British Journal of Pharmacology

British Journal of Pharmacology (2018) **175** 1999–2012 1999

Themed Section: Nicotinic Acetylcholine Receptors

RESEARCH PAPER

Loops D, E and G in the *Drosophila* Dα1 subunit contribute to high neonicotinoid sensitivity of Dα1-chicken β2 nicotinic acetylcholine receptor

Correspondence Kazuhiko Matsuda, Department of Applied Biological Chemistry, Faculty of Agriculture, Kindai University, 3327-204 Nakamachi, Nara 631-8505, Japan. E-mail: kmatsuda@nara.kindai.ac.jp

Received 4 January 2017; **Revised** 2 May 2017; **Accepted** 5 June 2017

Makoto Ihara[1], Mai Hikida[1], Hiroyuki Matsushita[1], Kyosuke Yamanaka[1], Yuya Kishimoto[1], Kazuki Kubo[1], Shun Watanabe[1], Mifumi Sakamoto[1], Koutaro Matsui[1], Akihiro Yamaguchi[1], Daiki Okuhara[1], Shogo Furutani[1], David B Sattelle[2] and Kazuhiko Matsuda[1]

[1]*Department of Applied Biological Chemistry, Faculty of Agriculture, Kindai University, Nara, Japan, and* [2]*Centre for Respiratory Biology, UCL Respiratory, University College London, London, UK*

BACKGROUND AND PURPOSE

Neonicotinoid insecticides interact with the orthosteric site formed at subunit interfaces of insect nicotinic ACh (nACh) receptors. However, their interactions with the orthosteric sites at α–non α and α–α subunit interfaces remain poorly understood. The aim of this study was to elucidate the mechanism of neonicotinoid actions using the *Drosophila* Dα1-chicken β2 hybrid nACh receptor.

EXPERIMENTAL APPROACH

Computer models of the $(D\alpha1)_3(\beta2)_2$ nACh receptor in complex with imidacloprid and thiacloprid were generated. Amino acids in the Dα1 subunit were mutated to corresponding amino acids in the human α4 subunit to examine their effects on the agonist actions of neonicotinoids on $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *Xenopus laevis* oocytes using voltage-clamp electrophysiology.

KEY RESULTS

The $(D\alpha1)_3(\beta2)_2$ nACh receptor models indicated that amino acids in loops D, E and G probably determine the effects of neonicotinoids. The amino acid mutations tested had minimal effects on the EC_{50} for ACh. However, the R57S mutation in loop G, although having minimal effect on imidacloprid's actions, reduced the affinity of thiacloprid for the $(D\alpha1)_3(\beta2)_2$ nACh receptor, while scarcely affecting thiacloprid's action on the $(D\alpha1)_2(\beta2)_3$ nACh receptor. Both the K140T and the combined R57S;K140T mutations reduced neonicotinoid efficacy but only for the $(D\alpha1)_3(\beta2)_2$ nACh receptor. Combining the E78K mutation with the R57S;K140T mutations resulted in a selective reduction of thiacloprid's affinity for the $(D\alpha1)_3(\beta2)_2$ nACh receptor.

CONCLUSIONS AND IMPLICATIONS

These findings suggest that a triangle of residues from loops D, E and G contribute to the selective actions of neonicotinoids on insect-vertebrate hybrid nACh receptors.

LINKED ARTICLES

This article is part of a themed section on Nicotinic Acetylcholine Receptors. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v175.11/issuetoc

Abbreviations

I_{max}, normalized maximum response; nACh receptor, nicotinic ACh receptor

DOI:10.1111/bph.13914

Introduction

Neonicotinoid insecticides such as imidacloprid and thiacloprid (Figure 1) have been used to control crop pests and pests of farm animals (Matsuda *et al.,* 2001, 2005, 2009; Tomizawa and Casida, 2003, 2005; Jeschke *et al.,* 2013; Casida and Durkin, 2017). Neonicotinoids interact with the orthosteric site of insect **nicotinic ACh receptors** (nACh receptors) mainly as partial and full agonists, although for certain compounds, super agonist and antagonist actions have also been described (Ihara *et al.,* 2004, 2006; Brown *et al.,* 2006; Tan *et al.,* 2007; Matsuda *et al.,* 2009). Unlike **ACh**, neonicotinoids are not hydrolyzed by acetycholinesterase and hence can persistently modulate nACh receptors (Matsuda *et al.,* 2001). Neonicotinoids show selectivity for insect over vertebrate nACh receptors (Matsuda *et al.,* 2001); they are broad spectrum insecticides taken up by roots and subsequently translocated into all plant tissues (Kagabu, 1997; Jeschke *et al.,* 2013). Neonicotinoids currently make up >25% of world insecticide sales (Jeschke *et al.,* 2013).

The reduced numbers of honey bees, bumble bees and other insect pollinators are a threat to the effective pollination of crop plants. Neonicotinoids have been suggested as one possible contributor to Colony Collapse Disorder, because they modulate the nACh receptors of bees as well as pest insect species (Gill *et al.,* 2012; Whitehorn *et al.,* 2012; Rundlof *et al.,* 2015). In view of the possible risk to bees, the EU restricted the use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) in 2013 and continues to restrict their deployment subject to further assessment of the risk. Therefore, it is extremely important to understand the structural features that determine the selectivity and potency of neonicotinoids at nACh receptors.

The nACh receptors are membrane-spanning proteins containing an integral cation channel and play a crucial role in fast cholinergic neurotransmission in vertebrates and invertebrates (Changeux, 2012). Channel opening in response to ACh binding results in depolarization of nerve and muscle membranes (Miyazawa *et al.,* 2003; Taly *et al.,* 2005, 2006; Unwin, 2005; Taly, 2007; Unwin and Fujiyoshi, 2012; Nemecz *et al.,* 2016). Although some nACh receptors are homomers or heteromers of α subunits (defined by a YXCC motif in loop C of the ACh binding site), most are heteromers of α and non-α subunits (Changeux, 2012). The N-terminal extracellular six loops (typically A, B, C from the α subunit and D, E, F from the non-α subunit) form the orthosteric site of such α/non-α heteromers (Corringer *et al.,* 2000) to which ACh and neonicotinoids bind (Matsuda *et al.,* 2005). Based on computational studies, we proposed that negatively charged nitro or cyano groups of neonicotinoids (Figure 1) interact with basic residues that are selectively present in insect nACh receptors (Matsuda *et al.,* 2001, 2005, 2009). The resulting planar imidazolidine or related moieties facilitate interaction with π-electron-rich aromatic residues (Matsuda *et al.,* 2009). Mutations of Gln^{79} in chicken α7 (Shimomura *et al.,* 2002) and Thr^{77} in chicken β2 subunit (Shimomura *et al.,* 2006), both of which are located in loop D, to basic residues, markedly enhanced imidacloprid sensitivity of nACh receptors containing these subunits, suggesting a possible interaction of the nitro group of imidacloprid with the added basic residues.

To demonstrate an interaction of loop D with neonicotinoids, we co-crystallized wild-type and the Q55R

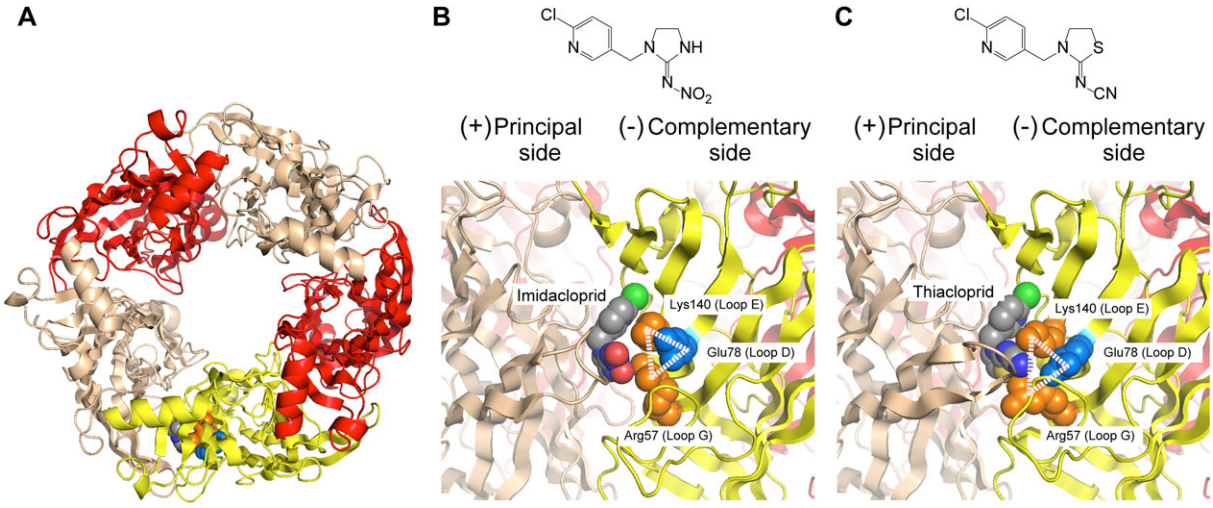

Figure 1

Homology models of the ligand binding domain of fruit fly Dα1/chicken β2 nACh receptor in complex with imidacloprid and thiacloprid. (A) Overall top view of the $(D\alpha1)_3(\beta2)_2$ nACh receptor model generated from human $(\alpha4)_2(\beta2)_3$ nACh receptor docked with imidacloprid. The Dα1 subunits at principal and complementary sides are coloured tan and yellow, respectively, whereas the β2 subunits are coloured red. (B) Chemical structure of imidacloprid and close-up view of the imidacloprid binding site. (C) Chemical structure of thiacloprid and close-up view of the thiacloprid binding site. Arg^{57} and Lys^{140} interacted electrostatically with the nitro group of imidacloprid (B) and the cyano group of thiacloprid (C). Glu^{78} made salt bridges with Arg^{57} and Lys^{140} to form a 'loop D-E-G triangle' (B, C). In each panel, the main chains of the nACh receptors are drawn as cartoon, whereas Arg^{57} (coloured orange), Lys^{140} (coloured orange), Glu^{78} (coloured blue) and the neonicotinoids are drawn as space filling models. For neonicotinoids, carbon-, nitrogen-, oxygen-, sulfur- and chlorine-atoms are coloured grey, blue, red, tan and green, respectively.

mutant of the ACh binding protein (AChBP) of *Lymnaea stagnalis*, which provides a surrogate of the nACh receptor orthosteric binding site, with neonicotinoids (imidacloprid, clothianidin, thiacloprid and the nitromethylene analogue of imidacloprid [CH-IMI]) (Ihara *et al.,* 2008, 2014a). The X-ray crystal structures revealed that basic residues in loop D interacted electrostatically with the nitro or cyano group of neonicotinoids. Also, we showed that desnitro-imidacloprid, an imidacloprid metabolite lacking the nitro group, bound to the AChBP, placing its guanidine tip in the opposite direction against loop D, supporting a role for loop D in the interactions of nACh receptors with neonicotinoids (Ihara *et al.,* 2014a).

We found that T77R;E79V mutations in loop D in the β2 subunit of the avian α4β2 nACh receptor resulted in enhanced neonicotinoid sensitivity, whereas they had no significant effect on the concentration–response curve for ACh, and hence, we predicted that inverse mutations in loop D in insects would lead to resistance (Shimomura *et al.,* 2006). In fact, an R81T mutation was later found in the β1 subunit in a neonicotinoid-resistant field population of aphid *Myzus persicae*, supporting our proposal that neonicotinoids interact strongly with loop D (Bass *et al.,* 2011).

In the structural work, we unexpectedly discovered that CH-IMI, clothianidin and thiacloprid interacted with Lys^{34} on the β1 strand (loop G) of the AChBP. Interestingly, insect nACh receptor α subunits possess basic residues in loop G, and thus, such residues could also underlie the potency and insect selectivity of neonicotinoids. Indeed, mutations of Ser^{58} in the avian α7 nACh receptor, which corresponds to Lys^{34} in the *Lymnaea* AChBP, led to enhanced agonist actions of neonicotinoids, while reducing the actions of ACh, (−)-nicotine and desnitro-imidacloprid (Ihara *et al.,* 2014a). Since loop G is located in the complementary side of α subunits, we predicted that the α–α subunit interface may also contribute to interactions with neonicotinoids (Ihara *et al.,* 2015). However, no evidence for this hypothesis has been provided using heteromers. In a relevant study, we showed that insect nACh receptor α subunits possess structural features that are favourable for interactions with neonicotinoids and identified loop C and the region upstream of loop B as potential contributors (Shimomura *et al.,* 2005). However, it is unclear as to precisely which amino acids in the region upstream of loop B underpin the selective interactions with neonicotinoids.

To address these questions, we computationally modelled a full length fruit fly (*Drosophila melanogaster*) Dα1-chicken (*Gallus gallus*) β2 nACh receptor in complex with imidacloprid, since this insect-vertebrate hybrid nACh receptor exhibits a higher neonicotinoid sensitivity than the chicken α4β2 nACh receptor (Ihara *et al.,* 2003, 2014b). The model showed that not only Arg^{57} in loop G, but also Lys^{140} in loop E at the Dα1-Dα1 subunit interface interact with the nitro group of imidacloprid and Glu^{78} supports this interaction by forming salt bridges. Thus, we mutated Arg^{57}, Lys^{140} and Glu^{78} in the *Drosophila* Dα1 subunit, as a representative of insect nACh receptor α subunits, to corresponding amino acids in the human α4 subunit, as a representative of mammalian nACh receptor α subunits, and investigated the effects of these mutations on the agonist actions of imidacloprid and thiacloprid on $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors. By this means, we sought to address whether the amino acids in the Dα1–Dα1 subunit interface contribute to the selectivity of neonicotinoids for insect nACh receptors.

Methods

Xenopus laevis oocytes

Female *Xenopus laevis* were anaesthetized with benzocaine (ethyl 4-aminobenzoate) to reduce animal suffering as much as possible according to the UK Animals (Scientific Procedures) Act, 1986, and minimum amounts of oocytes were removed from anaesthetized frogs. *Xenopus* oocytes were treated for 30 min with 2 $mg{\cdot}mL^{-1}$ Type IA collagenase (Sigma Aldrich, St. Louis, MO, USA) in Ca^{2+}-free standard oocyte saline Ca-free SOS: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES; (pH 7.6 adjusted with NaOH) and transferred to SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES; pH 7.6) (Ihara *et al.,* 2003; Shimomura *et al.,* 2006). The follicle cell layer was removed from oocytes manually with fine forceps, and defolliculated oocytes were injected with cRNAs. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.,* 2010; McGrath and Lilley, 2015).

cRNA preparation and injection into oocytes

The *Drosophila* Dα1 subunit and the chicken β2 subunit containing amino acid sequences in the Refseq database (Dα1: NP_524481; β2: NP_990144) were used as the wild-type subunits. Each cDNA was cloned into the pcDNA3.1 (+) vector (Thermo Fisher Scientific, Waltham, MA, USA). The nucleotide sequence of the Dα1 subunit sequence was mutated by PCR. The cRNAs of wild-type and mutant Dα1 subunits as well as of a wild-type chicken β2 subunit were prepared by *in vitro* transcription from each respective cDNA, cloned in the pcDNA 3.1 (+) vector using the mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific) (Furutani *et al.,* 2014). cRNAs were dissolved in RNase-free water at a concentration of 1 $mg{\cdot}mL^{-1}$. Dα1 and β2 cRNA solutions were mixed at a ratio of 5:1 or 1:5 for reconstituting $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors, respectively, in *Xenopus* oocytes. To each oocyte, 50 nL of cRNA mixture solutions were injected, and the injected oocytes were incubated for 4–5 days prior to electrophysiology in SOS supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (20 μg ml^{-1}) and 2.5 mM sodium pyruvate (pH 7.6).

Electrophysiology

Two-electrode voltage-clamp electrophysiology was conducted using a GeneClamp 500B amplifier (Molecular Devices, Sunnyvale, CA, USA) at a holding potential of −100 mV (Matsuda *et al.,* 1998; Ihara *et al.,* 2003; Shimomura *et al.,* 2006). Oocytes were perfused extracellularly at a flow rate of 7–10 $mL{\cdot}min^{-1}$ with SOS containing 0.5 μM **atropine** to suppress the muscarinic receptor-mediated responses of oocytes. Neonicotinoids were dissolved in DMSO at 100 mM and diluted with SOS for preparing test solutions. DMSO concentrations in each test solution were 0.1% or

lower at which DMSO had no apparent effect on the nACh receptor response to ACh and neonicotinoids. ACh was directly dissolved in SOS immediately before the experiments. After successive applications of 10 μM ACh to confirm reproducibility of the response, neonicotinoids were applied for 5 s, from lower to higher concentrations, at 3 min intervals. No irreversible nACh receptor desensitization was observed with treatments of 10 μM or lower concentrations of ACh and 100 μM or lower concentrations of neonicotinoids using this protocol.

Chemicals

Imidacloprid and thiacloprid were donated by Bayer CropScience. ACh was purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

Homology modelling of the fruit fly-chicken hybrid nACh receptors in complex with neonicotinoids

Homology modeling of $(D\alpha1)_3(\beta2)_2$ and docking with neonicotinoids were performed using Modeller version 9.17 (Webb and Sali, 2014). Amino acid sequences of the ligand binding domain of the $(D\alpha1)_3(\beta2)_2$ nACh receptor were aligned to those of the human $(\alpha4)_2(\beta2)_3$ nACh receptor taken from the PDB file of 5KXI (Morales-Perez *et al.*, 2016). Then, structural coordinates for corresponding amino acid regions were excised to make a structural template for the homology model. To generate an initial docking model, structural coordinates for imidacloprid (PDB: 2ZJU) and thiacloprid (3WTK) were manually transferred to that of 5KXI by using PyMOL (Schrödinger, New York, NY, USA), where only one neonicotinoid molecule was placed at the Dα1-Dα1 interface. The model was then refined by molecular dynamics combined with simulated annealing (Kirkpatrick *et al.*, 1983).

Electrophysiology data analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Peak current amplitude of each inward current recorded from oocytes in response to bath-applied neonicotinoid and ACh was measured repeatedly. The peak amplitude of the response of oocytes expressing wild-type and mutant Dα1β2 nACh receptors fluctuated considerably from several thousand nA to greater than 10 μA and was therefore normalized to that induced by 10 μM ACh. The concentration-normalized response relationships (ACh, n = 5; imidacloprid and thiacloprid, n = 6) were analysed by nonlinear regression using Prism 5 (GraphPad Software, La Jolla, CA, USA) to determine an EC_{50} (M) and normalized maximum response (I_{max}) using oocytes from at least two female frogs according to the following equation:

$$Y = I_{max}\left(1 + 10^{(\log EC_{50} - X)nH}\right)^{-1}$$, where Y is normalized response, X is log[agonist concentration (M)] and n_H is the Hill coefficient. The peak current amplitude of the response of the nACh receptors to 10 μM ACh is presented as mean ± SEM of 17 experiments. Differences between the values obtained for the wild-type and mutant nACh receptors were analysed by one-way ANOVA (Dunnett's test, $P < 0.05$).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Results

Homology modelling of $(D\alpha1)_3(\beta2)_2$ nACh receptors in complex with neonicotinoids

To investigate a role for the Dα1-Dα1 orthosteric site of Dα1β2 hybrid nACh receptors in its interactions with neonicotinoids, the wild-type $(D\alpha1)_3(\beta2)_2$ nACh receptors with imidacloprid and thiacloprid bound to them were modelled using the crystal structure of the human $(\alpha4)_2(\beta2)_3$ nACh receptor (Figure 1A–C). In these nACh receptor-neonicotinoid complexes, the two oxygens in the nitro group of imidacloprid formed hydrogen bonds with Arg^{57} in loop G and Lys^{140} in loop E (Figure 1B), whereas the cyano group of thiacloprid interacted mainly with Arg^{57} (Figure 1C). In addition, Glu^{78} in loop D was found to form salt bridges with Arg^{57} and Lys^{140}, making a 'loop D-E-G triangle' (Figure 1B, C).

Effects of mutations on agonist actions of ACh and neonicotinoids on $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors

To explore the nACh receptor-neonicotinoid interactions observed in the $(D\alpha1)_3(\beta2)_2$ nACh receptor models (Figure 1A, B), Arg^{57} in loop G, Lys^{140} in loop E and Glu^{78} in loop D were mutated to serine, threonine and lysine, respectively, which are corresponding amino acids in the human α4 subunit (Figure 2), and the agonist actions of ACh, imidacloprid and thiacloprid on the wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *Xenopus* oocytes were measured by voltage-clamp electrophysiology.

ACh showed agonist actions on the mutant as well as the wild-type $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors tested (Figure 3). For the wild-type nACh receptors, ACh showed slightly higher affinity, as measured by the EC_{50} value, for the $(D\alpha1)_3(\beta2)_2$ nACh receptor than the $(D\alpha1)_2(\beta2)_3$ nACh receptor (Table 1). The R57S, K140T, R57S;K140T and R57S;E78K;K140T mutations in the Dα1 subunit had a minimal effect on the EC_{50} values of ACh, not only for the $(D\alpha1)_3(\beta2)_2$ nACh receptor (Figure 4A) but also for the $(D\alpha1)_2(\beta2)_3$ nACh receptor (Figure 4B, Table 1). However, the E78K mutation slightly increased the ACh EC_{50} value of the $(D\alpha1)_2(\beta2)_3$ nACh receptor (Figure 4B, Table 1).

When the peak current amplitude of the response to ACh was compared between the wild-type and mutant nACh receptors, the E78K and R57S;K140T mutations significantly reduced the peak current amplitude of the response to 10 μM ACh of the $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors (Figures 3 and 4C, D, Table 1). In addition, the R57S and K140T mutations also reduced the peak current amplitude of the response to 10 μM ACh in the $(D\alpha1)_2(\beta2)_3$ nACh receptor.

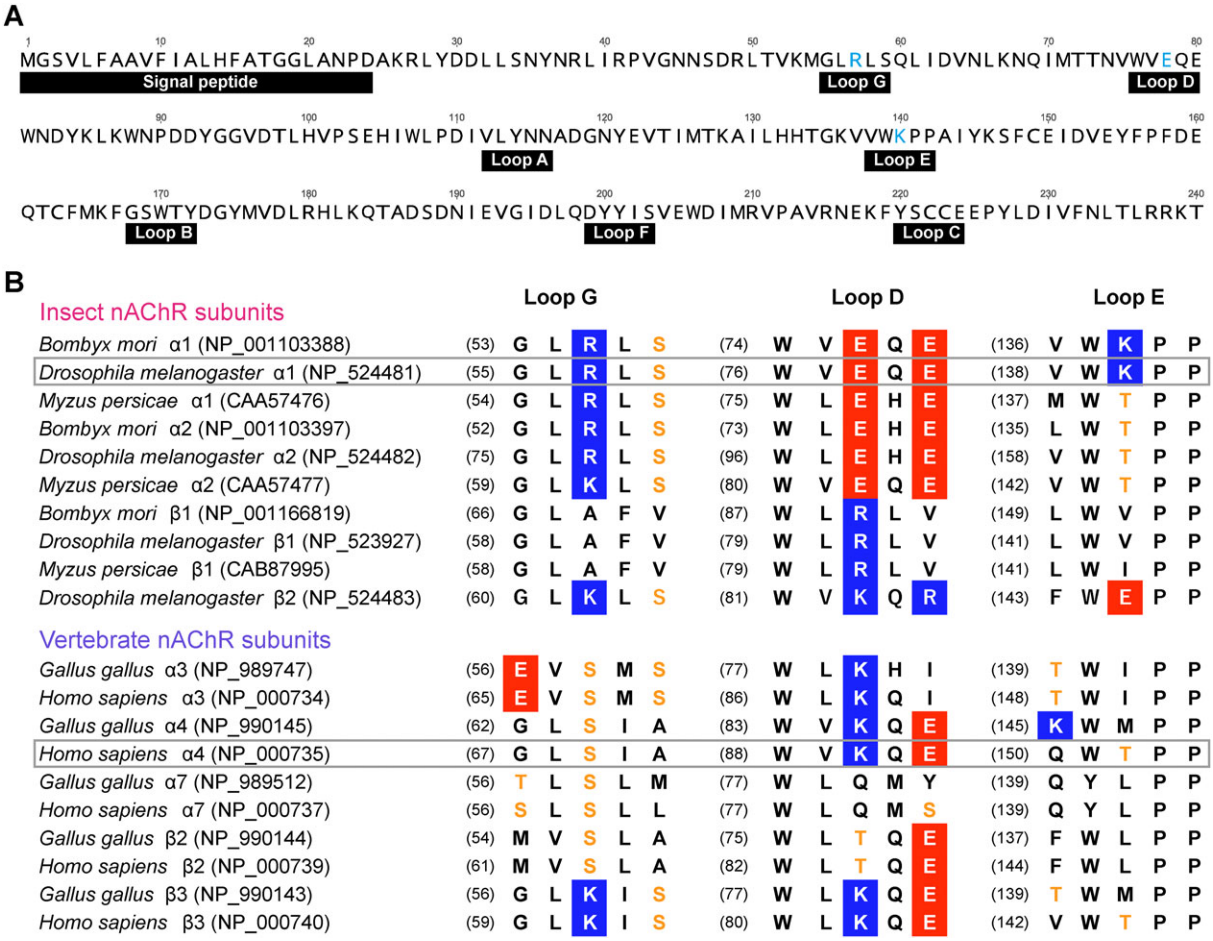

Figure 2

Amino acid sequences of nACh receptors. (A) Amino acid sequences of the ligand binding domain of *Drosophila melanogaster* α1 (Dα1) subunit (accession number: NP_524481). Mutated amino acids in loops D, E and G region are coloured cyan. (B) Multiple sequence alignments of loops D, E and G regions of insect, chicken and human nACh receptor subunits. Numbers in parentheses indicate the amino acid residue numbers taken from the sequence database. Basic- and acidic-residues are in blue and red boxes, respectively. Serine and threonine residues are coloured orange. Amino acid sequences of the *Drosophila* Dα1 and human α4 subunits are boxed.

Effects of mutations in loops G and E of the Dα1 subunit on agonist actions of neonicotinoids on $(Dα1)_3(β2)_2$ and $(Dα1)_2(β2)_3$ nACh receptors

Imidacloprid and thiacloprid were agonists (Figure 5) with a lower efficacy than ACh on the wild-type Dα1β2 nACh receptor (Table 2). The R57S mutation in the Dα1 subunit had a minimal impact on the EC_{50} and I_{max} of imidacloprid (Figure 6), while reducing the pEC_{50} value of thiacloprid from 7.47 (EC_{50} = 33.8 nM) to 6.53 (EC_{50} = 295 nM) in the $(Dα1)_3(β2)_2$ nACh receptor (Figure 7, Table 2). However, no such effect of the R57S mutation on the EC_{50} and I_{max} values of thiacloprid or imidacloprid was observed in the $(Dα1)_2(β2)_3$ nACh receptor (Figures 6 and 7, Table 2).

In contrast to the R57S mutation, the K140T mutation in the $(Dα1)_3(β2)_2$ nACh receptor reduced the efficacy of the neonicotinoids with a minimal shift of EC_{50} (Table 2), having a greater effect on the actions of imidacloprid than those of thiacloprid (Figures 6 and 7). A more profound effect of this mutation on the efficacy of imidacloprid was observed for the $(Dα1)_3(β2)_2$ compared to the $(Dα1)_2(β2)_3$ nACh receptors (Figure 6, Table 2). Also, similar effects on the agonist actions of neonicotinoids were observed in the R57S; K140T mutations with a higher impact on the I_{max} than the EC_{50} (Figures 6 and 7, Table 2).

Effects of the E78K mutation in loop D of the Dα1 subunit on agonist actions of neonicotinoids on $(Dα1)_3(β2)_2$ and $(Dα1)_2(β2)_3$ nACh receptors

In the $(Dα1)_3(β2)_2$ nACh receptor model (Figure 1), Glu^{78} forms salt bridges with Arg^{57} and Lys^{140}. We examined the role of this interaction on the effects of the neonicotinoids; the E78K mutation was predicted to lead to an electrostatic repulsion by Arg^{57} and Lys^{140}, resulting in an adverse effect on the orthosteric site. Indeed, ACh induced a much smaller amplitude current response than that seen in the wild-type nACh receptor (Figure 4C, D). Furthermore, imidacloprid

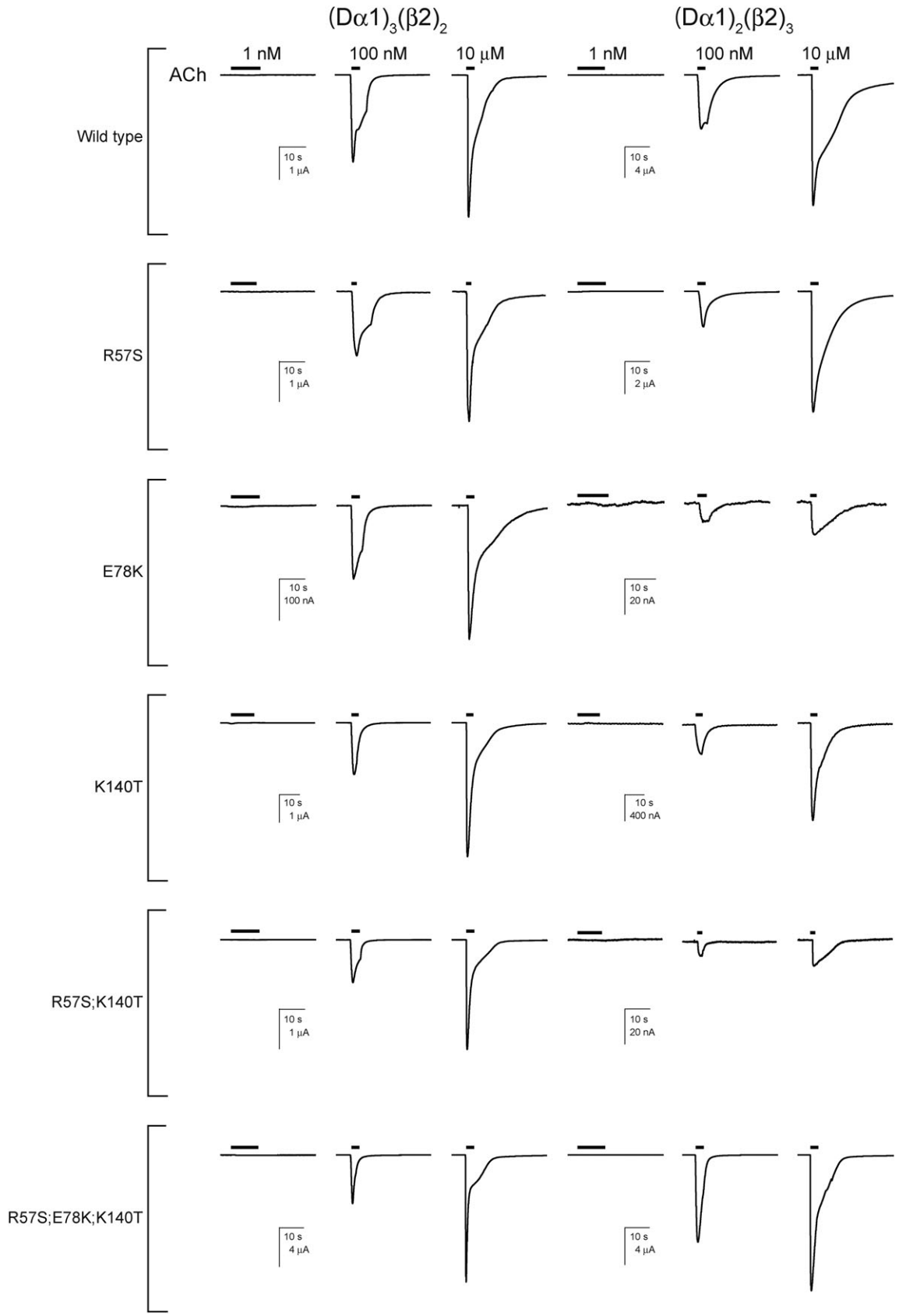

Figure 3

Currents recorded in response to ACh from wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *X. laevis* oocytes.

Table 1

Agonist actions of ACh on wild-type and mutant Dα1β2 nicotinic ACh receptors expressed in *Xenopus* oocytes[a]

	$(D\alpha1)_3(\beta2)_2$		$(D\alpha1)_2(\beta2)_3$	
nACh receptors	**pEC_{50}**	**I_{max}**	**pEC_{50}**	**I_{max}**
Wild type	7.05 ± 0.06	0.981 ± 0.031	6.74 ± 0.05	1.026 ± 0.031
R57S	6.97 ± 0.06	0.985 ± 0.031	6.67 ± 0.05	1.035 ± 0.031
K140T	6.89 ± 0.05	0.978 ± 0.026	6.66 ± 0.04	1.009 ± 0.024
R57S;K140T	6.90 ± 0.05	1.006 ± 0.030	6.81 ± 0.07	1.071 ± 0.038
E78K	7.04 ± 0.04	0.979 ± 0.021	7.02 ± 0.05*	1.036 ± 0.023
R57S;E78K;K140T	7.00 ± 0.04	0.977 ± 0.024	6.83 ± 0.08	0.995 ± 0.043

[a]Data are presented as mean ± SEM of repeated experiments ($n = 5$).
*The pEC_{50} (-log EC_{50}) value differed significantly from that determined in the wild type ($P < 0.05$).

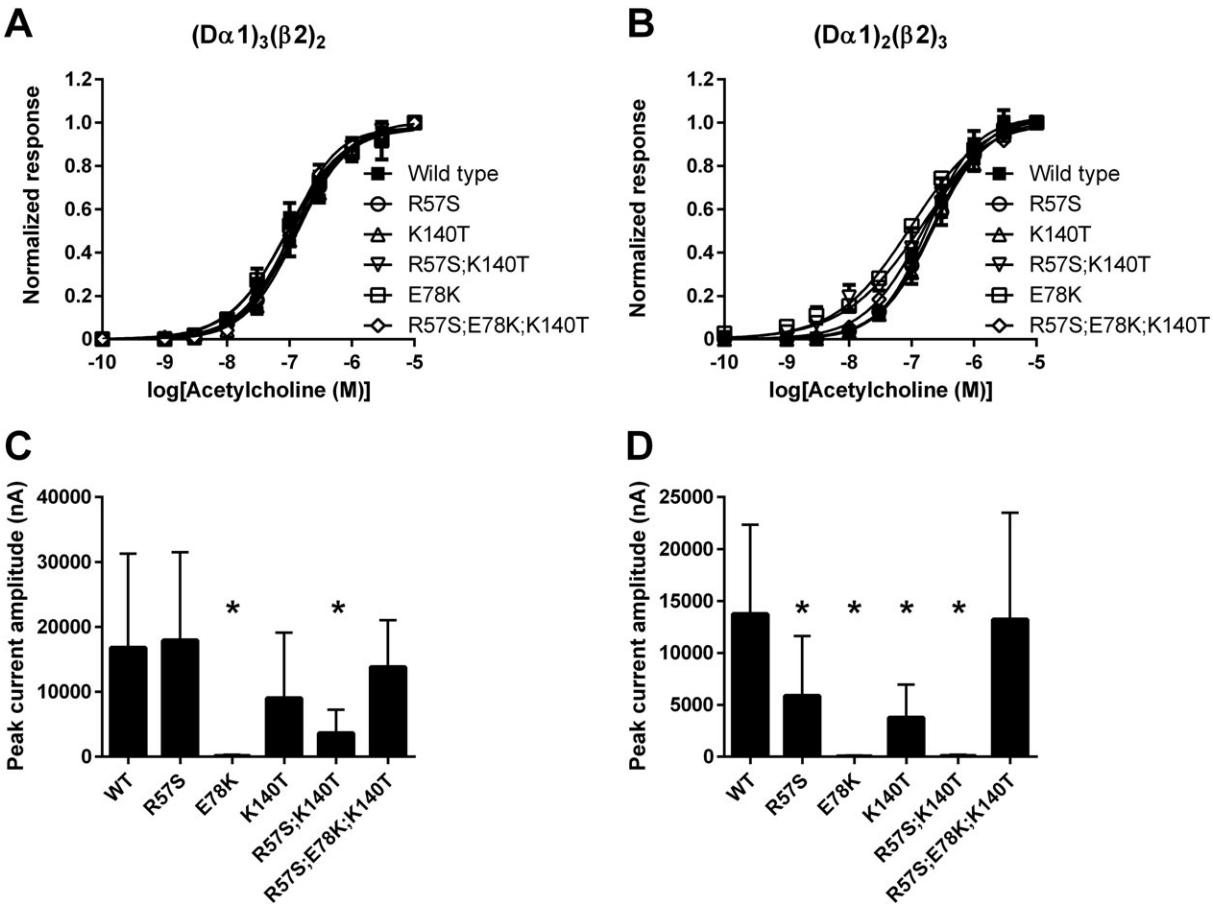

Figure 4

Concentration–response curves for ACh observed in $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ hybrid nACh receptors expressed in *X. laevis* oocytes and peak current amplitude of the ACh-induced responses. (A) Concentration–response curve for the $(D\alpha1)_3(\beta2)_2$ nACh receptor; (B) concentration–-response curve for the $(D\alpha1)_2(\beta2)_3$ nACh receptor. (C) Peak current amplitude of the response of $(D\alpha1)_3(\beta2)_2$ nACh receptor to 10 μM ACh. (D) Peak current amplitude of the response of $(D\alpha1)_2(\beta2)_3$ nACh receptor to 10 μM ACh. Each data point in panels (A, B) represents mean ± SEM ($n = 5$), whereas each bar graph in panels (C, D) represents mean ± SEM ($n = 17$). In panels (C) and (D), significant differences of the peak current amplitude of the response induced by 10 μM ACh between the wild-type and mutant nACh receptors are indicated by an asterisks (* $P < 0.05$).

and thiacloprid were inefficient in activating the E78K mutant of $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors even at 100 μM (Figure 5), hence the EC_{50} could not be determined. However, when the E78K mutation was combined with the R57S;K140T double mutations, the ability of the hybrid nACh receptor to respond to ACh (Figures 3 and 4) and

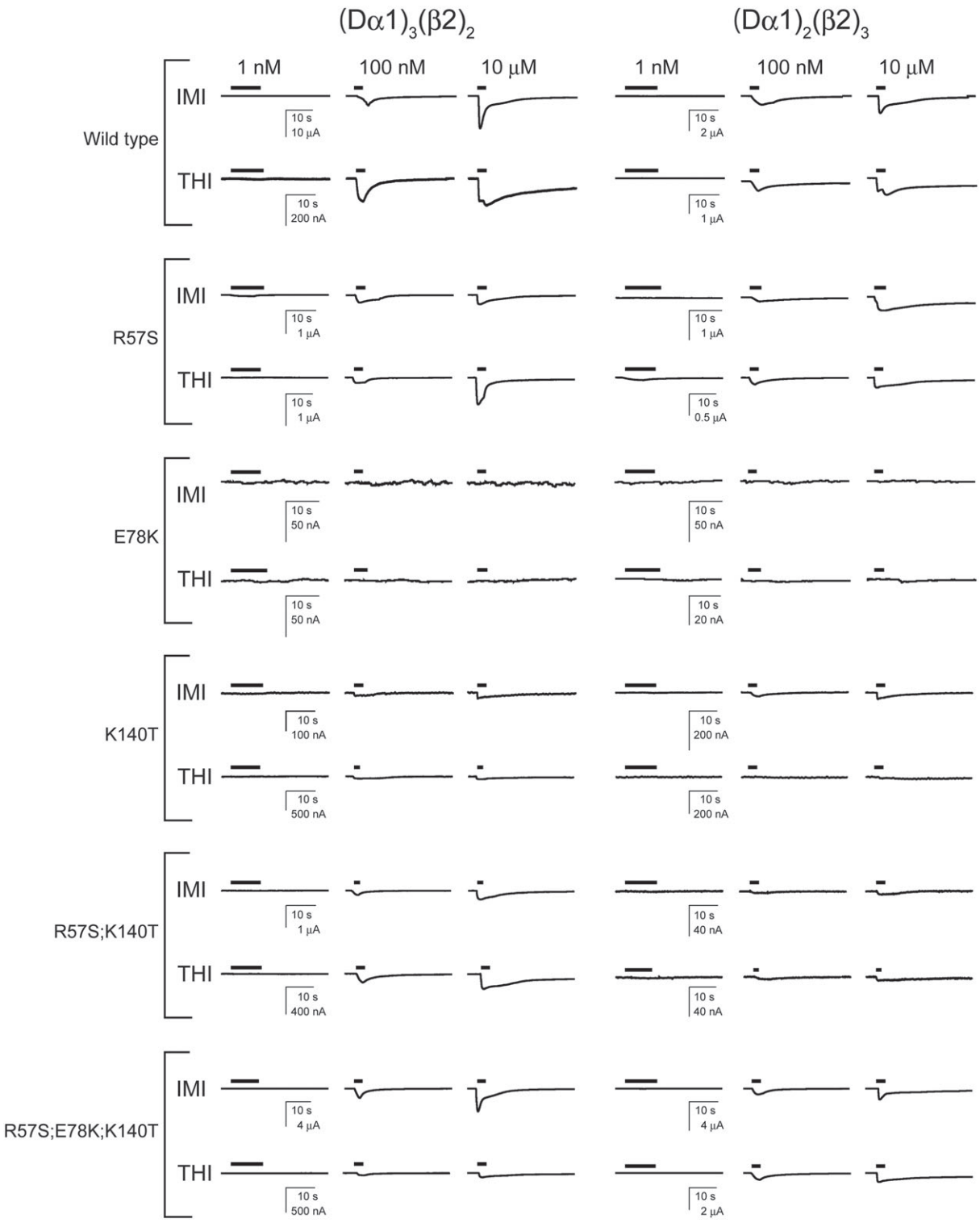

Figure 5

Currents recorded in response to imidacloprid (IMI) and thiacloprid (THI) from wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *X. laevis* oocytes.

neonicotinoids was restored (Figures 5–7). The triple mutations significantly reduced the pEC_{50} value of thiacloprid from 7.47 to 6.37 (EC_{50} = 424 nM) and the I_{max} value of imidacloprid from 0.165 to 0.119 for the $(D\alpha1)_3(\beta2)_2$ nACh receptor (Figures 6 and 7, Table 2). In contrast, they had a minimal effect on the pEC_{50} and I_{max} values of the neonicotinoids for the $(D\alpha1)_2(\beta2)_3$ nACh receptor (Figures 6 and 7, Table 2).

Table 2
Agonist actions of imidacloprid and thiacloprid on wild-type and mutant Dα1β2 nicotinic ACh receptors expressed in *X. laevis* oocytes[a]

nACh receptors	$(D\alpha1)_3(\beta2)_2$				$(D\alpha1)_2(\beta2)_3$			
	Imidacloprid		Thiacloprid		Imidacloprid		Thiacloprid	
	pEC_{50}	I_{max}	pEC_{50}	Imax	pEC_{50}	I_{max}	pEC_{50}	I_{max}
Wild type	7.20 ± 0.14	0.165 ± 0.009	7.47 ± 0.21	0.054 ± 0.005	7.24 ± 0.14	0.089 ± 0.005	7.70 ± 0.39	0.039 ± 0.003
R57S	6.99 ± 0.22	0.185 ± 0.015	6.53 ± 0.28*	0.064 ± 0.007	7.32 ± 0.15	0.058 ± 0.003*	7.53 ± 0.27	0.038 ± 0.004
K140T	7.29 ± 0.17	0.032 ± 0.002*	7.60 ± 0.26	0.018 ± 0.002*	7.05 ± 0.20	0.048 ± 0.003*	7.92 ± 0.15	0.019 ± 0.001*
R57S;K140T	7.08 ± 0.17	0.075 ± 0.004*	7.71 ± 0.14	0.017 ± 0.001*	7.07 ± 0.17	0.115 ± 0.006*	7.64 ± 0.21	0.045 ± 0.004
E78K	ND[b]	ND	ND	ND	ND	ND	ND	ND
R57S;E78K;K140T	6.71 ± 0.32	0.119 ± 0.014*	6.37 ± 0.29*	0.043 ± 0.005	6.92 ± 0.25	0.083 ± 0.008	7.36 ± 0.17	0.031 ± 0.002

[a]Data are presented as mean ± SEM of repeated experiments ($n = 6$).
[b]ND: could not be determined because no neonicotinoid-induced currents were observed in *Xenopus* oocytes expressing the E78K mutant nACh receptor.
*The pEC_{50} (-log EC_{50}) and I_{max} values differed significantly from those determined in the wild type ($P < 0.05$).

Discussion

In this study, we showed for the first time that mutations of Arg^{57} in loop G, Lys^{140} in loop E and Glu^{78} in loop D in the complementary (−) side of the Dα1 subunit, which are all located in the region upstream of loop B, are involved in determining the actions of neonicotinoids on the Dα1β2 hybrid nACh receptors heterologously expressed in *X. laevis* oocytes. The stoichiometry of the Dα1 and β2 subunits as well as the mutations in the Dα1 subunit examined had minimal effects on the EC_{50} value of ACh, pointing to selective interactions of ACh with the Dα1-β2 orthosteric site rather than the Dα1-Dα1 orthosteric site. The result is attributable to an electrostatic repulsion between ACh, which contains a quaternary ammonium, and the basic residues Arg^{57} and Lys^{140} that provide a positive charge at the Dα1-Dα1 site.

Although the $(D\alpha1)_3(\beta2)_2$ nACh receptor model (Figure 1A) indicates the proximity of the Arg^{57} to the bound imidacloprid, the effect of the R57S mutation on the EC_{50} was small. This does not contradict the model because the serine residue can form a hydrogen bond with the nitro group oxygen, thus permitting the access of imidacloprid. In contrast, the R57S mutation significantly shifted the EC_{50} of thiacloprid to a higher concentration (Table 2). An interpretation of this result is that the cyano group of thiacloprid more selectively interacts with the Arg^{57} than the nitro group of imidacloprid, as shown by the $(D\alpha1)_3(\beta2)_2$ nACh receptor model (Figure 1B, C). This provides one explanation for the R57S mutation having a more profound effect on the agonist action of thiacloprid than that of imidacloprid. Alternatively, the ability of the cyano group to form a hydrogen bond with the added serine residue is weaker than that of the nitro group, and thus, the R57S mutation may selectively reduce the action of thiacloprid.

We previously showed that the L118K mutation in loop E markedly enhanced the efficacy of imidaloprid, while reducing that of ACh for the chicken α7 nACh receptor. Therefore, we predicted that the lysine corresponding to Leu^{118} in insect nACh receptor α subunits (Amiri *et al.*, 2008) may partly account for the selective action of neonicotinoids. Indeed, the reduction in efficacy of imidacloprid and thiacloprid resulting from the K140T mutation was more profound in the $(D\alpha1)_3(\beta2)_2$ nACh receptor than in the $(D\alpha1)_2(\beta2)_3$ nACh receptor (Figures 6 and 7, Table 2), supporting an interaction with the Dα1-Dα1 interface. If efficacy represents interaction with the activated state of the nACh receptor, the results suggest that Lys^{140} interacts more strongly with imidacloprid than with thiacloprid in the activated state of the hybrid nACh receptor, as indicated by the homology models (Figure 1).

The nACh receptor model (Figure 1) predicts that the E78K mutation leads to repulsion in the triangle of Lys^{78}, Arg^{57} and Lys^{140}, adversely altering the function of the nACh receptor. Indeed, the E78K mutation markedly reduced the capacity of the nACh receptor to respond to agonists (Figures 3–5, Tables 1 and 2), validating the model. The model also suggests that combining the E78K mutation with the R57S;K140T mutations will have a much smaller effect on the function of the nACh receptor compared with the single E78K mutation, since no electrostatic repulsion occurs within the triangle; this accords with the minimal impact these mutations then have on the agonist action of ACh in terms of affinity as well as efficacy (Figures 3 and 4, Table 1).

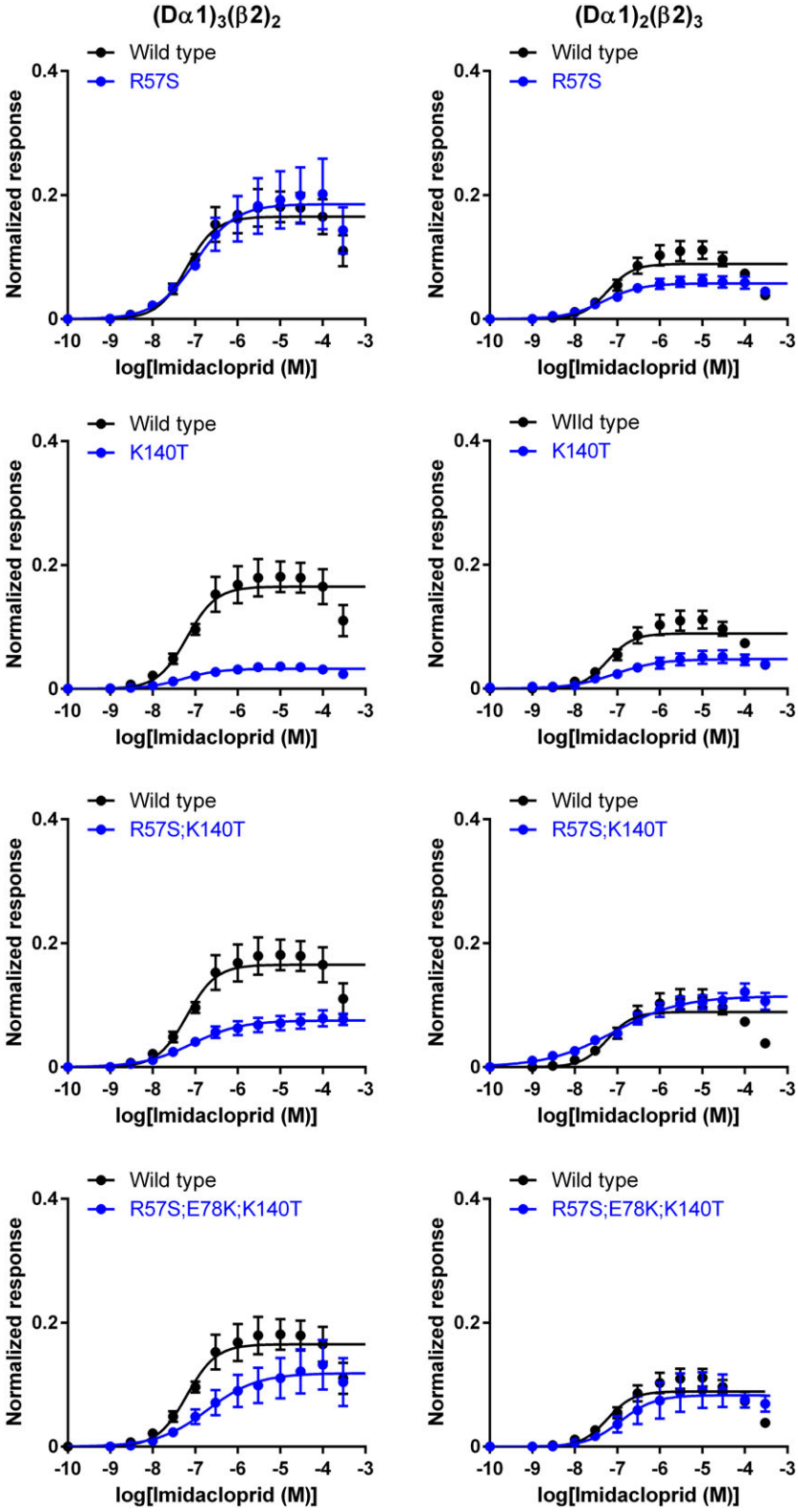

Figure 6

Concentration–response relationships for imidacloprid observed in wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *X. laevis* oocytes. Each data point represents the mean ± SEM (n = 6).

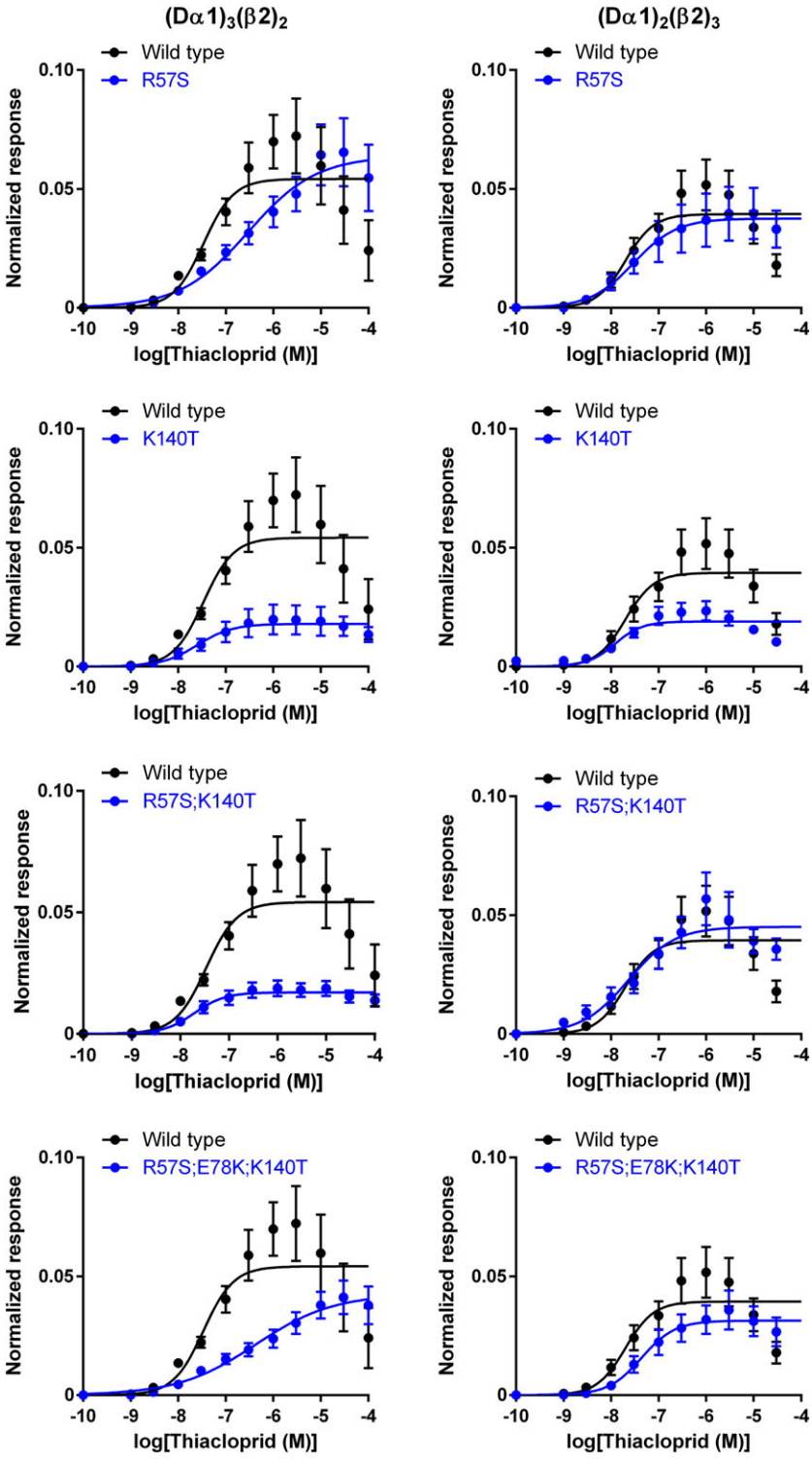

Figure 7

Concentration–response relationships for thiacloprid observed in wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nACh receptors expressed in *X. laevis* oocytes. Each data point represents the mean ± SEM ($n = 6$).

The increased effect of the triple mutations in the Dα1 subunit on the pEC_{50} value of thiacloprid when compared with imidacloprid for the $(D\alpha1)_3(\beta2)_2$ nACh receptor (Figures 6 and 7, Table 2) may arise from lower capacity of the cyano group compared with the nitro group to form hydrogen bonds with the added serine and theronine residues.

We previously showed that the region upstream of loop B in the *Drosophila* Dα2 subunit underlies the high neonicotinoid

sensitivity of the Dα2β2 hybrid nACh receptor (Shimomura *et al.,* 2005). In the Dα1 subunit, the amino acids in the loop D-E-G triangle located upstream of loop B accounts, at least in part, for the higher sensitivity of the insect-avian hybrid nACh receptors to neonicotinoids than the avian α4β2 nACh receptor (Matsuda *et al.,* 1998; Ihara *et al.,* 2003).

The R57S mutation significantly reduced the affinity (the pEC_{50} value) of thiacloprid for the $(D\alpha1)_3(\beta2)_2$ nACh receptor (Figure 7, Table 2). Hence, it is predicted that mutations of corresponding basic residues in nACh receptors of pest insect species may result in resistance to this neonicotinoid as it had a minimal effect on the ACh concentration–response curve. However, we did not employ expressed nACh receptors composed only of subunits from insects due to the difficulty of robust functional nACh receptor expression, not only in *Xenopus* oocytes, but also in various cell lines (Lansdell *et al.,* 1997). A solution to this problem, exploring resistant field population for this mutation in pests controlled solely with neonicotinoids, is urgently needed.

It has been shown that the use of nACh receptor concatemers allows nACh receptor-ligand interactions to be analysed at specified orthosteric sites (Carbone *et al.,* 2009; Mazzaferro *et al.,* 2011, 2014; Benallegue *et al.,* 2013). In this study, we did not define the sequence of the Dα1 and β2 subunit in the hybrid nACh receptors expressed in oocytes using a concatemer and, therefore, we cannot demonstrate unequivocally that the reduced neonicotinoid sensitivity resulting from the mutations tested can be attributed to the interactions with the Dα1-Dα1 interface. It will be necessary in the future to employ insect nACh receptor concatamers to study the mode of action of neonicotinoids. Nevertheless, it is reasonable to conclude that the structural changes in the complementary side of the Dα1 subunit selectively affect neonicotinoid interactions with the hybrid nACh receptors and thus are likely to play an important role in determining the selective neonicotinoid actions on insect nACh receptors.

In summary, we have shown for the first time that triple rather than single mutations of amino acids in the loop D-E-G triangle of the *Drosophila* Dα1 subunit to corresponding amino acids in the human α4 subunit significantly and selectively reduce the agonist actions of imidaloprid and thiacloprid on the $(D\alpha1)_3(\beta2)_2$ nACh receptor. The results add to our understanding of the mechanism of selectivity of neonicotinoids and provide new insights into the interactions of neonicotinoids with hybrid nACh receptors.

Acknowledgements

M.I. was supported by JSPS KAKENHI (Grant-in-Aid for Young Scientists (B), grant number 16K21507). K.M. was supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research (A), grant number 17H01472) and by MAFF Genomics-Based Technology for Agricultural Improvement (grant number PRM-3002). D.B.S. was supported by University College London.

Author contributions

M.I., M.H., H.M., K.Y., Y.K., K.K., S.W., M.S., K.M. (Matsui and Matsuda), A.Y., D.O. and S.F. conducted the experiments; M.I., D.B.S. and K.M. designed the experiments and wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Alexander SPH, Peters JA, Kelly E, Marrion N, Benson HE, Faccenda E *et al.* (2015). The Concise Guide to PHARMACOLOGY 2015/16: Ligand-gated ion channels. Br J Pharmacol 172: 5870–5903.

Amiri S, Shimomura M, Vijayan R, Nishiwaki H, Akamatsu M, Matsuda K *et al.* (2008). A Role for Leu118 of Loop E in agonist binding to the α7 nicotinic acetylcholine receptor. Mol Pharmacol 73: 1659–1667.

Bass C, Puinean AM, Andrews M, Cutler P, Daniels M, Elias J *et al.* (2011). Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid *Myzus persicae*. BMC Neurosci 12: 51.

Benallegue N, Mazzaferro S, Alcaino C, Bermudez I (2013). The additional ACh binding site at the α4(+)/α4(−) interface of the $(\alpha4\beta2)_2\alpha4$ nicotinic ACh receptor contributes to desensitization. Br J Pharmacol 170: 304–316.

Brown LA, Ihara M, Buckingham SD, Matsuda K, Sattelle DB (2006). Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. J Neurochem 99: 608–615.

Carbone AL, Moroni M, Groot-Kormelink PJ, Bermudez I (2009). Pentameric concatenated $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nicotinic acetylcholine receptors: subunit arrangement determines functional expression. Br J Pharmacol 156: 970–981.

Casida JE, Durkin KA (2017). Pesticide chemical research in toxicology: lessons from nature. Chem Res Toxicol 30: 94–104.

Changeux JP (2012). The nicotinic acetylcholine receptor: the founding father of the pentameric ligand-gated ion channel superfamily. J Biol Chem 287: 40207–40215.

Corringer PJ, Le Novere N, Changeux JP (2000). Nicotinic receptors at the amino acid level. Annu Rev Pharmacol Toxicol 40: 431–458.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA *et al.* (2015). Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol 172: 3461–3471.

Furutani S, Nakatani Y, Miura Y, Ihara M, Kai K, Hayashi H *et al.* (2014). GluCl a target of indole alkaloid okaramines: a 25 year enigma solved. Sci Rep 4: 6190.

Gill RJ, Ramos-Rodriguez O, Raine NE (2012). Combined pesticide exposure severely affects individual- and colony-level traits in bees. Nature 491: 105–108.

Ihara M, Matsuda K, Otake M, Kuwamura M, Shimomura M, Komai K *et al.* (2003). Diverse actions of neonicotinoids on chicken α7, α4β2 and *Drosophila*-chicken SADβ2 and ALSβ2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. Neuropharmacology 45: 133–144.

Ihara M, Matsuda K, Shimomura M, Sattelle DB, Komai K (2004). Super agonist actions of clothianidin and related compounds on the SADβ2 nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. Biosci Biotechnol Biochem 68: 761–763.

Ihara M, Brown LA, Ishida C, Okuda H, Sattelle DB, Matsuda K (2006). Actions of imidacloprid, clothianidin and related neonicotinoids on nicotinic acetylcholine receptors of American cockroach neurons and their relationships with insecticidal potency. J Pestic Sci 31: 35–40.

Ihara M, Okajima T, Yamashita A, Oda T, Hirata K, Nishiwaki H *et al.* (2008). Crystal structures of *Lymnaea stagnalis* AChBP in complex with neonicotinoid insecticides imidacloprid and clothianidin. Invert Neurosci 8: 71–81.

Ihara M, Okajima T, Yamashita A, Oda T, Asano T, Matsui M *et al.* (2014a). Studies on an acetylcholine binding protein identify a basic residue in loop G on the β1 strand as a new structural determinant of neonicotinoid actions. Mol Pharmacol 86: 736–746.

Ihara M, Shimazu N, Utsunomiya M, Akamatsu M, Sattelle DB, Matsuda K (2014b). A single amino acid polymorphism in the *Drosophila melanogaster* Dα1 (ALS) subunit enhances neonicotinoid efficacy at Dα1-chicken β2 hybrid nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. Biosci Biotechnol Biochem 78: 543–549.

Ihara M, Sattelle DB, Matsuda K (2015). Probing new components (loop G and the α-α interface) of neonicotinoid binding sites on nicotinic acetylcholine receptors. Pestic Biochem Physiol 121: 47–52.

Jeschke P, Nauen R, Beck ME (2013). Nicotinic acetylcholine receptor agonists: a milestone for modern crop protection. Angew Chem Int Ed Engl 52: 9464–9485.

Kagabu S (1997). Chloronicotinyl insecticides – discovery, application and future perspective. Rev Toxicol 1: 75–129.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kirkpatrick S, Gelatt CD Jr, Vecchi MP (1983). Optimization by simulated annealing. Science 220: 671–680.

Lansdell SJ, Schmitt B, Betz H, Sattelle DB, Millar NS (1997). Temperature-sensitive expression of *Drosophila* neuronal nicotinic acetylcholine receptors. J Neurochem 68: 1812–1819.

Matsuda K, Buckingham SD, Freeman JC, Squire MD, Baylis HA, Sattelle DB (1998). Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. Br J Pharmacol 123: 518–524.

Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M, Sattelle DB (2001). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol Sci 22: 573–580.

Matsuda K, Shimomura M, Ihara M, Akamatsu M, Sattelle DB (2005). Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular biology, and receptor modeling studies. Biosci Biotechnol Biochem 69: 1442–1452.

Matsuda K, Kanaoka S, Akamatsu M, Sattelle DB (2009). Diverse actions and target-site selectivity of neonicotinoids: structural insights. Mol Pharmacol 76: 1–10.

Mazzaferro S, Benallegue N, Carbone A, Gasparri F, Vijayan R, Biggin PC *et al.* (2011). Additional acetylcholine (ACh) binding site at α4/α4 interface of $(\alpha4\beta2)_2\alpha4$ nicotinic receptor influences agonist sensitivity. J Biol Chem 286: 31043–31054.

Mazzaferro S, Gasparri F, New K, Alcaino C, Faundez M, Iturriaga Vasquez P *et al.* (2014). Non-equivalent ligand selectivity of agonist sites in $(\alpha4\beta2)_2\alpha4$ nicotinic acetylcholine receptors: a key determinant of agonist efficacy. J Biol Chem 289: 21795–21806.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

Miyazawa A, Fujiyoshi Y, Unwin N (2003). Structure and gating mechanism of the acetylcholine receptor pore. Nature 423: 949–955.

Morales-Perez CL, Noviello CM, Hibbs RE (2016). X-ray structure of the human α4β2 nicotinic receptor. Nature 538: 411–415.

Nemecz A, Prevost MS, Menny A, Corringer PJ (2016). Emerging molecular mechanisms of signal transduction in pentameric ligand-gated ion channels. Neuron 90: 452–470.

Rundlof M, Andersson GK, Bommarco R, Fries I, Hederstrom V, Herbertsson L *et al.* (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. Nature 521: 77–80.

Shimomura M, Okuda H, Matsuda K, Komai K, Akamatsu M, Sattelle DB (2002). Effects of mutations of a glutamine residue in loop D of the α7 nicotinic acetylcholine receptor on agonist profiles for neonicotinoid insecticides and related ligands. Br J Pharmacol 137: 162–169.

Shimomura M, Satoh H, Yokota M, Ihara M, Matsuda K, Sattelle DB (2005). Insect-vertebrate chimeric nicotinic acetylcholine receptors identify a region, loop B to the N-terminus of the *Drosophila* Dα2 subunit, which contributes to neonicotinoid sensitivity. Neurosci Lett 385: 168–172.

Shimomura M, Yokota M, Ihara M, Akamatsu M, Sattelle DB, Matsuda K (2006). Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site. Mol Pharmacol 70: 1255–1263.

Southan C, Sharman JL, Benson HE, Faccenda E, Pawson AJ, Alexander SPH *et al.* (2016). The IUPHAR/BPS Guide to PHARMACOLOGY in 2016: towards curated quantitative interactions between 1300 protein targets and 6000 ligands. Nucleic Acids Res 44: D1054–D1068.

Taly A (2007). Opened by a twist: a gating mechanism for the nicotinic acetylcholine receptor. Eur Biophys J 36: 911–918.

Taly A, Delarue M, Grutter T, Nilges M, Le Novere N, Corringer PJ *et al.* (2005). Normal mode analysis suggests a quaternary twist model for the nicotinic receptor gating mechanism. Biophys J 88: 3954–3965.

Taly A, Corringer PJ, Grutter T, Prado De Carvalho L, Karplus M, Changeux JP (2006). Implications of the quaternary twist allosteric model for the physiology and pathology of nicotinic acetylcholine receptors. Proc Natl Acad Sci U S A 103: 16965–16970.

Tan J, Galligan JJ, Hollingworth RM (2007). Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. Neurotoxicology 28: 829–842.

Tomizawa M, Casida JE (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu Rev Entomol 48: 339–364.

Tomizawa M, Casida JE (2005). Neonicotinoid insecticide toxicology: mechanisms of selective action. Annu Rev Pharmacol Toxicol 45: 247–268.

Unwin N (2005). Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. J Mol Biol 346: 967–989.

Unwin N, Fujiyoshi Y (2012). Gating movement of acetylcholine receptor caught by plunge-freezing. J Mol Biol 422: 617–634.

Webb B, Sali A (2014). Protein structure modeling with MODELLER. Methods Mol Biol 1137: 1–15.

Whitehorn PR, O' Connor S, Wackers FL, Goulson D (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336: 351–352.