Identification of the potential function-specific sites in subunits of vertebrate neuronal nicotinic acetylcholine receptors

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Abstract

The nicotinic acetylcholine receptors (nAChR) are complexes comprising of subunits evolved from common ancestor. Although the subunits are similar in sequences and structures, their molecular function varies significantly. Therefore, detecting the molecular sites specific to each subunit is important to understand the property of the subunits and the receptors formed by them. As we know, the molecular sites critical to the structure and molecular function of a protein family usually are conserved in evolution, and those specific to each member of the family are often closely related to its structural and functional specificity. In this study, we analyzed the sequence specific sites in nAChR subunits by adopting the evolutionary trace method and the two-state model, and explored the relationship between structure and function in combination with the spatial location of the sites. The results showed that the detected sites in α7 subunit were closely related to ligand binding and conformational changes of the energetic coupling pathway. The conserved sites tended to be distributed in the interior of the spatial structure of protein molecules, and the sites potentially related to new functions were distributed on the surface of the spatial structure. In summary, our results could be helpful to understanding the molecular features related to the function specificity and diversity of the nAChR subunits.

Keywords: nAChRs; molecular structure; functional differentiation; molecular evolution

Introduction

Acetylcholine (ACh) is a neurotransmitter synthesized, stored, and released by cholinergic neurons, acting on the central nervous system (CNS) and peripheral nervous system (PNS) through two different types of receptors, i.e., the muscarinic and nicotinic ACh receptors (mAChRs and nAChRs)(Wessler and Kirkpatrick 2008; Gotti, Clementi et al. 2009). nAChRs are mainly involved in functions including learning and memory, arousal, reward, motor control, and analgesia (Zoli, Pucci et al. 2018). Molecularly, nAChRs belong to the super-family of Cys-loop ligand-gated ion channels that consist of five subunits arranged around a central pore. The nAChRs are diverse in properties, which is mainly due to the large number of the possible combinations of the known subunits. Till now, seventeen nAChR subunits have been discovered in the genome of vertebrate, among which the α2-α7 and β2-β4 subunits are usually referred to as the neuronal nAChR subunits (Wonnacott, Bermudez et al. 2018). The nAChR subunits have similar structure, including a extracellular amino terminal portion that carries the ACh binding site and faces the synaptic cleft, followed by three hydrophobic transmembrane domains (M1–M3), a large intracellular loop, and then a fourth hydrophobic transmembrane domain (M4)(Unwin 2005). These subunits can assemble into homo- or hetero- pentameric receptors, like the α7 homomeric and αnd meric, like hsubtypes. Compared with homomeric receptors, the heteromeric receptors contain at least two orthosteric ACh-binding sites at the interface between the primary (+) side of an α subunit and the complementary (−) side of a β subunit. In

each subunit, a number of amino acid residues are involved in ACh binding by grouping into short sequences that form loops A, B and C (the primary component) and D, E and F (the complementary component). Loops A, B, D and F, are in the middle of the interface of two adjacent subunits and provide a hydrophobic pocket to nicotinic ligands bind (Taly, Corringer et al. 2009). When the agonist binds to nAChRs, the conformation of the ion channel changes and the central pore opens, allowing ion flux to pass through the channel for a few milliseconds, after which the recipient closes to a non-conductive state (Gotti, Clementi et al. 2009). There is an energetic coupling pathway (ECP) between extracellular domain (ECD) and the transmembrane channel to transfer the conformational change of ECD to the ion gated region, and the ECP includes Cys-loop, β8-β9 loop, TM2-TM3 loop.

According to the neutral theory (Kimura 1979), some protein sites may change in evolution without affecting the general function of the protein, but a few sites subject to strong evolutionary constraints are much conservative, a replcement on these sites may cause modification in protein structure or function. In many families, the amino acids at a small number of sequence positions determine the particular functional specificity of member proteins. Analysis of these functional specific sites is helpful for protein function prediction, drug design and experimental analysis. In recent years, with the continuous development of technology, a large number of algorithms have been developed to identify protein sites responsible for functional specificity, mainly based on relative entropy, rate of evolution, amino acid physical and chemical properties, etc (Chakraborty and Chakrabarti 2015).

Previously we performed phylogenetic analysis on nine neuronal nAChR subunits and verified the homology of them (Pan, Zhao et al. 2019). In this study, we used the evolutionary trace (ET)(Lichtarge, Bourne et al. 1996) and the two-state model (Gu 2006) to identify and analyze the functional specific sites in nAChR subunits. The ET method relied on the conserved pattern of amino acids in protein sequence alignment to delineate the functional epitope and identify residues critical to ligand binding specificity. Based on mutation evolutionary analysis and structural homology of protein family, ET provides an evolutionary perspective for judging the function or structural role of each residue in protein structure. The two-state model divides the types of sites into four categories, and identifies the radical and conserved replacement sites based on the classification of the physical and chemical properties of amino acids. This method combines biochemistry with sequence evolution model to construct a protein functional evolution model.

By analyzing the amino acid sequences of neuronal nAChR subunits expressed in human and other vertebrate brain, we detected potential functional specific sites in each subunit, which could provide useful clues to understand their functional differences.

Materials and methods

Identification of functional conserved sites

Based on results of sequence alignment and phylogenetic analysis of 105 protein sequences in 9 neuronal nAChR subunits of 12 vertebrates (Pan, Zhao et al.

2019)(Supplemental Table S1), the ET method was used to identify the functional conserved sites in the nine neuronal nAChR subunits. ET uses phylogenetic analysis to identify amino acid sites important to protein structure or function based on alignment of homologous amino acid sequences (Lichtarge, Bourne et al. 1996).The idea of this method is to systematically define family subgroups of similar sequence (and, by implication, of similar functional expression) with the aid of phylogenetic tree, and then identify those residue positions that are invariant within each subgroup. These sites are conserved in subgroups involution, implicating that they may be vital to some shared characteristics of the subgroups (Pritchard and Dufton 1999). With the increase of branching differences, the correlation between branching sites and functional differences between proteins became stronger (Madabushi, Yao et al. 2002).

The steps of ET to identify functional conserved sites are as follows: (1) the phylogenetic tree of homologous proteins was constructed by multiple sequence alignment; (2) partition identity cutoffs (PICs) are used to define partitions of phylogenetic tree; (3) each partition can generate a consensus sequence, and if the site has not changed within the group, the type of its conserved residue is assigned, otherwise it is left blank. At low PIC, sequences of group vary widely. At high PIC, proteins in the same group are increasingly similar in sequence and function. Finally, the tracked functional sites were mapped to the tertiary structure of the protein, and the spatial positions of amino acid sites were combined for further analysis (Lichtarge, Bourne et al. 1996; Pritchard and Dufton 1999).

We used TraveSuite platform (Innis, Shi et al. 2000) to construct a phylogenetic tree using Kitsch algorithm and divide it into 10 partitions. Then, TraceSeq and TraceScript algorithms were used to track the site in each partition (Tsunoyama and Gojobori 1998). Finally，we use JDet (http://csbg.cnb.csic.es/JDet/) to represent the identified sites (Muth, García-Martín et al. 2012).

Identification of sites related to functional divergence

Differences in function between family members after gene replication are usually accompanied by changes in the rate of sites evolution (Gu 1999; Gaucher, Gu et al. 2002; Chakrabarti, Bryant et al. 2007). According to Gu et al., there are four types of amino acid sites for two duplicate gene clusters, i.e., type 0 sites are generally conserved throughout the gene family; type I sites are conservative in one cluster and variable in another; type II sites are conservative in both clusters, but with large functional differences; type U sites are variable in both clusters.

Some specific conserved sites different from other subgroups were not directly related to functional differences. In the standard of site mutation experiment, if the physical and chemical properties of amino acids change greatly after the site mutation, it is likely to have a greater impact on the functional change (Corringer, Le Novère et al. 2000). Based on the physicochemical properties of amino acids, we screened the sites of type □ functional divergence（θ_1）, then calculated the coefficient of type □ functional divergence between subunits using two-state model and maximum likelihood method, and calculated the correlation values with functional divergence for different amino acid substitution sites (Gu 1999; Wang and Gu 2001; Gu 2006).

The two-state model assumes that the site may experience two states early in gene duplication：F_0 (type II unrelated) and F_1 (type II related). The probability of a residue being under F_1 is P(F_1) = θ_I and that being under F_0 is P(F_0)=1-θ_I. In the later stages, an amino acid residue is always under the state of F_0, amino acid substitutions in this stage are mainly under purifying selection.

In this study, we used DIVERGE 3.0 to calculate θ_I, and Z-test was used to identify whether the two gene subgroups had obvious type □ functional divergence. For the subgroup with obvious functional divergence, Bayesian algorithm is used to calculate the posterior probability and predict the sites of type □ functional divergence. Compared with ET, DIVERGE divides the amino acids into four groups according to their physical and chemical properties, i.e., charge positive (K, R, and H), charge negative (D and E), hydrophilic (S, T, N, Q, C, G, and P), and hydrophobic (A, I, L, M, F, W, V, and Y). An amino acid substitution is called radical if it changes from one group to another; otherwise it is called conserved, that is, within the group (Gu 2006).

Mapping of sites to α7 tertiary structure

The Cryo-EM structures of the a7 nicotinic acetylcholine receptor was recently determined and the structure information was acquired from the Protein Data Bank (PDB; https://www.rcsb.org; ID: 7KOQ)(Noviello, Gharpure et al. 2021). The identified sites were projected onto the A chain of α7 using PyMol (https://pymol.org/) to observe the relationship between site function and spatial position.

Results

Conserved sites in the vertebrate neuronal nAChR subunit

The phylogenetic tree of neuronal nAChR subunits were divided into 10 partitions using the ET method (Supplementary Figure S1). In the consequent analysis, we tracked the three partitions of P01-P03 and analyze the identified sites.

A total of 55 completely conserved sites in the vertebrate neuronal nAChR subunits were traced by P01 partition (Supplementary Figure S2). Of these sites, 29 were distributed in ECD, 24 sites were distributed in TM1-TM3, and 2 sites (Val290 and MET305) were located in the α helix region of the first segment of ICD connecting TM3. Projecting these sites onto the tertiary structure of α7, three groups of densely distributed sites were identified (Figure 1A)(Marotta, Rreza et al. 2014). The sites of the first group (Figure 1B) were mainly distributed on loops A-C, β2-β3, β3-β4 linker, which overlapped with the positive binding interface of ligand binding domain (LBD) in space. The second set of sites (Figure 1C) was located between the LBD and the ion channel, of which 7 sites were distributed in the Cys-loop (Cys128-Cys142). Ala96, located on the β4-β5 linker, and Lys125, located on β6, were sequentially distant but closely linked in tertiary structure. A salt bridge was formed between the residues of Asp138 and Arg206, thus connecting the conformational transfer between LBD and ion channel (Galzi, Devillers-Thiery et al. 1992). The third group of sites was distributed in the transmembrane helical region of TM1-TM3. Although the sites of TM1 and TM3 were far apart in sequence, they were closely connected in tertiary structure. Notably, for Phe230 on TM1, Phe253 on TM2,

and Met279 on TM3, side-chain benzene rings or sulfur atoms were close to residues on other TM segments, resulting in stable chemical structures (Figure 1D). As can be seen from the distribution of these three groups of sites, the positive binding pocket of LBD, TM1-TM3 transmembrane helix, and the energetic coupling pathway between LBD and ion channel remained stable in the evolution of vertebrate neuronal nAChR (Corringer, Le Novère et al. 2000).

Conserved sites specific in α7 subunit

P02 tracked 22 specific conserved sites in the α7 subunit, as well as in other subunits, but different from the α7 subunit (Supplementary Figure S2). These sites were distributed in ECD, TM1-TM3, C-terminal and the α helix region of the first segment of ICD connecting TM3 (Figure 2A). The β4-β5 linker connected to loopA at the sequence site and was spatially adjacent to the β6' fold, which continued loopE in the sequence. In the case of nicotine molecule binding, loopA was related to loop E being an LBD pocket. Four sites of the α7 subunit were located in ECD, and Tyr118 was located at loop E, adjacent to the conserved Leu119, which was conserved non-polar tryptophan in other subunits. Ser95 was attached to Glu98 on the β4-β5 linker, and Glu98 was spatially closely linked to Lys125 on β6' (Figure 2B). In other subunits, Glu98 was replaced by polar glycine, which attenuated the intersite forces (Figure 2C). The weak acid Thr208 and Tyr210 at the entrance of α7 subunit were replaced by non-polar proline and phenylalanine, which probably attenuated the negative charge environment of ion channel on the surface of cell membrane, and was related to the decrease of calcium ion permeability. The α7 subunit was highly

conserved in the α helix region of the first segment of ICD connected to TM3, which was specific to the α7 subunit. We found that this structure was important for the expression and function of the α7 subunit (Jaiteh, Taly et al. 2016). P469 and N470 distributed at the C-terminus of the α7 subunit were space-aligned in other subunits. These two sites can also be regarded as specifically conserved in the α7 subunit and may be involved in the interaction between TM4 and the cell membrane.

Conserved sites specific to subunit subsets

P03 traced a total of 13 sites, which identified α2-α6 and β3 subunits as group 1, β2 and β4 subunits as group 2, and α7 subunits as group 3. These sites were conserved in each of the three groups. Asn107 and Ala226 in group 3 were replaced by basic lysine and polar threonine in group 1. Asn107, located in β5 and adjacent to loopE, was polar amino acid, asparagine, in both α7 and β2β4 subunits. After the second evolution, it was replaced by conserved alkaline lysine in the first group. In fact, when nicotine was bound to the ligand, the hydrophobic properties of the negative subunit loop E were more conducive to the binding of the ligand, which explained that nicotine can be bound to the nicotine molecule as a negative binding interface. However, the α4 subunit replaced the three hydrophobic subunits of loop E by one basic amino acid and two polar amino acids, thus rejecting ligand binding (Bouzat, Gumilar et al. 2004).

The conserved sites in the second group corresponded to Phe100, Asp131, Gly147, Ser223, and Ser249 in the α7 subunit. The non-polar amino acid Phe100 was located in the β4-β5 linker, adjacent to 1oopA (Leu92). Because Tyr93 of loop A was

an important site for binding ligand molecules such as nicotine, the non-polar aromatic amino acid was replaced by the weak acid polar aromatic amino acid at Phe100. It was likely to affect the ligand binding of loopA or the structural changes after ligand binding. The polar amino acid Gly147 at loop B corresponds to positively charged arginine in the second group. It has been shown that in α4β2 receptors, if the β2 subunit acts as a positive binding interface, the arginine group at this site will be wedged to loop C and loop A at the β2 subunit, thus occupying the binding site of the nicotine ligand molecule.

Asp131 was located in the Cys-loop region and plays an important role in energy transfer from LBD to ion gated channels. Arg133, Met261, Ala263, Ser267, Gln273 and Ile281 in the α7 subunit were conserved in the three groups, but not in different groups. Arg133, a basic amino acid located in the Cys-loop region, was a conserved basic amino acid in the second and third group; at the same site in the first group, amino acid threonine was presented, which was presumed to play different regulatory role in the structural changes of energy transfer. Lys274 was the site with the greatest change in the physical and chemical properties of amino acids between the three groups, located at the entrance of TM3 and adjacent to the spatial location of Cys-loop. The site in this region was related to the conformational transmission of LBD to ion channel, as well as the charge accumulation near the cell membrane (Paterson and Nordberg 2000).

Functional divergence sites between α7 subunit and other neural subunits

We calculated the functional divergence coefficients of type among nine

nAChR pairwise groups. The functional divergence coefficients of type among α2α4, α3α6 and β2β4 were not statistically significant, but the functional divergence coefficients of other subunits were significant. Notably, all subunits had the highest degree of type divergence relative to the α7 subunit (Table 1). We calculated the posterior probability ratio of sites of functional divergence between the α7 subunit and other neuronal subunits. Based on the posterior probability ratio of sites, two classes of sites conserved within the α7 subunit and in one or more of the other subunits, were identified. Compared with α7 subunit amino acids, the first type of sites had radical substitution, and the second type of sites had conserved substitution. Altogether, 74 substitution sites were identified between α7 and other subunits, which were mainly distributed in β4-β5 linker, Cys-loop, β10-TM1 linker, TM2-TM3 loop, and the initiation region of ICD (Table 2). These sites were likely involved in functional differences relative to the α7 subunit.

By mapping 74 class I sites to α7 tertiary structures and combining with 55 fully conserved sites tracked by ET, we can see that the fully conserved sites of subunits are mainly distributed in the interior of the receptor, while the new conserved functional sites that are easy to emerge in evolution are mainly distributed on the surface of the receptor (Figure 3A). There are eight sites (Tyr129, Ala258, Ala263, Asp266, Ser267, Gln273, Asp299, Gly303) that are conserved within each subunit but have altered amino acid properties relative to the α7 subunit (Figure 3B). Cys-loop is adjacent to the TM2-TM3 loop in spatial position, and plays an important role in the energy transfer from LBD to ion channel. Tyr129 is located in the Cys-loop, while Asp266

and Ser267 are located in the TM2-TM3 loop. Asp266 is replaced by lysine in the α5β3 subunit and leucine in the other six subunits. Asp266 was used to distinguish the three groups of subunits. The functional difference lies in that the acidic α7 subunit can form homomers at this site, while the non-polar subunit can only form heteromers in pairs. The basic α5 and β3 subunits at this site do not participate in ligand binding. Arg133 on the Cys-loop is a basic lysine in the β2β4 subunit and a polar threonine in the α2-α6 and β3 subunits, which may also be related to the fact that the three subunits provide negative binding interfaces (Figure 3C).

Functional divergence sites in subunit subsets

By calculating the posterior probability ratio of functional divergence sites of α7 subunit to other 8 subunits, 5 qualitative variant substitutions and 15 conservative substitutions were obtained, which were similar to those traced by P02 partition of ET (Supplementary Figure S3). Compared with ET, this method classifies the sites tracked by P02 and quantitatively calculates the posterior probability ratio. The sites where the properties of the first group of amino acids changed are more likely to be related to differences in the basic biological functions of the two subunits. According to the posterior probability ratio, a total of 29 radical substitution sites and 43 conserved substitution sites were obtained between α7 and β2β4 subunits. These sites include all sites tracked by P02 partition of ET except c-terminal and some sites tracked by P03 partition. The functional divergence sites between α7 subunit and β2β4 subunit were mainly distributed in "Cys-loop", TM2-TM3 loop, loopC and LBD binding interface near the connected linker region. Therefore, it can be speculated that

the two groups of subunits have great functional differences in the selective binding ability of ligand and the response coefficient of ion channel after ligand binding. There were 11 functional divergence sites between α7 subunit and α2-α6 and β3 subunit groups, all of which were identified in the P03 partition of ET, and no functional divergence sites of conserved substitution were found. Among the functional disambiguation sites between β2β4 and α2-α6 and β3 subunits, only I291 in TM3 was not identified in RT-P03. Although the posterior probability ratio of Glu259 was low, multi-sequence alignment revealed that the basic amino acids in β2β4 subunit were conserved, and the acidic amino acids in α3-α7 and β3 subunit were conserved, and the replacement of acidic amino acids only occurred in α2 subunit. This acid-base difference was likely related to the characteristics of receptor channels.

Finally, we divided α2α4, α3α6 and α5β3 subunits into three groups. The posterior probability values of α2α4 and α5β3, α3α6 and α5β3 showed significant functional divergence. The functional divergence sites were distributed in TM3 linker, ICD, β7β8 and β9'. It can be seen that the large functional differences among the three subunits are related to the characteristics of ion channels.

Discussion

As the major receptors in cholinergic system, nAChRs play important roles in a series of physiological processes. They are also involved in neurological disorders like nicotine addiction, Alzheimer's disease and Parkinson's disease (Dani and Bertrand 2007; Gundisch and Eibl 2011; Alexander, Peters et al. 2015; Dineley,

Pandya et al. 2015), thus, are promising targets of therapeutic agents (Bertrand and Terry 2018; Chuang, Paul et al. 2019; Diabasana, Perotin et al. 2020). The diversity in function and types of nAChRs relies on variety of properties of the subunits and their combinations. Since the neuronal subunits in vertebrates were descended from common ancestors, comparing these subunits in the context of evolution will provide valuable clues to understand their specificity.

By using ET, we identified 55 completely conserved sites in nine vertebrate nAChR subunits. These sites are mainly distributed in the positive binding surface of ligand binding domain, the input interface region of "LBD-TMD energy pathway" (β8-β9 connection loop and Cys-loop), the outer membrane inlet and outlet end of transmembrane helices, and the interconnecting sites between three transmembrane helices. These regions are important for the basic structure or function of the protein family. Previous experiments have shown that Loop A-C and Loop D-F provide positive and negative binding interface for binding domain, and loopsD-F has higher sequence variability than Loops A-C (Brejc, van Dijk et al. 2001; Millar and Gotti 2009; Cymes and Grosman 2021). The distribution region of one of the loci identified by P01 partition of ET overlapped with the positive binding interface, which verified that the positive binding interface played a stronger role in the binding of nAChR to the ligand. Compared with the positive binding surface, the negative binding surface is more suitable for the regulation of ligand binding parameters. The energetic coupling pathway between LBD and ion channel gated region confirmed by Lee WY (Lee and Sine 2005) and the 7 highly conserved sites in the superfamily measured by

Ortells (Ortells and Lunt 1995) are consistent with the results obtained by P01, thus confirming the important role of energetic coupling pathway in nicotinic receptor channel protein (Marcovich, Moglie et al. 2020). In addition, β4-β5 is adjacent to β4 and β6', β4 is a component of loop A, β6' is a continuation of loop E, β4-β5 loop is approximately located in the middle of positive and negative binding interfaces in space, which may affect the conformation of the two binding interfaces.

Multiple sites on the TM2-TM3 loop, the outer end of TM2 membrane and the outer beginning of TM3 membrane have been proved to be important components of the energetic coupling pathway, participating in the conformational transfer of LBD to the gated region of the receptor protein ion channel. In addition, this region is related to the concentration of ions that the receptor accumulates around the cell membrane, thus affecting the conductivity and calcium permeability of the channel. For example, the site near the entrance of TM3 of α7 subunit is polar, but less polar than other subunits, similar to the intermediate state of β2β4 subunit and other subunits. It is inferred that the difference in this region is related to the better permeability of calcium ions, lower binding efficiency with nicotine molecules and faster desensitization of α7 subunit compared with other subunits. Through the spatial distribution of sites, we found that all the conserved sites in the α7 subunit were concentrated in the positive binding interface of LBD, the transmembrane helical region, the LBD-TMD energetic coupling pathway and the α helical segment in ICD cells that connected TM3. Finally, the total subunit conserved sites are mainly distributed in the inner region of protein spatial structure, while the functional

divergence sites tend to be distributed in the surface region of protein surface. It is speculated that the subunits constantly generate new functions to adapt to changes in the external environment during the evolutionary process.

In summary, by using the statistical models such as evolutionary trace and the two-state model, we identified the conserved sites in nAChR subunits, and then analyzed the spatial distribution and their potential influence on the function of the subunits. The results showed that the conserved sites were concentrated in important regions related to ligand binding and ion channel energy transfer, and were closely related to the structure and function of the receptor. It is of great significance to analyze the sites of functional divergence among each subunit for understanding the functional differences among subunits, which will provide useful information for exploring the action mechanism of nAChR and targeted treatment of diseases.

Competing interest:
The authors declare that they have no competing interests.

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Figure legends:

Figure 1. Spatial distribution of functional conserved sites of nine neuronal nAChR subunits in vertebrates. (A) 55 completely conserved sites tracked by P01 were mapped to α7 tertiary structure. (B) The first set of sites (Trp60, Trp86, Pro88, Asp89, Leu92, Phe146, Ser148, Trp149, Tyr151, Tyr195), loopsA-C, L92 at loopA, F146 at loopB, and disulfide bonds between Cys on loopC are highlighted in yellow. (C) The second set of sites (Ala96, Lys125, Ser126, Cys128, Phe135, Pro136, Phe137, Asp138, Gln140, Cys142, Glu173, Trp174, Arg206, Tyr211) located in LBD and TMD were highlighted.Interlinked Ala96 and Lys125,Asp138 and Arg206 are labeled.(D) By highlighting Phe230, Phe253 and Met279, it is speculated that these sites play an important role in the connection of the three TM regions.

Figure 2. Spatial distribution of α7 subunit specific conserved sites identified by P02. (A) The 55 completely conserved sites identified by P01 were highlighted in green, and the 20 α7 subunit specific conserved sites identified by P02 are highlighted in red. (B) Rotate figure A by an Angle. (C) Glu98 conserved in the α7 subunit is replaced with glycine conserved in other subunits, and the substituted glycine is highlighted in yellow.

Figure 3. Spatial distribution of conserved sites of total subunit and specific functional divergence sites of α7 subunit. (A) The completely conserved sites measured by ET were highlighted in green, and the specific functional divergence sites of the α7 subunit were highlighted in red. (B) Figure (A) was rotated to indicate yellow sites that were conserved in each subunit but changed in physicochemical properties relative to the α7 subunit. (C) The area in the blue box in Figure (A), the detail between the "Cys-loop" and the TM2-TM3 loop.

Supplementary Figure S1. Phylogenetic trees and evolutionary divisions of the nine neuronal nAChR subunits from 12 vertebrates. As PIC increases, from P01 to P10, partitions comprise more groups, each with fewer sequences.

Supplementary Figure S2. Functional conserved sites identified by ET. The site number corresponds to the amino acid site number of the α7 subunit protein sequence. (A) There were 55 completely conserved sites in P01 partition. (B) Specific conserved sites within the α7 subunit traced by P02. (3) Specific conserved sites traced by P03. The α4 subunit sites represent highly

conserved in the entire first group, the β2 sites represent highly conserved in the entire second group, and the third group is α7.

Supplementary Figure S3. Functional divergence sites within subunit subsets identified by DIVERGE. The overlapping sites identified by ET were highlighted in red. The site number corresponds to the amino acid site number of the α7 subunit protein sequence. (A)Functional divergence sites between α7 subunit and other 8 subunits. (B) Functional divergence sites between α7 subunit and β2β4 subunits. (C) Functional divergence sites between α7 subunits and α2-α6, β3 subunits. (D) Functional divergence sites between β2β4 subunits and α2-α6, β3 subunits.

Table 1. Type □ functional divergence coefficients of 9 nAChR subunits in 12 vertebrates (θ_I ±SE)

	α7	**α2**	**α3**	**α4**	**α5**	**α6**	**β2**	**β3**
α2	0.43± 0.04*							
α3	0.46± 0.04*	0.12± 0.04*						
α4	0.45± 0.04*	0.00± 0.04	0.11 ± 0.04*					
α5	0.52± 0.04*	0.24± 0.04*	0.29 ± 0.04*	0.24± 0.04*				
α6	0.45± 0.04*	0.15± 0.05*	0.06 ± 0.05	0.13± 0.05*	0.25± 0.05*			
β2	0.49± 0.04*	0.26± 0.04*	0.29 ± 0.04*	0.27± 0.04*	0.40± 0.04*	0.32± 0.05*		
β3	0.49± 0.04*	0.24± 0.04*	0.29 ± 0.04*	0.23± 0.04*	0.08± 0.05#	0.26± 0.05*	0.37± 0.04*	
β4	0.42± 0.04*	0.20± 0.05*	0.24 ± 0.05*	0.19± 0.04*	0.33± 0.05*	0.29± 0.05*	0.02± 0.04	0.29± 0.05*

θ_I is given by the maximum likelihood method, and SE represents the standard deviation of θ_I.

P value is calculated according to Z test of (θ_I /SE, where * represents p <0.005, # represents 0.01<p<0.05, indicating the difference between the two subunits is statistically significant.

Table 2. Functional divergence sites of intercomparison between each subunit and α7 subunit.

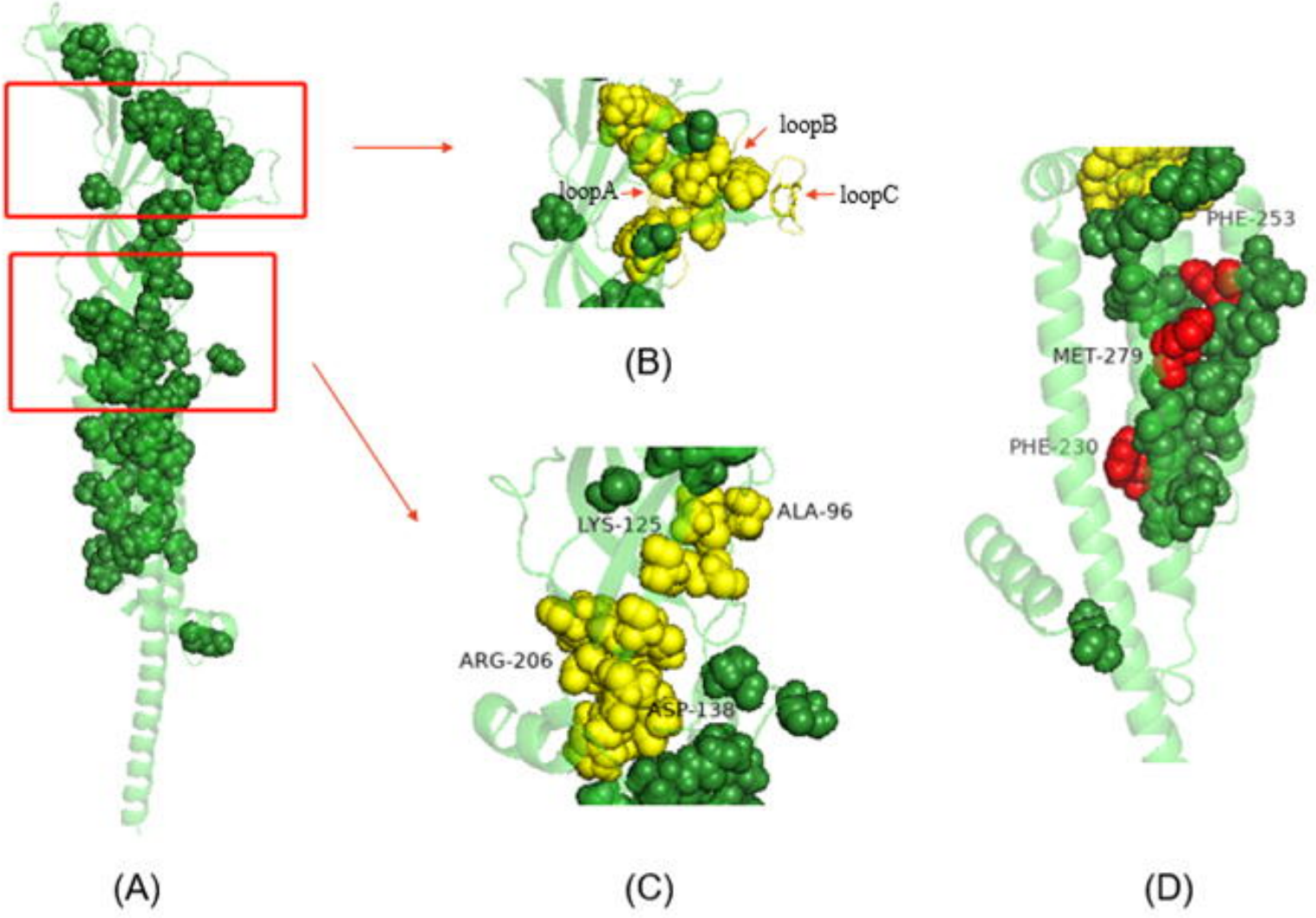
Residue	Subunit	Domain	Secondary structure
Tyr64	α2, α3, α4, α5, β2, β4	ECD	B2-β3 linker
Asp82	α2, α4, α5, β2, β3	ECD	B3-β4 linker
Lys87	β2, β3, β4	ECD	B3-β4 linker
Asn94	α5	ECD	B4-β5 linker
Asp97	α6	ECD	B4-β5 linker
Glu98	α2, α3, α4, α5, β2, β3, β4	ECD	B4-β5 linker
Asp101	α3, α4	ECD	B4-β5 linker
Ala102	β3	ECD	B4-β5 linker
His105	α2, α4, β2, β3	ECD	B4-β5 linker
Asn107	α2, α3, α4, α5, α6, β3	ECD	β5 strand
Leu109	α2	ECD	β5 strand
Asn111	α2, α4	ECD	β5 strand
Cys116	α3, α6	ECD	β6 strand
Gln117	β2	ECD	β6 strand
Gly122	α2, α3, α4, α6, β2, β4	ECD	β6' strand
Ile123	α5, β3	ECD	β6' strand
Tyr129	α2, α3, α4, α5, α6, β2, β3, β4	ECD	β6'-β7 strand
Arg133	α2, α3, α4, α5, α6, β3	ECD	β6'-β7 strand
Trp134	β2	ECD	β6'-β7 strand

Gly147	β2, β4	ECD	β7 strand
Gly153	α3, α6, β2	ECD	β7-β8 strand
Trp154	α5	ECD	β7-β8 strand
Ser155	α3, β2, β3, β4	ECD	β7-β8 strand
Gln159	α3, β2	ECD	β8 fold
Asp164	α6, β2	ECD	β8-β9 linker
Ile169	β2, β4	ECD	β8-β9 linker
Asp175	α2, α4	ECD	β9 strand
Arg183	α2, α4	ECD	β9' strand
Tyr188	α5	ECD	β9'-β10 strand
Lys192	α6, β2, β4	ECD	β9'-β10 strand
Thr201	β2, β4	ECD	β10 strand
Arg207	α2, α3, α4, α5, α6	ECD	β10-TM1 linker
Asn214	α5, β3		TM1
Leu221	α5, β3		TM1
Ala226	α2, α3, α4, α5, α6, β3		TM1
Ala234	α2, α3, α4, α5, α6, β2, β3, β4		TM1
Asp235	α5		TM1-TM2 linker
Ser236	β3		TM1-TM2 linker
Ile244	α5, β3		TM2
Ser249	β2, β4		TM2
Ala258	α2, α3, α4, α5, α6, β2, β3, β4		TM2
Glu259	β2, β4		TM2
Ile260	α6		TM2

Ala263	α2, α3, α4, α5, α6, β2, β3, β4	TM2-TM3 linker
Asp266	α2, α3, α4, α5, α6, β2, β3, β4	TM2-TM3 linker
Ser267	α2, α3, α4, α5, α6, β2, β3, β4	TM2-TM3 linker
Ala272	α2, α3, α4, α5, α6, β2, β3, β4	TM3
Gln273	α2, α3, α4, α5, α6, β2, β3, β4	TM3
Thr278	β3	TM3
Asp299	α2, α3, α4, α5, α6, β2, β3, β4	ICD
Gly303	α2, α3, α4, α5, α6, β2, β3, β4	ICD
Lys304	α3, β2	ICD
Pro306	α5, β3	ICD
Lys307	α5, β2, β3	ICD
Thr309	α2, α3, α4, α5, α6, β2, β3, β4	ICD
Asn315	α5	ICD
Trp316	β2	ICD
Cys317	α3, α5, β2, β3, β4	ICD
Ala318	α2, α3, α4, α5, α6, β2, β3, β4	ICD
Trp319	α2, α4	ICD
Arg322	α3, α5, β2, β3, β4	ICD
Arg325	α5, β2	ICD
Pro326	α5	ICD
Asp329	α5	ICD
Lys330	α5	ICD
Lys413	β4	ICD
Glu417	α2, α4, β2, β3, β4	ICD
Tys424	α3, α6	ICD
Gln428	α4, α5, β2, β3	ICD
Asp429	α6	ICD

Cys435	α2, α5, β3	ICD
Cys449	α2, α3, α4, α5, β2, β3, β4	TM4
Ala468	α2, α3, α4, β2, β4	TM4
Glu473	α4, α5, β2, β4	C-terminal

The radical functional divergence sites of α7 subunit and other subunits are shown in the table, with the residue numbers corresponding to the functional divergence sites of mature Hs _ α7 subunit protein sequence.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loopC
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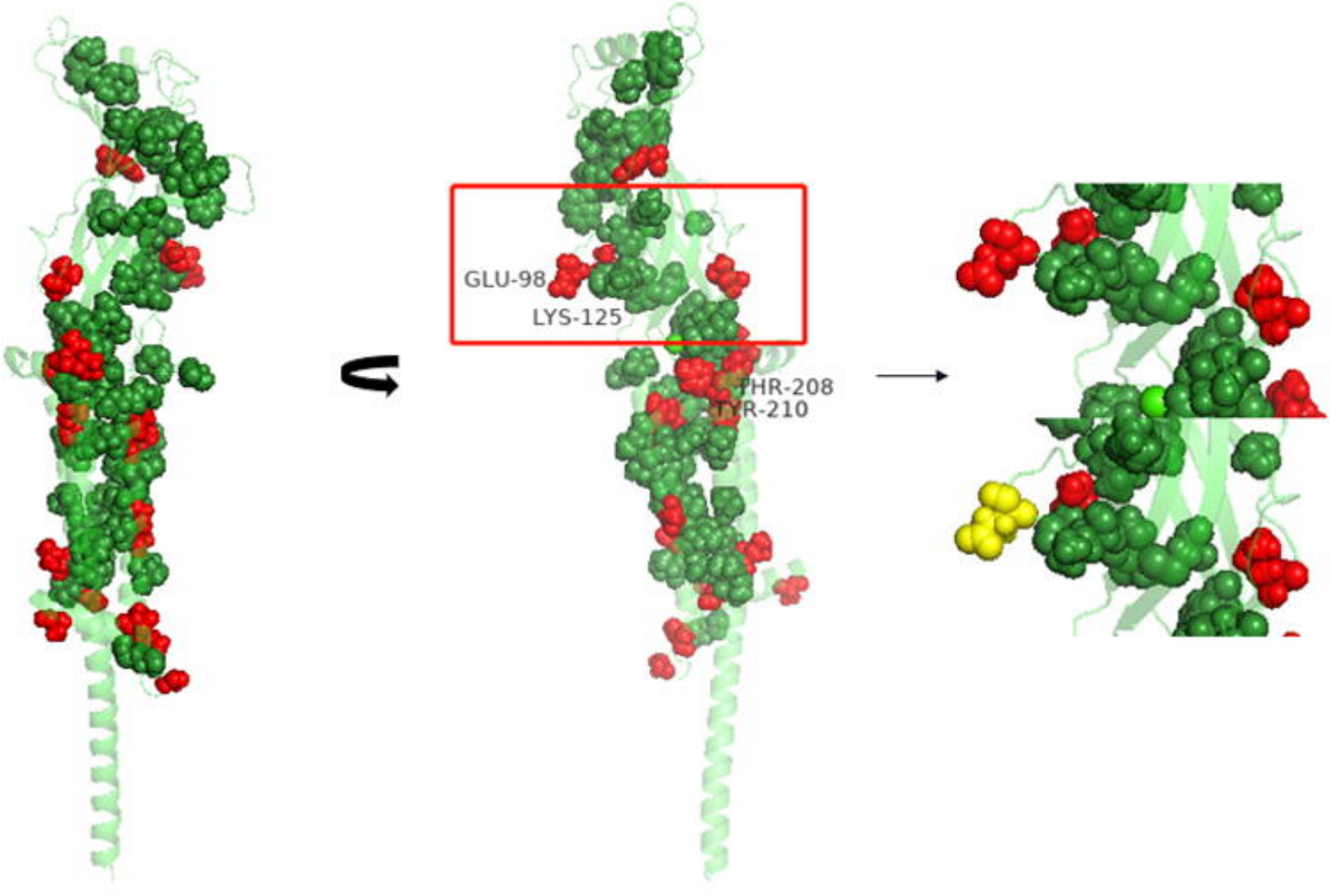
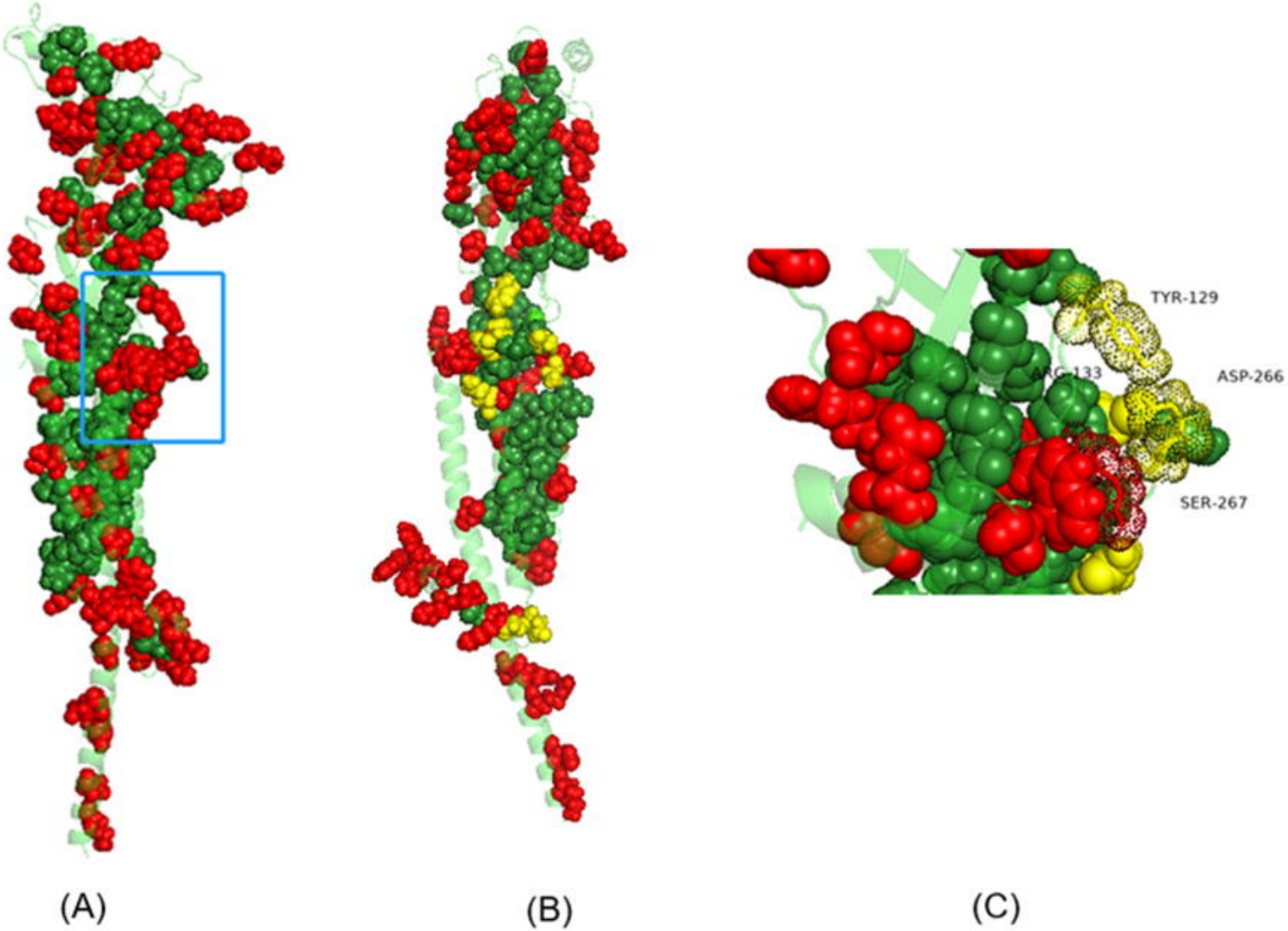
(D)



(A) (B) (C)


TYR-129
ASP-266
SER-267
(A)
(B)
(C)