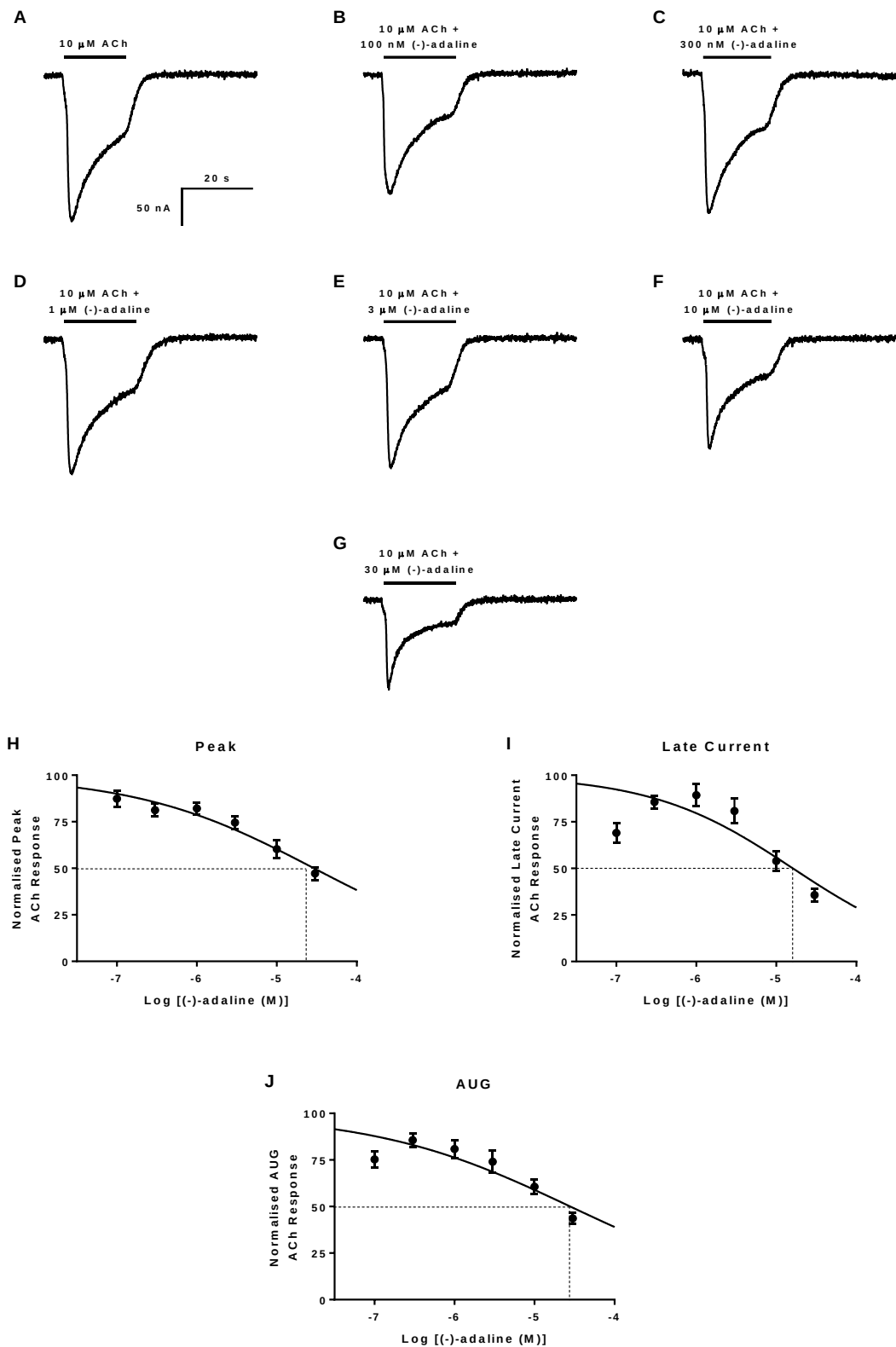


Fig. 3.3.7: Traces of the response to 10 μM ACh of rat $\alpha 4\beta 2$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 100 nM (-)-adaline, (C) 300 nM (-)-adaline, (D) 1 μM (-)-adaline, (E) 3 μM (-)-adaline (F) 10 μM (-)-adaline and (G) 30 μM (-)-adaline. Concentration-inhibition curves of the peak, late current and AUG responses gave IC_{50} values of 29 μM for peak response (H) , 16 μM for late current (I) and 28 μM for AUG (J). All responses were normalised to the control 10 μM ACh response and plotted as mean $\pm$ SEM ($n = 6$).

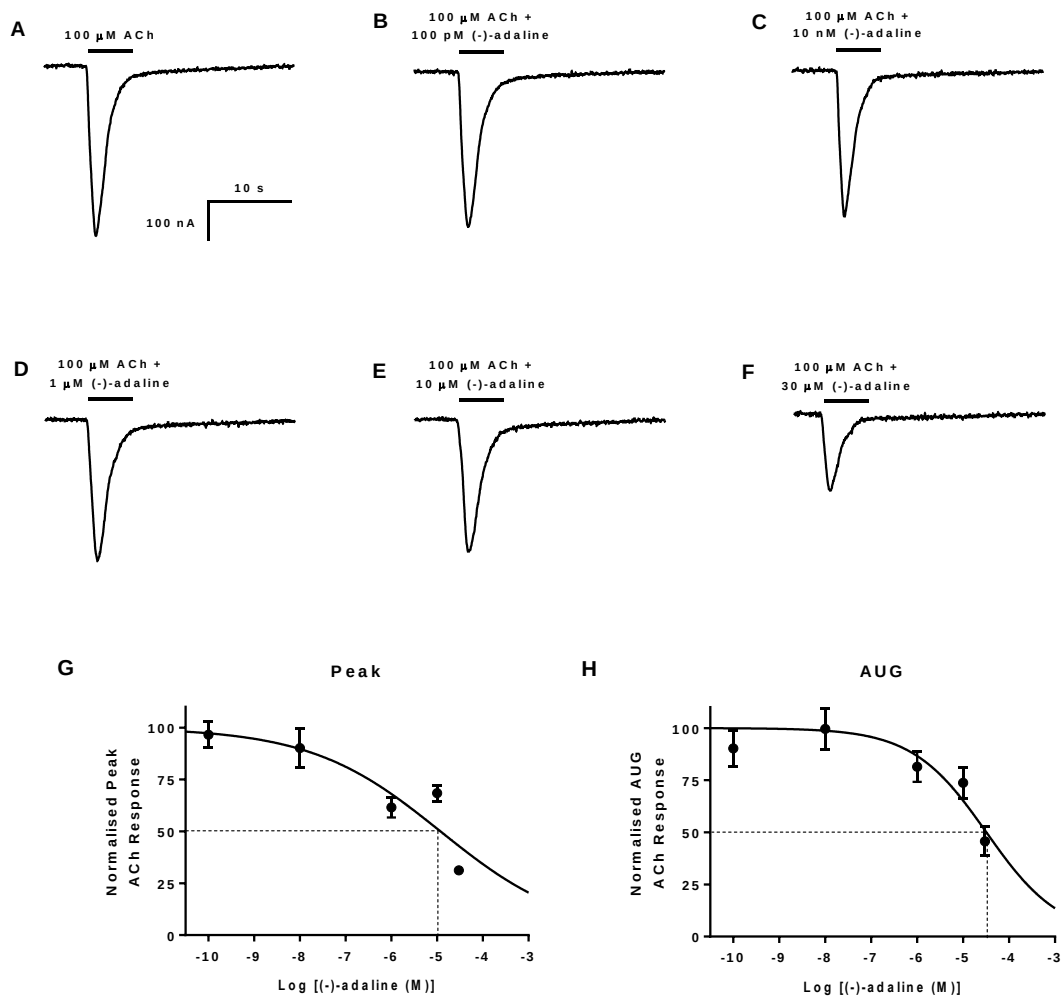


Fig. 3.3.8: Traces of the response to 100 μM ACh of human $\alpha 7$ nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 100 pM (-)-adaline, (C) 100 nM (-)-adaline, (D) 1 μM (-)-adaline, (E) 10 μM (-)-adaline and (F) 30 μM (-)-adaline. Concentration-inhibition curves of the peak current and AUG responses gave IC_{50} values of 11.8 μM for peak response (G) and 32.2 μM for AUG (H). All responses were normalised to the control 10 μM ACh response and plotted as mean $\pm$ SEM ($n = 5$).

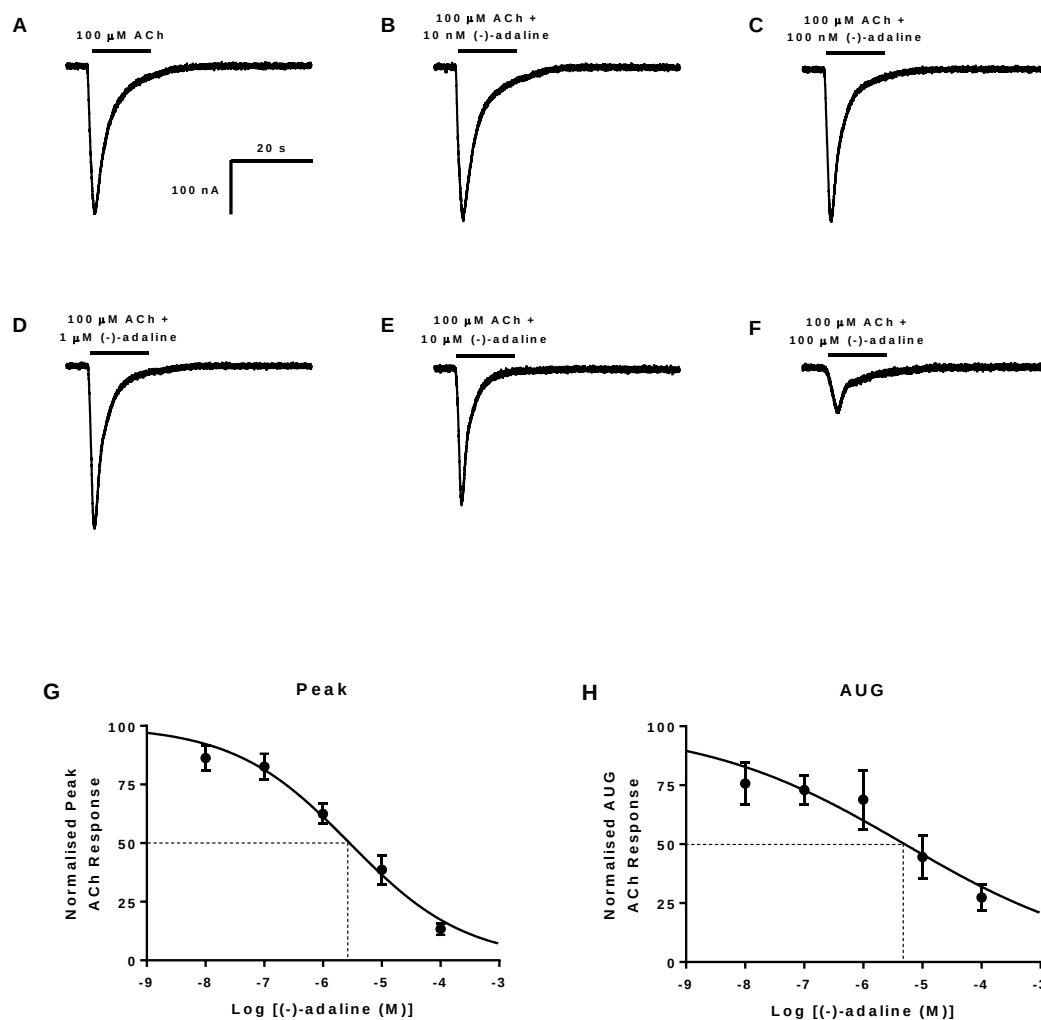


Fig. 3.3.9: Traces of the response to 100 μM ACh of hybrid Dα2/β2 nAChRs expressed in a single *Xenopus* oocyte at $V_h = -75$ mV upon co-application (represented by black bars) of (A) ACh alone, (B) 10 nM (-)-adaline, (C) 100 nM (-)-adaline, (D) 1 μM (-)-adaline, (E) 10 μM (-)-adaline and (F) 100 μM (-)-adaline. Concentration-inhibition curves of the peak current and AUG responses gave IC_{50} values of 2.84 μM for peak response (G) and 5 μM for AUG (H). All responses were normalised to the control 100 μM ACh response and plotted as mean $\pm$ SEM ($n = 5$).

Concentration-dependent inhibition was observed for all nAChRs tested with (-)-adaline having the the lowest IC₅₀ values on rat $\alpha 3\beta 4$ having (Table 3.3.2). The IC₅₀ values can be used to establish which compounds are inhibited more strongly by (-)-adaline. Human $\alpha 7$ and rat $\alpha 4\beta 2$ were the least affected followed by D $\alpha 2/\beta 2$ and then rat $\alpha 3\beta 4$ having the lowest IC₅₀ of those tested so the most affected by (-)-adaline. No significant differences (P>0.05) were observed when comparing the IC₅₀ values of peak, late current and AUG for each receptor using an extra sum-of-squares F-test.

Table 3.3.2 – Peak, late current and AUG IC₅₀ of nAChRs to (-)-adaline

nAChR	IC ₅₀ (μ M) (95% CI)		
	Peak	Late Current	AUG
$\alpha 7$	11.8 (3.86 – 62.1)	-	32.0 (13.7 – 60.1)
$\alpha 4\beta 2$	29.0 (17.1 – 65.5)	16.0 (8.57 – 55)	28.0 (13.8 – 102)
$\alpha 3\beta 4$	0.66 (0.24 – 1.67)	0.25 (0.08 – 0.71)	0.33 (0.07 – 1.17)
D $\alpha 2/\beta 2$	2.84 (1.6 – 5.03)	-	5.02 (1.14 – 40)

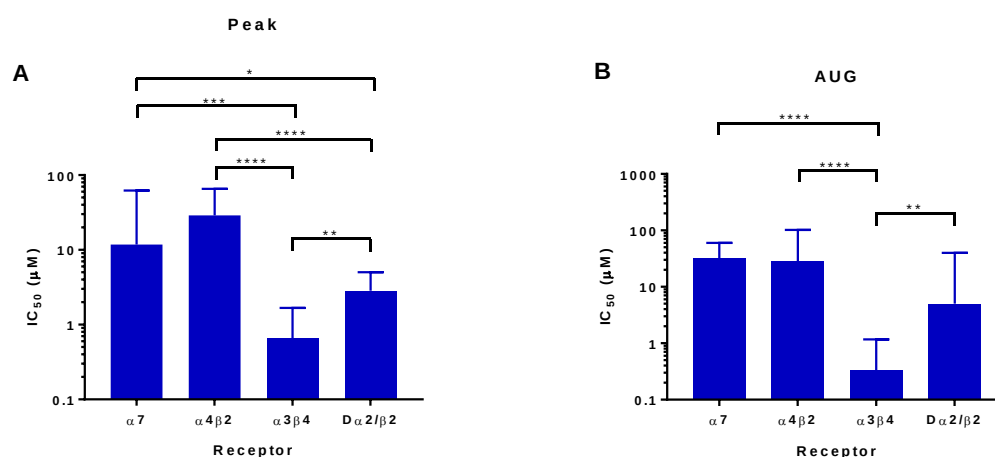


Fig. 3.3.10: IC₅₀ values for (A) peak current and (B) AUG were plotted as mean $\pm 95\%$ CI and compared using an extra sum-of-squares F-test. Significant differences were observed in the peak current IC₅₀ for $\alpha 3\beta 4$ vs. $\alpha 4\beta 2$ (P<0.0001), $\alpha 3\beta 4$ vs. $\alpha 7$ (P=0.0002), $\alpha 3\beta 4$ vs. D $\alpha 2/\beta 2$ (P=0.0065), D $\alpha 2/\beta 2$ vs. $\alpha 4\beta 2$ (P<0.0001) and D $\alpha 2/\beta 2$ vs. $\alpha 7$ (P=0.0159). Significant differences were observed in the AUG IC₅₀ for $\alpha 3\beta 4$ vs. $\alpha 4\beta 2$ (P<0.0001), $\alpha 3\beta 4$ vs. $\alpha 7$ (P<0.0001) and $\alpha 3\beta 4$ vs. D $\alpha 2/\beta 2$ (P=0.0069). (* = P<0.05, ** = P<0.01, *** = P<0.001, **** = P<0.0001).

Statistical comparison of (-)-adaline peak current IC_{50} values using an extra sum-of-squares F-test showed a significant selectivity for $D\alpha 2/\beta 2$ when compared to rat $\alpha 4\beta 2$ only ($P < 0.0001$). Rat $\alpha 3\beta 4$ had a significantly lower peak current IC_{50} than $D\alpha 2/\beta 2$ ($P = 0.0065$), rat $\alpha 4\beta 2$ ($P < 0.0001$) and human $\alpha 7$ ($P = 0.0002$) showing (-)-adaline to be selective for rat $\alpha 3\beta 4$ rather than $D\alpha 2/\beta 2$ (Fig 3.3.10A). (-)-adaline AUG IC_{50} s also showed a significantly lower IC_{50} for rat $\alpha 3\beta 4$ when compared to $D\alpha 2/\beta 2$ ($P = 0.0069$), rat $\alpha 4\beta 2$ ($P < 0.0001$) and human $\alpha 7$ ($P < 0.0001$) (Fig. 3.3.10B). Late current IC_{50} comparison also confirmed this selectivity for rat $\alpha 3\beta 4$ with a significantly lower value than rat $\alpha 4\beta 2$ ($P < 0.0001$).

3.4 Current-voltage relationship of HAE and (-)-adaline on mammalian neuronal nAChRs

The effect of membrane potential on the inhibition caused by HAE and (-)-adaline was investigated on rat $\alpha 3\beta 4$, rat $\alpha 4\beta 2$ and human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes. Whether inhibition is dependent on voltage or not can give insight into the binding region of the active component of HAE. If the alkaloids inhibit more strongly at more hyperpolarised (more negative) membrane potentials then this would indicate that they bind within the pore region of the channel. This was investigated by applying repeated voltage ramps to a nAChR ($\alpha 3\beta 4$ and $\alpha 4\beta 2$ only) expressing oocyte clamped at $V_h = -75$ mV. Over a period of 1 s with a 0.5 s delay the V_h was repeatedly ramped up from -100 to +50 mV. The effects of ACh and ACh co-applied with HAE on the current during the voltage ramp was observed (Fig. 3.4.1 & 3.4.2).

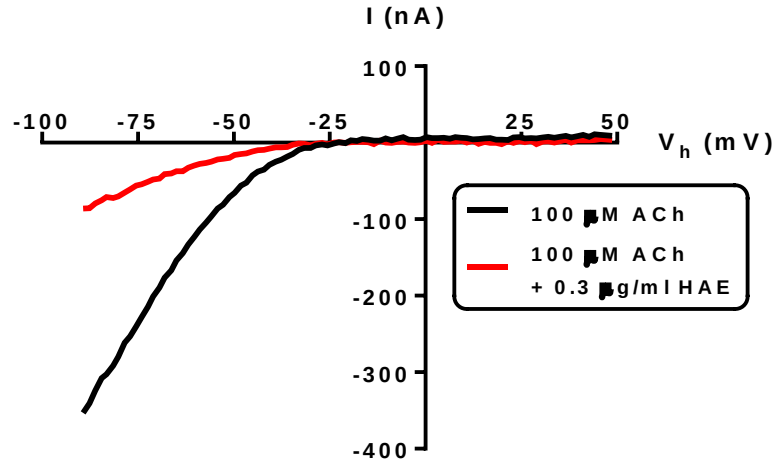


Fig 3.4.1: IV relationship of rat $\alpha 3\beta 4$ in response to 100 μM ACh and 100 μM ACh co-applied with 0.3 $\mu\text{g/ml}$ HAE from one oocyte. For each oocyte ($n=5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during drug applications. Leakage current was subtracted from stabilised current.

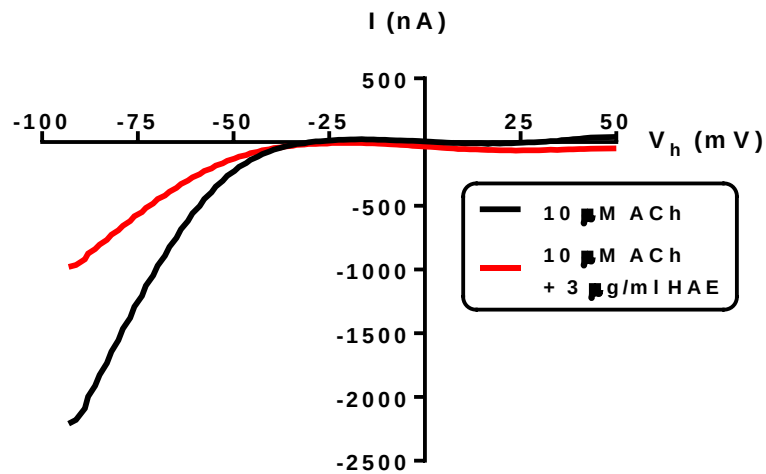


Fig 3.4.2: IV relationship of rat $\alpha 4\beta 2$ in response to 10 μM ACh and 10 μM ACh co-applied with 3 $\mu\text{g/ml}$ HAE from one oocyte. For each oocyte ($n=5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during drug applications. Leakage current was subtracted from stabilised current.

Measurable inward currents were observed when the V_h reached approximately -40 mV or less. The measurable portion of the current response in the presence of HAE was normalised to the control ACh response (Fig 3.4.3 & 3.4.4). Changes in V_h do not strongly affect the level of inhibition with an approximate 25% of the control response being observed across the V_h range tested for rat $\alpha 3\beta 4$ and so

antagonism by HAE can be concluded to be voltage independent (Fig. 3.4.3). An increase from approximately 44% to 75% in the percentage of the control response when the V_h was ramped from -90 mV to -40 mV was observed for rat $\alpha 4\beta 2$. A significant difference was observed when comparing the -50 to -40 mV range to the -90 to -80 mV range ($P=0.0006$, paired t-test). No significant differences were observed for rat $\alpha 3\beta 4$. As there are significant differences in the level of HAE inhibition across this V_h range, HAE inhibition on rat $\alpha 4\beta 2$ can be described as voltage-dependent (Fig. 3.4.4).

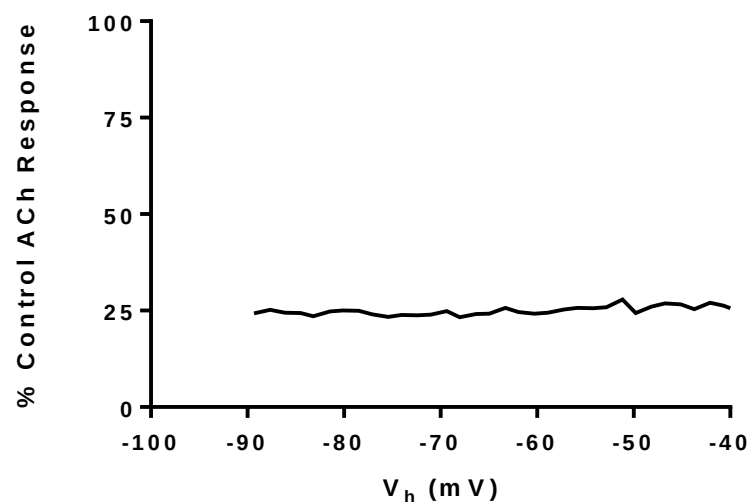


Fig 3.4.3: Response of rat $\alpha 3\beta 4$ to ACh co-applied with 0.3 $\mu\text{g/ml}$ HAE. Current responses from $V_h = -40$ mV onwards were obtained and normalised to the control ACh response.

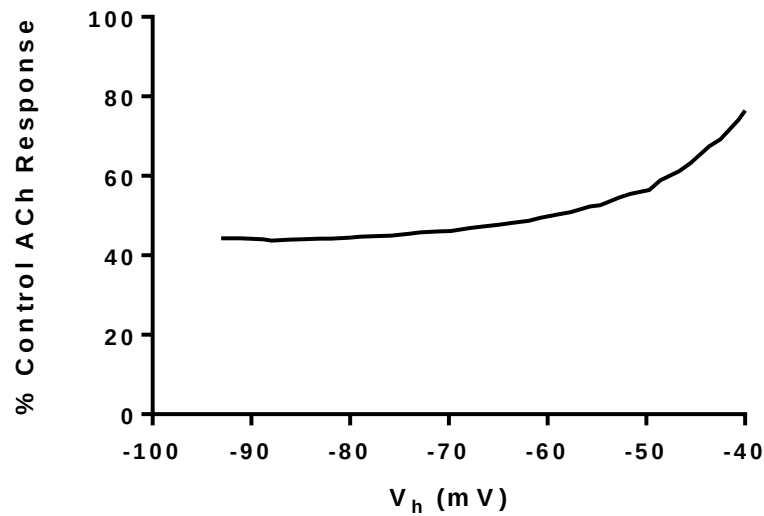


Fig 3.4.4: Response of rat $\alpha 4\beta 2$ to ACh co-applied with 3 $\mu\text{g/ml}$ HAE. Current responses from $V_h = -40$ mV onwards were obtained and normalised to the control ACh response.

Due to human $\alpha 7$ rapidly desensitising after the peak response has been reached, a voltage ramp could not be applied as the channel has opened and closed before the required number of voltage ramps could be applied. An alternative protocol was used where the V_h was manually changed from -25 mV to -125 mV in 25 mV steps with a 3 min wash interval. At each V_h , 100 μM ACh and 100 μM ACh with HAE was applied and the peak response was measured (Fig 3.4.5).

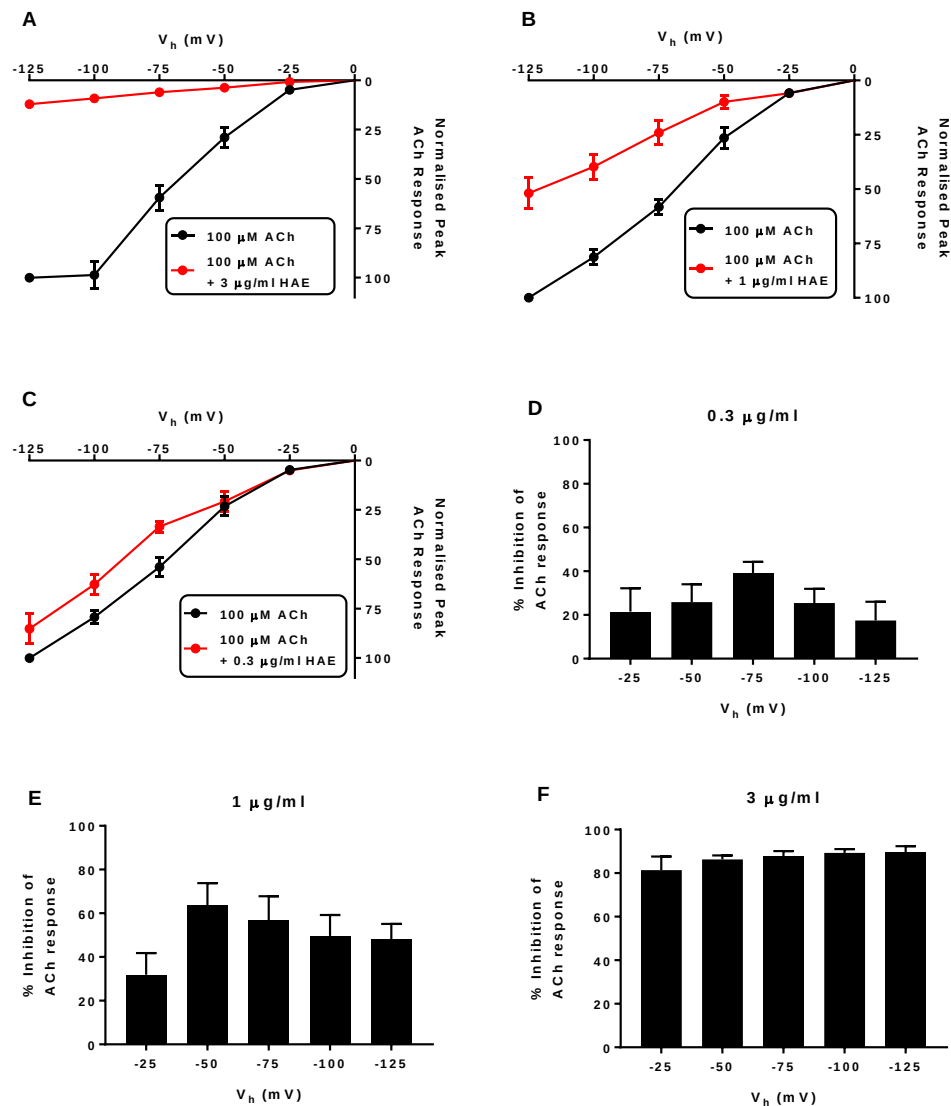


Fig 3.4.5: Responses of human $\alpha 7$ receptors to 100 μ M ACh and 100 μ M ACh co-applied with (A) 3 μ g/ml, (B) 1 μ g/ml and (C) 0.3 μ g/ml HAE as the V_h is changed showing the I-V relationship of HAE inhibition. All points are normalised to the -125 mV control ACh response and plotted as mean $\pm$ SEM ($n=5$) with some error bars not visible due to being smaller than the data point symbol. The percentage of the control response inhibited by HAE at each V_h was plotted as mean $\pm$ SEM for (D) 0.3 μ g/ml, (E) 1 μ g/ml and (F) 3 μ g/ml. One-way ANOVA followed by Tukey's multiple comparisons test showed no significant differences in the percentage of inhibition across the V_h range for each HAE concentration tested.

As the V_h reduced to more hyperpolarised membrane potentials, inward rectification was observed due to the ACh response increasing at the more hyperpolarised membrane potentials. A one-way ANOVA test followed by Tukey's multiple comparisons test was carried out to ascertain whether the percentage of

inhibition was altered by the change in V_h (Fig 4.4.5). Tukey's test was used after determining the data was normally distributed using the Shapiro-Wilk normality test. No significant differences were found for each of the three concentrations of HAE tested.

Whether or not the inhibition caused by (-)-adaline was affected by changes in V_h was investigated using the same method as used for HAE. This was again used to ascertain whether inhibition by (-)-adaline is dependent on voltage and give insight into the binding region of (-)-adaline on the nAChR.

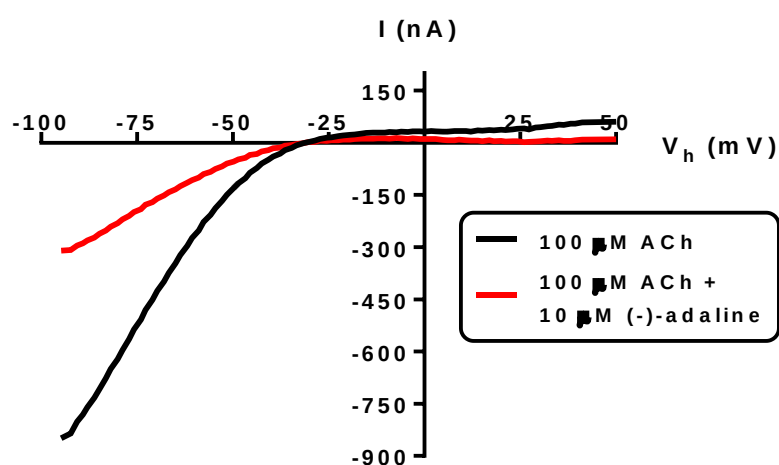


Fig 3.4.6: IV relationship of rat $\alpha 3\beta 4$ in response to 100 μ M ACh and 100 μ M ACh co-applied with 10 μ M (-)-adaline from one oocyte. For each oocyte ($n=5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during drug applications. Leakage current was subtracted from stabilised current.

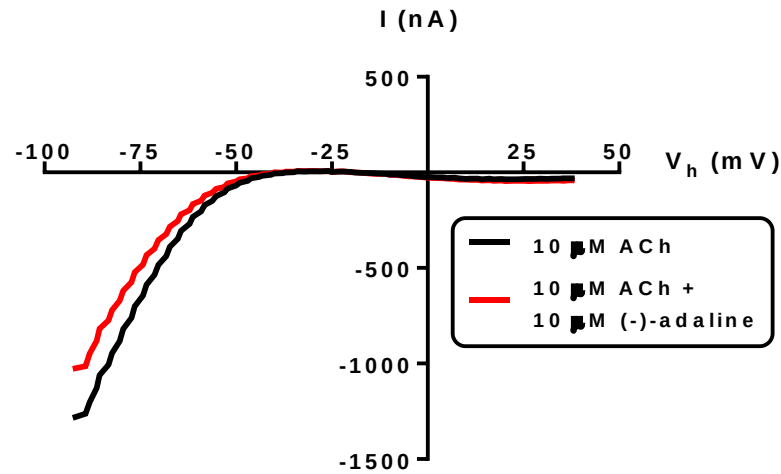


Fig 3.4.7: IV relationship of rat $\alpha 4\beta 2$ in response to 10 μM ACh and 10 μM ACh co-applied with 10 μM (-)-adaline from one oocyte. For each oocyte ($n=5$), responses were averaged over the readings of 3 voltage ramps of leakage current and stabilised current during drug applications. Leakage current was subtracted from stabilised current.

The response to ACh for both rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ increased at more hyperpolarised V_h in a non-linear fashion. This was also observed for the response to ACh co-applied with (-)-adaline in both cases (Fig 3.4.6 & 3.4.7). The responses of ACh co-applied with (-)-adaline to the more negative V_h from $V_h = -50$ mV onwards were normalised to the control ACh response (Fig 3.4.8 & 3.4.9). For both rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ the level of inhibition by 10 μM (-)-adaline was not strongly affected by changes in V_h and so the antagonism by (-)-adaline is voltage-independent across the V_h range tested.

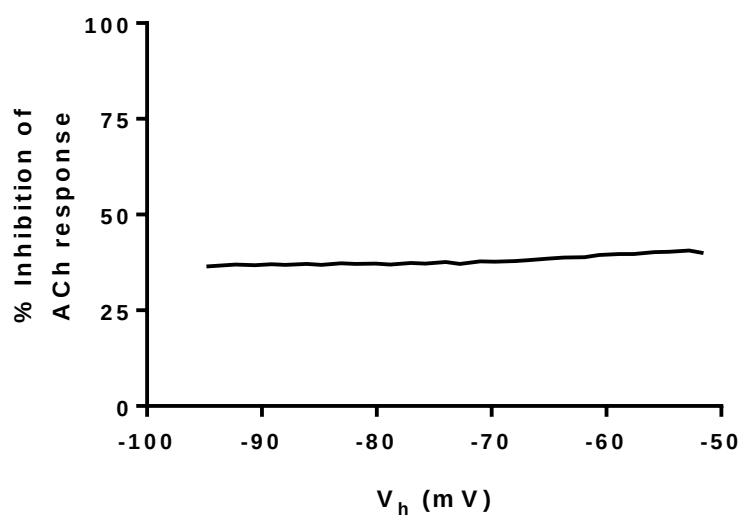


Fig 3.4.8: Response of rat $\alpha 3\beta 4$ to ACh co-applied with 10 μ M (-)-adalin. Current responses from $V_h = -50$ mV onwards were obtained and normalised to the control ACh response.

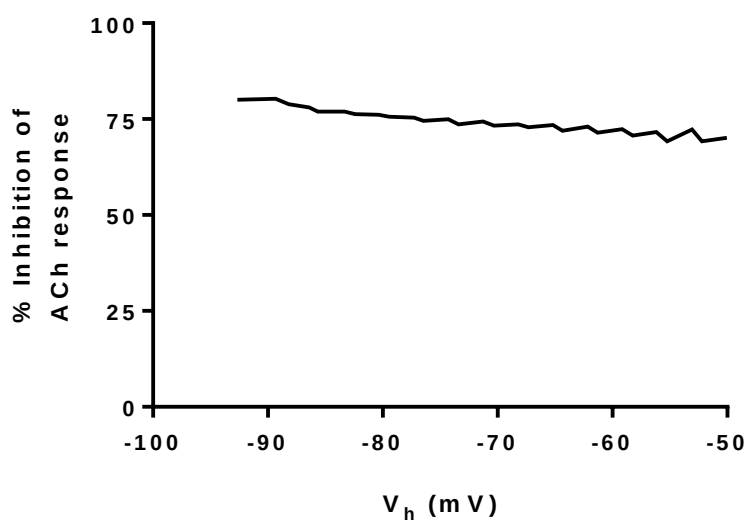


Fig 3.4.9: Response of rat $\alpha 4\beta 2$ to ACh co-applied with 10 μ M (-)-adalin. Current responses from $V_h = -50$ mV onwards were obtained and normalised to the control ACh response.

Repeated voltage ramps could not be applied to human $\alpha 7$ due to the rapid desensitisation of the ACh response. The same method used for investigating the I-V relationship of HAE inhibition was used where the V_h was manually changed in 25 mV steps from -125 to -25 mV. At each V_h , ACh and ACh co-applied with 10 μM (-)-adaline was applied, and the peak response measured (Fig 3.4.10).

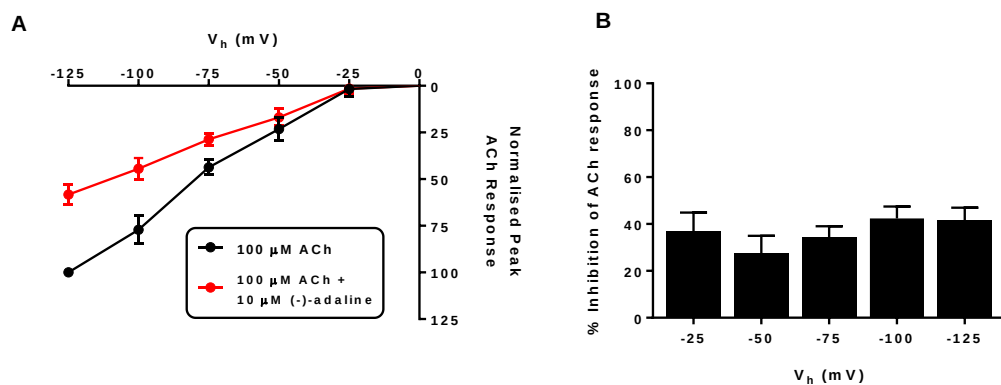


Fig 3.4.10: (A) Responses of human $\alpha 7$ receptors to 100 μM ACh and 100 μM ACh co-applied with 10 μM (-)-adaline as the V_h is changed showing the IV relationship of (-)-adaline inhibition. All points are normalised to the -125 mV control ACh response and plotted as mean $\pm$ SEM (n=5) with some error bars not being visible due to being smaller than the data point symbol. (B) The percentage of (-)-adaline inhibition at each V_h was plotted as mean $\pm$ SEM (n=5). One-way ANOVA followed by Tukey's multiple comparisons test showed no significant differences ($P>0.05$) in the percentage of inhibition across the V_h range tested.

Inhibition was observed when ACh was co-applied with (-)-adaline at each V_h tested. A one-way ANOVA followed by Tukey's multiple comparisons test showed no significant differences ($P>0.05$) in the level of (-)-adaline inhibition across the V_h range tested after determining the data was normally distributed using the Shapiro-Wilk normality test. Therefore the inhibition of (-)-adaline on human $\alpha 7$ can be described as voltage-independent similar to the results seen with rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$.

3.5 Effect of HAE and (-)-adaline on ACh EC₅₀ of mammalian neuronal nAChRs

To examine whether the activity of HAE and (-)-adaline is competitive or non-competitive the inhibition of a single concentration of HAE was tested against a range of concentrations of ACh. The responses were then compared to the responses of the ACh concentrations alone in order to construct 2 concentration-response curves showing the effect of the ladybird alkaloids on the EC₅₀ of ACh (Fig 3.5.1). EC₅₀ and top parameter values were then obtained (Table 3.5.1) and compared in order to determine whether the EC₅₀ had shifted and whether the top level of the plateau had reduced. A shifting of the EC₅₀ with no reduction in the top plateau would indicate that an increase in the concentration of ACh overcomes the inhibition of the ladybird alkaloids. Therefore binding could occur at the same site in order for more ACh to overcome the effects of ladybird alkaloids. A reduction in the top plateau would indicate that the ladybird alkaloids could continue to have an inhibitory effect despite the increase in ACh concentration. Therefore this is indicative of different binding site to the ACh binding site.

Table 3.5.1 – EC₅₀ and top plateau values from concentration-response curves of mammalian nAChRs in presence and absence of HAE

nAChR	EC ₅₀ (95 % CI) (μM)		Top Plateau	
	ACh	ACh + HAE	ACh	ACh + HAE
α7	194 (176 – 213)	199 (163 – 260)	100	85.0
α3β4	161 (138 – 187)	184 (98.8 – 254)	100	73.4
α4β2	19.9 (9.66 – 38.4)	241 (100 – 386)	100	100

The EC_{50} values for rat $\alpha 4\beta 2$ significantly shifted to a higher value in the presence of HAE when the 2 curves were compared using an extra sum-of-squares F-test ($P < 0.0001$) which indicates that HAE inhibition is competitive. There were no significant differences between the EC_{50} 's for rat $\alpha 3\beta 4$ and human $\alpha 7$. The top plateau value reduced to 73.4 for rat $\alpha 3\beta 4$ and 85.0 for human $\alpha 7$. This would indicate that non-competitive inhibition is occurring however this reduction was not found to be statistically significant when analysed using an extra sum-of-squares F-test.

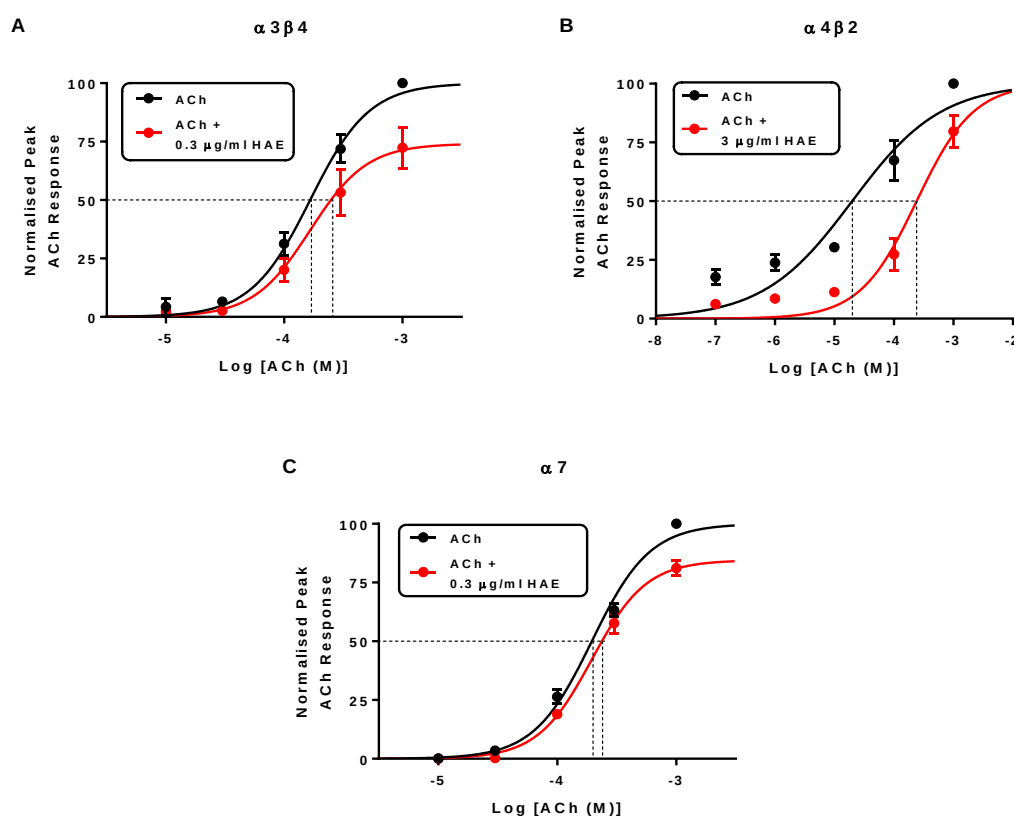


Fig 3.5.1: Concentration-response curves of ACh alone (black) and ACh co-applied with HAE (red) on (A) rat $\alpha 3\beta 4$, (B) rat $\alpha 4\beta 2$ and (C) human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes. 0.3 $\mu\text{g/ml}$ HAE was used for human $\alpha 7$ /rat $\alpha 3\beta 4$ and 3 $\mu\text{g/ml}$ HAE was used for rat $\alpha 4\beta 2$. EC_{50} values of 194 μM (ACh)/199 μM (ACh + HAE) for $\alpha 7$, 161 μM (ACh)/184 μM (ACh + HAE) for $\alpha 3\beta 4$ and 19.9 μM (ACh)/241 μM (ACh + HAE) for $\alpha 4\beta 2$ were obtained. The presence of HAE reduced the top plateau of the dose-response curve to 85% for $\alpha 7$ and 73.4% for $\alpha 3\beta 4$ with no effect on $\alpha 4\beta 2$. An extra sum-of-squares F-test showed only the difference in EC_{50} for $\alpha 4\beta 2$ to be statistically significant ($P < 0.0001$) with no significant differences in top plateau values. Each point was normalised to the control 1 mM ACh response and plotted as mean $\pm$ SEM ($n=5$).

Table 3.5.2 – EC₅₀ and top plateau values from concentration-response curves of mammalian nAChRs in presence and absence of (-)-adaline

nAChR	EC ₅₀ (95 % CI) (μM)		Top Plateau (% ACh response)	
	ACh	+ (-)-adaline	ACh	+ (-)-adaline
α7	171 (149 – 364)	393 (102 – 96.6)	100	85.1
α3β4	124 (94 – 156)	1415 (164 – 2859)	100	100
α4β2	11.6 (6.11 – 21.0)	122 (15.7 – 161)	100	100

The EC₅₀ value for ACh was significantly increased when co-applied with 10 μM (-)-adaline for rat α3β4 (P=0.0117) and rat α4β2 (P=0.0140) when compared using an extra sum-of-squares F-test. Human α7 showed no significant differences (P>0.05) and so the inhibition of rat α3β4 and rat α4β2 can be at least partially attributed to a competitive mode of action. Human α7 did however show a decrease in the top plateau value but was not statistically significant when analysed using an extra sum-of-squares F-test (P>0.05).

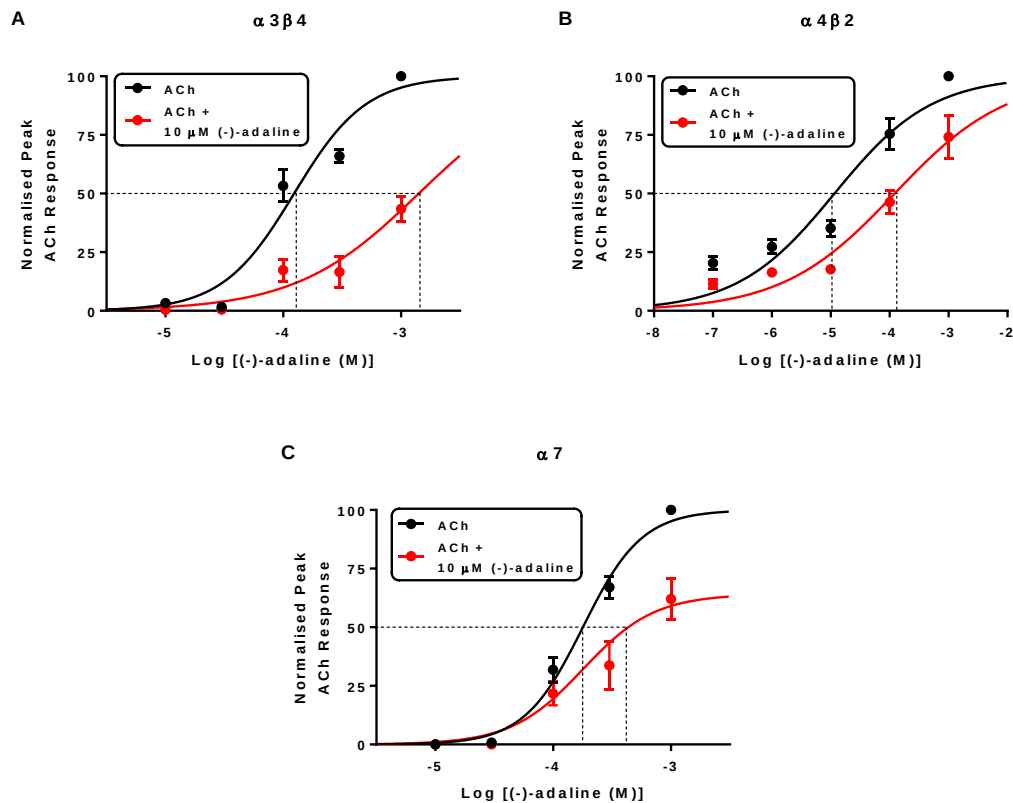


Fig 3.5.2: Concentration-response curves of ACh alone (black) and ACh co-applied with 10 μM (-)-adaline (red) on (A) rat α3β4, (B) rat α4β2 and (C) human α7 nAChRs expressed in *Xenopus* oocytes. EC₅₀ values of 171 μM (ACh)/393 μM (ACh + (-)-adaline) for α7, 124 μM (ACh)/1415 μM (ACh + (-)-adaline) for α3β4 and 11.6 μM (ACh)/122 μM (ACh + (-)-adaline) for α4β2 were obtained. The presence of (-)-adaline reduced the top plateau of the dose-response curve to 85.1% for α7 with no effect on α3β4 and α4β2. An extra sum-of-squares F-test showed significant differences in the IC₅₀ for α3β4 (P=0.0117) and α4β2 (P=0.0140) and no significant differences in the top plateau values. Each point was normalised to the control 1 mM ACh response and plotted as mean ± SEM (n=5).

3.6 Effect of HAE and (-)-adaline on glutamate LGICs

As HAE is a mixture of different chemicals there is the potential for more than one component being responsible for the inhibitory activity shown on nAChRs. There is also the potential for a component to target other families of receptors and so HAE was tested against rat GluN1-1a/2A NMDA receptors expressed in *Xenopus* oocytes.

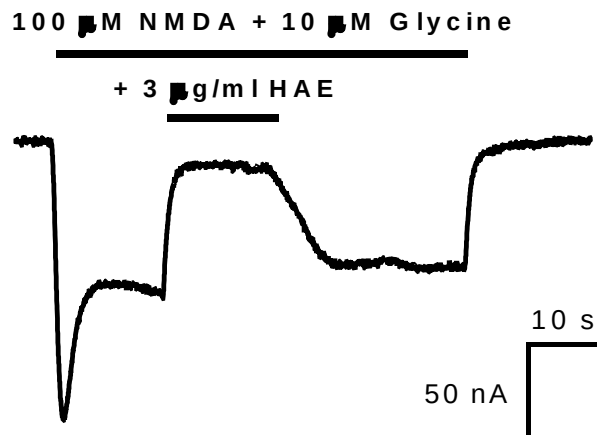


Fig 3.6.1: Response of rat GluN1-1a/2A NMDA receptors expressed in a single *Xenopus* oocyte to 100 μ M NMDA/10 μ M glycine. After a plateau was reached co-application with 3 μ g/ml HAE began till the response plateaued. Finally 100 μ M NMDA/10 μ M glycine alone was applied until the response again plateaued.

100 μ M NMDA/10 μ M glycine was chosen as these concentrations have been used in other experiments with the same receptor to investigate other NMDA antagonists (McClymont et al., 2012). Application of NMDA/glycine causes a steady state response; co-application with 3 μ g/ml HAE during this steady state response caused a reduction which recovered once NMDA/glycine alone was applied again (Fig 3.6.1).

This inhibition was concentration-dependent (Fig 3.6.2 & 3.6.3) with an IC_{50} (95% CI) of 0.407 μ g/ml (0.23 – 0.72) at $V_h = -50$ mV, 0.179 μ g/ml (0.116 – 0.278) at $V_h = -75$ mV and 0.0868 μ g/ml (0.056 – 0.133) at $V_h = -100$ mV (Fig. 3.6.3). The IC_{50} values for each V_h were compared using an extra sum-of-squares F-test and found to be significantly different ($P = 0.0002$) and comparison of -50 vs -75 mV ($P=0.0208$), -75 vs -100 mV ($P=0.0203$) and -50 vs -100 mV ($P<0.0001$) showed significant differences for

each IC_{50} compared to the other. Changes in IC_{50} show that HAE inhibition is voltage dependent across the range of voltages tested.

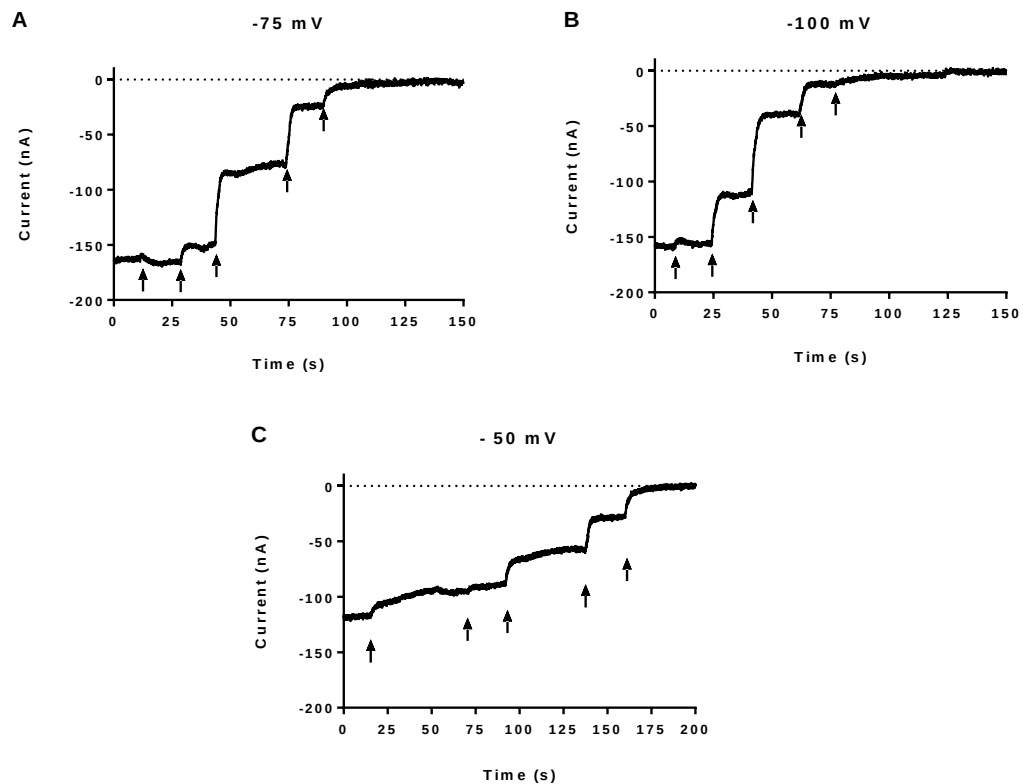


Fig 3.6.2: Concentration-dependent inhibition of GluN1-1a/2A NMDA receptors expressed in a single *Xenopus* oocyte at (A) $V_h = -75$ mV, (B) $V_h = -100$ mV and (C) $V_h = -50$ mV. Trace showing the steady state response to 100 μ M NMDA/10 μ M glycine followed by co-application (black arrows) with 0.0003 μ g/ml, 0.003 μ g/ml, 0.03 μ g/ml, 0.3 μ g/ml and 3 μ g/ml HAE.

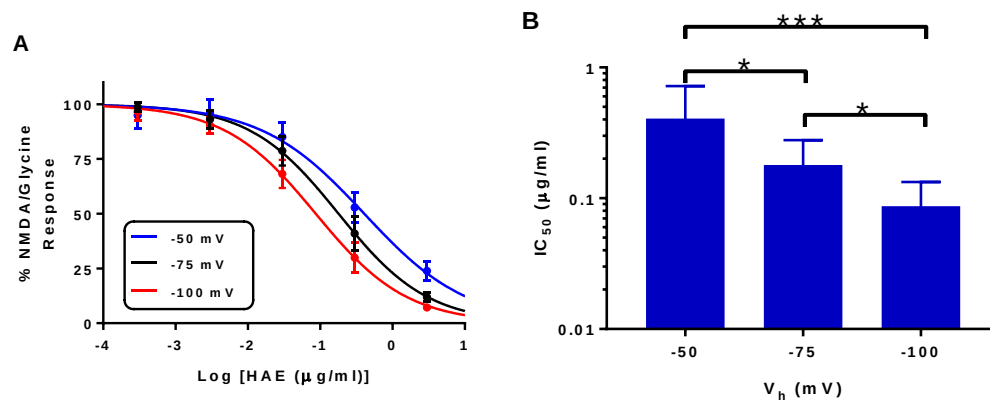


Fig 3.6.3: (A) Concentration-inhibition curves of GluN1-1a/2A with HAE at $V_h = -50$ mV (blue), -75 mV (black) and -100 mV (red). IC_{50} values of 0.407 (-50 mV), 0.179 (-75 mV) and 0.0868 (-100 mV) $\mu\text{g/ml}$ were obtained. Each point was normalised to the steady state NMDA/glycine response and plotted as the mean $\pm$ SEM ($n=4-5$). (B) IC_{50} values for each V_h were plotted as mean $\pm$ 95% CI and compared using an extra sum-of-squares F-test showing significant differences between the IC_{50} for each V_h : -50 vs -75 mV ($P=0.0208$), -75 vs -100 mV ($P=0.0203$) and -50 vs -100 mV ($P<0.0001$). (* = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, **** = $P<0.0001$).

As HAE was shown to antagonise the GluN1-1a/2A NMDA glutamate receptor, the effects on a different type of glutamate receptor, the GluA1 (flop) AMPA receptor, was investigated. $100 \mu\text{M}$ kainic acid (KA) was chosen to elicit a control response as KA does not induce a rapid desensitisation of current and has been used to investigate other AMPA receptor antagonists (Franzyk et al., 2014). KA was applied until a steady current was observed followed by co-application with different concentrations of HAE. No inhibition was observed when up to $30 \mu\text{g/ml}$ HAE was co-applied (Fig 3.6.4).

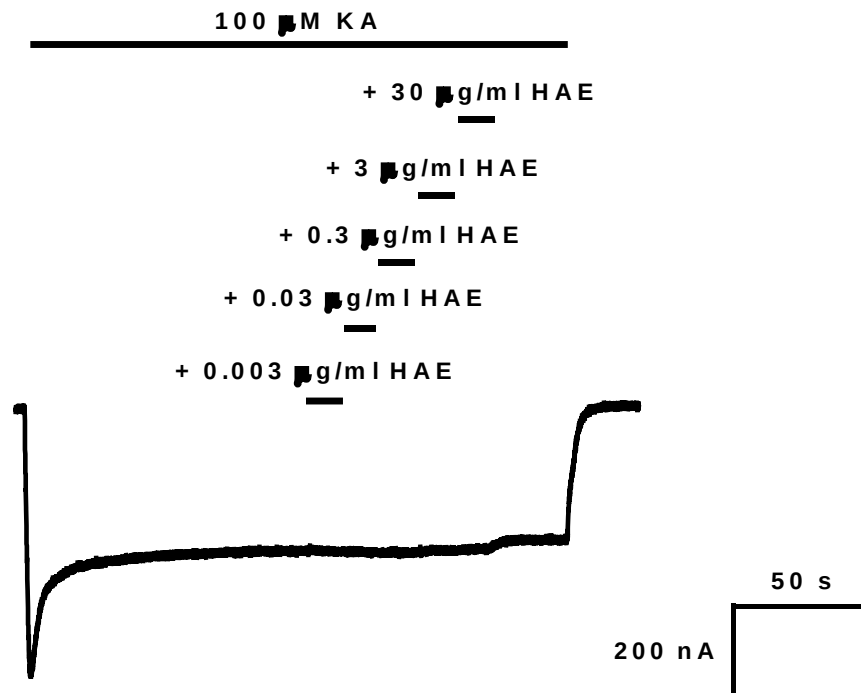


Fig 3.6.4: Effect of HAE on the response of GluA1 (flop) to $100 \mu\text{M KA}$ with concentrations increasing from 0.003 to $30 \mu\text{g/ml}$. No observable difference in the response is observed when KA is co-applied with HAE.

The effect of (-)-adalone was also investigated on GluA1 (flop) AMPA receptors expressed in *Xenopus* oocytes. HAE was shown not exclusively to target nAChRs and so AMPA receptors were investigated to ascertain whether (-)-adalone showed activity too. The same protocol used for investigating the effects of HAE on GluA1 (flop) was used where $100 \mu\text{M KA}$ was applied to the oocytes until a steady current was reached. $10 \mu\text{M}$ (-)-adalone was then co-applied but no effect on the response was observed (Fig 3.6.5).

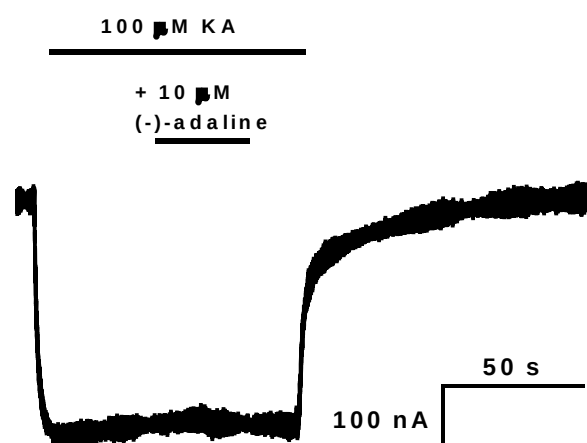


Fig 3.6.5: Effect of 10 μ M (-)-adaline on the response of GluA1 (flop) with no observable difference in the response is observed when KA is co-applied with (-)-adaline.

CHAPTER 4 - Discussion

4.1 Effects of ACh on mammalian and hybrid mammalian nAChRs

All combinations of nAChR subunits injected into *Xenopus* oocytes produced a response to the application of ACh characterised by a change in current when measured using the TEVC technique. Co-injection of rat $\alpha 3$ /rat $\beta 4$ or rat $\alpha 4$ /rat $\beta 2$ resulted in a depolarisation of current followed by slow desensitisation back to the baseline (Fig 3.1.2.1 & 3.1.2.2). To ensure that there were no effects of desensitisation after ACh application, the appropriate time interval between applications was investigated and a consistent response to ACh and a time interval of 3 min was found to give stable ACh responses and so was chosen for all experiments (Fig.3.1.4.1). Peak response EC_{50} values of 163 μM (95% CI = 131 – 199) for rat $\alpha 3\beta 4$ and 32.4 μM (95% CI = 25.4 – 41.3) for rat $\alpha 4\beta 2$ were obtained (Table 3.1.2.1). EC_{50} values for $\alpha 4\beta 2$ reported in previous studies of 35 μM (Demuro et al. 2001) and 11.9 μM (Kachel et al. 2016) are similar to the 32.4 μM EC_{50} reported in this study. A reported EC_{50} value of 103 μM for rat $\alpha 3\beta 4$ (Kachel et al., 2016) is also similar to the 163 μM EC_{50} reported in this study.

Human $\alpha 7$ subunit injection resulted in rapidly desensitising responses that are characteristic human $\alpha 7$ nAChR when expressed in *Xenopus* oocytes (Chavez-Noriega et al. 1997; Kachel et al. 2016). Previous studies on the EC_{50} of human $\alpha 7$ expressed in *Xenopus* oocytes were collated and found to range from 107 – 334 μM with an average of 200 μM (Papke & Porter Papke 2002). The EC_{50} value of 209 (95% CI = 187 – 234) obtained in this study falls within this range. Co-injection of D $\alpha 2$ /chick $\beta 2$ also resulted in depolarising currents upon the application of ACh. The EC_{50} value of 12 μM (95% CI = 7.24 – 20) was also similar to a reported 35 μM EC_{50} (Bertrand et al. 1994).

As the EC₅₀ values in this study fall within range or are similar to EC₅₀ values obtained in previous studies, the responses to ACh can be trusted as responses to expressed functional nAChRs and representative of the desired nAChR subtype.

The IV relationship of the mammalian nAChRs was studied in order to see the effect of changing the V_h on the response to ACh (Fig 3.3.1, 3.3.2 & 3.3.3). Each receptor showed inward rectification at more negative V_h and so the potential antagonistic effects of HAE and (-)-adaline on the ACh response over a range of V_h can be examined. Whether the degree of antagonism of ladybird alkaloids is dependent on voltage or not can give insight into the mode of action of HAE and (-)-adaline. An increase in the level of inhibition at more negative membrane potentials can indicate that the alkaloid is binding in the pore region of the channel (Leonard et al. 1988).

4.2 *Harmonia axyridis* alkaloid extract inhibits neuronal-type nicotinic acetylcholine receptors expressed in *Xenopus* oocytes

Gas chromatographic analysis of the fractions that tested positive using the Dragendorff's reagent test showed a mixture of compounds in HAE confirming the results found in a previous study (Richards, 2016). The alkaloids harmonine and 3-hydroxypiperidin-2-one are known to be present in *H. axyridis* haemolymph (Alam et al. 2002) (Fig. 4.2.1) and further mass spectrophotometer analysis of HAE would confirm the presence of these alkaloids as well as any other potential compounds.

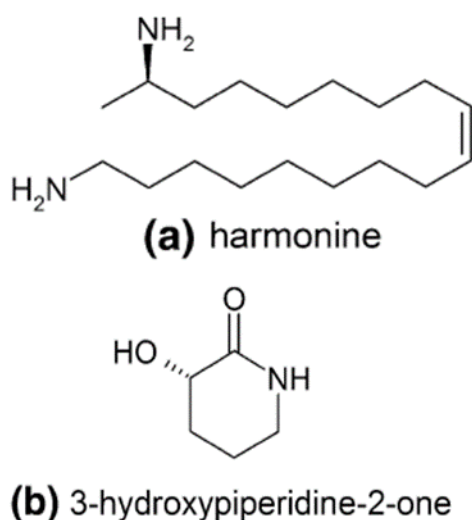


Fig. 4.2.1: Chemical structures of the alkaloids (A) harmonine and (B) 3-hydroxypiperidin-2-one from the harlequin ladybird *Harmonia axyridis*. Taken from (Sloggett et al. 2011).

The previous study showed antagonism of the ACh response of nAChRs expressed in TE671 muscle cells and locust neurons with selectivity for the locust nAChR (Richards 2016). TE671 cells are known to contain m-nAChRs and have been used in studies to investigate the effects of toxins on the m-nAChR (Shao et al. 1998; Brier et al. 2003; Mellor et al. 2003). However, the comparison between nAChRs of TE671 cells and locust neurons is not a direct one as they contain different subtypes of nAChR. As shown in this study, HAE also inhibits the ACh response of mammalian n-nAChRs and a hybrid *Drosophila*/chick $\text{D}\alpha 2/\beta 2$ receptor that is representative of an insect n-nAChR (Fig 3.3.1 – 3.3.4). Investigation of HAE on the n-nAChRs in this study revealed significant selectivity for $\text{D}\alpha/\beta 2$. Analysis of the peak current IC_{50} values showed that the IC_{50} for $\text{D}\alpha 2/\beta 2$ was significantly lower than the mammalian nAChRs and therefore most sensitive to HAE. HAE was approximately 3-fold more selective for $\text{D}\alpha 2/\beta 2$ than $\alpha 3\beta 4$, 5-fold more selective for $\text{D}\alpha 2/\beta 2$ than $\alpha 7$ and 7-fold more selective for $\text{D}\alpha 2/\beta 2$ than $\alpha 4\beta 2$ (Table 3.3.1) (Fig. 3.3.5). These findings are similar to the

findings when comparing TE671 cells and locust neurons, an approximate 3-fold selectivity for locust neurons over TE671 cells was observed (Richards, 2016). HAE appears to non-selectively target both n-nAChRs and m-nAChRs and further investigations on different subtypes that can also be expressed in *Xenopus* oocytes will be needed to confirm this. Expression and testing of m-nAChRs in *Xenopus* oocytes can also confirm whether any selectivity for m-nAChRs over n-nAChRs exists as this system can express both foetal and adult type m-nAChRs (Rasoulpour et al. 2012). Expression of other mammalian and insect n-nAChR subtypes in *Xenopus* oocytes can also be carried out to further investigate selectivity. The $\text{D}\alpha 1/\text{ALS}$ subunit can also form functional hybrid nAChRs (Ihara et al., 2014) and expression of functional homomeric $\text{D}\alpha 5/\text{D}\alpha 7$ nAChRs is also possible (Lansdell et al., 2012). Expression of nAChRs of other invertebrate species such as the desert locust *Schistocerca gregaria* (Amar et al., 1995), the brown dog tick *Rhipicephalus sanguineus* (Lees et al. 2014) and *Caenorhabditis elegans* (Boulin et al. 2008) is also possible. Testing of multiple *Drosophila* nAChRs can elucidate whether there is selectivity between different *Drosophila* nAChRs. Examination of other invertebrate nAChRs can also be carried out to determine whether selectivity exists between different invertebrate species.

Analysis of the late current and AUG IC_{50} compared to the peak current IC_{50} using an extra sum-of-squares F-test showed significant differences for all compared IC_{50} values. Rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ showed significant reductions in the late current and AUG IC_{50} when compared to the peak current while the IC_{50} was significantly larger for human $\alpha 7$ and $\text{D}\alpha 2/\beta 2$ (Fig. 3.3.5). Reduction of the late current and AUG indicate a larger effect on the decay of the response, indicating HAE could have an effect on the desensitisation of the channel. The effect on the AUG IC_{50} is lesser when compared

to the peak current IC_{50} for human $\alpha 7$ and $D\alpha 2/\beta 2$. The opposite is true for rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ indicating a differential effect of HAE depending on the subunits involved and whether the receptor slowly desensitises or not. The effect on the slowly desensitising receptors indicates that longer opening of the channel allows HAE to have a greater effect. As HAE is a mixture of alkaloid compounds the different effects could be explained by the presence of alkaloids that exhibit different pharmacological activity. Separation of HAE and individual testing of each component would be needed in order to find out which component(s) of HAE to attribute nAChR activity to and to provide a true value for the molar concentration of active alkaloid that is being used. This would then allow a more direct comparison to IC_{50} values obtained when investigating other ladybird alkaloids (Leong et al., 2014).

4.3 Mechanism of inhibition of *Harmonia axyridis* extract on nicotinic acetylcholine receptors differs depending on the receptor subtype

Investigations into the IV relationship of HAE inhibition on rat $\alpha 4\beta 2$ showed an increase in the level of HAE inhibition at more negative V_h (Fig. 3.4.3). No difference was observed for rat $\alpha 3\beta 4$ and human $\alpha 7$ (Fig. 3.4.1 & 3.4.5). The increase in HAE inhibition at lower V_h for rat $\alpha 4\beta 2$ indicates an open-channel blocking mechanism. As there is an increased electrochemical gradient across the membrane at more negative membrane potentials, there is a greater driving force allowing pore-binding compounds to bind more easily. The level of inhibition would then increase as the V_h is lowered as more HAE can bind. This is observed with HAE inhibition of rat $\alpha 4\beta 2$ and so this inhibition can at least be partially attributed to open-channel block. Open-channel blockers of nAChRs that only bind to the open-channel state such as QX-222

(Charnet et al. 1990) exist but others such as chlorpromazine can also bind to closed channels (Leonard et al. 1988). Separation of HAE and testing of individual alkaloids would allow this to be investigated on rat $\alpha 4\beta 2$.

IV investigations of HAE inhibition on rat $\alpha 3\beta 4$ (Fig. 4.4.3) and human $\alpha 7$ (Fig. 4.4.5) showed HAE inhibition was voltage-independent indicating that HAE inhibition is caused by binding to the nAChR at a site distinct from the pore. There appears to be a change in the level of inhibition when 1 $\mu\text{g/ml}$ HAE is used on human $\alpha 7$ when the V_h is changed from -25 mV to -50 mV but this did not show a significant difference using one-way ANOVA followed by Tukey's multiple comparisons test (Fig. 4.4.5E). Examination of the level of inhibition on human $\alpha 7$ when 3 $\mu\text{g/ml}$ and 0.3 $\mu\text{g/ml}$ HAE is used also showed no statistically significant differences and so the inhibition can be described as voltage-independent.

Competition assays were carried out on mammalian nAChRs to ascertain whether HAE inhibition is competitive or non-competitive. EC_{50} curves were constructed in the presence and absence of HAE and EC_{50} values were compared using an extra sum-of-squares F-test (Fig. 4.5.1). Only rat $\alpha 4\beta 2$ showed a significant difference ($P < 0.0001$) with a significant increase from 19.9 μM to 241 μM but no decrease in the top plateau level. An increase in EC_{50} alone indicates that ACh and the active component(s) of HAE are competing for the same binding site on rat $\alpha 4\beta 2$. As the HAE concentration is kept constant, increasing the concentration of ACh allows ACh to out-compete HAE for the same binding site negating the inhibitory effects of HAE at higher concentrations. The top plateau levels for rat $\alpha 3\beta 4$ and human $\alpha 7$ decreased indicating that HAE inhibition on these receptors is non-competitive. Increasing the concentration of ACh has no further effect as ACh is not competing with

HAE for the same binding site. ACh cannot out-compete HAE so the effect of HAE binding is not altered by changing the concentration of ACh. However, statistical analysis using an extra sum-of-squares F-test showed the decrease in top plateau values to not be significant (Fig. 4.5.1). A lower concentration was used for rat $\alpha 3\beta 4$ and human $\alpha 7$ due to the limited availability of HAE. 0.3 $\mu\text{g/ml}$ HAE was used for rat $\alpha 3\beta 4$ and human $\alpha 7$ compared to 3 $\mu\text{g/ml}$ used for rat $\alpha 4\beta 2$. Increased availability of HAE would allow a repeat investigation on rat $\alpha 3\beta 4$ and human $\alpha 7$ using 3 $\mu\text{g/ml}$ which is nearer the IC_{50} value for each receptor (Table 4.3.1). This higher concentration would then elucidate whether the reduction in top plateau observed is significant or not. A more direct comparison with the effects on rat $\alpha 4\beta 2$ can be made to establish whether or not the mode of action of HAE on rat $\alpha 3\beta 4$ and human $\alpha 7$ is non-competitive

4.4 Voltage-dependent inhibition of NMDA glutamate receptors by *Harmonia axyridis* extract

HAE has been shown to act on both mammalian n-nAChRs and m-nAChRs as well as insect (and hybrid insect) n-nAChRs. Due to there being little selectivity between nAChR subtypes the potential effects on other LGICs were investigated. Compounds that target the nAChR can also potentially target NMDARs, a LGIC family that is of clinical significance in the pathology of neurodegenerative diseases (Zhou & Sheng 2013a). The NMDAR antagonist memantine has been approved for the treatment of Alzheimer's disease and NMDAR antagonists are being investigated as a treatment option for other neurodegenerative diseases (Olivares et al. 2012). Memantine also inhibits nAChRs (Buisson & Bertrand 1998) and so if HAE activity

extends to NMDARs there is a potential clinical interest in the effects of HAE. Investigation of HAE on the GluN1-1a/2A NMDAR revealed inhibition (Fig. 4.6.1) with an IC_{50} of 0.179 $\mu\text{g/ml}$ at $V_h = -75 \text{ mV}$. This is lower than all IC_{50} obtained for the nAChRs investigated in this study including the hybrid insect nAChR (Table 3.3.5). Isolation of the active component(s) needs to be carried out in order to reliably compare the potency of HAE inhibition to other NMDAR antagonists being investigated for their clinical significance. Further investigation of the effects of HAE on other NMDAR subtypes is also warranted in order to determine whether any selectivity exists between NMDAR subtypes. As HAE has been demonstrated to be more potent on NMDARs than the nAChRs tested, any further investigation into the use of HAE as a pesticide will need to improve selectivity for nAChRs over NMDARs. Structure-activity studies where the structure of an active compound is altered in an attempt to alter its activity could be carried out in order to achieve this. Modifications to the structure of PhTX-343 has altered the selectivity of the toxin and also improved potency (Kachel et al. 2016). Insects also possess glutamate receptors and so the potential for HAE to target them also exists (Usherwood 1994).

Investigation of HAE on the GluA1 (flop) AMPAR showed no effects on the response to KA up to a concentration of 30 $\mu\text{g/ml}$ indicating that there is selectivity between different LGIC receptors (Fig. 4.7.1). Other toxins and their derivatives such as philanthotoxin target nAChRs, NMDARs and AMPARs through non-competitive open-channel block (Kachel et al., 2016; Franzyk et al. 2014; McClymont et al., 2012). HAE appears not to target AMPARs but further studies on different AMPAR subtypes need to be carried out in order to confirm this. Kainate receptors of the GluK family of glutamate receptors distinct from AMPARs and NMDARs can be expressed and

investigated using the TEVC technique in *Xenopus* oocytes (Straub et al. 2016). As HAE targets a subtype of glutamate receptor, there is a strong possibility that GluK receptors could also be targeted. There is also the potential for other LGICs to be targeted as HAE could contain multiple compounds effective against different LGICs.

4.5 (-)-adaline inhibits neuronal-type nicotinic acetylcholine receptors expressed in *Xenopus* oocytes

(-)-adaline inhibited the ACh response of all nAChRs when expressed in *Xenopus* oocytes in a concentration-dependent manner. The peak current IC_{50} value for rat $\alpha 3\beta 4$ (0.66 μM) was the lowest with values of 2.84 μM for $D\alpha 2/\beta 2$, 11.8 μM for human $\alpha 7$ and 29 μM for rat $\alpha 4\beta 2$ (Table 5.3.1). Selectivity for insect n-nAChRs was demonstrated in a previous study examining the effects of (-)-adaline on the ACh responses of TE671 cells and locust neurons. (-)-adaline was found to be approximately 14-fold more selective for n-nAChRs found in locust neurons over the $(\alpha 1)_2\beta 1\gamma \delta$ m-nAChRs found in TE671 cells when examining the AUG IC_{50} and approximately 19-fold more selective for the late current IC_{50} with little effect on the peak current (Richards, 2016). This selectivity was not replicated when comparing the mammalian n-nAChRs and hybrid insect n-nAChR in this study. Comparison of the IC_{50} values for AUG (Table 5.7.1) showed (-)-adaline having the least effect on the m-nAChRs in TE671 cells. Rat $\alpha 4\beta 2$, human $\alpha 7$ and $D\alpha 2/\beta 2$ fall between the values for TE671 and locust neurons with rat $\alpha 3\beta 4$ having the lowest IC_{50} values. As no mammalian n-nAChR was examined in the aforementioned study, expression and subsequent investigation of a mammalian m-nAChR in *Xenopus* oocytes could be carried out. Direct comparison using the same electrophysiological technique can be

made to see whether this selectivity is due to the difference between m-nAChRs and n-nAChRs or if selectivity of between insect and mammalian nAChRs exists. The small effect observed on the peak current can also be investigated.

Table 5.7.1 – Comparison of AUG and late current IC_{50} at $V_h = -75$ mV with values obtained in Richards (2016)

nAChR	AUG IC_{50} (μ M)	Late Current IC_{50} (μ M)
Richards Study:		
($\alpha 1$) ₂ $\beta 1\gamma\delta$ (expressed in TE671 cells)	51.75	24.4
This Study:		
n-nAChR (expressed in locust neurons)	3.64	1.28
Rat $\alpha 3\beta 4$	0.33	0.25
Rat $\alpha 4\beta 2$	28	16
Human $\alpha 7$	32	-
Drosophila/chick D $\alpha 2/\beta 2$	5.02	-

A similar peak current IC_{50} for the structurally different alkaloid precocinelline from the 7-spot ladybird *Coccinella septempunctata*. The effect of Precocinelline was examined on human $\alpha 7$ nAChRs expressed in *Xenopus* oocytes and gave an IC_{50} of 36.9 μ M when 100 μ M ACh was applied to oocytes (Leong et al., 2014). This is similar to the IC_{50} for the inhibition of peak current for rat $\alpha 4\beta 2$, human $\alpha 7$ and D $\alpha 2/\beta 2$ by (-)-adaline but larger than the IC_{50} seen with rat $\alpha 3\beta 4$. Many ladybird alkaloids have been isolated and screened for activity on nAChRs using various techniques (Leong et al., 2014; Richards, 2016) but not on the hybrid D $\alpha 2/\beta 2$ and heteromeric $\alpha 4\beta 2$ and $\alpha 3\beta 4$ receptors. Screening of different nAChR subtypes would give further insight into the selectivity of the examined ladybird alkaloids and therefore their potential pesticidal/therapeutic use.

There were no significant differences between the peak current, late current and AUG IC_{50} responses when analysed using an extra sum-of-squares F-test ($P > 0.05$).

This indicates that there is no larger effect on the decay of the response when compared to the peak of the response. A decrease in the late current and AUG IC_{50} would point towards (-)-adalone affecting the desensitisation of the receptor by reducing the time taken for the channel to reach the closed state after channel opening. This is not the case so this means that (-)-adalone could cause inhibition by affecting the opening of the channel.

4.6 Mechanism of inhibition of (-)-adalone on neuronal-type nicotinic acetylcholine receptors

Analysis of the IV relationship of (-)-adalone inhibition of ACh receptors showed no significant differences in the level of inhibition as the V_h was changed for rat $\alpha 3\beta 4$ (Fig. 3.4.6), rat $\alpha 4\beta 2$ (Fig. 3.4.7) and human $\alpha 7$ (Fig. 3.4.8). A voltage-independent block indicates that the level of inhibition is not altered by more channels being open, hence the binding site of (-)-adalone lies outside the open channel for these receptors. This is in contrast to previous findings/conclusions from studies using (-)-adalone on TE671 cells and locust neurons. These studies determined voltage-dependence using the Woodhull equation and (-)-adalone inhibition was found to be strongly voltage-dependent on the mammalian m-nAChR and insect n-nAChRs expressed in these cells (Richards, 2016). This method can be employed using TEVC (Kachel et al., 2016) and could be used on both m-nAChRs and n-nAChRs expressed in *Xenopus* oocytes, to determine voltage-dependency of (-)-adalone inhibition using the same method.

The effect of (-)-adalone on the ACh EC_{50} of mammalian nAChRs was carried out where ACh concentration-response curves were created in the presence and absence of 10 μM (-)-adalone (Fig. 3.5.2). Analysis of the EC_{50} concentrations showed

statistically significant increases in the EC₅₀ in the presence of (-)-adaline for rat $\alpha 4\beta 2$ (P=0.0117) and rat $\alpha 3\beta 4$ (P=0.0140). No reduction in the top plateau value was observed strongly indicating a competitive mode of action with ACh on these receptors. This is in contrast to previous work on TE671 cells and locust neurons that showed results consistent with non-competitive inhibition (Richards, 2016). Studies where all receptors can be examined using the same technique would elucidate whether the use of different investigative methods causes these observed differences or whether (-)-adaline does have different modes of action on different nAChR subtypes. This could be the case for human $\alpha 7$ when compared to others, as a reduction in the top plateau value is observed, however this is not statistically significant. Increasing the range of ACh concentrations tested as well as using a higher concentration of (-)-adaline would be informative. Due to limited availability of (-)-adaline, concentrations could not exceed 10 μ M for competition assays.

The GluA1 (flop) AMPAR was also tested and no effect on the response to KA was observed (Fig 5.6.1). This is consistent with the effects observed with HAE (Fig. 4.7.1) and so any potential effects of (-)-adaline on NMDARs should also be investigated as antagonism of NMDARs is being investigated as a treatment for multiple neurodegenerative diseases (Olivares et al., 2012).

4.7 Some selectivity was observed for insect nAChRs over mammalian nAChRs for *Harmonia axyridis* alkaloid extract but not (-)-adaline

HAE showed significant selectivity for the hybrid insect nAChR over the mammalian-nAChRs tested (Table 3.3.5). The peak current IC₅₀ value for D $\alpha 2/\beta 2$ was

the lowest for all the receptors tested however HAE appears to act in a much more potent manner on the decay of the response of rat $\alpha 3\beta 4$. Late current and AUG IC_{50} s of HAE on rat $\alpha 3\beta 4$ were orders of magnitude lower than other obtained IC_{50} s (Table 3.3.5). $D\alpha 2/\beta 2$ did not have the lowest peak current IC_{50} value for (-)-adoline and similar to HAE, rat $\alpha 3\beta 4$ produced the lowest IC_{50} values for peak current, late current and AUG (Table 3.3.10). The insect nAChR selectivity of (-)-adoline demonstrated in Richards (2016) did not extend to the hybrid $D\alpha 2/\beta 2$ receptor when compared to the mammalian n-nAChRs investigated in this study. Richards (2016) did demonstrate an approximate 3-fold selectivity for locust neurons over TE671 cells and this was consistent with the 3-fold selectivity observed for $D\alpha 2/\beta 2$ over rat $\alpha 3\beta 4$. 5-fold selectivity was observed when compared to human $\alpha 7$ and 7-fold selectivity when compared to rat $\alpha 4\beta 2$.

Other insect receptors can be investigated as the ALS/ $D\alpha 1$ subunit can also form hybrid receptors when co-expressed in *Xenopus* oocytes (Ihara et al., 2015). Investigation of homopentameric $D\alpha 5/D\alpha 6/D\alpha 7$ can also be carried out to provide a comparison with insect heteromeric and homomeric subtypes (Watson et al., 2010; Lansdell et al., 2012). Due to the environmental concerns caused by neonicotinoids on the honeybee (*Apis mellifera*) population among other factors (Gross, 2013), new insecticide candidates can be screened for activity on the honeybee as part of the development process. Electrophysiological recordings from Kenyon cells located in the mushroom bodies of the honeybee can be carried out to ascertain any risk of potential insecticide candidates at the early stage of development (Schäfer et al. 1994). Bioassays using honeybees can also be carried out. This has been the case for the development of Flupyradifurone, a novel insecticide that is a partial agonist of the

insect nAChR. Investigation into its effects on the foraging behaviour of honeybees revealed no adverse effects (Nauen et al. 2015). Addressing these concerns at the early stage of development can aid the search for novel insecticidal compound and allay fears that new candidates will raise the same concerns as the neonicotinoids have done.

4.8 Bioinformatic analysis of nAChR subunits

The difference in activity observed for HAE and (-)-adoline could be explained by differences in nAChR structure and the residues that the alkaloids interact with. There is evidence that HAE causes competitive inhibition on $\alpha 4\beta 2$ (Fig. 3.5.1) and (-)-adoline causes competitive inhibition on $\alpha 4\beta 2$ and $\alpha 3\beta 4$ (Fig. 3.5.2) and so the loops on the subunits that make up the binding site of ACh were examined (Fig. 4.8.1).

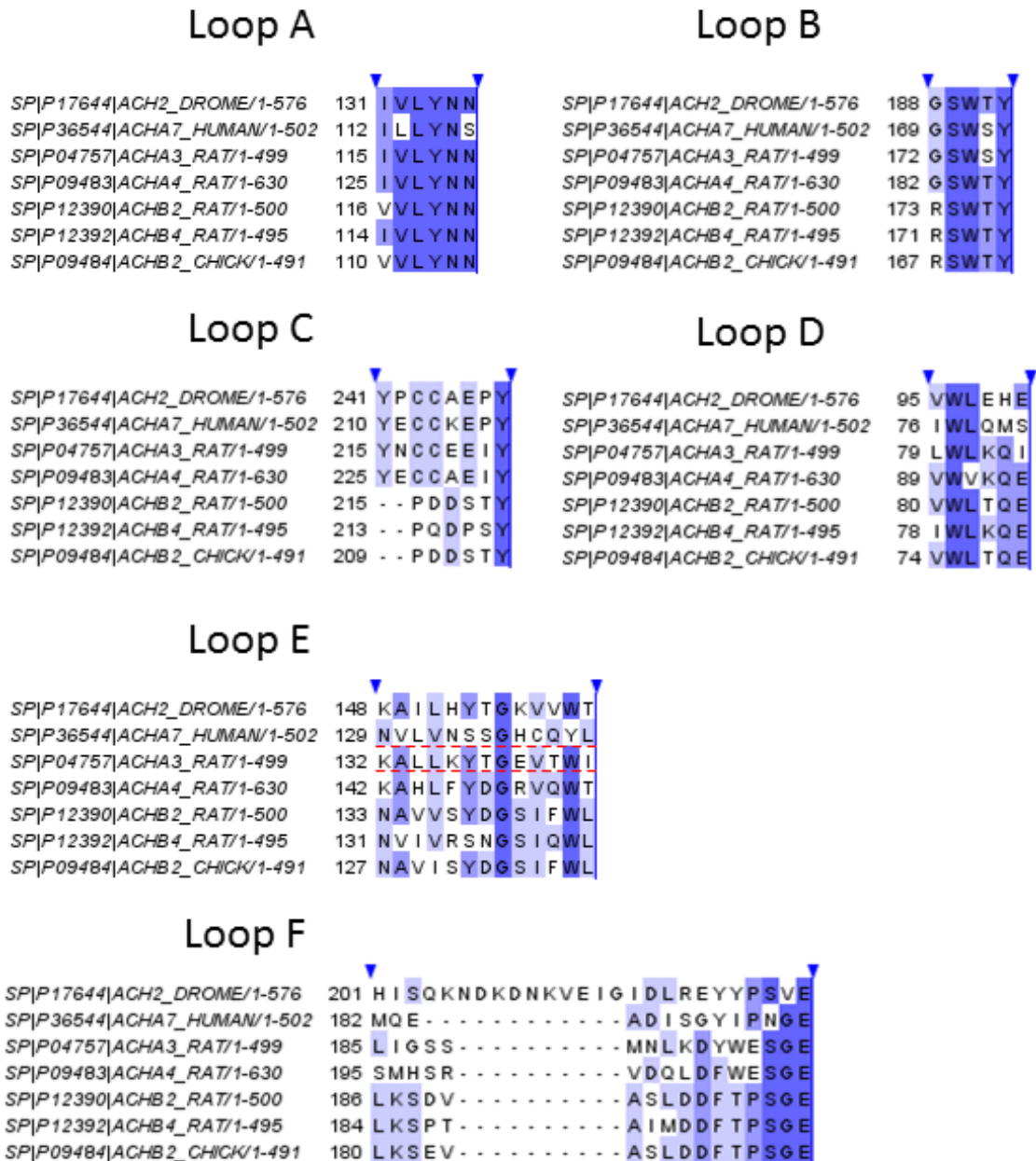


Fig. 4.8.1 – The binding loops from the principal (A-C) and complementary (D-F) sides of the $\alpha 2/\beta 2$, human $\alpha 7$, rat $\alpha 3$, rat $\alpha 4$, rat $\beta 2$, rat $\beta 4$ and chick $\beta 2$ nAChR subunits. Sequences were obtained from and aligned using the UniProt database and loops were identified using (Kouvatsos et al. 2016).

Analysis of differences in the amino acid sequence could give insight into the regions where binding could take place. Loops A, B and C appear to be highly conserved, loop D shows differences with human $\alpha 7$ and $\alpha 2/\beta 2$. The polar, hydrophilic glutamine is replaced with the positively charged histidine in $\alpha 2/\beta 2$ and

the non-polar, hydrophobic methionine in human $\alpha 7$. Loop E shows a region with the non-polar tryptophan that is highly conserved across the subunits examined. However this is substituted with the polar tyrosine in human $\alpha 7$ at position 140 on the $\alpha 7$ subunit. Loop F shows a glycine residue that is highly conserved across the mammalian nAChR subunits but is replaced by valine in D $\alpha 2/\beta 2$. Valine is hydrophobic as opposed to glycine which is hydrophilic and so this could be potentially cause differences in activity once the same IV and ACh EC₅₀ experiments can be carried out on D $\alpha 2/\beta 2$. As the data suggests that human $\alpha 7$ binds to a site separate from the ACh binding site, this change in residue could explain why $\alpha 7$ does not seem to bind. In order to confirm this, site-directed mutagenesis could be carried out on the subunit to change the tyrosine to a tryptophan and then examine the effects of the alkaloids on the mutated form of the channel once expressed in *Xenopus* oocytes. This technique has been used to investigate the role of different residues in channel function (Mishina et al. 1985) and for investigating the mode of action of NMDA receptor open-channel blockers (Abu 2016).

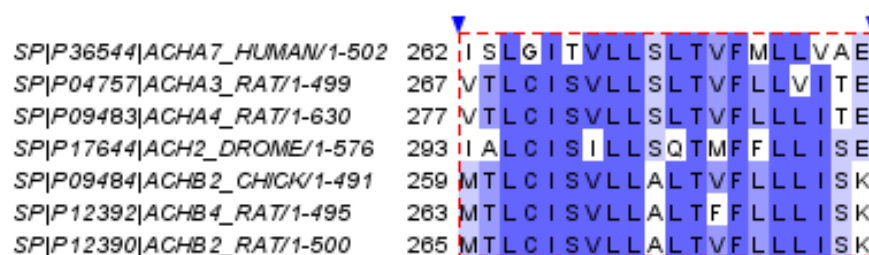


Fig. 4.8.2 – The pore-lining M2 transmembrane regions of the D $\alpha 2/\beta 2$, human $\alpha 7$, rat $\alpha 3$, rat $\alpha 4$, rat $\beta 2$, rat $\beta 4$ and chick $\beta 2$ nAChR subunits. Sequences were obtained from and aligned using the UniProt database and loops were identified using (Doucette-Stamm et al. 1993).

The M2 regions of the investigated subunits appear to be highly conserved (Fig. 4.8.2). A key change in D $\alpha 2/\beta 2$ exists where a non-polar, hydrophobic leucine present in all other mammalian receptors examined is replaced by a polar, hydrophilic

glutamine. Again, this could be a key residue in causing different effects of nAChR targeting compounds.

4.9 Investigation of potential therapeutic applications of HAE is promising as inhibition of NMDARs is exhibited

NMDA excitotoxicity is associated with neurodegeneration in Alzheimer's disease (Hynd et al. 2004) and other neurodegenerative diseases (Zhou & Sheng 2013b). The NMDAR antagonist memantine has been approved for the treatment of Alzheimer's disease and NMDAR antagonists are being investigated as a treatment option for other neurodegenerative diseases (Olivares et al. 2012). Memantine also inhibits nAChRs (Buisson & Bertrand 1998) and so if HAE activity extends to NMDARs there is a potential clinical interest in the effects of HAE. Separation of the active component(s) using liquid chromatography is vital to ensure a direct comparison of the potency on NMDARs can be studied. This will give a clearer idea into the potential of HAE for clinical investigation.

4.10 Alkaloids contribute to the chemical defence of *Harmonia axyridis* and *Adalia bipunctata*

HAE and (-)-adaline are now known to act on n-nAChRs of insects and so can contribute to the chemical defence of *H. axyridis* and *A. bipunctata*. The alkaloids of *H. axyridis* are known to be present in the haemolymph of adults and larvae (King and Meinwald, 1996) and coating eggs (Winters et al. 2014). Toxic effects are observed when eggs are consumed by other organisms (Phoofolo and Obrycki 1998; Yasuda and Ohnuma 1999; Cottrell 2007) and ingestion of n-nAChR targeting alkaloids can explain

this toxicity. Targetting of nAChRs can also possibly explain the toxicity observed when beetles are consumed by bird predators (Marples et al., 1989). The bright elytral colouring of *H. axyridis* (Bezzarides et al., 2007) and *A. bipunctata* can therefore be confirmed as an aposematic warning of the beetle's chemical defence. As there are different coloured variants of each of the investigated species in this study there could potentially be differences in the strength/abundance of defensive chemicals depending on the colouration. This has already been examined on the eggs of *C. septempunctata* and the coloration of the eggs was found to signal the strength of the chemical defence (Winters et al. 2014). The selectivity by HAE for the hybrid D α 2/ β 2 nAChR could be due to other insects being the natural predators and competitors of *H. axyridis*. Competition with other ladybird species of the same resources involves intraguild predation (Polis et al. 1989) where eggs of competitor species are consumed in an effort to reduce the population of competitor species. *H. axyridis* in particular has been extremely successful when invading areas in the US (Michaud 2002) and UK (Ware and Majerus, 2008). A combination of increased aggressiveness to other ladybird species (Michaud, 2002) and a better chemical defence of eggs (Yasuda and Ohnuma, 1999, Kajita et al., 2010) help *H. axyridis* become an effective intraguild predator. Competition with insect predators over a long period of time puts a large selection pressure on the species involved and so development of a chemical defence effective specifically against insect competitors and predators would give *H. axyridis* a competitive advantage and explain why the alkaloids appear to be more selective for insect nAChRs over mammalian nAChRs. Interestingly, the level of alkaloids on the surface of eggs can be controlled by the mother as alkaloids can also attract predators. *A. bipunctata* was found to lay eggs with higher levels of alkaloid only in areas with

poor resources and absent of predators (Paul et al. 2018). The level of alkaloid content can also seem to vary depending on the stage of invasion a population of *H. axyridis* is at. Comparisons between native and invasive populations showed differences in the abundance of defensive chemicals across the different populations indicating another factor determining the levels of defensive chemicals present (Magro et al. 2018). This indicates that use of defensive alkaloids can be more situational and is not the main form of defence in every situation.

The action of the alkaloids on nAChRs shows similarities with toxins from multiple species such as PhTX from the solitary wasp, *Philanthus triangulum* (Brier et al., 2003; Kachel et al., 2016), nereistoxin (Raymond Delpech et al. 2003) and toxins from other wasps and ants (Zalat et al. 2005). The shared mode of action indicates that targeting the nAChR is an effective method for a chemical protection. Although alkaloids are toxic and repellent to other organisms they have no noticeable effects on the beetle itself. This could be due to nerve sheaths in insects that shield neurons (Hill & Usherwood 1961) or differences in the amino acid sequences of nAChRs/other possible molecular targets could also account for the resistance. Differences in the amino acid sequence of the binding site of a toxin can confer resistance to a toxin targeting an ion channel. For example the L1014F point mutation in the voltage-gated sodium channel of pest insects such as the moth *Heliothis virescens* confers resistance to insecticides. This is achieved by the mutation promoting inactivation of the channel which works against the prolonging of channel inactivation which is the mechanism of action of pyrethroids (Burton et al. 2011). This has also been demonstrated in puffer fish where mutations in the target site of tetrodotoxin, endogenously produced by the

organism, confers resistance and allows accumulation with no effect (Venkatesh et al. 2005).

4.11 Conclusions

HAE and (-)-adoline are inhibitors of the hybrid insect $D\alpha 2/\beta 2$, rat $\alpha 3\beta 4$, rat $\alpha 4\beta 2$ and human $\alpha 7$ nAChRs. This is a different mode of action from the neonicotinoid group of insecticides that work through full or partial agonist activity and so the potential adverse side effects on honeybee populations could be avoided. Further investigation of HAE or (-)-adoline as a pesticide would need to be mindful of this even though there is a different mode of action.

HAE selectively inhibited $D\alpha 2/\beta 2$ but the HAE inhibition of rat $\alpha 3\beta 4$ was much stronger than $D\alpha 2/\beta 2$ on the decay of the response than on the peak. Inhibition was voltage-independent for rat $\alpha 3\beta 4$ as well binding to the closed state. Block of $\alpha 4\beta 2$ was voltage-dependent indicating open-channel block as the mechanism of inhibition. However, $\alpha 4\beta 2$ was found to have a competitive mode of action. Differing modes of action can be explained by HAE being a mixture of compounds and so there is likely more than one active component. Rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ could potentially have a non-competitive mode of action but further studies are needed to confirm this. (-)-adoline inhibition was the strongest for rat $\alpha 3\beta 4$ and all tested receptors were found to be voltage-independent indicating binding to the closed state. (-)-adoline inhibition of rat $\alpha 3\beta 4$ and rat $\alpha 4\beta 2$ is competitive with further studies being needed to investigate whether human $\alpha 7$ inhibition is non-competitive. The selectivity between insect and mammalian nAChRs demonstrated in Richards (2016) did not persist when

mammalian n-nAChRs were examined. As only one subtype of insect nAChR was examined the ladybird alkaloids could be selective for other subtypes. Locust neurons contain multiple subtypes of nAChR and so the effects of the ladybird alkaloids could have been exerted through a subtype pharmacologically different to the $D\alpha 2/\beta 2$ subtype. Modifying the structure of HAE and (-)-adaline could also lead to the development of compounds with increased activity and/or selectivity.

NMDARs were inhibited by HAE in a voltage-dependent manner consistent with open channel block. This shared a similar mechanism to a currently available Alzheimer's disease drug and other drugs currently in clinical trials indicating a potential use for some component(s) of HAE. HAE would need to demonstrate selectivity for the therapeutic target as activity on other targets could cause serious side effects. No activity was shown against AMPA receptors but other subtypes of AMPA receptors and glutamate receptors in general exist. Investigation into whether HAE has effects on other receptors, especially those of the Cys-loop family, needs to be carried out as they are closely related. There also exists the possibility that HAE could act on G-protein coupled receptors so this would also need to be investigated. Separation of HAE into its individual components would allow a true comparison to the other investigated drugs in order to see whether HAE can serve as a lead for a developing a novel clinical drug.

References

- Abu, I.F., 2016. Exploring block and permeation of N-methyl-D-Aspartate (NMDA) receptor channels for treatment of neurodegenerative disorders. Available at: <http://eprints.nottingham.ac.uk/38591/> [Accessed September 29, 2017].
- Alam, N. et al., 2002. A new alkaloid from two coccinellid beetles *Harmonia axyridis* and *Aiolocaria hexaspilota*. *Bulletin of the Korean Chemical Society*, 23(3), pp.497–499.
- Alaux, C. et al., 2010. Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology*, 12(3), pp.774–782. Available at: <http://doi.wiley.com/10.1111/j.1462-2920.2009.02123.x> [Accessed September 4, 2018].
- Allen, T.D. et al., 2007. A protocol for isolating *Xenopus* oocyte nuclear envelope for visualization and characterization by scanning electron microscopy (SEM) or transmission electron microscopy (TEM). *Nature Protocols*, 2(5), pp.1166–1172. Available at: <http://www.nature.com/doifinder/10.1038/nprot.2007.137> [Accessed September 29, 2017].
- Amar, M. et al., 1995. A nicotinic acetylcholine receptor subunit from insect brain forms a non-desensitising homo-oligomeric nicotinic acetylcholine receptor when expressed in *Xenopus* oocytes. *Neuroscience letters*, 199(2), pp.107–110. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8584235> [Accessed September 29, 2017].
- Anon, The Coccinellidae (Coleoptera) of America North of Mexico by Robert D. Gordon. Available at:

https://www.zin.ru/Animalia/Coleoptera/addpages/Andrey_Ukrainsky_Library/References_files/Gordon85a.htm [Accessed September 29, 2017].

Attygalle, A.B. et al., 1993. Azamacrolides: a family of alkaloids from the pupal defensive secretion of a ladybird beetle (*Epilachna varivestis*). *Proceedings of the National Academy of Sciences of the United States of America*, 90(11), pp.5204–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8506367> [Accessed September 29, 2017].

Bai, D. et al., 1991. Actions of Imidacloprid and a Related Nitromethylene on Cholinergic Receptors of an Identified Insect Motor Neurone. *Pesticide Science*, 33(April), pp.197–204.

Barnard, E. a, Miledi, R. & Sumikawa, K., 1982. Translation of exogenous messenger RNA coding for nicotinic acetylcholine receptors produces functional receptors in *Xenopus* oocytes. *Proceedings of the Royal Society of London*, 215(1199), pp.241–246.

Barthelme, A. et al., 2013. A two-directional approach to pyrrolizidines: total syntheses and biological evaluation of alkaloid cis-223B and (±)-xenovenine. *Chemical communications (Cambridge, England)*, 49(89), pp.10507–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24084985> [Accessed October 12, 2016].

Bass, C. et al., 2015. The global status of insect resistance to neonicotinoid insecticides. *Pesticide Biochemistry and Physiology*, 121, pp.78–87.

Berec, M., 2011. CalPhotos: *Epipedobates anthonyi*; Phantasmal Poison Frog. Available at:

https://calphotos.berkeley.edu/cgi/img_query?enlarge=0000+0000+0211+0577

[Accessed September 4, 2018].

Bertrand, D. et al., 1994. Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate beta 2 subunit and *Drosophila* alpha subunits. *The European journal of neuroscience*, 6, pp.869–875.

Betz, H., 1982. Interaction of histrionicotoxin with the putative nicotinic acetylcholine receptor of the chick visual system. *Neuroscience letters*, 33(2), pp.153–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7155458>

[Accessed September 4, 2018].

Bezzerides, A.L. et al., 2007. Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*. *Behavioral Ecology and Sociobiology*, 61(9), pp.1401–1408.

Bianchi, L. & Driscoll, M., 2006. Heterologous expression of *C. elegans* ion channels in *Xenopus* oocytes. *WormBook*, pp.1–16. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18050441> [Accessed September 29, 2017].

Blacquière, T. et al., 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology (London, England)*, 21(4), pp.973–92. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22350105> [Accessed September 30, 2016].

Boulin, T. et al., 2008. Eight genes are required for functional reconstitution of the *Caenorhabditis elegans* levamisole-sensitive acetylcholine receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 105(47), pp.18590–18595. Available at: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2587545/> <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2587545/>

i.nlm.nih.gov/pmc/articles/PMC2587545/pdf/zpq18590.pdf.

Boulter, J. et al., 1987a. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proceedings of the National Academy of Sciences of the United States of America*, 84(21), pp.7763–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2444984> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC299381>.

Boulter, J. et al., 1987b. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proceedings of the National Academy of Sciences of the United States of America*, 84(21), pp.7763–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2444984> [Accessed September 27, 2017].

Bowman, W.C., 2006. Neuromuscular block. *British journal of pharmacology*, 147 Suppl 1(Suppl 1), pp.S277-86. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16402115> [Accessed September 4, 2018].

Breer, H. & Sattelle, D., 1987. Molecular properties and functions of insect acetylcholine receptors. *Journal of Insect Physiology*, 33(11), pp.771–790. Available at: <http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:MOLECULAR+PROPERTIES+AND+FUNCTIONS+OF+INSECT+ACETYLCHOLINE+RECEPTORS#0>.

Brejci, K. et al., 2001. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature*, 411(6835), pp.269–276.

Brier, T.J. et al., 2003. Contrasting actions of philanthotoxin-343 and philanthotoxin-

(12) on human muscle nicotinic acetylcholine receptors. *Molecular pharmacology*, 64(4), pp.954–64. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/14500752> [Accessed September 30, 2016].

Briggs, C.A. et al., 2006. High- and Low-Sensitivity Subforms of $\alpha 4\beta 2$ and $\alpha 3\beta 2$ nAChRs. *Journal of Molecular Neuroscience*, 30(1–2), pp.11–12. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/17192606> [Accessed September 4, 2018].

Brown, L.A. et al., 2006. Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. *Journal of Neurochemistry*, 99(2), pp.608–615.

Buisson, B. & Bertrand, D., 1998. Open-Channel Blockers at the Human $\alpha 4\beta 2$ Neuronal Nicotinic Acetylcholine Receptor. *Molecular Pharmacology*, 53(3), pp.555–563. Available at:
<http://molpharm.aspetjournals.org/content/53/3/555.abstract>.

Burton, M.J. et al., 2011. Differential resistance of insect sodium channels with kdr mutations to deltamethrin, permethrin and DDT. *Insect Biochemistry and Molecular Biology*, 41(9), pp.723–732. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/21640822> [Accessed October 6, 2017].

Camarano, S., González, A. & Rossini, C., 2009. Biparental endowment of endogenous defensive alkaloids in *epilachna paenulata*. *Journal of Chemical Ecology*, 35(1), pp.1–7.

Casida, J.E. & Quistad, G.B., 1998. Golden age of insecticide research: past, present, or future? *Annual review of entomology*, 43, pp.1–16.

- Celie, P.H.N. et al., 2004. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron*, 41(6), pp.907–914.
- Changeux, J.P., Devillers-Thiéry, A. & Chemouilli, P., 1984. Acetylcholine receptor: an allosteric protein. *Science (New York, N.Y.)*, 225(4668), pp.1335–1345.
- Changeux, J.P. & Edelstein, S.J., 2006. Allosteric receptors after 30 years. *Rendiconti Lincei*, 17(1–2), pp.59–96.
- Changeux, J.P., Kasai, M. & Lee, C.Y., 1970. Use of a snake venom toxin to characterize the cholinergic receptor protein. *Proceedings of the National Academy of Sciences of the United States of America*, 67(3), pp.1241–7.
- Available at: <http://www.ncbi.nlm.nih.gov/pubmed/5274453> [Accessed September 30, 2016].
- Charnet, P. et al., 1990. An open-channel blocker interacts with adjacent turns of α -helices in the nicotinic acetylcholine receptor. *Neuron*, 4(1), pp.87–95. Available at: <https://www.sciencedirect.com/science/article/pii/089662739090445L> [Accessed September 5, 2018].
- Chavez-Noriega, L.E. et al., 1997. Pharmacological characterization of recombinant human neuronal nicotinic acetylcholine receptors h alpha 2 beta 2, h alpha 2 beta 4, h alpha 3 beta 2, h alpha 3 beta 4, h alpha 4 beta 2, h alpha 4 beta 4 and h alpha 7 expressed in *Xenopus* oocytes. *J.Pharmacol.Exp.Ther.*, 280(1), pp.346–356.
- Claudio, T. et al., 1983. Nucleotide and deduced amino acid sequences of Torpedo californica acetylcholine receptor gamma subunit. *Proceedings of the National Academy of Sciences of the United States of America*, 80(4), pp.1111–5.

Available at:

<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=393538&tool=pmc-entrez&rendertype=abstract>.

Cooper, E., Couturier, S. & Ballivet, M., 1991. Pentameric structure and subunit stoichiometry of a neuronal nicotinic acetylcholine receptor. *Nature*, 350(6315), pp.235–238. Available at: <http://www.nature.com/doifinder/10.1038/350235a0> [Accessed September 29, 2017].

Cottrell, T.E., 2007. Predation by adult and larval lady beetles (Coleoptera: Coccinellidae) on initial contact with lady beetle eggs. *Environmental entomology*, 36(2), pp.390–401.

Dale, R.P. et al., 2010. Identification of ion channel genes in the *Acyrtosiphon pisum* genome. *Insect Molecular Biology*, 19, pp.141–153. Available at: <http://doi.wiley.com/10.1111/j.1365-2583.2009.00975.x> [Accessed September 4, 2018].

Daloze, D., Braekman, J.-C. & Pasteels, J.M., 1994. Ladybird defence alkaloids: Structural, chemotaxonomic and biosynthetic aspects (Col.: Coccinellidae). *Chemoecology*, 5–6(3–4), pp.173–183. Available at: <http://link.springer.com/10.1007/BF01240602> [Accessed September 29, 2017].

Dani, J.A., 2015. Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine. *International review of neurobiology*, 124, pp.3–19. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26472524> [Accessed September 29, 2017].

Demuro, A. et al., 2001. Inhibition of nicotinic acetylcholine receptors by bicuculline.

Neuropharmacology, 41, pp.854–861. Available at:

www.elsevier.com/locate/neuropharm [Accessed September 27, 2017].

Devillers-Thiery, a et al., 1983. Complete mRNA coding sequence of the acetylcholine binding alpha-subunit of *Torpedo marmorata* acetylcholine receptor: a model for the transmembrane organization of the polypeptide chain. *Proceedings of the National Academy of Sciences of the United States of America*, 80(7), pp.2067–71. Available at:

<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=393754&tool=pmc&rendertype=abstract>.

DolenskÁ, M. et al., 2009. What constitutes optical warning signals of ladybirds (Coleoptera: Coccinellidae) towards bird predators: Colour, pattern or general look? *Biological Journal of the Linnean Society*, 98(1), pp.234–242.

Doucette-Stamm, L. et al., 1993. Cloning and sequence of the human $\alpha 7$ nicotinic acetylcholine receptor. *Drug Development Research*, 30(4), pp.252–256. Available at: <http://doi.wiley.com/10.1002/ddr.430300408> [Accessed September 5, 2018].

Elliott, M., 1976. Properties and applications of pyrethroids. *Environmental health perspectives*, 14, pp.1–13. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/789056> [Accessed September 30, 2016].

Finlayson, C. et al., 2010. Differential Consumption of Four Aphid Species by Four Lady Beetle Species. *Journal of Insect Science*, 10(31), pp.1–10. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20578952> [Accessed September 29, 2017].

Franzyk, H. et al., 2014a. The Effects of Conformational Constraints in the Polyamine

Moiety of Philanthotoxins on AMPAR Inhibition. *ChemMedChem*, 9(8), p.n/a-n/a. Available at: <http://doi.wiley.com/10.1002/cmdc.201402109> [Accessed September 30, 2016].

Franzyk, H. et al., 2014b. The Effects of Conformational Constraints in the Polyamine Moiety of Philanthotoxins on AMPAR Inhibition. *ChemMedChem*, 9(8), p.n/a-n/a. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25044789> [Accessed September 27, 2017].

G. Lognay, *,[†] et al., 1996. Adalinine, a New Piperidine Alkaloid from the Ladybird Beetles *Adalia bipunctata* and *Adalia decempunctata*.

Gill, J.K. et al., 2011. Agonist activation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proceedings of the National Academy of Sciences of the United States of America*, 108(14), pp.5867–72. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21436053> [Accessed September 4, 2018].

Glisan King, A. & Meinwald, J., 1996. Review of the Defensive Chemistry of Coccinellids. *Chemical reviews*, 96(3), pp.1105–1122. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11848782>.

Goldin, A.L., 2006. Expression of Ion Channels in *Xenopus* Oocytes. In *Expression and Analysis of Recombinant Ion Channels*. Weinheim, FRG: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 1–25. Available at: <http://doi.wiley.com/10.1002/3527608095.ch1> [Accessed September 29, 2017].

Gordon, R., 1985. The Coccinellidae (Coleoptera) of America North of Mexico by Robert D. Gordon. *Journal of the New York Entomological Society*. Available at: https://www.zin.ru/Animalia/Coleoptera/addpages/Andrey_Ukrainsky_Library/

References_files/Gordon85a.htm [Accessed September 4, 2018].

Gross, M., 2013. EU ban puts spotlight on complex effects of neonicotinoids. *Current Biology*, 23(11), pp.R462–R464.

Guan, B., Chen, X. & Zhang, H., 2013. Two-Electrode Voltage Clamp. In Humana Press, Totowa, NJ, pp. 79–89. Available at:

http://link.springer.com/10.1007/978-1-62703-351-0_6 [Accessed September 29, 2017].

GURDON, J.B. et al., 1971. Use of Frog Eggs and Oocytes for the Study of Messenger RNA and its Translation in Living Cells. *Nature*, 233(5316), pp.177–182. Available at: <http://www.nature.com/doi/10.1038/233177a0> [Accessed September 30, 2016].

H. Thany, S. & Tricoire-Leignel, H., 2011. Emerging Pharmacological Properties of Cholinergic Synaptic Transmission: Comparison between Mammalian and Insect Synaptic and Extrasynaptic Nicotinic Receptors. *Current Neuropharmacology*, 9(4), pp.706–714. Available at:

<http://www.ncbi.nlm.nih.gov/pubmed/22654728> [Accessed September 29, 2017].

Hagen, K.S., 1962. Biology and Ecology of Predaceous Coccinellidae. *Annual Review of Entomology*, 7(1), pp.289–326. Available at:

<http://www.annualreviews.org/doi/10.1146/annurev.en.07.010162.001445> [Accessed September 29, 2017].

Happ, G.M. & Eisner, T., 1961. Hemorrhage in a Coccinellid Beetle and Its Repellent Effect on Ants. *Science*, 134(3475), pp.329–331. Available at:

<http://www.sciencemag.org/content/134/3475/329>.

- Harel, M. et al., 2001. The Binding Site of Acetylcholine Receptor as Visualized in the X-Ray Structure of a Complex between α -Bungarotoxin and a Mimotope Peptide. *Neuron*, 32(2), pp.265–275. Available at: <https://www.sciencedirect.com/science/article/pii/S0896627301004615> [Accessed September 4, 2018].
- Haulotte, E., Laurent, P. & Braekman, J.C., 2012. Biosynthesis of defensive coccinellidae alkaloids: Incorporation of fatty acids in adaline, coccinelline, and harmonine. *European Journal of Organic Chemistry*, (10), pp.1907–1912.
- Hautier, L. et al., 2008. Intraguild predation by *Harmonia axyridis* on coccinellids revealed by exogenous alkaloid sequestration. *Chemoecology*, 18(3), pp.191–196.
- Hilf, R.J.C. & Dutzler, R., 2009. Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature*, 457(7225), pp.115–118. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18987630> [Accessed September 4, 2018].
- Hill, R.B. & Usherwood, P.N., 1961. The action of 5-hydroxytryptamine and related compounds on neuromuscular transmission in the locust *Schistocerca gregaria*. *The Journal of physiology*, 157(2), pp.393–401. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16992083> [Accessed September 29, 2017].
- Hillmer, A.T. et al., 2013. The effects of lobeline on $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor binding and uptake of [(18)F]nifene in rats. *Journal of neuroscience methods*, 214(2), pp.163–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23370310> [Accessed September 4,

2018].

- Højland, D.H. et al., 2015. Incidence, Spread and Mechanisms of Pyrethroid Resistance in European Populations of the Cabbage Stem Flea Beetle, *Psylliodes chrysocephala* L. (Coleoptera: Chrysomelidae). *PloS one*, 10(12), p.e0146045. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26717570> [Accessed September 30, 2016].
- Holloway, G.J. et al., 1991. Chemical defence in ladybird beetles (Coccinellidae). I. Distribution of coccinelline and individual variation in defence in 7-spot ladybirds (*Coccinella septempunctata*). *Chemoecology*, 2(1), pp.7–14.
- Hopkins, C. et al., 1995. A New Family of Conus Peptides Targeted to the Nicotinic Acetylcholine Receptor. *Journal of Biological Chemistry*, 270(38), pp.22361–22367. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.270.38.22361> [Accessed September 30, 2016].
- HYND, M., Scott, H.L. & Dodd, P.R., 2004. Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. *Neurochemistry International*, 45(5), pp.583–595. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15234100> [Accessed September 29, 2017].
- Ihara, M. et al., 2014. A single amino acid polymorphism in the *Drosophila melanogaster* D α 1 (ALS) subunit enhances neonicotinoid efficacy at D α 1-chicken β 2 hybrid nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. *Bioscience, biotechnology, and biochemistry*, 78(4), pp.543–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25036948>.
- Ihara, M. et al., 2003. Diverse actions of neonicotinoids on chicken α 7, α 4 β 2 and *Drosophila*-chicken SAD β 2 and ALS β 2 hybrid nicotinic

acetylcholine receptors expressed in *Xenopus laevis* oocytes.

Neuropharmacology, 45(1), pp.133–44. Available at:

<http://www.ncbi.nlm.nih.gov/pubmed/12814666> [Accessed September 4, 2018].

Ihara, M., Sattelle, D.B. & Matsuda, K., 2015. Probing new components (loop G and the α - α interface) of neonicotinoid binding sites on nicotinic acetylcholine receptors. *Pesticide biochemistry and physiology*, 121, pp.47–52. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26047111>.

Indurthi, D.C. et al., 2016. Ligand Binding at the $\alpha 4$ - $\alpha 4$ Agonist-Binding Site of the $\alpha 4\beta 2$ nAChR Triggers Receptor Activation through a Pre-Activated Conformational State T. Arendt, ed. *PLOS ONE*, 11(8), p.e0161154. Available at: <http://dx.plos.org/10.1371/journal.pone.0161154> [Accessed September 4, 2018].

Jacobsen, R. et al., 1997. Differential Targeting of Nicotinic Acetylcholine Receptors by Novel A-Conotoxins. *Journal of Biological Chemistry*, 272(36), pp.22531–22537. Available at: <http://www.jbc.org/cgi/doi/10.1074/jbc.272.36.22531> [Accessed September 30, 2016].

Jeschke, P. & Nauen, R., 2008. Neonicotinoids-from zero to hero in insecticide chemistry. *Pest Management Science*, 64(11), pp.1084–1098. Available at: <http://doi.wiley.com/10.1002/ps.1631> [Accessed September 30, 2016].

Jones, A.K. et al., 2005. Sgbeta1, a novel locust (*Schistocerca gregaria*) non-alpha nicotinic acetylcholine receptor-like subunit with homology to the *Drosophila melanogaster* Dbeta1 subunit. *Invertebrate neuroscience : IN*, 5(3–4), pp.147–55. Available at: <http://link.springer.com/10.1007/s10158-005-0007-6>

[Accessed September 29, 2017].

Jones, A.K. et al., 2010. The cys-loop ligand-gated ion channel gene superfamily of the parasitoid wasp, *Nasonia vitripennis*. *Heredity*, 104(3), pp.247–259.

Available at: <http://www.nature.com/articles/hdy200997> [Accessed September 4, 2018].

Jones, A.K. & Sattelle, D.B., 2010. Diversity of Insect Nicotinic Acetylcholine Receptor Subunits. In Springer, New York, NY, pp. 25–43. Available at: http://link.springer.com/10.1007/978-1-4419-6445-8_3 [Accessed September 29, 2017].

Jones, a K. & Sattelle, D.B., 2010. Diversity of insect nicotinic acetylcholine receptor subunits. *Advances in experimental medicine and biology*, 683, pp.25–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20737786>.

Kachel, H.S. et al., 2016. Block of nicotinic acetylcholine receptors by philanthotoxins is strongly dependent on their subunit composition. *Scientific Reports*, 6(1), p.38116. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/27901080> [Accessed September 28, 2017].

Kao, P.N. & Karlin, A., 1986. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. *Journal of Biological Chemistry*, 261(18), pp.8085–8088.

Karlin, A., 2002. Ion Channel Structureemerging Structure of the Nicotinic Acetylcholine Receptors. *Nature Reviews Neuroscience*, 3(2), pp.102–114. Available at: <http://www.nature.com/doifinder/10.1038/nrn731>.

Karlin, A. et al., 1983. The arrangement of the subunits of the acetylcholine receptor of *Torpedo californica*. , 258, pp.6678–6681.

Keyser, K.T. et al., 1993. Three subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors are expressed in chick retina. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 13(2), pp.442–54. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8426223> [Accessed September 29, 2017].

Koch, R.L., 2003. The multicolored Asian lady beetle, *Harmonia axyridis*: a review of its biology, uses in biological control, and non-target impacts. *Journal of insect science (Online)*, 3, p.32. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15841248> [Accessed September 29, 2017].

Kouvatsos, N. et al., 2016. Crystal structure of a human neuronal nAChR extracellular domain in pentameric assembly: Ligand-bound $\alpha 2$ homopentamer. *Proceedings of the National Academy of Sciences of the United States of America*, 113(34), pp.9635–40. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/27493220> [Accessed October 5, 2016].

Labriola, J.M. et al., 2013. Structural sensitivity of a prokaryotic pentameric ligand-gated ion channel to its membrane environment. *The Journal of biological chemistry*, 288(16), pp.11294–303. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23463505> [Accessed September 4, 2018].

Lansdell, S.J. et al., 2012. The *Drosophila* nicotinic acetylcholine receptor subunits D α 5 and D α 7 form functional homomeric and heteromeric ion channels. *BMC Neuroscience*, 13(1), p.73. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22727315> [Accessed September 29,

2017].

Laurent, P. et al., 2002. In vitro production of adaline and coccinelline, two defensive alkaloids from ladybird beetles (Coleoptera: Coccinellidae). *Insect biochemistry and molecular biology*, 32(9), pp.1017–23. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12213238> [Accessed September 29, 2017].

Lebbe, E.K.M. et al., 2014. Conotoxins targeting nicotinic acetylcholine receptors: an overview. *Marine drugs*, 12(5), pp.2970–3004. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24857959> [Accessed September 4, 2018].

Lees, K. et al., 2014. Functional characterisation of a nicotinic acetylcholine receptor α subunit from the brown dog tick, *Rhipicephalus sanguineus*. *International Journal for Parasitology*, 44(1), pp.75–81. Available at: <http://dx.doi.org/10.1016/j.ijpara.2013.11.002>.

Leonard, R.J. et al., 1988. Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. *Science (New York, N.Y.)*, 242(4885), pp.1578–81. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2462281> [Accessed September 28, 2017].

Leong, R.L. et al., 2015. Non-competitive Inhibition of Nicotinic Acetylcholine Receptors by Ladybird Beetle Alkaloids. *Neurochemical Research*, 40(10), pp.2078–2086. Available at: <http://link.springer.com/10.1007/s11064-014-1466-0>.

Lustig, L.R. et al., 2001. Molecular Cloning and Mapping of the Human Nicotinic Acetylcholine Receptor $\alpha 10$ (CHRNA10). *Genomics*, 73(3), pp.272–283. Available

at: <http://www.ncbi.nlm.nih.gov/pubmed/11350119> [Accessed September 29, 2017].

Magro, A. et al., 2018. The evolution of chemical defenses along invasion routes: *Harmonia axyridis* Pallas (Coccinellidae: Coleoptera) as a case study. *Ecology and Evolution*. Available at: <http://doi.wiley.com/10.1002/ece3.4299> [Accessed September 5, 2018].

Majerus, M.E.N., 1994. *Ladybirds*, HarperCollins.

Marks, M.J. et al., 2010. John Daly's compound, epibatidine, facilitates identification of nicotinic receptor subtypes. *Journal of molecular neuroscience : MN*, 40(1–2), pp.96–104. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19672723> [Accessed September 4, 2018].

MARPLES, N.M., BRAKEFIELD, P.M. & COWIE, R.J., 1989. Differences between the 7-spot and 2-spot ladybird beetles (Coccinellidae) in their toxic effects on a bird predator. *Ecological Entomology*, 14(1), pp.79–84. Available at: <http://doi.wiley.com/10.1111/j.1365-2311.1989.tb00756.x> [Accessed September 29, 2017].

Matsuda, K. et al., 2009. Diverse actions and target-site selectivity of neonicotinoids: structural insights. *Molecular pharmacology*, 76(1), pp.1–10. Available at: <http://molpharm.aspetjournals.org/content/76/1/1.long>.

McClymont, D.W., Harris, J. & Mellor, I.R., 2012. Open-channel blockade is less effective on GluN3B than GluN3A subunit-containing NMDA receptors. *European journal of pharmacology*, 686(1–3), pp.22–31. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22564863> [Accessed September 30, 2016].

- McLane, K.E. et al., 1991. Identification of sequence segments forming the alpha-bungarotoxin binding sites on two nicotinic acetylcholine receptor alpha subunits from the avian brain. *J Biol Chem*, 266(23), pp.15230–15239.
- Mellor, I.R. et al., 2003. Modification of the philanthotoxin-343 polyamine moiety results in different structure-activity profiles at muscle nicotinic ACh, NMDA and AMPA receptors. *Neuropharmacology*, 44(1), pp.70–80. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12559123> [Accessed September 30, 2016].
- Michaud, J.P., 2002. Invasion of the Florida Citrus Ecosystem by *Harmonia axyridis* (Coleoptera: Coccinellidae) and Asymmetric Competition with a Native Species, *Cycloneda sanguinea*. *Environmental Entomology*, 31(5), pp.827–835. Available at: <https://academic.oup.com/ee/article-lookup/doi/10.1603/0046-225X-31.5.827> [Accessed September 29, 2017].
- Millar, N.S. & Denholm, I., 2007. Nicotinic acetylcholine receptors: Targets for commercially important insecticides. *Invertebrate Neuroscience*, 7(1), pp.53–66. Available at: <http://link.springer.com/10.1007/s10158-006-0040-0> [Accessed September 30, 2016].
- Mishina, M. et al., 1985. Location of functional regions of acetylcholine receptor α -subunit by site-directed mutagenesis. *Nature*, 313(6001), pp.364–369. Available at: <http://www.nature.com/doifinder/10.1038/313364a0>.
- Morales-Perez, C.L., Noviello, C.M. & Hibbs, R.E., 2016. X-ray structure of the human $\alpha 4\beta 2$ nicotinic receptor. *Nature*. Available at: <http://www.nature.com/doifinder/10.1038/nature19785> [Accessed October 4, 2016].

- Nauen, R. et al., 2015. Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest management science*, 71(6), pp.850–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25351824> [Accessed September 29, 2017].
- Nauen, R. & Denholm, I., 2005. Resistance of insect pests to neonicotinoid insecticides: Current status and future prospects. *Archives of Insect Biochemistry and Physiology*, 58(4), pp.200–215.
- Nemecz, Á. et al., 2016. Emerging Molecular Mechanisms of Signal Transduction in Pentameric Ligand-Gated Ion Channels. *Neuron*, 90(3), pp.452–470. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S089662731630023X> [Accessed September 4, 2018].
- Noda, M. et al., 1982. Primary structure of alpha-subunit precursor of Torpedo californica acetylcholine receptor deduced from cDNA sequence. *Nature*, 299, pp.793–797.
- Noda, M. et al., 1983. Structural homology of Torpedo californica acetylcholine receptor subunits. *Nature*, 302(5908), pp.528–532. Available at: <http://dx.doi.org/10.1038/302528a0>.
- Olivares, D. et al., 2012. N-methyl D-aspartate (NMDA) receptor antagonists and memantine treatment for Alzheimer's disease, vascular dementia and Parkinson's disease. *Current Alzheimer research*, 9(6), pp.746–58. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21875407> [Accessed September 28, 2017].
- Olsen, C.A. et al., 2006. Tuning Wasp Toxin Structure for Nicotinic Receptor Antagonism: Cyclohexylalanine-Containing Analogues as Potent and Voltage-

Dependent Blockers. *ChemMedChem*, 1(3), pp.303–305. Available at: <http://doi.wiley.com/10.1002/cmdc.200500067> [Accessed September 30, 2016].

Olsen, J.A. et al., 2014. Molecular Recognition of the Neurotransmitter Acetylcholine by an Acetylcholine Binding Protein Reveals Determinants of Binding to Nicotinic Acetylcholine Receptors S. P. Chellappan, ed. *PLoS ONE*, 9(3), p.e91232. Available at: <http://dx.plos.org/10.1371/journal.pone.0091232> [Accessed October 28, 2016].

Panagis, G. et al., 2000. Effects of methyllycaconitine (MLA), an $\alpha 7$ nicotinic receptor antagonist, on nicotine- and cocaine-induced potentiation of brain stimulation reward. *Psychopharmacology*, 149(4), pp.388–96. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10867966> [Accessed September 4, 2018].

Papke, R.L., Dwoskin, L.P. & Crooks, P.A., 2007. The pharmacological activity of nicotine and nornicotine on nAChRs subtypes: relevance to nicotine dependence and drug discovery. *Journal of Neurochemistry*, 101(1), pp.160–167. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17241116> [Accessed September 4, 2018].

Papke, R.L. & Porter Papke, J.K., 2002. Comparative pharmacology of rat and human $\alpha 7$ nAChR conducted with net charge analysis. *British Journal of Pharmacology*, 137(1), pp.49–61. Available at: <http://doi.wiley.com/10.1038/sj.bjp.0704833> [Accessed September 27, 2017].

Pasteels, J.M., 2007. Chemical defence, offence and alliance in ants-aphids-ladybirds relationships. *Population Ecology*, 49(1), pp.5–14.

Paul, S. et al., 2018. *Frontiers in ecology and evolution.*, Frontiers Media. Available at:
<https://ore.exeter.ac.uk/repository/handle/10871/30982> [Accessed September 5, 2018].

Phoofolo, M.W. & Obrycki, J.J., 1998. Potential for intraguild predation and competition among predatory Coccinellidae and Chrysopidae. *Entomologia Experimentalis et Applicata*, 89(1), pp.47–55. Available at:
<http://doi.wiley.com/10.1046/j.1570-7458.1998.00380.x> [Accessed September 29, 2017].

Polis, G.A., Myers, C.A. & Holt, R.D., 1989. The Ecology and Evolution of Intraguild Predation: Potential Competitors That Eat Each Other. *Annual Review of Ecology and Systematics*, 20(1), pp.297–330. Available at:
<http://www.annualreviews.org/doi/10.1146/annurev.es.20.110189.001501>
[Accessed September 29, 2017].

Poorani, J., 2008. Adult Morphology. Available at:
http://www.angelfire.com/bug2/j_poorani/morphology.htm.

Poutsma, J. et al., 2008. Predicting the potential geographical distribution of the harlequin ladybird, *Harmonia axyridis*, using the CLIMEX model. *From Biological Control to Invasion: the Ladybird Harmonia Axyridis as a Model Species*, pp.103–125.

Di Prisco, G. et al., 2013. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, 110(46), pp.18466–71. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/24145453> [Accessed September 4,

2018].

Procopiou, G. et al., 2014. Two-directional synthesis and biological evaluation of alkaloid 5-epi-cis-275B'. *Chemical communications (Cambridge, England)*, 50(97), pp.15355–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25348081> [Accessed October 12, 2016].

Rasoulpour, R.J. et al., 2012. A Novel Mode-of-Action Mediated by the Fetal Muscle Nicotinic Acetylcholine Receptor Resulting in Developmental Toxicity in Rats. *Toxicological Sciences*, 127(2), pp.522–534. Available at: <https://academic.oup.com/toxsci/article-lookup/doi/10.1093/toxsci/kfs118> [Accessed September 5, 2018].

Ray, D.K.D. et al., 2013. Yield Trends Are Insufficient to Double Global Crop Production by 2050 J. P. Hart, ed. *PLoS ONE*, 8(6), p.e66428. Available at: <http://dx.plos.org/10.1371/journal.pone.0066428> [Accessed September 30, 2016].

Raymond Delpech, V. et al., 2003. Action of nereistoxin on recombinant neuronal nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Invertebrate neuroscience : IN*, 5(1), pp.29–35. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14608492>.

Reynolds, J.A. & Karlin, A., 1978. Molecular Weight in Detergent Solution of Acetylcholine Receptor from *Torpedo californica*. *Biochemistry*, 17(11), pp.2035–2038.

Richards, D.P., 2016. Investigating ladybird alkaloids as potential leads for novel insecticides. Available at: <http://eprints.nottingham.ac.uk/33024/> [Accessed September 28, 2017].

- Röhrich, C.R. et al., 2012. Harmonine, a defence compound from the harlequin ladybird, inhibits mycobacterial growth and demonstrates multi-stage antimalarial activity. *Biology letters*, 8(2), pp.308–311. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3297383&tool=pmcentrez&rendertype=abstract>.
- Rozental, R. et al., 1989. Allosteric inhibition of nicotinic acetylcholine receptors of vertebrates and insects by philanthotoxin. *The Journal of pharmacology and experimental therapeutics*, 249(1), pp.123–30. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2468760> [Accessed September 30, 2016].
- Santi, F. & Maini, S., 2006. Predation upon *Adalia bipunctata* and *Harmonia axyridis* eggs by *Chrysoperla carnea* larvae and *Orius laevigatus* adults. *Bulletin of Insectology*, 59(1), pp.53–58. Available at: <http://www.bulletinofinsectology.org/pdfarticles/vol59-2006-053-058santi.pdf> [Accessed September 29, 2017].
- Sato, S. & Dixon, A.F.G., 2004. Effect of intraguild predation on the survival and development of three species of aphidophagous ladybirds: consequences for invasive species. *Agricultural and Forest Entomology*, 6(1), pp.21–24. Available at: <http://doi.wiley.com/10.1111/j.1461-9555.2004.00197.x> [Accessed September 29, 2017].
- SATTELLE, D.B. et al., 1985. Nereistoxin: Actions on a CNS Acetylcholine Receptor/Ion Channel in the Cockroach *Periplaneta Americana*. *Journal of Experimental Biology*, 118(1).
- Schäfer, S., Rosenboom, H. & Menzel, R., 1994. Ionic currents of Kenyon cells from

the mushroom body of the honeybee. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 14(8), pp.4600–12. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/7519255> [Accessed September 29, 2017].

Schröder, F.C. & Tolasch, T., 1998. Psylloborine A, a new dimeric alkaloid from a ladybird beetle. *Tetrahedron*, 54(40), pp.12243–12248. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0040402098007534> [Accessed September 29, 2017].

Scott, J.G. et al., 2014. Genome of the house fly, *Musca domestica* L., a global vector of diseases with adaptations to a septic environment. *Genome biology*, 15(10), p.466. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25315136> [Accessed September 4, 2018].

Seago, A.E. et al., 2011. Phylogeny, classification and evolution of ladybird beetles (Coleoptera: Coccinellidae) based on simultaneous analysis of molecular and morphological data. *Molecular Phylogenetics and Evolution*, 60(1), pp.137–151.

Shao, Z. et al., 1998. Potentiation and inhibition of nicotinic acetylcholine receptors by spermine in the TE671 human muscle cell line. *The Journal of pharmacology and experimental therapeutics*, 286(3), pp.1269–76. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9732388> [Accessed September 28, 2017].

Simon-Delso, N. et al., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental science and pollution research international*, 22(1), pp.5–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25233913> [Accessed September 29,

2016].

Sloggett, J.J. et al., 2011. The chemical ecology of *Harmonia axyridis*. *BioControl*, 56(4), pp.643–661.

Smit, A.B. et al., 2001. A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature*, 411(6835), pp.261–268. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11357121> [Accessed September 29, 2017].

Steppuhn, A. et al., 2004. Nicotine's Defensive Function in Nature Michael Levine, ed. *PLoS Biology*, 2(8), p.e217. Available at: <http://dx.plos.org/10.1371/journal.pbio.0020217> [Accessed September 30, 2016].

Straub, C. et al., 2016. Distinct Subunit Domains Govern Synaptic Stability and Specificity of the Kainate Receptor. *Cell Reports*, 16(2), pp.531–544. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/27346345> [Accessed September 29, 2017].

Takahashi, T. et al., 1985. A physiological study on acetylcholine receptor expressed in *Xenopus* oocytes from cloned cDNAs. *Journal de physiologie*, 80(4), pp.229–32. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/3834076> [Accessed September 30, 2016].

Thany, S.H. et al., 2007. Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*, 28(1), pp.14–22.

Tomizawa, M., Millar, N.S. & Casida, J.E., 2005. Pharmacological profiles of recombinant and native insect nicotinic acetylcholine receptors. *Insect Biochemistry and Molecular Biology*, 35(12), pp.1347–1355.

- Tursch, B. et al., 2010. Chemical Ecology of Arthropods. VIII. Synthesis and Absolute Configuration of Adaline. *Bulletin des Sociétés Chimiques Belges*, 82(9–10), pp.699–703. Available at: <http://doi.wiley.com/10.1002/bscb.19730820911> [Accessed September 29, 2017].
- Unwin, N., 2013. Nicotinic acetylcholine receptor and the structural basis of neuromuscular transmission: insights from Torpedo postsynaptic membranes. *Quarterly reviews of biophysics*, 46(4), pp.283–322. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24050525> [Accessed October 3, 2016].
- Unwin, N., 2005. Refined structure of the nicotinic acetylcholine receptor at 4 ?? resolution. *Journal of Molecular Biology*, 346(4), pp.967–989.
- Unwin, N. & Fujiyoshi, Y., 2012. Gating Movement of Acetylcholine Receptor Caught by Plunge-Freezing. *Journal of Molecular Biology*, 422(5), pp.617–634.
- Usherwood, P.N.R., 1994. Insect Glutamate Receptors. *Advances in Insect Physiology*, 24, pp.309–341. Available at: <https://www.sciencedirect.com/science/article/pii/S0065280608600867> [Accessed September 5, 2018].
- Venkatesh, B. et al., 2005. Genetic Basis of Tetrodotoxin Resistance in Pufferfishes. *Current Biology*, 15(22), pp.2069–2072. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16303569> [Accessed September 29, 2017].
- Ware, R.L. & Majerus, M.E.N., 2008. Intraguild predation of immature stages of British and Japanese coccinellids by the invasive ladybird *Harmonia axyridis*. *BioControl*, 53(1), pp.169–188. Available at: <http://link.springer.com/10.1007/s10526-007-9135-8> [Accessed September 29,

2017].

Watson, G.B. et al., 2010. A spinosyn-sensitive *Drosophila melanogaster* nicotinic acetylcholine receptor identified through chemically induced target site resistance, resistance gene identification, and heterologous expression. *Insect Biochemistry and Molecular Biology*, 40(5), pp.376–384. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19944756> [Accessed September 29, 2017].

Webster, J.P.G., Bowles, R.G. & Williams, N.T., 1999. Estimating the economic benefits of alternative pesticide usage scenarios: Wheat production in the United Kingdom. *Crop Protection*, 18(2), pp.83–89.

Williams, G.R. et al., 2015. Neonicotinoid pesticides severely affect honey bee queens. *Scientific Reports*, 5, p.14621. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4602226&tool=pmcentrez&rendertype=abstract>.

Winters, A.E. et al., 2014. Maternal effects and warning signal honesty in eggs and offspring of an aposematic ladybird beetle K. McGraw, ed. *Functional Ecology*, 28(5), pp.1187–1196. Available at: <http://doi.wiley.com/10.1111/1365-2435.12266> [Accessed September 5, 2018].

Wonnacott, S. & Gallagher, T., 2006. The Chemistry and Pharmacology of Anatoxin-a and Related Homotropans with respect to Nicotinic Acetylcholine Receptors. *Marine Drugs*, 4(3), p.228. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3663412/> [Accessed September 4, 2018].

Yarbrough, J.A. et al., 1999. Allergic rhinoconjunctivitis caused by *Harmonia axyridis*

(Asian lady beetle, Japanese lady beetle, or lady bug). *The Journal of allergy and clinical immunology*, 104(3 Pt 1), pp.704–5. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/10482851> [Accessed September 29, 2017].

Yasuda, H. & Ohnuma, N., 1999. Effect of cannibalism and predation on the larval performance of two ladybird beetles. *Entomologia Experimentalis et Applicata*, 93(1), pp.63–67.

Zalat, S. et al., 2005. Modulation of nicotinic acetylcholine and N-methyl-d-aspartate receptors by some Hymenopteran venoms. *Toxicon : official journal of the International Society on Toxinology*, 46(3), pp.282–90. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/15970304> [Accessed September 30, 2016].

Zhang, W., Jiang, F. & Ou, J., 2011. Global pesticide consumption and pollution : with China as a focus. *Proceedings of the International Academy of Ecology and Environmental Sciences*, 1(2), pp.125–144.

Zhou, Q. & Sheng, M., 2013a. NMDA receptors in nervous system diseases. *Neuropharmacology*, 74, pp.69–75. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/23583930> [Accessed September 29, 2017].

Zhou, Q. & Sheng, M., 2013b. NMDA receptors in nervous system diseases. *Neuropharmacology*, 74, pp.69–75. Available at:
<http://linkinghub.elsevier.com/retrieve/pii/S0028390813001263> [Accessed September 29, 2017].

Appendix

Poster abstract for the Egyptian Society of Natural Toxins 6th International Conference on Natural Toxins, Suez Canal University, Ismailia, Egypt, 15th – 16th December 2014

Investigating ladybird alkaloids as potential leads for novel insecticides

Ladybird beetles (Coleoptera:Coccinellidae) represent a diverse group of chemically defended predators. Toxicity of whole beetles and extracts has been demonstrated towards passerine birds, other ladybirds and *Daphnia* but no mechanism of toxicity has been put forward. Structurally unique alkaloids, found in high concentrations within the haemolymph, are thought to be responsible. Ecological data suggests that ladybirds are intraguild predators, regularly predated upon by insects but rarely consumed by vertebrates. As defensive toxins are often honed to the taxa most regularly encountered as predators; we hypothesize that ladybird alkaloids will show selectivity for insect over vertebrate targets.

We used patch-clamp electrophysiology of human muscle (TE671) and insect (*Schistocerca gregaria*) neuron cells expressing mammalian muscle-type and insect neuronal-type nicotinic acetylcholine receptors (nAChRs) respectively. Our results show that synthetic (-)-adoline, produced by the 2-spot ladybird (*Adalia bipunctata*) is an antagonist of acetylcholine induced nAChR currents in both human and insect cells. Despite having activity towards both receptors, (-)-adoline is between 15-19 fold more

potent to the insect nAChR with IC₅₀ values of 24.4 and 1.28 µM for human and locust cells respectively at V_h-75 mV. Two-electrode voltage clamp of *Xenopus* oocytes expressing recombinant mammalian and insect neuronal-type receptors was also used to compare the selectivity of the alkaloids. The acetylcholine response of recombinant rat α4β2 nAChRs was also inhibited with an IC₅₀ of 13.2 µM for the peak current at V_h-75 mV.

Given the results of this study, we propose that ladybird alkaloids represent an, as yet, untapped source of potential insecticide lead structures.

Poster abstract for the 18th World Congress of the International Society on Toxinology, University of Oxford, Oxford, UK, 25th – 30th September 2015

Investigating the inhibition of nicotinic acetylcholine receptors by ladybird alkaloids

Ladybird beetles (Coleoptera:Coccinellidae) represent a diverse group of chemically defended predators. Toxicity of whole beetles and extracts has been demonstrated towards birds, other ladybirds and *Daphnia*. Structurally unique alkaloids, found in within the haemolymph, are thought to be responsible. Ecological data suggests that ladybirds are intraguild predators, regularly predated upon by insects but rarely by vertebrates. As defensive toxins are often honed to the taxa most regularly encountered as predators; we hypothesize that ladybird alkaloids may show selectivity for insect targets. Recent research has shown that several ladybird alkaloids

target nicotinic acetylcholine receptors (nAChRs) to achieve toxicity. We have used patch-clamp electrophysiology of human TE671 and insect (*Schistocerca gregaria*) neuron cells expressing mammalian muscle-type and insect neuronal-type nAChRs. All ladybird alkaloid extracts and synthetic analogues antagonised vertebrate and insect nAChRs in a concentration dependent manner, but some compounds showed a high level of differential toxicity. (-)-adaline, produced by the 2-spot ladybird (*Adalia bipunctata*) was an antagonist of nAChRs in both human and insect cells. (-)-adaline was between 15-19 fold more potent to the insect nAChR with IC₅₀ values of 24.4 and 1.28 μ M for human and locust cells respectively at V_h -75 mV. Two-electrode voltage clamp of *Xenopus* oocytes expressing recombinant mammalian and insect neuronal-type nAChRs was also used to compare the selectivity of the alkaloids. The acetylcholine response of recombinant rat α 4 β 2 nAChRs was also inhibited by (-)-adaline with an IC₅₀ of 13.2 μ M for the peak current at V_h -75 mV. All the compounds tested so far show non-competitive activity and voltage dependence, consistent with open-channel block. Given the results of this study, we propose that ladybird alkaloids represent an as yet untapped source of novel nAChR antagonists that show promise as insecticide leads.