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Selectivity of lynx proteins on insect nicotinic acetylcholine receptors in the brown planthopper, *Nilaparvata lugens*

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Abstract

Neuronal nicotinic acetylcholine receptors (nAChRs) are major excitatory neurotransmitter receptors in both vertebrates and invertebrates. Two lynx proteins (Nl-lynx1 and Nl-lynx2) have been identified in the brown planthopper, *Nilaparvata lugens*, which act as modulators on insect nAChRs. In the present study, two lynx proteins were found to act on the triplet receptor Nlα1/Nlα2/β2 expressed in *Xenopus* oocytes, increasing agonist-evoked macroscopic currents, but not changing agonist sensitivity and desensitization properties. Nl-lynx1 and Nl-lynx2 increased I_{max} (maximum responses) of acetylcholine to 4.85-fold and 2.40-fold of that of Nlα1/Nlα2/β2 alone, and they also increased I_{max} of imidacloprid to 2.57-fold and 1.25-fold. Although, on another triplet nAChRs Nlα3/Nlα8/β2, Nl-lynx2 increased I_{max} of acetylcholine and imidacloprid to 3.63-fold and 2.16-fold, Nl-lynx1 had no effects on I_{max} of either acetylcholine or imidacloprid. The results demonstrate the selectivity of lynx proteins for different insect nAChR subtypes. This selectivity was also identified in native *N. Lugens*. Co-immunoprecipitation was found between Nlα1/Nlα2-containing receptors and both Nl-lynx1 and Nl-lynx2, but was only found between Nlα3/Nlα8-containing receptors and Nl-lynx2. When the previously identified Nlα1^{Y151S} and Nlα3^{Y151S} mutations were included (Nlα1^{Y151S}/Nlα2/β2 and Nlα3^{Y151S}/Nlα8/β2), the increase in I_{max} of imidacloprid, but not acetylcholine, caused by co-expression of Nl-lynx1 and Nl-lynx2 was more noticeable than that of their wildtype counterparts. Taken together, these data suggest that two modulators, Nl-lynx1 and Nl-lynx2, might serve as an influencing factor in target site insensitivity in *N. lugens*, such as Y151S mutation.

Keywords: nicotinic acetylcholine receptors (nAChRs), nAChR modulator, *Nilaparvata lugens*, neonicotinoids resistance.

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Introduction

Nicotinic acetylcholine receptors (nAChRs) are neurotransmitter-gated ion channels, which mediate fast cholinergic synaptic transmission in insect and vertebrate nervous systems (Sattelle, 1980; Matsuda *et al*., 2001). Seventeen nAChR subunits (α1-α10, β1-β4, γ, δ and ε) have been identified in vertebrate species which generate a diverse family of nAChR subtypes (Millar, 2003). In the model insect species *Drosophila melanogaster*, 10 nAChR subunits (Dα1-Dα7 and Dβ1-Dβ3) have been identified, and a similar number of subunits have been identified in other insect species (Jones *et al*., 2007; Millar & Denholm, 2007). Heterologous expression of cloned nAChR subunits has provided a powerful approach to characterizing these receptors, but difficulties have been encountered in heterologous expression of insect nAChRs (Millar, 1999). Despite this, expression of functional recombinant nAChRs has been reported for several insect nAChR subunits when co-expressed with vertebrate nAChR subunits such as rat β2 subunit (Bertrand *et al*., 1994; Lansdell & Millar, 2000).

Because no functional nAChRs have been obtained when different insect subunits are combined in heterologous expression systems, it is thought that some proteins other than nAChRs themselves might play important roles in insect nAChR formation *in vivo* and *in vitro*, such as the chaperones or modulators (Choo *et al.*, 2008; Lansdell *et al.*, 2008; Millar, 2008). Recently, two members (Nl-lynx1 and Nl-lynx2) of the Ly-6/neurotoxin superfamily were identified in *Nilaparvata lugens*, which acted as modulators on insect nAChRs and increased agonist-evoked macroscopic currents of hybrid receptors Nlα1/β2, but did not change agonist sensitivity and desensitization properties (Liu *et al.*, 2009b). These two lynx proteins showed more notable effects on mutant receptors $Nl\alpha1^{Y151S}/\beta2$, which were associated with neonicotinoid resistance in *N. lugens* (Liu *et al.*, 2005, 2006). Because the mutation Y151S was found in two *N. lugens* α subunits (Nlα1 and Nlα3), and these two subunits were found to be involved in different receptors (Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2) (Zhang *et al.*, 2009; Liu *et al.*, 2009a), we wish to know whether there is selectivity of two lynx proteins on Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 receptors. Besides this main aim, the different influences of two lynx proteins on wildtype and mutant receptors (containing Y151S mutation) were also included.

Results

Co-expression of nicotinic acetylcholine receptors and lynx proteins in Xenopus *oocytes*

In our previous study, two lynx proteins (Nl-lynx1 and Nl-lynx2) were identified in *N. lugens* and were co-expressed with Nlα1/β2 receptor in *Xenopus* oocytes (Liu *et al.*, 2009b). In the present study, co-expression of these two lynx proteins and two triplet nAChRs (three subunits in one receptor: Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2) was performed in *Xenopus* oocytes.

On the triplet nAChRs, co-expression of lynx proteins changed the agonist-evoked macroscopic currents of receptors, but did not change agonist sensitivity, which is similar to that found in co-expression of lynx proteins and Nlα1/β2 (Liu *et al.*, 2009b). Nl-lynx1 and Nl-lynx2 increased the maximum responses (I_{max}) of acetylcholine to 4.85-fold (1240.6 ± 99.4 nA; $n = 4$) and 2.40-fold (613.9 ± 83.1 nA; $n = 4$) of that of Nlα1/Nlα2/β2 (255.8 ± 22.6 nA; $n = 3$) alone (Fig. 1A), and they also increased I_{max} of imidacloprid to 2.57-fold (477.0 ± 60.5 nA; $n = 4$) and 1.25-fold (232.0 ± 38.6 nA; $n = 4$) of that of Nlα1/Nlα2/β2 (185.6 ± 20.8 nA; $n = 3$) alone (Fig. 1B). Although Nl-lynx2 increased I_{max} of acetylcholine and of imidacloprid to 3.63-fold (1276.3 ± 95.0 nA; $n = 4$) and 2.16-fold (603.5 ± 53.3 nA; $n = 4$) of that of Nlα3/Nlα8/β2 (351.6 ± 33.2 nA for acetylcholine and 279.4 ± 33.1 nA for imidacloprid; $n = 3$) alone, Nl-lynx1 had no effect at all on I_{max} of either acetylcholine (343.9 ± 40.8 nA; $n = 3$) or imidacloprid (283.3 ± 37.6 nA; $n = 3$) (Fig. 1C, D). Co-expression of Nl-lynx1 and Nl-lynx2 had no effect on agonist sensitivity (EC_{50}) of acetylcholine or imidacloprid on two receptors Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 (Table 1). Co-expression of Nl-lynx1 and Nl-lynx2 did not change desensitization properties of receptors (data not shown) either, which is similar to the results of our previous study (Liu *et al.*, 2009b).

Co-immunoprecipitation of lynx proteins and nicotinic acetylcholine receptors in native proteins

The electrophysiological data described above provides evidence that Nl-lynx1 and Nl-lynx2 act as modulators on Nlα1/Nlα2/β2, but that only Nl-lynx2 acts on Nlα3/Nlα8/β2. To examine whether these interactions occurred in native proteins, immunoprecipitation studies were performed with subunit-specific antibodies. Our previous studies showed that Nlα3 and Nlα8 co-assembled in *N. lugens* native nAChRs (Zhang *et al.*, 2009), and recently Nlα1 and Nlα2 were also found to be co-assembled in native nAChRs (data not shown).

Detergent extracts of *N. lugens* head membranes were immunoprecipitated with Nlα1-specific antiserum N1-I and immunoprecipitated proteins were then analysed by immunoblotting with either L1-I (Fig. 2C) or L2-I (Fig. 2E). N1-I uniquely detected Nlα1 protein (Fig. 2A, indicated by asterisk) in immunopellet precipitated by N1-I, which was blocked by addition of excess Nlα1 fusion protein (Fig. 2B, corresponding to the band indicated by a double asterisk). In immunopellet precipitated by N1-I, L1-I (Fig. 2C, indicated by a black triangle) and L2-I (Fig. 2E) detected a single specific band, which could be blocked by the corresponding fusion proteins of Nl-lynx1 (Fig. 2D) and Nl-lynx2 (Fig. 2F). The same immunoprecipitation and immunodetection procedure was performed between Nlα2 and Nl-lynx1/Nl-lynx2 with specific antiserum N2-I, L1-I and L2-I, which provided similar results to that observed between Nlα1 and Nl-lynx1/Nl-lynx2 (Table 2).

Immunoprecipitation and immunoblotting were also performed using Nlα3-specific antiserum N3-I and lynx-specific antisera (L1-I and L2-I). Similarly, N3-I uniquely detected Nlα3 protein (Fig. 3A) in immunopellet precipitated by N3-I, which was blocked by addition of excess Nlα3 fusion protein (Fig. 3B). In immunopellet precipitated by N3-I, L2-I (Fig. 3D) detected a single specific band, which could be blocked by the corresponding fusion protein of Nl-lynx2 (Fig. 3E). In contrast, L1-I did not detect a specific band for Nl-lynx1 in immunopellet precipitated by N3-I (Fig. 3C). In immunopellet precipitated by N8-I (Nlα8 specific antiserum), only L2-I, but not L1-I, detected a single specific band too (Table 2). Both L2-I and L1-I did not detect the specific bands in immunopellet precipitated by other subunits specific antisera, such as N4-I for Nlα4, N6-I for Nlα6 and N7-I for Nlα7 (Table 2).

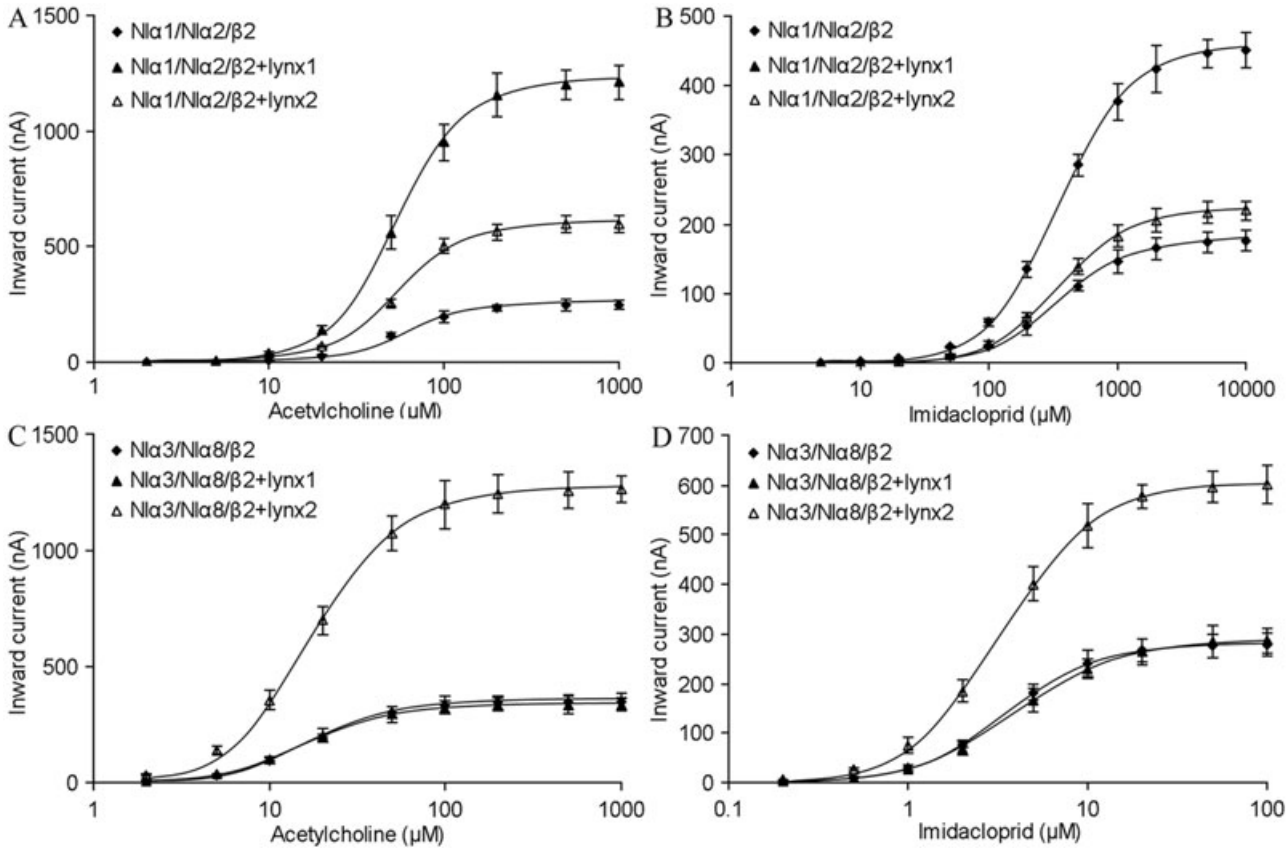

Figure 1. The effects of Nl-lynx1 and Nl-lynx2 on agonist-evoked responses of hybrid receptors Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 expressed in *Xenopus* oocytes. (A) Concentration-response curves for acetylcholine on Nlα1/Nlα2/β2 with or without Nl-lynx1 and Nl-lynx2. (B) Concentration-response curves for imidacloprid on Nlα1/Nlα2/β2 with or without Nl-lynx1 and Nl-lynx2. (C) Concentration-response curves for acetylcholine on Nlα3/Nlα8/β2 with or without Nl-lynx1 and Nl-lynx2. (D) Concentration-response curves for imidacloprid on Nlα3/Nlα8/β2 with or without Nl-lynx1 and Nl-lynx2. Data are means of at least three independent experiments (different batches of oocytes) ±SEM.

Effects of Nl-lynx1 and Nl-lynx2 on Y151S-containing nicotinic acetylcholine receptors

Previous studies of an imidacloprid-resistant population of *N. lugens* identified a resistance-associated point mutation (Y151S) in two nAChR subunits, Nlα1 and Nlα3 (Liu *et al.*, 2005). Because Nlα1 and Nlα3 were involved in two different receptors Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2, the effects of Nl-lynx1 and Nl-lynx2 on mutant receptors were also examined,

Table 1. Maximum current (I_{max}) and EC_{50} values for nicotinic acetylcholine receptor and modulator combinations

	Acetylcholine			Imidacloprid		
Combination	I_{max} (nA)	EC_{50} (μM)	*n*	I_{max} (nA)	EC_{50} (μM)	*n*
Nlα1/Nlα2/β2	255.8 ± 22.6	52.7 ± 7.5	3	185.6 ± 20.8	352.9 ± 31.5	3
Nlα1/Nlα2/β2+lynx1	1240.6 ± 99.4	54.3 ± 6.5	4	477.0 ± 60.5	346.8 ± 42.1	4
Nlα1/Nlα2/β2+lynx2	613.9 ± 83.1	51.7 ± 9.4	4	232.0 ± 38.6	356.9 ± 38.2	4
Nlα1^{Y151S}/Nlα2/β2	248.3 ± 35.2	64.8 ± 5.3	3	64.1 ± 7.6	610.5 ± 79.1	3
Nlα1^{Y151S}/Nlα2/β2+lynx1	1193.5 ± 94.6	62.1 ± 7.8	4	271.1 ± 30.5	598.3 ± 60.4	4
Nlα1^{Y151S}/Nlα2/β2+lynx2	591.0 ± 72.5	65.5 ± 6.2	4	148.1 ± 27.6	622.7 ± 93.6	4
Nlα3/Nlα8/β2	351.6 ± 33.2	18.2 ± 3.3	3	279.4 ± 33.1	3.5 ± 0.6	3
Nlα3/Nlα8/β2+lynx1	343.9 ± 40.8	16.9 ± 4.2	3	283.3 ± 37.6	3.9 ± 0.8	3
Nlα3/Nlα8/β2+lynx2	1276.3 ± 95.0	17.6 ± 4.5	4	603.5 ± 53.3	3.3 ± 0.5	4
Nlα3^{Y151S}/Nlα8/β2	342.7 ± 45.5	32.1 ± 3.9	3	45.8 ± 6.0	28.7 ± 3.4	3
Nlα3^{Y151S}/Nlα8/β2+lynx1	356.8 ± 58.7	30.9 ± 5.2	3	47.3 ± 8.1	27.9 ± 4.2	3
Nlα3^{Y151S}/Nlα8/β2+lynx2	1230.3 ± 98.7	34.3 ± 6.4	5	177.7 ± 26.2	29.5 ± 6.0	5

Data are means of three to five independent experiments (different batches of oocytes) ±SEM.

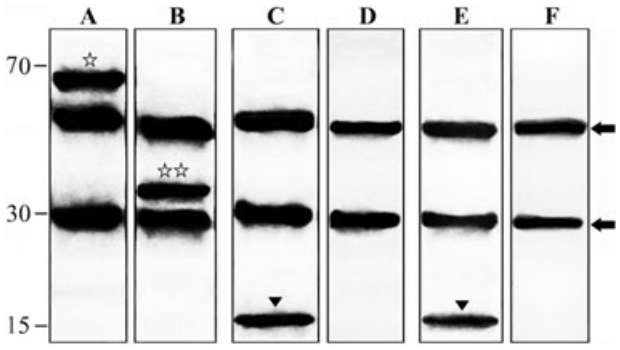

Figure 2. Co-immunoprecipitation of Nlα1 and Nl-lynx1/Nl-lynx2. (A) Nlα1-specific antibody N1-I uniquely detected Nlα1 protein (indicated by asterisk) in immunopellet precipitated by N1-I. The immunoglobulin heavy and light chains were also detected, because the same antibody was used for precipitation and immunodetection (in all experiments; indicated by arrow heads), which was confirmed by being probed only with secondary anti-mouse antibody (data not shown). (B) The unique Nlα1 protein detected by N1-I in A was blocked by addition of excess Nlα1 fusion protein (corresponding to the band indicated by a double asterisk). (C) In immunopellet precipitated by N1-I, Nl-lynx1 specific antibody L1-I detected Nl-lynx1 protein (indicated by black triangle). (D) Nl-lynx1 protein detected by L1-I in C was blocked by addition of excess Nl-lynx1 fusion protein. (E) In immunopellet precipitated by N1-I, Nl-lynx2 specific antibody L2-I detected Nl-lynx2 protein (indicated by black triangle). (F) Nl-lynx2 protein detected by L2-I in E was blocked by addition of excess Nl-lynx2 fusion protein.

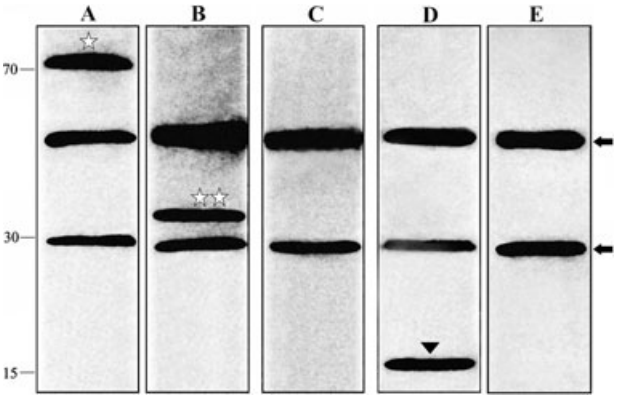

Figure 3. Co-immunoprecipitation of Nlα3 and Nl-lynx1/Nl-lynx2. (A) Nlα3-specific antibody N3-I uniquely detected Nlα3 protein (indicated by asterisk) in immunopellet precipitated by N3-I. The immunoglobulin heavy and light chains were also detected (in all experiments; indicated by arrow heads). (B) The unique Nlα3 protein detected by N3-I in A was blocked by addition of excess Nlα1 fusion protein (indicated by double asterisk). (C) In immunopellet precipitated by N3-I, Nl-lynx1-specific antibody L1-I detected no specific band. (D) In immunopellet precipitated by N3-I, Nl-lynx2 specific antibody L2-I detected Nl-lynx2 protein (indicated by black triangle). (E) Nl-lynx2 protein detected by L2-I in D was blocked by addition of excess Nl-lynx2 fusion protein.

respectively, and compared with that on their wildtype counterparts.

Similar to the effects on Nlα1/Nlα2/β2 nAChRs, Nl-lynx1 and Nl-lynx2 increased I_{max} values of ACh and imidacloprid on Nlα1^{Y151S}/Nlα2/β2 receptors, but did not change sensitivities to acetylcholine and imidacloprid, with similar EC_{50} values (Table 1). The fold effects on I_{max} of acetylcholine were also close to that of Nlα1/Nlα2/β2 nAChRs (Figs 1A, 4A). In contrast, both Nl-lynx1 and Nl-lynx2 showed different effects on I_{max} of imidacloprid on Nlα1^{Y151S}/Nlα2/β2, compared with that on Nlα1/Nlα2/β2 (Figs 1B, 4B). Co-expression of Nlα1^{Y151S}/Nlα2/β2 with Nl-lynx1 or Nl-lynx2 increased I_{max} of imidacloprid to 4.23- or 2.31-fold of that of Nlα1^{Y151S}/Nlα2/β2 alone, which was significantly higher than 2.57- or 1.25-fold of the co-expression of Nlα1/Nlα2/β2 with Nl-lynx1 or Nl-lynx2.

On Nlα3^{Y151S}/Nlα8/β2 nAChRs, only Nl-lynx2 caused the increase in I_{max} of acetylcholine and imidacloprid (Fig. 4C, D), which was similar to that on Nlα3/Nlα8/β2 (Fig. 1C, D). Nl-lynx2 caused the similar increase in I_{max} of acetylcholine on Nlα3^{Y151S}/Nlα8/β2 (Fig. 4C; 3.59-fold) and Nlα3/Nlα8/β2 (Fig. 1C; 3.63-fold). In contrast, Nl-lynx2 showed more notable effects on I_{max} of imidacloprid on Nlα3^{Y151S}/Nlα8/β2 (Fig. 4D; 3.88-fold) than on Nlα3/Nlα8/β2 (Fig. 1D; 2.16-fold).

Table 2. Co-immunoprecipitation between subunits and lynx proteins

	Nl-lynx1	Nl-lynx2
Nlα1	+	+
Nlα2	+	+
Nlα3	–	+
Nlα4	–	–
Nlα6	–	–
Nlα7	–	–
Nlα8	–	+

+, co-immunoprecipitation; –, no co-immunoprecipitation.

Discussion

Our understanding of insect nAChRs subunit diversity has been greatly enhanced by advances in genome sequencing and molecular cloning. Such approaches have identified ten subunits in *D. melanogaster* and a similar level of nAChR subunit diversity in other insect species (Jones *et al.*, 2007; Millar & Denholm, 2007). Different subunit combinations lead to high diversity in receptor subtypes with distinctive pharmacological profiles (Tomizawa & Casida, 2001). In our previous studies, four α subunits from *N. lugens* were found to assemble into two different hybrid nAChRs with rat β2 subunit, which were Nlα1/Nlα2/β2 (Liu *et al.*, 2009a) and Nlα3/Nlα8/β2 (Zhang *et al.*, 2009). We also found that two Ly-6/neurotoxin superfamily proteins Nl-lynx1 or Nl-lynx2 acted as the modulators on Nlα1-containing receptor Nlα1/β2 (Liu *et al.*, 2009b). Because the co-assembly of Nlα1 and Nlα2 had been identified in heterologous expression system (Liu *et al.*, 2009a) and in native nAChRs (manuscript submitted), it is more rational to evaluate the effects of lynx proteins on Nlα1-containing triplet nAChR Nlα1/Nlα2/β2, and compare with the doublet nAChR Nlα1/β2. The effects

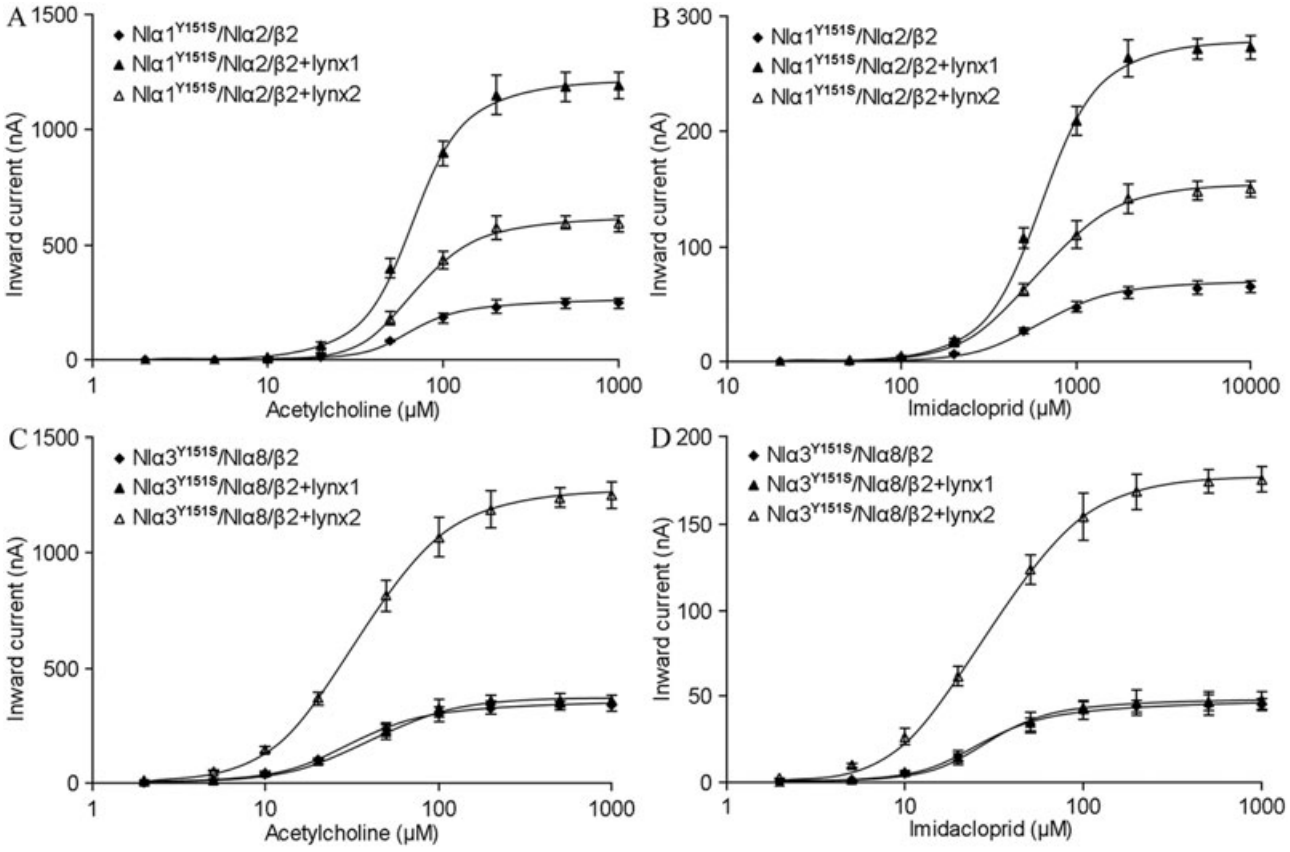

Figure 4. The effects of Nl-lynx1 and Nl-lynx2 on agonist-evoked responses of Y151S mutation containing receptors Nlα1^{Y151S}/Nlα2/β2 and Nlα3^{Y151S}/Nlα8/β2 expressed in *Xenopus* oocytes. (A) Concentration-response curves for acetylcholine on Nlα1^{Y151S}/Nlα2/β2 with or without Nl-lynx1 and Nl-lynx2. (B) Concentration-response curves for imidacloprid on Nlα1^{Y151S}/Nlα2/β2 with or without Nl-lynx1 and Nl-lynx2. (C) Concentration-response curves for acetylcholine on Nlα3^{Y151S}/Nlα8/β2 with or without Nl-lynx1 and Nl-lynx2. (D) Concentration-response curves for imidacloprid on Nlα3^{Y151S}/Nlα8/β2 with or without Nl-lynx1 and Nl-lynx2. Data are means of at least three independent experiments (different batches of oocytes) ±SEM.

of two lynx protein on another triplet nAChR Nlα3/Nlα8/β2 were also examined, and compared to Nlα1/Nlα2/β2.

As observed on Nlα1/β2, co-expression of Nl-lynx1 and Nl-lynx2 increased the agonist-evoked macroscopic currents of receptors, but did not change agonist sensitivity and desensitization properties of two triplet nAChR Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2. Nl-lynx1 caused the 4.85- and 2.57-fold increases in I_{max} of acetylcholine and imidacloprid on Nlα1/Nlα2/β2, which were significantly higher than those (3.56- and 1.72-fold) on Nlα1/β2 (Liu *et al.*, 2009b). In contrast, Nl-lynx2 led to a lower increase in I_{max} of acetylcholine and imidacloprid on Nlα1/Nlα2/β2 (2.40- and 1.25-fold) than on Nlα1/β2 (3.25- and 1.51-fold). The results demonstrate the addition of Nlα2 into Nlα1/β2 could increase the effects of Nl-lynx1, but decrease the effects of Nl-lynx2. This finding indicates modulator action of lynx proteins had preference on nAChR subtypes, such as Nl-lynx1 acting preferentially on the triplet receptor Nlα1/Nlα2/β2 and Nl-lynx2 on the doublet receptor Nlα1/β2. Nevertheless, the reason for such selectivity needs further study.

Another interesting finding was that Nl-lynx1 acted on the triplet receptor Nlα1/Nlα2/β2, but not on another triplet receptor Nlα3/Nlα8/β2, which showed the selectivity among receptor subtypes. In order to find out whether this selectivity occurred in native receptors, co-immunoprecipitation was performed between two lynx proteins and two triplet receptors. The results showed the co-immunoprecipitation between Nlα1/Nlα2 and two lynx proteins. In contrast, only co-immunoprecipitation was found between Nlα3/Nlα8 and Nl-lynx2. These results indicated that, in native nAChRs, both Nl-lynx1 and Nl-lynx2 acted on Nlα1/Nlα2-containing receptors, but only Nl-lynx2 acted on Nlα3/Nlα8-containing receptors. The lack of evidence for association between Nlα3/Nlα8 and Nl-lynx1 *in vivo* might provide a possible explanation for the lack of potentiation of Nlα3/Nlα8/β2 receptors in *Xenopus* oocytes. Comparing the effects of Nl-lynx2 on two triplet receptors Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2, it was found that the effects on Nlα3/Nlα8/β2 were significantly higher (3.63- and 2.16-fold increase in I_{max} of acetylcholine and imidacloprid) than on Nlα1/Nlα2/β2

(2.40- and 1.25-fold), which indicated Nl-lynx2 also showed preference among two triplet receptors. Taking co-immunoprecipitation results together, the data indicated that Nl-lynx1 selectively acted on Nlα1/Nlα2 containing receptors, Nl-lynx2 preferentially acted on Nlα3/Nlα8-containing receptors and these two lynx proteins did not interact with Nlα4, Nlα6 or Nlα7 subunits.

Two triplet receptors have been reconstituted in *Xenopus* oocytes, and imidacloprid showed a large difference (109.5-fold) in apparent affinity on recombinant Nlα1/Nlα2/β2 and Nlα3/Nlα8/β2 nAChRs (EC_{50} = 350.4 ± 16.8 μM and 3.2 ± 0.5 μM, respectively) (Zhang *et al*., 2009; Liu *et al*., 2009a). The Nlα3^{Y151S} mutation in Nlα3^{Y151S}/Nlα8/β2 caused a 9.2-fold increase in EC_{50}, which was much bigger than 1.8-fold increase of the Nlα1^{Y151S} mutation in Nlα1^{Y151S}/Nlα2/β2 (Zhang *et al*., 2009). These results indicated Nlα3^{Y151S} mutation might be more important in target site insensitivity in *N. lugens*. So, the effects of two lynx proteins on mutant receptors containing Y151S mutation were also examined, and compared with their wildtype counterparts. On two mutant receptors Nlα1^{Y151S}/Nlα2/β2 and Nlα3^{Y151S}/Nlα8/β2, two lynx proteins showed nearly identical effects on I_{max} of acetylcholine, which was similar to our previous study on Nlα1^{Y151S}/β2 (Liu *et al*., 2009b). In contrast, Nl-lynx1 and Nl-lynx2 caused a larger increase in I_{max} of imidacloprid on Nlα1^{Y151S}/Nlα2/β2 (4.23- and 2.31-fold) than on Nlα1/Nlα2/β2 (2.57- or 1.25-fold), and Nl-lynx2 also caused a larger increase in I_{max} of imidacloprid on Nlα3^{Y151S}/Nlα8/β2 (3.88-fold) than on Nlα3/Nlα8/β2 (2.16-fold). Because both Nlα1^{Y151S} and Nlα3^{Y151S} mutations caused a dramatic reduction in I_{max} of imidacloprid, and Nl-lynx1 and Nl-lynx2 could compensate for such a reduction, the importance of the Y151S mutation in conferring resistance to neonicotinoid insecticides might be reduced by *N. lugens* lynx proteins. The results indicated that Nl-lynx1 and Nl-lynx2 might serve as an influencing factor in target-site insensitivity in *N. lugens*.

Although heterologous expression of insect nAChR subunits (α subunits) with vertebrate nAChR subunits such as rat β2 subunit has provided a powerful approach to characterizing these receptors (Bertrand *et al*., 1994; Lansdell & Millar, 2000), it is unsuccessful in expressing recombinant receptors assembled exclusively from insect nAChR subunits. Some proteins, other than nAChRs themselves, might play important roles in insect nAChRs function *in vivo* and *in vitro*, such as the chaperones or modulators (Choo *et al*., 2008; Lansdell *et al*., 2008; Millar, 2008; Liu *et al*., 2009b). Although these proteins play essential roles in hybrid nAChRs including insect and mammalian subunits, we have been unable, as yet, to successfully recombine insect nAChRs with recruitment of these accessory proteins. It appears therefore that the successful heterologous expression of insect nAChRs may require the identification of additional accessory proteins (Lansdell *et al*., 2008).

Experimental procedures

Materials

Acetylcholine and imidacloprid were purchased from Sigma-Aldrich (St Louis, MO, USA).

Expression and electrophysiological recording in Xenopus oocytes

Nilaparvata lugens nAChR α subunits, Nlα1, Nlα2, Nlα3, Nlα8 and *Rattus norvegicus* β subunit rat β2 were subcloned into the expression vector pGH19 as described previously (Liu *et al*., 2006; Zhang *et al*., 2009). The coding regions of Nl-lynx1 and Nl-lynx2 cDNAs were also subcloned into *Eco*RI and *Xba*I sites of pGH19 (Liu *et al*., 2009b). All plasmid and mutant constructs were verified by nucleotide sequencing. *N. lugens* nAChR subunits and lynx cRNAs were generated using the mMESSAGE mMACHINE T7 transcription kit (ABI-Ambion, Foster, CA, USA). *Xenopus* oocyte preparation and cRNA injection were performed as described previously (Liu *et al*., 2006). Electrophysiological recordings were made using a two-electrode voltage clamp (Multiclamp 700B Amplifier, Axon Instruments, Foster, CA, USA) as previously described (Liu *et al*., 2006).

Antibodies and immunoprecipitation experiments

Polyclonal antisera (N3-I and N8-I), specific for the *N. lugens* α3 (Nlα3) and α8 (Nlα8), were prepared as described previously (Zhang *et al*., 2009). Polyclonal antisera (N1-I, N2-I, N4-I, N6-I and N7-I), specific for α1 (Nlα1), α2 (Nlα2), α4 (Nlα4), α6 (Nlα6) and α7 (Nlα7) subunits were raised against bacterially expressed fusion proteins containing the large cytoplasmic loop of Nlα1 (Val^{372}-Ala^{430}), Nlα2 (Phe^{424}-Tyr^{485}), Nlα4 (Val^{394}-Ala ^{450}), Nlα6 (Phe^{392}-Phe ^{439}) and Nlα7 (Phe^{399}-Val ^{445}). Polyclonal antisera (L1-I and L2-I) specific for Nl-lynx1 and Nl-lynx2 were raised against bacterially-expressed fusion proteins of Nl-lynx1 (Phe^{49}-Thr^{106}) and Nl-lynx2 (Phe^{47}-Val^{105}). Fusion proteins were purified as described previously (Schloß *et al*., 1988) and purified on protein G-Sepharose (GammaBind plus, Pharmacia, Germany). Detergent extracts of *N. lugens* head membranes were prepared as described previously for *Drosophila* (Schloß *et al*., 1988) and immunoprecipitation performed by methods described previously (Schulz *et al*., 2000; Liu *et al*., 2009b). GammaBind plus Sepharose was equilibrated in buffer A and 50 μl of a 1:1 GammaBind plus Sepharose/buffer A (10 mM Tris-HCl, pH 7.5, 280 mM sucrose, 0.01% w/v NaN_3, 100 μg/ml PMSF) suspension incubated for 2 h at 4 °C with 10 mg of antibody. Detergent extract was preabsorbed with GammaBind plus Sepharose to eliminate nonspecific binding and then the preabsorbed detergent extract (2.5 mg of protein) incubated overnight with antibody-coupled GammaBind plus Sepharose in a final volume of 1 ml of buffer A. In competition experiments, a ~1000-fold molar excess of fusion protein (described above) was included in the incubation mixture. Immunoprecipitates were collected by centrifugation and pellets were washed twice with buffer A, twice with buffer B (buffer A containing 1 M NaCl), and once with buffer C (50 mM Tris-HCl, pH 6.8). Proteins were eluted from the GammaBind plus

Sepharose, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, electroblotted on to nitrocellulose and then detected using ECL detection system, following the manufacturer's instructions (Millipore Corporation, Billerica, MA, USA).

Data analysis

Dose-response curves were fitted with the Hill equation:

$$I = I_{max} \Big/ \left[1 + (EC_{50}/x)^{nH}\right]$$

where I = response, I_{max} = maximum response, EC_{50} = half-maximal activation concentration, x = agonist concentration, and nH = Hill coefficient.

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