The *Apis mellifera* alpha 5 nicotinic acetylcholine receptor subunit expresses as a homomeric receptor that is sensitive to serotonin

Eleanor L Mitchell[a], Franco Viscarra[a], Isabel Bermudez[a], Joseph Hawkins[a1], Jim A Goodchild[b] and Andrew K Jones[a]

[a]Department of Biological and Medical Sciences, Faculty of Health and Life Sciences, Oxford Brookes University, Headington, Oxford, OX3 0BP, United Kingdom.

[b]Syngenta, Jealotts Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

[1]Present address: Syngenta, Jealotts Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom.

Authors' email addresses:

Eleanor Mitchell eleanor.mitchell-2018@brookes.ac.uk

Franco Viscarra franco.viscarra@stx.ox.ac.uk

Isabel Bermudez ibermudez@brookes.ac.uk

Joseph Hawkins Joe.Hawkins@syngenta.com

Jim Goodchild jim.goodchild@syngenta.com

Andrew Jones* a.jones@brookes.ac.uk

*corresponding author

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Abstract

Insect nicotinic acetylcholine receptors (nAChRs) are molecular targets of highly effective insecticides such as neonicotinoids. Functional expression of these receptors provides useful insights into their functional and pharmacological properties. Here, we report that the α5 nAChR subunit of the honey bee, *Apis mellifera*, functionally expresses in *Xenopus laevis* oocytes, which is the first time a homomeric insect nAChR has been robustly expressed in a heterologous system without the need for chaperone proteins. Using two-electrode voltage-clamp electrophysiology we show that the α5 receptor has low sensitivity to acetylcholine with an EC_{50} of 2.37 mM. However, serotonin acts as an agonist with a considerably lower EC_{50} at 119 μM that is also more efficacious than acetylcholine in activating the receptor. Molecular modelling indicates that residues in the complementary binding site may be involved in the selectivity towards serotonin. This is the first report of a ligand-gated ion channel activated by serotonin from an insect and phylogenetic analysis shows that the α5 subunit of *A. mellifera* and other non-Dipteran insects, including pest species, belong to a distinct subgroup of subunits, which may represent targets for the development of novel classes of insecticides.

Keywords

Apis mellifera, functional expression, insecticide, molecular model, nicotinic acetylcholine receptor, serotonin

Abbreviations

cysLGIC – cys-loop ligand-gated ion channel; 5-HT – 5-hydroxytryptamine or serotonin; nAChR – nicotinic acetylcholine receptor; OR2 – oocyte dissecting solution; SOS – standard oocyte saline solution

1. Introduction

We are delighted to be contributing a paper as part of a special issue honoring David Sattelle. His work has been instrumental in enhancing our understanding of the interactions of insecticides with their molecular targets and has paved the way towards this study, which has potentially identified a novel subgroup of insect ligand-gated ion channel subunits.

Cys-loop ligand-gated ion channels (cysLGICs) are a superfamily of receptors most widely known for mediating signalling in the nervous system (Connolly and Wafford, 2004). In vertebrates, these receptors include nicotinic acetylcholine receptors (nAChRs, which are gated by acetylcholine), GABA receptors (gated by γ-aminobutyric acid), glycine receptors and 5-HT_3 receptors (gated by serotonin). In insects, cysLGICs are known to be gated by acetylcholine, GABA, glutamate, histamine and zinc (Jones, 2018; Redhai et al., 2020). Insect cysLGICs are of interest as they are targets of highly effective insecticides (Jones, 2018). For example, neonicotinoids and spinosyns act on nAChRs (Ihara et al., 2017; Jones, 2018). As is typical for cysLGICs, nAChRs consist of five subunits arranged around a central ion channel (Zoli et al., 2018). Each nAChR subunit is encoded for by a separate gene and possesses an N-terminal extracellular domain with six distinct regions (loops A-F) forming the ligand-binding site (Corringer et al., 2000). Loops A-C from one subunit forms the principal side of the ligand-binding domain whilst loops D-F from an adjacent subunit forms the complementary side. A subunit is denoted as an α type if two adjacent cysteine residues are present in loop C, which are important for acetylcholine binding (Corringer et al., 2000; Kao and Karlin, 1986), whilst subunits lacking these two cysteines are referred to as β. For the nAChR to be functional, at least two of the subunits must be α. nAChRs can be homomeric, consisting of just one α subunit type, whilst heteromeric receptors are made up of at least two different subunits.

Genome sequencing projects have allowed for the characterization of complete nAChR gene families. In general, insect nAChR families are fairly compact when compared with those of the nematode *Caenorhabditis elegans* and vertebrates, commonly consisting of 10-12 genes (Jones, 2018; Jones and Sattelle, 2010), although larger nAChR families have been identified in the genomes of the parasitoid wasp, *Nasonia vitripennis*, with 16 subunits (Jones et al., 2010), and in the cockroaches *Blattella germanica* and *Periplaneta americana* where there are 17 and 19 subunits, respectively (Jones et al., 2021). Insect nAChR gene families can be divided into core groups of subunits that are highly conserved between species (Jones and Sattelle, 2010). Thus, insects possess α1, α2, α3, α4, α5, α6, α7, α8 (or β2 in some Dipteran species) and β1 subunits whilst the remaining subunits, which do not have clear orthologous relationships between different insects, are denoted as divergent. The α5, α6 and α7 subunits have been placed in a single core group due to their notable sequence homology with vertebrate α7 nAChR subunits (Grauso et al., 2002; Jones et al., 2010; Jones and Sattelle, 2010; Thany et al., 2005). The presence of vertebrate α7-like subunits in other organisms such as trematodes (Bentley et al., 2004), nematodes (Mongan et al., 2002) and molluscs (van Nierop et al., 2006) indicates an ancient lineage for this receptor subtype. As with vertebrate α7 nAChR subunits (Couturier et al., 1990), *Drosophila melanogaster* α5 or α7 subunits can form homomeric receptors in *Xenopus laevis* oocytes, although the presence of the chaperone

protein, RIC-3, is required for functional expression (Lansdell et al., 2012). More recently, the α7 nAChR subunit of another insect, the cockroach *Periplaneta americana*, was shown to express as a homomeric receptor in *X. laevis* oocytes, although very high concentrations of acetylcholine or nicotine were required to elicit a response, leading to the suggestion that co-expression with chaperone proteins may be required for more efficient expression (Cartereau et al., 2020). With the characterization of more insect nAChR gene families, it is becoming apparent that the α5 subunit of Diptera (such as *Aedes aegypti*, *Anopheles gambiae*, *D. melanogaster* and *Musca domestica*) and those of non-Dipteran insects including Blattodea (*B. germanica* and *P. americana*) Coleoptera (*Triboilum castaneum*), Hymenoptera (*Apis mellifera, Bombus terrestris and N. vitripennis*) and Lepidoptera (*Bombyx mori* and *Cydia pomonella*) may not be orthologous (Jones, 2018; Jones et al., 2010; Jones et al., 2021; Jones and Sattelle, 2010; Martin and Garczynski, 2016; Shao et al., 2007). For example, the α5 subunit of *An. gambiae* shares 88% amino acid sequence identity with *D. melanogaster* α5 and 49% identity with the chicken (*Gallus gallus*) α7 subunit (Jones et al., 2005). In contrast, *A. mellifera* α5 shares only 34% identity with *D. melanogaster* α5 and 37% identity with *G. gallus* α7 (Jones et al., 2006). Phylogenetic analysis shows that Dipteran α5 subunits cluster with α6 and α7 subunits of diverse insect species whereas this is not the case for non-Dipteran α5, which do not appear to be as closely associated with any other subunits (Jones et al., 2010; Jones et al., 2021; Jones et al., 2006; Jones and Sattelle, 2007; Martin and Garczynski, 2016; Shao et al., 2007). The non-Dipteran α5 subunit, therefore, may be a new nAChR subgroup that has yet to be functionally characterized. We report here that α5 of *A. mellifera*, as a representative non-Dipteran species, expresses robustly as a homomeric receptor in *X. laevis* oocytes with unusual pharmacological characteristics for a nAChR subunit.

2. Materials and methods

2.1 Reagents

Calcium chloride and sodium chloride were purchased from VWR Amresco Life Sciences (Lutterworth, UK), HEPES from Melford (Ipswich, UK), magnesium chloride from Merck (Gillingham, UK) and potassium chloride from VWR Prolabo Chemicals (Lutterworth, UK). Acetylcholine chloride, choline chloride, collagenase type I from *Clostridium histolyticum*, spinosad, imidacloprid and thiacloprid were purchased from Sigma-Aldrich (Gillingham, UK), atropine from Scientific Laboratory Supplies (Nottingham, UK) whilst serotonin (5-hydroxytryptamine) hydrochloride was obtained from Alfa Aesar (Heysham, UK).

2.2 Cloning of *A. mellifera* nAChR subunits

Total RNA was extracted from whole adult *A. mellifera* using the RNeasy Mini Kit (Qiagen, UK), as previously described (Jones et al., 2006). First strand cDNA was synthesized from the total RNA using the GoScript™ Reverse Transcription System (Promega, Southampton, UK). Nested reverse-transcription PCR reactions were performed using PCRBIO HiFi Polymerase (PCRBiosystems, London, UK) or Q5 High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK) to amplify complete coding regions of *A. mellifera* α1, α2, α3, α5, α6, α7, α8, α9 and β1 nAChR subunits (Jones et al., 2006) (for the complete α5 coding sequence Accession number AJE70263 was used). See Supplemental material Table 1 for PCR primer sequences. Amplified coding regions were then cloned into the pCI vector (Promega) using restriction sites incorporated into the PCR primers (see Supplemental material Table 1). Sequences of clones were verified at SourceBioscience (https://www.sourcebioscience.com/home).

2.3 Expression of *A. mellifera* nAChR subunits in *Xenopus laevis* oocytes and two-electrode voltage-clamp electrophysiology

Functional studies of the *A. mellifera* α5 nAChR subunit along with other honey bee nAChR subunits were performed using the *X. laevis* oocyte expression system and two-electrode voltage-clamp electrophysiology. Oocytes were either obtained ready to inject from Ecocyte Europe (https://ecocyte-us.com/products/xenopus-oocyte-delivery-service/) or *X. laevis* frogs were purchased from Xenopus 1, Dexter, Michigan, USA. Frogs were handled strictly adhering to the guidelines of the Scientific Procedure Act, 1986, of the United Kingdom, from which oocytes were either prepared for injection by incubation with collagenase type I at 2 mg/ml in oocyte dissecting solution (OR2) (sodium chloride 82 mM, potassium chloride 2 mM, magnesium chloride 2 mM, HEPES 5 mM, pH 7.6) for 45 minutes at room temperature with shaking at 150 RPM or by manual defolliculation followed by incubation with 0.5 mg/ml collagenase type I in OR2 for 6 minutes at room temperature at 150 RPM. The oocytes were then injected with 23 nl of pCI with the cloned cDNA. The α5 subunit alone was injected at a concentration of 498 ng/nl whilst multiple subunits were injected in equal ratios at final concentrations ranging from 90-610 ng/nl. Oocytes were stored in Ca^{2+} OR2 (sodium chloride 82 mM, potassium chloride 2 mM, calcium chloride 2 mM, HEPES 5 mM, pH 7.6), supplemented with 1X antibiotic antimycotic solution (Sigma, Gillingham, UK) and neomycin 0.05 mg/ml (Sigma, Gillingham, UK).

Oocytes were tested for responses 2-5 days after injection, using two-electrode voltage clamp, with borosilicate glass microelectodes filled with 3 mM potassium chloride (resistance 0.5-1 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments, CT, USA). Oocytes were clamped

at -80 mV and responses were recorded on a flatbed chart recorder (Kipp & Zonen BD-11E, Delft, The Netherlands). The oocytes were continuously perfused with standard oocyte saline solution (SOS; 100 mM sodium chloride, 2 mM potassium chloride, 1.8 mM calcium chloride, 1 mM magnesium chloride, 5 mM HEPES, pH 7.6) at a flow rate of 10 ml/min. Oocytes were selected for experiments if responses were consistent for two or more applications of 5 mM acetylcholine. Agonist EC_{50} concentration was determined using agonist concentration response curves, which were generated by challenging oocytes to different concentrations of agonist in SOS, with 3 min between challenges. Curves were calculated by normalizing the current response to maximal responses induced by the agonist. Spinosad (10 mM), imidacloprid (100 mM) and thiacloprid (100 mM) were initially dissolved in DMSO (BDH Laboratory supplies, Poole, UK) then at working concentrations in a 1:1000 ratio in SOS. The working DMSO concentration did not affect electrophysiological readings. Test chemicals in SOS were applied at 3 minute intervals.

2.4 Data analysis

Concentration-response data were fitted with the Hill equation: y = Imax/[1/(EC_{50}/[agonist])^nH], where y is the normalized current amplitude, Imax is the maximal response ($I_{max}/I_{agonistMax}$), EC_{50} is the agonist concentration at half-maximal efficacy, [agonist] is the agonist concentration, and nH is the Hill coefficient. Curve fitting was carried out using GraphPad software version 8 (GraphPad Software, La Jolla, CA United States) and nH was constrained to 1, unless specified. An F-test determined whether the data were best fitted to one-site or biphasic model; the simpler one-component model was preferred unless the extra sum-of-squares F test had a value of *p* less than 0.05. The peak of the current responses was normalized to the maximal agonist concentration: 1 mM serotonin, 5 mM acetylcholine or 12 mM choline, as appropriate. Error bars on graphs show standard error, 'n' indicates the number of experiments and 'N' indicates the number of frogs these oocytes came from. EC_{50} values are shown as mean ± 95% confidence limits whilst other values are shown as mean ± standard error.

2.5 Homology modelling and molecular docking

The amino acid sequence of the *A. mellifera* α5 nAChR subunit (Accession number A0A0H3V1C5) was acquired from the UniProt database (UniProt Consortium, 2021). The RCSB PDB database was searched for homologous proteins using the BLASTp tool (Altschul et al., 1990). The human α7 nAChR structure (7EKP) (Zhao et al., 2021) was selected as a template based on the best E value calculated by BLAST. Then, the template and target mature peptide sequences were aligned using MUSCLE server (Edgar, 2004) (Supplementary material Fig. 2) and the homology model of the full receptor was generated by Modeller (Sali and Blundell, 1993). Next, the model was embedded in a

lipid bilayer made of POPC:POPA:CHOL in a 3:1:1 ratio (Sali and Blundell, 1993) using CHARMM-GUI (Jo et al., 2008) and energy minimized for 5000 steps using GROMACS (Abraham et al., 2015) with the Amber ff19SB and lipid17 force fields for protein and lipids respectively.

For the molecular docking, acetylcholine and serotonin structures were retrieved from the PubChem database (PubChem CIDs 187 and 5202 respectively). The proteins and ligands were prepared for docking with the AutoDockTools suite (Morris et al., 2009) then both ligands were docked into the *A. mellifera* α5 or human α7 nAChR binding sites with AutoDock Vina (Trott and Olson, 2010). Protein structure figures were generated using Pymol (Lilkova et al., 2015).

2.6 Sequence analysis

Peptide sequences were used to construct the phylogenetic tree with MEGAX software (Kumar et al., 2018) using the Maximum Likelihood method and Jones-Taylor-Thornton matrix model (Jones et al., 1992). The tree with the highest log likelihood after 1000 bootstrap replications is shown.

3. Results

3.1 Sensitivity of *A. mellifera* α5 to acetylcholine

X. laevis oocytes were injected with the pCI plasmid encoding the *A. mellifera* α5 nAChR subunit. Two-electrode voltage-clamp electrophysiology showed that oocytes injected with this construct responded to acetylcholine in a concentration-dependent manner (Fig. 1A). Expression was robust and consistent, with responses observed in 100% of batches of oocytes injected and maximal currents up to about 2.30 μA.

In order to ensure that the response to acetylcholine was not caused by muscarinic acetylcholine receptors endogenous to the oocytes, atropine, an inhibitor of muscarinic acetylcholine receptors, was applied at 1 μM concentration. This did not ablate the response (see Supplementary material Fig. 1), demonstrating that the currents observed were not muscarinic and are likely due to the injected α5 subunit, which was supported by the absence of currents in oocytes injected with water alone (Fig. 1A).

An acetylcholine concentration response curve was generated (Fig. 1B) from which an EC_{50} of 2.366 ± 0.112 mM was determined (Table 1), showing an unusually low sensitivity to acetylcholine for a nAChR. We co-expressed the α5 subunit with other *A. mellifera* nAChR subunits in *X. laevis* oocytes to see if this affects sensitivity to acetylcholine (Table 1). As shown by EC_{50} values recorded in Table 1 and an example concentration response curve in Fig. 1D, the addition of several other

nAChR subunits in various combinations did not alter sensitivity to acetylcholine from that observed for α5 alone. This indicates that various nAChR subunits were not assembling with α5 under our expression conditions to form a receptor that is responsive to physiological concentrations of acetylcholine.

3.2 Actions of nicotine, neonicotinoids and spinosad on *A. mellifera* α5

We tested the actions of insecticides that act as agonists on nAChRs (Ihara et al., 2017). Spinosad did not elicit a response from *X. laevis* oocytes expressing the *A. mellifera* α5 subunit (Fig. 2A). Spinosad also did not act as an antagonist or modulator when 1-10 μM was applied with 1-5 mM acetylcholine (Fig. 2B).

Nicotine acted as an agonist on *A. mellifera* α5 expressed in *X. laevis* oocytes eliciting a response that is concentration dependent (Fig. 3A). However, responses were only observed at high concentrations of nicotine in the millimolar range. As for neonicotinoids, 100 μM imidacloprid and thiacloprid did not act as agonists on *A. mellifera* α5 (Fig. 3B and 3C respectively) despite 10 μM or lower concentrations being able to elicit a response from other honey bee nAChR subunits (Ihara et al., 2020). They did, however, act as antagonists when 100 μM was coapplied with 2 mM acetylcholine (Fig. 3B and 3C) with imidacloprid reducing the response to 57.4 ± 2.4% (n=3 oocytes, N=3 frogs) and thiacloprid to 58.6 ± 2.6% (n=3, N=3) of the response when compared to 2 mM acetylcholine alone. Using imidacloprid, it was shown that this inhibition was dose dependent (Fig. 3B) with 10 μM coapplication reducing the response to 76.3 ± 2.3% (n=3, N=3) whereas 1 μM coapplication appeared to have no effect as the response was 107.3% ± 1.5% (n=3, N=3).

3.3 Sensitivity of *A. mellifera* α5 to choline

In order to determine whether a ligand other than acetylcholine may possibly be acting as a natural endogenous agonist of the honey bee α5 nAChR subunit, we measured the actions of choline on *A. mellifera* α5 expressed in *X. laevis* oocytes since it has been found to act as an agonist on nAChRs such as the vertebrate α7 (Alkondon et al., 1997) and nematode DEG-3/DES-2 (Yassin et al., 2001). Choline acted as an agonist with response size being concentration dependent (Fig. 4A). From a concentration response curve (Fig. 4B), an EC_{50} of 6.806 ± 0.345 mM (Table 1) was estimated using the maximal concentration of choline used (12 mM). *A. mellifera* α5, therefore, appears to be less sensitive to choline than to acetylcholine.

3.4 Sensitivity of *A. mellifera* α5 to serotonin

With *A. mellifera* α5 showing low sensitivity to acetylcholine and choline, we measured the actions of another neurotransmitter, serotonin, on the honey bee nAChR subunit expressed in *X. laevis* oocytes. Serotonin acted as an agonist with current amplitude being concentration dependent (Fig. 5A). An EC_{50} of 119.0 ± 18.9 µM was determined from a concentration response curve (Table 1, Fig. 5B), showing that the *A. mellifera* α5 subunit is more sensitive to serotonin than to acetylcholine or choline. The maximum current responses evoked by serotonin (at 1 mM) were greater than those evoked by acetylcholine (5 mM) (mean 238 ± 24.0% (n=18, N=8), Fig. 5C), indicating that serotonin is more efficacious than acetylcholine at activating the receptor.

3.5 Homology modelling and molecular docking

A three-dimensional model of a homomeric *A. mellifera* α5 nAChR was generated using the human α7 nAChR structure (Zhao et al., 2021) as a template. The honey bee and human nAChRs shared sequence identity of 37.87% and a query cover of 93% (Supplementary material Fig. 2). Since there is structural information missing for the intracellular domain of the α7 receptor, the equivalent region of *A. mellifera* α5 was filled with a string of 13 glycine and threonine residues to maintain continuity within the peptide chain (Supplementary material Fig. 2). The stereochemical quality of the *A. mellifera* α5 model was assessed using a Ramachandran plot (Supplementary material Fig. 3), which showed that 89.6% of residues were in the most favored regions, 10.2% of residues were in allowed regions and 0.2% of residues were on disallowed regions. This shows a reasonable stereochemical quality of the model to continue with the docking studies.

Acetylcholine and serotonin were docked onto the *A. mellifera* α5 and human α7 structures, which yielded between 16 and 20 conformations for each combination. In the case of the acetylcholine docking, the best ranked conformation for each receptor was chosen, because the second-best conformation was highly similar and the amino group was directly interacting with the aromatic box (Trp77, Tyr213 and Tyr220 (*A. mellifera* α5 numbering)) through cation-π interactions, as expected for nAChRs (Fig. 6A and D) (Zhong et al., 1998). On the other hand, two conformations from the serotonin docking were examined, since the same two binding modes were the best ranked in each receptor and the binding energies predicted by Vina were too close to differentiate. One of these conformations has the amino group interacting with the aromatic box (Fig. 6B and E) and the other one has the indole ring oriented towards the aromatic box (Fig. 6C and F).

4. Discussion

Since their identification and cloning, insect nAChR subunits have proven to be frustratingly difficult to express in heterologous systems, presenting a major barrier in their functional and pharmacological characterization (Millar and Lansdell, 2010; Sattelle et al., 2005). Recently, however, the use of several chaperone proteins (RIC-3, UNC-50 and TMX3) have resulted in two landmark publications reporting the robust expression in *X. laevis* oocytes of heteromeric receptors consisting entirely of insect or crustacean nAChR subunits, these being α1, α2, α3, α8, β1 and β2 subunits (Ihara et al., 2020; Rufener et al., 2020). Here, we report for the first time the robust functional expression of an insect nAChR without the need for chaperone proteins. We show that the *A. mellifera* α5 nAChR subunit expresses as a homomeric ion channel in *X. laevis* oocytes that is not sensitive to the actions of two classes of insecticides (spinosyns and neonicotinoids, Figs. 2 and 3). Millimolar concentrations of nicotine were required to elicit a response from *A. mellifera* α5 (Fig. 3A) showing unusually low sensitivity considering the EC_{50S} for nicotine of α1+α2+β1+β2 and α3+β1+β2 nAChRs from *Lepeophtheirus salmonis* were 3.95 μM and 9.98 μM, respectively (Rufener et al., 2020). *A. mellifera* α5 also showed low sensitivity to acetylcholine, which is highlighted by its EC_{50} of 2.366 mM (Table 1) being considerably different to that of *D. melanogaster* α5, also expressed as a homomer in *X. laevis* oocytes, which was measured at 8.8 μM (Lansdell et al., 2012). Most strikingly, serotonin (5-hydroxytryptamine) acts as an agonist on the α5 ion channel evoking greater responses than to acetylcholine (Fig. 5C). Also, *A. mellifera* α5 is more sensitive to serotonin than to acetylcholine as indicated by EC_{50} values (Table 1). Since the EC_{50} measured for acetylcholine was exceptionally high, it is unlikely that *A. mellifera* α5 will respond to acetylcholine at physiological concentrations. In contrast, the EC_{50} for serotonin was considerably lower at 119 μM. With EC_{50} values of vertebrate 5-HT_3 receptors being in the micromolar range (Corradi et al., 2015), serotonin may be an endogenous neurotransmitter acting on *A. mellifera* α5. If this is found to be the case, *A. mellifera* α5 will be the first insect serotonin ligand-gated ion channel to be reported (Tierney, 2018).

To further investigate the interactions of *A. mellifera* α5 with acetylcholine and serotonin, we generated a three-dimensional homology model of the honey bee receptor based on the human α7 nAChR crystal structure (Zhao et al., 2021). In both the *A. mellifera* α5 and the human α7 nAChRs, acetylcholine adopted a conformation with the amino group positioned in the centre of the aromatic box, interacting through cation-π interactions with the two tyrosines in loop C and the tryptophans in loops B and loop D (Fig. 6A and D). The tyrosine in loop A (Tyr115) interacts with acetylcholine bound to α7 receptor, but in *A. mellifera* α5 it is too far away to be considered a cation-π interaction, since the common cut-off distance for said interaction is 6.0 Å (Gallivan and Dougherty, 1999). The cation-π interaction with the loop A tyrosine has been reported to be

essential for receptor gating in the α7 nAChR (Puskar et al., 2011), therefore the absence of this interaction in the *A. mellifera* α5 receptor may contribute towards the decreased potency observed for acetylcholine in the electrophysiological recordings (Table 1). Another striking difference between the two receptors is that the hydrogen bond between the carbonyl oxygen of acetylcholine and glutamine 139 in human α7 is absent in the honey bee α5 receptor (Fig. 6A and D). Instead, in *A. mellifera* α5 there is a valine, which cannot form hydrogen bonds with its side chain. This absent hydrogen bond would effectively decrease the binding affinity of the ligand, which could impact the potency of the agonist.

In the case of serotonin binding, the two best conformations predicted by Vina, for both receptors, were oriented in opposite directions, one with the amino group pointed towards the aromatic box (Fig. 6B and E) and the other with the indole ring interacting with the aromatic box (Fig. 6C and F). In both conformations, notable differences between *A. mellifera* α5 and human α7 lie in the complementary side of the binding site. For instance, in loop D of the α7 nAChR there is glutamine 79, the equivalent of which is a much smaller threonine in the α5 receptor (Fig. 6B and E). Furthermore, near loop E where α7 has proline 142, a residue that is well conserved in other nAChR subunits (Amiri et al., 2008), the α5 receptor possesses serine 143, which changes the secondary structure of this region (Fig. 6C and F). Mutations in the complementary side of the binding site, including the loop D glutamine, have been shown to affect the efficacy of neonicotinoid insecticides (Amiri et al., 2008; Matsuda et al., 2000; Shimomura et al., 2002; Shimomura et al., 2003). It has been suggested that a positively charged residue (lysine or arginine) in loop D of several insect nAChR subunits (such as α1, α2 and β1) at a position equivalent to human α7 glutamine 79 may increase affinity for neonicotinoids via electronic attraction (Shimomura et al., 2002). In *A. mellifera* α5, absence of a positive charge at this positon with a threonine residue may contribute to the lack of agonist actions seen for thiacloprid and imidacloprid (Fig. 3B and C). In accord with this, an arginine to threonine mutation (R81T) at this position in the aphid β1 nAChR subunit has been found to be associated with resistance to neonicotinoids (Bass et al., 2011). Taken together, these findings highlight the importance of the complementary side in agonist recognition and indicates a potential focal point for future exploration into the unique pharmacology of the *A. mellifera* α5 nAChR.

Previous analysis of insect nAChR protein sequences have indicated that the α5 sequences of Dipteran insects are not orthologous to α5 subunits of non-Dipteran species (Jones, 2018; Jones et al., 2010; Jones et al., 2021; Martin and Garczynski, 2016; Shao et al., 2007). This is highlighted here by a phylogenetic tree showing that *D. melanogaster* and *An. gambiae* α5 subunits cluster tightly with α7 subunits of a wide variety of insects and are also closely associated with α6 subunits

and vertebrate α7 and α8 nAChRs (Fig. 7). In contrast, the α5 subunit of non-Dipteran species (*A. mellifera*, *B. mori*, *B. terrestris*, *P. americana* and *T. castaneum*) are located on a separate branch indicating that they belong to a distinct receptor subgroup. It is therefore interesting to speculate that the α5 subunit in Diptera and non-Diptera arose from two different events, where gene duplication of the α7 subunit gave rise to α5 in Diptera and a gene duplication event preceding the evolution of α6 and α7 subunits gave rise to α5 in non-Dipteran insects. The appearance of the α5 subunit also occurred relatively late compared to the other core subunits as it is absent in the aphid, *Acyrthosiphon pisum*, a more evolutionary ancient insect species (Fig. 7) (Dale et al., 2010). Sequence-wise, non-Dipteran α5 more closely resembles some other nAChR subunits than it does vertebrate 5-HT_3 receptors. For example, *A. mellifera* α5 shares 35% identity with *D. melanogaster* α6 whilst sharing 28% identity with the rat 5-HT_{3A} subunit but has lower identity to other insect nAChR subunits such as *D. melanogaster* α4 with 26%. Interestingly, *A. mellifera* α5 shares notable identity to vertebrate α9 and α10 subunits, for example 34% and 35% to rat α9 and α10, respectively. Vertebrate heteromeric receptors containing α9 and α10 subunits show atypical characteristics for nAChRs including shared pharmacological properties with serotonin receptors where acetylcholine-evoked currents are blocked by 5-HT_3 receptor antagonists although serotonin itself showed no agonist actions (Rothlin et al., 2003). Evolutionary analysis of vertebrate α9 and α10 sequences has led to the suggestion that these subunits form a unique subgroup of nAChRs evolved to perform specialized roles in hair cells (Marcovich et al., 2020). In line with this, the vertebrate α9 and α10 subunits cluster separately in our phylogenetic tree (Fig. 7) between 5-HT_3 receptors and neuronal nAChRs perhaps reflecting, along with *A. mellifera* α5, early evolutionary steps determining the fate of whether cys-loop ligand-gated ion channels became nAChRs or serotonin receptors.

In situ hybridization showed that *A. mellifera* α5 nAChR (referred to as Apisα7-2) subunit transcripts were expressed in the dorsal lobes, the inner chiasma of optic lobes, the outer compact Kenyon cells and in the antennal lobes in pupae (stage P6) and adult brains, and there was expression in more layers of the optic lobes (lamina and medulla) in adults only (Thany et al., 2005). Interestingly, antibody staining revealed serotonin-immunoreactive fibers in the optic lobes of the adult honey bee restricted to the lamina and medulla (Schurmann and Klemm, 1984) and serotonin immunostaining was first seen in the lamina and medulla at pupal stages 4 and 7, respectively (Seidel and Bicker, 1996). Serotonin staining has also been observed in other neurons of *A. mellifera* including those of the dorsal and antennal lobes (Schurmann and Klemm, 1984; Seidel and Bicker, 1996). Serotonin mediates many physiological and behavioral processes in insects including sensory response, olfactory learning, feeding, place memory, circadian rhythms and sleep, which have been

attributed to the activity of metabotropic G-protein-coupled 5-HT receptors (Blenau and Thamm, 2011; French et al., 2014). It will be of interest to see if *A. mellifera* α5 mediates some of the actions of serotonin considering they have overlapping expression patterns and whether these actions are more particular to honey bees, such as modulating sociability in the hive (Hewlett et al., 2018) or fulfilling certain roles where, for instance, higher serotonin levels were found in the antennal lobes of forager compared with nurse bees (Schulz and Robinson, 1999).

It remains to be determined whether the *A. mellifera* α5 subunit functions as a homomeric receptor *in vivo*. Attempts to co-express *A. mellifera* α5 with the rat β2 nAChR subunit in *X. laevis* oocytes did not result in any detectable responses (Chen et al., 2019). However, this may be due to the highest concentration of acetylcholine applied being 500 μM, which we have found to evoke only a marginal response (Fig. 1A). *In situ* hybridization has shown that *A. mellifera* α5 co-localises with α2, α3 and α7 (referred to as Apisα7-1) nAChR subunits indicting that they may co-assemble to give rise to nAChRs with various combinations (Thany et al., 2005; Thany et al., 2003). Our co-expression studies, which include the α3 and α7 subunits, did not provide any evidence that α5 co-assembles with other subunits to form a different nAChR subtype as shown by unaltered acetylcholine EC_{50} values (Table 1). However, it may be that different combinations of subunits are required or that the use of chaperone proteins such as RIC-3, UNC-50 and TMX3 (Ihara et al., 2020; Lansdell et al., 2012; Rufener et al., 2020) is needed to ensure that subunits other than α5 are assembled into functional proteins. It may be that other nAChR subunits assemble with α5 to modulate its activity as a serotonin receptor as it was observed that the vertebrate α4 nAChR subunit altered the functional properties of the $5\text{-}HT_{3A}$ receptor expressed in *X. laevis* oocytes (van Hooft et al., 1998).

In conclusion, we have identified for the first time an insect ligand-gated ion channel that is activated by serotonin. It remains to be tested whether the expressed *A. mellifera* α5 responds to other biogenic amines such as dopamine or octopamine. Our phylogenetic tree shows that the α5 nAChR subunit from insects of orders other than Hymenoptera, these being Blattodea (*P. americana*), Coleoptera (*T. castaneum*) and Lepidoptera (*B. mori*), cluster with *A. mellifera* α5 (Fig. 7). It will be of importance to determine whether these α5 nAChR subunits are also sensitive to serotonin, highlighting this group of subunits as a novel subtype of receptors, perhaps calling for reclassification as ligand-gated 5-HT receptors. In this regard, it is interesting to note that unusual features observed in the complementary side when comparing homology models of human α7 and *A. mellifera* α5 are present in *P. americana*, *T. castaneum* and *B. mori* (Jones et al., 2021; Jones and Sattelle, 2007; Shao et al., 2007). For instance, the α5 subunit of these three species possess a serine

instead of P142 in human α7. The potential presence of these serotonin-gated α5 subunits in pest species such as cockroaches, beetles and moths raises the prospect that they may be targets for novel classes of insecticides with modes of action that differ to those of existing compounds. As with *A. mellifera* α5, if the α5 subunit from pests robustly express in heterologous systems this can provide a useful screening tool for the discovery of novel insecticidal compounds. A counter-screen with the expressed *A. mellifera* α5 subunit may identify compounds with greater activity towards the receptor in target species than from the honey bee. The search for insecticides that spare beneficial species can be aided further still by utilizing *in silico* screening approaches using three-dimensional models.

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Figure legends

Fig. 1. Reponses to acetylcholine in *X. laevis* oocytes expressing the *A. mellifera* α5 nAChR subunit. A. Representative current traces showing responses to different concentrations of acetylcholine (0.1 – 10 mM). The first trace shows the response of an oocyte injected with water alone whilst the remaining traces show responses from oocytes injected with α5. B. Acetylcholine concentration response curve. Data are normalized to the maximal response (5 mM acetylcholine) and have a mean EC_{50} of 2.366 ± 0.112 mM from 15 oocytes from 7 different batches of eggs. C. Responses to acetylcholine in *X. laevis* oocytes injected with *A. mellifera* α5, α3 and α8 nAChR DNA. D. Acetylcholine concentration response curves in *X. laevis* oocytes expressing α5 alone and co-expressing α5+α3+α8 subunits.

Fig. 2. Reponses to spinosad in *X. laevis* oocytes expressing the *A. mellifera* α5 nAChR subunit. A. Spinosad showed no agonist actions. 10 μM spinosad was applied 3 min after 5 mM acetylcholine (ACh) and did not elicit a response B. Spinosad does not modulate the action of acetylcholine. 10 μM spinosad coapplied with 2 mM acetylcholine (EC_{50} concentration) 3 min after 2 mM acetylcholine was applied alone.

Fig. 3. Reponses to nicotine and neonicotinoids (imidacloprid and thiacloprid) in *X. laevis* oocytes expressing the *A. mellifera* α5 nAChR subunit. A. Current traces showing responses to high concentrations of nicotine. B. Imidacloprid showed no agonist actions when 100 μM was applied 3 min after 5 mM acetylcholine. Imidacloprid showed antagonistic actions when coapplied with 2 mM acetylcholine (EC_{50} concentration) in a dose dependent manner. C. Thiacloprid showed no agonist actions when 100 μM was applied 3 min after 5 mM acetylcholine. Thiacloprid showed antagonistic actions when 100 μM was coapplied with 2 mM acetylcholine.

Fig. 4. Reponses to choline in *X. laevis* oocytes expressing the *A. mellifera* α5 nAChR subunit. A. Representative current traces showing responses to different concentrations of choline (0.3 – 12 mM). B. Choline concentration response curve. Data are normalized to the response to 12 mM choline and have a mean EC_{50} of 6.806 ± 0.345 mM from 5 oocytes from 3 different batches of eggs.

Fig. 5. Reponses to serotonin in *X. laevis* oocytes expressing the *A. mellifera* α5 nAChR subunit. A. Representative current traces showing responses to different concentrations of serotonin (1– 1000 μM). B. Serotonin concentration response curve. Data are normalized to the response to 1 mM serotonin and have a mean EC_{50} 119.0 ± 18.9 μM from 7 oocytes from 5 different batches of eggs. C. Sample traces comparing the response to maximal concentrations of acetylcholine (5 mM), serotonin (1 mM) or 1 mM acetylcholine.

Fig. 6. Homology modelling of the *A. mellifera* α5 nAChR based on the human α7 crystal structure (Zhao et al., 2021). Binding conformations generated by Autodock Vina with the interactions between acetylcholine (yellow) and serotonin (magenta) and the main binding site amino acids (red = hydrogen bond, orange = cation-π, and pale cyan = π-π) for *A. mellifera* α5 (A, B, and C) and human α7 (D, E, and F).

Fig. 7. Tree showing relationships of insect and vertebrate α7-10 nAChR subunit protein sequences as well as vertebrate 5-HT_3 subunits. Numbers at each node signify bootstrapping 1000 times represented as a percentage of trees in which the associated taxa clustered together and the scale bar represents substitutions per site. ELIC (Accession number P0C7B7), from *Dickeya chrysanthemi*, a bacterial ancestor of cysLGICs, was used as an outgroup. Species subunit sequences used in the tree are as follows: Agam (*Anopheles gambiae*) see (Jones et al., 2005); Amel (*Apis mellifera*) α5 (AJE70263) otherwise see (Jones et al., 2006); Apis (*Acyrthosiphon pisum*) see (Dale et al., 2010); Bmor (*Bombyx mori*) see (Shao et al., 2007); Bter (*Bombus terrestris*) see (Sadd et al., 2015); Dmel (*Drosophila melanogaster*) ; Pame (*Periplaneta americana*) see (Jones et al., 2021); Tcas (*Tribolium castaneum*) see (Jones and Sattelle, 2007); Ggal (*Gallus gallus*) α7 (NP989512), α8 (CAA36544), α9 (NP990091), α10 (NP001094506), 5-HT_{3A} (XP040508006); Hsap (*Homo sapiens*) α7 (CAA69697), α9 (NP060051), α10 (CAC20435), 5-HT_{3A} (NP998786), 5-HT_{3B} (NP006019.), 5-HT_{3C} (AF459285), 5-HT_{3D} (AY159812), 5-HT_{3E} (AY159813); Mmus (*Mus musculus*) α7 (AAF35885), α9 (NP001074573), α10 (NP001074893), 5-HT_{3A} (6Y59_A), 5-HT_{3B} (NP064670).

690 **Table 1.** Effects of agonists on membrane currents from *X. laevis* oocytes expressing *A. mellifera* α5
691 and other honey bee nAChR subunits. EC_{50} values are displayed as the mean ± 95% confidence limits.

Receptor	EC_{50} (μM)	n=oocytes, N=frogs
α5	acetylcholine: 2366 ± 112 choline: 6806 ± 345 serotonin: 119.0 ± 18.9	n=15, N=7 n=5, N=3 n=7, N=5
α5 + α3 + α8	acetylcholine: 2410 ± 262	n=3, N=2
α5 + α1	acetylcholine: 2570 ± 142	n=6 N=4
α5 + α9	acetylcholine: 2134 ± 244	n=3, N=2
α5 + β1	acetylcholine: 2117 ± 257	n=3, N=2
α5 + α6 + α7	acetylcholine: 2022 ± 257	n=3, N=2

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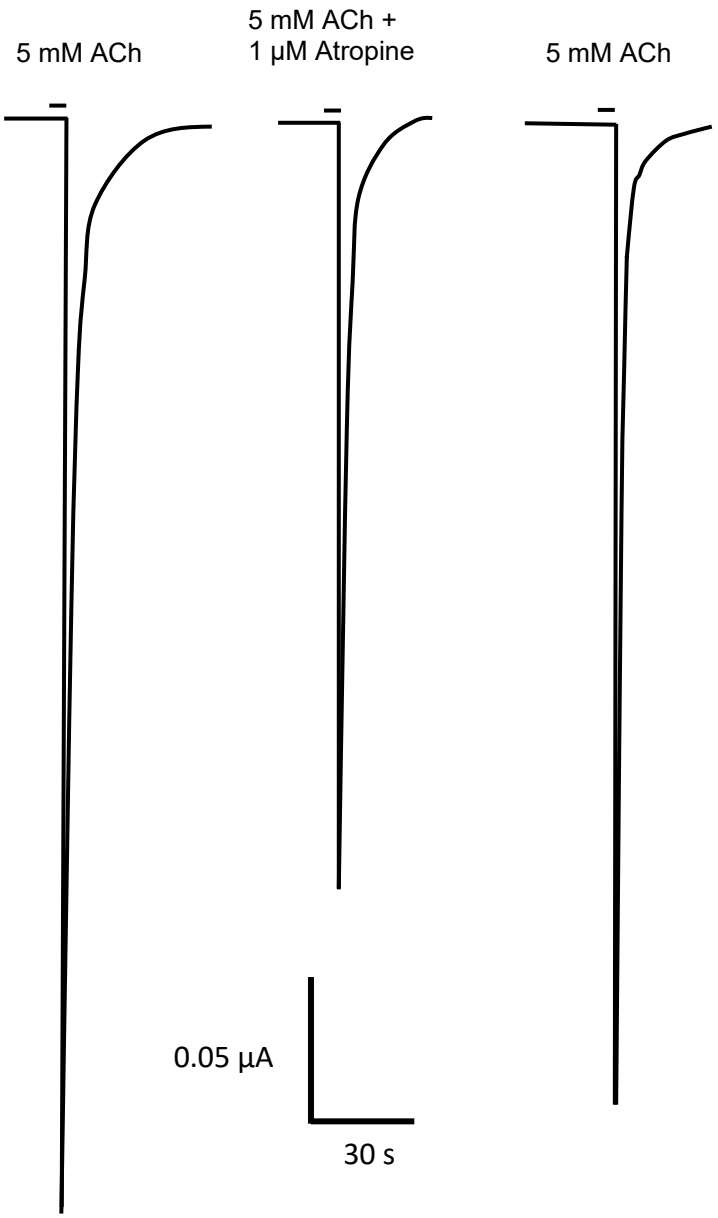
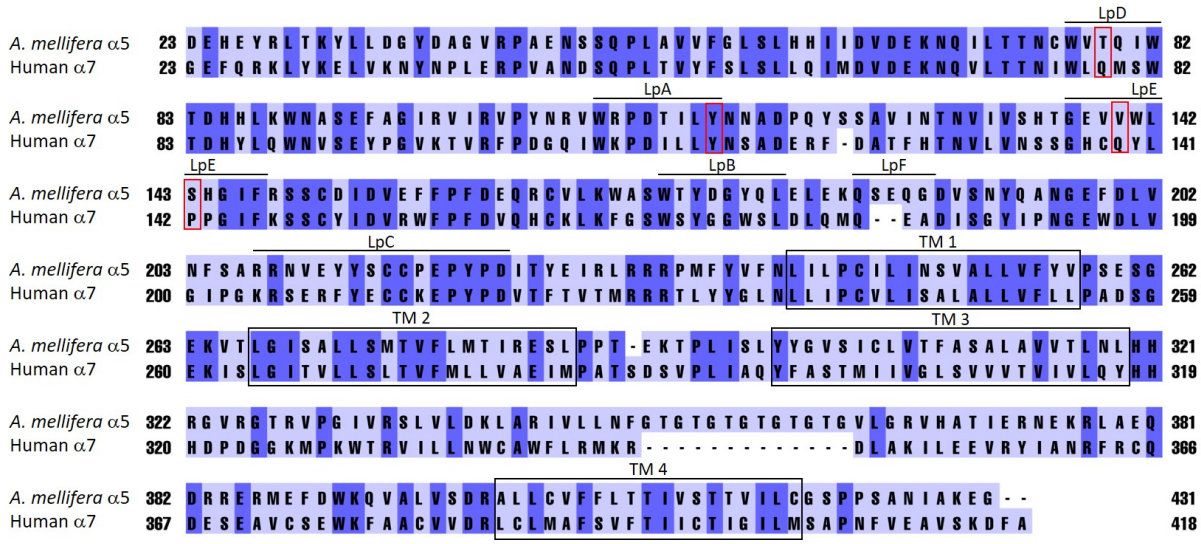
Supplementary material Table 1. Sequences for primers used in nested reverse transcription PCR to amplify *A. mellifera* (Amel) nAChR subunits. Primers 1 forward (for) and reverse (rev) were used in the first PCR reactions before primers 2 forward and reverse were used for the second, nested reaction. Restriction sites used for cloning into the pCI vector are underlined.

Primer name	Primer 1 Sequences 5'-3'	Primer 2 Sequences 5'-3'
Amel α1 for	GGCGGATTGCTGGCGATG	CGATCGGAATTCATGGCGACGGCCATTTCCTG
Amel α1 rev	CGCGGGATCGTCGACTAG	CGATCGTCTAGACTAGTCCTCCTCGGGACCC
Amel α2 for	CCTACTATTCAAAATGATACTCCAG	CGATCGGAATTCATGATACTCCAGACGATCATC
Amel α2 rev	CCATTCTCTCCGACACGAG	CGATCGTCTAGAATTTCTACACGAGAGTTTCCAAA
Amel α3 for	GTTGGACGTGACACAATGACATG	CGATCGGAATTCATGATGAAGAGCCTGGTGG
Amel α3 rev	ATGCGTTGTACGTGAAGAATAACG	CGATCGTCTAGATCAGAGGCTCGTAACGATG
Amel α5 for	TCTCGCGTTTAAGTGGTCCATCAA	ATCGCTCGAGATGTCGCCTTTGGTCCTGTTC
Amel α5 rev	CCGTATTTCTACCATCGTCCATT	CGATCGTCTAGATTAACCCTCTTTGGCAATGTTCG
Amel α6 for	CAAACGGCGCAGAAGCGA	CGATCGGAATTCATGCTTAGCGCAAGTAGTGTATTAC
Amel α6 rev	GGTGACTTGACCGGGGCA	CGATCGTCTAGATTGGACGATTATGTGTGGCG
Amel α7 for	CGATTCCTCTCTCGTGCAAAC	CGATCGGAATTCATGAGACGTTGGACTCTCATG
Amel α7 rev	GACCACCGACTCGGTCGA	CGATCGTCTAGATCACGTGACGATGATGTGTGG
Amel α8 for	TGGTCATTGCCATCTCAACC	CGATCGGAATTCATGTTTAAAATGCAAATATTGAC
Amel α8 rev	TATAGTCTTTTTACGCGTAT	CGATCGTCTAGATTATCCTTCTGGAGAAATGTCTATAT
Amel α9 for	GTGGCTGTTGGCTCATTTCA	CGATCGTCTAGAATGAAAATGAGAATAATAACAGCT
Amel α9 rev	GATTACTGATGAAGAACGTAGG	TCTGCGGCCGCTCACGTGGATGGTACAAGAG
Amel β1 for	CACCGCCTGAAACCTGTCCA	CGATCGTCTAGAATGCATAATATTTGCTCGAG
Amel β1 rev	ATTCCCTTTCAATTTCCTAGATT	TCTGCGGCCGCTTATTTTCCACGGTAGATCT



Supplementary material Fig. 1. Traces showing *Apis mellifera* α5 nAChR expressed in *Xenopus oocytes* in response to 5 mM acetylcholine (ACh), then with 1 µM atropine following pre-incubation with 1 µM atropine for 5 min followed by ACh only again after a wash with SOS solution for 5 min.



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708 **Supplementary material Fig. 2.** Alignment of the *A. mellifera* α5 and human α7 nAChR subunit
709 sequences used in homology modelling with the conserved amino acids highlighted in dark blue. The
710 loops involved in ligand binding (LpA-F) as well as the four transmembrane domains (TM1-TM4) are
711 indicated. Particular residues highlighted by three-dimensional modelling that were discussed in the
712 text are shown in red boxes.

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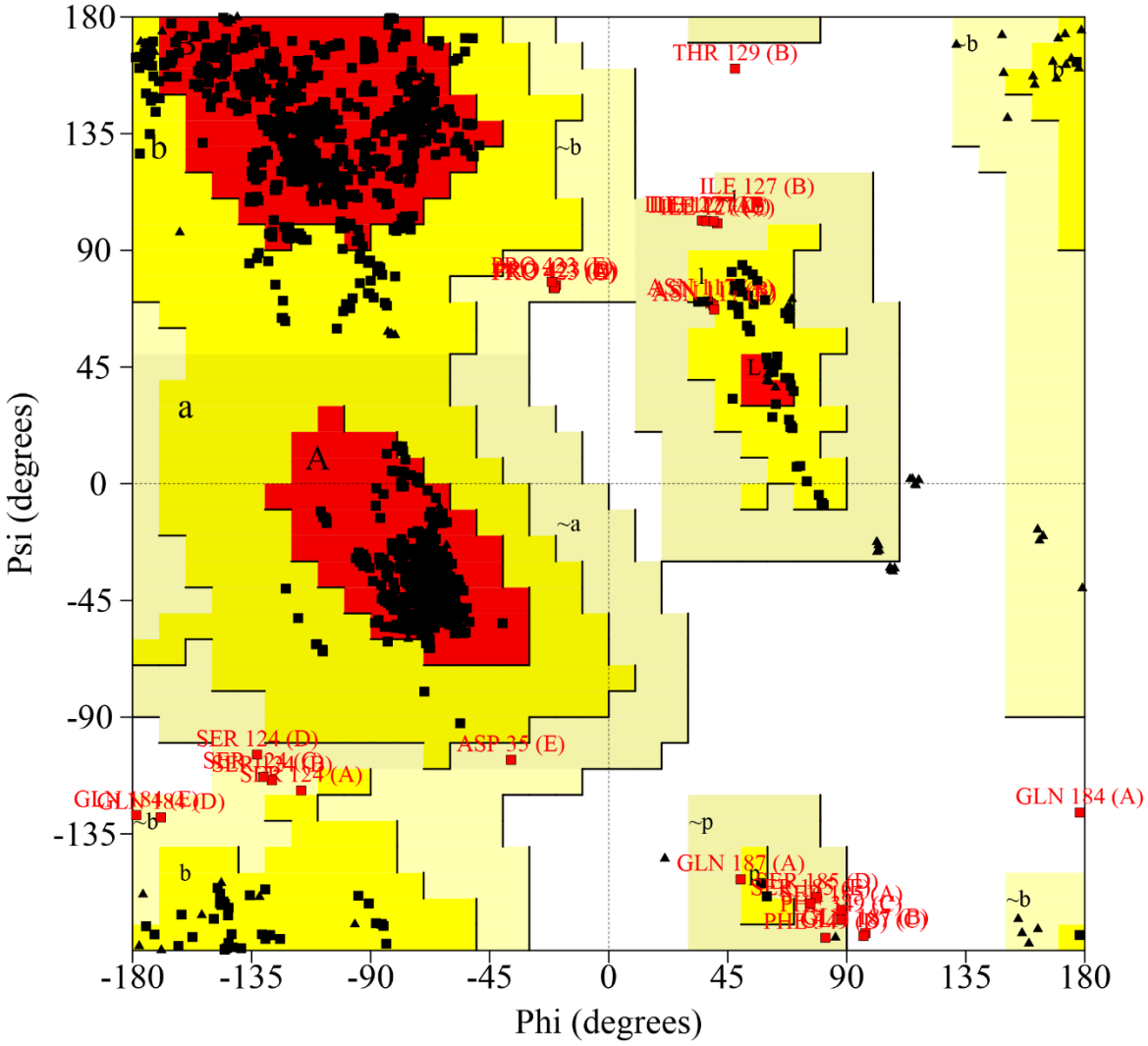

Plot statistics

Residues in most favoured regions [A,B,L]	1635	89.6%
Residues in additional allowed regions [a,b,l,p]	165	9.0%
Residues in generously allowed regions [~a,~b,~l,~p]	21	1.2%
Residues in disallowed regions	4	0.2%
Number of non-glycine and non-proline residues	1825	100.0%
Number of end-residues (excl. Gly and Pro)	5	
Number of glycine residues (shown as triangles)	125	
Number of proline residues	90	
Total number of residues	2045	

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716 **Supplementary material Fig. 3.** Ramachandran plot of the *A. mellifera* α5 nAChR homology model.