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Molecular Recognition of Neonicotinoid Insecticides: The Determinants of Life or Death

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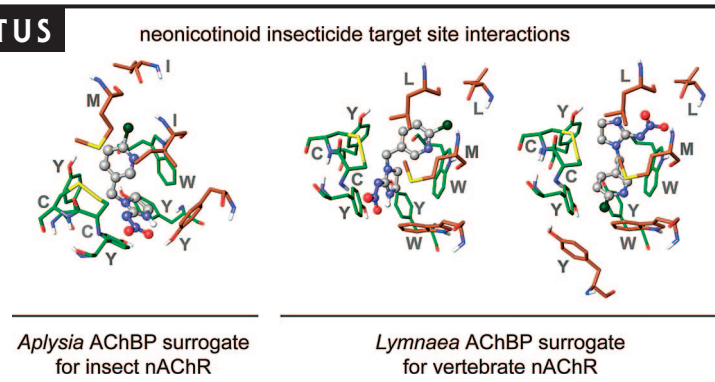

Until the mid-20th century, pest insect control in agriculture relied on largely inorganic and botanical insecticides, which were inadequate. Then, the remarkable insecticidal properties of several organochlorines, organophosphates, methylcarbamates, and pyrethroids were discovered, leading to an arsenal of synthetic organics. The effectiveness of these insecticides, however, diminished over time due to the emergence of resistant insect strains with less sensitive molecular targets in their nervous systems. This created a critical need for a new type of neuroactive insecticide with a different yet highly sensitive target.

Nicotine in tobacco extract was for centuries the best available agent to prevent sucking insects from damaging crops, although this alkaloid was hazardous to people and not very effective. The search for unusual structures and optimization revealed a new class of potent insecticides, known as neonicotinoids, which are similar to nicotine in their structure and action as agonists of the nicotinic acetylcholine receptor (nAChR). Fortunately, neonicotinoids are much more toxic to insects than mammals due in large part to differences in their binding site interactions at the corresponding nAChRs. This Account discusses the progress that has been made in defining the structural basis of neonicotinoid and nicotinoid potency and selectivity.

The findings are based on comparisons of two acetylcholine binding proteins (AChBPs) with distinct pharmacological profiles that serve as structural surrogates for the extracellular ligand-binding domain of the nAChRs. Saltwater mollusk (*Aplysia californica*) AChBP has high neonicotinoid sensitivity, whereas freshwater snail (*Lymnaea stagnalis*) AChBP has low neonicotinoid and high nicotinoid sensitivities, pharmacologies reminiscent of insect and vertebrate nAChR subtypes, respectively.

The ligand−receptor interactions for these AChBPs were established by photoaffinity labeling and X-ray crystallography. Both azidopyridinyl neonicotinoid and nicotinoid photoprobes bind in a single conformation with *Aplysia* AChBP; this is consistent with high-resolution crystal structures. Surprisingly, though, the electronegative nitro or cyano moiety of the neonicotinoid faced in a reversed orientation relative to the cationic nicotinoid functionality. For the *Lymnaea* AChBP, the azidoneonicotinoid probes modified two distinct and distant sites, while the azidonicotinoid probes, surprisingly, derivatized only one point. This meant that the neonicotinoids have two bound conformations in the vertebrate receptor model, which are completely inverted relative to each other, whereas nicotinoids appear buried in only one conserved conformation. Therefore, the unique binding conformations of nicotinic agonists in these insect and vertebrate receptor homologues define the basis for molecular recognition of neonicotinoid insecticides as the determinants of life or death.

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Introduction

This is a competitive world. Every year there are more people to compete with pests for a limited supply of food and fiber. Pest insect control was inadequate until the mid-1940s with largely inorganic and botanical insecticides. Then the remarkable insecticidal properties of DDT were discovered, leading to a 1948 Nobel Prize in Physiology or Medicine for Paul Müller. Soon thereafter a multitude of polychlorocycloalkanes were introduced. These chlorinated hydrocarbons provided excellent control of chewing insect pests, and more than five billion pounds were used before they were restricted or banned because of toxicological and environmental problems. Synthetic pyrethroids, modeled on the botanical insecticide pyrethrin I, were then introduced as replacements for DDT and other chlorinated hydrocarbons. The addition of organophosphates and methylcarbamates greatly expanded the armamentarium for control of not only chewing but also sucking insect pests. Crops were easily protected from pest attack and humans from insect-transmitted diseases. Malaria was practically eradicated. But these gains were short-lived.[1]

Resistance is a constant problem with intensive and extensive use of bioactive chemicals. Only a few years after DDT was introduced, resistant strains were selected for many pests often with cross-resistance to some pyrethroids due to a common low-sensitivity modified binding site in the voltage-activated sodium channel. All the polychlorocycloalkane insecticides also lost their initial effectiveness with cross-resistance this time largely due to a low-sensitivity target site in the γ-aminobutyric acid (GABA)-gated chloride channel. Even the organophosphates and methylcarbamates became ineffective for some pests as resistant strains were selected with a less-sensitive acetylcholinesterase and enhanced detoxification systems. There was an urgency, almost desperation, to find replacements working at different targets without cross-resistance.[1,2]

Nicotine (NIC) or tobacco extract was used for centuries before the organophosphates and methylcarbamates were introduced to control sucking insects on plants. However, by current standards this botanical alkaloid was hazardous to people and not very effective. Attempts to discover better insecticides based on the nicotine structure were not successful. However, screening chemicals of novel structures in the 1970s and optimization of a lead compound gave an interestingly potent nitromethylene with a thiazine ring designated nithiazine. This nitromethylene heterocycle was more effective and safer than nicotine but was unfortunately photolabile and therefore not appropriate for crop protection.[3] Further optimization introduced a chloropyridinylmethyl substituent and replaced the nitromethylene group with a nitroguanidine or cyanoamidine moiety conferring greatly enhanced photostability while retaining much of the potency.[4,5] This new class of insecticides was given the name neonicotinoids based on similarities to nicotine in structure and action.[6] Selective toxicity is critical for insecticide use, combining high potency for pests with low risk for humans and wildlife. Fortunately the neonicotinoids are much more active on insects than mammals leading to very extensive use (currently about one-fifth of the global insecticide market)[7,8] and an intense interest in their mechanism of selective toxicity.

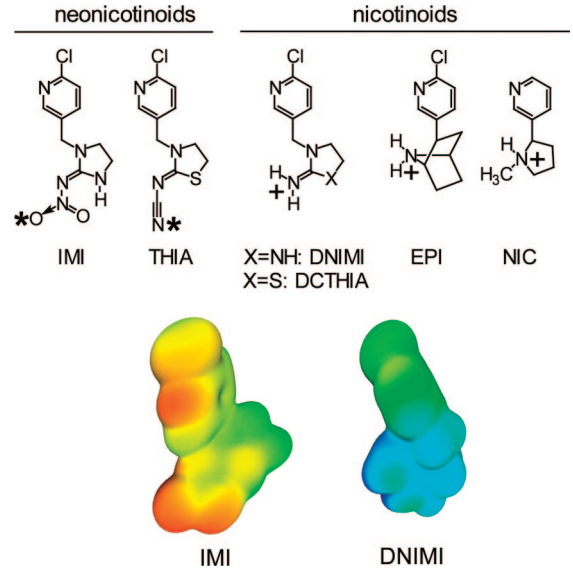

FIGURE 1. Chemical structures and electrostatic potential (ESP) mapping on the molecular surfaces of the neonicotinoid and nicotinoid chemotypes of nicotinic agonists with an electronegative pharmacophore and cationic moiety, respectively. Asterisks indicate functional tip oxygen and nitrogen of the neonicotinoids. ESP mapping of neonicotinoid IMI and nicotinoid desnitroimidacloprid (DNIMI) (protonated at physiological pH) was obtained by B3LYP/6-311G**.[9] ESP surfaces are displayed in red for negative and are graded through orange, yellow, and green to blue for positive with an overall energy range of −60 to 160 kcal/mol.

Neonicotinoid and Nicotinoid Chemotypes

Neonicotinoids and nicotinoids are structurally similar in some ways but not others. Neonicotinoid insecticides represented here by imidacloprid (IMI) and thiacloprid (THIA) and nicotinoids exemplified by NIC and epibatidine (EPI) (isolated from the skin of a tropical poison frog) have in common a pyridin-3-yl moiety with or without chlorine at the 6-position (Figure 1). However these two chemotypes are distinctly different being nonprotonated and predominantly protonated, respectively, at physiological pH. IMI is <0.0002% protonated at pH 7.4.[9] The neonicotinoid nitrogen atom bridging to the chlo-

ropyridinylmethyl substituent is of sp^2 nature in contrast to the sp^3 protonatable nitrogen of NIC or EPI.[9,10] The neonicotinoids are coplanar between the guanidine or amidine plane and the nitro or cyano substituent[9,10] providing electronic conjugation, which facilitates partial negative charge (δ^-) flow toward the tip.[9] However, the equivalent region of positive charge to balance the partial negative charge on the tip is not localized on any specific atom but instead is dispersed in the guanidine or amidine moiety.[9,11,12] The nitroso analogues retain the potency of the nitro compounds, thereby defining the functional tip oxygen.[9] Interestingly, the desnitro and descyano derivatives (DNIMI and DCTHIA) (Figure 1), which are metabolites in mammals,[13] are protonated (imine cation) at physiological pH as with NIC and EPI.[11] On this basis, neonicotinoids can yield nicotinoid metabolites.

Structures of Nicotinic Receptors and Acetylcholine Binding Proteins

Many drugs of therapy and abuse acting on the nervous system target neurotransmitter receptors and their intrinsic or associated channels or coupling proteins. Neonicotinoids and nicotinoids are agonists of nicotinic acetylcholine (ACh) receptors (nAChRs), which are prototypical agonist-gated ion channels responsible for rapid excitatory neurotransmission. They belong to the superfamily of Cys-loop receptors, which also includes GABA, glycine, and serotonin (5-HT) type-3 receptors. The vertebrate nAChR is a pentameric transmembrane structure consisting of diverse subtypes assembled from different sets of subunits expressed in skeletal muscle or electric ray (*Torpedo*) [α1, β1, γ (ε), and δ], neurons (α2−α10 and β2−β4), and sensory epithelia (α9 and α10).[14] The insect counterparts also have diverse nAChR subunits across many species.[15,16] However, the pentameric stoichiometries of the various insect nAChRs have not been resolved, and they can be examined functionally only as recombinant hybrids consisting of various insect α subunits and a vertebrate β2 subunit,[17−21] except for desert locust (*Schistocerca gregaria*) nAChR coexpressed as *Sg*α1 and *Sg*β1 subunits.[22] Native insect nAChRs are partially understood based on chemical approaches using neonicotinoid structures including affinity chromatography and photoaffinity labeling, allowing for purification and identification of the neonicotinoid binding subunit but not for definition of the binding site interactions.[23−27]

The functional architecture of the *Torpedo* nAChR was visualized by electron microscopy (Figure 2),[28] although not with adequate resolution to understand the recognition properties of the ligand binding sites. The nicotinic agonist or competitive antagonist binding pocket is localized at interfacial

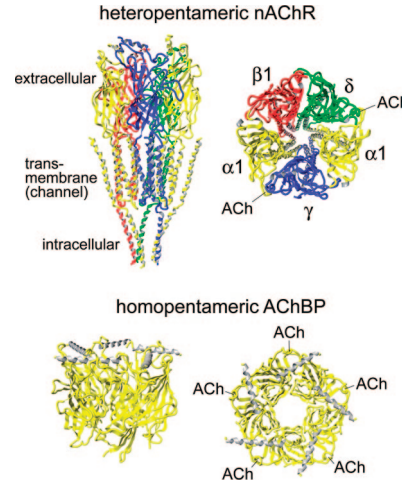

FIGURE 2. Protein structures of *Torpedo* heteropentameric nAChR (top) and *Aplysia* homopentameric AChBP (bottom) [views from side (left) and top (right) in each protein] based on electron microscopy and X-ray crystallography data (PDB ID codes 2BG9 and 2BYN, respectively).[28,31] AChBP lacks the transmembrane domain (ion channel pore) and cytoplasmic end of the nAChR.

regions between subunits and consists of several discontinuous loops (A−F). Specific subunit combinations confer differences in sensitivity to ACh and in pharmacological profiles. Understanding drug−nAChR interactions was greatly facilitated by the discovery and crystallization of soluble ACh binding proteins (AChBPs) from the saltwater mollusk *Aplysia californica* and the freshwater snail *Lymnaea stagnalis* as structural surrogates for the extracellular ligand-binding domain of the nAChR[29−31] (Figure 2 and Supporting Information). The AChBP can be expressed in functional form when combined with transmembrane spans of a Cys-loop receptor.[32] The nAChR is hetero- or homopentameric with two or five ACh binding sites, respectively, while AChBP with a defined high-resolution crystal structure is homopentameric encompassing five ligand binding sites.

Target Site Specificity

Neonicotinoids are selective for insects and nicotinoids for vertebrates. This opposite selectivity profile is based largely on the differential sensitivity of the insect and vertebrate nAChR subtypes (Table 1), which is attributable to their unique chemical features. Neonicotinoids with a nitro or cyano pharmacophore have high affinity for the insect nAChR with low agonist potency at the vertebrate receptor. As with NIC and EPI bearing a cationic functionality, desnitro DNIMI and descyano DCTHIA show diminished affinity at the insect nAChR but simultaneously gain enhanced agonist potency at the mammalian receptor.[11,33,34]

Aplysia AChBP is highly sensitive to neonicotinoids and nicotinoids (Table 1). Interestingly, the two chemotypes of ago-

TABLE 1. Affinity of Neonicotinoids and Nicotinoids for nAChRs and AChBPs

	K_i (nM)			
	nAChR		AChBP	
compound[a]	insect[b]	vertebrate[c]	*Aplysia*[d]	*Lymnaea*[e]
	Neonicotinoids			
IMI-CHNO$_2$	0.12	60	1.7	80
THIA	1.2	240	3.9	219
IMI	3.0	970	19	970
acetamiprid	7.2	680	32	1180
	Nicotinoids			
(±)-EPI	290	0.01	1.0	0.3
DCTHIA	130	1.2	0.6	16
DNIMI	1000	2.2	15	18
(−)-NIC	2700	1.9	30	100

[a] Chemical structures are given in Figure 1 and Supporting Information. [b] *Drosophila* assayed with [^{3}H]IMI. [c] Chick $\alpha4\beta2$ determined with [^{3}H]NIC. [d] Y55W mutant evaluated with [^{3}H]acetamiprid. [e] Assayed with [^{3}H]EPI.

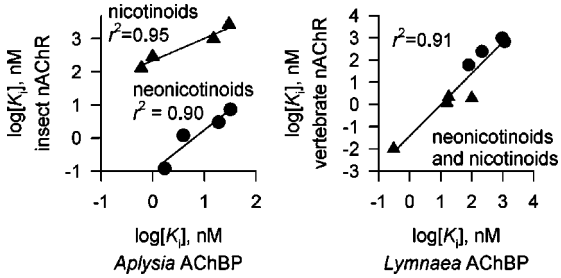

FIGURE 3. Correlation plots for affinities of neonicotinoids (●) and nicotinoids (▲) to the *Aplysia* AChBP versus insect (*Drosophila*) nAChR (left) and *Lymnaea* AChBP versus vertebrate (chick $\alpha4\beta2$) nAChR (right). Data points are from Table 1.

nists act at the same *Aplysia* site in the same way based on individual displacement determination with the neonicotinoid and nicotinoid radioligands [^{3}H]acetamiprid and [^{3}H]EPI.[35] In marked contrast, the *Lymnaea* AChBP subtype has lower affinity for neonicotinoids than nicotinoids.[36] These observations indicate that two AChBP subtypes from mollusks have distinct pharmacology suggestive of the nAChRs from species as divergent as insects and vertebrates. Thus, the *Aplysia* AChBP serves as a plausible structural surrogate for interactions of both neonicotinoids with the insect nAChR and nicotinoids with the vertebrate receptor (Figure 3). Further, the *Lymnaea* AChBP may be a surrogate for the vertebrate nAChR.

Agonist Binding Site Interactions

Photoaffinity labeling combined with mass spectrometry technology provides a direct and physiologically relevant chemical biology method for three-dimensional structural investigation of drug−receptor interaction. On the other hand, atomic resolution crystallography defines geometries of functional amino acids in the drug-bound state and conformational rearrangement of the binding pocket upon ligand interaction.

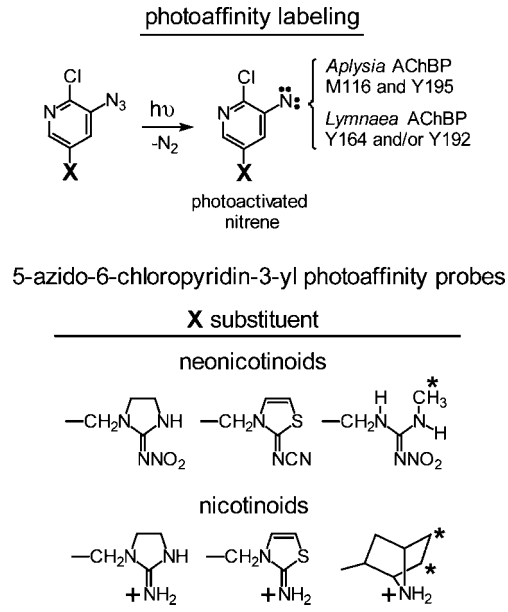

FIGURE 4. Scheme for photoaffinity labeling (top) and structures of 5-azido-6-chloropyridin-3-yl neonicotinoid and nicotinoid photoaffinity probes used for photoderivatization studies (bottom). In principle, the probe binds to the specific site and then the reactive nitrene intermediate, generated by photoirradiation at 300 nm, reacts covalently with the target molecule. Asterisks indicate the positions of tritium label.

Therefore comparison of both solution- and crystal-based determinations with the *Aplysia* AChBP confers a more comprehensive structural insight into the neonicotinoid−receptor interactions.

Photoaffinity Labeling. The studies used two chemotypes of nicotinic photoaffinity probes that share the 5-azido-6-chloropyridin-3-yl moiety but have distinct pharmacophores, that is, nitro- or cyanoimino neonicotinoids versus desnitro or descyano iminium or ammonium nicotinoids (Figure 4). The azido substituent of the photoprobes does not appreciably alter the potency at the insect and vertebrate $\alpha4\beta2$ nAChRs and AChBPs.[27,35−40] Photoaffinity labeling of *Aplysia* AChBP enabled precise comparison of the binding site interactions of neonicotinoids versus nicotinoids. Both chemotypes of probes adequately and specifically modified the AChBP with up to one agonist molecule for each subunit based on analysis of the intact derivatized protein.[35,36,40] In addition, direct measurement of ligand binding through quenching of native tryptophan fluorescence of AChBP revealed simple bimolecular association and unimolecular dissociation of the neonicotinoids and nicotinoids consistent with homogeneity of the binding sites (full occupation of the five binding pockets; K_d's of IMI, THIA, DNIMI, and EPI are 63, 14, 18, and 14 nM, respectively).[41,42] Both the neonicotinoid and nicotinoid photoprobes labeled *Aplysia* AChBP at only one position at the

TABLE 2. Photoaffinity Probes and Derivatized Site(s) in AChBPs

	Aplysia AChBP		*Lymnaea* AChBP	
chemotype[a]	loop	site	loop	site
neonicotinoids	C	Tyr195	C	Tyr192
	E	Met116	F	Tyr164
nicotinoids	C	Tyr195	C	Tyr192
	E	Met116		

[a] The same results were obtained with each of the neonicotinoid probes and each of the nicotinoid probes (see chemical structures in Figure 4). *Aplysia* AChBP Tyr195 and Met116 are spatial neighbors to the azido substituent of the probes, whereas *Lymnaea* AChBP Tyr192 and Tyr164 are distinct and distant sites. *Lymnaea* AChBP Tyr192 corresponds to *Aplysia* AChBP Tyr195. Protein sequence alignment between the two AChBP subtypes is given in Supporting Information.

interface between loop C, Tyr195 on the principal or (+)-face subunit, and loop E, Met116 on the partnering or (−)-face subunit (Table 2 and Figure 5). These findings establish structural models defining interactions between the ligand and functional amino acids in the binding pocket.

Aplysia AChBP accommodates both neonicotinoids and nicotinoids in the same subunit interfacial binding pocket (Figure 5). Each of their chloropyridine rings is buried in the same position in the same way (interacting with the loop E amino acids) and serves as the fulcrum for the rest of the molecule. The IMI guanidine or THIA amidine (not shown) plane π-stacks with the loop C Tyr188 aromatic side chain and possibly with that of loop B Trp147. The electronegative tip nitro oxygen or cyano nitrogen hydrogen bonds primarily with loop C Cys190 NH, Ser189 OH or both. This is an obvious deviation from the usual quaternary ammonium or protonated cationic agonist ligands acting on the superfamily of Cys-loop receptors. Instead the nicotinoid cationic functionality (iminium or ammonium ion of DNIMI or EPI, respectively) critically contacts the carbonyl oxygen of loop B Trp147 via hydrogen bonding, and this interaction is stabilized by cation$-\pi$ contacts with Trp147 and other aromatic side chains from loops A, C, and D. These two very different interactions of neonicotinoids and nicotinoids occur in the same binding pocket. Accordingly, the neonicotinoid electronegative pharmacophore is nestled in a reverse direction compared with the nicotinoid cationic functionality.[35,40]

Crystallography. The high-resolution crystal structures of *Aplysia* AChBP−neonicotinoid complexes with IMI and THIA[42] are consistent with the results of solution-based photoaffinity labelings. Common water molecule positions are captured in the crystal structures liganded with neonicotinoids and nicotinoids. A water or solvent molecule is observed near the pyridine nitrogen of IMI or THIA[42] and EPI[31] bridging to loop B or loop E amino acids or both. An additional water bridge around the tip nitrogen of the THIA cyano substituent presumably

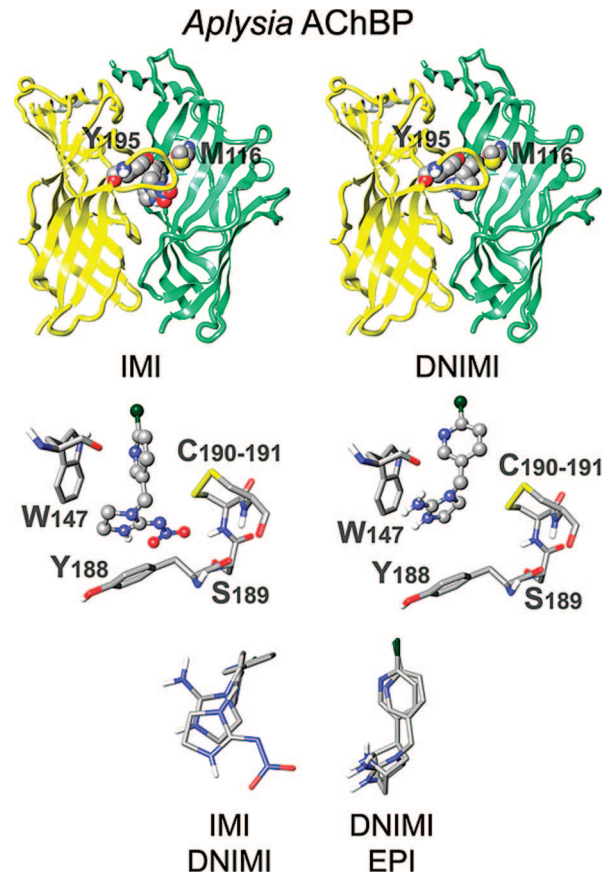

FIGURE 5. Binding site interactions of neonicotinoid IMI and nicotinoid DNIMI at the *Aplysia* AChBP as an insect nAChR surrogate. IMI and DNIMI (in CPK display) are buried in the interfacial agonist binding pocket between the primary or (+)-face (yellow) and complementary or (−)-face (green) subunits. Sites for photoderivatization (Tyr195 and Met116) are emphasized by CPK models (top). Differential ligand binding interactions of neonicotinoids and nicotinoids are illustrated by featuring the amino acids on loop C (Tyr188, Ser189, Cys190, and Cys191) and loop B (Trp147) (middle). *Aplysia* AChBPs complexed with THIA and DCTHIA (not shown) are also consistent with those of IMI and DNIMI.[35,42] Superimposition of bound ligand conformations of IMI overlaid with that of DNIMI and of DNIMI with that of EPI as observed in the agonist binding pocket (bottom).

enhances the interaction of this electronegative tip with loop C.[42] Notably, Gln57 (not shown) on loop D (sequence given in Supporting Information) faces toward the tip oxygen of IMI but not the cyano nitrogen of THIA,[42] suggesting that the Gln57 geometry may be flexible. This unusual geometry of Gln57 in the AChBP-IMI complex is not observed with AChBP-EPI.[31] Gln57 of *Aplysia* AChBP is spatially equivalent to arginine, lysine, or asparagine of the insect nAChR β subunit. In the insect receptors, the arginine or lysine, which is a few atom units longer than glutamine, cannot assume the same side chain orientation as with Gln57 of AChBP due to interactions with the loop C tip region.[35] These observations indicate that

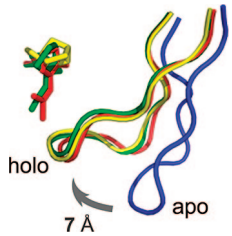

FIGURE 6. Conformational rearrangement of loop C region induced by agonist occupation based on crystal structures of apo and agonist-bound (holo) *Aplysia* AChBPs. The loop C region of the ligand vacant apo-form (blue, PDB ID code 2BYN) is overlaid with those of the IMI-bound (red, 3C79), THIA-bound (green, 3C84), and EPI-bound (yellow, 2BYQ) forms.[31,42]

Gln57 of the *Aplysia* protein plays a supplemental (not a crucial) role in IMI binding.

Comparing AChBP−ligand complexes with an apo structure provides direct evidence of differential conformational rearrangement particularly of the loop C domain induced by agonist and antagonist occupation.[31] The loop C movement tightly capping the binding pocket, characteristic of agonist occupation, is also proven for the high-affinity *Aplysia* AChBP−neonicotinoid complexes (about 7 Å difference between apo and agonist-bound states),[42] therefore indicating stabilization of the neonicotinoid electronegative pharmacophore interaction with the closed loop C region (Figure 6). This can be rationalized as an initial event for the ligand-induced channel opening mechanism of the nAChR.[31]

Distinctive Molecular Recognition Confers Selectivity

Subtype Selectivity. The use of nicotinic agents as insecticides or therapeutics requires high species or subtype selectivity. Understanding the structural determinants of nAChR subtype selectivity was greatly advanced using a family of peptide antagonists, the α-conotoxin variants, with a binding region extending over a large interfacial surface to embrace a unique moiety of the antagonist molecule.[31,43−45] However, the molecular mechanism of selectivity for small agonist molecules is less well resolved since most of the key amino acids in the nAChR binding pocket are conserved in all of the receptor subtypes and species (Supporting Information). The amino acids forming the binding pockets are structurally or functionally consistent not only in the diverse nAChR subtypes but also in the AChBPs, yet there is considerable neonicotinoid selectivity. This points out a distinct limitation for the classical site-directed mutagenesis or chimeric approach. More precise chemical-scale study is required to define a basis for the subtype selectivity of small agonist molecules.[46]

The *Lymnaea* AChBP subtype, which is poorly sensitive to neonicotinoids but highly sensitive to nicotinoids, serves as a model for the vertebrate nAChR.[36] This subtype has been crystallized not only with bound NIC[30] but also with bound IMI and clothianidin (see structure in Supporting Information), both with nitroimine moieties and affinities of 1600 and 7300 nM, respectively.[47] One water molecule is observed near the pyridine or thiazole nitrogen in the three crystals.[30,47] The *Lymnaea* AChBP Gln55 NH_2 faces outside of the binding pocket in the NIC-bound crystal.[30] The Gln55 side chain assumes an anomalous and wobble geometry to contact the alternative nitro oxygen (not the tip oxygen) of IMI. This is observed in two of five binding pockets within the same pentamer, although neither one of the clothianidin nitro oxygens contacts Gln55.[47] Further, in the low-affinity *Lymnaea* AChBP−neonicotinoid complexes, there is little if any loop C movement.[47]

Selectivity Mechanism. Photoaffinity labeling with *Lymnaea* AChBP subtype in physiological medium helps structurally define the mechanism of selectivity. In sharp contrast to *Aplysia* AChBP in both solution- and crystal-based investigations, neonicotinoid photoaffinity labeling of *Lymnaea* AChBP specifically yields two distinct and distant modification sites at loop F Tyr164 and loop C Tyr192. The stoichiometry of photoincorporation is one ligand molecule per binding site based on MS analysis of intact modified subunit protein. However, only one site Tyr192 is pinpointed in nicotinoid photolabeling (Table 2), leading to the proposed unique neonicotinoid binding conformations[36] (Figure 7). *Lymnaea* AChBP accommodates the neonicotinoids in two distinct bound conformations. One binding orientation is completely inverted compared with the common conformation (which is the one observed in the *Aplysia* subtype). In the inverted bound conformation, the IMI nitro oxygen or THIA cyano nitrogen tip hydrogen bonds with the backbone Met114 NH (loop E) on the (−)-face subunit or possibly forms a water bridge to Leu102 and Met114. However, the inverted bound position as compellingly evident in solution[36] is not reported in the less stringent *Lymnaea* AChBP−neonicotinoid crystals;[47] perhaps nucleation and crystal growth may force the packing orientation. Hence, a mixture of two very disparate binding conformations at the *Lymnaea* AChBP and vertebrate nAChR corresponds to the inferior affinity of neonicotinoids at these sites. Only a single tight binding conformation at the *Aplysia* AChBP and insect nAChR model leads to their high neonicotinoid sensitivity. In nicotinoids, a single binding orientation is conserved for all AChBP and nAChR subtypes.[35,36] The final binding constant represents a combination of multiple indi-

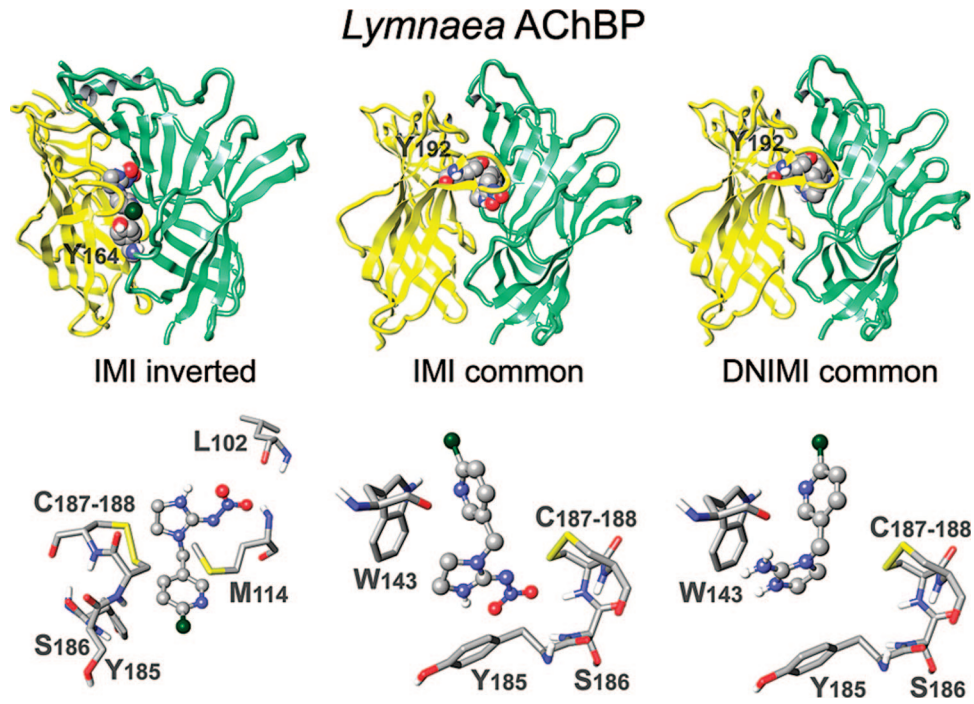

FIGURE 7. Binding site interactions of neonicotinoid IMI and nicotinoid DNIMI with *Lymnaea* AChBP as a vertebrate nAChR surrogate. IMI binds in two conformations designated "inverted" and "common", while DNIMI tightly associates in only the "common" binding orientation (top). The photoderivatization site for azidopyridinyl neonicotinoid probes is either Tyr164 on the complementary (green) subunit in the inverted bound conformation or Tyr192 on the primary (yellow) subunit in the common orientation. Nicotinoid probes modify only Tyr192. Binding site interactions of IMI and DNIMI are also shown in more detail with several important amino acids on loop B (Trp143), C (Tyr185−C188), or E (Leu102 and Met114) (bottom). Neonicotinoid THIA or nicotinoids DCTHIA and EPI are positioned in identical ways as that of IMI or DNIMI, respectively (not shown).[36]

vidual constants unique to different conformations. The same agonist molecule can also adopt different binding directions at other Cys-loop receptors depending upon the nature and position of the aromatic amino acid side chains, that is, 5-HT at 5-HT_3 versus MOD-1 (*Caenorhabditis elegans*) receptors and GABA at $GABA_A$ versus $GABA_C$ receptors.[46,48,49] Although the relationships between binding conformation and subsequent ion channel opening and toxicity are not defined, the atypical molecular recognition of neonicotinoid insecticides clearly serves as a determinant for their selective toxicity.

Receptor Structure-Guided Insecticide Design

The AChBP or nAChR structure in the neonicotinoid-bound state indicated a unique niche, which extends from the nitro oxygen or cyano nitrogen tip of IMI or THIA toward the loop D region on the (−)-face or β subunit. Replacement of the nitro- or cyanoimino moiety by an extended substituent may provide points for hydrogen-accepting or van der Waals contacts at the targeted regional domain. A series of compounds was therefore examined with extended N-substituted-imine pharmacophores to see whether they fitted the cavity in the insect nAChR (Figure 8). Indeed, *N*-pyrazinoylimine and *N*-tri-

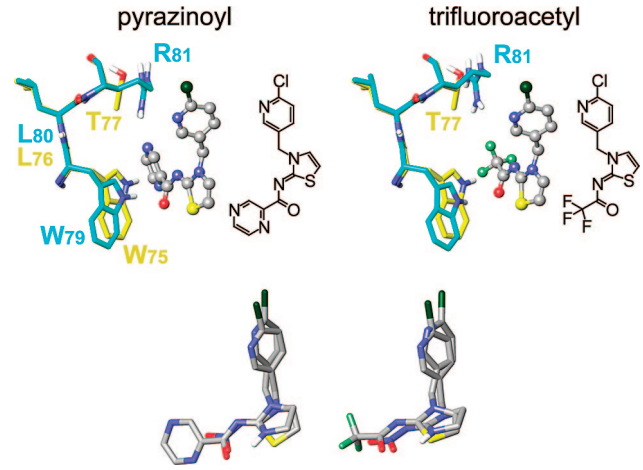

FIGURE 8. Selective nicotinic agonists with extended N-substituted pharmacophores interacting with the loop D niche of the insect nAChR. Structural comparison of loop D amino acids from insect and vertebrate β subunits with Arg81 and Thr77, respectively (top), showing their interactions with pyrazinoylimine (left) and trifluoroacetylimine compounds (right). Loop D amino acids Trp79, Leu80, and Arg81 (aquamarine) on the β subunit of the insect agonist-binding pocket (aphid *Myzus persicae* $\alpha 2\beta 1$) are overlaid onto the equivalent region Trp75, Leu76, and Thr77 (yellow) on the β subunit of the vertebrate (chick $\alpha 4\beta 2$ interface) receptor. Superimposition of bound conformations of pyrazinoylimine or trifluoroacetylimine compounds onto that of IMI as observed in the insect receptor binding pocket[50] (bottom).

TABLE 3. Selectivity of Neonicotinoids and Nicotinoids between Insects and Vertebrates

	target site potency,[b] IC_{50} (nM)			intrinsic toxicity,[c] LD_{50} (mg/kg)		
compound[a]	insect	vertebrate	ratio	housefly	mouse	ratio
			Neonicotinoid			
IMI	4.3	2600	605	0.021	45	2142
THIA	2.7	860	319	0.032	28	875
pyrazinoyl	1.5	900	600	0.035	>24 (0%)[d]	>685
trifluoroacetyl	3.1	500	161	0.027	>36 (0%)[d]	>1333
			Nicotinoid			
NIC	4000	7.0	0.0018	>100	7.0	<0.07
EPI	430	0.04	0.00009	>25	0.08	<0.003

[a] Structures for the neonicotinoids and nicotinoids are given in Figures 1 and 8. [b] Assayed as [^{3}H]IMI or [^{3}H]NIC binding to the *Drosophila* or chick $\alpha4\beta2$ nAChRs, respectively. [c] Insecticidal activity against adult houseflies via injection with metabolic inhibitor (synergist) and toxicity to mice via intraperitoneal administration. [d] Percent lethality at the indicated dose (maximal dose administered due to the solubility limitation in vehicle) with no poisoning signs.

fluoroacetylimine compounds had high affinity at the insect nAChR but rather low potency at the vertebrate $\alpha4\beta2$ nAChR (Table 3).

In a molecular dynamics simulation of the insect nAChR liganded with the pyrazinoylimine, the two pyrazine nitrogens and NC(O) oxygen undergo hydrogen bonding with the loop D Arg81 guanidine NH_2 and Trp79 indole NH. Similarly the trifluoroacetylimine compound variously interacts with loops C and D regions: that is, the fluorine atoms hydrogen bond to loop D Arg81 and Trp79 (directly or possibly via water bridges) and to loop C Cys226 (not shown) and also make van der Waals contact with the Trp79 side chain; the NC(O) oxygen hydrogen bonds with the Trp79 indole NH. When the loop D regions on the β subunits from the insect (Trp79, Leu80, and Arg81) and vertebrate (Trp75, Leu76, and Thr77) receptors are overlaid, the insect Arg81 more intimately faces the pyrazine or trifluoromethyl moiety compared with the vertebrate Thr77 ($\geq$4 Å difference), presumably serving as a determinant for target site selectivity[50] (Figure 8). This excellent target site selectivity accounts for their intrinsic toxicity to the insect versus mammal, a relationship similar to that of IMI or THIA but opposed to that of NIC or EPI (Table 3). Fascinatingly, the trifluoroacetylimine compounds under laboratory conditions show potent insecticidal activity even without a synergist, rivaling that of other chemotypes of commercial insecticides.[50]

Concluding Remarks

Neonicotinoids are selectively toxic to insects and nicotinoids to mammals attributable in large part to differences in target site interactions. Chemical neurobiology approaches were essential in defining the structural basis of the species or subtype selectivity. Two AChBP subtypes proved to be suitable structural surrogates because they have differential sensitivity to the two chemotypes of nicotinic agonists, resulting in pharmacological profiles reminiscent of insect and vertebrate nAChRs. For the neonicotinoids, the nitroguanidine or cyanoamidine pharmacophore is nestled in a reversed position compared with the cationic functionality of nicotinoids. A single dominant binding orientation is conceivably attributable to the high affinity for neonicotinoids at the insect nAChR and different positioning for nicotinoids at the vertebrate nAChR. However, the inferior potency of neonicotinoids at the vertebrate nAChR model is associated with multiple binding conformations in the agonist-binding pocket, perhaps contributing to the poor binding constant, which reflects a weighted average of a multiplicity of binding orientations. In conclusion, these findings in molecular recognition regarding neonicotinoid and nicotinoid target site potency and selectivity may facilitate rational design of insecticides with enhanced effectiveness and maximal safety.

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Supporting Information Available. Chemical structures of neonicotinoids and nicotinoids showing the position of tritium label and protein sequence alignments among mollusk AChBP and extracellular domains of vertebrate and insect nAChR subunits. This material is available free of charge via the Internet at http://pubs.acs.org.

BIOGRAPHICAL INFORMATION

Motohiro Tomizawa was born in Kanagawa, Japan, in 1965. He obtained his Ph.D. degree in agricultural chemistry at the Tokyo University of Agriculture (1993) and then moved to the University of California at Berkeley as a postdoctoral fellow. He is currently a Specialist and Codirector of the Environmental Chemistry and Toxicology Laboratory. His research focuses on neurotoxicological mechanisms of environmental chemicals particularly insecticides with chemical biology approaches.

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FOOTNOTES

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REFERENCES

1 Casida, J. E.; Quistad, G. B. Golden age of insecticide research: Past, present, or future. *Annu. Rev. Entomol.* **1998**, *43*, 1–16.

2 ffrench-Constant, R. H.; Daborn, P. J.; Goff, G. L. The genetics and genomics of insecticide resistance. *Trends Genet.* **2004**, *20*, 163–170.

3 Kollmeyer, W. D.; Flattum, R. F.; Foster, J. P.; Powell, J. E.; Schroeder, M. E.; Soloway, S. B. Discovery of the nitromethylene heterocycle insecticides. In *Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor*; Yamamoto, I., Casida, J. E., Eds; Springer: Tokyo, 1999; pp 71−89.

4 Kagabu, S. Molecular design of neonicotinoids: Past, present, and future In *Chemistry of Crop Protection*; Voss, A., Ramos, G., Eds.; Wiley-VCH: Weinheim, Germany, 2003; pp 193−212.

5 Kagabu, S.; Medej, S. Stability comparison of imidacloprid and related compounds under simulated sunlight, hydrolysis conditions, and to oxygen. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 980–985.

6 Tomizawa, M.; Yamamoto, I. Structure-activity relationships of nicotinoids and imidacloprid analogs. *J. Pestic. Sci.* **1993**, *18*, 91–98.

7 Tomizawa, M.; Casida, J. E. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu. Rev. Entomol.* **2003**, *48*, 339–364.

8 Tomizawa, M.; Casida, J. E. Neonicotinoid insecticide toxicology: Mechanisms of selective action. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 247–268.

9 Tomizawa, M.; Zhang, N.; Durkin, K. A.; Olmstead, M. M.; Casida, J. E. 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* **2003**, *42*, 7819–7827.

10 Kagabu, S.; Matsuno, H. Chloronicotinyl insecticides. 8. Crystal and molecular structures of imidacloprid and analogous compounds. *J. Agric. Food Chem.* **1997**, *45*, 276–281.

11 Tomizawa, M.; Lee, D. L.; Casida, J. E. Neonicotinoid insecticides: Molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *J. Agric. Food Chem.* **2000**, *48*, 6016–6024.

12 Wang, Y.; Cheng, J.; Qian, X.; Li, Z. 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.* **2007**, *15*, 2624–2630.

13 Ford, K. A.; Casida, J. E. Chloropyridinyl neonicotinoid insecticides: Diverse molecular substituents contribute to facile metabolism in mice. *Chem. Res. Toxicol.* **2006**, *19*, 944–951.

14 Changeux, J.-P.; Edelstein, S. J. *Nicotinic Acetylcholine Receptors: From Molecular Biology to Cognition*; Odile Jacob: New York, 2005; 284 pp.

15 Thany, S. H.; Lenaers, G.; Raymond-Delpech, V.; Sattelle, D. B.; Lapied, B. Exploring the pharmacological properties of insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* **2006**, *28*, 14–22.

16 Millar, N. S.; Denholm, I. Nicotinic acetylcholine receptors: Targets for commercially important insecticides. *Invertebr. Neurosci.* **2007**, *7*, 53–66.

17 Bertrand, D.; Ballivet, M.; Gomez, M.; Bertrand, S.; Phannavong, B.; Gundelfinger, E. D. Physiological properties of neuronal nicotinic receptors reconstituted from the vertebrate β2 subunit and *Drosophila* α subunits. *Eur. J. Neurosci.* **1994**, *6*, 869–875.

18 Lansdell, S. J.; Millar, N. S. The influence of nicotinic receptor subunit composition upon agonist, α-bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology* **2000**, *39*, 671–679.

19 Liu, Z.; Williamson, M. S.; Lansdell, S. J.; Denholm, I.; Han, Z.; Millar, N. S. A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (brown planthopper). *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 8420–8425.

20 Tomizawa, M.; Millar, N. S.; Casida, J. E. Pharmacological profiles of recombinant and native insect nicotinic acetylcholine receptors. *Insect Biochem. Mol. Biol.* **2005**, *35*, 1347–1355.

21 Shimomura, M.; Yokota, M.; Ihara, M.; Akamatsu, M.; Sattelle, D. B.; Matsuda, K. Role in the selectivity of neonicotinoids of insect-specific basic residues in loop D of the nicotinic acetylcholine receptor agonist binding site. *Mol. Pharmacol.* **2006**, *70*, 1255–1263.

22 Jones, A. K.; Marshall, J.; Blake, A. D.; Buckingham, S. D.; Darlison, M. G.; Sattelle, D. B. Sgβ1, a novel locust (*Schistocerca gregaria*) non-α nicotinic acetylcholine receptor-like subunit with homology to the *Drosophila melanogaster* Dβ1 subunit. *Invertebr. Neurosci.* **2005**, *5*, 147–155.

23 Tomizawa, M.; Latli, B.; Casida, J. E. Novel neonicotinoid-agarose affinity column for *Drosophila* and *Musca* nicotinic acetylcholine receptors. *J. Neurochem.* **1996**, *67*, 1669–1676.

24 Latli, B.; Tomizawa, M.; Casida, J. E. Synthesis of a novel [^{125}I]neonicotinoid photoaffinity probe for the *Drosophila* nicotinic acetylcholine receptor. *Bioconjugate Chem.* **1997**, *8*, 7–14.

25 Tomizawa, M.; Casida, J. E. [^{125}I]Azidonicotinoid photoaffinity labeling of insecticide-binding subunit of *Drosophila* nicotinic acetylcoline receptor. *Neurosci. Lett.* **1997**, *237*, 61–64.

26 Tomizawa, M.; Casida, J. E. Structure and diversity of insect nicotinic acetylcholine receptors. *Pest Manage. Sci.* **2001**, *57*, 914–922.

27 Tomizawa, M.; Wen, Z.; Chin, H.-L.; Morimoto, H.; Kayser, H.; Casida, J. E. Photoaffinity labeling of insect nicotinic acetylcholine receptors with a novel [^{3}H]azidoneonicotinoid. *J. Neurochem.* **2001**, *78*, 1359–1366.

28 Unwin, N. Refined structure of the nicotinic acetylcholine receptor at 4 Å resolution. *J. Mol. Biol.* **2005**, *346*, 967–989.

29 Brejc, K.; van Dijk, W. J.; Klaassen, R. V.; Schuurmans, M.; van der Oost, J.; Smit, A. B.; Sixma, T. K. Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **2001**, *411*, 269–276.

30 Celie, P. H. N.; van Rossum-Fikkert, S. E.; van Dijk, W. J.; Brejc, K.; Smit, A. B.; Sixma, T. K. Nicotine and carbamylcholine binding to nicotinic acetylcholine receptors as studied in AChBP crystal structures. *Neuron* **2004**, *41*, 907–914.

31 Hansen, S. B.; Sulzenbacher, G.; Huxford, T.; Marchot, P.; Taylor, P.; Bourne, Y. Structures of *Aplysia* AChBP complexes with nicotinic agonists and antagonists reveal distinctive binding interfaces and conformations. *EMBO J.* **2005**, *24*, 3635–3646.

32 Bouzat, C.; Gumilar, F.; Spitzmaul, G.; Wang, H.-L.; Rayes, D.; Hansen, S. B.; Taylor, P.; Sine, S. M. Coupling of agonist binding to channel gating in an ACh-binding protein linked to an ion channel. *Nature* **2004**, *430*, 896–900.

33 Tomizawa, M.; Casida, J. E. Minor structural changes in nicotinoid insecticides confer differential subtype selectivity for mammalian nicotinic acetylcholine receptors. *Br. J. Pharmacol.* **1999**, *127*, 115–122.

34 Tomizawa, M.; Cowan, A.; Casida, J. E. Analgesic and toxic effects of neonicotinoid insecticides in mice. *Toxicol. Appl. Pharmacol.* **2001**, *177*, 77–83.

35 Tomizawa, M.; Talley, T. T.; Maltby, D.; Durkin, K. A.; Medzihradszky, K. F.; Burlingame, A. L.; Taylor, P.; Casida, J. E. Mapping the elusive neonicotinoid binding site. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9075–9080.

36 Tomizawa, M.; Maltby, D.; Talley, T. T.; Durkin, K. A.; Medzihradszky, K. F.; Burlingame, A. L.; Taylor, P.; Casida, J. E. Atypical nicotinic agonist bound conformations conferring subtype selectivity. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 1728–1732.

37 Kagabu, S.; Maienfisch, P.; Zhang, A.; Granda-Minones, J.; Haettenschwiler, J.; Kayser, H.; Maetzke, T.; Casida, J. E. 5-Azidoimidacloprid and an acyclic analogue as candidate photoaffinity probes for mammalian and insect nicotinic acetylcholine receptors. *J. Med. Chem.* **2000**, *43*, 5003–5009.

38 Zhang, N.; Tomizawa, M.; Casida, J. E. Structural features of azidopyridinyl neonicotinoid probes conferring high affinity and selectivity for mammalian α4β2 and *Drosophila* nicotinic receptors. *J. Med. Chem.* **2002**, *45*, 2832–2840.

39 Zhang, N.; Tomizawa, M.; Casida, J. E. 5-Azidoepibatidine: An exceptionally potent photoaffinity ligand for neuronal α4β2 and α7 nicotinic acetylcholine receptors. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 525–527.

40 Tomizawa, M.; Maltby, D.; Medzihradszky, K. F.; Zhang, N.; Durkin, K. A.; Presley, J.; Talley, T. T.; Taylor, P.; Burlingame, A. L.; Casida, J. E. Defining nicotinic agonist binding surfaces through photoaffinity labeling. *Biochemistry* **2007**, *46*, 8798–8806.

41 Hansen, S. B.; Talley, T. T.; Radić, Z.; Taylor, P. Structural and ligand recognition characteristics of an acetylcholine-binding protein from *Aplysia californica*. *J. Biol. Chem.* **2004**, *279*, 24197–24202.

42 Talley, T. T.; Harel, M.; Hibbs, R. H.; Radić, Z.; Tomizawa, M.; Casida, J. E.; Taylor, P. Atomic interactions of neonicotinoid agonists with AChBP: Molecular recognition of the distinctive electronegative pharmacophore. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 7606–7611.

43 Ulens, C.; Hogg, R. C.; Celie, P. H.; Bertrand, D.; Tsetlin, V.; Smit, A. B.; Sixma, T. K. Structural determinants of selective α-conotoxin binding to a nicotinic acetylcholine receptor homolog AChBP. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3615–3620.

44 Talley, T. T.; Olivera, B. M.; Han, K.-H.; Christensen, S. B.; Dowell, C.; Tsigelny, I.; Ho, K.-Y.; Taylor, P.; McIntosh, J. M. α-Conotoxin OmIA is a potent ligand for the acetylcholine-binding protein as well as α3β2 and α7 nicotinic acetylcholine receptors. *J. Biol. Chem.* **2006**, *281*, 24678–24686.

45 Dutertre, S.; Ulens, C.; Büttner, R.; Fish, A.; van Elk, R.; Kendel, Y.; Hopping, G.; Alewood, P. F.; Schroeder, C.; Nicke, A.; Smit, A. B.; Sixma, T. K.; Lewis, R. J. AChBP-targeted α-conotoxin correlates distinct binding orientations with nAChR subtype selectivity. *EMBO J.* **2007**, *26*, 3858–3867.

46 Dougherty, D. A. Cys-loop neuroreceptors: Structure to the rescue. *Chem. Rev.* **2008**, *108*, 1642–1653.

47 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, D. B.; Matsuda, K. Crystal structures of *Lymanea stagnalis* AChBP in complex with neonicotinoid insecticides imidacloprid and clothianidin. *Invertebr. Neurosci.* **2008**, *8*, 71–81.

48 Mu, T.-W.; Lester, H. A.; Dougherty, D. A. Different binding orientations for the same agonist at homologous receptors: A lock and key or a simple wedge. *J. Am. Chem. Soc.* **2003**, *125*, 6850–6851.

49 Padgett, C. L.; Hanek, A. P.; Lester, H. A.; Dougherty, D. A.; Lummis, S. C. R. Unnatural amino acid mutagenesis of the $GABA_A$ receptor binding site residues reveals a novel cation-π interaction between GABA and β_2Tyr97. *J. Neurosci.* **2007**, *27*, 886–892.

50 Tomizawa, M.; Kagabu, S.; Ohno, I.; Durkin, K. A.; Casida, J. E. Potency and selectivity of trifluoroacetylimino and pyrazinoylimino nicotinic insecticides and their fit at a unique binding site niche. *J. Med. Chem.* **2008**, *51*, 4213–4218.