Comparative and statistical analysis of nAChR sequences: An *ab initio* approach to the origin of molecular discrimination

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Compared with traditional structure-based approaches for the identification of species-specific ligands, the *ab initio* approach, based on large-scale protein sequences from different species, has been used to locate specific sites that may be important to the molecular selectivity of species. Statistically significant differences in the distribution of residues in different species and differences in the physicochemical properties of residue-specific sites may largely account for species selectivity. The nicotinic acetylcholine receptor (nAChR), an important neuro-receptor with significantly different ligand selectivity in different species, was used to test our method. Because of the lack of nAChR structural information, the mechanism of ligand discrimination is unclear which hinders attempts at novel molecular design. In this study, the specific site residues 186 and 189 in the principal subunits and residues 34, 55, 56, 57, 106 and 112 in complementary subunits of nAChR were identified by applying our method with stringent statistical cutoffs. These sites were predicted to contribute to ligand selectivity and this result coincides well with the known experimental data.

bioinformatics, molecular discrimination, nAChR, statistical analysis, species selectivity

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Research into the mechanisms of molecular discrimination is important for understanding bimolecular interactions and in aiding drug design. Currently, species-specific molecular discrimination studies have used both theoretical and experimental methods: experimental methods are extremely time-consuming and laborious, and traditional theoretical methods are limited by the availability of effective protein structure information. Genome sequencing projects have shown that it is experimentally simpler to sequence a gene than to determine the structure of the protein it codes for. Therefore, with the aim of logically analyzing the available genomic data, an *ab initio* method based on translated protein sequences was developed to reveal the mechanisms of species-specific discrimination. Some researchers [1–3] used only primary sequence information and evolutionary methods to predict bio-functional differences between subfamily; however, using nicotinic acetylcholine receptor (nAChR) as an

example, we have correlated species-specific residues with ligand discrimination mechanisms in an attempt to establish a bridge between the biology and chemistry of the receptor, and to demonstrate the species selective mechanism.

nAChR is an agonist-regulated ion channel complex essential to synaptic transmission. It is a pentameric assembly of various combinations of different subunits (α , β , γ , ϵ and δ) [4–6]. Because of its role in a wide range of brain activities and its influences on a number of physiological functions, nAChR is viewed as an important target. Particularly, invertebrate nAChRs are the primary target sites for several major insecticides and anti-parasitic drugs [7–9]. As an important receptor for human health, crop-protection, and animal health [9–15], the application of species-selective molecular design to the nAChR would be of great value. However, because it is difficult to obtain pure samples of nAChR, its structure remains mostly unresolved. To date, the only available structure of nAChR (PDB ID: 2BG9) [16] has been solved using cryo-electron microscopy at low

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resolution (4 Å). This is a muscle-type nAChR; insect nAChRs are all neuro-type [17]. The X-ray structures of acetylcholine binding protein (AChBP) from the *Bulinus truncatus*, *Lymnaea stagnalis* and *Aplysia californica* [18–22], a homology protein to nAChR, have been solved at middle resolution (2.0–3.0 Å); however, the sequence identities between AChBPs and nAChRs are very low (no more than 25%). Another difficulty is that, in invertebrates, the subunit assembly of nAChRs is not as clear as in vertebrates because no insect subunit combinations have been expressed as robust functional receptors in vertebrates [23–25]. Until now, robust, functional responses have only been obtained when an α -subunits of insect nAChR is co-expressed with a vertebrate β -subunit.

Neonicotinoids, one of the most successful insecticides used today, show remarkable selectivity toward insect nAChRs over mammalian nAChRs [26,27]. Therefore, understanding its excellent selectivity and improving its selectivity and potency are essential issues related to environment protection, human health and the insecticide resistance [28,29]. In 2003, Tomizawa et al. [30] reported that Lys and Arg were prominent in the extracellular domain of the D α 2 subunit of nAChR and proposed that a putative cationic subsite (possibly Lys or Arg) in insect nAChRs may interact with the electronegative tip of neonicotinoids through electrostatic interactions and/or hydrogen bonds. Through mutation experiments, Shimomura et al. identified residues 55 and 164 [31–33] in the complementary subunit, and residues 163 and 186 [34] in the principal subunit that affected sensitivity to imidacloprid in a SAD-chicken β 2 hybrid nAChR. In 2008, Talley et al. [20] and Ihara et al. [21] published X-ray structures of AChBPs complexed with neonicotinoids (PDB ID: 3C79, 3C84, 2ZJU, 2ZJV) which gave more information about the interactions between neonicotinoids and AChBPs. However, a clear understanding of molecular discrimination is still elusive, because, until now, no effective nAChR structural information is available.

Because of this lack of structural and subunit combination information, we aim to investigate nAChR species selectivity in arthropods and vertebrates by deciphering the available sequence information.

Here, using statistical analysis, we explored differences in the distribution of residues in the active sites of nAChRs in arthropods and vertebrates, and successfully identified species-specific sites that agree well with known experimental data. Compared to the time-consuming and expensive chemistry, biochemistry, molecular biology and electrophysiology experiments, our method is computationally feasible and may have wide applications.

1 Methods

1.1 Data collection and preprocessing

The neuronal nAChR genes encode two types of subunits: α

subunits (α 2– α 10) and β subunits (β 2– β 4) [5]. Of these, α 5 and β 3 do not participate in ligand binding, and the function of α 9 and α 10 is not clear until now [5]. Therefore, these subunits were not included in the present study. Based on the known ligand binding roles of the subunits, the sequences were divided into two sets: principal subunits (α 2, α 3, α 4, α 6, α 7 and α 8 subunits) and complementary subunits (β 2, β 4, α 7 and α 8 subunits). The α 7 and α 8 subunits appear in both groups because they can assemble into homomeric structures and have two different regions that can both interact with the ligand.

All available neuronal nAChR sequences were retrieved from the NCBI (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Redundant sequences were removed by clustering analysis using the BLASTClust program (<ftp://ftp.ncbi.nih.gov/blast/documents/blastclust.txt>) with the similarity threshold set to 95% (sequence identity). The numbers of redundant and nonredundant sequences that resulted from this procedure are shown in Table 1. Totally, 59 nonredundant nAChR sequences from arthropods and 74 nonredundant sequences from vertebrates were selected to study the species-specific residues.

1.2 Multiple sequences alignment

Multiple sequences alignments (MSAs) of the two group sequences (principal and complementary subunits) were constructed using ClustalX [35], with AChBP as the reference sequence. All alignment results were manually refined to make them more consentaneous and correct. Each of the sites close to the binding pocket was carefully examined based on the structure alignments with *Torpedo* nAChR (PDB ID: 2BG9) and AChBPs. To investigate the factors involved in molecular discrimination, the ligand binding sites identified from the available structures were selected for our research.

1.3 Construction of the residue distribution map

All residues were clustered into four non-intersecting sets based on the physicochemical properties of the residues: non-polar residues (NR), Ala, Val, Leu, Ile, Pro, Phe, Met, Trp, Gly and Cys; uncharged polar residues (PR), Asn, Gln, Ser, Thr and Tyr; acidic residues with negative charge (AR), Asp and Glu; basic residues with positive charge (BR), Lys, Arg and His. The residue distribution map (percentage) for each site in the binding domain was constructed and is shown

Table 1 Neuronal nAChR sequences from the NCBI

Taxonomy	Principal subunits		Complementary subunits	
	Redundant	Nonredundant	Redundant	Nonredundant
Arthropods	48	39	32	20
Vertebrates	118	44	77	30

in Figure 1. For clarity, all residue numbers mentioned in this paper refer to the analogous residue in Ac-AChBP.

1.4 Data analysis

Based on the MSAs, the residue distribution differences in the selected sites were statistically analyzed to identify specific sites that might be important for molecular discrimination in species. Statistical analysis was done using SPSS v13 statistical software; Fisher's exact test was used to test for significant differences between species.

2 Results and discussion

As shown in Figure 1, the residue distribution in the selected sites of nAChRs differs in arthropods and vertebrates. Before carrying out the statistical analysis, the residues that were conserved in all the sequences were excluded. The universally conserved sites in the principal subunits were, residues 87–89, 141–144, and 187 and 188, and in the complementary subunits the conserved residues were 53, 54, 58, 103, 105, 109, 111, 113 and 115 residues. These conserved sites are likely to co-contribute to the function of all nAChRs.

2.1 Species-specific sites

To clarify site differences in the arthropod and vertebrate nAChRs, percent differences in residue-type composition

were calculated and are shown in Figure 2. The significance of the residue distribution differences in the nAChRs was evaluated using Fisher's exact tests. Bioinformatics and statistical analysis of the sites with statistically significant species differences ($P < 0.01$) identified residues 92, 94, 95, 147, 183, 184, 186 and 189 in the principal subunits and residues 34, 55, 56, 57, 106, 110, 112, 160, 161 and 163 in the complementary subunits (shown in bold in Table 2) as species-specific, indicating that these sites may contribute to nAChR species-specific molecular discrimination.

2.2 Roles of the species-specific sites

In addition to residue type, the spatial locations of the sites are also important. Because no useful neuro-nAChR structure information is available, we derived the spatial information for these sites from the analogous ones in the X-ray structure of AChBP. The sites that are analogous to the species-specific ones in neuro-nAChR are shown in the structure of AChBP using a stick representation (Figure 3). We select AChBP as the reference structure because it is homologous to nAChR and, importantly, because the X-ray structure of AChBP is for a complex it provides useful information about the protein-ligand interactions that can be used to examine the possible roles of the specific sites in nAChR.

Considering the locations of the analogous sites in AChBP (Figure 3), the specific sites in nAChRs were classified as: (1) located far from the ligand; (2) located close to the ligand but unable to interact with it directly; (3) located close to

Figure 1 Distribution (in percent) of the physicochemical properties of the residues in the ligand binding sites of the nAChRs. Vertical bars represent the distribution of residue properties at each site. The displayed residue positions are, from left to right: 87–90, gap, 91–95, 141–147, 182–194 in the principal subunits (left panel), and 32–36, 53–59, 73–76, 100, 102–116, 160–165 in the complementary subunits (right panel). The residues that differ most between arthropod nAChRs (upper row) and vertebrate nAChRs (lower row) are marked with a red dot. NR represents non-polar residues, Ala, Val, Leu, Ile, Pro, Phe, Met, Trp, Gly and Cys; PR represents uncharged polar residues, Asn, Gln, Ser, Thr and Tyr; AR represents acidic residues with negative charge, Asp and Glu; BR represents basic residues with positive charge, Lys, Arg and His.

Figure 2 Site differences in residue-type composition in arthropod and vertebrate nAChRs. Vertical bars represent the residue-type composition at each site for arthropod nAChRs minus vertebrate nAChRs. Residue positions are the same as those described in Figure 1. The residues that differ most between arthropod nAChRs (upper section of the graph) and vertebrate nAChRs (lower section of the graph) are marked with a red dot. Overall, the site differences in the complementary subunits (upper panel) are much more than of the differences in the principal subunits (lower panel). NR represents non-polar residues; PR represents uncharged polar residues; AR represents acidic residues with negative charge; BR represents basic residues with positive charge.

Figure 3 Residues in the X-ray structure of AChBP analogous to the identified species-specific sites in the nAChRs. The residues analogous to residues 92, 94, 95, 147, 183, 184, 186 and 189 in the principal subunit are in red and residues analogous to residues 34, 55, 56, 57, 106, 110, 112, 160, 161 and 163 in the complementary subunit are in green. Residues 186, 189 (principal subunit) and 34, 55, 56, 57, 112 (complementary subunit) that are located close to the nicotine ligand (shown in yellow) are labeled.

the ligand and in a position to directly interact with it. In the first two cases, although the residues do not interact with the ligand directly, they may contribute to molecular discrimination through their affect on the other residues. In the third case, because the residues can play a direct role in ligand binding, they are likely to be the ones that contribute most to molecular discrimination. In Figure 3, these residues are labeled in pink.

Table 2 Statistically significant results (*P* values) of the residue distribution differences in active sites of nAChR^{a)}

Subunit		Residue position and <i>P</i> value					
Principal subunit	Residue position	87	88	89	90	Gap	91
	<i>P</i> value	/	/	0.469	/	/	/
	Residue position	92	93	94	95	141	142
	<i>P</i> value	0	0.82	0	0	/	/
	Residue position	143	144	145	146	147	182
	<i>P</i> value	/	/	0.118	0.618	0	0.042
	Residue position	183	184	185	186	187	188
	<i>P</i> value	0.004	0	/	0	/	/
	Residue position	189	190	191	192	193	194
	<i>P</i> value	0	0.469	0.729	0.211	0.193	0.699
Complementary subunit	Residue position	32	34	36	53	54	55
	<i>P</i> value	0.211	0	0.139	/	/	0.001
	Residue position	56	57	58	59	73	74
	<i>P</i> value	0.01	0	/	0.029	0.474	/
	Residue position	75	76	100	102	103	104
	<i>P</i> value	0.062	/	0.417	0.058	0.145	0.145
	Residue position	105	106	107	108	109	110
	<i>P</i> value	/	0	0.132	0.123	/	0
	Residue position	111	112	113	114	115	116
	<i>P</i> value	0.569	0	0.569	0.155	/	/
	Residue position	160	161	162	163	164	165
	<i>P</i> value	0	0	1	0.001	0.395	0.155

a) “/” indicates that the residue type at this position was conserved in both species and therefore was excluded from the statistical analysis. Residues with low *P* values (*P* values ≤0.01) were in bold together with its *P* values.

Table 3 lists the residues-type composition of the species-specific residues and, based on the classification described above, indicates their probable role in the molecular discrimination. Residues 186 and 189 in the principal subunits and residues 34, 55, 56, 57, 112 in the complementary subunits belong to the third case and, therefore, may be primary factors for molecular discrimination. These species-specific sites may be among the original factors for the species selectivity of nAChR in arthropods and vertebrates.

2.3 Correlation between the species-specific sites and the ligands

The nAChR ligands usually bind to the interface of two subunits of nAChR. According to their selectivity, the ligands were divided into two sets: mammalian-selective molecules and insect-selective molecules. It is generally accepted that, like ACh (acetylcholine), mammalian-selective molecules contain a cationic center that ionizes at physiological pH [36]; on the other hand, insect-selective molecules, with a nitroimine, cyanoimine, or nitromethylene group, are not protonated at physiological pH. In the protonated form, mammalian-selective molecules interact with nAChR through a prominent cation- π interaction, similar to the way that ACh interacts [37–39]. The selective mechanism for insect-selective molecules is not as clear as for the mammalian-selective ones. Thus, by successfully correlating the

properties of the residues at the species-specific sites with the features of the ligands it selects for, we have provided further support to our proposal that the sites we identified are critical to ligand discrimination.

It is well known that electrostatic and hydrophobic interactions play a major role in binding between molecules. While protonated mammalian-selective molecules assume an apparent positive electrostatic character, insect-selective molecules have a negative electrostatic character because of the electronegative tip which is common to all insect-selective molecules. Log P is related to the hydrophobic character of a molecule. We calculated the Log P values for the insect and mammalian-selective molecules and found that the Log P values for insect-selective molecules were much higher than for *homo*-selective molecules (Figure 4).

The physicochemical properties of the residues that were predicted to directly interact with the ligand are listed in Table 4. The differences are remarkable. To illustrate the pocket environment differences caused by these species-specific sites, the residue types were mapped onto the X-ray structure of AChBP (Figure 5). The differences between the species-specific arthropod and vertebrate sites are clearly revealed.

Figure 5 and Table 4 clearly show the different pocket environments in arthropods and vertebrates nAChR. The pocket environment of vertebrate nAChR is polar and negative, and non-polar and positive in arthropod nAChR. The

Table 3 Residues type composition of the nAChR species-specific sites and their probable roles in molecular discrimination^{a)}

Subunits	Residue position	<i>P</i> value	Arthropods				Vertebrates				Role in ligand discrimination
			NR	PR	AR	BR	NR	PR	AR	BR	
Principal subunit	186	0.000	0.410	0.513	0.000	0.000	0.000	0.372	0.628	0.000	Directly
	189	0.000	0.513	0.179	0.282	0.000	0.186	0.070	0.395	0.349	
	92	0.000	0.000	0.026	0.974	0.000	0.302	0.000	0.674	0.023	Indirectly
	94	0.000	0.000	0.769	0.103	0.103	0.000	0.023	0.651	0.326	
	95	0.000	0.615	0.333	0.026	0.000	1.000	0.000	0.000	0.000	
	147	0.000	0.000	1.000	0.000	0.000	0.023	0.326	0.000	0.651	
	183	0.004	0.333	0.000	0.205	0.410	0.488	0.023	0.000	0.488	
	184	0.000	0.308	0.641	0.000	0.000	0.256	0.070	0.000	0.674	
Complementary subunit	112	0.000	0.950	0.000	0.000	0.050	0.367	0.533	0.067	0.033	Directly
	55	0.001	0.000	0.300	0.000	0.650	0.033	0.733	0.000	0.167	
	56	0.010	0.850	0.100	0.000	0.000	0.500	0.433	0.000	0.000	
	57	0.000	0.350	0.000	0.450	0.150	0.000	0.467	0.433	0.033	
	34	0.000	0.350	0.450	0.000	0.100	0.033	0.900	0.000	0.000	
	106	0.000	0.000	0.300	0.000	0.700	0.067	0.767	0.000	0.167	Indirectly
	110	0.000	0.000	0.450	0.500	0.050	0.067	0.633	0.033	0.267	
	163	0.000	0.000	0.900	0.050	0.000	0.000	0.500	0.500	0.000	
	161	0.000	0.000	0.000	0.950	0.000	0.100	0.400	0.500	0.000	
	160	0.001	0.550	0.400	0.000	0.000	1.000	0.000	0.000	0.000	

a) For residues that can directly interact with ligand, its largest residue type compositions rendered in bold type.

Figure 4 Log P values, as a measure of hydrophobic character, for insect-selective and homo-selective compounds. The Log P values of seven insect-selective molecules and seven homo-selective molecules were calculated using Alogp98 in the Cerius2 software package (version 4.8; Accelrys, San Diego, USA). The chemical structures of the molecules are shown in Figure 6. All the compounds studied were selected for their high nAChR selectivity and potency.

Table 4 Different residue types in arthropod nAChR and vertebrate nAChRs of the residues predicted to directly interact with the ligands

Subunits	Residue position	Arthropod nAChRs	Vertebrate nAChRs
Complementary subunit	112	Hydrophobic	Hydrophilic
	55	Positive charged	Hydrophilic
	56	Hydrophobic	Hydrophilic
	57	Hydrophobic	Hydrophilic
	34	Hydrophobic	Hydrophilic
	106	Positive charged	Hydrophilic
Principal subunit	186	Hydrophobic	Negative charged
	189	Hydrophobic	Negative charged

Figure 5 The pocket environment created by differences in the physicochemical properties of the species-specific residues mapped onto the AChBP structure. The X-ray structure of AChBP (PDB ID: 1UW6) was used. Residues 186, 189 (principal subunits) and residues 34, 55, 56, 57, 106, 112 (complementary subunits) of vertebrate nAChR are shown on the left part and the same residues of arthropods nAChR are shown on the right. Nicotin binds in the ligand-binding pocket. Species-specific residues are colored according to their amino acid properties. NR represents non-polar residues; PR represents uncharged polar residues; AR represents acidic residues with negative charge; BR represents basic residues with positive charge. Conserved aromatic active site residues are in gray.

correlation between the pocket environment and the ligand features for arthropod and vertebrate nAChRs is shown in Table 5. This correlation successfully explains the species-specific mechanism of the well-known neonicotinoid insecticides because the electronegative group and the hydrophobic effect fit the pocket environment of insect nAChR better than the homo nAChR pocket.

The identification of the essential species-specific sites that explain species selectivity will greatly advance our understanding of the molecular basis of the nAChR ligand discrimination mechanism and facilitate the design of novel species-specific agents.

2.4 Comparison of the theoretical and experimental results

The results that we obtained using an *ab initio* approach coincide well with published mutation experiments. Shimomura et al. [32,34] undertook series mutation experiments at sites 186 and 163 in the principal subunit, and at sites 55 and 164 in the complementary subunit. Residue 163 is located in functional loop B-C interval, far from the pocket. Because it probably does not interact directly with the ligand, we did not consider it in this paper. P186E (α), Q55E (β), G164E (β) mutations reduced the imidacloprid sensitivity of nAChR, and Q55K (β), Q55R (β) mutations increased the imidacloprid sensitivity of nAChR. All these mutation experiments coincide well with our finding that the non-polar residue Pro (P) will preferentially interact with insect-selective molecules compared to the negatively charged residue, Glu (E), which will preferentially interact with mammal-selective molecules. Our result is also consistent with the results for the Q55K and Q55R mutations; the positive residues Lys (K) and Arg (R) will prefer to interact with the electronegative group of the imidacloprid as Glu (E) does. The other statistically significant species-specific residues for which no experimental data is available await further experimental test to validate them.

We found that the sequences of the complementary subunits differ much more between species than the sequences of the principal subunits (Table 3 and Figure 2). Interestingly, this observation suggests that the complementary subunits may play a more important role in the nAChR species-specific ligand discrimination.

Another interesting observation is that the sequences

Table 5 Correlation of ligand features and the pocket environment at the species-specific sites of arthropod and vertebrate nAChRs

Pocket environment	Arthropod		Vertebrate	
	Positive	Negative	Hydrophobic	Hydrophilic
Ligand feature	Negative electrostatic	Positive electrostatic	High Log P	Low Log P

Figure 6 Chemical structures of the molecules in Figure 4 that were studied for their high nAChR selectivity and potency. A1–A7 are insect-selective; B1–B7 are *homo*-selective.

differences that exist between species (arthropods and vertebrates) are well “conserved” in almost all nAChR subunits ($\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 6$, $\alpha 7$, $\alpha 8$, $\beta 2$, $\beta 4$). Thus species-selective molecular design without the need to consider the complex assembly of the nAChR becomes more feasible. This observation is important because the assembly of insect nAChR is unknown up to now [23–25].

3 Conclusion

To tackle the problem of insecticide resistance [28], the discovery of a novel family of nicotinoid derivatives is one of the main challenges in pest management science. However, because of the lack of information about the structure of nAChR, up to now, nicotinoid molecular design has been based on the molecules known to be active. Our study extends the understanding of the ligand discrimination mechanism using only the available sequence-based data. The results that we obtained successfully interpreted the structure requirements of nAChR insect-selective ligands and explained the sensitivity of neonicotinoids for insect nAChRs. Our results also identified the ligand selective factors of the insect nAChR. The important species-specific sites identified using the *ab initio* approach correspond well with the results of mutation experiments. Although experimental studies of the nAChR can reveal important ligand binding and discrimination information, unless the experiments cover all the residues near the pocket, a complete view of

the binding site cannot be obtained. The identification of all residues involved in the binding specificity of the ligands is a primary requirement to fully understand which sequence and structural components are essential to the selective mechanism of the nAChR.

The success of our approach shows that the method is of practical value in interpreting the structure information of the selective mechanism of the receptor using only sequence-based data, and thus makes sequence-based molecular design feasible.

The detailed sequence analysis of the reasons behind molecular discrimination of nAChR presented here should contribute to the design of the new specific and potent molecules for nAChRs.

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