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# Role of loop D of the α7 nicotinic acetylcholine receptor in its interaction with the insecticide imidacloprid and related neonicotinoids

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**1** The nitroguanidine insecticide imidacloprid along with a second generation of related compounds including nitenpyram, all nicotinic acetylcholine (ACh) receptor ligands, are used increasingly in many countries. Site-directed mutagenesis and heterologous expression in *Xenopus laevis* oocytes have been deployed to investigate mutants (G189D and G189E) of the chicken α7 homomer-forming nicotinic receptor subunit which are predicted to enhance the negative charge at the negative subsite (loop D) of the ACh binding site.

**2** *Xenopus* oocytes expressing wild-type α7 nicotinic receptors respond to imidacloprid with rapid inward currents. Imidacloprid and nitenpyram are partial agonists, whereas ACh, (−)-nicotine and (+)-epibatidine are full agonists.

**3** Compared to wild-type α7, the mutant G189D and G189E receptors are much less sensitive to the insecticides, whereas their sensitivity to (−)-nicotine, ACh and (+)-epibatidine is only slightly reduced. In contrast, G189N and G189Q mutants are sensitive not only to ACh, (−)-nicotine and (+)-epibatidine, but also to the two insecticides. Thus reduction of the insecticide-sensitivity by the mutations G189D and G189E are attributed to an increase in negativity of loop D. Desnitro-imidacloprid (DN-IMI), an imidacloprid derivative lacking the nitro group is a potent agonist on the G189D and G189E mutants suggesting an important role of loop D in nicotinic receptor interactions with the nitro group of nitroguanidine insecticides.
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**Keywords:** Nicotinic acetylcholine receptor; α7 subunit; negative subsite mutations; neonicotinoids; imidacloprid; nitenpyram; (+)-epibatidine

**Abbreviations:** ACh, acetylcholine; DN-IMI, desnitro-imidacloprid; $EC_{50}$, concentration giving half the maximum normalized response; $I_{max}$, maximum normalized response; $I_{min}$, minimum normalized response

## Introduction

The insecticide imidacloprid (1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidine-2-ylideamine) is chemically related to nicotine and epibatidine (Figure 1) and acts on insect nicotinic acetylcholine (ACh) receptors (Bai *et al*., 1991; Tomizawa & Yamamoto, 1992; 1993; Liu & Casida, 1993; Tomizawa *et al*., 1996). At certain native and recombinant heteromeric nicotinic ACh receptors, imidacloprid is a partial agonist, whereas (+)-epibatidine is a full agonist (Matsuda *et al*., 1998; Nagata *et al*., 1996), indicating that, since both molecules share an identical chloronicotinyl group, it is the imidazolidine moiety that is likely to contribute to the partial agonist activity of imidacloprid.

A recent model for agonist binding sites on the nicotinic ACh receptor postulates that negative subsites (loop D) contributed by the non-α subunits in the case of heteromeric receptors (and by the α subunit in the case of homomer-forming α7-9 subunits) recognize the quarternary nitrogen atom of ACh (Karlin & Akabas, 1995; Arias, 1997). Based on structure-activity studies using imidacloprid analogues and the electron deficiency predicted for imidacloprid from its $^{15}N$-NMR chemical shift, Yamamoto *et al*. (1995) predicted that the nitrogen atom of the imidazolidine group corresponds to the positively charged, protonated form of nicotine. If this is the case, then an interaction between this electron-deficient nitrogen and the nicotinic receptor negative subsite is predicted.

In this study we have utilized the well-studied vertebrate neuronal nicotinic receptor subunit α7 from chicken which is able to form functional homomeric receptors when heterologously expressed in *Xenopus laevis* oocytes (Couturier *et al*., 1990). Preliminary results demonstrated that the chicken α7 receptor is a highly neonicotinoid-sensitive recombinant nicotinic ACh receptor and forms homomeric receptors which facilitate the interpretation of experiments combining site-directed mutagenesis and pharmacology. We have investigated the interactions of imidacloprid, nitenpyram, ACh, (−)-nicotine, (+)-epibatidine and DN-IMI (Figure 1), with wild-type α7 and mutants designed to examine a contribution of loop D to nitroguanidine insecticide recognition.

## Methods

### *Preparation of the G189E mutant cDNA expression plasmid*

The chicken nicotinic α7 subunit cDNA (Couturier *et al*., 1990) in the pMT3 vector (Swick *et al*., 1992) was generously gifted

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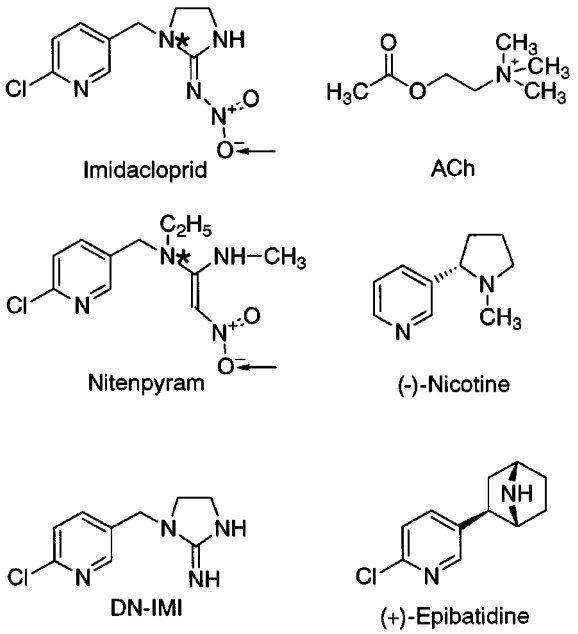


**Figure 1** Chemical structures of neonicotinoid insecticides and natural ligands. The electron-deficient nitrogen and negative oxygen of the insecticides are marked with an asterisk and an arrow, respectively.

by Dr M. Ballivet. Oligonucleotides for mutagenesis KM008 (5′-CAGATATATCCGAGTATATTTCAAATG-3′) and KM009 (5′-CATTTGAAATATACTCGGATATATCTG-3′) were based on the negative subsite sequence but contained the G189E mutation. Forward primer KM001 (5′-TGTCCACTCCCAGGTCCAACTG-3′) was based on the sequence flanking the multiple cloning site of the pMT3 vector; reverse primer KM012 (5′-CTCCATGCTTGACAGGCTG-CATC-3′) was based on α7 cDNA about 1.1 kb downstream of start codon. These four oligonucleotides were used to perform a PCR-based mutagenesis (Higuchi, 1989). A pair of first round PCRs were carried out using Expand™ enzyme (Boehringer, Tokyo, Japan), 500 ng of the wild-type pMT3-α7 as a template, 0.3 μM primers (KM001 and KM009; KM008 and KM012) and 0.2 mM dNTP mixture in a 50 μl solution for 30 cycles of 95°C 45 s, 48°C 60 s and 72°C 90 s. Bands of the correct size were purified by agarose (Promega, Tokyo, Japan) digestion from a low melting point agarase gel. The second round PCR was performed using KOD Dash polymerase (Toyobo, Tokyo, Japan), 15 ng each of the first round PCR product and 0.2 μM primers (KM001 and KM012) for 30 cycles of 98°C 30 s, 60°C 3 s and 74°C 30 s, yielding a single band of the predicted size. After gel purification, the isolated fragment was digested with *Not*I and *Eco*RI (Takara, Shiga, Japan) and subcloned into *Eco*RI and *Not*I complete sites of pMT3-α7. This plasmid was cut with *Eco*RI and ligated with a 1.2 kb *Eco*RI fragment of pMT3-α7 to complete the full length mutant α7. Orientation of the 1.2 kb insert was confirmed by restriction enzyme analysis. Other DNA constructs coding G189D, G189N and G189Q mutants were prepared in the same manner and complete sequences of all mutants were confirmed by automated DNA sequencing.

### *Preparation and nuclear injection of* Xenopus *oocytes*

Mature *Xenopus laevis* females were anaesthetized by immersion in 1.5 g $l^{-1}$ tricaine for 30–45 min (depending on body weight) before surgical removal of part of the ovary. Oocytes at stage V or VI of development were separated from the follicle cell layer by treatment with 2 mg $ml^{-1}$ collagenase (Sigma type IA) for 30 min at room temperature (19–25°C). The follicle cell layer was removed manually using fine forceps. The nucleus of each defolliculated oocyte was injected with 20 nl of cDNA in distilled water (0.1 ng $nl^{-1}$) and incubated at 18°C in standard oocyte saline (SOS) of the following composition (in mM): NaCl 100, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0 and HEPES 5.0, pH 7.6, supplemented with penicillin (100 units $ml^{-1}$), streptomycin (100 μg $ml^{-1}$), gentamycin (50 μg $ml^{-1}$) and 2.5 mM sodium pyruvate. The incubation medium was changed daily. Electrophysiology was performed 3–6 days after nuclear injection.

### *Electrophysiology*

*Xenopus* oocytes were secured in a Perspex recording chamber (80 μl volume) with a Sylgard base and perfused continuously with normal oocyte saline (5 ml $min^{-1}$) by a gravity-fed system (Buckingham *et al*., 1994). To suppress any responses resulting from activation of endogenous muscarinic acetylcholine receptors (Kusano *et al*., 1977; Lupu-Meiri *et al*., 1990), 0.5 μM atropine was included in the saline. Membrane currents were recorded by the two-electrode voltage-clamp method using 2.0 M KCl-filled electrodes (resistances 0.5–5.0 MΩ) and either a GENECLAMP 500 (Axon Instruments, U.S.A.), or an OOCYTE CLAMP 275C (Warner Instruments, U.S.A.) amplifier. The oocyte membrane was clamped at −100 mV. Current signals were recorded by a pen recorder or digitized by a TL-1 interface (Axon Instruments, U.S.A.) and stored on an IBM computer with Axotape software (version 1.2.01, Axon Instruments, U.S.A.), followed by off-line analyses.

To prepare test solutions, stock solutions of ligands were diluted with SOS containing 0.5 μM atropine. Stock solutions of (−)-nicotine (100 mM), (+)-epibatidine (10 mM), imidacloprid (3 mM), nitenpyram (100 mM) and DN-IMI (10 mM) in SOS were stored at −20°C, whereas solutions of ACh in SOS were prepared immediately prior to experiments. Oocytes were challenged with compounds at intervals of 3–5 min to minimize effects of desensitization. (−)-Nicotine, (+)-epibatidine, and DN-IMI resulted in desensitization that was not fully reversible at concentrations close to or higher than 1 mM. However, at all lower doses tested, stable responses were repeatedly observed after wash of oocytes with the saline. Oocytes which gave stable responses to two or more successive applications of 200 or 400 μM ACh were used. Dose-response data were obtained by challenging oocytes with increasing concentrations of an agonist and the maximum amplitude of the current recorded in response to each challenge was normalized to the maximum amplitude of the saturating current response to ACh. As the dose-response curves for ACh were slightly shifted to higher concentrations by the mutations, data from the wild-type receptor and mutants were normalized by the current response to 1 mM and 3 mM ACh, respectively. Using GraphPad 'Prism' (GraphPad Software, U.K.) normalized data were fitted to the following equation:

$$\psi = I_{min} + (I_{max} - I_{min})/[1 + 10^{(\log EC_{50} - [A])n_H}] \qquad (1)$$

where $\psi$ is the normalized response to a compound applied at concentration [A]. $I_{max}$ and $I_{min}$ are the maximum and the minimum normalized responses respectively, $EC_{50}$ is the concentration giving half the maximum normalized response and $n_H$ is the Hill coefficient. Experiments were performed at room temperature (19–25°C). Imidacloprid and DN-IMI were synthesized *de novo* and nitenpyram was donated by Takeda Chemical Industries, Ltd. ACh (chloride salt), (−)-nicotine

(free base) and (+)-epibatidine (HCl salt) were obtained from Sigma-Aldrich Co. (Dorset, U.K.).

## Results

The insecticide imidacloprid (1 mM) evoked maximal amplitude inward currents in *Xenopus* oocytes expressing chicken neuronal wild-type α7 nicotinic ACh receptors when the membrane potential was clamped at −100 mV. Whereas (+)-epibatidine ($n=4$) and nicotine ($n=4$) were full agonists (ACh is defined as a full agonist), imidacloprid was a partial agonist ($n=4$, Figure 2). When the mutants α7G189D ($n=5$) and G189E ($n=7$) were tested, the response to imidacloprid was substantially reduced relative to that of ACh (Figure 2a). Reduction of the maximum currents was not only seen for imidacloprid but also for another neonicotinoid nitenpyram ($n=6$ for G189D mutant and $n=6$ for G189E mutant, Figure 2b). To examine if these findings resulted from either an increase of a negative change, or a change of the steric conformation of loop D, responses of G189N and G189Q mutants were tested for their sensitivity to the insecticides (Figure 3). The efficacy of imidacloprid ($n=4$ for G189N mutant and $n=6$ for G189Q mutant) and that of nitenpyram ($n=5$ for G189N mutant and $n=6$ for G189Q mutant) was scarcely affected by the G189N mutation and only slightly reduced by the G189Q mutation (Figure 3). The reduced insecticide efficacy resulting from the G189Q mutation was, however, much smaller than that conferred by the G189E mutation shown in Figure 2.

DN-IMI is an imidacloprid derivative lacking the nitro group (Figure 1). Since the maximum current amplitude of the wild-type α7 nicotinic ACh receptor in response to a saturating concentration of this compound was as large as that induced by ACh, it is a full agonist. This pharmacology was not limited to its action on the wild-type α7 receptor but extended even to that on the G189D ($n=7$) and G189E ($n=10$) mutants (Figure 4).

Figure 5 shows the agonist profiles of wild-type and the four mutant α7 receptors for the insecticides, DN-IMI and natural ligands. The $pEC_{50}$ ($=-\log EC_{50}$) and $I_{max}$ values obtained from the dose-response curves are summarized in Table 1. (+)-Epibatidine is the most potent of the compounds tested and the rank order of the agonist potency following this agonist is DN-IMI ≥ (−)-nicotine > ACh > Imidacloprid > nitenpyram on the wild-type and mutant α7 receptors. Imidacloprid and nitenpyram were always partial agonists. The $pEC_{50}$ values for ACh, (−)-nicotine and (+)-epibatidine appeared to be slightly reduced by all mutations and, in most cases, the shifts were significant (*t*-test, $P<0.05$). However, the $pEC_{50}$ values of imidacloprid and nitenpyram were not significantly reduced.

The $I_{max}$ values (nA) before normalization for ACh were $1370\pm214$ ($n=36$), $1072\pm161$ ($n=37$), $1852\pm224$ ($n=48$), $2659\pm476$ ($n=24$) and $2962\pm479$ ($n=27$) for the wild-type, G189D, G189E, G189N and G189Q α7 nicotinic receptors, respectively. The $I_{max}$ value was not affected by the G189D and G189E mutations but it was significantly increased by G189N and G189Q mutations (*t*-test, $P<0.05$). The $I_{max}$ values for (−)-nicotine, (+)-epibatidine and DN-IMI are close to the values for ACh (Table 1), regardless of either the presence or absence of mutations in loop D. In contrast, $I_{max}$ values for the insecticides were markedly reduced by the G189D and G189E mutations. Thus the reduction in $I_{max}$ produced by the mutations was selective to the insecticides.

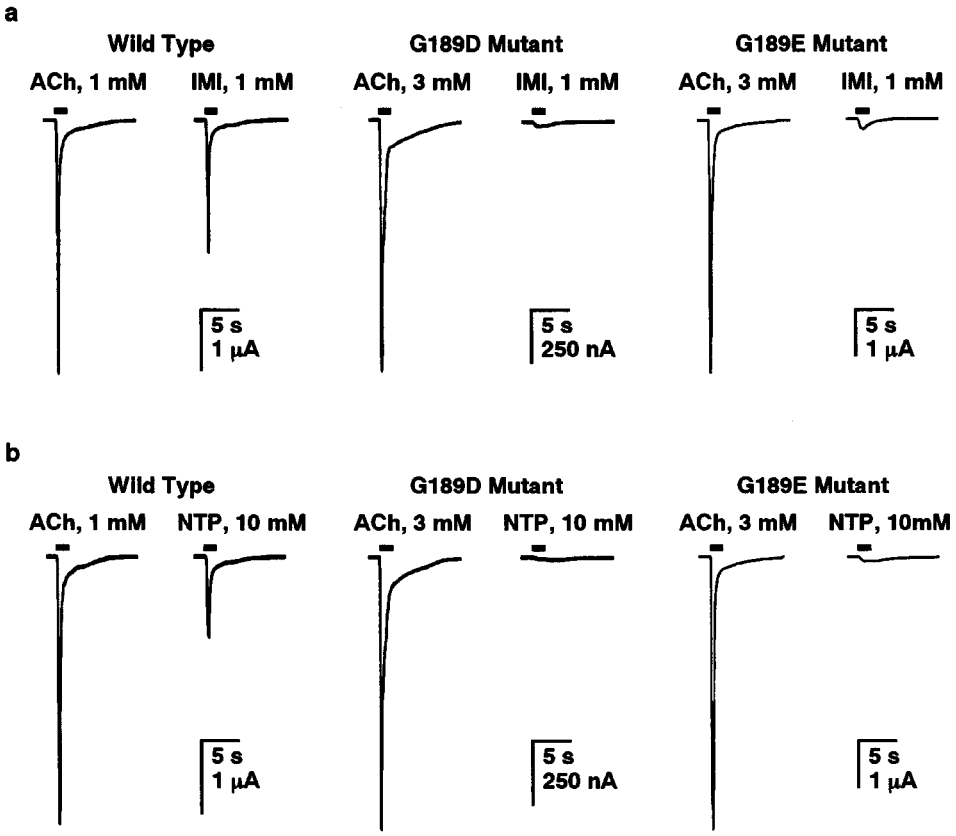


**Figure 2** Actions of ACh and two neonicotinoid insecticides (imidacloprid (IMI) and nitenpyram (NTP)) on the wild-type and two point mutations (G189D and G189E) of the chicken α7 nicotinic receptor expressed in *Xenopus laevis* oocytes. Both mutations reduced the efficacy of (a) imidacloprid ($n=5$ for G189D mutant and $n=7$ for G189E mutant) and (b) nitenpyram ($n=6$ for G189D mutant and $n=6$ for G189E mutant) more than that of ACh. The peak current amplitude of each ACh-evoked response was scaled to have the same size to facilitate comparisons.

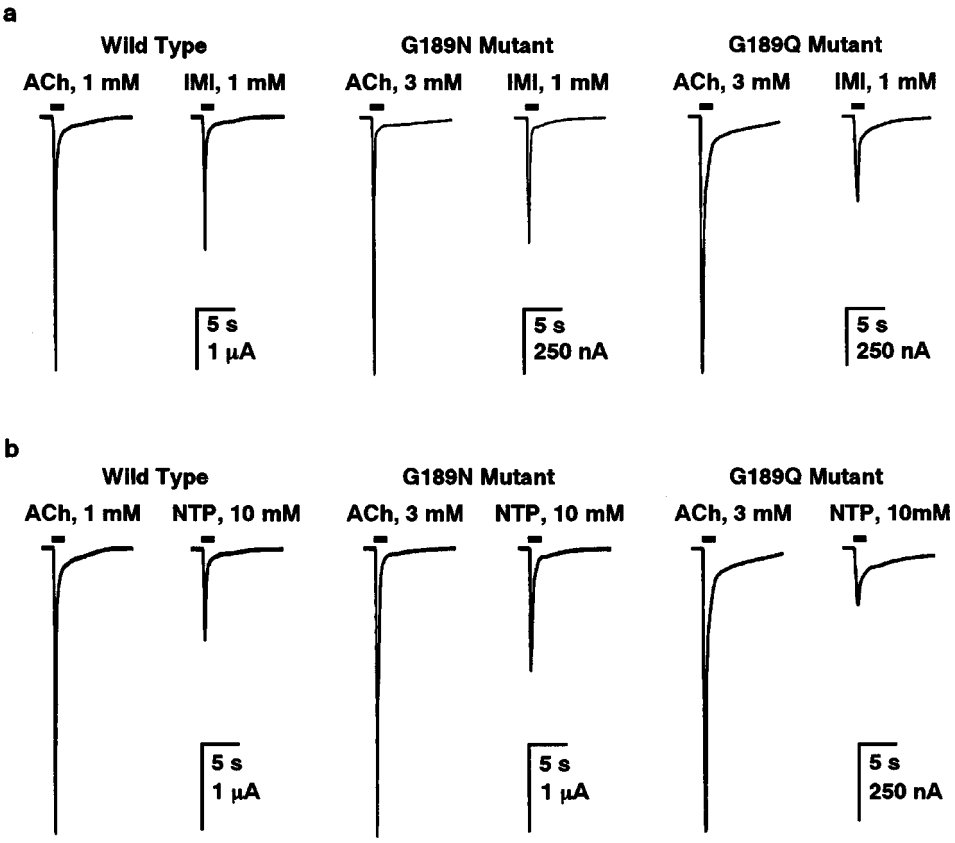


**Figure 3** Actions of ACh and two neonicotinoid insecticides (imidacloprid (IMI) and nitenpyram (NTP)) on the wild-type and two point mutations (G189N and G189Q) of the chicken $\alpha 7$ nicotinic receptor expressed in *Xenopus laevis* oocytes. The G189N mutation was without effect on the efficacy of (a) imidacloprid ($n=4$) and (b) nitenpyram ($n=5$), whereas the G189Q mutation slightly reduced their efficacy (imidacloprid , $n=6$; nitenpyram, $n=6$). The peak current amplitude of each ACh-evoked response was scaled to have the same size to facilitate comparisons.

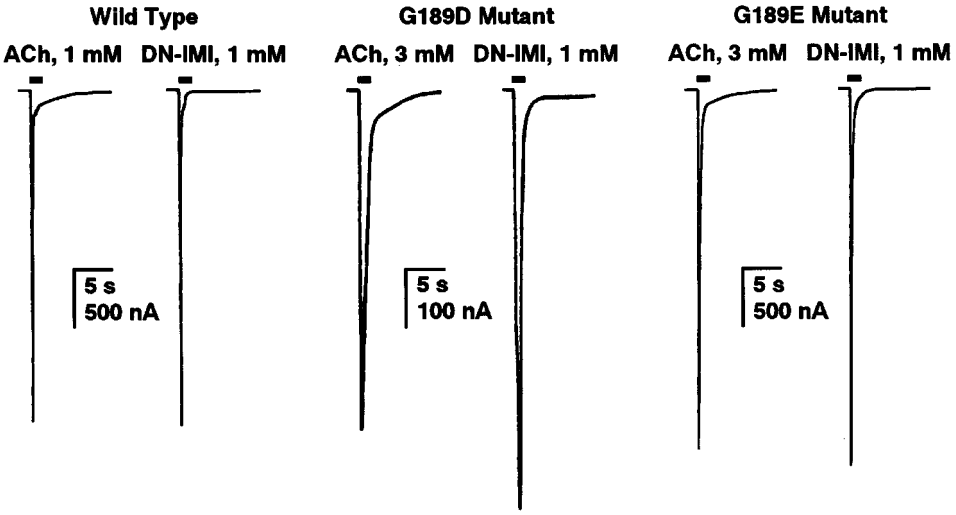


**Figure 4** Actions of desnitro-imidacloprid on the wild-type ($n=4$) and two point mutations (G189D ($n=7$) and G189E ($n=10$)) of the chicken $\alpha 7$ nicotinic receptor expressed in *Xenopus laevis* oocytes.

## Discussion

Imidacloprid is a partial agonist of both wild-type and the mutant G189E $\alpha 7$ nicotinic receptors, as is also the case for chicken $\alpha 4\beta 2$ and *Drosophila* SAD chicken $\beta 2$ hybrid receptors (Matsuda *et al*., 1998). Epibatidine, which has the 6-chloro-3-pyridyl moiety, also found in imidacloprid, is a full agonist of both the wild-type and the mutant G189D and G189E $\alpha 7$ receptors, whereas the maximum current amplitudes evoked by imidacloprid and nitenpyram are markedly reduced by these mutations. This suggests that the nitroguanidine moiety of the insecticides is a key determinant of the partial agonism and that this moiety is likely to interact with loop D.

Negatively charged residues are found not only in vertebrate non-$\alpha$ subunits but also in insect non-$\alpha$ subunits at positions corresponding G189 of the $\alpha 7$ subunit (Table 2).

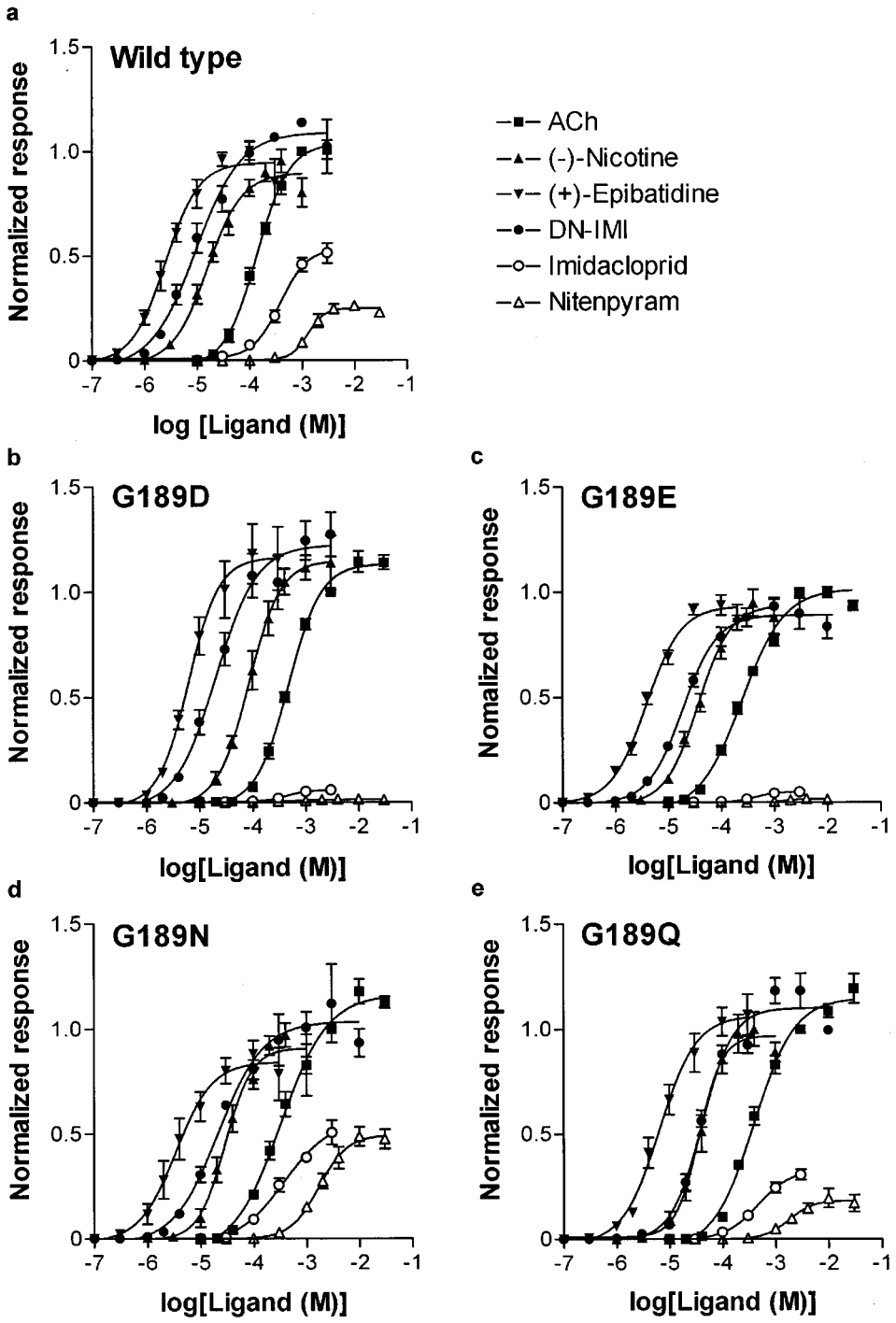


**Figure 5** Dose-response relationships for ACh, (−)-nicotine, (+)-epibatidine, DN-IMI, imidacloprid and nitenpyram obtained for wild-type (a), G189D (b), G189E (c), G189N (d) and G189Q (e) mutant α7 nicotinic ACh receptors expressed in *Xenopus laevis* oocytes.

**Table 1** $pEC_{50}$ and $I_{max}$ values of neonicotinoid insecticides and other ligands

| | *Wild-type* | | *G189D* | | *G189E* | | *G189N* | | *G189Q* | |
|---|---|---|---|---|---|---|---|---|---|---|
| *Ligands* | $pEC_{50}$ | $I_{max*}$ | $pEC_{50}$ | $I_{max*}$ | $pEC_{50}$ | $I_{max*}$ | $pEC_{50}$ | $I_{max*}$ | $pEC_{50}$ | $I_{max*}$ |
| ACh | 3.86±0.04 | 1.04±0.03 | 3.32±0.03 | 1.13±0.02 | 3.61±0.03 | 1.01±0.02 | 3.49±0.05 | 1.16±0.03 | 3.40±0.04 | 1.15±0.03 |
| Nicotine | 4.80±0.07 | 0.90±0.03 | 4.07±0.06 | 1.15±0.05 | 4.44±.006 | 0.94±0.04 | 4.54±0.07 | 0.91±0.04 | 4.44±0.06 | 0.97±0.04 |
| Epibatidine | 5.60±0.07 | 0.95±0.04 | 5.21±0.08 | 0.16±0.06 | 5.42±0.04 | 0.93±0.02 | 5.47±0.10 | 0.84±0.05 | 5.19±0.07 | 1.07±0.05 |
| DN-IMI | 5.02±0.06 | 1.09±0.03 | 4.69±0.08 | 1.22±0.05 | 4.73±0.05 | 0.89±0.02 | 4.66±0.10 | 1.04±0.05 | 4.38±0.04 | 1.10±0.03 |
| Imidacloprid | 3.42±0.07 | 0.53±0.04 | 3.36±0.18 | 0.06±0.01 | 3.35±0.08 | 0.05±0.01 | 3.43±0.15 | 0.57±0.09 | 3.33±0.09 | 0.33±0.03 |
| Nitenpyram | 2.89±0.04 | 0.25±0.01 | 3.24±0.51 | 0.01±0.01 | 2.81±0.09 | 0.02±0.01 | 2.77±0.06 | 0.49±0.03 | 2.74±0.10 | 0.18±0.02 |

The values shown are the result of a fit of the dose-response data (mean±s.e.mean, $n=4-10$) illustrated in Figure 2. The maximum currents are shown as a fraction of the response in the same oocyte to saturating concentrations of ACh (wild-type 1 mM; mutants 3 mM).

**Table 2** Alignment of loop D sequences

| *Subunit* | *Amino acid sequences* |
|---|---|
| | 189 |
| Chicken $\alpha$7 | ADIS**G**YISNGEWDI |
| Chicken $\beta$2 | ASLD**D**FTPSGEWDI |
| Chicken $\beta$4 | ASMD**D**FTPSGTWDI |
| *Drosophila* ARD | VDLS**D**YWKSGTWDI |
| *Drosophila* SBD | IDLT**E**FYLSVEWDI |
| *Locust* $\beta$1 | VDLS**D**YWKSGTWDI |

Since the neonicotinoids alone do not have any positively charged nitrogens (Figure 1), the impact of mutations G189D and G189E on the agonist actions of the insecticides and the natural ligands was predicted to differ and this was found to be the case in our experiments. These mutations alter the conformation as well as increase the negative charge of loop D. To evaluate how the electronic factor contributes to reduction of the agonist efficacy of the insecticides, the sensitivity of the G189N and G189Q mutants to the insecticides has been examined. Much higher sensitivity of these mutants to the insecticides (Figure 3) compared to the G189D and G189E mutants demonstrates that the results shown in Figure 2 are mainly attributable to an increase of the negative charge on the receptor. Nevertheless, a slight reduction in the insecticide efficacy resulting from the G189Q mutation indicates a contribution from the steric change in loop D. Kagabu *et al*. (1998) have shown that the nitroguanidine moiety of imidacloprid has a rigid co-planar configuration. The selective reduction of imidacloprid sensitivity by the mutations G189D and G189E may be due in part to inflexibility of the insecticide molecule, thereby preventing a good fit into the altered agonist binding site.

If an increase of the negative charge resulting from mutations G189D and G189E is the main factor accounting for the reduction in the insecticide efficacy, then the question arises as to which moiety of the nitroguanidine molecule contributes to such an electronic interaction. The finding that DN-IMI is a full agonist irrespective of these mutations (Figure 4) is therefore of interest. The nitro group of the insecticides is electron withdrawing and accumulates a negative charge at the oxygen (Figure 1, arrowed). Thus, for both nitroguanidines this oxygen, rather than the electron deficient nitrogen (Figure 1, shown with an asterisk), is most likely to result in an electronic repulsion with the added aspartate and glutamate residues in loop D, hence the observed interference with insecticide-receptor interaction in both G189D and G189E mutants. In contrast DN-IMI with no such nitro group is readily protonated resulting in a positive charge and is also sterically smaller than imidacloprid, both factors favouring an improved fit to loop D, 'negative subsite', compared to that of the insecticides containing the functional group. The full agonist actions of DN-IMI may therefore reflect this enhanced interaction.

In conclusion we show for the first time that point mutations G189D and G189E in loop D of the $\alpha$7 nicotinic ACh receptor selectively reduce the sensitivity of this receptor to neonicotinoid insecticides. The high sensitivity of these mutants to the desnitro derivative of imidacloprid provides evidence for an important role of this loop in interacting with the nitro moiety of these nitroguanidine insecticides.

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