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Modulation by neonicotinoids of honeybee α1/chicken β2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes

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ABSTRACT

Neonicotinoids targeting insect nicotinic acetylcholine (ACh) receptors (insect nAChRs) are used for crop protection, but there is a concern about adverse effects on pollinators such as honeybees (*Apis mellifera*). Thus, we investigated the agonist actions of neonicotinoids (imidacloprid, thiacloprid and clothianidin) on *A. mellifera* α1 (Amα1)/chicken β2 hybrid nAChRs in *Xenopus laevis* oocytes according to the subunit stoichiometry of $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ using voltage-clamp electrophysiology. ACh activated $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs with similar current amplitude. We investigated the agonist activity of imidacloprid, thiacloprid and clothianidin for the two hybrid nAChRs and found that: 1) imidacloprid showed higher affinity than clothianidin, whereas clothianidin showed higher efficacy than imidacloprid for the nAChRs; 2) Thiacloprid showed the highest agonist affinity and the lowest efficacy for the nAChRs. The Amα1/β2 subunit ratio influenced the efficacy of imidacloprid and thiacloprid, but hardly affected that of clothianidin. Hydrogen bond formation by the NH group in clothianidin with the main chain carbonyl of the loop B may account, at least in part, for the unique agonist actions of clothianidin on the hybrid nAChRs tested.

1. Introduction

Honeybees (*Apis mellifera*) pollinate crop plants as well as produce honey and wax. Therefore, honeybees are essential for sustainable food production. It has been reported since the 2000s that a large number of bees have disappeared in the US and Europe (VanEngelsdorp et al., 2007; Steinhauer et al., 2014; Lambert et al., 2013; Williams et al., 2010). This phenomenon is referred to as "Colony Collapse Disorder (CCD)": Workers decrease while food, larvae, and queens remain, but no corpses are found in the vicinity of the nest (Vanengelsdorp et al., 2009). CCD has been attributed to viruses (Chen et al., 2014), mites (Martin et al., 2012), agricultural chemicals (Zhu et al., 2014; Mesnage and Antoniou, 2018), environmental stresses (Li et al., 2018), and a combination of these factors (Kielmanowicz et al., 2015; Pettis et al., 2013; Straub et al., 2019), but the mechanism is not clearly understood. Among these risk factors, synthetic pesticides, notably neonicotinoids, have been demonstrated as a major risk to bees (Woodcock et al., 2017; Forfert et al., 2017).

Neonicotinoids with good plant systemic activity and pest control efficacy are used widely for crop protection (Jeschke et al., 2011). Neonicotinoids act on insect nicotinic acetylcholine receptors (nAChRs) as competitive modulators (Ihara and Matsuda, 2018). nAChRs are ligand-gated ion channels belonging to the Cys-loop superfamily and function as pentamers of subunits with four transmembrane domains (Changeux, 2012). Neonicotinoids bind to the orthosteric site formed by the N-terminal extracellular domain at subunit interfaces (Ihara et al., 2017; Matsuda et al., 2005; Matsuda et al., 2009; Matsuda et al., 2020). During prolonged exposure of poisoning, neonicotinoids desensitize nAChRs, since they bind much more strongly to the desensitized state (Salgado and Saar, 2004). Most nAChRs are heteromers consisting of α and non-α subunits. Neonicotinoids bind not only to α/non-α subunit interfaces but also to α/α subunit interfaces (Ihara and Matsuda, 2018; Matsuda et al., 2020). nAChRs are widely expressed in the central nervous systems and involved in learning and memory (Gauthier, 2010). This is a reason why neonicotinoids affect bee learning and memory, and are presumed to be a contributing cause of CCD (Farooqui, 2013). Hence, it is of value to clarify the mechanism of interactions of neonicotinoids with honeybee nAChRs at a molecular level.

Although it is difficult to express robust insect nAChRs in *Xenopus*

Abbreviations: ACh, acetylcholine; Amα1, *Apis mellifera* α1; DMSO, dimethyl sulfoxide; EC_{50}, half maximal concentration; I_{max}, normalized maximum concentration; nAChR, nicotinic acetylcholine receptor; n_H, Hill coefficient

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laevis oocytes or cell lines, α-subunits of some insect species have been shown to form hybrid nAChRs when co-expressed with vertebrate non-α subunits (Ihara and Matsuda, 2018; Ihara et al., 2017). Therefore, we co-expressed honeybee α1 (Amα1) subunit with chicken β2 subunit nAChRs in *Xenopus* oocytes to investigate the agonist actions of neonicotinoids (imidacloprid, thiacloprid and clothianidin) on the hybrid nAChRs, employing voltage clamp electrophysiology.

2. Methods

2.1. Xenopus laevis oocytes

In compliance with the UK Animals (Scientific Procedures) Act 1986, female *Xenopus* were anesthetized with benzocaine (ethyl 4-aminobenzoate) and minimum amounts of oocytes were used. Oocytes were treated with 2 mg mL^{-1} Type IA collagenase (Merck/Sigma-Aldrich, St. Louis, MO, USA) in Ca^{2+}-free standard oocyte saline (Ca^{2+} free SOS: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES; pH 7.6), then 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) to remove the follicle layers.

2.2. cRNAs

The amino acid sequences of the honeybee α1 subunit (XP_026298411) and chicken β2 subunit (NP_990144) on the Refseq database were used. cRNAs encoding each subunit was prepared using an mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and diluted to 1 mg mL^{-1} with RNase-free water (Ihara et al., 2018). Injection of 50 nL of the cRNA with a mixture ratio of honeybee α1 and chicken β2 subunits at 5:1 and 1:5 ratios was performed to express $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs in oocytes (Hikida et al., 2018). Incubation at 16 °C was carried out for 3 days in SOS with penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), gentamicin (20 μg mL^{-1}), and sodium pyruvate (2.5 mM).

2.3. Chemicals

Neonicotinoids were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan), and ACh was purchased from Merck/Sigma-Aldrich. Each neonicotinoid was dissolved in dimethyl sulfoxide (DMSO) to prepare a 100 mM stock solution, and test solutions were prepared by diluting the stock solution with SOS containing 0.5 μM atropine (to suppress the response of endogenous muscarinic receptors). The final concentration of DMSO was 0.1% or lower, and 0.1% DMSO had no effect on the agonist effects of neonicotinoids as well as of ACh.

2.4. Voltage-clamp electrophysiology

Oocytes expressing nAChRs were subjected to two-electrode voltage clamp electrophysiology using an Axoclamp900A amplifier (Molecular Devices, San Jose, CA, USA) (Ihara et al., 2003; Shimomura et al., 2006). SOS supplemented with 0.5 μM atropine was perfused at a flow rate of 7–10 mL min^{-1} using gravity (Shimomura et al., 2002; Toshima et al., 2009; Matsuda et al., 1998). Membrane potential was fixed at −100 mV with an electrode filled with 2 M KCl, and inward currents induced by agonist treatment were recorded using Clampex (Molecular Devices). The recorded data digitized by Digidata 1550B A/D converter (Molecular Devices) were then analyzed using Clampfit (Molecular Devices).

2.5. Modeling the Amα1/chicken β2 subunit interface in complex with clothianidin

A homology model of the Amα1/chicken β2 nAChR was constructed using Modeller (Webb and Sali, 2016) with the crystal structure of *Lymnaea stagnalis* ACh binding protein (*Ls*-AChBP) complexed with clothianidin (PDB id: 2zjv) (Ihara et al., 2008) as a structural template. To build the model, amino acid sequences of the N-terminal extracellular region of the Amα1 nAChR subunit (XP_026298411: residue number 44–261) and chicken β2 nAChR subunit (NP_990144: residue number 19–226) were aligned with the amino acid sequences of *Ls*-AChBP using MAFFT (Katoh and Standley, 2013). Details of the alignment were adjusted manually, and then the homology model of $(Amα1)_2$(chicken $β2)_3$ nAChR was built using the Modeller automodel algorithm, where clothianidin were placed at the Amα1(+)-chicken β2(−) subunit interfaces as a rigid body. The structure coordinate of the homology model was visualized using PyMOL.

2.6. Data analysis

The peak current amplitude for each concentration of agonist was normalized with an amplitude of responses to 10 and 100 μM ACh of $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ hybrid nAChRs, respectively, a concentration–response curve was prepared from the equation below using Prism 6 (GraphPad Software, San Diego, CA, USA), where Y is the normalized response amplitude, I_{max} is the normalized maximum response, EC_{50} is the half-maximum effect concentration (M), X is the log [agonist concentration (M)], and n_H is the Hill coefficient. Response data at each concentration was tested at $n = 4$ using oocytes from at least two frogs.

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

3. Results and discussion

We co-injected cRNAs of Amα1 and chicken β2 subunits at ratios of 5:1 and 1:5 to express $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ hybrid nAChRs, respectively, in *Xenopus* oocytes. As a result, robust hybrid nAChRs were formed in oocytes with similar amplitude responses to ACh (Fig. 1A, B). Hence, the agonist efficacy of neonicotinoids represented by the I_{max} value reflected the current amplitude of the response to the compounds tested. ACh showed higher agonist affinity in terms of the pEC_{50} (-logEC_{50} (M)) values for $(Amα1)_3(β2)_2$ nAChR (6.95 ± 0.03) than $(Amα1)_2(β2)_3$ nAChR (6.50 ± 0.03) (Table 1).

Having found robust expression of $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs, the agonist actions of neonicotinoids (imidacloprid, thiacloprid and clothianidin) were investigated with these recombinant nAChRs. Imidacloprid activated both nAChRs with similar pEC_{50} values [$(Amα1)_3(β2)_2$ nAChR: 7.22 ± 0.16; $(Amα1)_2(β2)_3$ nAChR: 7.18 ± 0.10], whereas its agonist efficacy in terms of I_{max} for $(Amα1)_3(β2)_2$ nAChR was 2.6-fold larger than that for $(Amα1)_2(β2)_3$ nAChR (Fig. 2, Table 1), indicative of a selective interaction of imidacloprid with the Amα1/Amα1 subunit interface. The agonist affinity in pEC_{50} of imidacloprid was comparable with that reported previously for the recombinant nAChRs formed by the *Drosophila melanogaster* Dα1 subunit and the chicken β2 subunit [$(Dα1)_3(β2)_2$ nAChR: 7.25; $(Dα1)_2(β2)_3$ nAChR: 7.12] (Hikida et al., 2018), suggesting that the structural features of the Amα1 subunit involved in the interactions with imidacloprid resembled those of the Dα1 subunit.

Thiacloprid showed the highest agonist affinity for the $(Amα1)_3(β2)_2$ nAChR (pEC_{50} = 7.73 ± 0.18) and the $(Amα1)_2(β2)_3$ nAChR ((pEC_{50} = 7.52 ± 0.31) among the neonicotinoids tested (Fig. 3, Table 1). Also, its affinity for the $(Amα1)_3(β2)_2$ nAChR was higher than those observed for the $(Dα1)_3(β2)_2$ nAChR (7.16) (Hikida et al., 2018), suggesting that the Amα1 subunit is more favorable than

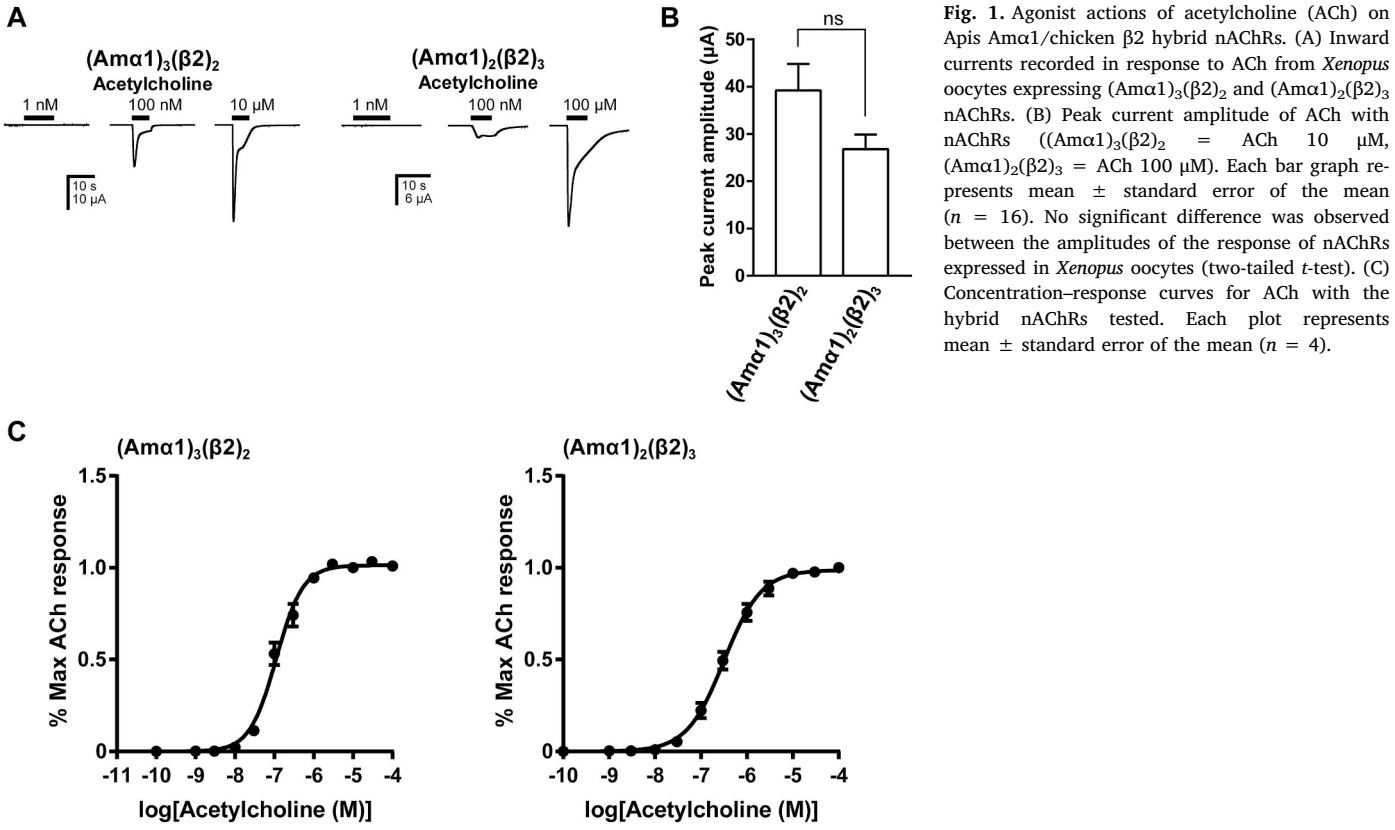

Fig. 1. Agonist actions of acetylcholine (ACh) on Apis Amα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to ACh from *Xenopus* oocytes expressing $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs. (B) Peak current amplitude of ACh with nAChRs ($(Amα1)_3(β2)_2$ = ACh 10 μM, $(Amα1)_2(β2)_3$ = ACh 100 μM). Each bar graph represents mean ± standard error of the mean (n = 16). No significant difference was observed between the amplitudes of the response of nAChRs expressed in *Xenopus* oocytes (two-tailed t-test). (C) Concentration–response curves for ACh with the hybrid nAChRs tested. Each plot represents mean ± standard error of the mean (n = 4).

the Dα1 subunit for binding thiacloprid. This appears to indicate that using thiacloprid for crop protection is a risk for bees. However, thiacloprid is metabolized by cytochrome P450 enzymes in honeybees (Manjon et al., 2018), which plays an equally important role as intrinsic nAChR potency/efficacy in determining whole-organism activity. The agonist efficacy of thiacloprid was lowest among the neonicotinoids tested (Fig. 3, Table 1). It is therefore suggested that the antagonist action is more important than the agonist action in determining the toxicity.

As in the case of imidacloprid, the efficacy of thiacloprid reduced with decrease of the Amα1/β2 subunit ratio, pointing to selective binding of thiacloprid to the Amα1/Amα1 subunit interface. The agonist efficacy (I_{max}) not only of imidacloprid, but also of thiacloprid, for the $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs was lower than that for the $(Dα1)_3(β2)_2$ and $(Dα1)_2(β2)_3$ nAChRs (Hikida et al., 2018). Hence, it is conceivable that the interactions of these neonicotinoids with the Amα1 subunit at concentrations where the response amplitude attained a maximum are weaker than those with the Dα1 subunit.

Clothianidin was also a partial agonist of $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs with lower affinity but higher efficacy than imidacloprid (Fig. 4, Table 1). Provided that pEC_{50} and I_{max} reflect the interactions with the resting and activated state of nAChRs, respectively, the result may suggest that clothianidin can bind to the orthosteric site more potently than imidacloprid in the activated state. The efficacy of clothianidin was hardly affected by the Amα1/β2 subunit ratio, indicating an interaction unique to this neonicotinoid. To clarify the mechanism for the unique actions of clothianidin, we modeled the Amα1/β2 subunit interfaces complexed with clothianidin (Fig. 5). The models revealed a hydrogen bond formed between the main chain of loop B in the Amα1 subunit and the NH group in clothianidin, which imidacloprid and thiacloprid lacks (Fig. 2, 3), accounting, at least in part, for the similar efficacy of clothianidin for the $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs. (See Fig. 5).

Despite lacking the NH group, however, ACh can activate the nAChRs with the highest efficacy. This is because ACh is most flexible and smallest among the ligands tested and therefore has capacity to persistently bind to the orthosteric site even if the conformation of the site changes dramatically in response to activation (Nemecz et al., 2016; Unwin, 2013). On the other hand, the conformationally-restricted imidazolidine ring of imidacloprid and thiazolidine ring of thiacloprid interact with the orthosteric site in a totally different way from ACh. These rings as well as the guanidine moiety of clothiandin stack with a tyrosine residue in loop C. Furthermore, the methylene (CH_2-CH_2) moiety of the imidazolidine and thiazolidine rings undergoe the CH-π interactions with a tryptophan ring in loop B to enhance the binding to the orthosterci site (Ihara et al., 2008; Ihara et al., 2014). It is

Table 1
Agonist actions of acetylcholine and neonicotinoids on honeybee Amα1/chicken β2 hybrid nAChRs expressed in *Xenopus laevis* oocytes.[a]

	$(Amα1)_3(β2)_2$		$(Amα1)_2(β2)_3$	
	pEC_{50}	I_{max}	pEC_{50}	I_{max}
Acetylcholine	6.95 ± 0.03	1.01 ± 0.02	6.50 ± 0.03	0.988 ± 0.016
Imidacloprid	7.22 ± 0.16	0.013 ± 0.001	7.18 ± 0.10	0.0046 ± 0.0003
Thiacloprid	7.73 ± 0.18	0.0021 ± 0.0002	7.52 ± 0.31	0.00065 ± 0.00009
Clothianidin	6.21 ± 0.14	0.262 ± 0.019	6.03 ± 0.10	0.353 ± 0.018

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

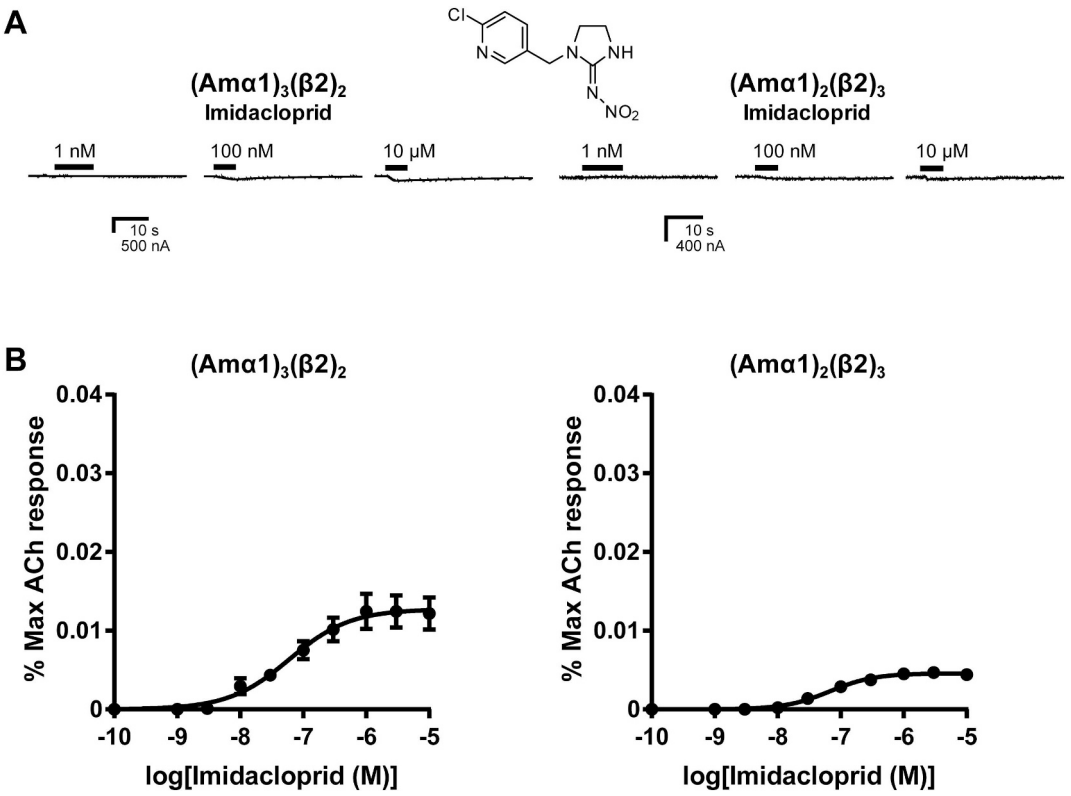

Fig. 2. Agonist actions of imidacloprid on Amα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to imidacloprid from *Xenopus* oocytes expressing $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs. (B) Concentration–response curves of imidacloprid with the hybrid nAChRs tested. Each plot represents mean ± standard error of the mean (n = 4).

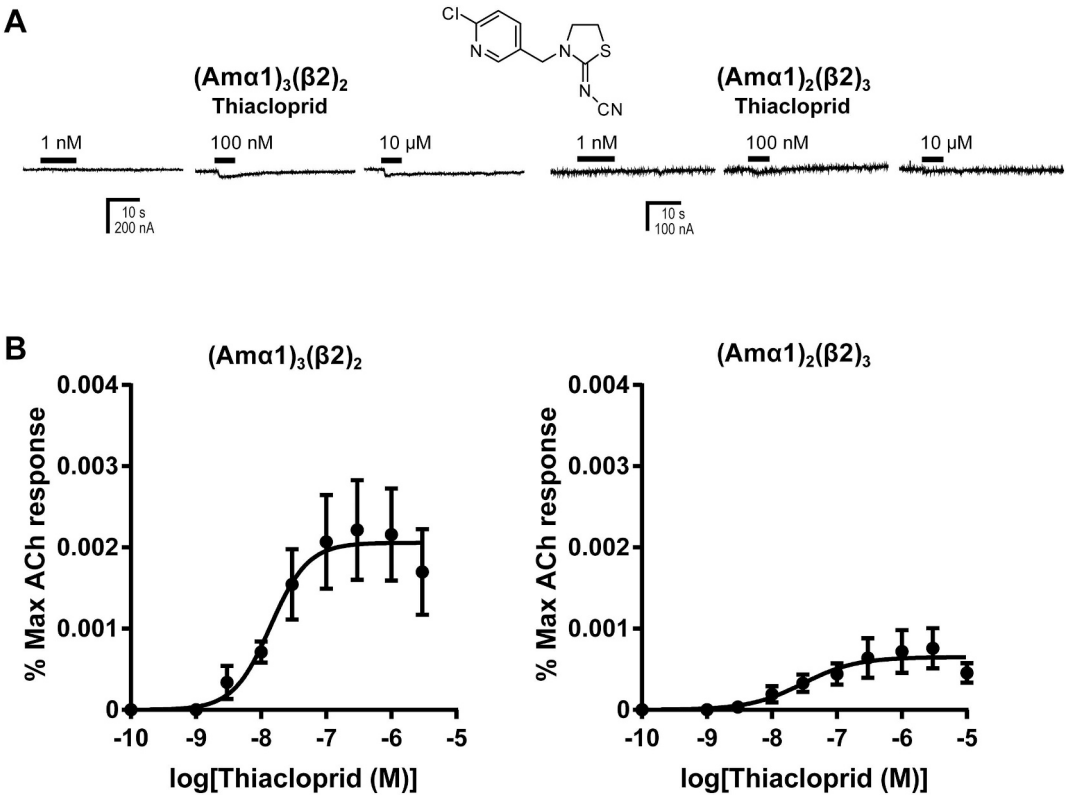

Fig. 3. Agonist actions of thiacloprid on Amα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to imidacloprid from *Xenopus* oocytes expressing $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs. (B) Concentration–response curves of imidacloprid with the hybrid nAChRs tested. Each plot represents mean ± standard error of the mean (n = 4).

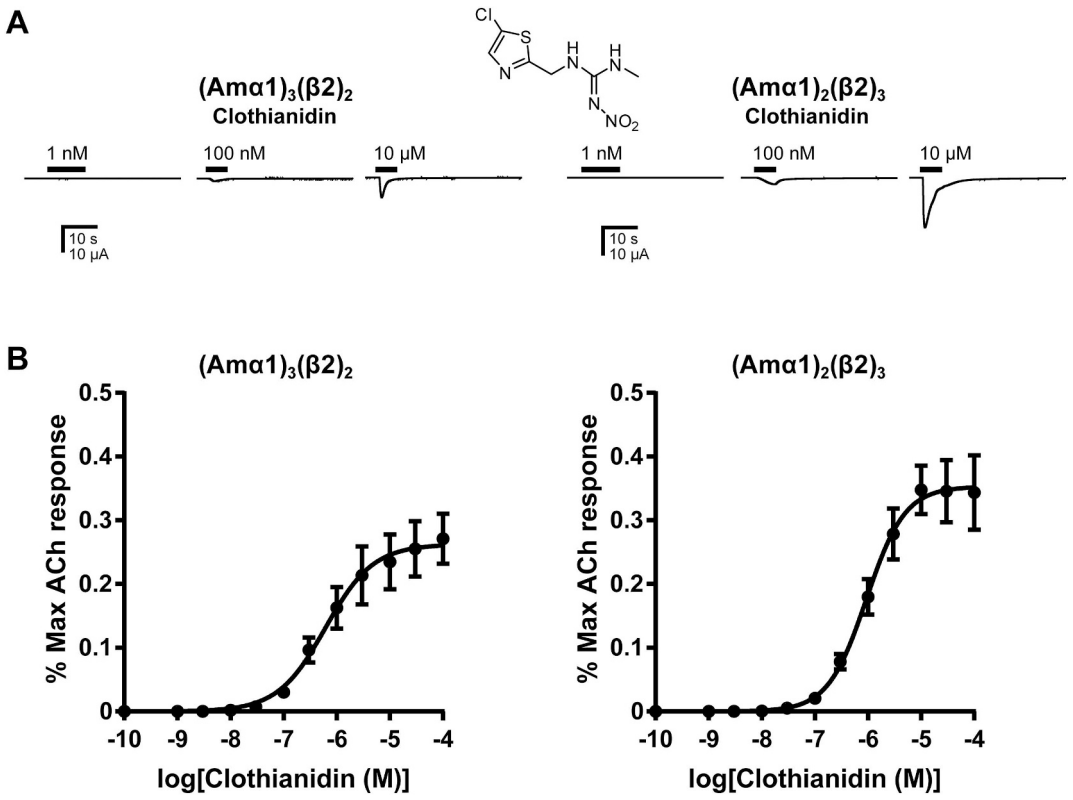

Fig. 4. Agonists action of clothianidin on Amα1/chicken β2 hybrid nAChRs. (A) Inward currents recorded in response to clothianidin from *Xenopus* oocytes expressing $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs. (B) Concentration–response curves of clothianidin with the hybrid nAChRs tested. Each plot represents mean ± standard error of the mean (n = 4).

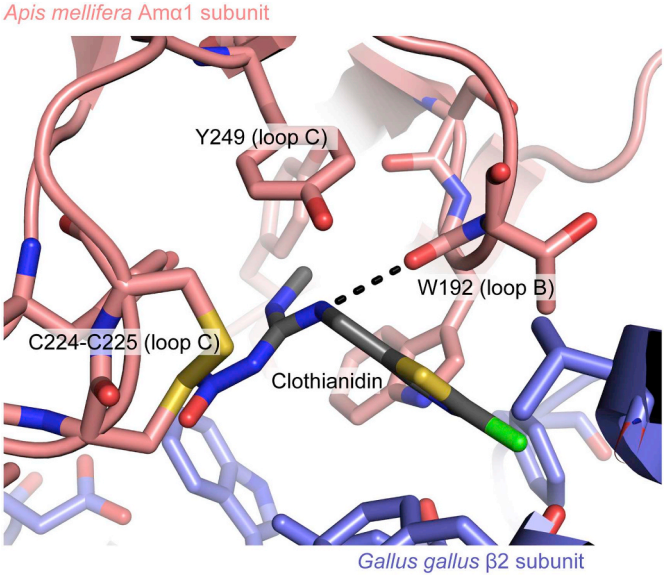

Fig. 5. Model of the honeybee Amα1/chicken β2 subunit interface in complex with clothianidin. Main chain of the honeybee (*Apis mellifera*) Amα1 subunit and chicken (*Gallus gallus*) β2 subunit illustrated as cartoon colored salmon pink and slate blue, respectively. In clothianidin, the carbon, nitrogen, oxygen, chlorine, and sulfur atoms are colored dark grey, blue, red, green, and yellow, respectively. Clothianidin formed a hydrogen bond shown as a broken line between its NH and the main chain carbonyl of Trp192 in loop B of the Amα1 subunit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

postulated that the methylene moiety of these two neonicotinoids moves away from the position where the moiety can contact the tryptophan ring, resulting in reduced efficacy. Alternatively, the methylene moiety may hinder their binding to the orthosteric site by steric contacts. By contrast, clothianidin does not rely much on the CH-π interactions of the methyl group and therefore showed lower affinity for the resting nAChRs compared to imidacloprid and thiacloprid.

Imidacloprid was shown to modulate the desensitizing and non-desensitizing components of nAChRs with higher affinity for the desensitizing component in the American cockroach neurons (Salgado and Saar, 2004). Similarly, the neonicotinoids tested are likely to modulate the two components of $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs with distinct affinities. Indeed, imidacloprid appeared to evoke a slowly-desensitizing single component (Fig. 2), whereas clothianidin evoked the two components in the $(Amα1)_2(β2)_3$ nAChR (Fig. 4). Although these findings are interesting, we did not further study the effects because the efficacy of thiacloprid was too low to evaluate with accuracy and such effects were varied with the concentrations tested. However, it is of value to study the role of the two components in determining affinity and efficacy of neonicotinoids using other nAChRs to enhance our understanding of the action mechanism of neonicotinoids.

In conclusion, we have shown for the first time that the Amα1 subunit can form robust nAChRs with the chicken β2 subunit when co-expressed in *Xenopus* oocytes. Therefore, we investigated the agonist actions of imidacloprid, thiacloprid and clothianidin on $(Amα1)_3(β2)_2$ and $(Amα1)_2(β2)_3$ nAChRs expressed in oocytes and found that these neonicotinoids act as partial agonists with different affinity and efficacy. Clothianidin showed higher efficacy than imidacloprid and thiacloprid regardless of the subunit composition, probably relying on hydrogen bond formation with the main chain in loop B and/or its steric

property. However, our data were obtained only with honeybee α1-avian β2 hybrid nAChRs; therefore, it is essential to examine in the future the effects of neonicotinoids on nAChRs consisting purely of honeybee subunits as well as on the other hybrid nAChRs to understand their toxicity to honeybees in detail.

Declaration of competing interest

There is no conflict of interest to declare.

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