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Actions on mammalian and insect nicotinic acetylcholine receptors of harmonine-containing alkaloid extracts from the Harlequin ladybird *Harmonia axyridis*

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ABSTRACT

The harlequin ladybird, Harmonia axyridis (H. axyridis), possesses a strong chemical defence that has contributed to its invasive success. Ladybird beetle defensive chemicals, secreted in response to stress and also found on the coating of laid eggs, are rich in alkaloids that are thought to be responsible for this beetle's toxicity to other species. Recent studies have shown that alkaloids from several species of ladybird beetle can target nicotinic acetylcholine receptors (nAChRs) acting as receptor antagonists, hence we have explored the actions of alkalous of the ladybird H. axyridis on both mammalian and insect nAChRs. Electrophysick gical studies on native and functionally expressed recombinant nAChRs were upon to establish whether an alkaloid extract from *H. axyridis* (HAE) targeted nAChRs and whether any selectivity exists for insect over mammalian receptors of this type. HAE was found to be an inhibitor of all nAChRs tested with the voltage-dependence of inhibition and the effect on ACh EC₅₀ differing between nAChR subtype: Our finding that an HAE fraction consisting almost entirely of harmonine had a strong inhibitory effect points to this alkaloid as a key component of nAChR inhilitory actions. Comparison of HAE inhibition between the mammalian and insect n. ChRs investigated indicates some preference for the insect nAChR supporting the view that investigation of ladybird alkaloids shows promise as a method for identifying natural product leads for future insecticide development.

KEYWORDS

Nicotinic acetylcholine receptor; alkaloid; harmonine; antagonist; patch-clamp; voltageclamp.

ABBREVIATIONS

HAE, Harlequin alkaloid extract.

1. INTRODUCTION

Ladybird beetles (Coleoptera: Coccinellidae) chemically defend themselves by "reflex bleeding" and by coating laid eggs with a defensive fluid that can repel insect predators and competing insect species (Happ and Eisner, 1961). They also cause toxicity when ingested by other insect species (Phoofolo and Obrycki, 1998; Hemptinne et al., 2000; Santi and Maini, 2006) and birds (Marples et al., 1989). Endogenously produced alkaloids are a component of these defensive secretions (Laurent et al., 2002; Haulotte et al., 2012) and are postulated to account for this toxicity. The defensive chemistry of the harlequin ladybird, *Harmonia axyridis*, is of particular interest, as this species has successfully invaded from the Far Fast and established itself across the Asian and European continents (Roy et al., 2016). When H. axyridis was introduced for biological control purposes, unfortunately *t* ourcompeted other ladybird beetle species (Roy and Wajnberg, 2008). Examination of the interactions between *H. axyridis* and competing predator species has shown that *H. axyridis* eggs have a broader range of toxicity to competitors in comparison to eggs of competing ladybird species (Sato and Dixon, 2004; Kajita et al., 2010, Sloggett and Davis, 2010; Katsanis et al., 2013). This has led to multiple investigations into the defensive chemicals of *H. axyridis*. Of particular interest has been the all aloid harmonine, with its broad antimicrobial activity (Alam et al., 2002; Rohrich et al., 2012), toxicity to parasitic trematodes of the genus Schistosoma and inhibition of the enzyme acetylcholinesterase (Kellershohn et al., 2019).

Ladybird beetles themselves have been deployed as a home remedy to soothe toothache (Majerus, 2016) and ion channels including nAChRs are analgesic targets (Umana *et al.*, 2013) as well as being targets for alkaloids (Wink, 2000), leading to the hypothesis that ladybird alkaloids could target ion channels. This was confirmed when investigation of several azaphenalene alkaloids from multiple ladybird beetle species

were found to inhibit mammalian nicotinic acetylcholine receptors (nAChRs) (Leong *et al.*, 2015). The nAChRs are members of the Cys-loop family of ligand-gated ion channels (Cys-loop LGICs) (Thompson *et al.*, 2010). The neurotransmitter roles of these pentameric, transmembrane molecules are well documented and individual receptors consist of 5 highly homologous subunits, further sub-divided into α and non- α types. The α subunits possess a pair of adjacent cysteines in loop C, one of the 6 loops (A-F) that make up the ACh binding site located at the interface between adjacent subunits (Changeux and Edelstein, 2006). Typically loops A, B and C are provided by the α subunit and loops D, E and F are donated by the non- α subunit. 9 α , 4 β , 1 γ , 1 δ and 1 ϵ subunits are found in humans with specific combinations of subunits occurring in particular areas within the nervous system. (Wooltorton *et al.*, 2003) and at neuromuscular junctions (Kalamida *et al.*, 2007).

Insect nAChRs play important relies in behaviour as ACh is the primary afferent neurotransmitter in these organizms (Breer and Sattelle, 1987) and they are also targets for insecticides (Gepner et al., 1978; Sattelle, 1980; Ihara *et al.*, 2018). Cartap (Lee *et al.*, 2003), the commendial derivative of the natural annelid toxin, Nereistoxin (Sattelle *et al.*, 1985), Spulpsyns (Sparks *et al.*, 2001) and neonicotinoids, which show similarities to nicotine and epibatidine (Matsuda *et al.*, 2009), have all been successfully developed as commercial insecticides, with imidacloprid being the first commercially successful compound (Bai *et al.*, 1991; Kagabu, 2011). Since then acetamiprid, clothiandin, nitenpyram, dinotefuran, thiacloprid and thiamethoxam have been developed (Ihara *et al.*, 2018). Flupyradifurone is a butenolide insecticide that activates insect nAChRs (Nauen *et al.*, 2015). The sulfoximine, sulfoxaflor, is also an agonist of insect nAChRs (Oliveira *et al.*, 2011; Watson *et al.*, 2011). Triflumezopyrim is a recent addition to insecticides targeting insect nAChRs acting as an antagonist at the ACh binding site (Cordova *et al.*, 2016).

Other commercially successful groups of insecticides target other insect ion channels including GABA receptors (Buckingham *et al.*, 2017), ryanodine receptors (Cordova *et al.*, 2007) and sodium channels (Soderlund, 2010). However, the actions of all insecticides will ultimately suffer from the development of resistant pest insect populations resulting in a need for a pipeline of alternative insecticides (Soderlund, 2008; Sparks *et al.*, 2012; Bass *et al.*, 2015; Troczka *et al.*, 2015; Buckingham *et al.*, 2008; Sparks *et al.*, 2012; Bass *et al.*, 2015; Troczka *et al.*, 2015; Buckingham *et al.*, 2017) and improved pest management strategies (Sparks and Nauen, 2015). Neonicotinoids are now facing intense scrutiry due to their adverse actions on pollinators (Stanley *et al.*, 2015) and insectivorous birds (Hallmann *et al.*, 2014) hastening the need for alternatives. Investigation of natural products has proved to be a viable pathway for the development of ovel insecticide leads (Thompson *et al.*, 2000). As ladybird alkaloids target mammalian nAChRs, there exists the possibility that insect nAChRs are also targeted due to target cations on other insect species.

Harmonine-containing alkaloid extracts from *H. axyridis* were therefore examined for their activity on both insect and mammalian nAChRs. Detection of any target-site selectivity for insects over mammalian nAChRs would make *H. axyridis* alkaloids worthwhile leads to pursue further in the search for novel insect control chemicals.

2. MATERIALS & METHODS

2.1 Chemical reagents and nucleic acids

ACh was obtained from Sigma-Aldrich. cDNA clones of rat neuronal nAChR subunits (α 3, α 4, β 2, β 4) were acquired from the laboratory of Professor Stephen

Heinemann at the Salk Institute for Biological Studies. The cDNA clone for the human α 7 and the chick β 2 nAChR subunits were a gift from Professor Marc Ballivet. The *Drosophila* nAChR D α 2 was provided by Professor Heinrich Betz. Plasmids were linearised and cRNA transcribed using the mMessage mMachine transcription kit (Ambion).

2.2 Whole alkaloid extraction

Adult H. axyridis beetles were collected from the grounds of University Park (Nottingham) and Rothamstead Research (Harpenden, Herurushire), and stored at -20 °C until extraction. A whole alkaloid extract of H. 1xyr dis (HAE) was obtained by initially freezing the beetles (ca. 5000 weighing ca. 187 g) in liquid nitrogen, crushing with a pestle and mortar and preparing a whole organism extract by soaking the crushed material in methanol (2 x 250 mL) for 24 h at room temperature. The methanolic extracts were filtered using 'Iter paper (Grade 4, Whatman), combined and dried using a rotary evaporator (PUCII, Switzerland). The residue (ca. 8 g) was then subjected to acid-base extraction at room temperature using 1 M HCl (50 mL) and diethyl ether (3 x 20 mL) to remove lipoidal material, followed by adjustment to ca. pH 10 – 12 using 2 M Naol' and extraction with dichloromethane (3 x 20 mL). The combined organic layers were washed with saturated NaCl solution, dried using anhydrous MgSO₄ and evaporated *in vacuo* to yield a residue (ca. 450 mg). The residue was re-dissolved in dichloromethane and aliquots of residue (300 µg and 500 µg) were measured for storage in glass ampoules sealed under nitrogen. The presence of alkaloids in HAE was confirmed using Dragendorff's reagent.

Crude fractions of HAE were prepared by using 5 mg HAE dissolved in methanol. HAE was loaded onto silica glass plates (20 cm³, Fluka) and plates were run using methanol with 1 % ammonium hydroxide as the eluting solvent. Two fractions were

clearly visible without staining, and Dragendorff's reagent confirmed that these fractions were discrete, one mobile and one that remained at the loading level. The silica containing each of the two fractions was collected into glass vials and extracted in methanol. The supernatant was removed to clean glass vials and dried under nitrogen to yield two dried extracts: the mobile fraction A (560 μ g) and the stationary fraction B (1.27 mg).

Coupled Gas Chromatography-Mass-Spectrometry (GC-MS) analysis of extracts was performed using a Waters Autospec Ultima mass spectrometer coupled to an Agilent 6890 GC fitted with a HP-1 capillary column (50 m x 0.32 mm id, 0.52 µm film thickness) and a cool on-column injector. Ionization was by electron impact (70 eV, source temperature 220 °C). Helium was the carrier gas. The oven temperature was maintained at 30 °C for 5 min, and then programmed at 5 °C/min to 250 °C. Identification was confirmed by composition of spectral data with published values (Abel et al., 2015).

2.3 TE671 cell and locust rearon culture

TE671 cells express embryonic human muscle-type nAChRs (composed of $(\alpha 1)_2\beta 1\delta\gamma$ subunits containing the γ embryonic subunit rather than the adult ε subunit) (Luther *et al.*, 1989). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and supplemented with 10 % fetal calf serum, 2 mM glutamine, 10 IU/mL penicillin and 10 µg/mL streptomycin (Sigma-Aldrich), and incubated at 36.5 °C in a 5 % CO₂ atmosphere. Cells were grown in 25 cm² flasks and divided 1:10 when they were approximately 75 % confluent. For whole-cell patch-clamp electrophysiological recordings, dividing cells were plated onto pieces of glass coverslip (5x20 mm) in 35-mm Petri dishes containing 2 mL DMEM.

Desert locusts (Schistocerca gregaria) were purchased from Livefoods UK Ltd and kept in locust breeding cages maintained at an ambient temperature of 26-28 °C with a 40 W incandescent bulb used to provide a basking spot of 38 °C and a 12:12 h light cycle. Locusts were selected at the 6th instar, cold anaesthetised at 4 °C for 10 min, dipped in 70 % ethanol and decapitated. Locust heads were transferred to cooled Ca²⁺/Mg²⁺ free Rinaldini's saline (135 mM NaCl, 25 mM KCl, 0.4 mM NaHCO₃, 0.5 mM glucose, 5 mM HEPES, pH 7.2 with NaOH). The brain was excised and placed in cooled Ca^{2+}/Mg^{2+} free Rinaldini's saline. The mushroom bodies were dissected and placed in 200 µL of Rinaldini's saline containing 2 mg/mL coll get ase (type 1A, Sigma-Aldrich) and 0.5 mg/mL dispase (Boehringer Mannheim 57K Ltd). After 15 min incubation at 36.5 °C, the tube was centrifuged at 800 x g for 1 min at room temperature. The supernatant was removed and replaced with 200 µL locust culture medium (5:4 DMEM [supplemented with 10 % FCS, 2 mM (utamine]:Schneider's insect medium, with 10 IU/mL penicillin and 20 µg/mL strent mycin). The mushroom bodies were then gently triturated through a 200 µL Giann pipette tip before being distributed over heatsterilised glass coverslips (5x29 mm) coated in 0.01 % poly-L-lysine (Sigma-Aldrich) that had been placed i ito 35 mL Petri dishes containing 2 mL locust culture medium. Dishes were incubated a : 36.5 °C in a 5 % CO₂ atmosphere and used within 24 h.

2.4 Whole-cell patch-clamp electrophysiology

Whole-cell patch-clamp electrophysiology was carried out on TE671 cells and locust neurons using an Axopatch 200A (Axon Instruments, US) patch-clamp amplifier and recorded to the disk of a PC using WinWCP V4.5.7 software provided by Dr John Dempster, Institute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK. Patch-pipettes were created using borosilicate glass capillaries (World Precision Instruments, US) using a programmable micropipette puller (P-97, Sutter Instruments

Co., US) and filled with a caesium chloride based pipette solution (140 mM CsCl, 1 mM MgCl₂, 11 mM EGTA and 5 mM HEPES, pH 7.2) giving resistances of 5-7 MΩ. Solutions were perfused using a DAD-12 Superfusion system (Adams & List Associates, US) fitted with a 100 µm polyamide coated quartz output tube with a solution exchange time of 30 - 50 ms and controlled by WinWCP. The perfusion system was pressurised with compressed nitrogen and solutions applied at 200 mm/Hg. TE671 cells prepared for patch-clamp studies were placed in a perfusion chamber and constantly perfused at a flow rate of 5 mL/min with mammalian Ringer (135 mM MaCl, 5.4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 10 mM D-glucose, pH 7.4 vith NaOH). Locust neuronal cultures were perfused with locust saline (180 raid NaCl, 10 mM KCl, 2 mM CaCl₂, 10 mM HEPES, pH 7.2 with NaOH).

2.5 Xenopus laevis oocyte preparation a: d ckNA injection

Xenopus laevis oocytes were ac uired from the European Xenopus Resource Centre (University of Portsmouth, UK). On arrival, excised ovaries were treated with 0.5 mg/mL collagenase (type 1A, Signer) in Ca²⁺-free modified Barth's saline (MBS) (96 mM NaCl, 2 mM KCl, 5 mM HEPES, 2.5 mM pyruvic acid & 0.5 mM theophylline, pH 7.5) for 1 h at 18 °C to remove t're follicular tissue surrounding the oocytes. After washing with Ca²⁺-free MBS the isolared oocytes were incubated at 18 °C in MBS supplemented with the antibiotic gentamicin (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES, 2.5 mM pyruvic acid & 0.5 mM theophylline, pH 7.5 followed by the addition of 0.05 mg/mL gentamicin). Healthy stage IV-V oocytes were selected and 50 nL cRNA was injected using a Nanoliter injector (World Precision Instruments, Inc.). Human α 7 was injected at a concentration of 100 ng/µL, rat α 3/ β 4 and rat α 4/ β 2 were injected in a 1:1 ratio at a concentration of 200 ng/µL each and *Drosophila* α 2/chick β 2 was injected in a 1:1 ratio at a concentration of 1 μ g/ μ L each. Oocytes were incubated for 3-4 days at 18 °C prior to electrophysiological recordings.

2.6 Two-electrode, voltage-clamp electrophysiology

Electrophysiological recordings were obtained from nAChR-expressing oocytes by two-electrode voltage clamp using an Axoclamp 2A voltage clamp amplifier (Axon instruments, USA). An oocyte was transferred to the perfusion chamber using a plastic Pasteur pipette and the bath was perfused (~5 mL/min) with standard oocyte saline (SOS) (100mM NaCl, 2mM KCl, 1.8mM CaCl₂, 1mM MgC¹₂, 5mM HEPES, pH 7.6). Microelectrodes were pulled from borosilicate glass cap.'laries (GC150TF-4, Harvard Apparatus) using a programmable micropipette puller (P-97, Sutter Instruments Co., US), and they had resistances between 0.5 and 2.5 $\times\Omega$ when filled with 3 M KCl. The oocyte was voltage-clamped at a holding priterual (V_h) of -75 mV. ACh was consistently used as the agonist, and it was applied v: thout or together with HAE extracts via a MPS-2 multi-channel gravity fed perfusion system (World Precision Instruments Inc.). Atropine (0.5 μ M) was added to the SOS to prevent any endogenous muscarinic ACh receptor response (Dascal and Landau, 1980). Output currents were transferred using a PCI-6221 A/D converter National Instrument Corp., US) to a PC and WinEDR software (Dr John Dempster, 1) stitute of Pharmacy & Biomedical Sciences, University of Strathclyde, UK) was used for recording and analysis.

2.7 Data Analysis

WinWCP software was used to measure the peak current and current 1 s after onset of response for patch clamp electrophysiology recordings. WinEDR software was used to measure the peak current and current 15 s after onset of response ("late" current) for two-electrode voltage clamp electrophysiology recordings. Data were normalised as a % of an ACh control response or % of maximal ACh response. Each

plotted data point is the mean ±SEM of recordings from 7-14 cells or 5-8 oocytes. GraphPad Prism 8 was used for all data analysis, graph plotting, curve fitting and statistical tests as well as application of the Woodhull equation. Concentrationinhibition and concentration-response curves were used to calculate IC₅₀s for HAE/HAE fractions and EC₅₀s for ACh using the following equations:

% control response = $100/(1 + 10^{(LogIC_{50} - X) * Hillslope})$

or

% max ACh response = $max/(1 + 10^{(LogEC_{50} - X)*Hillslope})$ where X is the concentration of ACh or HAE.

3. RESULTS

3.1 Actions of harmonine-containing all aloid extracts on human muscle nAChRs and insect neuronal nAChRs

Extraction of alkaloids from adult *H. axyridis* beetles resulted in an alkaloid extract containing multiple compounds (Fig. 1C) including the alkaloid harmonine (Fig. 1D). Further fractioning of the extract resulted in 2 alkaloid fractions, one mobile (fraction A) and one that rymained at the loading level (fraction B) (Fig. S1A & B). 5 mg was used for this process of which 560 µg was recovered as fraction A, 1.27 mg was recovered as fraction B and the remaining 3.17 mg was unaccounted for and may have been lost in the re-extraction procedure. Gas chromatography-Mass Spectrometry (GC-MS) analysis revealed fraction B to be harmonine and fraction A to be a mixture of unidentified alkaloids and a low concentration of harmonine (Fig. S1C & D).

The effect of both fractions on human muscle-type nAChRs expressed in TE671 cells and insect neuronal-type nAChRs in locust neurons was investigated using patchclamp electrophysiology. No inward currents were detected upon application of the

fractions A or B to TE671 cells and locust neurons (Fig. S2). Having established that there were no nicotinic stimulatory effects by the alkaloids, we co-applied the fractions with ACh. Inhibition of the ACh response was observed upon co-application of either fraction A or fraction B with ACh to both TE671 cells and locust neurons (Fig. 2A - D) and concentration-inhibition plots were created in order to obtain IC₅₀ values for each fraction (Fig. 3A - D, Table 1).

The peak current amplitude and the current amplitude after 1 s (1 s current) were both measured because some antagonists, notably Finchotoxin-343, can have a much larger effect on the late current than the peak current (Brier et al., 2003). A greater effect on the late current would imply that **II** E inhibition was dependent on the activation of the receptor. Comparison of IC50 values of the HAE fractions showed that the 1 s current IC₅₀ was lower than the pear current IC₅₀ in each case. For fraction A the 1 s IC₅₀ was 156-fold lower than the _L ·ak IC₅₀ for TE671 cells and 28-fold lower for locust neurons. For fraction B the $1 \sim IC_{50}$ was 17-fold lower than the peak IC₅₀ for TE671 cells and 74-fold lower to, locust neurons. This implies that the inhibition was activation-dependent. In addition to the comparison of the effect of the HAE fractions on the peak and 1 s current, comparison of the HAE fractions between TE671 cells and locust neurons was male to determine whether selectivity exists for insect neuronaltype nAChRs over mammalian muscle-type nAChRs (Fig. 3E & F). One-way ANOVA revealed that in all comparisons, fraction B was significantly more potent on locust neuron nAChRs than on the TE671 cell nAChR (peak P \leq 0.05 and 1 s P \leq 0.0001). Fraction B was also significantly more potent than fraction A in its action on nAChRs from both cell types (peak $P \le 0.05$ and $1 \le P \le 0.0001$).

Cell/nAChR type	Current	IC ₅₀ , μg/mL (95 % CI)	
		Fraction A	Fraction B
TE671 (human muscle-type)	Peak	745 (57.4 – 9669)	69.6 (24.9 – 194)
	1 s	4.77 (2.12 – 10.7)	1.68 (1.16 – 2.45)
Locust (insect neuronal-type)	Peak	90.2 (25.1 – 325)	5.30 (2.87 – 9.88)
	1 s	3.23 (1.69 - 6.18)	0.072 (0.038 - 0.140)

Table 1. IC₅₀ values for inhibition by HAE fractions A and B of the peak and 1 s current of locust neuronalnAChRs and human muscle-type nAChRs expressed TE671 cells.

3.2 Mode of action studies on human muscle-type nAC hRs

As there was a limited yield of fractions from the thin-layer chromatography process, the mode of action of whole HAE rawer than the separate fractions was investigated. Furthermore, the availability o (1) ole HAE was also limited so mode of action studies were conducted on TEF/1 cents only. The effect of HAE on the ACh EC₅₀ was explored in order to determine whether there was competition between HAE and ACh at the agonist binding site of the nAChR. Concentration-response plots in the presence and absence of HAE were constructed (Fig. 4A & B) with a HAE concentration of 30 µg/mL. Comparison of the plateau of the curves showed a lower plateau (80 % of ACh control for peak and 41 % of ACh control for 1 s) in the presence of HAE. EC₅₀ values of ACh control vs in the presence of HAE were $3.30 \,\mu\text{M}$ (95 % CI = 2.61 – 4.29) vs $6.31 \mu M$ (95 % CI = 3.61 - 11) for peak current and $2.26 \mu M$ (95 % CI = 1.6 - 3.19) vs 1.48 μ M (95 % CI = 0.07 – 31.2) for 1 s current. Observation of lowered plateau values indicate a non-competitive mode of action for HAE on mammalian muscle-type nAChRs in TE671 cells, however, a significant difference was observed (P = 0.0433, extra sumof-squares F-test, GraphPad Prism 8) for the increase in peak current EC₅₀ indicating a minor component of competitive action.

The effect of changes in V_h on the inhibitory activity of HAE was also investigated in order to determine potential binding of HAE to the channel pore (Fig. 4C & D). Concentration-inhibition plots were constructed over the HAE concentration range of $0.1 - 100 \mu g/mL$. HAE was co-applied with $10 \mu M$ ACh at V_h of +50 mV, -50 mV, -75 mV, -100 mV and -120 mV (Fig. 4C). The inhibition of the peak current was low (15 - 20 % inhibition with 100 $\mu g/mL$ HAE) such that accurate curves could not be fitted nor IC₅₀ values calculated. The 1 s current IC₅₀ values were reduced at more negative V_h values; IC₅₀ values were plotted against holding potential (Fig. 4D) and a regression fitted with the Woodhull equation (Woodhull, 1973). The mixed composition of HAE meant that an accurate Z value could not be assigned, so Z and C were combined. The slope of the Woodhull-fitted regression differed significantly from zero (P = 0.012) indicating strong voltage-dependence of inhibition by HAC on muscle-type nAChRs in TE671 cells. However, there was still substantial inhibition at +50 mV suggesting that either binding was not deep in the pore, or an add¹/₁ on a binding site outside of the pore is present.

3.3 Exploring selectivity oy comparing actions on receptor subtypes with different subunit composition

In order to further investigate selectivity between mammalian and insect nAChRs, a comparison of the effects of HAE on mammalian neuronal-type nAChRs was needed as the comparison between TE671 cells and locust neurons is a comparison between mammalian muscle-type and insect neuronal-type nAChRs. The *Xenopus* oocyte heterologous expression system and two electrode voltage-clamp electrophysiology technique was used to investigate the effects of HAE on mammalian neuronal-type human α 7, rat α 4 β 2, rat α 3 β 4 and the hybrid *Drosophila* α 2/chick β 2

 $(D\alpha 2/\beta 2)$ nAChRs clamped at V_h = -75 mV. Inhibition by HAE of each receptor was observed (Fig. 5A - D) and concentration-inhibition plots were constructed in order to obtain and compare IC₅₀ values (Fig. 6A - F, Table 2). For a more direct comparison with the 1 s current measurement from the TE671 cell and locust neuron studies, the current was measured 15 s from response onset (late current). This could only be achieved with the more slowly desensitising rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ nAChRs and late current IC₅₀ values are given in Table 2. When taking only peak current into account, the D $\alpha 2/\beta 2$ IC₅₀ was 3.34 fold lower than the lowest mammalian $\alpha \beta C_{h}R$ IC₅₀ indicating some selectivity for D $\alpha 2/\beta 2$. Statistical comparison of the IC₅ values (Fig. 6C) shows the D $\alpha 2/\beta 2$ peak current IC₅₀ to be significantly lower than all mammalian receptors investigated (P < 0.0001 for D $\alpha 2/\beta 2$ vs $\alpha 4\beta 2$. P = 0.0159 for D $\alpha 2/\beta 2$ vs $\alpha 7$, P = 0.0065 for D $\alpha 2/\beta 2$ vs $\alpha 3\beta 4$, extra sum-of-squares 7-test, Graphpad Prism 8). However, there is a substantial effect on the late current of $\alpha 3\beta 4$ with a reduction to 17 % of the ACh control response upon application z f the lowest concentration of HAE (0.003 µg/mL).

Table 2. IC₅₀ values for inhibition b, HAE of human α 7, rat α 4 β 2, rat α 3 β 4 and the hybrid *Drosophila* α 2/chick β 2 (D α 2/ β 2) nACh^P as pressed in *Xenopus* oocytes (* 95 % CI could not be calculated; N/A = no late current).

Receptor	IC ₅₀ , μg/mL (95 % CI)		
	Peak Current	Late Current	
α7	2.30 (1.67 – 3.17)	N/A	
α4β2	3.24 (1.47 - 8.65)	0.431 (0.233 - 0.804)	
α3β4	1.47 (0.708 – 2.88)	<<0.03 (*)	
Dα2/β2	0.440 (0.211 - 0.910)	N/A	

3.4 Further exploration of mode of action studies on receptor subtypes with different subunit compositions

The increased solution volumes required for investigation using the rapidly perfused *Xenopus* oocyte expression system was demanding on HAE availability. Hence multiple concentration-inhibition curves could not be constructed to investigate voltage-dependence of HAE inhibition. As an alternative approach, a voltage ramp from -90 mV to +50 mV over a period of 1 s was applied during the steady-state phase of the rat $\alpha 4\beta 2$ and rat $\alpha 3\beta 4$ nAChR currents. 0.3 µg/mL HAE co-applied with 100 µM ACh was used for $\alpha 3\beta 4$ and $3\mu g/mL$ HAE co-applied with 10 μM ACh was used for $\alpha 4\beta 2$. Measurable inward currents were observed when the Vh reached approximately -40 mV for both receptors and so the current in the range -40 mV to -90 mV was normalised to the control ACh response (Fig. 7A). The level of ILE inhibition for $\alpha 4\beta 2$ significantly changed from approximately 75 % of the control response at -40 mV to 44 % at -90 mV (comparison of the -50 mV to -40 mV and 0 mV to -80 mV ranges, P = 0.0006, paired ttest) indicating voltage-dependence of HAE inhibition on $\alpha 4\beta 2$. The level of HAE inhibition for $\alpha 3\beta 4$ remained consistent at approximately 25 % of the control ACh response for the entire V_h range maintaing HAE inhibition is not voltage-dependent on $\alpha 3\beta 4$. As human $\alpha 7$ is a reputly desensitising receptor a voltage ramp could not be applied. An alternative method of applying an ACh control (100 µM ACh) and manually changing the V_h in 25 n V steps from -25 mV to -125 mV followed by co-application of ACh and HAE (1 μ g/mL) at the same V_hs was used (Fig. 7B). One-way ANOVA revealed no significant differences in the percentage of inhibition across the V_h range tested.

The effect of HAE on the ACh EC₅₀ was also examined with $\alpha 3\beta 4$ and $\alpha 4\beta 2$ (Fig. 8A & B). HAE concentrations of 0.3 µg/mL and 3 µg/mL were used for $\alpha 3\beta 4$ and $\alpha 4\beta 2$ respectively in order to produce a more measurable level of inhibition. EC₅₀ values of ACh control vs in the presence of HAE were 161 µM (95 % CI = 138 – 187) vs 184 µM (95 % CI = 98.8 – 254) for $\alpha 3\beta 4$ and 19.9 µM (95 % CI = 9.66 – 38.4) vs 241 µM (95 % CI

= 100 – 386) for $\alpha 4\beta 2$. The maximum response was reduced to 73.4 % of the control response for $\alpha 3\beta 4$ with no effect on the maximum response of $\alpha 4\beta 2$. Only the increase in EC₅₀ value observed for $\alpha 4\beta 2$ was found to be significant (P < 0.0001, extra sum-of-squares F-test, GraphPad Prism 8) indicating competition between HAE and ACh at the agonist binding site of $\alpha 4\beta 2$. The observed reduction in maximum response with minimal (NS) shift in the ACh EC₅₀ for $\alpha 3\beta 4$ suggest non-competitive inhibition by HAE.

4. DISCUSSION

HAE was shown to be an inhibitor of the nAChF response of human α 7, rat α 3β4, rat α 4β2 and the hybrid D α 2/β2 nAChRs expressed in *Xenopus* oocytes as well as the nAChR responses of TE671 cells and locust neurons (Fig. 2 & 5). Examination of the inhibition of the observed peak current showed selectivity for a native insect and an insect/vertebrate hybrid nAChR in each comparison indicating potential selectivity of HAE for insect over mammalian n^Chi's (Fig. 3 & 6). The inhibition of the current 1 s after application and so thic was examined in the responses of the slowly desensitising nAChRs expressed in *Yenonus* oocytes by examining the current 15 s after application (late current) (Fig. 6B). This revealed a larger effect on the late current consistent with the results observed in TE671 cells and locust neurons. The inhibition of the late current of rat α 3β4 was so great that concentrations lower than the range tested in these studies will need to be applied in order to determine an appropriate IC₅₀. A greater inhibitory effect after the onset of response indicates HAE inhibition is at least partly activation-dependent.

Further studies were carried out on mammalian nAChRs in order to explore the mode of action of HAE inhibition. Inhibition was shown to be voltage-dependent in

TE671 cells indicating binding to the pore region of the open channel (Brier *et al.*, 2003) and analysis of voltage-dependency using the Woodhull equation (Woodhull, 1973) was consistent with actions as an open channel blocker. In addition to this, HAE still showed some inhibition at the positive V_h of +50 mV (Fig. 4C), albeit at lower levels than observed at negative V_hs, indicating that the binding site is either to a site not deep in the pore, or to an additional site outside of the pore. Voltage-dependent HAE inhibition was found to be inconsistent across all mammalian nAChRs investigated. For example, the inhibition of human α 7 and rat α 3 β 4 was found to be voltage-dependent while inhibition of rat α 4 β 2 was found to be voltage-dependent to the pore region only in muscle-type nAChRs and α 4 β 2 neuronal-type nAChRs with a distinct type of binding site being present on α 7 and α 3 β 4 nAChRs. This is consistent with the observation of inhibition at +50 mV pointing to a potential binding of the pore.

The possibility of binding to the ACh binding site was investigated by analysing the effect of the presence of H $\mu_{\rm E}$ on the EC₅₀ of ACh for each of the nAChRs. In TE671 cells (Fig. 4A & B) the upper plateau of the EC₅₀ plot was reduced by HAE rather than a large increase in the EC₅₀, which reveals no competition between HAE and ACh for the ACh binding site. This was not the case for all nAChRs tested as the same study revealed a reduction of the upper plateau for α 3β4 indicating no competition for the ACh binding site, however, a lack of reduced upper plateau and a significant increase in the EC₅₀ for α 4β2 did indicate competition for the ACh binding site for this nAChR subtype (Fig. 8). These investigations point to potential binding of HAE at both the pore and the ACh binding site of the nAChRs investigated, with the relative extent of each being dependent on receptor subtype. As HAE is a mixture of compounds, further separation needs to be carried out. This will assist in determining whether our findings are

attributable to one compound (e.g. harmonine) binding to different receptor regions, depending on nAChR subtype. Alternatively, our findings could be explained in terms of the binding of different HAE components to distinct binding sites.

Separating the components of HAE proved challenging for a number of reasons. Although it would have been desirable to isolate, identify and quantify all of the individual alkaloid compounds from the mixture, this was not possible. When subjected to reverse phase HPLC, it was possible to separate compounds, but harmonine, the major alkaloid present, could not be visualised with UV absorbance. We considered deploying HPLC coupled with secondary mass spect om try, but the low-throughput nature of the technique precluded this approach. Liquid chromatography was attempted to increase the quantity of alkaloid extract that could be separated and fractions collected. Multiple attempts with durerent stationary phases, solvents and solvent gradients failed to separate alk. oids effectively. Preparative scale TLC enabled separation of harmonine (fraction R) from the other alkaloid components (fraction A) within the alkaloid extract (Fig. S1 A & B); this was confirmed with mass spectrometry (Fig. S1C & D). From 5 mg of whole alkaloid extract, 1.27 mg of harmonine and 560 µg of unidentified alkaloids v/ith a very low concentration of harmonine were recovered. This confirmed that harmon is the predominant alkaloid within HAE and because fraction B showed a greater inhibitory effect (Fig. 3, Table 1), harmonine is the prime candidate alkaloid responsible for nAChR inhibition. An alternative method of obtaining harmonine to the isolation carried out in this study is to synthesise harmonine *de novo*. Initially 2 syntheses were published consisting of 19 and 15 steps (Braconnier et al., 1985; Enders and Bartzen, 1991) but more recently multiple separate shorter syntheses consisting of 5 - 6 steps have been published (Philkhana *et al.*, 2014; Abel *et al.*, 2015; Nagel *et al.*, 2015). Employment of these recent syntheses could prove a more reliable

and successful route of obtaining harmonine and permit the production of structural analogues that will address the issues of correct physicochemical properties as well as compound stability.

The results of this study along with the results of the investigation of several azaphenalene alkaloids from other species of ladybird beetle show that antagonist actions on nAChRs are characteristic of ladybird beetle alkaloids. The azaphenalene alkaloids displaced binding to the Torpedo nAChR of tritiated piperidyl-N-(1(2thienyl)cyclohexyl)-3,4-piperidine ([³H]-TCP), which bir.us are within the channel pore, but did not displace binding of [³H]-cytisine which binds to the ACh binding site indicating non-competitive inhibition (Leong et al., 2015). The effects of the azaphenalene alkaloids, precoccinelline and coccinelline, were also examined on muscle-type nAChRs expressed in TE67: cens and pre-coccinelline on human α 7 nAChRs expressed in *Xenopus* oocytes. Un-competitive inhibition was observed in each case (Leong et al., 2015). This resembles our own observations on the binding of HAE to the pore region in TE671 cells, however, this binding may be to a shallower region of the pore than the [³H]-TCF binding site. Both nereistoxin and the insecticide derived from it, cartap, are also AChR inhibitors that displace [³H]-TCP in honeybee (Apis *melifaria*) head membranes (Lee *et al.*, 2003), so HAE could have a different mechanism of inhibition to nereistoxin and cartap.

There exist over 4,200 species of ladybird beetle (Iperti, 1999) and alkaloids with a variety of different structures have been identified in a relatively small number of these species (King and Meinwald, 1996), therefore, investigation into these alkaloids could also yield many more chemicals of interest. Alkaloids are also present in many other arthropod species (Roberts and Wink, 2013) that could also yield new insecticide candidates. A number of arthropod alkaloids have shown activity on both mammalian

and insect ionotropic receptors such as Philanthotoxin from the Egyptian digger wasp *Philanthus triangulum* (Rozental *et al.*, 1989; Brier *et al.*, 2003; Mellor *et al.*, 2003; Kachel *et al.*, 2016). Alkaloids from the venom of the fire ant *Solenopsis saevissma* and *Solenopsis richteri* show activity on the insect nAChR but do not show insecticidal activity in whole insect toxicity tests (David *et al.*, 1984). Harmonine did not cause lethal toxicity when injected into the 7-spot ladybird, *Coccinella septempunctata* (Vilcinskas *et al.*, 2013), and it will be important to pursue such tests on pest species for this and other arthropod alkaloids.

Further investigation of HAE and harmonine a ter separation will allow a more detailed understanding of the binding region and mode of action. Further examination of their selectivity will be beneficial, as expression of other mammalian and insect neuronal-type nAChRs in *Xenopus* oocytes is possible. The *Drosophila* nAChR subunits $D\alpha 1$ (Ihara et al., 2014) and $D\alpha 5/D\alpha /$ (cansdell et al., 2012) can also be expressed in *Xenopus* oocytes. Expression of nAChK cubunits from other invertebrate species such as the desert locust *Schistocerca gregaria* (Marshall *et al.*, 1990; Amar *et al.*, 1995), the brown dog tick *Rhipicephalus cuparia* (Lees *et al.*, 2014) and the nematode worm *Caenorhabditis elegans* (Bellivet *et al.*, 1996; Boulin *et al.*, 2008) is also possible so in future it will be of interest to explore whether HAE is more or less selective for other insect/invertebrate neuronal-type nAChRs. As HAE appears to show a degree of selectivity for the insect nAChR, ladybird alkaloids may be of interest as potential sources for lead candidates in the search for novel insecticides.

FIGURE LEGENDS

Figure 1. Ladybird beetle alkaloid extraction overview. Adult *H. axyridis* beetles (A) were collected and used as the source of alkaloids for this study. The alkaloid extraction process (B) involved using methanol to extract the haemolymph (blood analogue in insects) then separating the alkaloids using an acid-base separation followed by thinlayer chromatography to generate fractions. Gas-chromatography – Mass Spectrometry analysis produced a chromatogram (C) showing multiple compounds present in HAE including the alkaloid harmonine (D) known to be present in *H. axyridis*.

Figure 2. HAE fractions A and B inhibit the ACh response of TE671 cells and locust neurons. Representative traces showing the inhibition of ACh induced whole-cell currents in both TE671 cells (A & C) and locust neurons (B & D) by fractions of HAE generated by thin-layer chromatography. Both fractions an equiver against locust receptors in both cells types, but fraction B was significantly more acuse against locust receptors than fraction A and showed selectivity for locust over TE6.71 (AChR (V_h = -75 mV).

Figure 3. HAE fractions A and B have a larger ink. bitory effect on the late current, with fraction B more effective than fraction A and showing greater selectivity for locust neuron nAChRs. Increasing concentrations of HAE fractions A and B were individually co-applied with ACh to both TE671 cells (blue) and locust neurons (red) which had been clamped at $V_h = -75$ mV (A, B, C & D). The peak and 1 s currents were measured and normalised against a control response. A 4 parameter logistic regression curve was fitted to the points, from which IC₅₀ values were derived (n = 10 for Locust Fr A, 9 for Locust Fr B, 9 for TE671 Fr A and 12 for TE671 Fr B). The IC₅₀ and selectivity values for Fractions A and B were then compared in column charts (E & F) against TE671 (blue) and locust neurons (and nAChRs. Asterisks (* P < 0.05, **** P < 0.0001) represent the result of one-way ANOVA comparison of IC₅₀ values. Fraction B is significantly more potent to locust neurons ($V_h = -75$ mV; error bars show 95 % CI).

Figure 4. HAE shows non-competitive, voltage-dependent inhibition of human muscle-type nAChRs in FE671 cells. Concentration-response plots (A & B) showing the current response of 7E671 cells to ACh (black) and to ACh co-applied with 30 μ g/mL HAE (red). Data vere normalised to the peak response to ACh alone. The results show that co-application of HAE with ACh causes the response to plateau at 80 % for peak current and 41 % for 1 s current compared to ACh alone. Rather than a shift to the right, as expected if ACh displaced alkaloid binding, curves instead reflect a noncompetitive inhibition of the ACh response. The EC₅₀ values were (control and in the presence of alkaloid respectively) 3.35 μ M and 6.31 μ M for peak and 2.26 μ M and 1.48 μ M for 1 s currents. Error bars show ±SEM, V_h = -75 mV and n = 12. (C) Concentrationinhibition plot showing the voltage-dependence of HAE inhibition of human muscletype nAChR. TE671 cells were clamped at holding potentials ranging from +50 to -120 mV and perfused with 10 μ M ACh followed by co-application of 10 μ M ACh and 0.1 – 100 µg/mL HAE. Responses were normalised against the peak response of an ACh only control. Mean values ±SEM are plotted and data is fitted with 4 parameter logistic regression curves (GraphPad, Prism 8). (D) IC₅₀s for 1 s data were plotted against V_h and fitted with the Woodhull equation. The slope of the curve (0.16) was significantly greater than 0 (P = 0.012) indicating voltage-dependent inhibition by HAE.

Figure 5. HAE inhibits the ACh response of 3 well characterised mammalian neuronal nAChRs and a hybrid insect/mammalian nAChR expressed in *Xenopus* **oocytes.** Representative traces showing the inhibition by 3 μ g/mL HAE of ACh-induced currents in the (A) hybrid *Drosophila* α 2/chick β 2, (B) rat α 4 β 2, (C) human α 7 and (D) rat α 3 β 4 nAChRs expressed in *Xenopus* oocytes at V_h = -75 mV.

Figure 6. HAE shows selectivity for the hybrid insect/mammalian receptor and potent action on the late current of the rat α3β4 nAChR. Concentration-inhibition curves showing the actions of HAE on (A) the peak ACh-induced current for the hybrid *Drosophila* α2/chick β2, human α7, rat α3β4 and rat α4β2; and (B) on late current responses for rat α3β4 and rat α4β2 expressed in *Xenopus* oocytes. A 4 parameter logistic regression curve was fitted (GraphPad, Prism 8) from which IC₅₀ values were derived. Peak current IC₅₀ values of 3.24 µg/mL for α4p⁻. 2.30 µg/mL for α7, 1.47 µg/mL for α3β4 and 0.440 µg/mL for Dα2/β2 with late corrent IC₅₀ values of 0.431 µg/mL for α4β2 and <<0.03 µg/mL for α3β4 were obtained. All responses were normalised to the control 100/10 µM ACh response and µlotted as mean ±SEM (n = 7-8). (C) IC₅₀ values for the inhibition of peak current IC₅₀ oy HAE were plotted as mean ±95 % CI and compared using an extra sum-of squares F-test (GraphPad, Prism 8). Significant differences were observed in the peak current IC₅₀ for Dα2/β2 vs. α7 (P = 0.0102), Dα2/β2 vs. α4β2 (P = 0.0016) and Dα2/β2 vs. α3β4 (P = 0.0217) (* = P<0.05, ** = P<0.01).

Figure 7. HAE shows voltage-deper dett inhibition of rat α4β2, but not rat α3β4 nor human α7 nAChRs. (A) A voltage is mp was applied to *Xenopus* oocytes expressing rat α4β2 or rat α3β4 nAChRs where the V_h was ramped from -90 mV to +50 mV over 1 s, ACh co-applied with 0.3 ug/mL HAE to α3β4 or ACh co-applied with 3 ug/mL HAE for α4β2 was applied in addition to AC'r control (n = 5). When examining the -90 mV to -40 mV range the % control response for α4β2 changed from approximately 44 % to 75 % and for α3β4 the level of inh bitson across the same voltage range remained consistent at approximately 25 % of the control response. (B) Responses of human α7 receptors expressed in *Xenopus* ooc, tes to 100 μM ACh and 100 μM ACh co-applied with 1 μg/mL HAE as the V_h was charged. All points are normalised to the -125 mV ACh response and plotted as mean ±SE⁴ (1, -5). One-way ANOVA showed no significant differences in the percentage of inhibition across the V_h range for each HAE concentration tested.

Figure 8. HAE shows competitive inhibition of rat $\alpha 4\beta 2$ **nAChR but not rat** $\alpha 3\beta 4$ **nAChR.** Concentration-response curves of ACh alone (black) and ACh co-applied with HAE (red) on (A) rat $\alpha 3\beta 4$ and (B) rat $\alpha 4\beta 2$ nAChRs expressed in *Xenopus* oocytes. 0.3 µg/mL HAE was used for rat $\alpha 3\beta 4$ and 3 µg/mL HAE was used for rat $\alpha 4\beta 2$. EC₅₀ values of 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 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 EC₅₀ for $\alpha 4\beta 2$ to be statistically significant (P < 0.0001). Each point was normalised to the 1 mM ACh response and plotted as mean ±SEM (n = 5).

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Highlights:

- Alkaloid extract from the harlequin ladybird (HAE) inhibited nAChRs
- HAE mode of action varied depending on nAChR subtype
- HAE showed some selectivity for insect nAChRs
- A fraction containing a high concentration of harmonine increased inhibition





Figure 2





Figure 4









Figure 6





Normalised Peak

Figure 7

Α

α3β4



Β

α4β**2**



Figure 8