**Running title: Role of loops D, E and G in neonicotinoid actions**

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

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**Plain Language Summary**

Neonicotinoid insecticides acting on insect nicotinic acetylcholine receptors (nACh receptors) can have adverse effects on beneficial insects such as pollinators and thus it is extremely important to understand the mechanism underpinning the potency and selectivity of neonicotinoids on insect nACh receptors. We show for the first time that amino acids in ACh binding site loops D, E and G of the *Drosophila* Dα1 subunit play an important role in determining neonicotinoid actions at Dα1-Dα1 subunit interface of Dα1-chicken β2 nACh receptor expressed in *Xenopus laevis* oocytes.

**Abstract**

**Background and Purpose**

Neonicotinoid insecticides interact with the orthosteric site formed at subunit interfaces of insect nicotinic acetylcholine receptors (insect 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 (Dα1)3(β2)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 (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus laevis* oocytes using voltage-clamp electrophysiology.

**Key Results**

The (Dα1)3(β2)2 nACh receptor models indicated that amino acids in loop D, E and G are likely to determine neonicotinoid actions. The amino acid mutations tested scarcely influenced the EC50 of ACh. However, the R57S mutation in loop G, although having minimal effect on imidacloprid actions, reduced the affinity of thiacloprid for the (Dα1)3(β2)2 nACh receptor, while scarcely affecting thiacloprid action on the (Dα1)2(β2)3 nACh receptor. Both the K140T and R57S;K140T mutations reduced neonicotinoid efficacy but only for the (Dα1)3(β2)2 nACh receptor. Combining the E78K mutation with the R57S;K140T double mutations resulted in a selective reduction of thiacloprid affinity for the (Dα1)3(β2)2 nACh receptor.

**Conclusions and Implications**

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

**Abbreviations**

ACh, acetylcholine; EC50, half maximum concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Imax, normalized maximum response; nACh receptor, nicotinic acetylcholine receptor, nH, Hill coefficient.

**Introduction**

Neonicotinoid insecticides such as imidacloprid and thiacloprid (Figure 1A) have been used to control crop pests and pests of farm animals (Casida *et al.*, 2016; Jeschke *et al.*, 2013; Matsuda *et al.*, 2001a; Matsuda *et al.*, 2009; Matsuda *et al.*, 2005; Tomizawa *et al.*, 2005; Tomizawa *et al.*, 2003). Neonicotinoids interact with the orthosteric site of insect [nicotinic acetylcholine receptors](http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=76) (insect nACh receptors) mainly as partial and full agonists, although for certain compounds super agonist and antagonist actions have also been described (Brown *et al.*, 2006; Ihara *et al.*, 2006; Ihara *et al.*, 2004; Matsuda *et al.*, 2009; Tan *et al.*, 2007). Unlike [acetylcholine](http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=294) (ACh), neonicotinoids are not hydrolyzed by ACh esterases and hence can persistently modulate nACh receptors (Matsuda *et al.*, 2001a). Neonicotinoids show selectivity for insect over vertebrate nACh receptors (Matsuda *et al.*, 2001a); they are broad spectrum insecticides with superior uptake by the roots and subsequent translocation in plants (Jeschke *et al.*, 2013; Kagabu, 1997). 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 the Colony Collapse Disorder, because they modulate nACh receptors of bees as well as pest insect species (Gill *et al.*, 2012; Rundlof *et al.*, 2015; Whitehorn *et al.*, 2012). In view of the possible risk to bees, the EU restricted the use of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) in 2012 and continues to restrict their deployment subject to further assessment of the risk. Therefore, it is extremely important to understand structural features determining 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 the fast cholinergic neurotransmission in vertebrates and invertebrates (Changeux, 2012). Channel opening in response to ACh binding (Miyazawa *et al.*, 2003; Nemecz *et al.*, 2016; Taly, 2007; Taly *et al.*, 2006; Taly *et al.*, 2005; Unwin, 2005; Unwin *et al.*, 2012) results in depolarization of nerve and muscle membranes. 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.*, 2001b; 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.*, 2001a; Matsuda *et al.*, 2009; Matsuda *et al.*, 2005). The resulting planar imidazolidine or related moieties facilitate interaction with π-electron rich aromatic residues (Matsuda *et al.*, 2009; Matsuda *et al.*, 2005; Shimomura *et al.*, 2006). Mutations of glutamine79 in chicken α7 (Shimomura *et al.*, 2002) and threonine in chicken β2 subunit (Shimomura *et al.*, 2006), both of which are located in loop D, to basic residues, markedly enhanced imidacloprid sensitivity of these nACh receptors, suggesting a possible interactions with 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 Q55R mutant of the acetylcholine binding protein (AChBP) of *Lymnaea stagnalis*, which provides a surrogate of the nACh receptor orthosteric binding site, with neonicotinoids (imidacloprid, clothianidin, thiacloprid and nitromethylene analog of imidacloprid (CH-IMI) (Ihara *et al.*, 2014a; Ihara *et al.*, 2008). 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 an opposite direction against loop D, supporting a role for loop D in its interactions 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 hardly affected 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 model where 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 Lys34 on the β1 strand (loop G) in 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 Ser58 in the avian α7 nACh receptor, which corresponds to Lys34 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 precisely which amino acids in the region upstream of loop B underpin the selective interactions with neonicotinoids.

To address these questions, we computationally modeled 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 chicken α4β2 nACh receptor (Ihara *et al.*, 2003; Ihara *et al.*, 2014b). The model showed that not only Arg57 in loop G, but also Lys140 in loop E, at the Dα1-Dα1 subunit interface interact with the nitro group of imidacloprid, and Glu78 supports such an interaction by forming salt bridges. Thus we mutated Arg57, Lys140 and Glu78 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 (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors to show that the amino acids in the Dα1–Dα1 subunit interface contribute to determining the selective neonicotinoid actions on insect nACh receptors.

**Methods**

***Xenopus laevis oocytes***

Female *Xenopus laevis* were anesthetized with benzocaine (ethyl 4-aminobenzoate) to reduce animal suffering as much as possible according to the U.K. Animals (Scientific Procedures) Act, 1986 and minimum amounts of oocytes were removed from anesthetized frogs. *Xenopus* oocytes were treated with 2 mg ml-1 Type IA collagenase (Sigma Aldrich, St. Louis, MO, USA) in Ca2+-free standard oocyte saline (Ca-free standard oocyte saline (SOS): 100 mM NaCl, 2 mM KCl, 1 mM MgCl2 and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.6 adjusted with NaOH)) and transferred to SOS (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2 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.

*cRNA preparation and injection to oocytes*

The *Drosophila* Dα1 subunit and the chicken β2 subunit containing amino acid sequences deposited in the GenBank (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 subunit as well as of wild type chicken β2 subunit were prepared by *in vitro* transcription from 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 ml-1. Dα1 and β2 cRNA solutions were mixed at a ratio of 5:1 or 1:5 for reconstituting (Dα1)3(β2)2 and (Dα1)2(β2)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.

*Electrophysiology*

Two-electrode voltage-clamp electrophysiology was conducted using a GeneClamp 500B amplifier (Molecular Devices, CA, USA) at a holding potential of -100 mV (Ihara *et al.*, 2003; Matsuda *et al.*, 1998; Shimomura *et al.*, 2006). Oocytes were perfused extracellularly at a flow rate of 7-10 ml min-1 with SOS containing 0.5 μM [atropine](http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=320) to suppress the muscarinic response of oocytes. Neonicotinoids were dissolved in dimethylsulfoxide (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 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 with 3 min interval. No irreversible nACh receptor desensitization was observed by treatment of 10 μM or lower concentrations of ACh and 100 μM or lower concentrations of neonicotinoids under 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 modeling of the fruit fly-chicken hybrid nACh receptors in complex with neonicotinoids*

Homology model of (Dα1)3(β2)2 and docking with neonicotinoids were performed using Modeller version 9.17 (Webb *et al.*, 2014). Amino acid sequences of the ligand binding domain of the (Dα1)3(β2)2 nACh receptor were aligned to those of the human (α4)2(β2)3 nACh receptor taken from 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*

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 analyzed by non-linear regression of using Prism 5 (GraphPad Software, La Jolla, CA, USA) to determine a half maximum concentration EC50 (M) and normalized maximum response Imax using oocytes from at least two female frogs according to the following equation:

Y = Imax (1+10(logEC50-X)nH)-1, where Y is normalized response, X is agonist concentration and nH is the Hill coefficient. The peak current amplitude of the response of the nACh receptors to 10 μM ACh was represented as mean ± standard error of 17 experiments. Differences between such values obtained for the wild type and mutant nACh receptors were analyzed 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 modeling of (Dα1)3(β2)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 (Dα1)3(β2)2 nACh receptors with imidacloprid and thiacloprid bound were modeled using the crystal structure of human (α4)2(β2)3 nACh receptor (Fig. 1, A-C). In these nACh receptor-neonicotinoid complexes, the two oxygens in the nitro group of imidacloprid formed hydrogen bonds with Arg57 in loop G and Lys140 in loop E (Fig. 1B), whereas the cyano group of thiacloprid interacted mainly with Arg57 (Fig. 1C). In addition, Glu78 in loop D was found to form salt bridges with Arg57 and Lys140, making a “loop D-E-G triangle” (Fig. 1, B, C).

*Effects of mutations on agonist actions of ACh and neonicotinoids on (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors*

To explore the nACh receptor-neonicotinoid interactions observed in the (Dα1)3(β2)2 nACh receptor models (Fig. 1A, B), Arg57 in loop G, Lys140 in loop E and Glu78 in loop D were mutated to serine, threonine and lysine, respectively, which are corresponding amino acids in the human α4 subunit (Fig. 2), and the agonist actions of ACh, imidacloprid and thiacloprid on the wild type and mutant (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus* oocytes were measured by voltage-clamp electrophysiology.

ACh showed agonist action for the mutant as well as the wild type (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors tested (Fig. 3). For the wild type nACh receptors, ACh showed slightly higher affinity as measured by EC50 value for the (Dα1)3(β2)2 nACh receptor than the (Dα1)2(β2)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 EC50 values of ACh, not only for the (Dα1)3(β2)2 nACh receptor (Fig. 4A), but also for the (Dα1)2(β2)3 nACh receptor (Fig. 4B, Table 1). However, the E78K mutation slightly increased the ACh EC50 value of the (Dα1)2(β2)3 nACh receptor (Fig. 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 (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors (Fig. 3, 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 (Dα1)2(β2)3 nACh receptor.

*Effects of mutations in loop 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 (Fig. 5) with 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 EC50 and Imax of imidacloprid (Fig. 6), while reducing the pEC50 value of thiacloprid from 7.47 (EC50 = 33.8 nM) to 6.53 (EC50 = 295 nM) in the (Dα1)3(β2)2 nACh receptor (Fig. 7, Table 2). However, no such effect of the R57S mutation on the EC50 and Imax values of thiacloprid or imidacloprid was observed in the (Dα1)2(β2)3 nACh receptor (Fig. 6, 7, Table 2).

In contrast with the R57S mutation, the K140T mutation in the (Dα1)3(β2)2 nACh receptor reduced the efficacy of neonicotinoids with a minimal shift of EC50 (Table 2), having a greater impact on the actions of imidacloprid than thiacloprid on (Fig. 6, 7). A more profound effect of this mutation on the efficacy of imidacloprid was observed in the (Dα1)3(β2)2 than (Dα1)2(β2)3 nACh receptors (Fig. 6, Table 2). Also, similar effects on neonicotinoids were observed in the R57S;K140T mutations with higher impact on Imax than EC50 (Fig. 6, 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 (Fig. 1), Glu78 forms salt bridges with Arg57 and Lys140. To examine the role of such an interaction in determining neonicotinoid actions, the E78K mutation was predicted to lead to an electrostatic repulsion by Arg57 and Lys140, 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 (Fig. 4C, D). Furthermore, imidacloprid and thiacloprid were inefficient in activating the E78K mutant of (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors even at 100 μM (Fig. 5), hence the EC50 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 (Fig. 3, 4) and neonicotinoids was restored (Fig. 5–7). The triple mutations significantly reduced the pEC50 value of thiacloprid to from 7.47 to 6.37 (EC50 = 424 nM) and the Imax value of imidacloprid from 0.165 to 0.119 for the (Dα1)3(β2)2 nACh receptor (Fig. 6, 7, Table 2). By contrast, they had a minimal effect on the pEC50 and Imax values of the neonicotinoids for the (Dα1)2(β2)3 nACh receptor (Fig. 6, 7, Table 2).

**Discussion**

In this study, we show for the first time that mutations of Arg57 in loop G, Lys140 in loop E and Glu78 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 neonicotinoid actions on the Dα1β2 hybrid nACh receptors heterologously expressed in *Xenopus* *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 EC50 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 containing a quaternary ammonium and basic residues Arg57 and Lys140 containing a positive charge at the Dα1-Dα1 site.

Although the (Dα1)3(β2)2 nACh receptor model (Fig. 1A) indicated proximity of the Arg57 to imidacloprid bound, the effect of the R57S mutation on EC50 was small. This does not contradict the model because the serine residue can form a hydrogen bond with the nitro group oxygen thus permitting access of imidacloprid. By contrast, the R57S mutation significantly shifted the EC50 of thiacloprid to higher concentration (Table 2). An interpretation of this result is that the cyano group of thiacloprid more selectively interacts with the Arg57 than the nitro group of imidacloprid as shown by the (Dα1)3(β2)2 nACh receptor model (Fig. 1B, C). This offers one explanation for the R57S having a more profound effect on the agonist action of thiacloprid than 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 Leu118 in insect nACh receptor α subunits 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 (Dα1)3(β2)2 nACh receptor than in the (Dα1)2(β2)3 nACh receptor (Fig. 6, 7, Table 2), supporting an interaction with the Dα1-Dα1 interface. If efficacy represents interaction at the activated state of nACh receptor, the results suggest that Lys140 interacts more strongly with imidacloprid than with thiacloprid in the activated state of the hybrid nACh receptor as indicated by the homology models (Fig. 1).

The nACh receptor model (Fig. 1) predicts that the E78K mutation leads to repulsion in the triangle of Lys78, Arg57 and Lys140, adversely altering the nACh receptor function. Indeed, the E78K mutation markedly reduced the capacity of the nACh receptor to respond to agonists (Fig. 3–5, Table 1, 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 nACh receptor function compared with the single E78K mutation since no electrostatic repulsion occurs within the triangle in accord with the minimal impact on the agonist action of ACh in terms of affinity as well as efficacy (Fig. 3, 4, Table 1). The higher impact of the triple mutations in the Dα1 subunit on the pEC50 value of thiacloprid when compared to imidacloprid for the (Dα1)3(β2)2 nACh receptor (Fig. 6, 7, Table 2) may arise from lower capacity of the cyano group compared to the nitro group to hydrogen bond with the added serine and theronine residues.

We previously showed that the region upstream of loop B in the *Drosophila* Dα2 subunit underlie the high neonicotinoid sensitivity of the Dα2β2 hybrid nACh receptor (Shimomura *et al.*, 2005). In the Dα1 subunit, the amino acids in loop D-E-G triangle are located in upstream of loop B accounts, at least in part, for higher neonicotinoid sensitivity of the insect-avian hybrid nACh receptors than the avian α4β2 nACh receptor (Ihara *et al.*, 2003; Matsuda *et al.*, 1998).

The R57S mutation significantly reduced the affinity in the pEC50 value of thiacloprid for the (Dα1)3(β2)2 nACh receptor (Fig. 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 as well as field monitoring of such mutation in pests controlled solely with neonicotinoids is urgently needed.

It has been shown that using nACh receptor concatemers permits analysis of nACh receptor-ligand interactions at specified orthosteric sites (Benallegue *et al.*, 2013; Carbone *et al.*, 2009; Mazzaferro *et al.*, 2011; Mazzaferro *et al.*, 2014). In this study, we did not define the sequence of the Dα1 and β2 subunit in expressing the hybrid nACh receptor 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 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 likely play an important role in determining the selective neonicotinoid actions on insect nACh receptors.

In summary, we have for the first time shown 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 (Dα1)3(β2)2 nACh receptor. The results add to our understanding the mechanism of selectivity of neonicotinoids and provide new insight into the neonicotinoid-hybrid nAChR interactions.

**Author contribution**

MI, MH, HM, KY, YK, KK, SW, MS, KM (Matsui and Matsuda), AY, DO, SF conducted experiments; MI, DBS and KM designed experiments and wrote ms.

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**Conflict of Interest**

We declare that there is no conflict of interest in this study.

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Table 1. Agonist actions of acetylcholine on wild type and mutant Dα1β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| nACh receptors | (Dα1)3(β2)2 | | (Dα1)2(β2)3 | |
|  | pEC50 | Imax | pEC50 | Imax |
| 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 |

1Data are represented as mean ± standard error of the mean of repeated experiments (n = 5).

\*The difference in pEC50 (-log EC50) was statistically significant from that determined in the wild type (P<0.05).

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Table 2. Agonist actions of imidacloprid and thiacloprid on wild type and mutant Dα1β2 nicotinic acetylcholine receptors expressed in *Xenopus* *laevis* oocytes1

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| nACh receptors | (Dα1)3(β2)2 | | | | (Dα1)2(β2)3 | | | |
|  | Imidacloprid | | Thiacloprid | | Imidacloprid | | Thiacloprid | |
|  | pEC50 | Imax | pEC50 | Imax | pEC50 | Imax | pEC50 | Imax |
| 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 | ND2 | 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 |

1Data are represented as mean ± standard error of the mean of repeated experiments (n = 6).

2ND: could not be determined because no neonicotinoid-induced currents were observed in *Xenopus* oocytes expressing the E78K mutant nACh receptor.

\*The difference in pEC50 (-log EC50) and Imax values was statistically significant from those determined in the wild type (P<0.05).

**Figure legends**

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 (Dα1)3(β2)2 nACh receptor model generated from human (α4)2(β2)3 nACh receptor docked with imidacloprid. The Dα1 subunits at principal and complementary sides are colored tan and yellow, respectively, whereas the β2 subunits are colored red. (B) Close-up view of the imidacloprid binding site. (C) Close-up view of the thiacloprid binding site. Arg57 and Lys140 interacted electrostatically with the nitro group of imidacloprid (B) and the cyano group of thiacloprid (C). Glu78 made salt bridges with Arg57 and Lys140 to form a “loop D-E-G triangle” (B, C). In each panel, the main chain of the nACh receptors are drawn as cartoon, whereas Arg57 (colored orange), Lys140 (colored orange), Glu78 (colored blue) and the neonicotinoids are drawn as space filling models. For neonicotinoids, carbon-, nitrogen-, oxygen-, sulfur- and chlorine-atoms are colored grey, blue, red, tan and green, respectively.

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 loop D, E and G region are colored cyan (B) Multiple sequence alignments of loop D, E and G region of insect, chicken and human nACh receptor subunits. Numbers in the parenthesis 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 colored orange. Amino acid sequences of the *Drosophila* Dα1 and human α4 subunits are boxed.

Figure 3. Current responses to ACh of wild-type and mutant (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus laevis* oocytes.

Figure 4. Concentration-response curves of ACh for (Dα1)3(β2)2 and (Dα1)2(β2)3 hybrid nACh receptors expressed in *Xenopus* *laevis* oocytes and peak current amplitude of the ACh-induced responses. A, Concentration-response curve for (Dα1)3(β2)2 nACh receptor; B, Concentration-response curve for (Dα1)2(β2)3 nACh receptor. C, Peak current amplitude of response of (Dα1)3(β2)2 nACh receptor to 10 μM ACh. D, Peak current amplitude of response of (Dα1)2(β2)3 nACh receptor to 10 μM ACh. Each data point in (A, B) represents mean ± standard error of the mean (n = 5), whereas Each bar graph in (C, D) represents mean ± standard error of the mean (n = 17). In C and D, statistical difference of the peak current amplitude of 10 μM ACh-induced response between the wild type and mutant nACh receptors are indicated by asterisks (\*, P<0.05).

Figure 5. Current responses to imidacloprid (IMI) and thiacloprid (THI) of wild-type and mutant (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus laevis* oocytes.

Figure 6. Concentration-response relationships of imidacloprid for wild type and mutant (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus* *laevis* oocytes. Each data point represents mean ± standard error of the mean (n = 6).

Figure 7. Concentration-response relationships of thiacloprid for wild type and mutant (Dα1)3(β2)2 and (Dα1)2(β2)3 nACh receptors expressed in *Xenopus* *laevis* oocytes. Each data point represents mean ± standard error of the mean (n = 6).