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Diverse actions and target-site selectivity of neonicotinoids: structural insights

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Running title: Running title: Nicotinic receptor - neonicotinoid interactions

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Number of text page: 34
Number of tables: 0
Number of figures: 8
Number of references: 91
Number of words in Abstract: 248
Number of words in Introduction: 746

Abbreviations: *Ac*, *Aplysia californica*; ACh, acetylcholine; AChBP, acetylcholine binding protein; LBD, ligand binding domain; *Ls*, *Lymnaea stagnalis*; nAChR, nicotinic acetylcholine receptor; TM, transmembrane region.

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Abstract

The nicotinic acetylcholine receptors (nAChRs) are targets for human and veterinary medicines as well as insecticides. Subtype-selectivity among the diverse nAChR family members is important for medicines targeting particular disorders and pest-insect selectivity is essential for the development of safer, environmentally-acceptable insecticides. Neonicotinoid insecticides selectively targeting insect nAChRs have important applications in crop protection and animal health. Members of this class exhibit strikingly diverse actions on their nAChR targets. Here we review the chemistry and diverse actions of neonicotinoids on insect and mammalian nAChRs. Electrophysiological studies on native nAChRs and on wild type and mutagenized recombinant nAChRs have shown that basic residues particular to loop D of insect nAChRs are likely to interact electrostatically with the nitro group of neonicotinoids. In 2008, the crystal structures were published showing neonicotinoids docking into the acetylcholine (ACh) binding site of molluscan acetylcholine binding proteins (AChBP) with homology to the ligand binding domain (LBD) of nAChRs. The crystal structures showed that Gln in loop D, corresponding to the basic residues of insect nAChRs, hydrogen bonds with the NO₂ group of imidacloprid, in addition to neonicotinoid-unique stacking and CH- π bonds at the LBD. A neonicotinoid-resistant strain obtained by laboratory-screening has been found to result from target site mutations and possible reasons for this are also suggested by the crystal structures. The prospects of designing neonicotinoids which are safe, not only to mammals, but also to beneficial insects such as honey bees (*Apis mellifera*), are discussed in terms of interactions with non- α nAChR subunits.

Introduction

Sustainable agriculture aims to supply sufficient food for the world population, while minimizing environmental impact. Neonicotinoids, targeting insect nicotinic acetylcholine receptors (insect nAChRs) have veterinary and crop protection applications, their fast actions providing economic benefits. However, their target selectivity must be strictly assessed to ensure safety and to limit adverse effects on beneficial insects such as honeybees.

The nicotinic acetylcholine receptors (nAChRs) (Fig. 1A) are pentameric membrane proteins that rapidly transduce the actions of the chemical neurotransmitter acetylcholine (ACh) to membrane depolarization at synapses. Nicotine (Fig. 1B), a major alkaloid of the tobacco plant *Nicotiana tabacum* is a non-hydrolyzable agonist of nAChRs and remains much longer at the synapses than ACh which is hydrolyzed by acetylcholine esterase, inducing complex modifications to neural signaling. Human drugs targeting nAChRs are clinically important because they may offer therapy for nicotine addiction, Alzheimer's disease and schizophrenia as well as treatment for some neuropathies resulting from mutations in nAChRs (Arneric et al., 2007; Changeux and Taly, 2008; Dani and Bertrand, 2007; Levin and Rezvani, 2007). Effective control of insect pests and helminth parasites has been achieved by targeting invertebrate nAChRs (Brown et al., 2006b; Matsuda et al., 2001, 2005; Tomizawa and Casida, 2003, 2005). This road from nicotine to neonicotinoids was long and tortuous. In general, compounds require appropriate lipophilicity to show high insecticidal actions because they can only access nAChRs after traversing a waxy cuticle membranes and cells enveloping the nervous system. However, nicotine is protonated at neutral or lower

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pH, yielding a water-soluble ammonium which lowers its insect toxicity, although this ammonium form is recognized by nAChRs. Additionally, the low field stability and adverse mammalian toxicity mean that nicotine is of historical interest only in pest control. The development of insecticides acting on insect nAChRs has posed a challenge. Indeed until recently the only successes were cartap, bensultap and thiocyclam based on a marine worm (*Lumbriconereis heteropoda*) toxin, nereistoxin. Cartap was shown to undergo hydrolytic activation to nereistoxin (Lee et al., 2004), which exerts toxicity by blocking nAChRs (Eldefrawi et al., 1980; Sattelle et al., 1985; Raymond et al., 2003; Lee et al., 2004). Although these nereistoxin derivatives are used for crop protection, their current market share is much smaller than that of organophosphates and pyrethroids.

The major commercial insecticides targeting nAChRs were not derived from natural products but rather from the discovery of synthetic nitromethylene heterocycles (Kagabu, 1997; Soloway et al., 1979). Although the leading compounds were not in the first wave, introducing the 6-chloro-3-pyridylmethyl and nitroimine moieties led to the development of the first new-type of nicotinic insecticide imidacloprid (Kagabu, 1997; Moriya et al., 1992). In parallel with synthesis, mode of action studies have been conducted to show that both early chemotypes, the nitromethylene heterocycles (Leech et al., 1991; Sattelle et al., 1989; Schroeder and Flattum, 1984), and imidacloprid (Bai et al., 1991) act on insect nAChRs. Imidacloprid rapidly expanded its share of the market and several analogs followed. Since the chloronicotinyl (6-chloro-3-pyridylmethyl) moiety is seen in the first generation of imidacloprid analogs, they were once called chloronicotinyl insecticides. However, neither this

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moiety, nor the imidazolidine ring, features in the second generation of neonicotinoids (Fig. 1B). The generic name “neonicotinoids” has been adopted now for all members of this class to show that they are new in terms of their mode of actions and their structural features that are clearly different from those of nicotine and nicotine-related compounds, “nicotinoids”.

Neonicotinoids show selective actions on insect nAChRs (Matsuda et al., 2001, 2005; Thany et al., 2007; Tomizawa and Casida, 2003, 2005). Electrophysiology, computational chemistry and site-directed mutagenesis in conjunction with homology modeling of the nAChR ligand binding domain (nAChR LBD)-imidacloprid complexes, have been employed to elucidate the nature and the diversity of their actions. To understand the structural factors involved in the selectivity and diversity of neonicotinoid actions, we have crystallized the mollusc *Lymnaea stagnalis* (*Ls*) acetylcholine binding protein (AChBP) in complex with neonicotinoids (imidacloprid and clothianidin) (Ihara et al., 2008). At about the same time, the crystal structures of the *Aplysia californica* (*Ac*)-AChBP in complex with imidacloprid and thiacloprid were elucidated (Talley et al., 2008). From these crystal structures, a common concept for the nAChR LBD-neonicotinoid interactions can be derived which clearly differs from the binding modes of nicotinoids. Despite these achievements, two major problems may threaten the future of neonicotinoids: (a) the development of resistance in pests and (b) the adverse effects on beneficial insect species. Structural insights relevant to these two problems are also discussed here.

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Neonicotinoids and nicotinic ligands defined by computational chemistry

Nicotine possesses two nitrogens, one in the pyridine ring and another in the pyrrolidine ring. The basicity of the pyridine nitrogen is low because its lone pair electrons participate in the aromatic system, whereas the pyrrolidine nitrogen can accept a proton to become a positively-charged ammonium, mimicking the quaternary ammonium of ACh (Fig. 2)

Unlike nicotine, neonicotinoids (Fig. 1) are largely devoid of protonation. In the case of imidacloprid, two nitrogens in its imidazolidine ring are conjugated through a C=N bond with the electron-withdrawing nitro (NO₂) group. Such a push-pull conjugation results in a co-planarity of the imidazolidine ring with the nitroimino (C=N-NO₂) group (Kagabu, 1997). Positive electrostatic potentials surround the ammonium form of pyrrolidine in nicotine and similar properties hold for other nicotinic ligands such as epibatidine (Fig. 1). In contrast, such strongly positive regions are not seen in neonicotinoids. Instead, the NO₂ oxygens and the CN nitrogen are negatively charged in neonicotinoids. In addition to their electrostatic nature, both groups can hydrogen bond with local hydrogen donors.

The changes in atomic charge of the imidazolidine ring before and after the NO₂ group-ammonium complex formation have been calculated (Matsuda et al., 2001). When complexed with ammonium, the imidazolidine ring, notably hydrogens of the CH₂-CH₂ moiety, become more electron deficient, resulting in enhanced positive charges. Another calculation has shown that the NO₂ group forms a stronger complex with a methylammonium than phenol and methanol (Ihara et al., 2003). The following

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predictions for imidacloprid can be made from these calculations: 1) basic or hydrogen bondable residues, which are selectively present in the ACh binding region of insect nAChRs, contact the NO₂ group of imidacloprid to strengthen the nAChR-neonicotinoid binding. 2) The complex formation also strengthens the electron-deficient nature of hydrogens in the imidazolidine CH₂-CH₂ as well as the π -electron-nature of the lone pairs on the imidazolidine nitrogens. 3) These electron-deficient hydrogens are predicted to interact with electron-rich amino acid residues. This three-step binding consisting of 1. first contact, 2. changing electrostatic properties and 3. attracting electron-rich residue is thought to be a kind of induced fit. In the case of ACh (Zhong et al., 1998) and nicotine (Cashin et al., 2005), cation- π interactions of the ammonium nitrogens with the aromatic ring of the tryptophan residue in loop B determine the binding affinity. Thus, it was predicted that the imidazolidine and related moieties may contact by cation- π electrostatic interactions the tryptophan residue in loop B (Ihara et al., 2007; Tomizawa et al., 2003). Another model was proposed based on *ab initio* molecular orbital calculations showing that the imidazolidine ring is likely to interact with the tryptophan by a π - π stacking (Wang et al., 2007). Conclusions from this *in silico* based approach have been harder to reconcile with the subsequent crystal structures.

Differential binding of nicotine and neonicotinoids

The nAChRs possess a long extracellular N-terminal ligand binding domain (LBD) and four transmembrane (TM) regions with the C-terminus also located extracellularly. Two classes of subunit are present among nAChRs (α and non- α), the α subunits, possessing a pair of adjacent cysteines in loop C of the ACh binding site (Karlin, 2002).

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The integral, cation-selective ion channel opens transiently upon binding of ACh. In the case of heteromeric nAChRs, ACh binds at the interface of the N-terminus regions of α and non- α subunits. However, in the case of either homomers ($\alpha 7$, $\alpha 8$, $\alpha 9$) (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994), or hetero α dimers such as $\alpha 9/\alpha 10$ (Elgoyhen et al., 2001) and *C. elegans* DEG-3/DES-2 (Treinin et al., 1998), the ACh binding site is formed at the interface of two adjacent α subunits. The α and non- α subunits respectively donate loops A-C and loops D-F to generate the ACh binding site (Corringer et al., 2000; Karlin, 2002). Site-directed mutagenesis and photoaffinity labeling of amino acids that contact directly with agonists and antagonists have been deployed extensively in the case of vertebrate $\alpha 7$ nAChRs (Corringer et al., 2000). A general principle derived from these “wet” experiments requires confirmation by crystallization of nAChRs, but it has not yet been achieved, although exciting progress has been made in crystallizing bacterial LGICs (Bocquet et al., 2008; Hilf and Dutzler, 2008a, b). However, water soluble acetylcholine binding proteins (AChBPs) from molluscs *Lymnaea stagnalis*, *Aplysia californica* (Ac) and *Bulinus truncatus* have added considerably to our understanding of nAChR-ligand interactions. The AChBPs are homologous to the N-terminal ligand binding domain of $\alpha 7$ and also form a pentamer. Unlike nAChRs, the AChBPs lack the TM regions and are thus water soluble. They act as an ACh-sink at molluscan synapses (Smit et al., 2001). The first AChBP crystal structure showed that the 6 binding site loops (A-F) are all located at subunit interfaces (Brejc et al., 2001) and AChBPs proved to be profitable surrogates of nAChRs with respect to exploring ligand interactions (Bourne et al., 2005, 2006; Celie et al., 2004, 2005; Hansen et al., 2005; Hansen and Taylor, 2007).

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In the crystal structure of *Lymnaea stagnalis* (*Ls*)- AChBP with nicotine bound (Celie et al, 2004), the proton on the pyrrolidine nitrogen of nicotine form a hydrogen bond with the backbone C=O of Trp143 (loop B), whereas N-CH₃ in the pyrrolidine ring points to the center of the tryptophan ring, forming a CH- π hydrogen bond (Fig. 4). The CH- π hydrogen bond involves not only the London's dispersion force but also electrostatic interaction (Nishio, 2005). This interaction resembles a conventional hydrogen bond and therefore should not be referred to simply as a hydrophobic contact. The proton of N-CH₃ also makes a CH- π interaction with Tyr192 (loop A; not shown in Fig. 4 to facilitate the view of nicotine-Trp interactions). Trp53 in loop D is located close to nicotine but only contributes to building a hydrophobic wall. In addition to these interactions, the cationic center of epibatidine, namely the protonated nitrogen, undergoes a cation- π interaction (Cashin et al., 2005) and the OH of Tyr93 (loop A) and the backbone C=O of Trp147 (loop B) form hydrogen bonds with the hydrogens on the bridge head nitrogen (Hansen et al., 2005). The pyridine nitrogen of nicotine and epibatidine forms a water bridge with the backbone C=O of two amino acids in loop E (Leu102 in both *Ls* and *Ac*-AChBPs; Met114 in *Ls*-AChBP and Ile118 in *Ac*-AChBP).

The crystal structures of *Ls*-AChBP (Fig. 5A) (Ihara et al., 2008) and *Ac*-AChBP (Fig. 5B) (Talley et al., 2008) in complex with imidacloprid were elucidated almost at the same time. The five binding pockets are fully occupied with imidacloprid in *Ls*-AChBP, whereas, in the crystal structure of *Ac*-AChBP, four of five sites are occupied the remaining site being complexed with an isopropyl alcohol molecule. Furthermore, one binding pocket of *Ac*-AChBP was bound by both imidacloprid and isopropyl alcohol. Although neonicotinoids show higher binding affinity for

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Ac-AChBP vs *Ls*-AChBP (Tomizawa et al., 2008), the binding modes of imidacloprid in these two crystals are quite similar. The pyridine ring forms a water bridge (Ihara et al., 2008; Talley et al., 2008) with the backbones of two amino acids in loop E similar to the binding seen for both nicotine (Celie et al., 2004) and epibatidine (Hansen et al., 2005). This result is in accord with photoaffinity labeling results obtained using azidopyridine analogs (Tomizawa et al., 2007). However, since the pyridine ring recognition pattern is conserved in nicotinoids and neonicotinoids, this cannot explain the selectivity of neonicotinoids.

Interactions particular to imidacloprid are observed for the 2-nitroimino-imidazolidine moiety (Fig. 5, C and D). This group stacks with Tyr185 and Tyr188 in loop C of *Ls*- and *Ac*-AChBPs, respectively, while two protons in the CH₂-CH₂ moiety of the imidazolidine ring and a proton on the C2 of the pyridine ring of imidacloprid form CH- π hydrogen bonds with the tryptophan ring in loop B (Fig. 5, C-F). As the tyrosine residue corresponding to Tyr185 of *Ls*-AChBP and Tyr188 of *Ac*-AChBP is conserved throughout vertebrate and invertebrate nAChRs (Fig. 3), its presence in itself is not the cause for selectivity. The nitro group of imidacloprid forms a hydrogen bond with a glutamine residue (Gln55 and Gln57 in loop D of *Ls*- and *Ac*-AChBPs, respectively) (Fig. 5, C, D, G, H) and the corresponding residues of insect nAChRs are basic (Fig. 3). Thus, they are able to tether the nitro group of neonicotinoids by an electrostatic force. Furthermore, if the distance between the NO₂ group and the basic residues is short, hydrogen bonds will add to the interaction. Therefore, the loop D basic residue (glutamine in AChBPs) plays a role in capturing neonicotinoids to strengthen the stacking and CH- π hydrogen bonds. Consistent with this, mutations of

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the corresponding loop D residues to basic residues were found to dramatically enhance the neonicotinoid sensitivity of the chicken $\alpha 7$ (Shimomura et al., 2002) and $\alpha 4\beta 2$ nAChRs (Shimomura et al., 2006; Toshima et al., 2009). The selectivity-determining role of this residue can also explain, at least in part, why $\alpha 7$ having a glutamine (Gln89) residue in loop D is more neonicotinoid sensitive than $\alpha 4\beta 2$ (Ihara et al., 2003). In this context, AChBPs, from *Lymnaea* or *Aplysia*, resemble insect nAChRs because they possess this important residue. At first sight, the finding that human $\beta 4$ has a lysine, at this otherwise highly conserved residue, is surprising yet interestingly $\beta 4$ -containing nAChRs are also less sensitive to imidacloprid than insect nAChRs (Lansdell and Millar, 2000). This too can be resolved by consideration of electrostatic interference, in this case involving a glutamate residue corresponding to Thr57 of *Ls*-AChBP which is located very close to the basic residue (Ihara et al., 2008).

In the crystal structure of the *Ls*-AChBP-clothianidin complex, the NO₂-Gln55 distance was outside the hydrogen bondable range. However, in the Q55R mutant, the basic residue contacts electrostatically with NO₂ in *Ls*-AChBP (Matsuda et al., unpublished). For thiacloprid (Fig. 1), its thiazolidine ring stacks with Tyr188 in loop C, the CN group pointing to Ser189 in *Ac*-AChBP (Talley et al., 2008). Although this appears to indicate that loop D is not essential for selectivity, in its Q57R mutant, the CN group may point to the introduced arginine residue. Also, appropriate care is required in interpretation of the crystal data because isopropyl alcohol used for crystallization binds in the vicinity of thiacloprid (see the pdb file 3C84). Thus, for *Ac*-AChBP, isopropyl alcohol-free crystals with all 5 LBDs filled with neonicotinoids are desirable for detailed comparison and homology modeling.

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Loop D alone is not the only determinant of selective neonicotinoid actions.

Employing the fruit fly *Drosophila melanogaster* $\alpha 2$ ($D\alpha 2$)/chicken $\beta 2$ hybrid nAChR (Bertrand et al., 1994) as well as the chicken $\alpha 4\beta 2$ nAChR, mutations of the X residue in the α -defining YXCC motif in loop C were found to strongly influence neonicotinoid sensitivity of the nAChRs (Shimomura et al., 2004). The *Drosophila* $D\alpha 2$ subunit has a proline at this position, whereas in vertebrate $\alpha 4$ subunits this is a glutamate (see Fig. 3). The E219P mutation enhanced the response amplitude of the chicken $\alpha 4\beta 2$ nAChR to imidacloprid, whereas a reverse mutation P242E markedly reduced the affinity and the efficacy of the $D\alpha 2\beta 2$ hybrid nAChR. The crystallographic data offer, at least in part, an explanation of these findings. In both *Ls*- and *Ac*-AChBPs, the corresponding residue is a serine (Ser186 in *Ls*-AChBP and Ser189 in *Ac*-AChBP). Ser186 in loop C contacts with Glu163 in loop F in *Ls*-AChBP (Fig. 3), whereas Ser189 forms a hydrogen bond with the NO₂ of imidacloprid in *Ac*-AChBP. It is conceivable from the crystal structure of the *Ls*-AChBP-imidacloprid complex that vertebrate $\alpha 2$ and $\alpha 4$ subunits with a glutamate residue in this motif (YECC) (Fig. 3) will lead to an electrostatic repulsion when in contact with acidic residues in loop F, corresponding to Glu163 of *Ls*-AChBP. As the consequence of loop C-F repulsion, an inter-subunit bridge is broken, resulting in a reduced affinity or efficacy of neonicotinoids.

Supporting this hypothesis, neither Glu219 in loop C, nor Thr77 in loop D contacts with the NO₂ of imidacloprid in the homology model of the wild-type $\alpha 4\beta 2$ LBD with imidacloprid bound (Toshima et al., 2009). An alternative explanation based on the crystal structure of *Ac*-AChBP-imidacloprid complex is that the acidic residue in loop C may directly repel the NO₂ or CN groups of neonicotinoids, lowering affinity. We

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have found that the addition of serine to the YXCC motif of the chicken $\alpha 4\beta 2$ nAChR scarcely influences the response to imidacloprid, and that, when combined together with the mutations in loop D, the X residue mutations to insect nAChR type amino acids result in enhanced efficacy but not affinity of imidacloprid (Toshima et al., 2009). Thus, it is apparent that the YXCC motif affects the neonicotinoid-sensitivity of nAChRs, yet a serine residue in this motif alone is not sufficient for the selective neonicotinoid actions on insect nAChRs, whether or not it contacts with the NO₂ or the CN group of neonicotinoids.

Structural factors and the diverse actions of neonicotinoids

Voltage-clamp electrophysiology has shown that neonicotinoids act as partial, full and, in particular cases, super agonists on nAChRs. Imidacloprid is a partial agonist of native nAChRs expressed by insect neurons (Deglise et al., 2002; Nagata et al., 1996; Nagata et al., 1998; Brown et al., 2006a) as well as the recombinant D $\alpha 2\beta 2$ hybrid nAChRs expressed in *Xenopus laevis* oocytes (Ihara et al., 2003; Matsuda et al., 1998). Opening of the imidazolidine ring leads to an enhanced efficacy (Ihara et al., 2003; Ihara et al., 2004; Tan et al., 2007). For example, dinotefuran (Kagabu et al., 2002) and nitenpyram (Ihara et al., 2003) (Fig. 1) are full or nearly full agonists of the D $\alpha 2\beta 2$ hybrid nAChR. On the other hand, clothianidin and its analog both show higher agonist efficacy than ACh on the D $\alpha 2\beta 2$ hybrid nAChR (Ihara et al., 2004) and native *Drosophila* nAChRs (Brown et al., 2006a). Patch-clamp electrophysiology has been used to demonstrate that the clothianidin analog opened the native nAChRs at the highest conductance state more frequently than ACh, offering a possible explanation for its super agonist action. The crystal structure of *Ls*-AChBP in complex with

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clothianidin shows that the NH of the guanidine moiety of clothianidin forms a hydrogen bond with the backbone C=O of Trp143 in loop B (Fig. 6), which is not seen in the AChBP-imidacloprid complex (Ihara et al., 2008). It has been demonstrated that the agonist binding to LBD is likely to induce a global twist of nAChRs to gate the ion channels (Cheng et al., 2006; Miyazawa et al., 2003; Taly et al., 2005; Unwin, 2005). In this event, the agonist-binding-induced inward motion of loop C is transmitted to the Cys loop through a structural rearrangement of loops D and A, resulting finally in the interaction of the Cys loop and β 2- β 3 linker with the TM2-TM3 linker for the channel opening. For neonicotinoids, interacting not only with loops B and C, but also with loop D, this structural rearrangement is likely to cause its release from the binding site. The NH-backbone hydrogen bond particular to clothianidin may help capture the ligand even after this structural rearrangement, thereby leading to enhanced channel opening.

Met114 (Fig. 5G) in loop E of *Ls*-AChBP and corresponding Ile118 in *Ac*-AChBP (Fig. 5H) are located in the vicinity of the nitroimine moiety of imidacloprid. Since these amino acids are predicted to play a role in the agonist recognition, the effects of Leu118 mutations on the responses to ACh and imidacloprid were investigated. The L118E mutation almost completely blocked the response to imidacloprid, leaving the response to ACh, whereas the reverse was the case for L118K and L118R mutations (Amiri et al., 2008), suggesting a contribution to efficacy. Some insect nAChR α subunits possess a basic residue at this position. The possibility that such residues are also involved in the selective actions of neonicotinoids cannot be excluded because some α nAChRs are functional when they serve as partners for another α subunit (e.g. α 10, which partners α 9). The special case of loop E in α/α heteromers remain to be clarified.

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Target based neonicotinoid resistance: a structural interpretation

Two issues may limit the long-term utility of neonicotinoids: (a) resistance in pest species; (b) adverse effects on beneficial insect species. Neonicotinoid resistance has been well described in rice plant hoppers (Matsumura et al., 2008; Wang et al., 2008), peach potato aphids (Foster et al., 2008) and whiteflies (Nauen et al., 2008). Neonicotinoid resistance is often the result of enhanced metabolism (Karunker et al., 2008; Nauen et al., 2008) but there are examples of reduced sensitivity to neonicotinoids at the target site. In the case of eastern US field populations of the Colorado potato beetle, *Leptinotarsa decemlineata*, imidacloprid sensitivity of the central nervous system in terms of excitation blocking action was found to be significantly reduced in resistant insects (Tan et al., 2008). Equally interesting is the study on the laboratory-selected neonicotinoid-resistant brown planthopper *Nilaparvata lugens*. Binding-assays using [³H]imidacloprid show a reduced binding affinity for membrane preparations from the resistant population. A comparison of α and non- α subunit genes from susceptible and resistant population has shown that one point mutation Y151S in loop B can account for the reduced imidacloprid sensitivity (Liu et al., 2005). To understand the mechanism and to examine whether this kind of mutation lowers the neonicotinoid sensitivity in other insect species, wild type and mutant $\alpha 2\beta 1$ nAChR from peach potato aphid *Myzus persicae* (M $\alpha 2\beta 1$ nAChR) has been modeled using the crystal structures of *Ls*-AChBP in complex with imidacloprid (Fig. 7). This subunit combination was adopted by the following four reasons. (1) Both $\alpha 2$ and $\beta 1$ subunits are important subunits and have a similar distribution in the *Drosophila* central nervous system (Jonas et al., 1994). (2) The *Drosophila* D $\alpha 2$ and D $\beta 1$ subunits were co-purified with the D $\alpha 1$ and D $\alpha 3$ subunits from the fly heads by

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α -bungarotoxin-affinity column (Chamaon et al., 2002). (3) The Mp α 2/rat β 2 hybrid nAChR is much more sensitive to imidacloprid than the Mp α 1/rat β 2 hybrid nAChR (Huang et al., 1999) and the Mp α 2 subunit co-assembles with the Mp β 1 subunit in the *Drosophila* S2 cell (Huang et al., 2000). (4) The Mp α 2 subunit has a valine at position X in the YXCC motif (Fig. 3), which obviates the need to consider possible NO₂ hydrogen bonding. In the wild type Mp α 2 β 2 nAChR, the imidazolidine ring stacks with loop C tyrosine while two protons in the CH₂-CH₂ bridge make CH- π contacts with loop B tryptophan as seen in the AChBPs (Fig. 7A). The *Myzus* tyrosine residue corresponding to Tyr151 of the *N. lugens* α subunit is tightly packed in a hydrophobic groove (not shown), thereby indirectly fixing the tryptophan residue in loop B. The tyrosine to serine mutation resulted in a reduced residue size, making the tryptophan residue wobble. As a consequence, the tryptophan residue has a reduced probability of proximity to the imidazolidine ring, thereby reducing the CH- π contacts with the imidazolidine ring and resulting in reduced neonicotinoid sensitivity. Among the commercial neonicotinoids, dinotefuran was found to act on the Y151S mutant of *N. lugens* nAChR as effectively as on the wild type (Liu et al., 2006). It will be of interest to examine in future whether or not dinotefuran can compensate for the movement of the tryptophan residue by particular contacts with the mutant nAChR. This could lead to a strategy for rational design of novel neonicotinoids effective on target-based resistant pests.

Prospects for design of species specific insecticides

Another issue confronting neonicotinoids is the adverse effects on honey bees (Decourtye et al., 2003, 2004; Faucon et al., 2005; Guez et al., 2001, 2003; Yang et al.,

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2008), though they are safe to mammals. To explore a solution to this issue, we have modeled in complex with imidacloprid, cotton peach aphid (*M. persicae*) and honey bee (*Apis mellifera*) $\alpha 2\beta 1$ nAChRs (Fig. 8). The stacking and CH- π interactions (Fig. 5) are conserved irrespective of insect species, yet marked differences between the binding sites of the two insect species are seen at a hidden groove extending from the NO₂ binding site, formed mainly by loops D and F, with particular reference to electrostatic (Fig. 8, A and B) and hydrogen-bond accepting/donating features (Fig. 8, C and D). First, the groove in the aphid nAChR is broader than that of the bee receptor. Secondly, higher electrostatic regions distribute more broadly in the bee receptor (Fig. 8A) than in the aphid receptor (Fig. 8B). Finally, the aphid nAChR groove (Fig. 8C) contains more hydrogen bond forming hooks than the bee nAChR groove (Fig. 8D). These predictions suggest a concept insecticide generation, in which designing a molecular fragment for optimal fit to the groove is the first step. Then linking this fragment with a traditional neonicotinoid framework using a functional group which is isosteric to the nitro group may yield new insect control chemicals highly selective for pest species nAChRs. Alternatively, “Crick Chemistry” (Kolb et al., 2001) may be applied to link the two fragments. By this mean, one fragment with an alkynyl end is reacted with another fragment containing an azide group on the pest nAChRs in aqueous solution at ambient temperature. Such PTS (pest target selective) neonicotinoids could help resolve a major issue in crop protection.

Concluding remarks

We have discussed the structural basis of how nAChRs recognize nicotinoids and neonicotinoids. Several hooks play important roles in either capturing, or repelling

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small but important features of these ligands. So far, the importance of non- α subunits in the interactions of nicotinic ligands with heteromeric nAChRs seems to have been underestimated. The interactions with loops A-C are common for nicotinic and neonicotinic ligands and selectivity is often donated by interactions with loops D-F. For nicotinoids or neonicotinoids, the hidden grooves and hydrogen bonding options offer a treasure trove of possibilities for generating novel ligands selective to nAChR subtypes. For rapid progress, the crystallization of an entire nAChR molecule is needed. Now that bacterial pentameric ligand-gated ion channels have been crystallized (Bocquet et al., 2008; Hilf and Dutzler, 2008a, b), a new era with rational design of a key component is an exciting and perhaps a not too distant prospect.

References

- Amiri S, Shimomura M, Vijayan R, Nishiwaki H, Akamatsu M, Matsuda K, Jones AK, Sansom MS, Biggin PC and Sattelle DB (2008) A role for Leu118 of loop E in agonist binding to the $\alpha 7$ nicotinic acetylcholine receptor. *Mol Pharmacol* **73**:1659-1667.
- Arneric SP, Holladay M and Williams M (2007) Neuronal nicotinic receptors: a perspective on two decades of drug discovery research. *Biochem Pharmacol* **74**:1092-1101.
- Bai D, Lummis S, Leicht W, Breer H and Sattelle D (1991) Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. *Pestic Sci* **33**:197-204.
- Bertrand D, Ballivet M, Gomez M, Bertrand S, Phannavong B and Gundelfinger ED (1994) Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate $\beta 2$ subunit and *Drosophila* α subunits. *Eur J Neurosci* **6**:869-875.
- Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux JP, Delarue M and Corringer PJ (2008) X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. *Nature* **457**:111-114.
- Bourne Y, Hansen SB, Sulzenbacher G, Talley TT, Huxford T, Taylor P and Marchot P (2006) Structural comparison of three crystalline complexes of a peptidic toxin with a synaptic acetylcholine recognition protein. *J Mol Neurosci* **30**:103-104.
- Bourne Y, Talley TT, Hansen SB, Taylor P and Marchot P (2005) Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake α -neurotoxins and nicotinic receptors. *EMBO J* **24**:1512-1522.
- Brejck K, van Dijk WJ, Klaassen RV, Schuurmans M, van Der Oost J, Smit AB and Sixma TK (2001) Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **411**:269-276.
- Brown LA, Ihara M, Buckingham SD, Matsuda K and Sattelle DB (2006a) Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors. *J Neurochem* **99**:608-615.
- Brown LA, Jones AK, Buckingham SD, Mee CJ, Sattelle DB (2006b) Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. *Int J Parasitol* **36**, 617-624.

MOL #55186

- Cashin AL, Petersson EJ, Lester HA and Dougherty DA (2005) Using physical chemistry to differentiate nicotinic from cholinergic agonists at the nicotinic acetylcholine receptor. *J Am Chem Soc* **127**:350-356.
- Celie PH, Kasheverov IE, Mordvintsev DY, Hogg RC, van Nierop P, van Elk R, van Rossum-Fikkert SE, Zhmak MN, Bertrand D, Tsetlin V, Sixma TK and Smit AB (2005) Crystal structure of nicotinic acetylcholine receptor homolog AChBP in complex with an α -conotoxin PnIA variant. *Nat Struct Mol Biol* **12**:582-588.
- Celie PH, van Rossum-Fikkert SE, van Dijk WJ, Brejc K, Smit AB and Sixma TK (2004) Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* **41**:907-914.
- Chamaon K, Smalla KH, Thomas H and Gundelfinger ED (2002) Nicotinic acetylcholine receptors of *Drosophila*: three subunits encoded by genomically linked genes can co-assemble into the same receptor complex. *J Neurochem* **80**:149-157.
- Changeux JP and Taly A (2008) Nicotinic receptors, allosteric proteins and medicine. *Trends Mol Med* **14**:93-102.
- Cheng X, Lu B, Grant B, Law RJ and McCammon JA (2006) Channel opening motion of $\alpha 7$ nicotinic acetylcholine receptor as suggested by normal mode analysis. *J Mol Biol* **355**:310-324.
- Corringer PJ, Le Novère N and Changeux JP (2000) Nicotinic receptors at the amino acid level. *Annu Rev Pharmacol Toxicol* **40**:431-458.
- Couturier S, Bertrand D, Matter JM, Hernandez MC, Bertrand S, Millar N, Valera S, Barkas T and Ballivet M (1990) A neuronal nicotinic acetylcholine receptor subunit ($\alpha 7$) is developmentally regulated and forms a homo-oligomeric channel blocked by α -BTX. *Neuron* **5**:847-856.
- Dani JA and Bertrand D (2007) Nicotinic acetylcholine receptors and nicotinic cholinergic mechanisms of the central nervous system. *Annu Rev Pharmacol Toxicol* **47**:699-729.
- Decourtye A, Lacassie E and Pham-Delègue MH (2003) Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag Sci* **59**:269-278.
- Decourtye A, Devillers J, Cluzeau S, Charreton M and Pham-Delègue MH (2004) Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicol Environ Saf* **57**:410-419.

MOL #55186

- Deglise P, Grunewald B and Gauthier M (2002) The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neurosci Lett* **321**:13-16.
- Eldefrawi AT, Bakry NM, Eldefrawi ME, Tsai MC and Albuquerque EX (1980) Nereistoxin interaction with the acetylcholine receptor-ionic channel complex. *Mol Pharmacol* **17**:172-179.
- Elgoyhen AB, Johnson DS, Boulter J, Vetter DE and Heinemann S (1994) $\alpha 9$: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell* **79**:705-715.
- Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF and Boulter J (2001) $\alpha 10$: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. *Proc Natl Acad Sci USA* **98**:3501-3506.
- Faucon JP, Aurières C, Drajnudel P, Mathieu L, Ribière M, Martel AC, Zeggane S, Chauzat MP and Aubert MF (2005) Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies. *Pest Manag Sci* **61**:111-125.
- Foster SP, Cox D, Oliphant L, Mitchinson S and Denholm I (2008) Correlated responses to neonicotinoid insecticides in clones of the peach-potato aphid, *Myzus persicae* (Hemiptera: Aphididae). *Pest Manag Sci* **64**:1111-1114.
- Gerzanich V, Anand R and Lindstrom J (1994) Homomers of $\alpha 8$ and $\alpha 7$ subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. *Mol Pharmacol* **45**:212-220.
- Guez D, Suchail S, Gauthier M, Maleszka R and Belzunces LP (2001) Contrasting effects of imidacloprid on habituation in 7- and 8-day-old honeybees (*Apis mellifera*). *Neurobiol Learn Mem* **76**:183-191.
- Guez D, Belzunces LP and Maleszka R (2003) Effects of imidacloprid metabolites on habituation in honeybees suggest the existence of two subtypes of nicotinic receptors differentially expressed during adult development. *Pharmacol Biochem Behav* **75**:217-222.
- Halgren TA (1999a) MMFF VI. MMFF94s option for energy minimization studies. *J Comput Chem* **20**:720-729.
- Halgren TA (1999b) MMFF VII. Characterization of MMFF94, MMFF94s, and other widely available force fields for conformational energies and for intermolecular-interaction energies and geometries. *J Comput Chem* **20**:730-748.

MOL #55186

- Hansen SB, Sulzenbacher G, Huxford T, Marchot P, Taylor P and Bourne Y (2005) Structures of *Aplysia* AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J* **24**:3635-3646.
- Hansen SB and Taylor P (2007) Galanthamine and non-competitive inhibitor binding to ACh-binding protein: evidence for a binding site on non- α -subunit interfaces of heteromeric neuronal nicotinic receptors. *J Mol Biol* **369**:895-901.
- Hilf RJ and Dutzler R (2008a) Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. *Nature* **457**:115-118.
- Hilf RJ and Dutzler R (2008b) X-ray structure of a prokaryotic pentameric ligand-gated ion channel. *Nature* **452**:375-379.
- Huang Y, Williamson MS, Devonshire AL, Windass JD, Lansdell SJ and Millar NS (1999) Molecular characterization and imidacloprid selectivity of nicotinic acetylcholine receptor subunits from the peach-potato aphid *Myzus persicae*. *J Neurochem* **73**:380-389.
- Huang Y, Williamson MS, Devonshire AL, Windass JD, Lansdell SJ and Millar NS (2000) Cloning, heterologous expression and co-assembly of Mp β 1, a nicotinic acetylcholine receptor subunit from the aphid *Myzus persicae*. *Neurosci Lett* **284**:116-120.
- Ihara M, Matsuda K, Otake M, Kuwamura M, Shimomura M, Komai K, Akamatsu M, Raymond V and Sattelle DB (2003) Diverse actions of neonicotinoids on chicken α 7, α 4 β 2 and *Drosophila*-chicken SAD β 2 and ALS β 2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Neuropharmacology* **45**:133-144.
- Ihara M, Matsuda K, Shimomura M, Sattelle DB and Komai K (2004) Super agonist actions of clothianidin and related compounds on the SAD β 2 nicotinic acetylcholine receptor expressed in *Xenopus laevis* oocytes. *Biosci Biotechnol Biochem* **68**:761-763.
- Ihara M, Okajima T, Yamashita A, Oda T, Hirata K, Nishiwaki H, Morimoto T, Akamatsu M, Ashikawa Y, Kuroda S, Mega R, Kuramitsu S, Sattelle DB and Matsuda K (2008) Crystal structures of *Lymnaea stagnalis* AChBP in complex with neonicotinoid insecticides imidacloprid and clothianidin. *Invert Neurosci* **8**:71-81.

MOL #55186

- Ihara M, Shimomura M, Ishida C, Nishiwaki H, Akamatsu M, Sattelle DB and Matsuda K (2007) A hypothesis to account for the selective and diverse actions of neonicotinoid insecticides at their molecular targets, nicotinic acetylcholine receptors: catch and release in hydrogen bond networks. *Invert Neurosci* **7**:47-51.
- Jonas PE, Phannavong B, Schuster R, Schröder C and Gundelfinger ED (1994) Expression of the ligand-binding nicotinic acetylcholine receptor subunit D α 2 in the *Drosophila* central nervous system. *J Neurobiol* **25**:1494-1508.
- Kagabu S (1997) Chloronicotiny insecticides - discovery, application and future perspective. *Rev Toxicol* **1**:75-129.
- Kagabu S, Matsuda K and Komai K (2002) Preparation of dinotefuran related compounds and agonistic actions on SAD β 2 hybrid nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *J Pestic Sci* **27**:375-377.
- Karlin A (2002) Emerging structure of the nicotinic acetylcholine receptors. *Nat Rev Neurosci* **3**:102-114.
- Karunker I, Benting J, Lueke B, Ponge T, Nauen R, Roditakis E, Vontas J, Gorman K, Denholm I and Morin S (2008) Over-expression of cytochrome P450 CYP6CM1 is associated with high resistance to imidacloprid in the B and Q biotypes of *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Insect Biochem Mol Biol* **38**:634-644.
- Kirkpatrick S, Gelatt CD and Vecchi MP (1983) Optimization by simulated annealing. *Science* **220**:671-680.
- Kolb HC, Finn MG and Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. *Angew Chem Int Ed Engl* **40**:2004-2021.
- Lansdell SJ and Millar NS (2000) The influence of nicotinic receptor subunit composition upon agonist, α -bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology* **39**:671-679.
- Lee SJ, Caboni P, Tomizawa M and Casida JE (2004) Cartap hydrolysis relative to its action at the insect nicotinic channel. *J Agric Food Chem* **52**:95-98.
- Leech CA, Jewess P, Marshall J and Sattelle DB (1991) Nitromethylene actions on in situ and expressed insect nicotinic acetylcholine receptors. *FEBS Lett* **290**:90-94.
- Levin ED and Rezvani AH (2007) Nicotinic interactions with antipsychotic drugs, models of schizophrenia and impacts on cognitive function. *Biochem Pharmacol* **74**:1182-1191.

MOL #55186

- Liu Z, Williamson MS, Lansdell SJ, Denholm I, Han Z and Millar NS (2005) A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *Proc Natl Acad Sci USA* **102**:8420-8425.
- Liu Z, Williamson MS, Lansdell SJ, Han Z, Denholm I and Millar NS (2006) A nicotinic acetylcholine receptor mutation (Y151S) causes reduced agonist potency to a range of neonicotinoid insecticides. *J Neurochem* **99**:1273-1281.
- Matsuda K, Buckingham SD, Freeman JC, Squire MD, Baylis HA and Sattelle DB (1998) Effects of the α subunit on imidacloprid sensitivity of recombinant nicotinic acetylcholine receptors. *Br J Pharmacol* **123**:518-524.
- Matsuda K, Buckingham SD, Kleier D, Rauh JJ, Grauso M and Sattelle DB (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **22**:573-580.
- Matsuda K, Shimomura M, Ihara M, Akamatsu M and Sattelle DB (2005) Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular biology, and receptor modeling studies. *Biosci Biotechnol Biochem* **69**:1442-1452.
- Matsumura M, Takeuchi H, Satoh M, Sanada-Morimura S, Otuka A, Watanabe T and Van Thanh D (2008) Species-specific insecticide resistance to imidacloprid and fipronil in the rice planthoppers *Nilaparvata lugens* and *Sogatella furcifera* in East and South-east Asia. *Pest Manag Sci* **64**:1115-1121.
- Miyazawa A, Fujiyoshi Y and Unwin N (2003) Structure and gating mechanism of the acetylcholine receptor pore. *Nature* **423**:949-955.
- Moriya K, Shibuya K, Hattori Y, Tsuboi S, Shiokawa K and Kagabu S (1992) 1-(6-Chloronicotiny)-2-nitroimino-imidazolidines and related compounds as potential new insecticides. *Biosci Biotechnol Biochem* **56**:364-365.
- Nagata K, Aistrup GL, Song JH and Narahashi T (1996) Subconductance-state currents generated by imidacloprid at the nicotinic acetylcholine receptor in PC 12 cells. *Neuroreport* **7**:1025-1028.
- Nagata K, Song JH, Shono T and Narahashi T (1998) Modulation of the neuronal nicotinic acetylcholine receptor-channel by the nitromethylene heterocycle imidacloprid. *J Pharmacol Exp Ther* **285**:731-738.
- Nauen R, Bielza P, Denholm I and Gorman K (2008) Age-specific expression of resistance to a neonicotinoid insecticide in the whitefly *Bemisia tabaci*. *Pest Manag Sci* **64**:1106-1110.

MOL #55186

- Nishio M (2005) CH/ π hydrogen bonds in organic reactions. *Tetrahedron* **61**:6923-6950.
- Raymond Delpech V, Ihara M, Coddou C, Matsuda K and Sattelle DB (2003) Action of nereistoxin on recombinant neuronal nicotinic acetylcholine receptors expressed in *Xenopus laevis* oocytes. *Invert Neurosci* **5**:29-35.
- Sattelle DB, Buckingham SD, Wafford KA, Sherby SM, Bakry NM, Eldefrawi AT, Eldefrawi ME and May TE (1989) Actions of the insecticide 2(nitromethylene)tetrahydro-1,3-thiazine on insect and vertebrate nicotinic acetylcholine receptors. *Proc R Soc Lond B Biol Sci* **237**:501-514.
- Sattelle DB, Harrow ID, David JA, Pelhate M, Callect JJ, Gepner JI and Hall LM (1985) Nereistoxin: action on a CNS acetylcholine receptor/ion channel in the cockroach *Periplaneta americana*. *J Exp Biol* **118**:37-52.
- Schroeder M and Flattum R (1984) The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pestic Biochem Physiol* **22**:148-160.
- Shimomura M, Okuda H, Matsuda K, Komai K, Akamatsu M and Sattelle DB (2002) Effects of mutations of a glutamine residue in loop D of the $\alpha 7$ nicotinic acetylcholine receptor on agonist profiles for neonicotinoid insecticides and related ligands. *Br J Pharmacol* **137**:162-169.
- Shimomura M, Yokota M, Ihara M, Akamatsu M, Sattelle DB and Matsuda K (2006) Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site. *Mol Pharmacol* **70**:1255-1263.
- Shimomura M, Yokota M, Matsuda K, Sattelle DB and Komai K (2004) Roles of loop C and the loop B-C interval of the nicotinic receptor α subunit in its selective interactions with imidacloprid in insects. *Neurosci Lett* **363**:195-198.
- Smit AB, Syed NI, Schaap D, van Minnen J, Klumperman J, Kits KS, Lodder H, van der Schors RC, van Elk R, Sorgedragter B, Brejc K, Sixma TK and Geraerts WP (2001) A glia-derived acetylcholine-binding protein that modulates synaptic transmission. *Nature* **411**:261-268.
- Soloway SB, Henry AC, Kollmeyer WD, Padgett WM, Powell JE, Roman SA, Tieman CH, Corey RA and Horne CA (1979) Nitromethylene Insecticides, in *Advances in Pesticide Science Part 2* (Geissbühler H, Brooks GT and Kearney PC eds) pp 206-217, Pergamon, Oxford.
- Taly A, Delarue M, Grutter T, Nilges M, Le Novère N, Corringer PJ and Changeux JP (2005) Normal mode analysis suggests a quaternary twist model for the nicotinic receptor gating mechanism. *Biophys J* **88**:3954-3965.

MOL #55186

- Talley TT, Harel M, Hibbs RE, Radic Z, Tomizawa M, Casida JE and Taylor P (2008) Atomic interactions of neonicotinoid agonists with AChBP: molecular recognition of the distinctive electronegative pharmacophore. *Proc Natl Acad Sci USA* **105**:7606-7611.
- Tan J, Galligan JJ and Hollingworth RM (2007) Agonist actions of neonicotinoids on nicotinic acetylcholine receptors expressed by cockroach neurons. *Neurotoxicology* **28**:829-842.
- Tan J, Salgado VL and Hollingworth RM (2008) Neural actions of imidacloprid and their involvement in resistance in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Pest Manag Sci* **64**:37-47.
- Thany SH, Lenaers G, Raymond-Delpech V, Sattelle DB and Lapied B (2007) Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. *Trends Pharmacol Sci* **28**:14-22.
- Tomizawa M and Casida JE (2003) Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol* **48**:339-364.
- Tomizawa M and Casida JE (2005) Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol* **45**:247-268.
- Tomizawa M, Maltby D, Talley TT, Durkin KA, Medzihradszky KF, Burlingame AL, Taylor P and Casida JE (2008) Atypical nicotinic agonist bound conformations conferring subtype selectivity. *Proc Natl Acad Sci USA* **105**:1728-1732.
- Tomizawa M, Talley TT, Maltby D, Durkin KA, Medzihradszky KF, Burlingame AL, Taylor P and Casida JE (2007) Mapping the elusive neonicotinoid binding site. *Proc Natl Acad Sci USA* **104**:9075-9080.
- Tomizawa M, Zhang N, Durkin KA, Olmstead MM and Casida JE (2003) The neonicotinoid electronegative pharmacophore plays the crucial role in the high affinity and selectivity for the *Drosophila* nicotinic receptor: an anomaly for the nicotinoid cation- π interaction model. *Biochemistry* **42**:7819-7827.
- Toshima K, Kanaoka S, Yamada A, Tarumoto K, Akamatsu M, Sattelle DB and Matsuda K (2009) Combined roles of loops C and D in the interactions of a neonicotinoid insecticide imidacloprid with the $\alpha 4\beta 2$ nicotinic acetylcholine receptor. *Neuropharmacology* **56**:264-272.
- Treinin M, Gillo B, Liebman L and Chalfie M (1998) Two functionally dependent acetylcholine subunits are encoded in a single *Caenorhabditis elegans* operon. *Proc Natl Acad Sci USA* **95**:15492-15495.

MOL #55186

- Unwin N (2005) Refined structure of the nicotinic acetylcholine receptor at 4Å resolution. *J Mol Biol* **346**:967-989.
- Wang Y, Chen J, Zhu YC, Ma C, Huang Y and Shen J (2008) Susceptibility to neonicotinoids and risk of resistance development in the brown planthopper, *Nilaparvata lugens* (Stal) (Homoptera: Delphacidae). *Pest Manag Sci* **64**:1278-1284.
- Wang Y, Cheng J, Qian X and Li Z (2007) Actions between neonicotinoids and key residues of insect nAChR based on an *ab initio* quantum chemistry study: hydrogen bonding and cooperative π - π interaction. *Bioorg Med Chem* **15**:2624-2630.
- Yang EC, Chuang YC, Chen YL and Chang LH (2008) Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae). *J Econ Entomol* **101**:1743-1748
- Zhong W, Gallivan JP, Zhang Y, Li L, Lester HA and Dougherty DA (1998) From *ab initio* quantum mechanics to molecular neurobiology: a cation- π binding site in the nicotinic receptor. *Proc Natl Acad Sci USA* **95**:12088-12093.

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Footnotes

KM was supported by in part by the “Academic Frontier” Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology (04F011), and by the Integrated Research Project for Plant, Insect and Animal using Genome Technology from the Ministry of Agriculture, Forestry and Fisheries of Japan (1302). DBS was supported by The Medical Research Council of the UK.

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Figure Legends

Figure 1 The nicotinic acetylcholine receptor (nAChR) (A) and ligands (nicotinoids and neonicotinoids) (B). The model of the well characterized muscle nAChR was generated based on a pdb file of 2BG9 (Unwin, 2005) using the Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO).

Figure 2 Electrostatic potentials (EPs) surrounding nicotinoids and neonicotinoids (A) and interactions of imidacloprid with the arginine residue in insect nAChRs predicted by computational chemistry (B). In A, the EPs were calculated by the MNDO semi-empirical molecular orbital method using the Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO). Red dots indicate positive EPs, whereas blue dots indicate negative EPs. In B, the NO₂ oxygens are highlighted in blue whereas the protonated guanidine moiety of the arginine is highlighted in red. The hydrogens on the CH₂-CH₂ moiety in the imidazolidine ring and on the C2 carbon in the pyridine ring are colored light green because they are more electron deficient than those on usual alkyl carbons. A part of these hydrogens form CH- π hydrogen bonds with the tryptophan ring in loop B in the crystal structures of acetylcholine binding proteins (see Fig. 4 for details).

Figure 3 Multiple sequence alignments of acetylcholine binding proteins (AChBPs) with nicotinic acetylcholine receptor α and non- α subunits. Amino acids which have been shown to interact directly with nicotine and neonicotinoids are highlighted with a yellow background, whereas those indirectly determining neonicotinoid sensitivity are shown with a light blue background (The X residue in the YXCC motif is tentatively highlighted with blue). The 6 loops comprising the ligand binding domain are shown

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above the sequences. Abbreviations used: Ls, *Lymnaea stagnalis*; Ac, *Aplysia californica*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Mp, *Myzus persicae*; Am, *Apis mellifera*

Figure 4 Amino acids playing a critical role in the interactions with nicotine in the crystal structure of *Lymnaea stagnalis* acetylcholine binding protein (PDB: 1UV6). The picture was generated using the Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO). Note that nicotine is captured by a hydrogen bond between NH and the backbone of Trp143 as well as a CH- π interaction between N⁺CH₂-H and the tryptophan ring. The backbone of the principal side donating loops A-C is colored yellow, whereas that of the complementary side donating loops D-F is colored cyan. Abbreviations: Nic, nicotine; HB, hydrogen bond.

Figure 5 Amino acids interacting with a neonicotinoid imidacloprid in *Lymnaea stagnalis* (Ls) and *Aplysia californica* (Ac) acetylcholine binding proteins (AChBPs). A and B shows side views of crystal structures of Ls-(A) and Ac-(B) AChBPs. All panels were prepared using the Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO). In A and B, imidacloprid and isopropylalcohol (colored magenta) are generated in spheres to highlight. In C and D, amino acids interacting with imidacloprid in Ls- and Ac-AChBPs are shown, respectively. Irrespective of the mollusc species, a common mechanism is involved in the neonicotinoid recognition by the ligand binding domain of AChBPs. The main chain donating loops A-C is colored yellow, whereas the main chain giving loops D-F is shown cyan. In E (Ls-AChBP) and F (Ac-AChBP), amino acids from loops B and C are shown, whereas in G and H those from loops D and

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E are shown in orientations facilitating view of interactions. In all panels, carbon, hydrogen, nitrogen, oxygen and chlorine atoms are colored white, light-blue, blue, red and blue-green, respectively. Abbreviations: IMI, imidacloprid; HB, hydrogen bond.

Figure 6 The ligand binding domain of *Lymnaea stagnalis* acetylcholine binding protein in complex with clothianidin. The figure was generated using the Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO). Carbon, hydrogen, nitrogen, oxygen and chlorine and sulfur atoms of clothianidin are colored white, light-blue, blue, red, blue-green and yellow, respectively. Note that the NH of the guanidine moiety of clothianidin forms a hydrogen bond (HB) with the backbone C=O of Trp143 in loop B. Such a clothianidin-unique hydrogen bond may be involved in the super agonist actions of clothianidin and its analog on native *Drosophila* nAChRs (Brown et al., 2006a) as well as recombinant *Drosophila* D α 2/chicken β 2 hybrid nAChRs expressed in *Xenopus laevis* oocytes (Ihara et al., 2004).

Figure 7 LBD homology models of (A) wild-type α 2 β 1 nicotinic acetylcholine receptor from the peach potato aphid *Myzus persicae* and (B) Y176S mutant. Models were constructed according to Toshima et al. (2009). Modeling of the N-terminal region of *Myzus persicae* α 2 β 1 and its Y176S mutant nAChRs was carried out using the molecular modeling software package Sybyl (version 7.3; Tripos Associates, Inc., St. Louis, MO) and the homology modeling software Protein Discovery Full Automatic Modeling System Pro (PDFAMS ver. 2.0; In-Silico Sciences, Inc., Tokyo, Japan) in the ligand and complex mode. Both α 2 and β 1 subunits were aligned with the *Ls*-AChBP bound by imidacloprid (PDB code 2ZJU). In the second step, the three dimensional

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structures of the wild-type and the Y176S mutant LBD-imidacloprid complexes were generated based on the sequence alignment and the coordinates of the AChBP and imidacloprid using the simulated annealing method (Kirkpatrick, 1983). The coordinates of imidacloprid were fixed during the simulated annealing. The receptor model constructed in this way was energy-minimized for 5,000 iterations of conjugated gradients using the force field and partial charges of the molecular mechanics MMFF94 (Halgren 1999a, b) using Sybyl. Residues within 10 Å radius of the centrally located imidacloprid, as well as imidacloprid itself, were treated as flexible entities except the C=N-NO₂ moiety which was fixed during energy-minimization. Also, residues within 10-15 Å radius of the ligand were considered rigid entities in order to speed up the computation. Other residues were ignored in energy-minimization. Carbon, hydrogen, nitrogen, oxygen and chlorine atoms of amino acids and imidacloprid are colored white, light-blue, blue, red and blue-green, respectively. Note that the CH- π hydrogen bonds with the tryptophan residue in loop B are reduced by this mutation, consistent with enhanced imidacloprid-resistance in pests.

Figure 8 Electrostatic (A and B) and hydrogen bonding (C and D) fields extended from the ligand binding site of the peach potato aphid, *Myzus persicae* (A and C) and the honey bee, *Apis mellifera* (B and D). Models were constructed according to Toshima et al. (2009). In A and B, regions with high (positive) and low (negative) electrostatic potentials are colored red and blue, respectively. In C and D, the hydrogen-bond accepting atoms such as nitrogens and oxygens are colored blue, whereas the hydrogen atoms attached to these hetero atoms are colored red. In all figures, carbon, hydrogen, nitrogen, oxygen and chlorine atoms of imidacloprid are

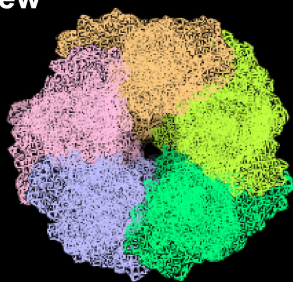
MOL #55186

colored white, light-blue, blue, red and blue-green, respectively. The grooves extending from the NO₂ binding site in the pest and beneficial species nAChRs differ in terms of the size, electrostatic and hydrogen bonding properties, which may lead to a generation of pest target selective (PTS) insecticides.

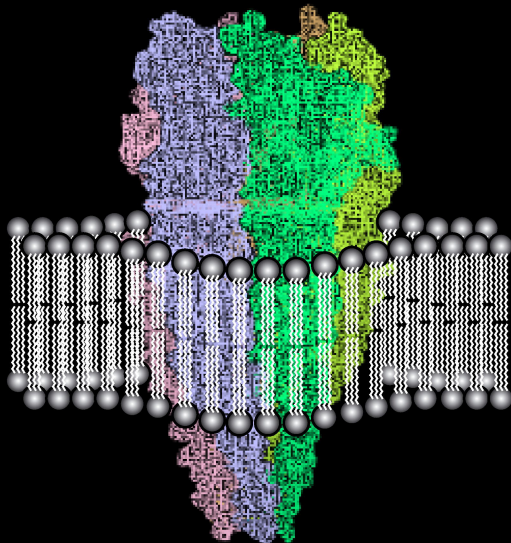
Figure 1

A

Top view

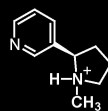


Side view

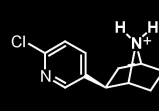


B

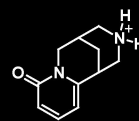
Nicotinoids



Nicotine

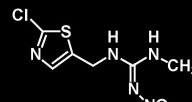


Epibatidine

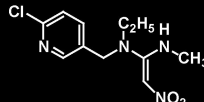


Cytisine

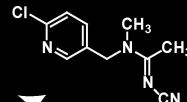
Neonicotinoids



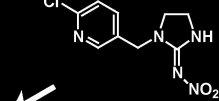
Clothianidin



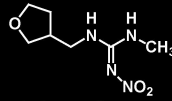
Nitenpyram



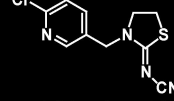
Acetamiprid



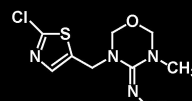
Imidacloprid



Dinotefuran



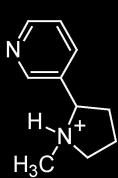
Thiacloprid



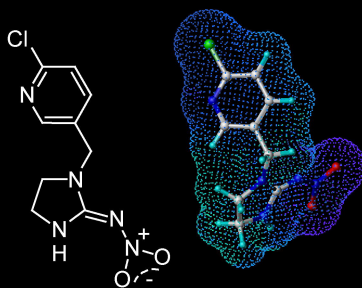
Thiamethoxam

Figure 2

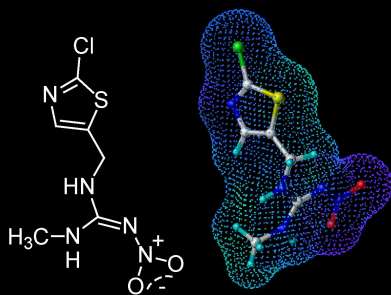
A



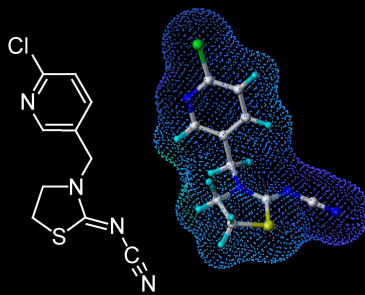
Nicotine



Imidacloprid

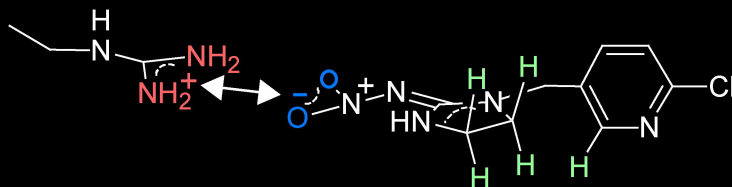


Clothianidin



Thiacloprid

B




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As  AchBP      . . . . . MRRNIFCLACLWIVQACLSDLRADILYNIQRTSRPDVIP. TQR
Ac  AchBP      . . . . . MLVSVYLALLVACVCGQAHSQANLMRLKSDLFNRSFPMYGPPTKG
Hs  o3          . . . . . MGSGPLSLPLALSPPRLLLLLLLLLSLLPVARASEAEHRLFERLFED. YNEIIRPVANV
Hs  o4          . . . . . MELGGPGAPRLLPLLLLLLGTGLRASSHVETRAHAERELKKLFSG. YNKWRSRPVANI
Hs  o7          . . . . . MRCSPGGVWALAAASLLHVSLOGEFGORKLYKELVKN. YNPLERPVAND
Dm  o1          . . . . . HSGVLFAAVFIALHFATGGLAN. PDAKRLYDDLLSN. YNRLIRPVGNV
Dm  o2          . . . . . MAPGCCCTTRPRPIALLAHIWHRCKPLCLLLLLLLLCETVQAN. PDAKRLYDDLLSN. YNRLIRPVSNV
Mp  o1          . . . . . LRAADVPALLLLSavgCLCN. PDAKRLYDDLLSN. YNRLIRPVSNV
Mp  o2          . . . . . LEKKTMKIIcAIFASVIVGQlAMPYASVYGsADAKRLYDDLLSN. YNRLIRPVGNV
Am  o1          . . . . . MATAISCLVAFfPGASAN. sEAKRLYDDLLSN. YNRLIRPVGNV
Am  o2          . . . . . MIlQTIIILISARiCHGN. PDAKRLYDDLLSN. YNRLIRPVSNV
Hs  beta        . . . . . MARRCGPVALLLGFGLLLRLCSGVWGTDTTEERLVEHLLDPSRYNKLIRPATNG
Hs  beta        . . . . . MRRAPSLVFLFLVALCGRCGNCRVANAEEKLMDLLLNKTRYNNKLIRPATSS
Hs  gamma       . . . . . MHGGQGFLLLLLLLLAVCLGAQRNGEERLLADLMQN. YDPNLRPAERD
Hs  epsilon     . . . . . MARAPLGVLLLLGLLGRGVGKNEERLRYHHLFNN. YDPKSRPVRFP
Dm  beta        . . . . . MESSCKSWLLCSlVLVfASLVSASEDEERLVRDLFRG. YNKLIRPVQNM
Mp  beta        . . . . . MNTSVGLLMAVfFISQfIRGCWCSEDEERLVRDLFRG. YNKLIRPVQNM
Am  beta        . . . . . MNtNC. sRLRGILLISAVFCVGLCSDEERLVRDLFRG. YNKLIRPVQNM

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loop A
loop D

30
40
50
60
70
80
90

Ls AchBP DRPVAVSVSLKFINILEVN EITNEVDVVFWQQTTSWSDRTLAWNSSSHSP. .DQVSVPISSLWVPDLAAYN.
 Ac AchBP DDPLTVTYLGTFTLQDQIVAKDSSTNEVDLVVYEQQTQKRWKLSLWMDPNEYGNITDFTSSAADITWPTDIATYSS
 Hs α3 SDPVIITHFEVSMSQLVKVDVEVQIMETNLNLKQIQWNDYKLRWNPSPDYGGAEFMVRVPAQKIWKPDPDVLVYNN
 Hs α4 SDVVLVRFGLSLIAQLIDVDDEKNQMMTTNVVVKQEWHDYKLRWDPADYENVTSIRIPSELILWRPDPDVLVYNN
 Hs α7 SQPLTVYFSLSLQLIMDVEDEKNQVLTTNIWLQMSWTDHYLRQWNVSEVPKTVTRFPDGGQIKWPKDILLVYNN
 Dm α1 SDRLTVMKGLRLSLQLIDNLNLKQIMTTNVVVEQEWNDYKLRWNPDDYGGVDTLHVPSEHIHWLPDVLVYNN
 Dm α2 TDTVLVKLGLRLSLQLIDNLNLKQDILTTNVVLEHEWQDHKFKWDPSEYGGVTETLYVPSEHIWLDPDVLVYNN
 Mp α1 TDTVLVKLGLRLSLQLIELNLKQDILTTNVVLEHEWADHKFLEWPELSEYGGVKELVVPSEHIWLDPDVLVYNN
 Mp α2 SDRLTVMKGLKLSQIEIVNLNLQIMTTNVVVEQEWNDYKLRWNPDEYGGVDTLHVPSEHIWLDPDVLVYNN
 Am α1 SDRLTVMKGLRLSLQLIDNLNLKQIMTTNVVVEQEWNDYKLRWNPDDYGGVDTLHVPSEHIWLDPDVLVYNN
 Am α2 NDTVVVKLGLRLSLQLIDNLNLKQDILTTNVVLEHEWQDHKQWDPAEYGGVTETLYVPSEHIWLDPDVLVYNN
 Hs β2 SELVTVQLMVSLAQLISVHEREQIMTTNVVWLQEQWEDYRLTWWKPEEFDNMKKVRLPSKHTLWPDVLVYNN
 Hs β4 SQLISIKLQLSLAQLISVNEREQIMTTNVVWLQEQWEDYRLTWNSSRYEGMKNLIRIPAKRWLPDVLVYNN
 Hs γ SDVVNVSLKLTTLTNLISLNEREEALTTNVVVICMQWCDYRLRWDPDRDEGLWVLVVPSTMVWRPDPDVLVNN
 Dm β1 EDTVTVISLKVTLTNLISLNEKEETLTTSVVICIDWQDYRLNLSPKDDFGGIETLRVPSSELVWLPEIVLENN
 TkQKVGVRFLAFLQILNVNEKNQVMSNSVWLRLVWYDYQLQWDEADYGGIGVLRLLPDKVWKPDVLVFN
 Mp β1 TEKVVNVQFLGAFVLQILNVNEKSIQMSNSVWLRLVWRDYQLQWDEADYGGIGVLRLLPDKVWKPDVLVFN
 Am β1 TEKVHVNFGLAFLVQLILNVNEKNQIMKNSVNWRLFTWTDYQLQWDEADYGGIGVLRLLPDKVWKPDVLVFN

	loop E										loop B																																																									
	100					110					120					130					140					150																																										
LsAChBP	A	I	S	K	P	E	V	L	T	P	Q	L	A	R	V	V	S	D	G	E	V	L	Y	M	P	S	I	R	Q	F	S	C	D	V	S	G	V	D	T	E	S	G	.	A	T	C	R	I	K	I	G	S	W	T	H	S	R	E	I	S	V	D	P	T	T	E	N	.
AcAChBP	T	R	.	P	V	Q	V	L	S	P	Q	L	A	V	V	T	H	D	G	S	V	M	F	I	P	A	Q	R	L	S	F	M	C	D	P	.	T	G	V	D	S	E	E	G	A	T	C	A	V	K	F	G	S	W	V	S	G	F	E	I	D	L	K	T	D	T	.	.
Hs α3	A	V	G	D	F	Q	V	D	D	K	T	K	A	L	L	K	T	G	B	V	T	W	I	P	P	A	I	F	K	S	S	C	I	D	V	T	F	F	D	Q	T	G	N	C	T	M	K	F	G	S	W	Y	D	K	A	I	D	L	V	L	G	S	.	.				
Hs α4	A	D	G	D	F	A	V	T	H	L	T	K	A	H	L	F	H	D	G	R	V	Q	W	T	P	P	A	I	F	K	S	S	C	I	D	V	T	F	F	D	Q	T	G	N	C	T	M	K	F	G	S	W	Y	D	K	A	I	D	L	V	N	M	H	S	.	.		
Hs α7	A	D	E	R	F	A	T	F	H	T	.	K	A	L	V	N	S	S	G	H	C	Q	Y	L	P	P	A	I	F	K	S	S	C	I	D	V	R	W	F	P	F	D	V	H	Q	K	L	K	F	G	S	W	Y	G	G	S	L	D	L	Q	M	Q	E	A	.	.		
Dm α1	A	D	G	N	E	V	T	I	M	T	K	A	L	L	H	T	G	K	V	V	W	K	P	P	A	I	F	K	S	C	B	I	D	V	E	Y	F	F	D	E	Q	T	C	F	M	K	F	G	S	W	Y	D	G	Q	I	V	D	L	R	H	L	K	I	Q	T	.	.	
Dm α2	A	D	G	E	V	V	T	T	M	K	A	L	L	H	T	G	K	V	V	T	P	P	A	I	F	K	S	C	B	I	D	V	R	Y	F	F	D	Q	T	C	F	M	K	F	G	S	W	Y	D	G	Q	I	V	D	L	R	H	S	Q	K	N	.	.					
Mp α1	A	D	G	E	V	V	T	T	M	K	A	L	V	H	S	G	K	V	M	T	P	P	A	I	F	K	S	C	B	I	D	V	R	Y	F	F	D	Q	T	C	F	M	K	F	G	S	W	Y	D	G	N	Q	I	N	L	K	H	I	Q	L	.	.						
Mp α2	A	D	G	N	E	V	T	I	M	T	K	A	L	L	H	T	G	K	V	V	W	K	P	A	I	F	K	S	C	B	I	N	V	E	Y	F	F	D	E	Q	T	C	M	K	F	G	S	W	Y	D	G	M	M	D	L	R	H	I	S	Q	A	.	.					
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Hs β2	A	D	M	G	E	V	S	F	F	S	.	N	A	V	V	S	Y	D	G	S	.	F	W	L	P	P	A	I	F	K	S	A	C	K	I	E	V	K																														

	loop F										loop C																																																					
	160					170					180					190					200					210																																						
LsAChBP	S	D	D	S	E	Y	F	S	Q	Y	S	R	F	E	I	L	D	V	T	Q	K	N	S	V	T	Y	S	C	P	E	A	Y	E	D	V	E	V	S	L	N	F	R	K	K	R	S	E	I	L													
AcAChBP	Q	V	D	L	S	S	Y	A	S	S	K	Y	E	I	L	S	A	T	Q	T	R	Q	V	Q	H	Y	S	C	P	E	Y	I	D	V	N	L	V	K	F	R	R	A	G	N	F	R	N	L	I	P												
Hs α3	S	M	N	L	K	D	Y	W	E	G	E	W	A	I	K	A	P	G	Y	K	H	D	I	K	Y	N	C	E	E	I	Y	P	D	I	T	S	Y	L	R	L	P	L	F	Y	T	I	N	L	I	P												
Hs α4	R	V	D	Q	L	D	F	W	E	G	E	W	I	V	D	A	V	G	T	Y	N	T	R	K	Y	E	C	A	E	I	Y	P	D	I	T	S	Y	A	F	V	I	R	L	P	L	F	Y	T	I	N	L	I	P									
Hs α7	D	I	S	G	Y	I	P	N	G	E	W	D	L	V	G	I	P	G	K	R	S	E	R	F	Y	E	C	K	E	E	P	Y	D	V	T	F	T	Y	T	M	R	R	T	L	Y	G	L	N	L	L	I	P										
Dm α1	A	D	S	D	N	I	E	V	G	I	D	L	Q	D	Y	I	S	V	E	W	D	I	M	R	V	P	A	V	R	N	E	K	F	Y	S	C	P	E	E	P	Y	L	D	I	V	F	N	L	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P
Dm α2	D	K	N	K	V	E	I	G	I	D	L	R	E	Y	P	S	V	E	W	D	I	L	G	V	P	A	E	R	H	E	K	Y	P	C	A	E	Y	P	D	I	F	F	N	I	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P				
Mp α1	V	G	T	N	K	V	D	V	G	I	D	L	S	A	Y	P	S	V	E	W	D	I	L	G	V	P	A	E	R	H	E	K	Y	P	C	A	E	Y	P	D	I	F	F	N	I	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P			
Mp α2	P	D	S	D	V	E	I	G	I	D	L	Q	D	Y	I	S	V	E	W	D	I	M	G	V	P	A	V	R	H	E	K	F	Y	V	C	P	E	E	P	Y	L	D	I	F	F	N	I	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P	
Am α1	B	D	S	N	Q	I	E	V	G	I	D	L	T	D	Y	I	S	V	E	W	D	I	K	V	P	A	V	R	N	E	A	F	Y	I	C	K	E	E	P	Y	P	D	I	F	F	N	I	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P	
Am α2	M	G	D	K	V	E	I	G	I	D	L	R	E	Y	P	S	V	E	W	D	I	L	G	V	P	A	E	R	H	K	K	Y	P	C	D	E	P	Y	P	D	I	F	F	N	I	T	L	R	R	K	T	L	F	Y	T	V	N	L	I	P		
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Hs β4	T	A	S	M	D	F	T	P	S	G	E	W	D	I	A	L	P	G	R	T	V	N	P	Q	D	S	Y	V	D	T	Y	D	F	I	R	K	P	L	F	Y	T	I	N	L	I	P															

Figure 4

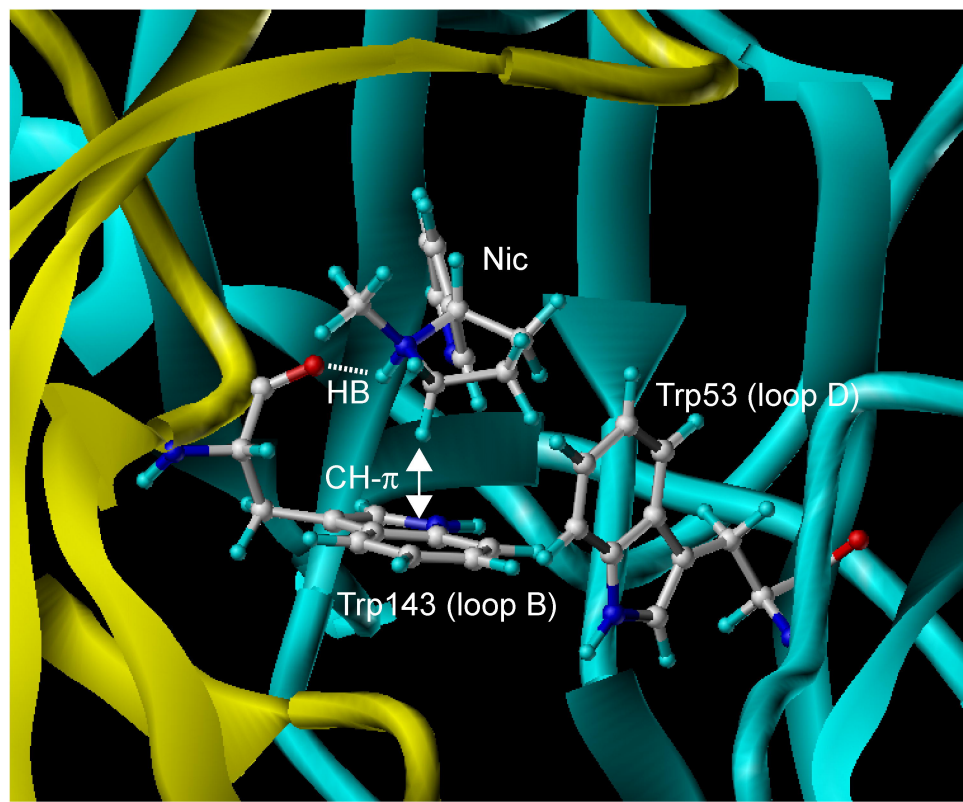


Figure 5

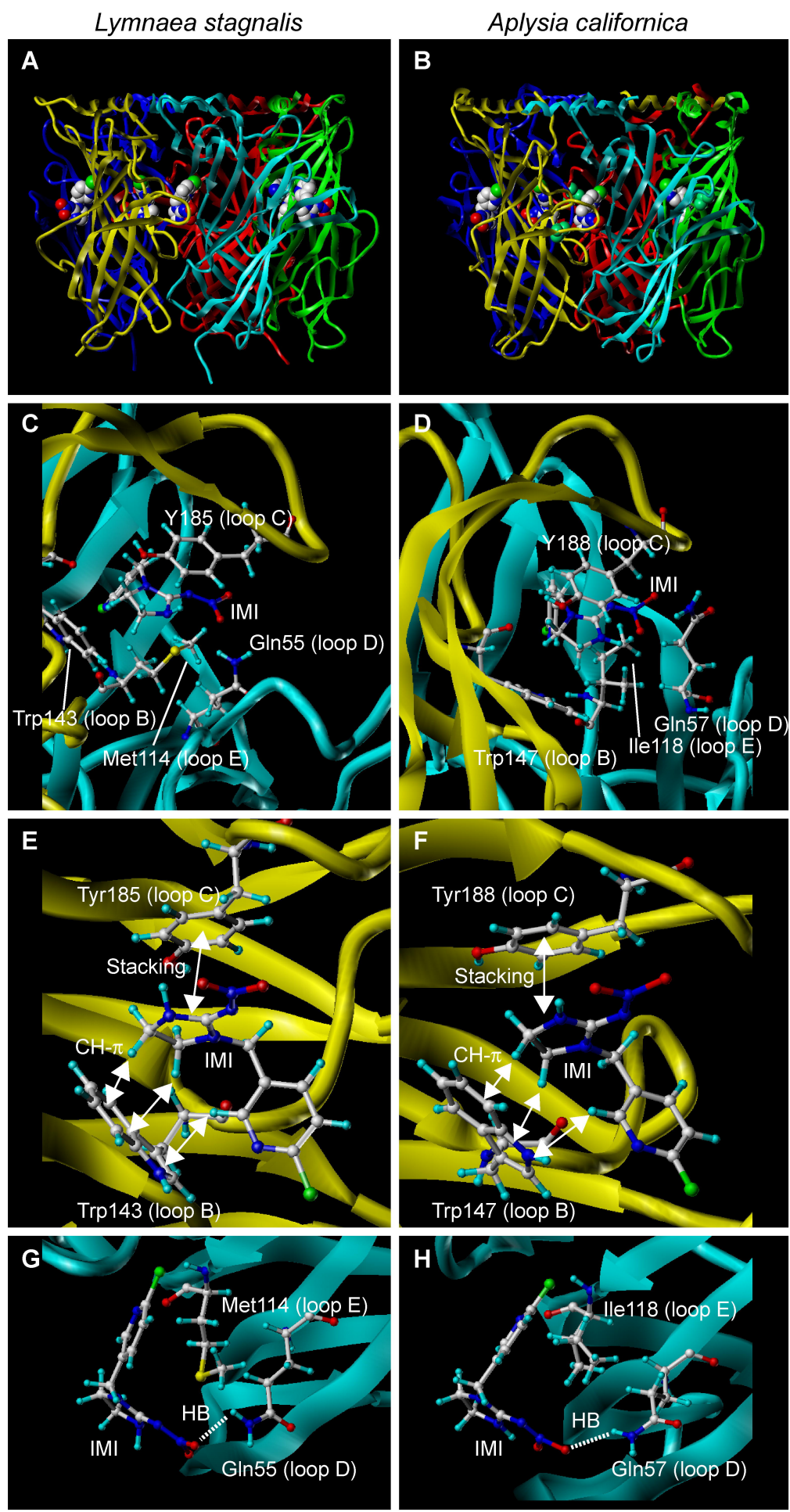


Figure 6

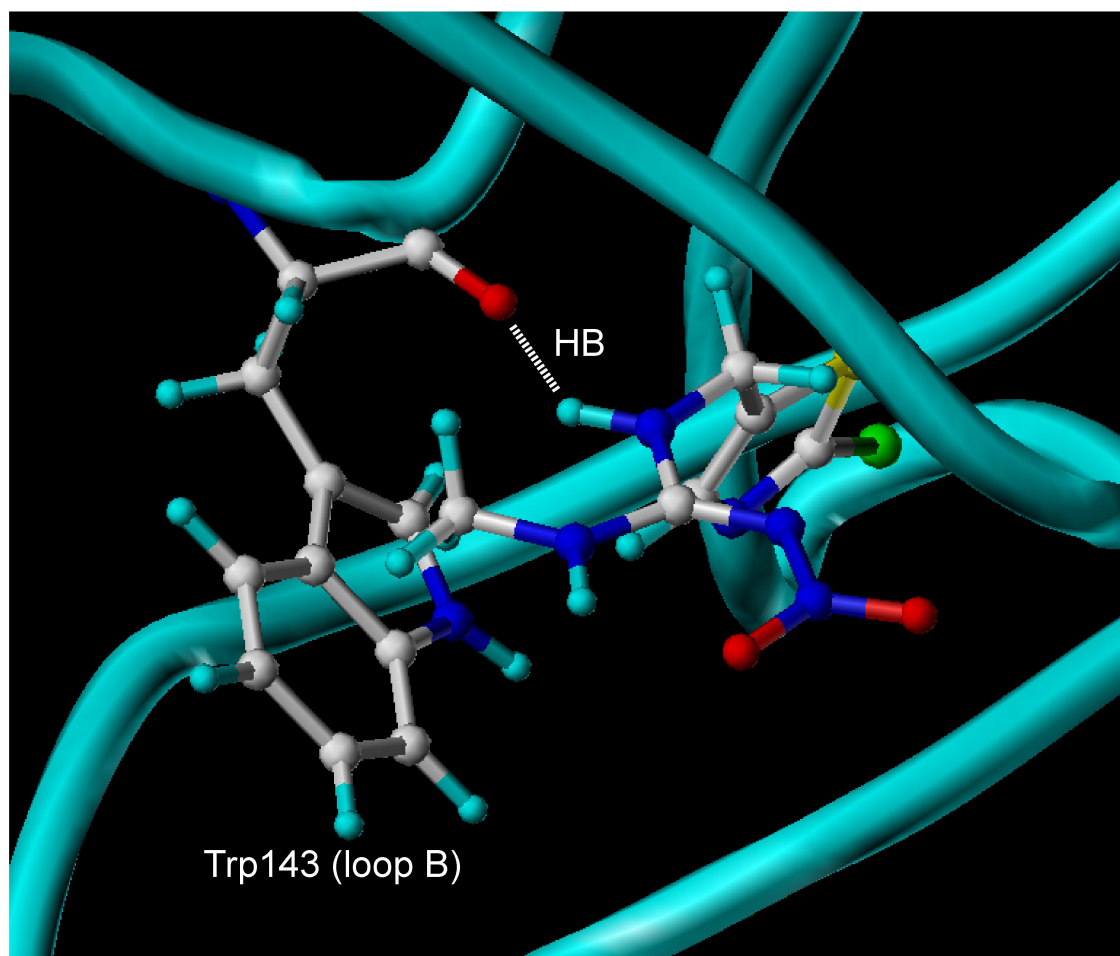


Figure 7

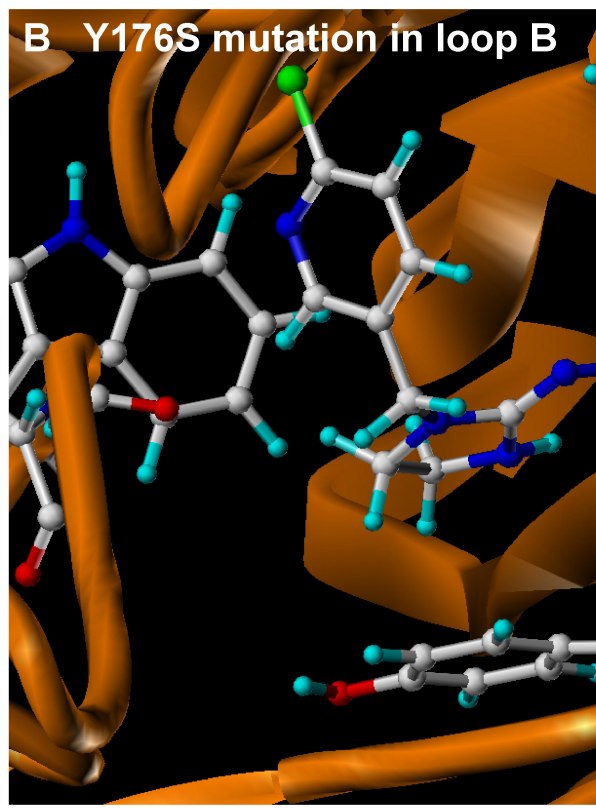
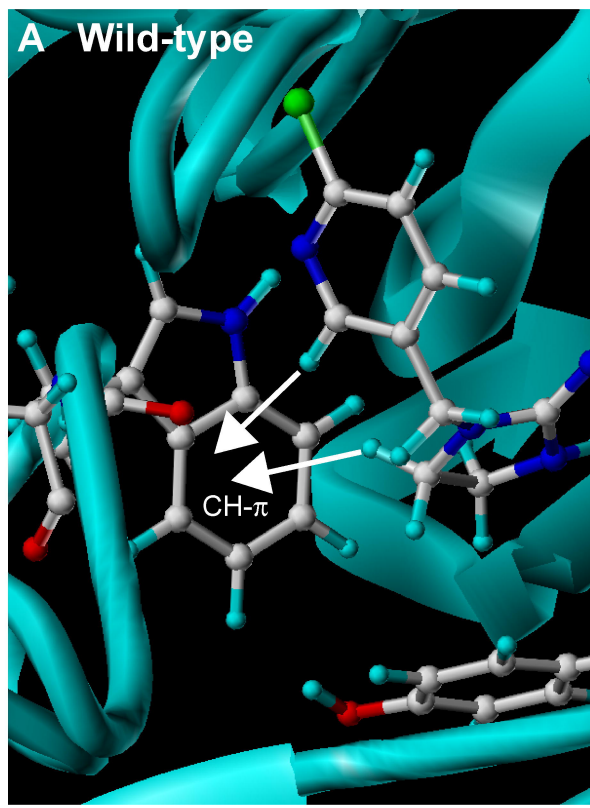


Figure 8

