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MINIREVIEW

Diverse Actions and Target-Site Selectivity of Neonicotinoids: Structural Insights

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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 binding site of molluscan acetylcholine binding proteins with homology to the ligand binding domain (LBD) of nAChRs. The crystal structures showed that 1) glutamine in loop D, corresponding to the basic residues of insect nAChRs, hydrogen bonds with the NO_2 group of imidacloprid and 2) 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 that are safe not only for mammals but also for beneficial insects such as honey bees (*Apis mellifera*) are discussed in terms of interactions with non-α nAChR subunits.

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, with their fast actions providing economic benefits. However, their target-selectivity is important to ensure safety and to limit adverse effects on beneficial insects such as honeybees.

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The 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 nonhydrolyzable 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 therapeutic approaches for nicotine addiction, Alzheimer's disease and schizophrenia as well as treatment for some neuropathies resulting from mutations in nAChRs (Arneric et al., 2007; Dani and Bertrand, 2007; Levin and Rezvani, 2007; Changeux and Taly, 2008). Effective control of insect pests and helminth parasites has been achieved by targeting invertebrate nAChRs (Matsuda et al., 2001, 2005; Tomizawa and Casida, 2003, 2005; Brown et al., 2006b). This

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; *Ac*, *Aplysia californica*; ACh, acetylcholine; AChBP, acetylcholine binding protein; LBD, ligand binding domain; *Ls*, *Lymnaea stagnalis*; TM, transmembrane region; EP, electrostatic potential; PDB, Protein Data Bank.

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 waxy cuticle membranes and cells enveloping the nervous system. However, nicotine is protonated at neutral or lower pH, yielding a water-soluble ammonium that lowers its insect toxicity, although this ammonium form is recognized by nAChRs. In addition, 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 Delpech 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 heterocyles (Soloway et al., 1979; Kagabu, 1997). 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 (Moriya et al., 1992; Kagabu, 1997). In parallel with synthesis, mode-of-action studies have been conducted to show that both early chemotypes, the nitromethylene heterocycles (Schroeder and Flattum, 1984; Sattelle et al., 1989; Leech et al., 1991) and imidacloprid (Bai et al., 1991) act on insect nAChRs. Imidacloprid rapidly expanded its share of the market, and several analogs followed. Because 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 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 action 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; Tomizawa and Casida, 2003, 2005; Thany et al., 2007). Electrophysiology, computational chemistry, and site-directed mutagenesis, in conjunction with homology modeling of the nAChR ligand binding domain (nAChR LBD)-imidacloprid complexes, have been used 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 crystallized the molluscan *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: 1) the develop-

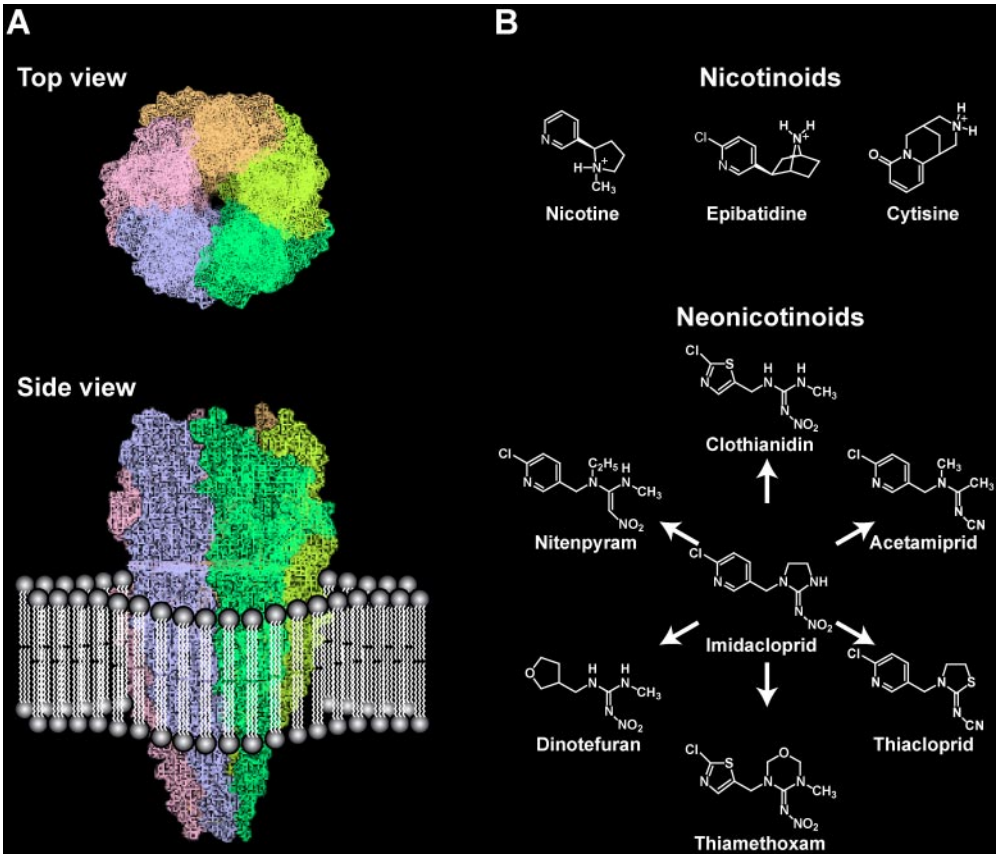

Fig. 1. The nAChR (A) and ligands (nicotinoids and neonicotinoids) (B). The model of the well characterized muscle nAChR was generated based on a PDB file of 2BG9 (Unwin, 2005) using Sybyl (version 7.1; Tripos Associates, Inc., St. Louis, MO).

ment of resistance in pests, and 2) the adverse effects on beneficial insect species. Structural insights relevant to these two problems are also discussed here.

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 (NO_2) group. Such a push-pull conjugation results in a coplanarity of the imidazolidine ring with the nitroimino (C=N-NO_2) 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 NO_2 oxygens and the CN nitrogen

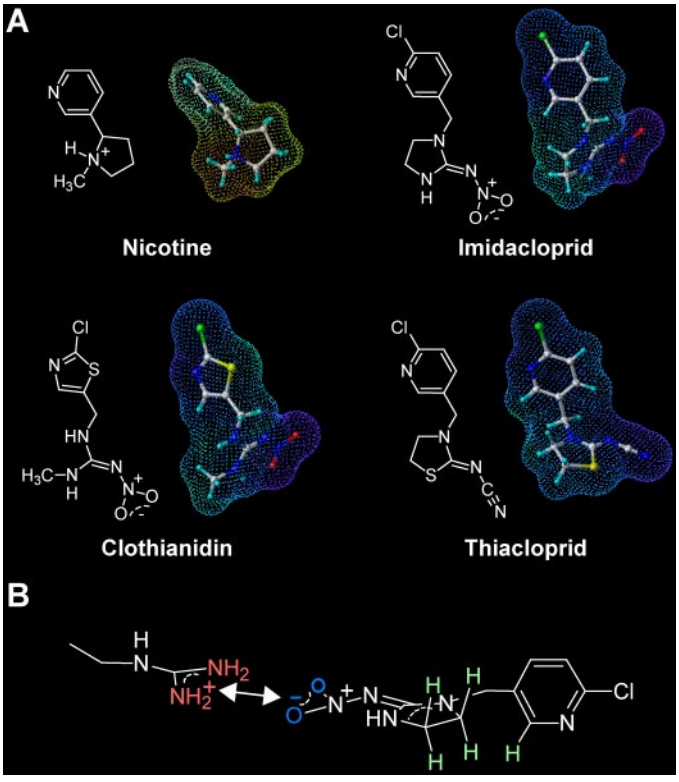

Fig. 2. Electrostatic potentials (EPs) surrounding nicotinoids and neonicotinoids (A), and interactions of imidacloprid with the arginine residue in insect nAChRs predicted by computational chemistry (B). In A, the EPs were calculated by the MNDO semiempirical molecular orbital method using the Sybyl (version 7.1; Tripos Associates, Inc.). Red dots indicate positive EPs, whereas blue dots indicate negative EPs. In B, the NO_2 oxygens are highlighted in blue, whereas the protonated guanidine moiety of the arginine is highlighted in red. The hydrogens on the CH_2-CH_2 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).

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 NO_2 group-ammonium complex formation have been calculated (Matsuda et al., 2001). When complexed with ammonium, the imidazolidine ring, notably hydrogens of the CH_2-CH_2 moiety, become more electron-deficient, resulting in enhanced positive charges. Another calculation has shown that the NO_2 group forms a stronger complex with a methylammonium than with phenol and methanol (Ihara et al., 2003). The following 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 NO_2 group of imidacloprid to strengthen the nAChR-neonicotinoid binding; 2) the complex formation also strengthens the electron-deficient nature of hydrogens in the imidazolidine CH_2-CH_2 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 believed 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 (Tomizawa et al., 2003; Ihara et al., 2007). 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 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). 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-terminal regions of α and non-α subunits. However, in the case of either homomers (α7, α8, and α9) (Couturier et al., 1990; Elgoyhen et al., 1994; Gerzanich et al., 1994) or hetero-α-dimers such as α9/α10 (Elgoyhen et al., 2001) and *Caenorhabditis 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 to C and loops D to 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 α7 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 ligand-

gated ion channels (Bocquet et al., 2009; Hilf and Dutzler, 2008a,b). However, water-soluble AChBPs from molluscs *Ls*, *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 α7 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 six binding site loops (A–F) (Fig. 3) 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 (Celie et al., 2004, 2005; Bourne et al., 2005, 2006; Hansen et al., 2005; Hansen and Taylor, 2007).

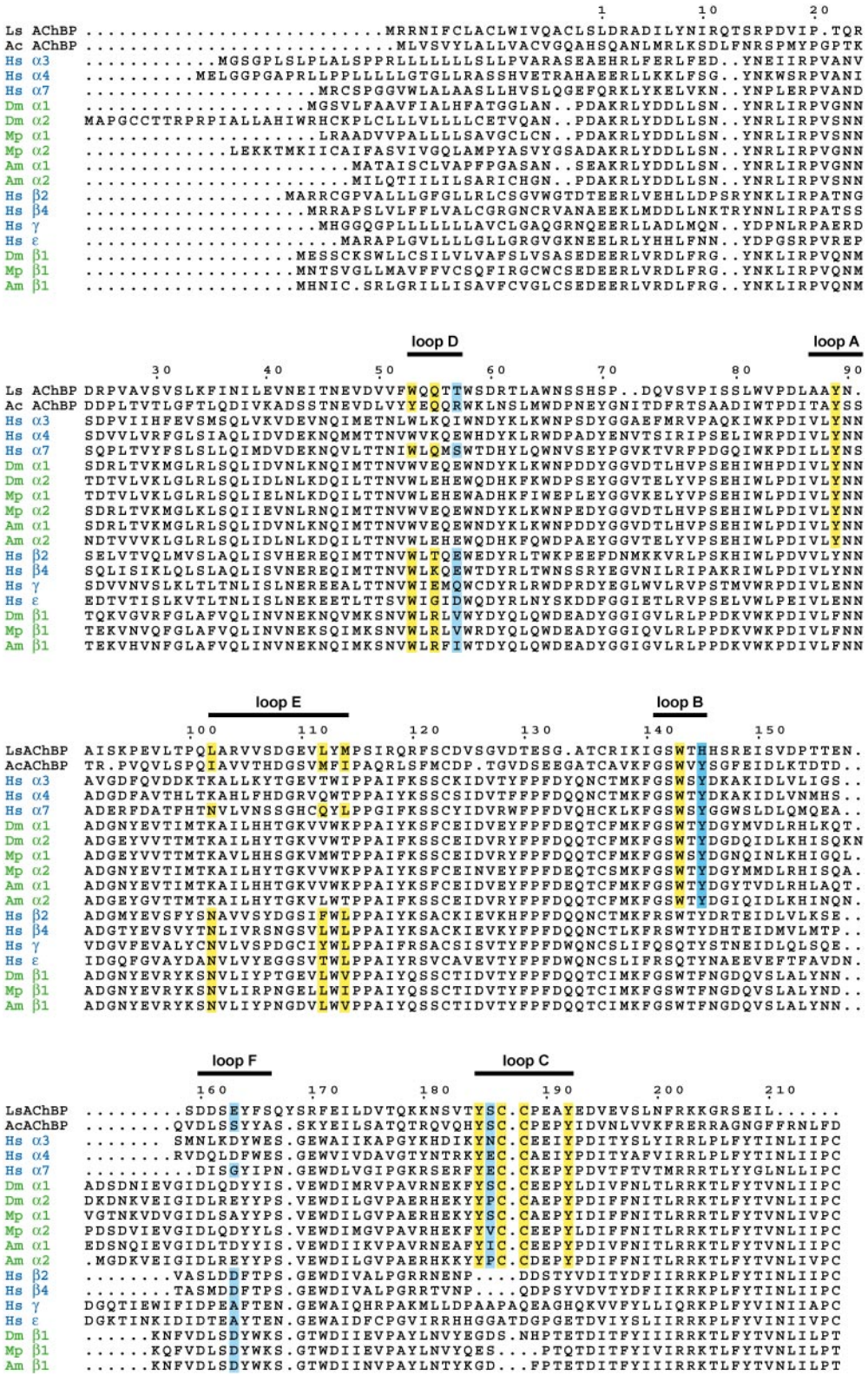

Fig. 3. Multiple sequence alignments of AChBPs with nicotinic acetylcholine receptor α and non-α subunits. Amino acids that have been shown to interact directly with nicotine and neonicotinoids are highlighted with a yellow background, whereas those indirectly determining neonicotinoid sensitivity are shown with a light blue background (The X residue in the YXCC motif is tentatively highlighted with blue). The six loops comprising the ligand binding domain are shown above the sequences. Ls, *Lymnaea stagnalis*; Ac, *Aplysia californica*; Hs, *Homo sapiens*; Dm, *Drosophila melanogaster*; Mp, *Myzus persicae*; Am, *Apis mellifera*.

In the crystal structure of *Ls*-AChBP with nicotine bound (Celie et al., 2004), the proton on the pyrrolidine nitrogen of nicotine forms a hydrogen bond with the backbone C=O of Trp143 (loop B), whereas N-CH_3 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-CH_3 also makes a CH-π interaction with Tyr192 (loop A; not shown in Fig. 4 to facilitate the view of nicotine-tryptophan 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, with 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 *Ac*-AChBP versus *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, because the pyridine ring recognition pattern is conserved in nicotinoids and neonicotinoids, this cannot explain the selectivity of neonicotinoids.

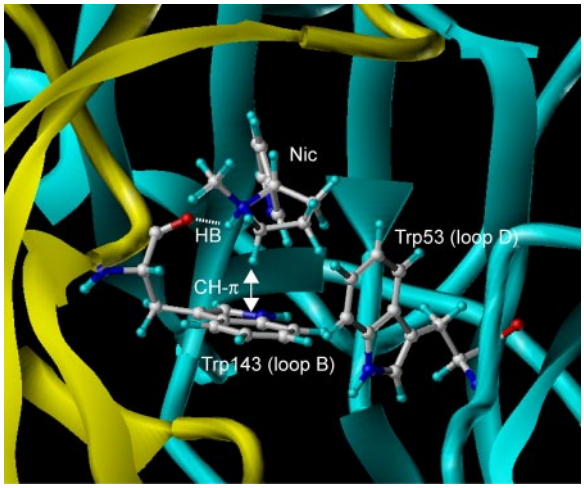

Fig. 4. Amino acids playing a critical role in the interactions with nicotine in the crystal structure of *Ls* acetylcholine binding protein (PDB, 1UV6). The picture was generated using Sybyl (version 7.1; Tripos Associates, Inc.). Note that nicotine is captured by a hydrogen bond between NH and the backbone of Trp143 as well as a CH-π interaction between N^+CH_2-H and the tryptophan ring. The backbone of the principal side donating loops A to C is colored yellow, whereas that of the complementary side donating loops D to F is colored cyan. Nic, nicotine; HB, hydrogen bond.

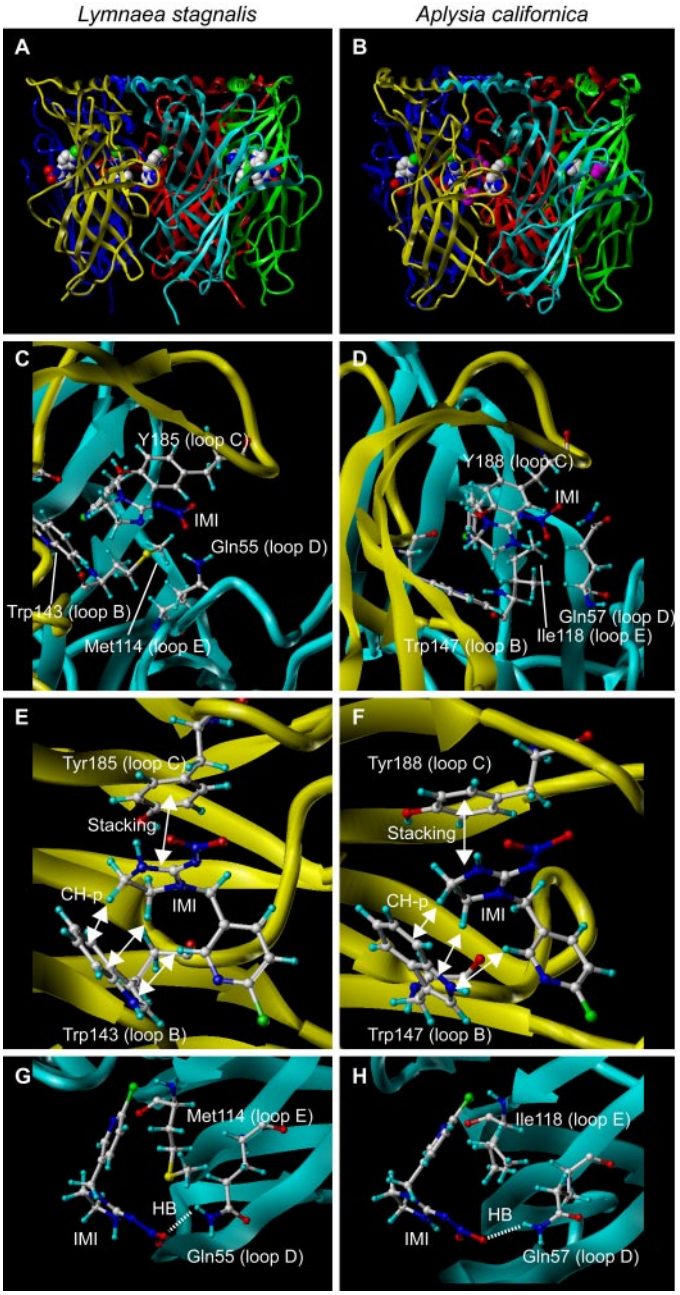

Fig. 5. Amino acids interacting with a neonicotinoid imidacloprid in *Ls*- and *Ac*-AChBPs. A and B, side views of crystal structures of *Ls*- (A) and *Ac*- (B) AChBPs. All were prepared using Sybyl (version 7.1; Tripos Associates, Inc.). In A and B, imidacloprid and isopropyl alcohol (colored magenta) 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 to C is colored yellow, whereas the main chain giving loops D to 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 E are shown in orientations facilitating view of interactions. Carbon, hydrogen, nitrogen, oxygen, and chlorine atoms are colored white, light blue, blue, red, and blue-green, respectively. IMI, imidacloprid; HB, hydrogen bond.

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, whereas two protons in the CH_2-CH_2 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). Because 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, and 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 NO_2 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 the corresponding loop D residues to basic residues were found to dramatically enhance the neonicotinoid sensitivity of the chicken α7 (Shimomura et al., 2002) and $\alpha4\beta2$ nAChRs (Shimomura et al., 2006; Toshima et al., 2009). The selectivity-determining role of this residue can also explain, at least in part, why α7 having a glutamine (Gln89) residue in loop D is more neonicotinoid-sensitive than $\alpha4\beta2$ (Ihara et al., 2003). In this context, AChBPs, from *L. stagnalis* or *A. californica*, resemble insect nAChRs because they possess this important residue. At first sight, the finding that human $\beta4$ has a lysine, at this otherwise highly conserved residue, is surprising, yet interestingly, β4-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 NO_2-Gln55 distance was outside the hydrogen bondable range. However, in the Q55R mutant, the basic residue contacts electrostatically with NO_2 in *Ls*-AChBP (M. Ihara and K. Matsuda, unpublished data). 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 seems to indicate that loop D is not essential for selectivity, in its Q57R mutant, the CN group may point to the introduced arginine residue. In addition, appropriate care is required in the 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 five LBDs filled with neonicotinoids are desirable for detailed comparison and homology modeling.

Loop D alone is not the only determinant of selective neonicotinoid actions. Using the fruit fly *Drosophila melanogaster* α2 (Dα2)/chicken β2 hybrid nAChR (Bertrand et al., 1994) and the chicken $\alpha4\beta2$ 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 *D. melanogaster* Dα2 subunit has a proline at this position, whereas in vertebrate $\alpha4$ subunits this is a glutamate (see Fig. 3). The E219P mutation enhanced the response amplitude of the chicken $\alpha4\beta2$ nAChR to imidacloprid, whereas a reverse mutation P242E markedly reduced the affinity and the efficacy of the D$\alpha2\beta2$ 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 NO_2 of imidacloprid in *Ac*-AChBP. It is conceivable from the crystal structure of the *Ls*-AChBP-imidacloprid complex that vertebrate α2 and α4 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 a consequence of loop C–F repulsion, an intersubunit 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 NO_2 of imidacloprid in the homology model of the wild-type $\alpha4\beta2$ 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 NO_2 or CN groups of neonicotinoids, lowering affinity. We have found that the addition of serine to the YXCC motif of the chicken $\alpha4\beta2$ 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 NO_2 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 (Nagata et al., 1996, 1998; Déglise et al., 2002; Brown et al., 2006a) as well as the recombinant D$\alpha2\beta2$ hybrid nAChRs expressed in *Xenopus laevis* oocytes (Matsuda et al., 1998; Ihara et al., 2003). Opening of the imidazolidine ring leads to an enhanced efficacy (Ihara et al., 2003, 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$\alpha2\beta2$ hybrid nAChR. On the other hand, clothianidin and its analog both show higher agonist efficacy than ACh on the D$\alpha2\beta2$ hybrid nAChR (Ihara et al., 2004) and native *D. melanogaster* 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 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 com-

plex (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 (Miyazawa et al., 2003; Taly et al., 2005; Unwin, 2005; Cheng et al., 2006). In this event, the agonist-binding-induced inward motion of loop C is transmitted to the cysteine loop through a structural rearrangement of loops D and A, resulting finally in the interaction of the cysteine loop and β2–β3 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. Because 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., α10, which partners α9). The special case of loop E in α/α heteromers remains to be clarified.

Target-Based Neonicotinoid Resistance: A Structural Interpretation

Two issues may limit the long-term utility of neonicotinoids: 1) resistance in pest species; and 2) 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 U.S. 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 [^{3}H]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 α2β1 nAChRs from peach potato aphid *Myzus persicae* (Mpα2β1

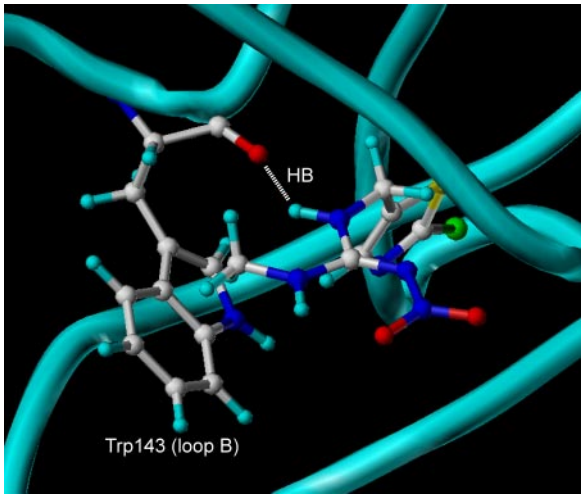

Fig. 6. The ligand binding domain of *Ls* acetylcholine binding protein in complex with clothianidin. The figure was generated using Sybyl (version 7.1; Tripos Associates, Inc.). Carbon, hydrogen, nitrogen, oxygen, and chlorine and sulfur atoms of clothianidin are colored white, light blue, blue, red, 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 *D. melanogaster* nAChRs (Brown et al., 2006a) as well as recombinant *D. melanogaster* Dα2/chicken β2 hybrid nAChRs expressed in *X. laevis* oocytes (Ihara et al., 2004).

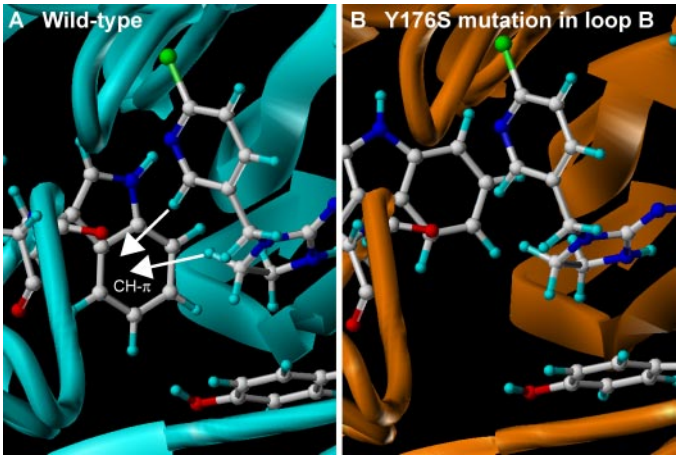

Fig. 7. LBD homology models of wild-type α2β1 nicotinic acetylcholine receptor from the peach potato aphid *M. persicae* (A) and Y176S mutant (B). Models were constructed according to Toshima et al. (2009). Modeling of the N-terminal region of *M. persicae* α2β1 and its Y176S mutant nAChRs was carried out using the molecular modeling software package Sybyl (version 7.3; Tripos Associates, Inc.) and the homology modeling software PDFAMS Ligands & Complex (version 2.1; In-Silico Sciences, Inc., Tokyo, Japan) in the ligand and complex mode. Both α2 and β1 subunits were aligned with the *Ls*-AChBP bound by imidacloprid (PDB code 2ZJU). In the second step, the three-dimensional 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 et al., 1983). The coordinates of imidacloprid were fixed during the simulated annealing. The receptor model constructed in this way was energy-minimized for 5000 iterations of conjugated gradients using the force field and partial charges of the molecular mechanics MMFF94 (Halgren 1999a,b) using Sybyl. Residues within a 10-Å radius of the centrally located imidacloprid, as well as imidacloprid itself, were treated as flexible entities except the C=N-NO_2 moiety, which was fixed during energy minimization. In addition, residues within 10 to 15 Å radius of the ligand were considered rigid entities 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.

nAChR) have been modeled using the crystal structures of *Ls*-AChBP in complex with imidacloprid (Fig. 7). This subunit combination was adopted for the following reasons: 1) both α2 and β1 subunits are important subunits and have a similar distribution in the *D. melanogaster* central nervous system (Jonas et al., 1994); 2) the *D. melanogaster* Dα2 and Dβ1 subunits were copurified with the Dα1 and Dα3 subunits from the fly heads by α-bungarotoxin-affinity column (Chamaon et al., 2002); 3) the Mpα2/rat β2 hybrid nAChR is much more sensitive to imidacloprid than the Mpα1/rat β2 hybrid nAChR (Huang et al., 1999), and the Mpα2 subunit coassembles with the Mpβ1 subunit in the *D. melanogaster* S2 cell (Huang et al., 2000); and 4) the Mpα2 subunit has a valine at position X in the YXCC motif (Fig. 3), which obviates the need to consider possible hydrogen bonding with NO_2. In the wild-type Mpα2β1 nAChR, the imidazolidine ring stacks with loop C tyrosine, whereas two protons in the CH_2-CH_2 bridge make CH-π contacts with loop B tryptophan as seen in the AChBPs (Fig. 7A). The *M. persicae* tyrosine residue (Tyr176) corresponding to Tyr151 of the *N. lugens* α subunit is tightly packed in a hydrophobic groove (data 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 the future whether 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 honeybees (Guez et al., 2001, 2003; Decourtye et al., 2003, 2004; Faucon et al., 2005; Yang et al., 2008), although 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 honeybee (*A. mellifera*) α2β1 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 NO_2 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. Second, 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 that 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 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 small but

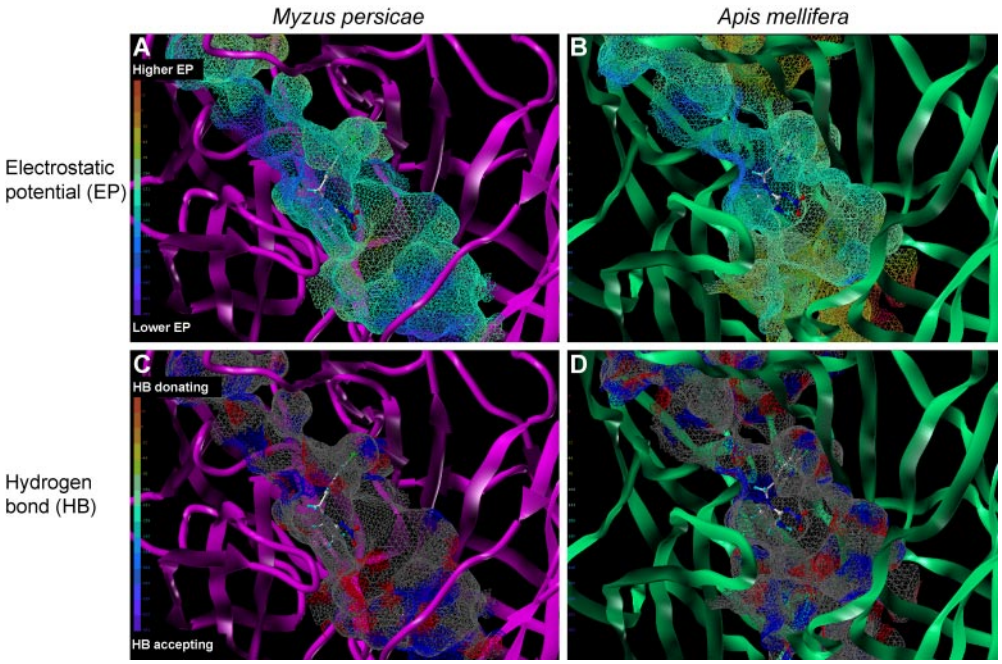

Fig. 8. Electrostatic (A and B) and hydrogen bonding (C and D) fields extended from the ligand binding site of the peach potato aphid *M. persicae* (A and C) and the honeybee *A. mellifera* (B and D). Models were constructed according to Toshima et al. (2009). In A and B, regions with high (positive) 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, carbon, hydrogen, nitrogen, oxygen, and chlorine atoms of imidacloprid are colored white, light blue, blue, red, and blue-green, respectively. The grooves extending from the NO_2 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 insecticides.

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 to C are common for nicotinic and neonicotinic ligands, and selectivity is often donated by interactions with loops D to 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., 2009; 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.

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