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ORIGINAL ARTICLE

Reduction in mRNA and protein expression of a nicotinic acetylcholine receptor α8 subunit is associated with resistance to imidacloprid in the brown planthopper, *Nilaparvata lugens*

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Abstract

Target-site resistance is commonly caused by qualitative changes in insecticide target-receptors and few studies have implicated quantitative changes in insecticide targets in resistance. Here we show that resistance to imidacloprid in a selected strain of *Nilaparvata lugens* is associated with a reduction in expression levels of the nicotinic acetylcholine receptor (nAChR) subunit Nlα8. Synergism bioassays of the selected strain suggested resistance was conferred, in part, by a target-site mechanism. Sequencing of *N. lugens* nAChR subunit genes identified no mutations associated with resistance, however, a decrease in mRNA and protein levels of Nlα8 was observed during selection. RNA interference knockdown of Nlα8 decreased the sensitivity of *N. lugens* to imidacloprid, demonstrating that a decrease in Nlα8 expression is sufficient to confer resistance *in vivo*. Radioligand binding assays revealed that the affinity of the high-affinity imidacloprid-binding site of native nAChRs was reduced by selection, and reducing the amount of Nlα8 cRNA injected into *Xenopus* oocytes significantly decreased imidacloprid potency on recombinant receptors. Taken together, these results provide strong evidence that a decrease in Nlα8 levels confers resistance to imidacloprid in *N. lugens,* and thus provides a rare example of target-site resistance associated with a quantitative rather than qualitative change.

Keywords: insecticide resistance, nicotinic acetylcholine receptors, quantity change, target insensitivity.

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Insecticide resistance is an ongoing constraint in insect pest management. As early as 1946, the first case of resistance to the insecticide dichlorodiphenyltrichloroethane was recorded (Barnes 1946). Since then, resistance has been described in a huge number of different insect species, involving a wide array of different insecticide classes (APRD, http://www.pesticideresistance.com). For most insecticide classes, resistance has been shown to result primarily from enhanced detoxification, termed metabolic resistance (Devonshire 1991; Scott 1999; Enayati *et al.* 2005; Li *et al.* 2007), or target-site insensitivity, termed target-site resistance (Mutero *et al.* 1994; Martinez-Torres *et al.* 1998). For target-site resistance, one or more mutations, often at conserved positions, have been shown to confer insensitivity. Classic examples of this, that have now been identified in a wide range of insect species, include a single amino acid mutation A302S in the GABA receptor *Rdl*, which confers resistance to cyclodiene insecticides (Ffrench-Constant *et al.* 1993), and point mutations in the voltage-gated sodium channel, which confer knockdown resistance to pyrethroid insecticides (Williamson *et al.* 1996). Examples for neonicotinoid insecticides, a globally important class of insecticides worldwide, include the Y151S substitution identified in two nicotinic acetylcholine receptor (nAChR) α subunits of

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Abbreviation used: nAChR, nicotinic acetylcholine receptor.

an imidacloprid selected strain of the brown planthopper, *Nilaparvata lugens* (Liu *et al.* 2005) and R81T in the nAChR β1 subunit of peach–potato aphid, *Myzus persicae*, field strains that are highly resistant to neonicotinoids (Bass *et al.* 2011a).

Beyond qualitative changes in insecticide target sites, few reports have described quantitative changes in target proteins associated with resistance. Exceptions are a handful of reports associating changes in expression of nAChRs, ryanodine receptors, and acetylcholinesterases with resistance. Investigation of neonicotinoid-resistant field populations and susceptible laboratory strains of *Musca domestica* L. revealed a reduction in the expression of nAChR subunit Mdα2 in the resistant populations (Markussen and Kristensen 2010). Changes in expression of nAChR subunits have also been described in the pea aphid, *Acyrthosiphon pisum*, with larvae treated with imidacloprid, thiamethoxam, or clothianidin, showing increase in the expression of Apisumα10 and Apisumβ1, and decreases in the expression of Apisumβ2 (Taillebois *et al.* 2014). The ryanodine receptor is the target-site of the newer diamide insecticides and the mRNA level of this receptor in resistant strains of diamondback moth (*Plutella xylostella* L.) were found to be 2.9-fold that of the susceptible strain (Yan *et al.* 2014), although resistant strains also carried the G4946E mutation known to confer high levels of resistance to diamides. In the case of acetylcholinesterases, the gene encoding this enzyme, *ace*, has been reported to be over-expressed in resistant strains of several arthropod species when compared with susceptible strains, in some cases as a result of gene duplication/amplification (Gao and Zhu 2002; Hsu *et al.* 2008; Pan *et al.* 2010; Shen *et al.* 2012; Edi *et al.* 2014; Shang *et al.* 2014; Weetman *et al.* 2015). Although over-expressed *ace* genes may also carry target-site mutations that confer resistance, the elevated expression has in certain cases also been shown to provide an adaptive advantage (Edi *et al.* 2014; Weetman *et al.* 2015).

With the exception of *ace*, the cases detailed above have reported a simple correlation between a change in mRNA or protein level of an insecticide target and insecticide resistance, however, firm evidence demonstrating a causative role in resistance has been lacking. In the present study, a decrease in the abundance of a nAChR subunit Nlα8 was observed in an imidacloprid-resistant strain of *N. lugens* and several lines of evidence provided support that this alteration is directly involved in resistance.

Materials and methods

Brown planthopper (*N. lugens*) strain

The original *N. lugens* population used in this study was collected from a paddy field in Nueva Ecija (Philippines) in August 2011. Bioassay and resistance selection were performed using the rice-stem dipping method with fifth-instar nymphs (Wang *et al.* 2008). In bioassays, six concentrations of imidacloprid were used to assess the sensitivity of each generation or population and to determine LC_{50} and LC_{75}. For resistance selection, the LC_{75} concentration of each parental generation was used to treat the insects of the following generation. For synergism bioassays, 6 μg of synergists (2 μg for each of triphenyl phosphate, diethyl maleate, and piperonyl butoxide) in 0.08 μL acetone was delivered onto the prothorax notum of fifth-instar nymphs 1 h before exposure to imidacloprid (Tang *et al.* 2010).

Assessment of mRNA and protein levels

Total RNA was extracted from three to six pools of five adult BPHs using Trizol reagent following the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The mRNA levels of the different nAChR subunit genes were measured by qRT-PCR, using the One Step SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China). In a previous study, five subunits (Nlα1, Nlα2, Nlα3, Nlα8, and Nlβ1) were found to constitute two binding sites for imidacloprid in *N. lugens* (Li *et al.* 2010), so these subunits were selected for qRT-PCR. Nlα6, which is not related to imidacloprid binding, was also selected as a negative control. For normalization, two reference genes, β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were validated experimentally for each generation and treatment, with the geometric mean of the selected genes then used for normalization according to the strategy described previously (Vandesompele *et al.* 2002). The sequences for qRT-PCR primers were included in Table S2. Protein levels for different subunits were measured using an immunoblotting method as previously described (Liu *et al.* 2005). The specific antibodies for different nAChR subunits and reference proteins were prepared as previously reported (Li *et al.* 2010). Membrane proteins were extracted from insects as described previously (Liu *et al.* 2005). *N. lugens* (50 mg) were homogenized in 50 mL of extraction buffer (pH 7.2, 0.32 mM sucrose, 100 mM EDTA, and 1% proteinase inhibitor mixture I; Sigma, St Louis, MO, USA). The homogenate was centrifuged at 1000 *g* for 30 min. The resultant supernatant was filtered through four layers of cheesecloth and centrifuged at 30 000 *g* for 60 min. The pellet was resuspended in incubation buffer (pH 7.4, 0.05 mM Tris, 0.12 mM NaCl, and 100 mM EDTA). Protein content was determined by a Bio-Rad Laboratories, Hercules, CA, USA DC protein assay using bovine serum albumin as a standard (Bradford 1976). Extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were blocked and then incubated with 1 : 1200 dilution of specific antibody for 1 h at room temperature (25 °C). The nitrocellulose membrane was washed thoroughly, incubated with 1 : 2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG solution (CWBIO, Beijing, China), and processed, using the enhanced chemiluminescence detection system (Amersham Pharmacia). GAPDH (C_NLTH5658 in BPH EST database (http://bphest.dna.affrc.go.jp/; Noda *et al.*, 2008), β-tubulin (C_NLTA0757) and β-actin (EU179846) were first selected as reference proteins. After evaluation of the stable expression in different generations (Figure S1), β-actin (EU179846) was finally employed as the reference standard to normalize the protein levels between samples. The intensity of each band was determined using Image J software (NIH,

http://rsb.info.nih.gov/ij/). The first band was framed with the rectangle selection tool in Image J, and then the 'Analyze-Gels-Select Next Lane' command was used to select an equal area in other lanes. Average pixel intensity was plotted across the selection box in each lane, with the protein band seen as a peak above the background pixel intensity. The area under the peak was calculated. For each protein, the band intensity (area under the peak) was normalized to the band intensity of β-actin band in each lane calculated by the same method.

RNA interference

RNA interference (RNAi) was performed as previously described (Liu *et al.*, 2010). The dsRNA for different genes was prepared using T7 Ribomax Express RNAi System (Promega, Madison, WI, USA). A fixed volume of 50 nL, with different dsRNA concentrations to obtain different dsRNA amounts, was microinjected into insects at the conjunctive between prothorax and mesothorax. Injected insects were placed on fresh rice seedlings to recover, and a sample of the injected insects was randomly selected and kept at −80°C for further tests.

[^{3}H]imidacloprid binding assays

Radioligand binding assays were performed as described previously (Liu *et al.* 2005; Li *et al.* 2010). In a total volume of 300 mL incubation buffer, the receptor preparation (0.2–0.3 mg protein per assay) was incubated for 120 min at 4°C with appropriate concentration of [^{3}H]imidacloprid. Samples were assayed by filtration onto Whatman GF/B filters pre-soaked in 0.5% polyethylenimine, followed by rapid washing with ice-cold saline buffer (pH 7.4, 20 mM Na_2HPO_4, 0.15 M NaCl, and 0.2% bovine serum albumin). The filters were transferred into the scintillation vials, and the radioactivity remaining on the filter was assayed after overnight incubation in 3 mL of scintillation cocktail (OptiPhase Supermix; PerkinElmer, Waltham, MA, USA) on an LS6500 Liquid Scintillation Counter (Beckman Coulter, Fullerton, CA, USA). Specific binding was defined as the difference in radioactivity in the absence and the presence of unlabeled imidacloprid with a 1000-fold molar excess (compared to [^{3}H]imidacloprid concentrations used).

Electrophysiological recording

N. lugens Nlα3 (AY378700), Nlα8 (FJ481979), and *Rattus norvegicus* β subunit rat β2 (L31622) were subcloned into the expression vector pGH19 as described previously (Liu *et al.* 2006; Yixi *et al.* 2009). Subunit 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). The amount of Nlα3 and rat β2 cRNAs injected into the oocyte was 50. The amount of injected cRNA of Nlα8 was varied from 50 to 10 ng. Electrophysiological recordings on *Xenopus* oocytes were made using a two-electrode voltage clamp (Multiclamp 700B Amplifier; Axon Instruments, Foster, CA, USA) as previously described (Liu *et al.* 2006).

Statistical analysis

Significant differences were analyzed by one-way ANOVA with at least three repeats. Multiple comparisons between the groups were performed using S-N-K method. The level of significance for results was set at $p < 0.05$.

Results

Selection of imidacloprid resistance and synergist bioassays

A field-derived strain of *N. lugens* from the Philippines was selected with imidacloprid for 26 generations. Resistance was found to develop slowly over the first 14 generations of selection reaching 49-fold resistance by the 14th generation (G14) (Fig. 1). The resistance then increased sharply in the following two generations (G15–16) before stabilizing with only a slight increase observed in the following nine generations (G17–26), from 426- to 448-fold. When three commonly used insecticide synergists, triphenyl phosphate, diethyl maleate and piperonyl butoxide, that inhibit detoxification enzymes were used in combination with imidacloprid, much of the resistance of the selected strain was overcome, suggesting a significant metabolic component to the resistance. The greatest synergistic effect was seen with the use of piperonyl butoxide (Figure S2), suggesting cytochrome P450s are the primary enzyme system involved in the resistance. The characterization of this metabolic component of the resistance was explored in a separate study. Despite this finding, a 'non-metabolic' component of the resistance remained after the use of synergists, especially in the increase from 7- to 57-fold from G14 to G16.

Imidacloprid resistance was associated with a reduction in Nlα8 subunit mRNA and protein levels

To investigate if the imidacloprid resistance of the selected *N. lugens* strain was associated with mutation of the target-site, the cDNA sequences for five nAChR subunits, Nlα1, Nlα2, Nlα3, Nlα8, and Nlβ1, related to imidacloprid binding were compared between the pooled individuals from G14 and G16 (Li *et al.* 2010). No non-synonymous mutations associated with imidacloprid resistance were observed in any of the subunit sequences (data not shown). To explore

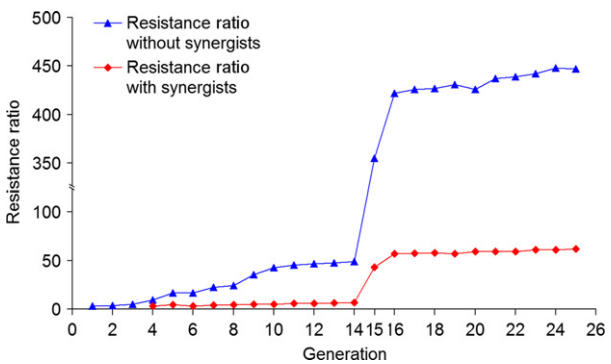

Fig. 1 Imidacloprid resistance of a strain of *Nilaparvata lugens* under selection with imidacloprid and the impact of co-application of three synergists (piperonyl butoxide, triphenyl phosphate, diethyl maleate) on resistance. Resistance is expressed as 'Resistance Ratios' calculated by dividing the LC_{50} of the selected population with the LC_{50} of the unselected population.

potential quantitative changes in the nAChR and association with resistance, mRNA and protein levels of these five nAChR subunits potentially involved in imidacloprid binding were analyzed by quantitative PCR and immunoblotting in certain key generations of the imidacloprid selected strain (G1, G14, G16, and G25). Nlα6, a subunit not related to imidacloprid binding, was also selected as a negative control. A significant decrease in both mRNA and protein levels was only detected for the Nlα8 subunit (Figure S3). Following this finding, the mRNA and protein levels of Nlα8 were tested for all generations. A significant decrease in Nlα8 subunit mRNA levels was found from G14 to G16. Small, but still significant, changes in Nlα8 mRNA levels were also observed before G14 (Fig. 2a). Similar significant decreases in Nlα8 protein levels were observed from G14 to G16. Overall protein levels showed a greater reduction than mRNA levels, falling more than 3-fold between G14 and G16 (Fig. 2a and b). No significant further decreases in mRNA or protein levels were observed from G16 to G25.

RNAi knockdown of Nlα8 reduces sensitivity to imidacloprid *in vivo*

In order to evaluate the effects of Nlα8 quantity on imidacloprid sensitivity *in vivo*, Nlα8 mRNA levels were knocked down by RNAi and the consequent reduction in Nlα8 protein levels were determined. The effect of knockdown on the imidacloprid sensitivity of injected insects was then determined following different injection regimes. When dsRNA of Nlα8 was applied to G1 of the *N. lugens* strain both Nlα8 mRNA and protein levels were significantly reduced only after a double injection (Fig. 3). The level of knockdown was concentration-dependent with the highest level of knockdown observed when 50 ng dsRNA was injected twice, with mRNA decreasing to 29% of controls.

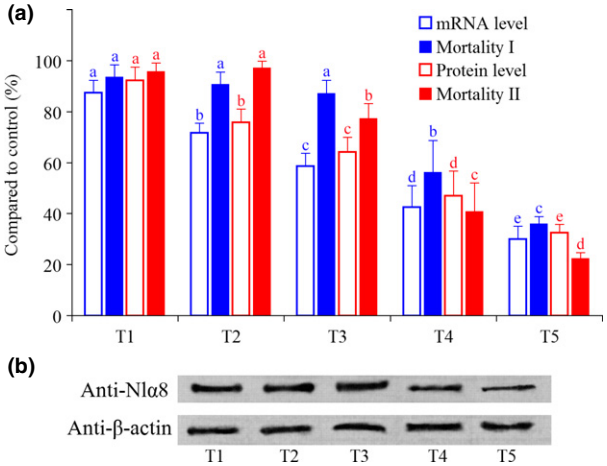

Fig. 3 The quantity of Nlα8 mRNA and expressed protein after injection with dsRNA and mortality of injected insects to imidacloprid. T1 and T2 were single injections of 25 and 50 ng dsRNA, respectively; T3, T4, and T5 were double injections of 30, 40, and 50 ng dsRNA, respectively. (a) Open blue columns represent Nlα8 mRNA levels 4 days after dsRNA injection at the third instar. Open red columns represent Nlα8 protein levels 8 days after dsRNA injection. Mortality I, indicated by filled blue columns, was determined 4 days after dsRNA injection when RNAi knockdown of Nlα8 mRNA was the highest. Mortality II, indicated by filled red columns, was determined 8 days after dsRNA injection when knockdown of Nlα8 protein level was greatest. Data are means of at least three independent experiments ± SEM. (b) Nlα8 protein detected by immunoblot over the same time period. Different letters above the same colour columns indicate significant differences among treatments.

The mortality of injected insects after exposure to 2 ng imidacloprid per hopper (determined 4 days after dsRNA injection when Nlα8 mRNA levels were lowest) showed a decrease to 35.9% of the controls. Similarly, mortality 8 days

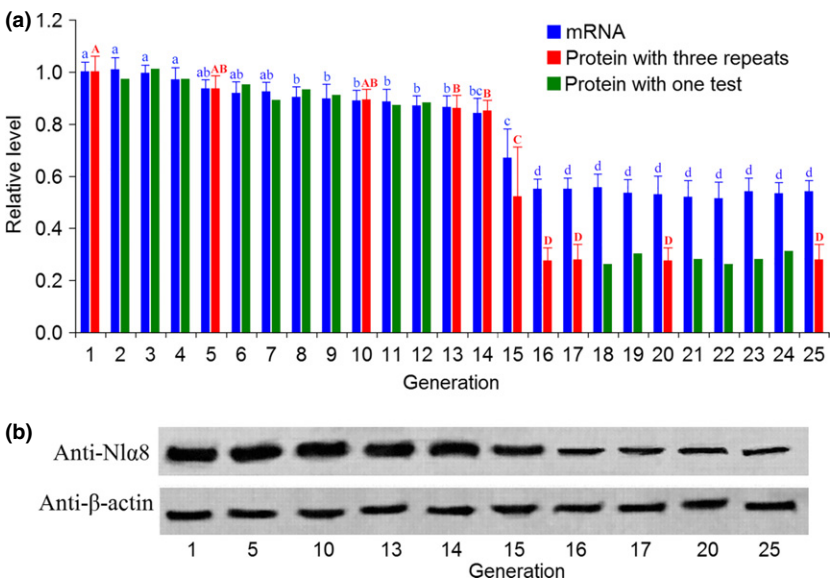

Fig. 2 (a) Expression of Nlα8 mRNA and protein levels during imidacloprid selection of a field-collected *Nilaparvata lugens* strain. Blue columns represent mRNA levels plotted as means ± SEM of at least three repeats. Red columns represent protein levels plotted as means ± SEM of three repeats in key generations. Green columns represent protein levels determined by a single test. (b) Levels of expressed Nlα8 subunit proteins in several key generations of the selected strain as assessed by immunoblot. Different lowercase and uppercase letters indicate significant differences in mRNA and protein levels between generations.

after dsRNA injection (when Nlα8 protein level was lowest) decreased to 22.3%. It is noteworthy that the biggest decreases in imidacloprid mortality corresponded to the time when Nlα8 protein levels were lowest, rather than the time when mRNA levels were lowest.

Radiolabeled imidacloprid-binding assays to native membrane preparations of the imidacloprid selected *N. lugens* strain

Previous studies on the native subunit composition of *N. lugens* nAChR subtypes revealed that nAChRs containing the Nlα3/Nlα8/Nlβ1 subunits constitute the high-affinity binding site for imidacloprid (Li *et al.* 2010). To investigate if the reduction in Nlα8 mRNA and protein levels seen in the selected strain was associated with changes in imidacloprid binding to the nAChR, [^{3}H]imidacloprid binding was performed on native membrane proteins extracted from G14 and G16 individuals of the imidacloprid selected strain. Two imidacloprid-binding sites with different affinities were observed in G14, in which the dissociation constant (K_d values) for the high- and low-affinity binding sites were 3.18 ± 0.43 pM and 1.78 ± 0.19 nM, respectively (Fig. 4a). These results are consistent with our previous study (Liu *et al.* 2005) and the findings in other Hemipteran insects, such as *M. persicae* and *Nephotettix cincticeps* (Lind *et al.*, 1998). Although two different affinity binding sites were also detected in G16, the K_d value (Table S1) of the high-affinity binding site was 10.8 times that of G14. A significant decrease in maximum binding capacity (B_{max}) for the high-affinity site was also observed (Table S1). In contrast, there were no significant differences in imidacloprid binding (K_d or B_{max} values) to the low-affinity binding sites between G14 and G16. These results are well illustrated by transformation of the data in the form of a Scatchard plot (Fig. 4b).

Influence of Nlα8 quantity on imidacloprid response on recombinant receptors

Since it is difficult to generate insect functional nAChRs in heterologous expression system, a successful strategy is to co-express insect nAChR α subunits with a vertebrate non-α subunit, such as rat β2 (Millar 2009). Previous studies of hybrid nAChRs expressed in *Xenopus* oocytes have indicated that Nlα3 and Nlα8 subunits are likely co-assembled with rβ2 into one receptor (Yixi *et al.* 2009). In order to confirm the influence of Nlα8 levels on agonist potencies on recombinant receptors, triplet nAChRs Nlα3/Nlα8/rβ2 with different quantities of Nlα8 subunit were generated in *Xenopus* oocytes by injection of different amounts of Nlα8 cRNA. When the amount of injected cRNA of Nlα8 was decreased from 50 to 30 ng, the inward currents evoked by acetylcholine and imidacloprid were both significantly decreased (Fig. 5a). However, there was no significant difference in the potency of either agonist (EC_{50} values) (Fig. 5b). Interestingly, further reducing Nlα8 cRNA to 20 ng resulted in a large rightward shift in the dose–response curve for imidacloprid (9.0-fold increase in EC_{50}). Under this injection regime, the maximal agonist response (I_{max}) for imidacloprid on the receptors recovered to a similar level to that of receptors when 50 ng of Nlα8 had been injected. The decrease in Nlα8 cRNA injection to 20 ng caused a small but statistically significant rightward shift in the dose–response curve of acetylcholine (1.8-fold increase in EC_{50}, Figure S4).

Discussion

As outlined in the introduction, a great deal of work has been done over the last 50 years to characterize, at the molecular level, insecticide resistance resulting from mutations in the insecticide target sites. In the majority of cases, rigorous functional analyses have been carried out to definitively prove the causal effect of these target-site alterations in reducing or modifying insecticide binding. However, to date, much less work has been carried out to examine the effect of quantitative changes in insecticide target-sites on resistance, and the few reports that have been published often describe simple correlations with resistance, and have lacked functional evidence that clearly supports a role in resistance.

In this study, we provide several lines of evidence that a change in the mRNA and protein levels of an insecticide

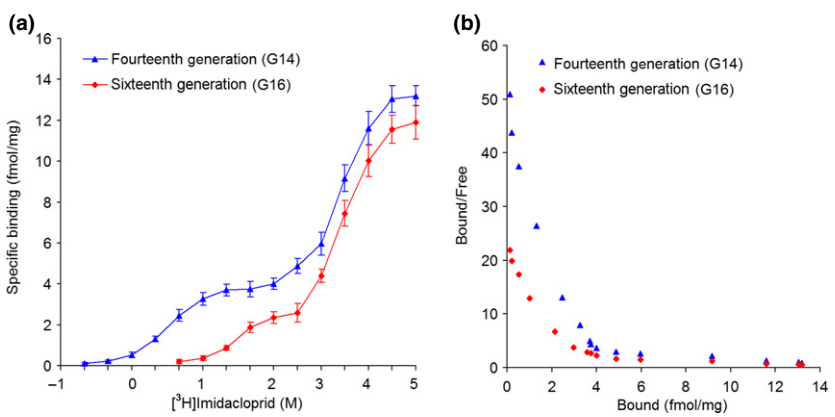

Fig. 4 Radioligand binding of [^{3}H]imidacloprid to *Nilaparvata lugens* native nAChRs from G14 and G16 of the imidacloprid selected strain. (a) Equilibrium saturation binding of [^{3}H]imidacloprid to membrane preparation extracts of *N. lugens*. (b) Scatchard plot of data presented in (a).

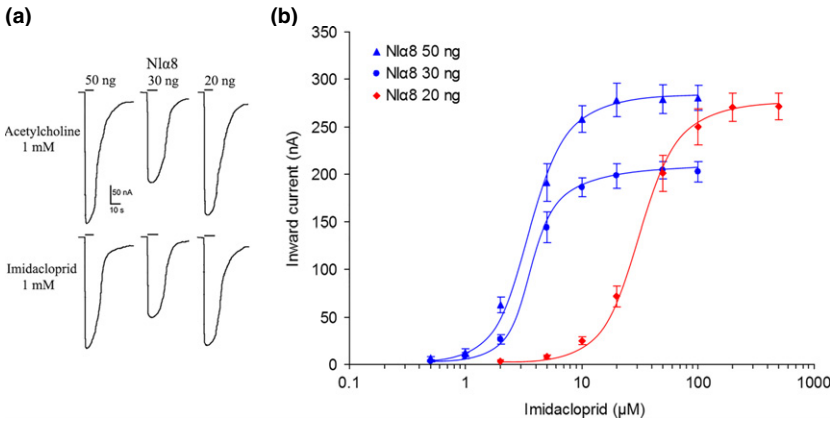

Fig. 5 The effect of different injection amounts of Nlα8 cRNA on agonist-evoked responses of triplet recombinant nAChRs Nlα3/Nlα8/rβ2 expressed in *Xenopus* oocytes. (a) Representative responses evoked by 1 mM acetylcholine or imidacloprid on Nlα3/Nlα8/rβ2. (b) Concentration–response curves for imidacloprid on Nlα3/Nlα8/rβ2 expressed with Nlα8 injection amounts of 50, 30, and 20 ng. In all experiments the injection amount of Nlα3 and rβ2 cRNA was held at 50 ng. Data are the means ± SEM of at least three independent experiments (different batches of oocytes).

target-site confers resistance. Continuous selection of a field-collected strain of *N. lugens* with imidacloprid resulted in a substantial increase in resistance with a particularly rapid increase observed from G14 to G16. Synergism bioassays using inhibitors of metabolic enzymes, strongly suggested that a considerable component of the resistance observed in this strain was metabolic, with cytochrome P450s particularly implicated. Metabolic resistance in *N. lugens* has been previously well characterized with two P450s, CYP6ER1, and CYP6AY1, thought to confer imidacloprid resistance in field populations worldwide (Bass *et al.* 2011b; Ding *et al.* 2013; Garrood *et al.* 2015). For this reason, in this study, we focused on investigating the molecular basis of the significant non-metabolic component of resistance. Sequencing of genes encoding nAChR subunits from imidacloprid selected and unselected *N. lugens* strains failed to identify any non-synonymous mutations associated with resistance. This included the Y151S and R81T amino acid substitutions described previously in neonicotinoid-resistant *N. lugens* and *M. persicae,* respectively (Liu *et al.* 2005; Bass *et al.* 2011a).

Although no alterations in the amino acid sequence of the imidacloprid selected strains were identified, assessment of mRNA and protein levels of each nAChR subunit revealed a significant decrease in Nlα8 from G14 to G16. Decreases in Nlα8 protein levels were greater than those of mRNA expression suggesting that the changes in the selected strain may result from transcriptional, post-transcriptional, or post-translational regulation of Nlα8. However, the mRNA level of a gene does not always precisely correlate with its protein level (Maier *et al.* 2009) and so further research is required to confirm or refute this.

To explore whether a reduction in this subunit subtype has any influence on imidacloprid susceptibility, RNAi was used to knockdown the Nlα8 expression and the effect of this on mortality after exposure to imidacloprid was examined. The results clearly revealed that silencing Nlα8 caused a reduction in imidacloprid sensitivity *in vivo* with mortality of injected insects reducing to 22% of controls when Nlα8 protein levels were lowest.

To investigate if the reduction in Nlα8 was associated with changes in the affinity of imidacloprid binding to nAChRs, we performed radiolabeled imidacloprid binding to native membrane proteins extracted from G14 and G16 individuals of the imidacloprid selected strain. An overall reduction in imidacloprid binding was seen to receptors of G16 individuals when compared with those of G14 individuals and this resulted from a 10-fold reduction in the affinity of imidacloprid binding to the high-affinity imidacloprid-binding site. Interestingly, no differences were observed in the affinity of the low-affinity imidacloprid-binding site. nAChRs comprise five subunits assembled from a diverse family of subtypes, which may associate to form homomeric or heteromeric receptors (Sattelle 1980; Liu *et al.* 2005; Millar and Denholm 2007). Recent study of nAChR subunits in *N. lugens* has suggested which subunits of the nAChR contribute to the formation the two imidacloprid-binding sites (Li *et al.* 2010). This work has shown that the Nlβ1 subunit is an absolute requirement for the imidacloprid binding and that nAChRs containing Nlα1, Nlα2, and Nlβ1 constitute the lower affinity site whereas nAChRs containing Nlα3, Nlα8, and Nlβ1 constitute the higher affinity site. Given these findings, it is particularly interesting that only the high-affinity imidacloprid-binding site that contains Nlα8 subunit, showed a reduction in affinity to imidacloprid consistent with a hypothesis that reduced expression of Nlα8 results in reduced imidacloprid binding and hence resistance.

To investigate this possibility further, the influence of Nlα8 levels on agonist potency of recombinant nAChRs receptors assembled from Nlα3/Nlα8/rβ2 was explored by functional expression in *Xenopus* oocytes. When the amount of Nlα8 cRNA injected into *Xenopus* oocytes was reduced from 50 to 20 ng, a significant increase in imidacloprid EC_{50} was observed, i.e. the potency of imidacloprid against recombinant receptors was reduced. In heteromeric receptors, such as native Nlα3/Nlα8/Nlβ1 receptors in *N. lugens* and recombinant Nlα3/Nlα8/rβ2 receptors in *Xenopus* oocytes, the various ratios of the different subunit subtypes may influence the pharmacological characteristics of the receptor and this may extend to its sensitivity to insecticides. In a study of human nAChRs, altering the various ratios of α4 and β2 subunits in α4/β2 nAChRs resulted in the generation of two functional classes of receptors in *Xenopus* oocytes (Zwart and Vijverberg 1998). When the injected cRNA ratio of α4 : β2 was 1 : 9, the resulting nAChRs were more sensitive to acetylcholine with an EC_{50} value of 1.84 μM. When the injected cRNA ratio of α4 : β2 was 9 : 1 or 1 : 1, the EC_{50} value was ~ 60 μM. Further studies using metabolic labeling with [^{35}S]methionine found that the low-affinity nAChRs had a stoichiometry of $(\alpha4)_3(\beta2)_2$ and the high-affinity nAChRs $(\alpha4)_2(\beta2)_3$ (Nelson *et al.* 2003). In the nematode *Ascaris suum*, changing the stoichiometry of the receptor reproduced two pharmacological subtypes of nAChRs (Williamson *et al.* 2009). When the ratio of Asu-unc-38 : Asu-unc-29 was 5 : 1, nicotine was a full agonist. However, nicotine became a partial agonist when this ratio was reversed. Taken together, these studies demonstrate that changes in the subunit ratios can alter the subunit composition of nAChRs and modify the pharmacological characteristics of the receptor. In our study, during imidacloprid selection of the resistant strain, the greatest shift in the resistance occurred when Nlα8 protein levels decreased by more than 60%. Consistent with this was the results of RNAi experiments where the mortality of *N. lugens* exposed to imidacloprid was substantially decreased only after a 60% reduction in Nlα8 protein was achieved. It is possible that a certain threshold of reduction in Nlα8 is required to confer resistance and this could be explained by a change in the composition of nAChRs after this threshold is crossed. In support of this, a 60% reduction in the amount of Nlα8 cRNA injected into *Xenopus* oocytes during the generation of Nlα3/Nlα8/rβ2 recombinant receptors resulted in a large rightward shift in the dose–response curve for imidacloprid.

In the present study, our findings provide a body of evidences that a reduction in Nlα8 results in resistance to imidacloprid. Further work is now required to investigate the precise genetic changes that result in the decreased expression of Nlα8 mRNA and protein levels. One possible scenario is a *cis*-acting mutation in an up- or down-stream regulatory region of the Nlα8 gene that modifies the binding of a transcription factor. Alternatively, a mutation may have occurred in a trans-acting factor that regulates the expression. In this regard, a diverse range of proteins have been shown to regulate the expression levels of nAChRs including ubiquilin-1, UBXD4, and 14-3-3 protein (Jones *et al.* 2010). Ubiquilin-1 was shown to directly interact with unassembled human nAChR α subunits, and to reduce the surface expression of α3/β2 nAChRs (Ficklin *et al.* 2005). UBXD4 was found to increase the surface expression of α3-containing nAChRs (Rezvani *et al.* 2009). Finally 14-3-3η, as a member of the 14-3-3 proteins family containing seven isoforms, increased the steady-state levels of α4 subunit and α4/β2 nAChRs (Jeanclos *et al.* 2001).

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Comparison of three reference proteins among generations under selection. From left to right, the columns were for the 1st (G1), 14th (G14), 15th (G15), 16th (G16) and 25th (G25) generations.

Figure S2. The synergistic effects of three different synergists on imidacloprid resistance levels at four key generations during selection.

Figure S3. mRNA and protein expression levels for different nAChR subunits from four representative generations of the selected strain. G1m, G14m, G16m and G25m show mRNA levels for each tested subunits in the 1st, 14th, 16th and 25th generations, and G1p, G14p, G16p and G25p show protein levels in these generations. Significant differences in mRNA and protein levels between generations are indicated by different lowercase and uppercase letters respectively.

Figure S4. Concentration-response curves for acetylcholine on Nlα3/Nlα8/rβ2 recombinant receptors expressed with 50, 30 and 20 ng Nlα8 cRNA. In all experiments, the injection amount ofNlα3 and rβ2 cRNA washeld at 50 ng. Data are means ± SEM of at least three independent experiments (different batches of oocytes).

Table S1. K_d and B_{max} values of imidacloprid binding to high- and low-affinity binding sites in the 14th (G14) and 16th (G16) generations of the imidacloprid selected strain of *N. lugens*.

Table S2. The sequences for qRT-PCR primers.

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