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Homology modeling of human $\alpha1\beta2\gamma2$ and house fly $\beta3$ GABA receptor channels and Surflex-docking of fipronil

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Abstract To further explore the mechanism of selective binding of the representative γ-aminobutyric acid receptors (GABARs) noncompetitive antagonist (NCA) fipronil to insect over mammalian GABARs, three-dimensional models of human $\alpha1\beta2\gamma2$ and house fly $\beta3$ GABAR were generated by homology modeling, using the cryo-electron microscopy structure of the nicotinic acetylcholine receptor (nAChR) of *Torpedo marmorata* as a template. Fipronil was docked into the putative binding site of the human $\alpha1\beta2\gamma2$ and house fly $\beta3$ receptors by Surflex-docking, and the calculated docking energies are in agreement with experimental results. The GABA receptor antagonist fipronil exhibited higher potency with house fly $\beta3$ GABAR than with human $\alpha1\beta2\gamma2$ GABAR. Furthermore, analyses of Surflex-docking suggest that the H-bond interaction of fipronil with Ala2 and Thr6 in the second transmembrane segment (TM2) of these GABARs plays a relatively important role in ligand selective binding. The different subunit assemblies of human $\alpha1\beta2\gamma2$ and house fly $\beta3$ GABARs may result in differential selectivity for fipronil.

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Introduction

γ-Aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the central nervous system and exerts its physiological effect by binding to GABA receptors (GABARs) [1]. Mammalian GABARs can be classified simply into two types: ionotropic receptors, which are ligand-gated ion channels (GABA$_A$R, GABA$_C$R); and metabotropic receptors (GABA$_B$R) [2]. The ionotropic GABARs are members of the superfamily of Cys-loop ligand-gated ion channels, which also includes nicotinic acetylcholine receptors (nAChR), strychnine-sensitive glycine receptors, and serotonin type 3 receptors [3].

Similar to the superfamily member nAChR, ionotropic GABARs are comprised of five subunits, which are arranged around the ligand-gated ion pore and span the lipid membrane. They can be assembled from five copies of a single subunit, but are more commonly assembled from several different subunits [4].

Vertebrate ionotropic GABARs are distributed widely in the nervous systems. Cloning from cDNA or genomic libraries has so far revealed 19 related GABAR subunits in the mammalian nervous system. These subunits are of the classes 6α, 3β, 3γ, 1δ, 1ε, 1π, 1θ, and 3ρ [5–7]. Subunits in the same class share rather high similarity (70–80%), while similarities between subunits in different classes are

relatively low (20–40%). One of the most common GABARs in the human brain is the α1β2γ2 subunit combination, and the proposed receptor stoichiometry is 2α2β1γ [8–10].

Far less is known about insect GABARs than those of vertebrates. To date, researchers have cloned three kinds of subunits of insect ionotropic GABARs: RDL (a subunit encoded by the resistant-to-dieldrin gene), LCCH3 (ligand-gated chloride channel 3), and GDR (the GABA_A and glycine receptor-like subtype of *Drosophila*). The RDL-encoded subunits of *Drosophila* and other insects were shown to form functional GABA-gated channels, indicating its contribution to the majority of insect GABARs [11–13]. The β3 subunit has a high sequence similarity with the house fly RDL subunit in the second transmembrane region [14].

The structures of all subunits contain three main domains: an N-terminal ligand-binding domain (LBD), transmembrane domains (TMDs), and a long cytoplasmic loop that links TM3 and TM4. The LBD is a hydrophilic long chain located on the extracellular side of the membrane, consisting primarily of β-strands linked by disulfide bonds between cysteine residues. In each subunit, the TMD is a four-α-helix bundle spanning the lipid bilayer four times, forming the ion channel containing the channel gate. From the N-terminus to the C-terminus, the four hydrophobic helices are clearly defined as TM1, TM2, TM3, and TM4 in sequence. Furthermore, the TM2s of five subunits make inner multiple-ring-like arrangements of amino acid residues within an ion pore, while the other three TMs shield the inner rings from the lipids. The large variable loop links TM3 and TM4 inside the membrane, and the structure of this domain is still ambiguous.

In recent years, a series of compounds acting on the GABARs has been discovered [15, 16]. Towards a better understanding of the molecular interactions between receptors and ligands and development of safer insecticides, scientists have focused their attention on four main sorts of GABAR noncompetitive antagonists (NCAs): (1) polychlorinated cycloalkanes; (2) picrotoxinin and related terpenoids analogues; (3) fipronil and its analogues; (4) trioxabicyclooctanes. Binding studies with [³H]ethynylbicycloorthobenzoate ([³H]EBOB) as the radioligand as well as electrophysiological studies have confirmed that some of insecticidal compounds act as NCAs at GABARs [15]. All these NCAs bind mammalian and insect GABARs to block the chloride ion channels, thus triggering physiological effects [17].

To date, the three-dimensional (3D) crystal structure of GABAR has not been confirmed as it is still difficult to obtain the membrane protein crystal. In this study, the TMD of human α1β2γ2 and house fly β3 GABARs were built by homology modeling. These models were optimized and validated using computational tools as well as by comparison with experimental results. The NCA fipronil was docked to the binding sites of both human α1β2γ2 and house fly β3 GABARs, and the results were used to explain and consolidate experimental data. In addition, the mechanism of the selective action of fipronil with the house fly versus human GABARs was studied.

Methods

All work on homology modeling and Surflex-docking studies was performed using the SYBYL 7.3 software package (http://www.tripos.com/) running on a Linux workstation [18].

Sequence selection

To build the homology models of the TMDs of human α1β2γ2 and house fly β3 GABARs, the sequences of the human α1 (P14867), β2 (P47870), γ2 (P18507) subunits, and the house fly β3 (β) subunit (Q75NA5) were obtained from the Swiss-Prot/TrEMBL database. These four sequences were all edited to remove the LBD and the long cytoplasmic loop linking TM3 and TM4.

Template selection

Template selection is an important starting point in homology modeling because the template directly determines the main folding of the target structures, and influences their quality. Due to the technological limitations of membrane-bound protein crystallization, few well-resolved structures of membrane-bound proteins have been obtained through X-ray crystallography and NMR methods. However, all GABAR subunits were modeled using the cryo-electron microscopy structure of nAChR of *Torpedo marmorata* as the template, which was obtained at 4 Å resolution in the resting state (Protein Data Bank identifier 1OED) [19]. The templates contain the TMD of α, β, γ, and δ subunits of nAChR.

Building the subunits

Each subunit was built using the following procedure. After aligning each target sequence with the template sequence using the Needleman and Wunsch method, a multiple sequence format (MSF) file was generated [20]. The sequences and structures are structurally aligned using ORCHESTRAR program of the BATON method [21]. All target peptide chains were built by recognizing structure conserved regions (SCR), searching the gaps and adding side chains.

Fig. 1 a Schematic presentation of subunit correspondence between nicotinic acetylcholine receptor (nAChR) and γ-aminobutyric acid receptor (GABA$_A$R). **b** Each target sequence (the transmembrane domain of human GABAR α1, β2, γ2, and house fly GABAR β3 subunits) was aligned with all four template sequences (nAChR α, β, δ, and γ subunits). Different types of amino acids are depicted in different colors. The four transmembrane domains (TMDs) of GABAR subunits are indicated with *bold lines*. The positions of the 2′, 6′ and 9′ residues in TM2 of each GABAR subunit are indicated
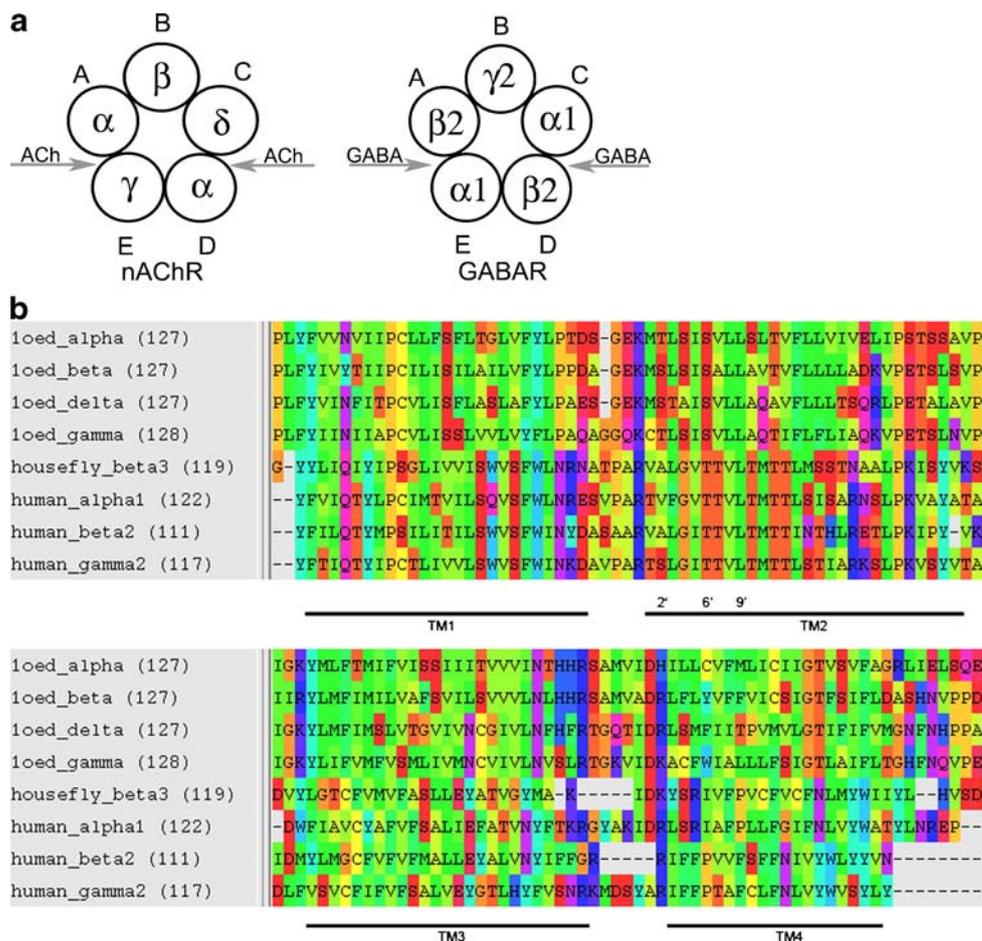
Model assembly

Models were assembled using the following method. According to the schematic presentation of subunit correspondence between nAChR and α1β2γ2 GABAR shown in Fig. 1a, the integral GABA receptor was obtained by aligning subunits of human GABAR to the corresponding subunits of the template. That is to say, the GABAR α subunit was aligned to nAChR subunits γ and δ, the GABAR γ subunit to nAChR subunit β, and the GABAR β subunit to nAChR subunit α. This method of model construction is based mainly on the fact that the agonist binding sites of both the GABA and nACh receptors are spatially similar. The nAChR α subunits form the principal part of the acetylcholine (ACh) binding pocket, while the GABAR β subunits form the principal side of the GABA binding pocket. The homology model of the house fly β3 GABAR was set up by aligning the β3 subunit to every nAChR subunit.

Model refinement

Both of the initial models were optimized energetically using the AMBER7 FF99 force field by performing a conjugate gradient minimization to reach a root-mean-square (RMS) gradient energy of 0.5 kcal mol^{-1} Å^{-1}. Subsequently, a dynamics simulation was performed to find the steady-state conformation of initial human α1β2γ2 and house fly β3 GABARs over 500 ps with a step size of 1 fs at a constant temperature 300 K [18].

Ligand docking

To validate the 3D homology models, the GABA receptor NCA fipronil was docked into the putative binding pocket of human α1β2γ2 and house fly β3 GABARs. Before performing ligand docking, it is critical to search for the binding pocket of the prepared protein. In this study, Residues Mode was adopted to generate the protomol in the program Surflex [18]. This mode defines the active site by considering a reasonable distance around chosen residues. In addition, two parameters that can significantly affect the size and extent of the protomol generated are the threshold and the bloat value.

The Surflex scoring function, which is based on the binding affinities of protein-ligand complexes, takes into account several terms, including hydrophobic, polar, repulsive, entropic and solvation [22]. The docking scores are expressed in $-\lg_{10}K_d$ units to evaluate the docking results, where K_d represents a dissociation constant of a ligand [18].
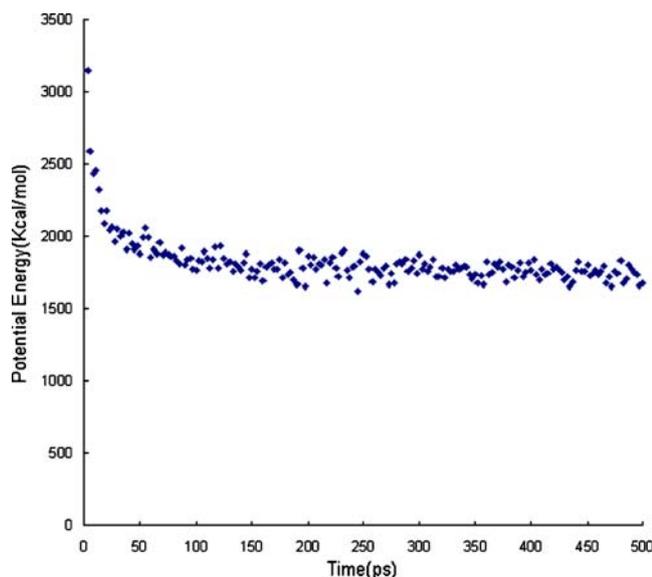
Fig. 2 Potential energy with respect to simulation time for 500 ps molecular dynamics on the human α1β2γ2 GABAR

In the study, the binding free energies (kcal mol^{-1}) of protein-ligand complexes would be obtained according to the calculation as follows, where RT=0.59 kcal mol^{-1}:

$$Free\ Energy\ of\ Binding = RT lg_e K_d \qquad (1)$$

Results and discussion

Building the homology model

Human hetero-oligomeric α1β2γ2 receptors have a wide distribution in the nervous system. A homology model was
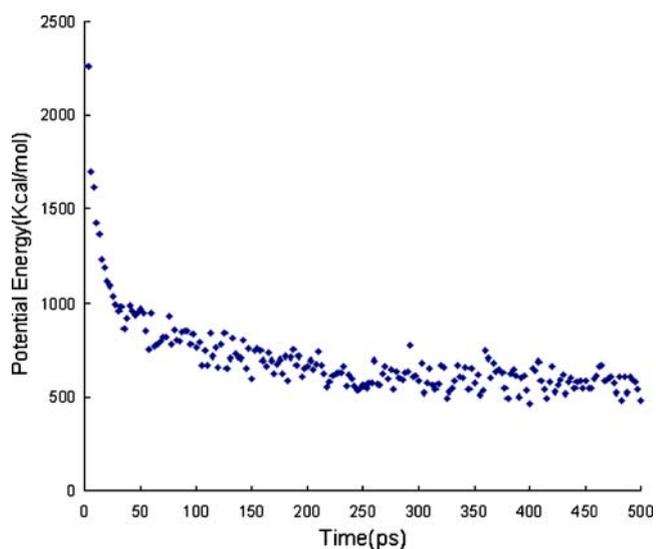
Fig. 3 Potential energy with respect to simulation time for 500 ps molecular dynamics on the house fly β3 GABAR

constructed to study the mechanism of action of insecticides with GABARs in the mammalian brain. Although the subunit composition of native insect receptors is unknown, it has been confirmed that the GABAergic insecticide binding potency on a pentameric receptor formed from the β3 subunit correlates well with that on the house fly receptor [17]. Therefore, house fly β3 homo-oligomeric GABAR was built to predict the interaction between insect GABAR and the test compounds. In the current study, all homology models were built based on the multiple structure-sequence alignment method, which utilized all subunits of nAChR. Details of the alignment are shown in
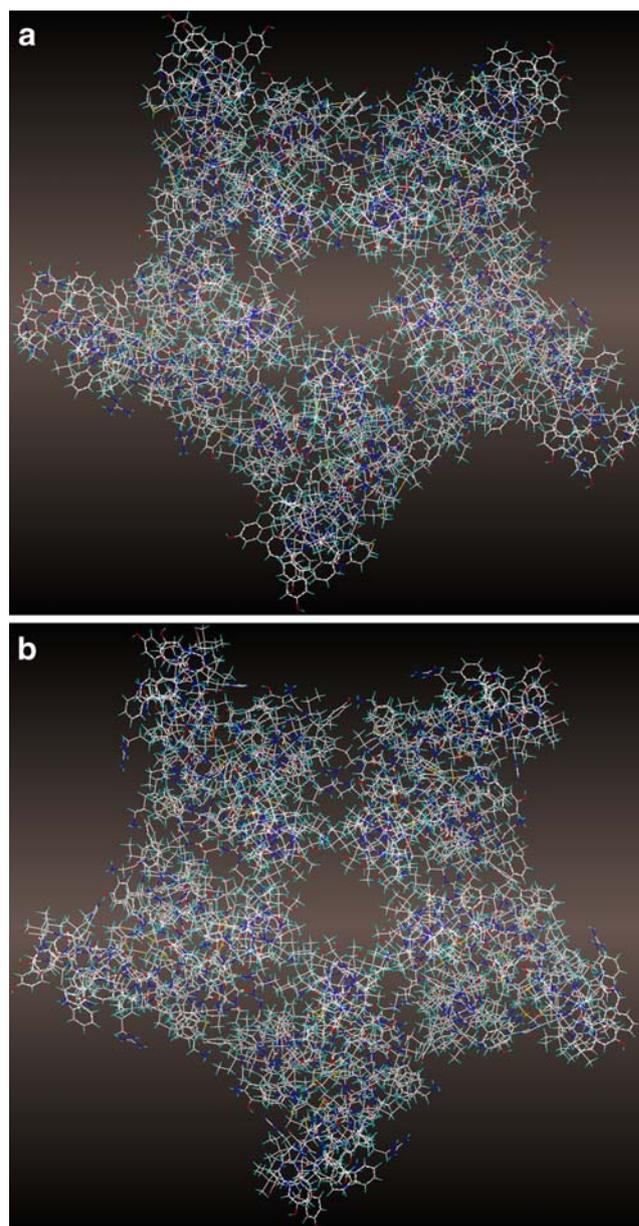
Fig. 4 **a** Three-dimensional (3D) molecular structure of the TMD of human α1β2γ2 GABAR. **b** 3D molecular structure of the TMD of house fly β3 GABAR

Fig. 1b. The percentage identity of the sequence of GABAR subunits with the nAChR subunits is 20–26%. Theoretically, many factors, such as template selection, the length of the sequences and alignment accuracy, may have a large influence on the accuracy of the model [23]. GABAR subunits share a low but definite amino acid sequence homology with the subunits of nAChRs [24]. However, there is evidence to indicate that it is reasonable to use nAChR subunits as the templates. First, it is known that nAChRs, which have many characteristics in common with GABARs, are also members of the superfamily of Cys-loop ligand-gated ion channels. They both contain a great many hydrophobic residues, especially in the four TM domains. In addition, 1OED has been repeatedly used as a template to build the GABA$_A$ ion channel protein. For example, the model built by Campagne-Slater and Weaver [25] has been used to study anaesthetic binding. Compared to the latter models, our models have a semblable alignment in TM1 and TM2, with the differences focused mainly in the alignments of TM3 and TM4.

The results of dynamics simulations on both the initial human α1β2γ2 and house fly β3 GABARs are shown in Figs. 2 and 3. Figure 2 indicates that the potential energy of the human homology model decreased in the first 250 ps

time period, and then reached a plateau in the subsequent simulation time. According to Fig. 3, the potential energy of the housefly model declined in the first 300 ps time period, and then remained stable. Both figures suggest that the models are reliable and can be used for subsequent study. The final models for human α1β2γ2 and house fly β3 GABARs are given in Fig. 4a and b, respectively.

Evaluating homology model

Conformational analyses

Two repeating torsion angles along the backbone chain, which are called Φ and Ψ, are used to describe the conformations of the models. By comparing the Φ and Ψ dihedral angles of the homology models to the statistical Ramachandran map obtained from the ProTable program, evaluation of the backbone conformation of the constructed model and detection of dissatisfactory residues is straightforward [18].

Conformationally unreasonable residues fall in the disallowed regions of the statistical Ramachandran map. Glycine residues often locate at the disallowed regions. As the structure of the glycine residue contains two hydrogen

Fig. 5 Φ–Ψ graph of the backbone of human α1β2γ2 GABAR. The conformationally disfavorable residues are labeled: *blue* proline, *magenta* glycine, *black* all other residues, *red* core regions, *yellow* allowed regions, *green* generous regions, *all other areas* disallowed regions
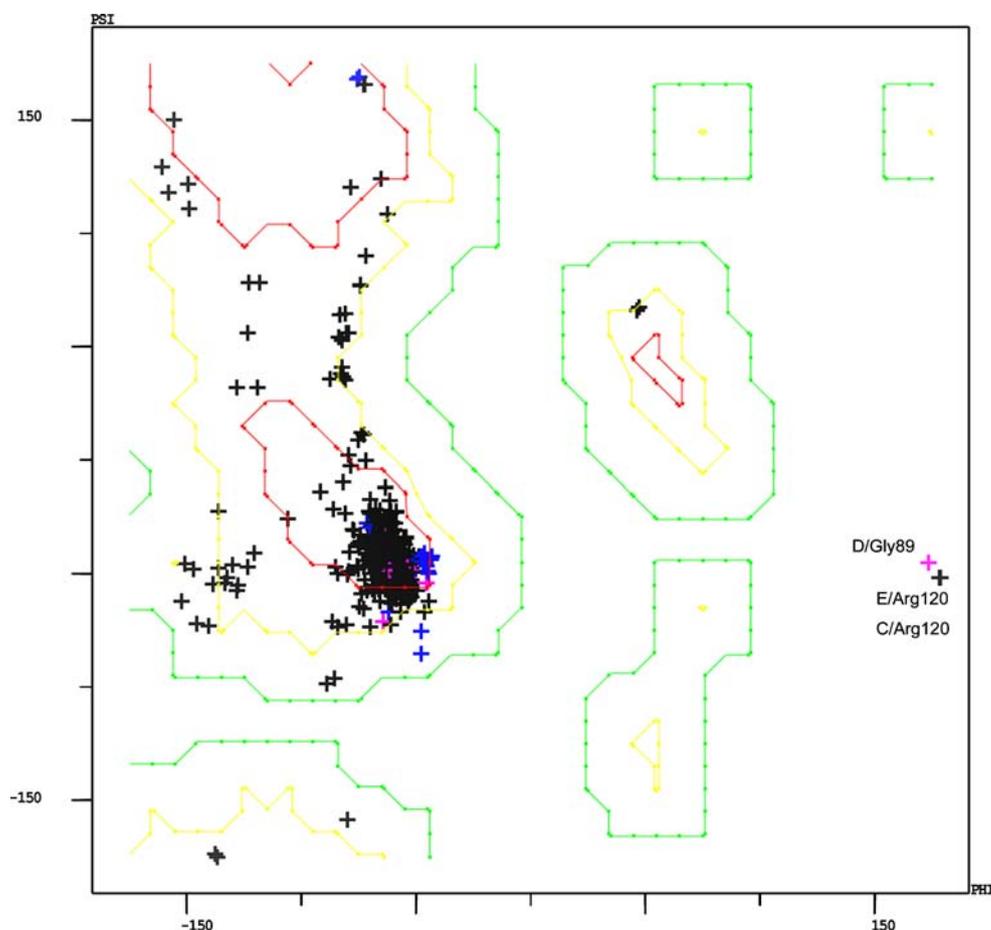
atoms in α-positions, one hydrogen atom in the side chain possesses an extremely small van der Waals radius and was more unrestricted than other residues.

Figure 5 indicates that approximately 97.77% of the residues in the α1β2γ2 GABAR model are either in the most accepted or in the additionally accepted regions of the Ramachandran plot. Figure 6 shows that 98.45% of residues in the β3 GABAR model are located in the satisfactory regions. In Figs. 5 and 6, most residues converge around a Φ value of −60° and a Ψ value of −45°. This phenomenon agrees well with the fact that two models are made up mainly of α helices. Accordingly, the two constructed models are conformationally reasonable and can be used for further studies.

Docking analyses

Considering that the receptor is a transmembrane channel, the protomol is defined by setting the threshold and the bloat value to 0.5 and 0 Å, respectively. The representative GABA receptor NCA fipronil, which has been prevalently applied as a highly efficient insecticide in agriculture, was chosen as the docked ligand to validate the quality of the

homology models of α1β2γ2 and β3 GABARs [26]. It has been reported that binding of EBOB was obviously reduced with mutations at two specific positions—Ala2 and Thr6 in the channel-lining region of TM2 of the human β3 homo-pentamer [27]. Two high-affinity sites for insecticidal GABAR antagonists are conserved in selectivity and potency for human recombinant homo-oligomeric β3 receptors and native receptors in house fly head membranes [17]. For human α1, β2, β3 and γ2 subunits, the Ala2 and Thr6 residues of TM2s are highly conserved, except in the 2′ residues of the TM2 of human α1 and γ2 subunits. Hence, these two critical residues are considered to be related to the NCA binding site. Based on the above observations, Residue Mode was used to define the putative binding site in the chloride ion channel. Hence, the 2′ and 6′ residues of the subunits were chosen as the active sites.

Using Surflex-docking, fipronil was docked to the human α1β2γ2 and house fly β3 GABARs, and the calculated binding free energies of the protein–ligand complexes were −3.60 kcal mol^{-1} and −4.24 kcal mol^{-1}, respectively. The calculated energy values demonstrate that the target-site specificity of house fly versus human is greater for fipronil, which is agreement with the IC$_{50}$ values

Fig. 6 Φ–Ψ graph of the backbone of house fly β3 GABAR. The conformationally disfavorable residues are labeled: *blue* proline, *magenta* glycine, *black* all other residues, *red* core regions, *yellow* allowed regions, *green* generous regions, *all other areas* disallowed regions
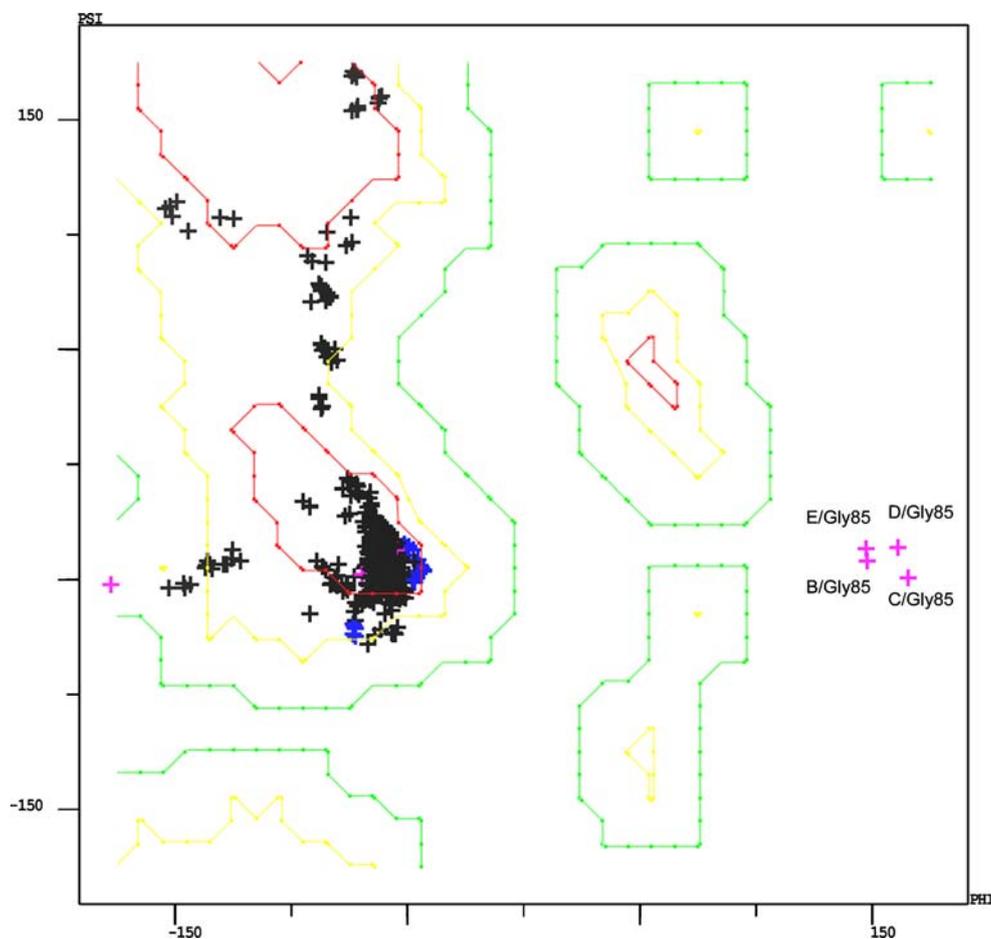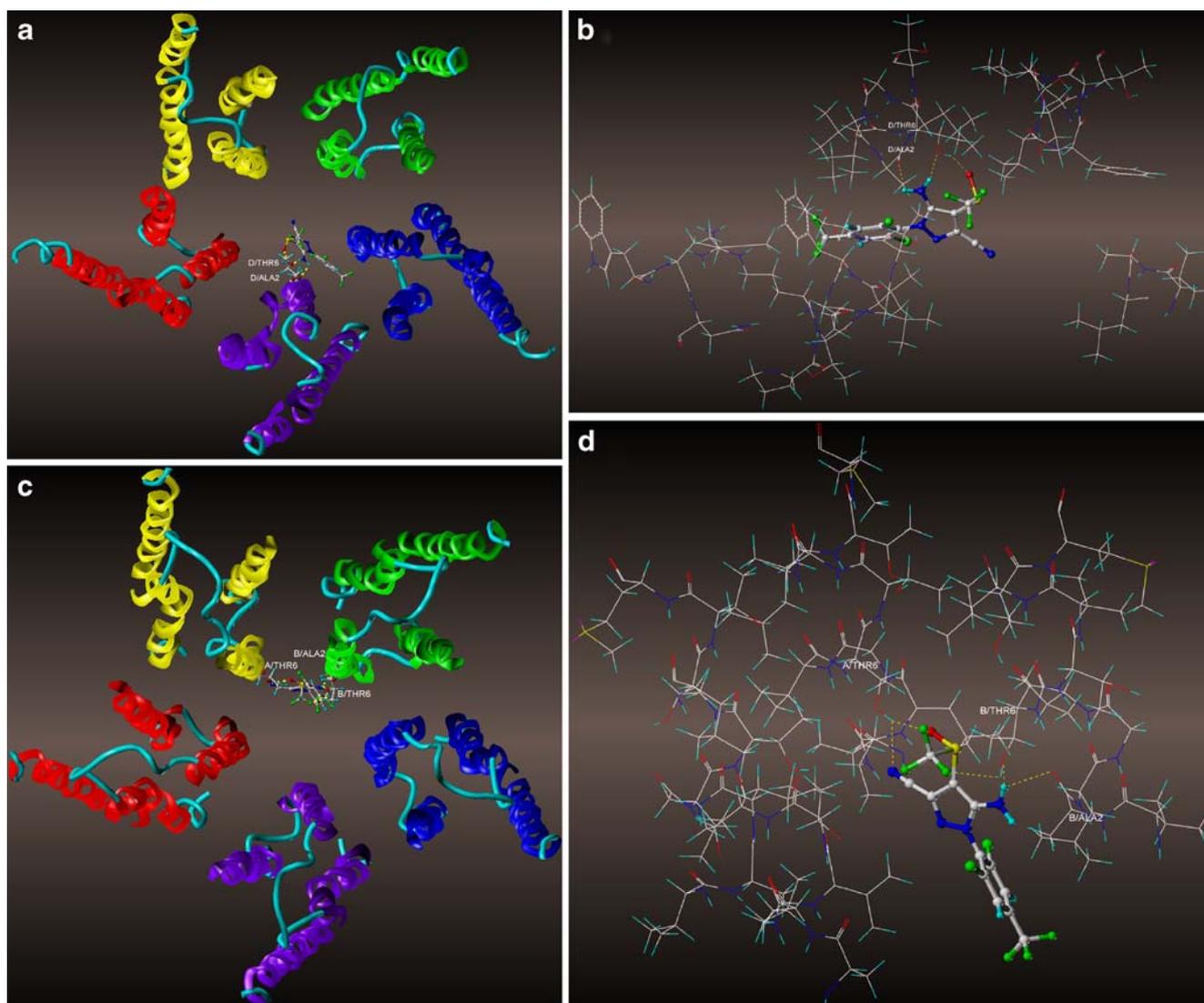
Fig. 7a–d Ligand binding analyses based on the refined homology model. **a** Ribbon representation of α1β2γ2 GABAR with fipronil. **b** Detailed view of the active site and hydrogen bonds of α1β2γ2 GABAR with fipronil. **c** Ribbon representation of housefly β3 GABAR with fipronil. **d** Detailed view of the active site and hydrogen bonds of housefly β3 GABAR with fipronil. Each subunit, which consists of a four-α-helix bundle, is colored: *yellow* chain A, *green* chain B, *blue* chain C, *purple* chain D, *red* chain E; detailed hydrogen bonds are depicted by *yellow dotted lines*

Table 1 H-bonds formed between γ-aminobutyric acid receptors (GABARs) and fipronil

Receptor	Atom 1	Atom 2 (fipronil)	Distance (Å)	Angle (°)
Human α1β2γ2 GABAR	D/Ala2: backbone O	Amino H	2.183	100.17
	D/Thr6: hydroxyl H	Trifluoromethylsulfinyl O	1.738	148.82
	D/Thr6: hydroxyl O	Amino H	1.858	159.85
House fly β3 GABAR	B/Ala2: backbone O	Amino H	2.444	103.49
	A/Thr6: hydroxyl H	Cyano N	2.674	111.08
	A/Thr6: hydroxyl H	Trifluoromethylsulfinyl O	1.931	159.45
	B/Thr6: hydroxyl H	Trifluoromethylsulfinyl F	2.473	88.47
	B/Thr6: hydroxyl O	Amino H	1.999	159.89

of fipronil in inhibiting [^{3}H]EBOB binding to house fly GABARs (IC$_{50}$=2.3 nM) and human GABARs (IC$_{50}$= 2,500 nM) [26].

Binding model for fipronil

The mechanism of the selective binding of fipronil to the house fly β3 and human α1β2γ2 GABARs was further explored with Surflex-docking, and the result is shown in Fig. 7. As illustrated in Fig. 7a and c, Surflex-docking analyses clearly show that fipronil fitted into the putative binding pockets located in the cytoplasmic half of TM2. Fipronil was located at the β(+)/α(−) subunit interface of the human homology model, and between the two β subunits of the house fly model.

Figures 7b and d show the detailed binding mode between fipronil and the active sites of human α1β2γ2 and house fly β3 GABARs. H-bonds were also introduced to help understand the interactions of the complexes, which allowed us to determine the amino acid residues involved in the recognition of GABAR NCA ligands. The distances and angles of H-bonds are given in Table 1.

According to the docking results, it can be speculated that fipronil has higher potency toward house fly GABARs than toward human GABARs. The main reason seems to be that the specific interaction between fipronil and the β subunit is reduced when α1 and γ2 subunits participate in the binding. In fact, the results obtained in this study are consistent with previously reported findings [28–30]. One important finding is that a specific EBOB binding is required between the β subunit alone or with other subunits [28, 29]. Therefore, GABARs containing the β2 or β3 subtype are a prerequisite for the potency of fipronil. Another finding is that the target site selectivity for fipronil is present in the house fly β3 GABA receptor, but not the human α1β2γ2 receptor, the reason being that there are subunits other than the β subunit in the human receptor, which could negatively influence the binding mode [30].

Additionally, analyses of H-bond interactions confirmed that Ala2 and Thr6 in TM2 play relatively important roles in binding potency (Table 1). The side chain of Thr6 and the backbone oxygen atom of Ala2 are inclined to form H-bonds with the polar moieties of fipronil such as the amino group and the oxygen atom of the trifluoromethylsulfinyl group. Specially, the side chains of Thr6 form the majority of the H-bonds with fipronil. Interestingly, the nitrogen atom of the cyano group of fipronil accepts an H-bond from the side chain of Thr6 in the house fly GABAR, which does not appear in the interaction between the human GABAR and fipronil. From these two results, it appears that Thr6 has a critical influence on the H-bond interactions of the protein–ligand complex. Ozoe et al. [27] also reported that

the 6′ amino acid of TM2 has more intense effects on EBOB binding than the 2′ amino acid.

Compared with our models, Casida et al. [31] proposed a different binding mode in which the 2,6-dichloro-4-trifluoromethylphenyl substituent of fipronil interacts with the methyl group of Thr6, whereas we suppose that this lipophilic part would more likely interact with Ala2. The reason for our supposition is that Ala2, which resides in proximity to the channel lumen, is more hydrophobic than Thr6. Meanwhile, ffrench-Constant et al. [32] confirmed that cross-resistance of insects to all classes of commercial NCA insecticides occurs when Ala2 is replaced by Ser via site-directed mutagenesis. This also indicates that a polar amino acid at the 2′ position of TM2 may influence the hydrophobic interaction with the 2,6-dichloro-4-trifluoromethylphenyl substituent of fipronil.

Conclusions

Three-dimensional models of human α1β2γ2 and housefly β3 GABARs were generated using the cryo-electron microscopy structure of the nAChR of *Torpedo marmorata* as a template. The results showed that the models of human α1β2γ2 and house fly β3 GABARs have a certain validity and practicality. Molecular docking and H-bond analyses allowed us to speculate on the mechanism of fipronil blocking the GABA-gated chloride ion channel. Docking studies demonstrate that the house fly GABAR has greater specificity for fipronil than the human target-site. H-bond interactions reveal Thr6 and Ala2 in TM2 as key residues in the ligand–receptor complex. Fipronil is sensitive to the β2 or β3 subunit, while the binding mode may be modulated by other subunits.

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