Manuscript Details

Manuscript number	PESTBIO_2018_41
Title	Combined effects of mutations in loop C and the loop D-E-G triangle on neonicotinoid interactions with Drosophila Dα1/chicken β2 hybrid nAChRs
Article type	Research Paper

Abstract

Neonicotinoid insecticides interact with the orthosteric sites of nicotinic acetylcholine receptors (nAChRs) formed at the interfaces of (a) two adjacent α subunits and (b) α and non-α subunits. However, little is known of the detailed contributions of these two orthosteric sites to neonicotinoid actions. We therefore applied voltage-clamp electrophysiology to the Dα1/chicken β2 hybrid nAChR expressed in Xenopus laevis oocytes to explore the agonist actions of imidacloprid and thiacloprid on wild type receptors and following binding site mutations. First, we studied the S221E mutation in loop C of the ACh binding site of the Dα1 subunit. Secondly, we explored the impact of combining this mutation in loop C with others in the loop D-E-G triangle (R57S; E78K; K140T; S221E). The S221E loop C mutation alone reduced the affinity of the neonicotinoids tested, while hardly affecting the concentration-response curve for acetylcholine. Addition of the three R57S; E78K; K140T mutations in the loop D-E-G triangle led to a further reduction in neonicotinoid sensitivity, suggesting that all four binding site loops (C, D, E, G) in the Dα1 subunit, which are located upstream of loop B in the N-terminal, extracellular domain, contribute to the selective actions of neonicotinoid insecticides.

Keywords	ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; Imax, maximum normalized response; EC50, half maximal concentration; nH, Hill coefficient.

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To Prof. John Clark
Chief Editor, *Pesticide Biochemistry and Physiology*

Dear Professor John Clark
Please find attached our ms entitle **"Combined effects of mutations in loop C and the loop D-E-G triangle on interactions of neonicotinoids with *Drosophila* Dα1/chicken β2 hybrid nAChRs"** for consideration for publication in *Pesticide Biochemistry and Physiology* in the special issue devoted to ACS award winner, Professor Jeffery Bloomquist.

Neonicotinoids now enjoy a quarter of world sales of insecticides, but their use in certain regions has been restricted due to potential adverse effects. Hence it is important to understand in detail how they act selectively on insect nicotinic acetylcholine receptors (nAChRs). We recently demonstrated that neonicotinoids are likely to interact with the α-α subunit interface as well as the α-β2 subunit interface of insect-vertebrate hybrid nAChRs. Such hybrid receptors, although imperfect models, have been used extensively as insect heteromeric receptors targeted by neonicotinoids have proved difficult to express. Here we provide further evidence to support this notion by examining the effects of a mutation in loop C alone and when combined with mutations in the "loop D-E-G" triangle. The results are novel and add to our understanding of neonicotinoid-nAChR interactions. Thus we hope that our paper will be of interest to the broad readership of *Pesticide Biochemistry and Physiology*.

We very much hope you will consider the paper for the special issue of *Pesticide Biochemistry and Physiology* devoted to the honor of Professor Jeffery Bloomquist.

Best regards,

Kazuhiko Matsuda, on behalf of all authors

Combined effects of mutations in loop C and the loop D-E-G triangle on neonicotinoid interactions with *Drosophila* Dα1/chicken β2 hybrid nAChRs

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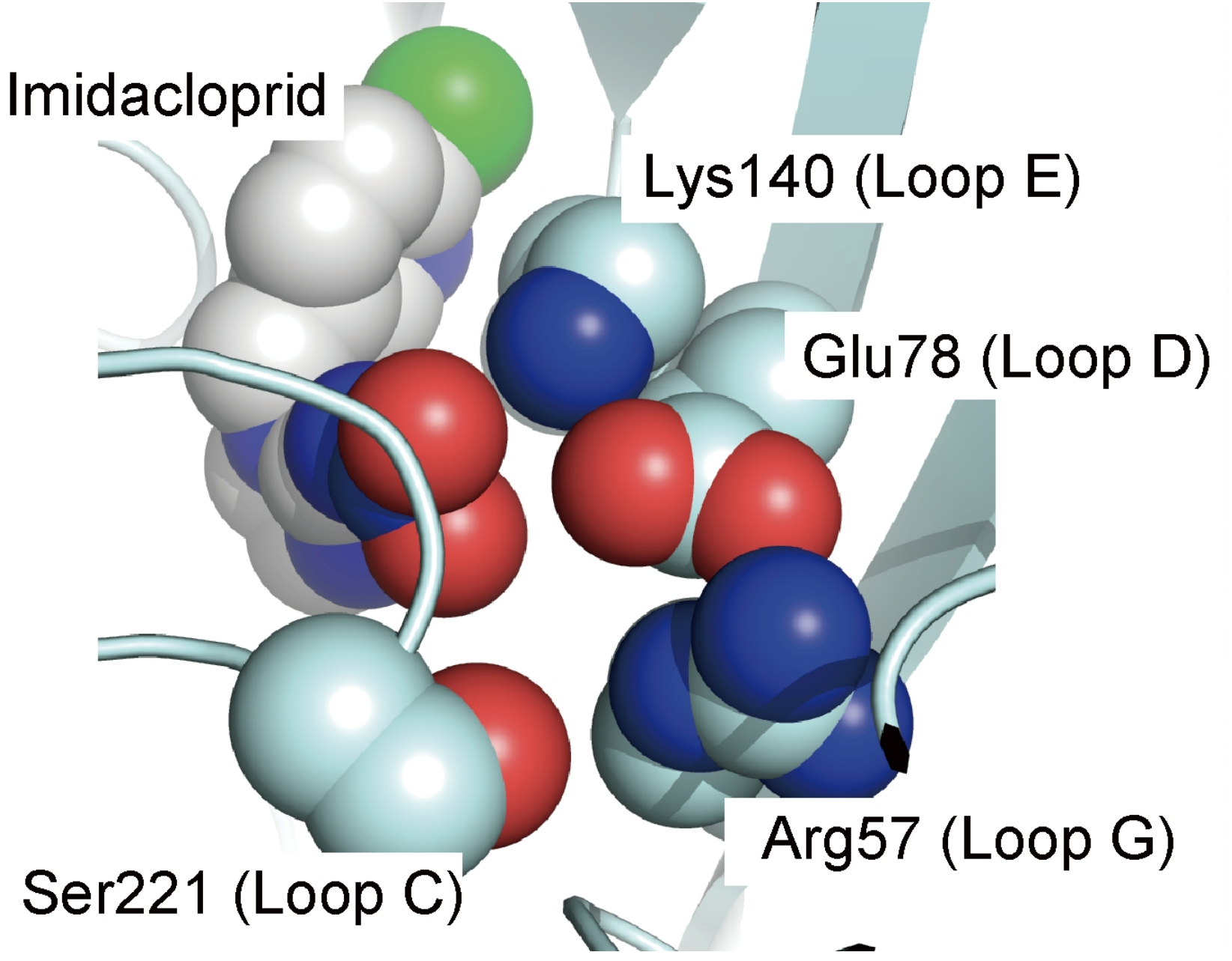
Highlights

- Serine221 was mutated to glutamate in loop C of *Drosophila* Dα1 subunit.
- The S221E mutation reduced neonicotinoid affinity for $(D\alpha1)_3$(chicken $\beta2)_2$ nAChR.
- R57S; E78K; K140T triple mutations were added to the S221E Dα1 mutant.
- The four amino acid mutations further reduced the neonicotinoid actions.
- Thus, all four residues play critical roles in the interactions with neonicotinoids.


Imidacloprid
Lys140 (Loop E)
Glu78 (Loop D)
Arg57 (Loop G)
Ser221 (Loop C)

Combined effects of mutations in loop C and the loop D-E-G triangle on neonicotinoid interactions with *Drosophila* Dα1/chicken β2 hybrid nAChRs

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Abstract

Neonicotinoid insecticides interact with the orthosteric sites of nicotinic acetylcholine receptors (nAChRs) formed at the interfaces of (a) two adjacent α subunits and (b) α and non-α subunits. However, little is known of the detailed contributions of these two orthosteric sites to neonicotinoid actions. We therefore applied voltage-clamp electrophysiology to the Dα1/chicken β2 hybrid nAChR expressed in *Xenopus laevis* oocytes to explore the agonist actions of imidacloprid and thiacloprid on wild type receptors and following binding site mutations. First, we studied the S221E mutation in loop C of the ACh binding site of the Dα1 subunit. Secondly, we explored the impact of combining this mutation in loop C with others in the loop D-E-G triangle (R57S; E78K; K140T; S221E). The S221E loop C mutation alone reduced the affinity of the neonicotinoids tested, while hardly affecting the concentration-response curve for acetylcholine. Addition of the three R57S; E78K; K140T mutations in the loop D-E-G triangle led to a further reduction in neonicotinoid sensitivity, suggesting that all four binding site loops (C, D, E, G) in the Dα1 subunit, which are located upstream of loop B in the N-terminal, extracellular domain, contribute to the selective actions of neonicotinoid insecticides.

6 keywords

Nicotinic acetylcholine receptor; neonicotinoid; imidacloprid; thiacloprid; orthosteric binding site; α subunit.

Abbreviations

ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; I_{max}, maximum normalized response; EC_{50}, half maximal concentration; n_H, Hill coefficient.

Introduction

Imidacloprid and related compound, known collectively as neonicotinoids, are insecticides developed by improving the structure and pest control efficacy of the original lead compound nithiazine [1-4]. Neonicotinoids act selectively on insect over mammalian nicotinic acetylcholine receptors (nAChRs) and show high systemic activity in crop plants with moderate persistence; they currently make up approximately one quarter of the world's insecticide market [3].

Neonicotinoids target insect nAChRs [1, 3-6] and studies on the more extensively characterized vertebrate nAChRs show that these membrane proteins are pentameric, ligand-gated cation channels possessing 4 transmembrane domains (4 TMs) as well as a di-cysteine loop in the N-terminal extracellular domain, which is a key signature motif of the Cys-loop ligand-gated ion channel superfamily [7]. Each nAChR molecule typically gates a cation channel in response to the binding of the neurotransmitter acetylcholine (ACh) or nicotinic agonists to the receptor orthosteric binding site [8-11]. Numerous α subunits (defined by a vicinal cysteine motif in loop C, one of 6 (A-F) loops of the ACh binding site) and non-α subunits make up the nAChR family [12]. Neonicotinids act as agonists, super agonists or antagonists depending on compound structure and nAChR subunit composition [4, 13]. Neonicotinoids interact with the orthosteric sites [1], which are formed, either by an α and non-α subunit interface, or by the interface between two adjacent α subunits. The orthosteric site at the α subunit/non-α subunit interface is composed of loops A, B, C on the α subunit and loops D, E, F on the non-α subunit [12], whereas it is formed by loops A, B, C on the principal side and loops D, E, F on the complementary side at the α subunit/α subunit interface [14]. We

have shown that not only basic residues in loop D [15-17], but also non-acidic (X) residues in the YXCC motif in loop C of the ACh binding site [18], are key determinants of the selective actions of neonicotinoids [4, 5, 13]. In addition, we have recently shown that Arg57 in loop G and Lys140 in loop E, both forming a salt bridge with Glu78 in loop D, in the *Drosophila melanogaster* Dα1 subunit underlie the interactions of imidacloprid and thiacloprid with the α/α subunit interface of Dα1-chicken β2 hybrid nAChRs [19]. However, it is not known if Ser221 in the X position in the YXCC motif in loop C also plays a role. To address this question, we used site-directed mutagenesis and voltage-clamp electrophysiology to investigate the effects of S221E alone and in combination with R57S;E78K;K140T;S221E mutations on the agonist actions of imidacloprid and thiacloprid on the $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nAChRs expressed in *Xenopus* oocytes.

Methods

Chemicals

Imidacloprid and thiacloprid were purchased from Wako Pure Chemical Industries (Osaka, Japan), while ACh was purchase from Merck/Sigma-Aldrich (St. Louis, MO, USA).

Computational modeling of Dα1β2 nAChR in complex with neonicotinoids at Dα1/Dα1 subunit interface

Homology models of Dα1β2 nAChR complexed with imidacloprid and thiacloprid were constructed using the automodel algorithm of Modeller version 9.19 [20], where structure coordinates 2zju and 3wtk were used as the template for the imidacloprid-

87 nAChR complex and the thiacloprid-nAChR complex, respectively. Imidacloprid and
88 thiacloprid interactions with key amino acid residues were illustrated using PyMOL
89 software (Schrödinger, New York, NY, USA).
90
91 **cRNAs**
92 cDNAs of the Dα1 subunit were mutated by the quick-change method and entire
93 nucleotide sequences of the mutants were confirmed by automated sequencing using a
94 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). cRNAs
95 encoding the chicken β2 subunit as well as the wild type and mutant Dα1 subunits were
96 prepared using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo
97 Fisher Scientific) and diluted to 1 mg ml^{-1}[19, 21].
98
99 ***Xenopus laevis* oocytes**
100 Females of *X. laevis* were anesthetized with benzocaine and minimum amounts of
101 oocytes were used for studies according to the Animals (Scientific Procedures) Act
102 1986, UK as described previously [22, 23]. The follicle layers were removed from
103 oocytes after collagenase treatment. cRNAs of the Dα1 and β2 subunits were mixed at
104 ratios of 5:1 and 1:5 to express $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nAChRs and 50 ng of the
105 cRNA mixtures were injected into oocytes [19]. The cRNA-injected oocytes were then
106 incubated in Standard Oocyte Saline (SOS) containing NaCl (100 mM), KCl (2 mM),
107 $CaCl_2$ (1.8 mM), $MgCl_2$ (1 mM) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic
108 acid (HEPES) (5 mM), pH 7.6, supplemented with penicillin (100 units ml^{-1}),
109 streptomycin (100 μg ml^{-1}), gentamycin (20 μg ml^{-1}) and sodium pyruvate (2.5 mM) at
110 16ºC for 4 days prior to electrophysiological recording of the receptor responses to ACh

and neonicotinoids.

Voltage-clamp electrophysiology

Xenopus oocytes expressing nAChRs were perfused at a flow rate of 7-10 ml min^{-1} by a gravity-fed system with SOS containing 0.5 μM atropine in order to suppress any endogenous muscarinic responses [16, 17, 22, 24]. Using 3MKCl-filled microelectrodes, the membrane potential was clamped at -100 mV and inward currents induced by ACh and neonicotinoids were recorded by a GeneClamp500B amplifier using Clampex 8 software (Molecular Devices, Sunnyvale, CA, USA). The current data were digitized by a Digidata 1322A A/D converter (Molecular Devices) and analyzed offline using Clampfit 9 software (Molecular Devices).

Data analysis

Peak current amplitudes of the responses to ACh and neonicotinoids (imidacloprid and thiacloprid) at each concentration were normalized to that of 10 μM ACh. The normalized data were fitted by non-linear regression using Prism 5 software (GraphPad, La Jolla, CA, USA) according to the following equation where Y is the amplitude of the normalized response, I_{max} is normalized maximum response, EC_{50} is the half maximal concentration (M), X is log[agonist concentration (M)] and n_H is the Hill coefficient. Experiments were repeated using at least n=4 replicates with experiments from two separate frogs for each data point plotted.

$$Y = \frac{I_{max}}{1 + 10^{(\log EC_{50} - X) n_H}}$$

Results and Discussion

We modeled the orthosteric site at a Dα1-Dα1 subunit interface of Dα1β2 nAChR in complex with imidacloprid and thiacloprid (Figure 1). Loop C donates Ser221, while loops E and G provide Lys140 and Arg57, respectively, to bind tightly imidacloprid and thiacloprid. Glu78 in loop D supports the architecture of loops E and G by electrostatic force, which is in accordance with our previous reports [18, 19]. To explore the role of the serine residue in loop C in interactions with neonicotinoids, we first investigated the effects of the Dα1 S221E mutation on the agonist actions of ACh on expressed $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nAChRs (Figure 2). ACh activated the mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_2(\beta2)_3$ nAChRs (Figure 2A, B) with pEC_{50} ($-\log EC_{50}$ (M)) value of 7.009 ± 0.034 (Table 1). The S221E mutation had a significant but a minimal impact on the agonist actions of ACh in the $(D\alpha1)_3(\beta2)_2$ nAChR, whereas the effect was insignificant in the $(D\alpha1)_2(\beta2)_3$ nAChR as indicated by the pEC_{50} value of 6.981 ± 0.080. Also, combining the S221E mutation with R57S; E78K; K140T mutations in the loop D-E-G triangle had only a small effect on the concentration-response curve of ACh (For $(D\alpha1)_3(\beta2)_2$ nAChR, 7.008 ± 0.028; For $(D\alpha1)_2(\beta2)_3$ nAChR, 6.491 ± 0.042, Table 1), suggesting that roles for Ser221 as well as Arg57, Glu78 and Lys140 in Dα1 interactions with ACh are not very important.

Next, we measured the agonist actions of imidacloprid and thiacloprid on the S221E mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_3(\beta2)_2$ nAChRs (Figure 3). Both neonicotinoids activated the mutant nAChRs in a concentration-dependent manner (Figures 3A) and their EC_{50} and I_{max} values were determined in the mutant nAChRs (Table 2). In contrast with the minimal effects observed on the agonist action of ACh, the S221E mutation shifted the

158 concentration-response curves for the neonicotinoids to the right in the case of the
159 $(D\alpha1)_3(\beta2)_2$ nAChR. Notably, the mutant nAChR response to imidacloprid did not
160 reach maximum and therefore pEC_{50} and I_{max} values could not be determined (Figure
161 3B). However, the effects of the S221E mutation on the concentration-response curves
162 of neonicotinoids were less pronounced in the case of the $(D\alpha1)_2(\beta2)_3$ nAChR (Figure
163 3B, Table 2). This suggests that the neonicotinoids tested interacted preferentially with
164 the Dα1/Dα1 subunit interface. The attenuated actions on the mutant nAChR is
165 probably due to electrostatic repulsion between the Glu221 with negative charges on the
166 neonicotinoid nitro and cyano groups as pointed out previously [18].
167
168 We further examined the effects of the R57S; E78K; K140T; S221E mutations on the
169 agonist actions the neonicotinoids. The four mutations combined shifted the
170 concentration-response curve of neonicotinoids more profoundly than the S221E
171 mutation alone in the case of the $(D\alpha1)_3(\beta2)_2$ nAChR but without affecting efficacy.
172 The same 4 mutations also reduced the affinity for neonicotinoids but the curve
173 saturated over the concentrations tested and reduced the I_{max} values (Figure 3B, Table
174 2). In the $(D\alpha1)_3(\beta2)_2$ nAChR, the newly added Ser57 and Thr140 are capable of
175 interacting with neonicotinoids by hydrogen bond formation and thus the R57S; E78K;
176 K140T; S221E mutant retained the efficacy of neonicotinoids (Figure 3B). In the
177 $(D\alpha1)_2(\beta2)_3$ nAChR, however, neonicotinoids mainly interact with the Dα1-β2 interface
178 where there is no Dα1 residue able to form a hydrogen bond, thereby leading to lower
179 efficacy (Figure 3). In future it will be of interest to test this hypothesis directly by
180 deploying concatamer nAChRs [25-27].
181

In this study, we have investigated for the first time the effects on the agonist actions of imidacloprid and thiacloprid on Dα1β2 hybrid nAChRs containing an S221E mutation in loop C alone and combined with mutations in the loop D-E-G triangle. The findings indicate that the insecticides preferentially interact with the orthosteric site at the Dα1 / Dα1subunit interface in the fly-chicken hybrid nAChR and that the effect of the S221E mutation is the result of electrostatic repulsion between the glutamate residue and the nitro / cyano groups of the neonicotinoids. Also, we note that the 4 amino acids mutated contribute, at least in part, to the structural features favorable for insect nAChR α subunit-neonicotinoid interactions. The present findings accord well with our earlier demonstration that important residues contributing to neonicotinoid actions are located upstream of loop B [28]. The present novel findings enhance our understanding of neonicotinoid actions and may assist in the design new compounds targeting insect nAChRs.

Acknowledgements

This study was supported by JSPS KAKENHI to Makoto Ihara (Grant number 16K21507) and Kazuhiko Matsuda (Grant number 17H01472).

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290 Table 2. Effects of mutations in the Dα1 subunit on EC_{50} and I_{max} values[1] of acetylcholine on wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_3(\beta2)_2$ nAChRs

291 expressed in *Xenopus laevis* oocytes

	$(D\alpha1)_3(\beta2)_2$		$(D\alpha1)_2(\beta2)_3$	
	pEC_{50}	I_{max}	pEC_{50}	I_{max}
Wild type	7.302 ± 0.048	0.964 ± 0.018	6.990 ± 0.039	0.938 ± 0.016
S221E	7.009 ± 0.034*	0.986 ± 0.016	6.981 ± 0.080	0.982 ± 0.032
R57S; E78K; K140T; S221E	7.008 ± 0.028*	0.989 ± 0.012	6.491 ± 0.042*	1.044 ± 0.020

292 [1]Data are the mean ± standard error of the mean (n = 4).

293 [2]Asterisk indicates the data significantly differ from those obtained from the wild type nAChR.

294

295 Table 2. Effects of mutations in the Dα1 subunit on EC_{50} and I_{max} values[1] of imidacloprid and thiacloprid on wild-type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_3(\beta2)_2$

296 nAChRs expressed in *Xenopus laevis* oocytes

	Imidacloprid				Thiacloprid			
	$(D\alpha1)_3(\beta2)_2$		$(D\alpha1)_2(\beta2)_3$		$(D\alpha1)_3(\beta2)_2$		$(D\alpha1)_2(\beta2)_3$	
	pEC_{50}	I_{max}	pEC_{50}	I_{max}	pEC_{50}	I_{max}	pEC_{50}	I_{max}
Wild type	7.248 ± 0.205	0.118 ± 0.009	7.118 ± 0.257	0.085 ± 0.010	7.156 ± 0.232	0.044 ± 0.005	7.795 ± 0.153	0.039 ± 0.003
S221E	ND[2]	ND	6.460 ± 0.238	0.116 ± 0.014	6.158 ± 0.222*[3]	0.085 ± 0.012*	6.941 ± 0.235	0.035 ± 0.005
R57S; E78K; K140T; S221E	ND	ND	6.407 ± 0.214	0.034 ± 0.004*	ND	ND	6.931 ± 0.291*	0.011 ± 0.002*

297 [1]Data are the mean ± standard error of the mean (n = 4).

298 [2]Could not be determined because neonicotinoid-induced response did not attain maximum.

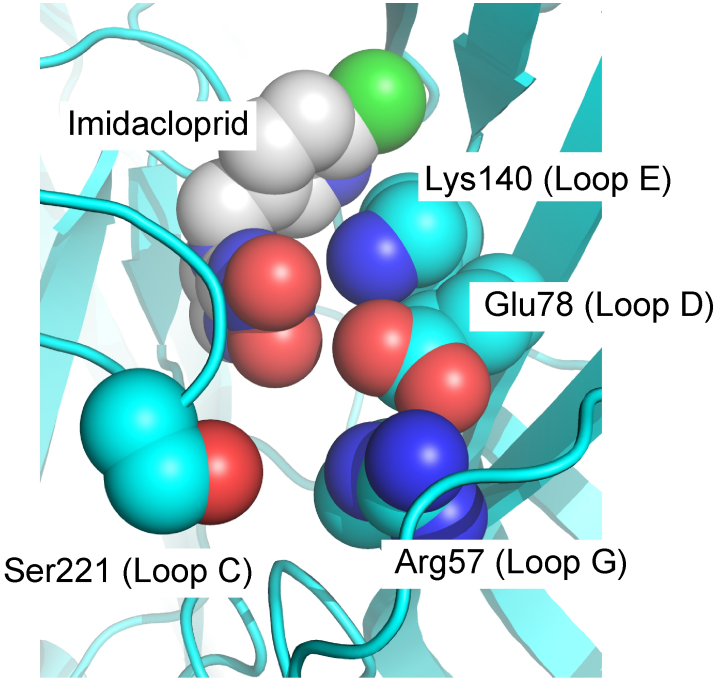
299 [3]Asterisk indicates the data significantly differ from those obtained from the wild type nACh.

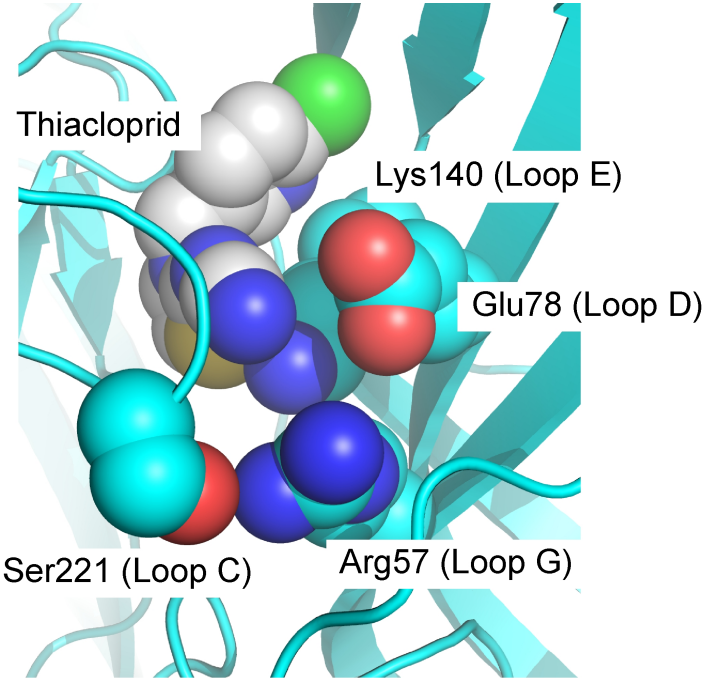
Figure legends

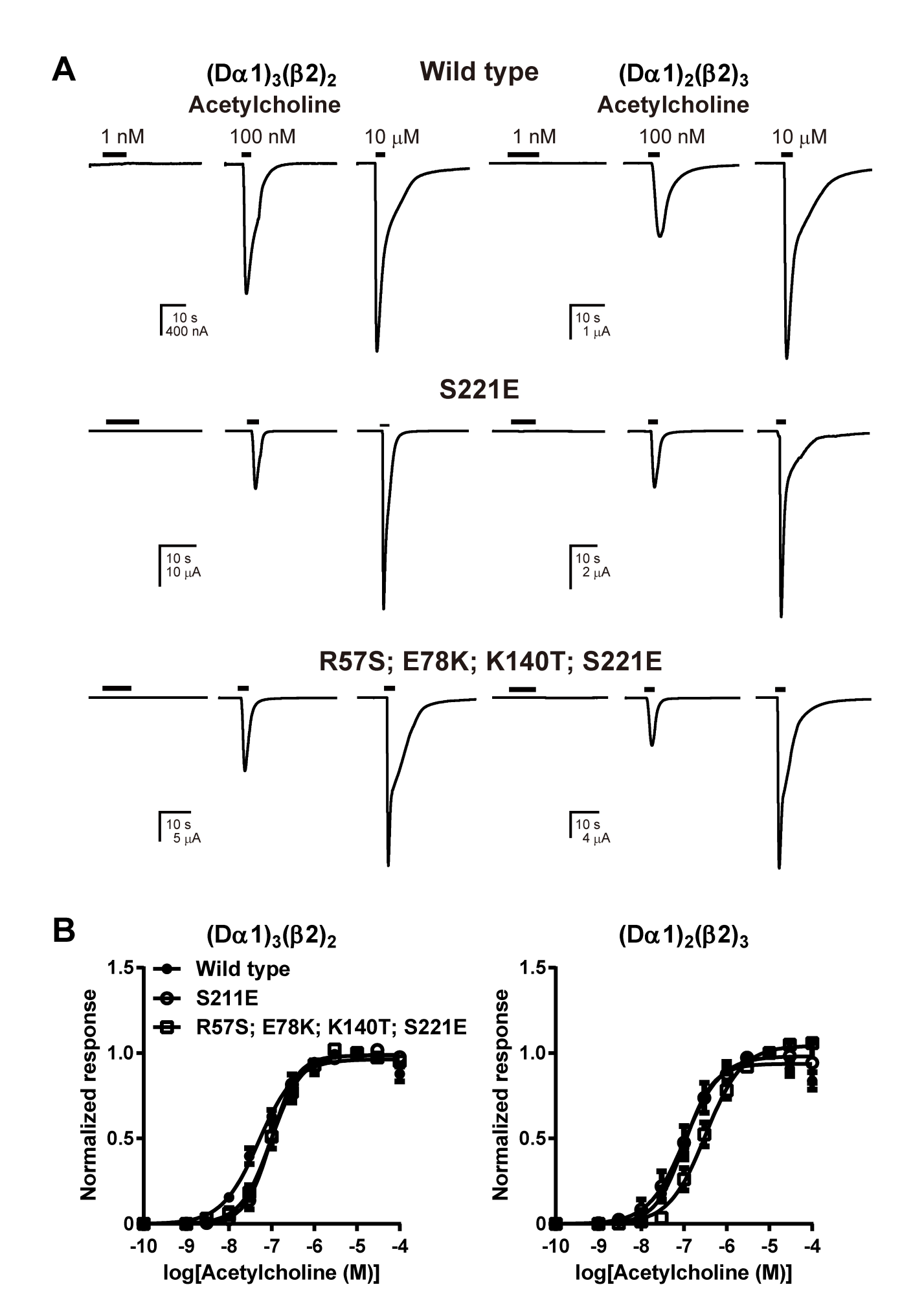
Figure 1. Computational models of the *Drosophila* Dα1 / Dα1 orthosteric site in complex with imidacloprid and thiacloprid. The models were constructed using Modeller version 9.19 [20]. The main chains are shown in cartoon format, whereas amino acid residues in loops C, D, E and G as well as imidacloprid and thiacloprid are drawn as space-fill models. Carbons, nitrogens and oxygens in the amino acid residues are colored cyan, blue and red, respectively, whereas carbons, chlorine, nitrogens and oxygens in the neonicotinoids are colored grey-white, green, blue and red respectively.

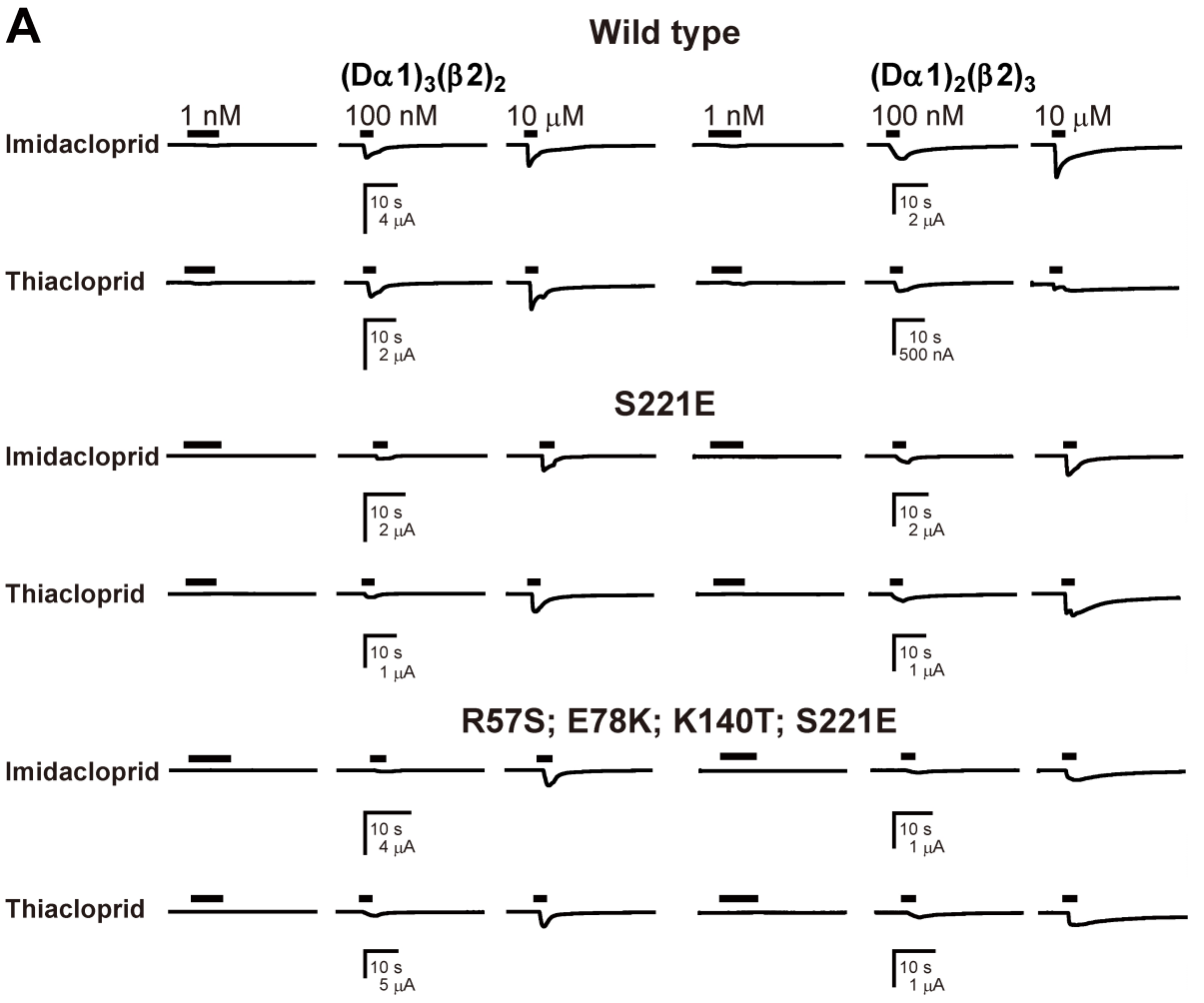
Figure 2. Agonist actions of acetylcholine (ACh) on wild type, Dα1 S221E mutant and R57S; E78K; K140T; S221E mutants of the *Drosophila* Dα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to ACh and the neonicotinoids from *Xenopus* oocytes expressing wild type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_3(\beta2)_2$ nAChRs. (B) Concentration-response curves for ACh for the hybrid nAChRs tested. Each plot represents mean ± standard error of the mean (n = 4 at least two frogs).

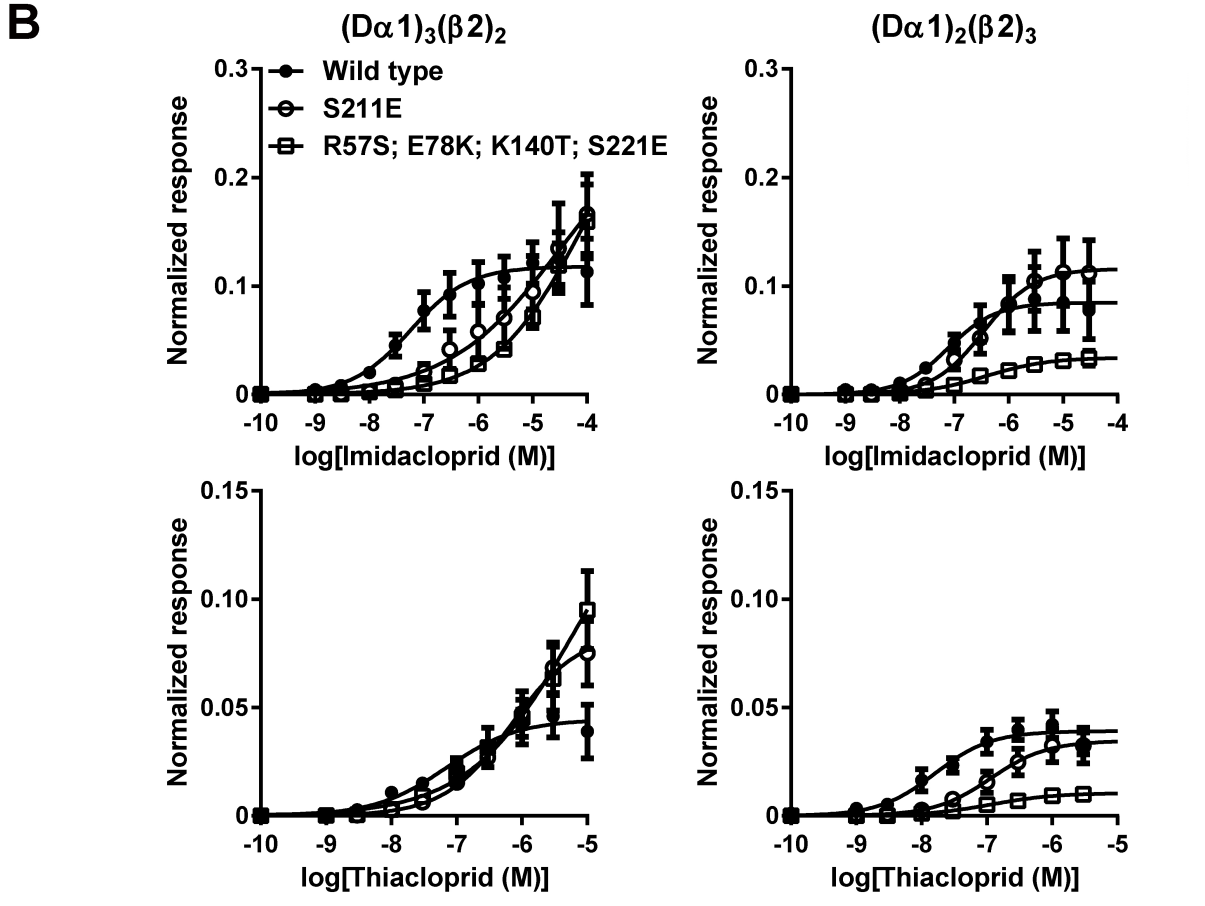
Figure 3. Agonist actions of imidacloprid and thiacloprid on wild type, S221E and R57S; E78K; K140T; S221E mutants of the *Drosophila* Dα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to the neonicotinoids from *Xenopus* oocytes expressing the wild type and mutant $(D\alpha1)_3(\beta2)_2$ and $(D\alpha1)_3(\beta2)_2$ nAChRs. (B) Concentration-response curves of imidacloprid and thiacloprid for the hybrid nAChRs studied. Each plot represents mean ± standard error of the mean (n = 4, at least two frogs).


Imidacloprid
Lys140 (Loop E)
Glu78 (Loop D)
Ser221 (Loop C)
Arg57 (Loop G)


Thiacloprid
Lys140 (Loop E)
Glu78 (Loop D)
Ser221 (Loop C)
Arg57 (Loop G)


A
(Dα1)3(β2)2
Acetylcholine
Wild type
(Dα1)2(β2)3
Acetylcholine
1 nM
100 nM
10 μM
1 nM
100 nM
10 μM
10 s
400 nA
10 s
1 μA
S221E
10 s
10 μA
10 s
2 μA
R57S; E78K; K140T; S221E
10 s
5 μA
10 s
4 μA
B
(Dα1)3(β2)2
(Dα1)2(β2)3
Wild type
S211E
R57S; E78K; K140T; S221E
Normalized response
1.5
1.0
0.5
0
-10
-9
-8
-7
-6
-5
-4
log[Acetylcholine (M)]


A
Wild type
(Dα1)3(β2)2
(Dα1)2(β2)3
1 nM
100 nM
10 μM
Imidacloprid
Thiacloprid
10 s
4 μA
2 μA
500 nA
S221E
1 μA
R57S; E78K; K140T; S221E
5 μA


B
(Dα1)3(β2)2
(Dα1)2(β2)3
Wild type
S211E
R57S; E78K; K140T; S221E
Normalized response
log[Imidacloprid (M)]
log[Thiacloprid (M)]