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Comparative Models of $GABA_A$ Receptor Extracellular and Transmembrane Domains: Important insights in pharmacology and function

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Running title: **Comparative Models of $GABA_A$ Receptor Transmembrane Domains**

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Abstract

Comparative models of the extracellular and transmembrane domains of $GABA_A$ receptors in the agonist free state were generated based on the recently published structures of the nicotinic acetylcholine receptor. The models were validated by computational methods and their reliability was estimated by analyzing conserved and variable elements of the cys-loop receptor topology. In addition, the methodological limits in the interpretation of such anion channel receptor models are discussed. Alignment ambiguities in the helical domain were resolved for helix 3 by placing two gaps into the linker connecting helices 2 and 3. The resulting models were shown to be consistent with a wide range of pharmacological and mutagenesis data from $GABA_A$ and glycine receptors. The loose packing of the models results in a large amount of solvent accessible space and offers a natural explanation for the rich pharmacology and the great flexibility of these receptors that are known to exist in numerous drug induced conformational states. Putative drug binding pockets found within and in between subunits are described and amino acid residues important for the action and subtype selectivity of volatile and intravenous anaesthetics, barbiturates and furosemide are shown to be part of these pockets. The entire helical domain, however, appears to be crucial not only for binding of drugs but also for transduction of binding to gating or of allosteric modulation. These models can now be used to design new experiments for clarification of pharmacological and structural questions as well as for investigating and visualizing drug induced conformational changes.

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Introduction

$GABA_A$ receptors mediate a large part of the fast inhibitory transmission in the CNS and are the targets for many clinically important drugs, such as sedatives, hypnotics, anxiolytics, anticonvulsives, muscle relaxants and anaesthetics (Sieghart 1995). They are composed of five subunits that can belong to different homologous subunit classes and form a chloride channel that can be opened by GABA. Individual neurons can express many different subunits, resulting in the formation of a large variety of functionally different receptor subtypes (Sieghart and Sperk 2002). Depending on the subunit composition, these receptors exhibit a distinct pharmacology (Sieghart 1995).

The subunit organization with the extracellular ligand binding domain containing the “signature” disulfide, four transmembrane (TM) segments and a large variable cytoplasmic domain (termed „cytoplasmic loop“) of unknown structure, as well as the receptor organization as a pentamer, are hallmarks of the superfamily of cys-loop receptors (pentameric ligand gated ion channels) comprising the cation conducting nicotinic acetylcholine (nACh) and serotonin type 3 ($5HT_3$-) receptors and the anion conducting $GABA_A$ and glycine receptors.

In 2001 the X-ray crystallographic structure of acetylcholine binding protein (AChBP) has revealed the fold in which the beta strand rich „extracellular domain“ of the superfamily is organized (Brejc, van Dijk et al. 2001). Subsequently, comparative models of the extracellular domain of different receptors based on this structure have been generated, for review see (Ernst, Brauchart et al. 2003).

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Following the release of the AChBP structure, cryo-EM images of ACh free and ACh bound preparations of electric fish organ nicotinic receptors have been analyzed by fitting the core of the AChBP X-ray structure into the two sets of EM density maps (Unwin, Miyazawa et al. 2002). The ACh bound state turned out to be pseudosymmetrical, with all subunits in the conformation that corresponds with the HEPES bound conformation of AChBP subunits. The ACh free (tense) state, on the other hand, was found to be conformationally asymmetrical. The extracellular domain of the two alpha subunits (that form the plus-sides of the ACh pockets) is in a conformation distinct from the beta, gamma and delta subunits' extracellular domain (Unwin, Miyazawa et al. 2002).

The structure of the ion pore domain fragment of the torpedo nACh receptor was published in 2003 (Miyazawa, Fujiyoshi et al. 2003), as a 4Å model in the resting state (Protein Data Bank (PDB) identifier 1OED). It also has been derived from cryo-EM images, and confirmed the notion that the TM domain of cys-loop receptors is organized as a 4-helix bundle (Bertaccini and Trudell 2001). Shortly thereafter, a first model combining the extracellular and transmembrane domains of the nicotinic receptor has been discussed (Unwin 2003), which was eventually published in a refined version (Unwin 2005) and released with the PDB identifier 2BG9.

This work is the first attempt to integrate all published structural information from the nACh receptor in the closed state into comparative models of $GABA_A$ receptors, with special emphasis on the helical domain. These models were then validated by computational tools as well as by comparison with

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experimental results. Finally, they were used to explain and consolidate experimental data.

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Methods

Nomenclature conventions

Amino acid sequence numbering corresponds to the mature protein. In the helix 2 segment the primed index numbering scheme is used additionally. Whenever individual amino acid residues are named in the text, their residue number in mature rat protein ($GABA_A$ receptors) or torpedo fish (nACh receptor) is indicated, the corresponding position in homologues is often provided in parenthesis as reading aid. Domains of cys-loop receptors have traditionally been termed “extracellular ligand binding domain”, “transmembrane domain” and “cytoplasmic loop”. Since it is known that the so-called “extracellular ligand binding domain” and the so-called ”transmembrane domain” form independent folding units that are of predominantly beta-stranded and helical character, respectively, we will refer to them as “beta-folded domain” and “helical domain”. This is particularly appropriate as the helical folding unit is only partially inserted into the membrane and extends significantly (~10Å) beyond the lipid bilayer into the extracellular compartment (Miyazawa, Fujiyoshi et al. 2003). Little structural data is available on the cytoplasmic “loop”, but there is evidence for significant helix contents (Peters, Kelley et al. 2004; Unwin 2005). We refer to this putative independent folding unit as “cytoplasmic domain”.

Modeling

Standard modeling techniques were used to perform the individual modeling steps. The structure files 1I9B (Brejc, van Dijk et al. 2001), 1OED (Miyazawa, Fujiyoshi et al. 2003), and 2BG9 (Unwin 2005) were obtained from the

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PDB. Subunit correspondence between the nACh receptor structures and $GABA_A$ receptors was assigned on the basis of functional homology. Thus, the nACh receptor alpha subunits, that form the principal side of the ACh binding pocket, and the $GABA_A$ receptor beta subunits, that forms the principal side of the GABA pocket, correspond to each other, as indicated in Figure 1b.

All other subunit correspondences follow then inevitably from the respective receptor subunit arrangements. The nACh and $GABA_A$ receptor subunit sequences were then aligned with the appropriate 1OED, 2BG9 and 1I9B subunits with FUGUE (Shi, Blundell et al. 2001). The resulting sequence-to-structure alignment problems have been discussed extensively for the beta-folded domain (Ernst, Brauchart et al. 2003). Here we focus on the helical domain, where the sequence identity shared between $GABA_A$ receptor subunits and the corresponding 1OED/ 2BG9 segments ranges from 13% to 21%. Additional alignments were scored and investigated with ClustalX (Thompson, Higgins et al. 1994). A representative multi sequence alignment and some possible alignment variants are provided in Figures 1a and 1c and will be discussed below in the section on variable regions.

Coordinate manipulations needed for rotation of the individual sheets of the nACh receptor alpha subunit (Unwin, Miyazawa et al. 2002) and for docking 1OED with nACh receptor extracellular domain models were performed with the Molecular Modeling Tool Kit MMTK (Hinsen 2000). Comparative modeling, model scoring and selection were performed as described previously (Ernst, Brauchart et al. 2003), using Modeller version 6 (Sali and Blundell 1993; Marti-Renom, Stuart et al. 2000). The $GABA_A$ receptor subtype $\alpha_1\beta_2\gamma_2$ was investigated most extensively,

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other subtypes, such as α_6 containing receptors, were based on the $\alpha_1\beta_2\gamma_2$ models. The resulting selection of $GABA_A$ receptor models that passed validation and for which uncertainty estimates have been made was subjected to putative active site analysis with PASS (Brady and Stouten 2000), and the cavities that were identified by PASS were examined. The results were analyzed in the light of published experimental studies.

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Results

Two generations of comparative models of the closed (tense) state of $GABA_A$ receptors are presented in this work. The older set, generated before 2BG9 (Unwin 2005) was released, is based on three published sources (Brejc, van Dijk et al. 2001), (Unwin, Miyazawa et al. 2002), (Miyazawa, Fujiyoshi et al. 2003), and was built in several steps: First, the AChBP structure (Brejc, van Dijk et al. 2001) was used to model the beta-folded domain of the nACh receptor's beta, gamma and delta subunits. Then, the tense state described in (Unwin, Miyazawa et al. 2002) was reconstructed for the nACh receptor alpha subunits. The individual subunits of this tense state model of the beta-folded domain of the nACh receptor were then joined with the 1OED model (Miyazawa, Fujiyoshi et al. 2003) of the helical domain to establish proper connectivity and "docked" at a distance just allowing van der Waals contacts. The resulting initial model of the combined domains of the nACh receptor was subjected to a standard simulated annealing protocol provided by the modeling program, without further refinement. This procedure was carried out repeatedly with different initial models to ensure that the domain junction converges to a consensus topology, which it did. Using this auxiliary nACh receptor model as template for the tense state, models of $GABA_A$ receptors (lacking the "cytoplasmic domain") were then constructed.

After the release of 2BG9 (Unwin 2005), this structure was used as single template to directly model $GABA_A$ receptors in the tense state, using the same alignments as with the "homebuilt" nACh receptor template, and the resulting models essentially replaced the first generation models. The two generations of models used in this study were analyzed in terms of similarities and differences.

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Within the limits of model uncertainty, the agreement was found to be very good. The older models, that were constructed by docking the extracellular domain (based on AChBP) and the helical domain (based on the nACh receptor helical domain fragment), displayed a small rigid-body shift in the relative orientation of the two domains compared to what is seen in 2BG9. This is often encountered when multiple templates are used (Marti-Renom, Stuart et al. 2000). However, most results obtained with the first generation models concerning interesting properties of the helical domain and the domain junction were confirmed by the second set of models. Thus, the results presented below reflect findings that agree between both generations of models, except when stated otherwise.

Prior to the interpretation of computationally derived models, the expected degree of reliability needs to be assessed. In the following, putative structurally conserved elements of cys-loop receptors are identified and analysed on the basis of our models of nACh and $GABA_A$ receptors, then the putative variable elements that lead to functional diversity are discussed for $GABA_A$ receptors. Figure 2 provides an overview of the topological features of the cys-loop receptor family.

Conserved topological properties of cys-loop receptors

All subunits that form cys-loop receptors share a common architecture, whose conserved elements are as follows: The “extracellular”, beta-rich domain consists of a variable N-terminus (not shown), and two beta sheets that form a “sandwich” (Brejc, van Dijk et al. 2001). They have been termed “inner” and “outer” sheet (Unwin, Miyazawa et al. 2002), indicating their luminal (inner) and abluminal (outer) localization, and are connected by the signature disulfide bridge.

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The details of the architecture of the beta-folded domain are shown in Figure 2a: The inner sheet, that is indicated by light blue lines, connects the minus side diagonally with the plus side of the subunit on the luminal face and contains several key elements of agonist and drug action: So-called binding site “loops” D, E (minus side) and loop 2 (plus side) belong topologically to the inner sheet. Several of the linking segments, that connect the inner sheet with the outer sheet, also have been shown to be key players in the mediation of ligand effects: “Loops” A, B, (plus side), loop F (minus side) and loop 7 (the cys-loop), as well as the signature disulfide bridge (yellow double arrow in the figure), interconnect the two sheets.

The outer sheet, indicated by red connections, diagonally connects the plus side with the minus side of the subunit on the abluminal face, and provides loop C as functional segment. Strand 10 of the outer sheet terminates the beta-folded domain and directly connects the plus side of the beta-folded domain (loop C) with the minus side (pre-M1) of the helical pore domain. The topology diagram that shows how the peptide chain is organized into the two sheets is provided in Figure 2b.

This particular topology, that couples the plus-side of the beta folded, agonist binding domain with the minus-side of the helical domain and vice versa would imply transducing elements that make use of these cross-connections. Indeed, experimental result on the transducing elements of $GABA_A$ receptors (Kash, Jenkins et al. 2003; Kash, Dizon et al. 2004) strongly suggest such a “diagonal transduction”: GABA binding occurs at the beta subunit’s plus side. For consecutive gating to occur properly, the pre-M1 region of the beta subunit (Kash,

Dizon et al. 2004), which is localized at the minus side, was shown to be crucial. The alpha subunit, on the other hand, where GABA interacts with the minus side (loops D and E), appears to couple via the inner sheet and loop 2, which is localized at the plus side, as well as loop 7 (the “cys-loop”) with the nearby M2/3 linker (Kash, Jenkins et al. 2003).

The helical pore-forming domain’s architecture and topology is most likely conserved. In the published structures 1OED and 2BG9 four helical segments that pass the membrane form an “up-down” bundle, which is interrupted by the “cytoplasmic domain” between helix 3 and helix 4. The positions of the 4 helices relative to the position within the pentameric complex are shown in Figure 2c: Helix 1 is located in continuation of the minus-side, helix 2 is pore-forming, helix 3 lies at the plus- side and helix 4 at the abluminal side of the subunit.

One of the striking features of the pore domain structure of the nACh receptors structural models 1OED and 2BG9 is the loose packing, which is likely to also be a conserved feature of this superfamily. This is not a general property of the 4-helix bundle fold (Pearl, Todd et al. 2005), which contains a number of superfamilies (for example certain cytokines), many of which are packed tightly. The loose packing is shown in Figure 3, where all solvent accessible space, that is enclosed in a model of a $GABA_A$ receptor, is filled with “pseudo-solvent”. If this buried pseudo solvent is quantified, the helical domain is found to contain much more such putative pocket volume than the beta-folded domain. The total enclosed pseudo solvent can be clustered into groups that represent different pockets, as indicated by different clusters of color in the figure. The possible roles of the cavities provided by the loosely packed helical domain will be discussed below.

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The topology of the domain junction is likely to be highly conserved. Evidence for a well conserved topology at the domain junction was also provided by our modeling studies: In our first generation models of both nACh receptors and $GABA_A$ receptors that were based on two different and independently determined structures, the AChBP and the helical fragment of the nACh receptor, the extracellularly located loop 7 (cys-loop) and loop 2 interdigitate with the "M2/3" linker of the helical domain (Fig 2a). This result was obtained by simply docking the two domains without using any extra restraints that would have forced a particular junction topology. The interlock of loops 2 and 7 with the M2/3 linker is in agreement with what has been suggested previously for the $GABA_A$ receptors, using combined experimental (Kash, Jenkins et al. 2003) and modeling (Kash, Trudell et al. 2004) approaches, and was also confirmed by the latest release of the nACh receptor structure 2BG9 (Unwin 2005). In fact, the agreement in the junction topology of our first generation models of the nACh receptor with the 2BG9 structural model was very good, and by far within the uncertainty that is intrinsic to the method.

Variable elements of the cys-loop receptor architecture

While architecture and topology of both the beta-rich and the helical domain are conserved, the large functional diversity among cys-loop receptors must have corresponding structural equivalents, located mainly in the variable regions. The structural differences between the AChBP subunits and the β, γ and δ subunits of the heteropentameric 2BG9 structure of the nACh receptor clearly demonstrate the variability of the beta-stranded domain: The short strands, the

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loops and the interface forming segments of the individual subunits show significant structural diversity. This correlates well with results from our recent sequence conservation analysis (Ernst, Brauchart et al. 2003). The conservation indices presented there can still be used as a first guide for defining conserved and variable regions for the purpose of estimating uncertainties in predictions. Additionally, the strands of the topologically conserved sheets are organized in a unique way for each subunit, which is common for beta-stranded fold families, but cannot be predicted in detail. The combined differences between AChBP and nACh receptor β, γ and δ C-alpha coordinates are reflected in a root mean square deviation (RMSD) of 2.5 to 3 Å. Maximum differences of up to 12 Å between individual C-alpha atom pairs from sequence aligned, highly variable regions are found. This is typically observed in beta-stranded fold families, and it is to be expected that other superfamily members will display at least the same degree of variation.

The domain junction is known to display high variability within the conserved topology among different superfamily members (Kash, Kim et al. 2004). Thus, in the conserved regions of the beta-stranded domain and its junction with the helical domain (Ernst, Brauchart et al. 2003, and see below), model structures will be accurate up to the level of sidechain orientation. In the structurally variable regions, they will be correct at the level of topological position of segments. Armed with this knowledge, predictions can be interpreted and used properly.

While in beta stranded folds structural variation manifests in different patterns of sheet organization, in helical folds structural variability results in less

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obvious and more local changes such as in different packing motifs. Thus, structural variability of this domain must be carefully accounted for in modeling anion channels: Figure 1a shows an alignment of the four helical segments of selected members of the superfamily. It is immediately obvious, that there are very few absolutely conserved positions, and these are predominantly in helix 1 and helix 2 as indicated by asterisks in Figure 1a. In helix 3 and helix 4, conservation among cation channels, as well as among anion channels is high, but low between cation and anion channels. The questions associated with identifying the homology model that „best“ predicts $GABA_A$ receptor structure will be adressed in more detail below.

Conservation in helices 1 and 2

Helix 1 is characterized by a conserved Pro residue, which presumably is structurally equivalent in all family members. A second position in helix 1 is highly conserved, namely the Leu marked in the alignment in Figure 1a by an asterisk. This position is hydrophobic in all superfamily members, most of them have a Leu in this position. The conserved Pro, together with proper alignment of hydrophobic and non-hydrophobic positions in all members of the superfamily, implies that helix 1 is closely conserved in the structural sense. This means, that the orientation of sidechains with respect to the surroundings of helix 1 will be similar to 1OED and 2BG9 in the other receptors.

In helix 2, the presence of two conserved residues (Leu and Pro at index positions 9’ and 23’, respectively, Figure 1a) strongly implies conserved structure. Indeed, in our models based on 1OED and 2BG9, the same sidechains that have been mapped as pore-lining by experimental approaches (Xu and Akabas 1996)

are found along the central pore, thus confirming close conservation.

Thus, close structural similarity can be assumed in helices 1 and 2 for all members of the superfamily.

Lack of conservation in helices 3 and 4

In aligning helix 3 between cation and anion channels, two problems arise: There are no absolutely conserved residues, and the contents of hydrophobic amino acids is high. This leads to significant ambiguities in the alignment. Therefore, multiple alignment variants, as well as multiple degrees of "homology", or "conservedness" have to be considered in computing and interpreting models. In homologues that share sequence identity below 30%, as is the case here, insertions/deletions (so-calles indels) are commonly found in linkers connecting secondary structure elements and must be considered in constructing alignments for modeling purposes.

In figure 1c, three variants of aligning helix 3 between cation channels and anion channels are shown. They are generated by placing no, one or two gaps into the 2/3 linker, and they all obtain roughly equal alignment scores due to the hydrophobic character of the aligned segments. Thus alignment scores do not help in selecting among these variants, but gap penalties become high if more than 2 gaps are introduced. A clear ranking also could not be established by computing and scoring the corresponding models.

Since it is not feasible to identify the most convincing helix 3 alignment by purely computational means, we have used a combined approach of relating local model properties to experimental data to discriminate between the possible variants. Extensive SCAM mapping data (Akabas 2004) on the accessibility of helix

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3 residues of $GABA_A$ receptor α1 subunits, in the resting state and under the influence of various ligands, is available (Williams and Akabas 2002; Akabas 2004; Jung, Akabas et al. 2005), see Figure 4. The following observations from the SCAM studies are informative for the alignment problem:

In the resting state, only α_1A290C and α_1Y293C, shaded in light blue in Figure 4 (gray in Figure 1), could be modified with Cys-modifying reagents. This yields the labelling pattern AxxY, where x denotes a presumably inaccessible position. This pattern was then compared with sidechain accessibility in the nACh receptor structures 1OED and 2BG9, as determined with the procheck program (Laskowski, MacArthur et al. 1993). Near the N-terminus of the nACh receptor's helix 3 there are also two significantly more solvent accessible residues, αF280 and αI282, yielding the pattern FxxI (shaded gray in Figure 1c). Thus, it is reasonable to align the AxxY motif in the $GABA_A$ receptor α_1 with the FxxI motif in the nACh receptor. This alignment corresponds to the variant with two gaps in the linker.

Further support for this 2-gap hypothesis comes from three additional observations: First, in this alignment variant an aspartate (α_1D286) that is conserved at the N-terminus of helix 3 in anion channels ends up aligned with a conserved basic position in the cation channels, as seen in Figure 1c. This makes more sense than what is seen in the other two alignment variants. Second, and even more convincing, α_1A290 and homologous residues near the N-terminus of helix 3 have been found to be part of a hypothetical binding pocket for anaesthetics (Jenkins, Greenblatt et al. 2001), and the two gap alignment places these residues

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indeed in a pocket-lining position, as will be discussed in detail below. Third, the position α_1E302C was found to be accessible for Cys- modifying reagents only in the presence of gating concentrations of GABA or propofol (Williams and Akabas 2002), see Figure 4, and thus is thought to be buried in the resting state. Only the 2-gap alignment models position this residue in a buried position in which the acidic side chain does not interfere with hydrophobic packing. The approximate position of this sidechain, as well as the two residues α_1A290 and α_1Y293, as seen in the 2-gap models is shown in Figure 1d. From all this, we conclude that the 2-gap alignment generates model structures that resemble the true structure better than other alignment variants. This conclusion is supported by even more experimental evidence, as will be discussed below.

Helix 4 is also characterized by very low sequence similarity between cation and anion channels, apart from the high degree of hydrophobic amino acids in the membrane spanning portion. An aspartate residue, which occurs in all superfamily members, suggests a conserved function for this position, which in turn suggests structural homology. Thus, the most plausible alignment variant for this segment is the one shown in Figure 1a, which also yields models with reasonable scores.

Nevertheless, in spite of the two gap model of helix 3, and the conserved aspartate hypothesis for helix 4 being highly plausible, high uncertainty is associated with coordinates of amino acid residues in these segments of the comparative models. This is due to the low homology between cation and anion channels in the helical domain, with sequence identity ranging only from 13 to

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21%. At such low sequence similarity, sidechain interactions and packing properties are generally not conserved. From structure comparison methods (Prlic, Domingues et al. 2000) it is known that structurally equivalent amino acids in pairs of related and superposed structures can have Cα and Cβ distances from each other of up to 5 Angstroms. This natural variation is shown schematically in Figure 1e for the Cα positions of helix 3 in the nACh receptor's α subunit. $GABA_A$ receptor residues corresponding to the nACh receptor residues αK276 and αY277 are shown according to the no, one or two gap alignment. This shows the changes in amino acid side chain positions resulting from different alignments. The additional variation that comes from low homology is sketched by the gray helices in Fig. 1e (different symbols are used to represent alternative C-α positions), indicating how far away from the αK276 position these residues could be located, and still be well within the definition of „structurally equivalent" (Prlic, Domingues et al. 2000). This results from the fact that the natural variation shown in Figure 1e can neither be predicted nor modeled. In general, comparative models have a tendency to resemble their "parent structures" too closely and energy minimization or simulation runs will not result in more "true" structures (Schonbrun, Wedemeyer et al. 2002). More sophisticated approaches are cost-prohibitive for models of this size and are still in the stage of development. The „true" structure, thus, can only be determined by experimental means. Despite these caveats, models still can be used to derive valuable predictions. At low sequence similarity, multiple models based on distinct input such as different alignments, loop conformations and templates (if available) must generally be used. In the conserved regions, these

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models will yield consensus predictions. In the variable regions, the different predictions that „survive“ standard validation must be analyzed on a statistical basis. Subsequently, suitable experimental data could be used as spatial restraints in building improved model structures.

Pockets in and around the helical domain in the closed, tense state

Putative binding sites can be identified in structural models using PASS (Brady and Stouten 2000) or similar tools. Even in models of moderate accuracy, the overall pocket organization can be determined with such an approach. The pockets contained in the extracellular domain at the inter-subunit interfaces have been discussed previously (Ernst, Brauchart et al. 2003). In this work, we focus on the pockets that are, fully or in part, formed by segments from the helical domain. As can be seen in Figure 3, two “types” of cavities are found by pocket finding algorithms: A rather large cavity is present inside of each of the 4 helix bundles, shown in green in Figure 3. Additionally, and as has been noted before (Miyazawa, Fujiyoshi et al. 2003), another large cavity exists between the subunits at and below the domain junction, shown in purple in Figure 3.

While the shape and volume of the pockets varies with different model variants, the cavities as such are present in the overwhelming majority of models and the identity of the segments that form the cavities is not affected by ambiguities in helices 3 and 4. However, predictions of the cavity forming segments now can be refined by subsequent experimental mapping, which in turn will lead to models with a more defined pocket geometry.

Interestingly, in some models the inter-subunit (purple) pockets of the

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helical domain appear to communicate with their extracellular counterparts. Thus, it might be that the interface between subunits contains a continuous groove that may well have more than one function.

The intra-subunit („green“) pockets, that are confined by the four helices of each subunit, contain a number of amino acid residues well investigated by various experimental means: In alpha subunits, residues in helices 1, 2 and 3 have been shown to be key components in binding or action of different modulatory drugs: α_6I228 of helix 1 (corresponding to α_1T229) determines alpha selectivity of furosemide (Thompson, Arden et al. 1999), and is part of the wall of the “green” pocket in alpha subunits. See Figure 3b for a view of this pocket from two directions. α_1S269 of helix 2 has been proposed to be part of a pocket for volatile anaesthetics (Jenkins, Greenblatt et al. 2001), this residue also lines this pocket. α_1A290 (rat sequence numbering, human: α_1A291) of helix 3 also has been proposed to be part of the “anaesthetics pocket” (Jenkins, Greenblatt et al. 2001), and is also found in the pocket wall in models derived from the alignment variant bearing two gaps. (see Figure 3b)

In beta subunits, residues in helix 2 and helix 3, in positions homologous to those discussed above, have also been shown to be key components in binding or action of modulatory drugs: Residue β_1S265 corresponds to residue α_1S269 in alpha subunits. β_2 and β_3 have an Asn at the homologous position. This S/N polymorphism is critical for the additional beta subunit selectivity of furosemide action (Thompson, Arden et al. 1999). The same S/N polymorphism of the beta subunits also accounts for the beta subtype selectivity of loreclezole, etomidate,

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and other related substances (Belelli, Muntoni et al. 2003). Thus, the S/N position at β_1S265 (corresponding to α_1S269) is part of the “green” pocket and may be part of a binding site for these substances.

Finally, the position for the sidechains of the pre-M1 point mutants β_2G219X could not be localized with any reliability in the old generation of models. But in the 2BG9 derived models these sidechains appear to be either part of the “green” pocket or to control the access pathway to the pocket. This could nicely explain that substitution of β_2G219 with larger residues leads to a reduced barbiturate sensitivity of the receptor, and that the reduction increases with increasing size of the substituent (Carlson, Engblom et al. 2000).

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Discussion

Models of the beta-folded and helical domains of $GABA_A$ receptors agree with experimental data

In the present study we have modeled the beta-folded “extracellular”and the helical “transmembrane” domain of electric organ nACh receptors in the closed, "tense" state on the basis of available structural data prior to the release of the 2BG9 coordinates. Using this model as a template, we then proceeded to model the combined beta-folded and helical domains of $GABA_A$ recepor subtypes. Additional models based on 2BG9 were added after these coordinates became available. Subsequently all $GABA_A$ receptor models were evaluated for model uncertainty and validated by comparison with known data. In agreement with experimental evidence, we find that the junction between the beta-folded and helical domains is formed by loops 2 and 7 of the extracellular domain spontaneously interdigitating with the 2/3 linker of the helical domain (Fig. 2a). This junction topology is likely to be conserved, as indicated by the observation that only loops 2, 7 (cys-loop) and 9 (F-loop) (see Fig. 2a) had to be modified in the AChBP portion of chimeras consisting of an N-terminal AChBP and the helical domain of the $5HT_3$-receptor in order to engineer receptor-like properties (Bouzat, Gumilar et al. 2004). Nevertheless, different superfamily members and even different subunits of a single receptor class, for example the α and β subunits of $GABA_A$ receptors (Kash, Jenkins et al. 2003; Kash, Dizon et al. 2004) employ slightly different mechanisms in transducing conformational changes.

Although the overall architecture and topology of the helical domain is

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conserved within the superfamily, alignment of the four helical segments of cys-loop receptors indicate that only helix 1 and helix 2 are closely conserved in a structural sense allowing a more or less correct prediction of amino acid side chain positions. In helix 3 and helix 4 there are quite some ambiguities in the alignment. We find the best overall performance for our $GABA_A$ receptor models when 2 gaps in the alignment are introduced in the M2/3 linker. Under these conditions, two amino acid residues, namely $GABA_A$ receptor α_1A290 and α_1Y293 within the resulting helix 3 that are solvent accessible in the resting state of $GABA_A$ receptors as indicated by experimental data, are located in positions homologous to those of solvent accessible residues of the nACh receptor.

The existence of inter- and intra-helical pockets suggests multiple drug binding sites

In contrast to a previous model of $GABA_A$ receptors which combines the beta-folded domain with a transmembrane domain based on the structures of nonrelated proteins, and was described at “tightly packed” (Trudell and Bertaccini 2004), the helical domain of models based on the nACh receptor structures is loosely packed (Miyazawa, Fujiyoshi et al. 2003). Consequently they feature a fairly large content of “putative pocket volume” (Fig. 3). The pockets can be grouped into two main clusters. One of these are located at the subunit interfaces. In some models they form a continuum from the extracellular domain to deep into the helical domain. They have been proposed to be needed by the movement of helix 2 in gating of the ion channels (Unwin 2003). Since the extracellular pockets between subunits contain the GABA and the benzodiazepine binding sites of

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$GABA_A$ receptors, it is quite conceivable that their extension into the junctional and helical domains can also be used by drugs to modulate the function of receptors.

Additional cavities are present inside of each of the 4 helix bundles of the subunits (green pockets in Figure 3). Assuming the two-gap alignment variant for helix 3, amino acid residues in helices 1 (α_1T229/ α_6I228), 2 (α_1S269/ β_1S265/ $\beta_{2/3}$N265) and 3 (α_1A290/ β_2M286) of alpha or beta subunits previously demonstrated to be important for the actions of volatile and intravenous anesthetics, furosemide, etomidate, and barbiturates, are found in our models to line the wall of the respective intra-helical pockets.

Independent evidence for this conclusion comes from experiments on glycine receptors. Cysteines replacing the glycine receptor residues homologous to α_1S269 (β_1S265) and α_1A290 (β_2M286) can form a disulfide bond (Lobo, Trudell et al. 2004). Since residue α_1S269 is oriented towards the center of the 4-helix bundle, as indicated by experimental evidence (Mascia, Trudell et al. 2000), and by the conserved side chain positions of helix 2, (see 1OED and 2BG9), disulfide formation is possible only if the glycine receptor residue homologous to α_1A290 (β_2M286) also is accessible from the pocket inside of the helical domain. Considering the uncertainty of model coordinates and the apparently significant flexibility of helix 3 (Akabas 2004), a disulfide bridge crossing the intra-subunit pocket can easily be envisioned in models based on the two gap alignment.

In $GABA_A$ receptor alpha subunits residues α_1S269 and α_1A290 have been proposed previously to be part of a binding pocket for volatile anaesthetics (Mascia, Trudell et al. 2000). Strong support for a role of this intra-subunit pocket

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as a possible drug binding site comes from the fact that in beta subunits helix 3 mutant β_2M286C (homologue of α_1A290) has been shown to be protected by propofol in a dose dependent manner against covalent modification by cys-reactive reagents (Bali and Akabas 2004).

The cumulative evidence for the existence of this pocket type as a conserved feature of $GABA_A$ and glycine receptor subunits is thus impressive. Since functional modulation of $GABA_A$ receptors by furosemide or certain anesthetics can be influenced by amino acid residues in α as well as β subunits, it is tempting to speculate that multiple binding sites for these compounds are present in the intra-subunit ("green") pockets of different $GABA_A$ receptor subunits. Depending on the specific electrostatic and steric interactions of drugs with these pockets, they could stabilize different conformations of the receptors, giving rise to their GABA-modulating, direct gating or inhibitory action at different drug concentrations. The entire helical domain, however, appears also to be crucial for transduction of ligand binding to gating or allosteric modulation. This is indicated by the effects of point mutants in this region on GABA action and benzodiazepine modulation (Boileau and Czajkowski 1999), and by conformational changes in the helical domain induced by drug binding (see below).

Structural models provide clues on conformational changes induced by ligand binding

Recently, helix 3 of $GABA_A$ receptor α_1 subunits has been point mutated in 15 positions and probed with cysteine modifying reagents under the influence of

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various modulatory and GABA-mimetic drugs (Williams and Akabas 2002; Akabas 2004; Jung, Akabas et al. 2005). Data indicate that the solvent accessibility of helix 3 residues changes differentially with different drugs (Fig. 4) pointing towards large conformational flexibility of helix 3. The resting state displays detectable Cys-modifying reactivity only in two positions near the N-terminus, α_1A290 and α_1Y293, the further C-terminal portion of the helix appears to be fairly well buried under this condition. GABA, benzodiazepines, ethanol, and propofol increase the total number of modifyable positions. The individual SCAM "fingerprints" (Fig. 4) imply that each substance induces a distinct functional state. Interestingly, position α_1E302C was found modifyable only in the presence of gating concentrations of GABA or propofol. Thus, either the segment of helix 3 where α_1E302 is located, or its neighbourhood, namely helix 2, must undergo major rearrangement upon gating in order for E302C to become modifyable. Helix 2 forms the ion gate, thus, in agreement with previous proposals (Unwin 2003; Goren, Reeves et al. 2004), it is plausible to assume that it is helix 2 that moves.

Benzodiazepine modulation induces a conformation very closely resembling the GABA induced gating conformation, lacking only E302C in its fingerprint. This supports the conclusion that benzodiazepine binding causes a conformational change that is directly transduced into the helical domain. In contrast, the states induced by two different concentrations of propofol are quite distinct from each other, supporting the notion that propofol interacts with an additional binding site at gating concentrations. In addition, gating concentrations of propofol appear to induce a conformation in alpha subunits that is completely

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different from the gating conformation induced by GABA, consistent with a completely different mechanism of action of these compounds.

Further mapping of drug-induced changes in solvent accessibility of all four helices as well as of other parts of the receptors using our models as a guide will delineate similarities and differences in drug action and drug-induced conformational changes. Model structures can then be used to visualize these changes and to describe the dynamics of these important receptors.

In summary, by using available information on the structure of the AChBP and the nACh receptor, we were able to develop models of the $GABA_A$ receptor that are not only consistent with most experimental data, but also could explain experimental observations and propose the location of putative drug binding sites. These models can now be used to design new experiments for clarification of pharmacological and structural questions as well as for shedding light on conformational changes during binding of agonists, gating and allosteric modulation of these receptors. Overall, these experiments will lead to an improvement in the accuracy of the models and finally pave the way for a structure based drug design.

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Footnotes

Comparative Models of $GABA_A$ Receptor Extracellular and Transmembrane Domains: Important insights in pharmacology and function

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Legends for Figures

Figure 1: Alignment of nACh and $GABA_A$ receptor helical domains

a: A subset of a superfamily alignment of the 4 segments that make up the helical (trans-membrane) domain of the subunit chains is shown. The position of the missing "cytoplasmic loop" between helices 3 and 4 is indicated by a double-gap and a bold dotted line across all columns. Note that for helices 3 and 4 other alignment variants can be found, depending on the alignment parameters and on the number and choice of sequences that are included in a multi-sequence alignment. Standard Clustal (Thompson, Gibson et al. 1997) symbols (***:.**) are used to indicate degree of sequence conservation, also indicated by the bar graph beneath the alignment.

b: Subunit correspondence between nACh receptors and $GABA_A$ receptors is indicated by the schematic pentamers, arrows indicate agonist binding site locations.

c: Three different alignments for helix 3 of $GABA_A$ receptor α_1 and β_2 subunits with the corresponding nACh receptor alpha subunit segment from 1OED are shown. They result from placing no, one or two gaps in the alignment of the 2/3 linker between cation and anion channels. $GABA_A$ receptor residues α_1D286 and α_1E302, corresponding to a conserved D and E in anion channels, are highlighted to emphasize how they align with residues in the nAChR in the three alignment variants. The highly solvent accessible nACh receptor residues αF280 and αI282 as well as $GABA_A$ receptor residues α_1A290 and α_1Y293, that can be cys-modified in appropriate mutants in the resting state, are also highlighted.

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d: Model corresponding to the 2-gap alignment of helix 3 of a $GABA_A$ receptor α subunit. Helix 3 is viewed from N-terminal and perpendicular to the paper plane, and all neighbouring protein segments are also shown. Approximate sidechain orientations for the two cys-modifyable positions α_1A290C and α_1Y293C (wild type sidechains shown), as well as the position for α_1E302 that is modifyable only under gating conditions, are depicted.

e: The helical wheel of the utmost N-terminus of helix 3 is shown as schematic C-alpha trace, the black trace indicates the nACh receptor. The residue correspondence according to the three alignment variants shown in 1c is indicated for the first two helix positions. Natural variation in C-alpha position of distant homologues is indicated by two additional (gray) alternative helical wheels, the first two C-alpha atoms are indicated by different symbols. The indicated deviations correspond to C-alpha position changes in the order of 2 Angstrom. Up to 5 Angstrom deviations are possible in structurally corresponding residue positions in homologues of low sequence identity.

Figure 2: Topology of cys-loop receptors

a: Topology of a single subunit of a cys-loop receptor. The secondary structure motifs that are likely to be conserved are shown in ribbon representation, strand numbering is shown in latin numerals, numbering of the membrane spanning helices in roman numerals. The important topologically conserved, but structurally variable regions are indicated only schematically. The inner sheet's hydrogen bonds are schematically depicted in light blue, the ones of the outer

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sheet in red. All features that are associated with the plus-side are shown in orange, the features that belong to the minus side are green, the cys-loop is yellow, the disulfide bond indicated as double arrow in yellow.

b:The topology diagram of the beta-folded domain is shown, with the inner sheet forming strands light blue, and the outer sheet forming strands in red. Note that strand length is not likely to be conserved in the superfamily, and that short strands may not be conserved altogether.

c: Pentamer architecture of a $GABA_A$ receptor's helical domain consisting of 2 α, 2 β and a γ subunit. The view corresponds to a projection view in order to correctly show the localization of the 4 helices, curled around one another, with respect to the subunit-interfaces. It can be seen that each interface is formed by helix 2 contacts, as well as by contacts of helix 1 of the minus-side with helix 3 of the plus-side subunit.

Figure 3: Solvent accessible space contained in $GABA_A$ receptor models

a: Two views of a $GABA_A$ receptor model are shown to illustrate the solvent accessible pockets, filled with "probe-solvent". The left view shows a dimer from the outside of the pore, the right view is from extracellular, with the beta-folded domain invisible. The protein is shown in ribbon representation, the putative pockets identified with PASS (Brady and Stouten 2000) are shown in dotted space filling representation. Clusters of connected solvent accessible volumes that may correspond to drug binding pockets are highlighted by colors: Pink is used for the

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space associated with the subunit-interface of the beta-folded domain, purple for the large cavity present at the subunit interface at the junction between the beta-folded and the helical domain, that extends into the interface of the helical domains, and green for the cavity that is present inside each of the 4-helix bundles of the subunit. Additional smaller clusters are shown in pale gray. It should be noted, that due to the high uncertainty in side chain positions, the total volume, shape and electrostatic properties of the pockets varies considerably among models, in some models some of the pockets may even be missing. Within the uncertainty of the method, it is also possible that there is significant communication between the “pink” and “purple”, as well as between “green” and “purple” pockets. For this illustration a representative model was used, whose properties correspond to what the majority of models display.

b: Helical domain of a $GABA_A$ receptor α_1 subunit shown from the side and from above. In the side view, helix 4 is broken to permit a view into the pocket enclosed by the four helices. The most likely positions of amino acid residues that mediate different ligand effects in $GABA_A$ receptor α_1 subunits are shown. According to our models, the intrasubunit “green” pocket appears to be lined, in α_1 subunits, by T229, which has been shown to account for lack of furosemide effect in α_1 , and has a corresponding Ile in α_6. Residues α_1S269 in helix 2 and α_1A290 in helix 3 have been associated with the action of volatile anaesthetics, and they are also part of the pocket. In glycine receptors, the residues homologous to S269 and A290 have been crosslinked in a double-Cys mutant, the residue positions shown in this figure are entirely consistent with this, the large apparent separation can

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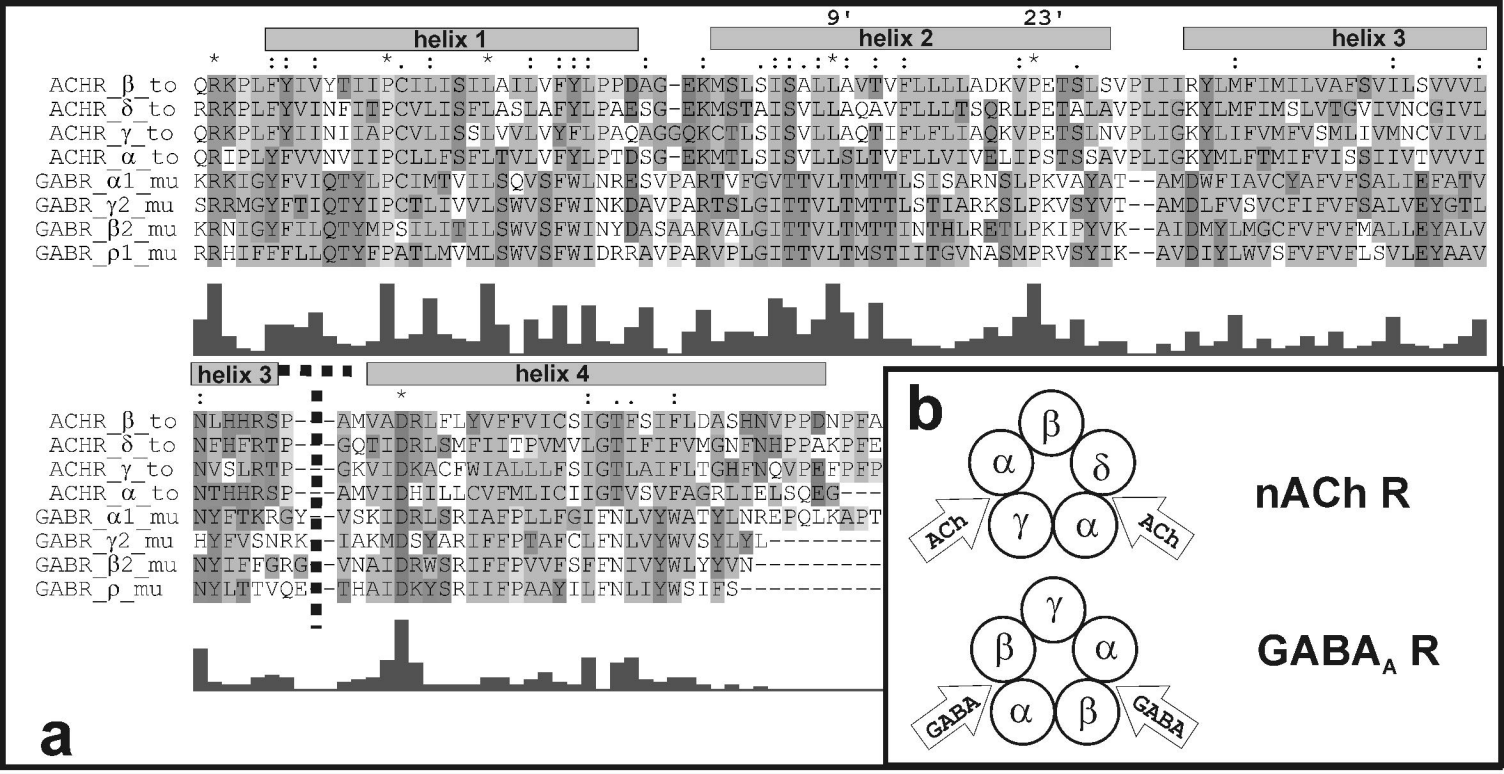
easily be overcome by flexibility in helix 3 and might also be exaggerated by position errors due to methodological limits discussed in the text.

Figure 4: Drug induced changes in the accessibility of amino acid residues in helix 3

The sequence of the $GABA_AR$'s α1 helix 3 segment is shown, with cystein modifyable positions color coded. The helix 3 sequence of the torpedo nACh receptor α subunit is shown in the bottom line, in the alignment variant featuring two gaps in the 2/3 linker. Each experimental condition is characterized by a unique set of modifyable positions in helix 3, indicating great conformational flexibility of this segment. In general, presence of agonistic or positively modulating agents increases accessibility of helix 3. The corresponding sidechains' Cα and Cβ positions are shown in a model of helix 3, viewed from N-terminal and pointing straight through the plane of the paper.


9'
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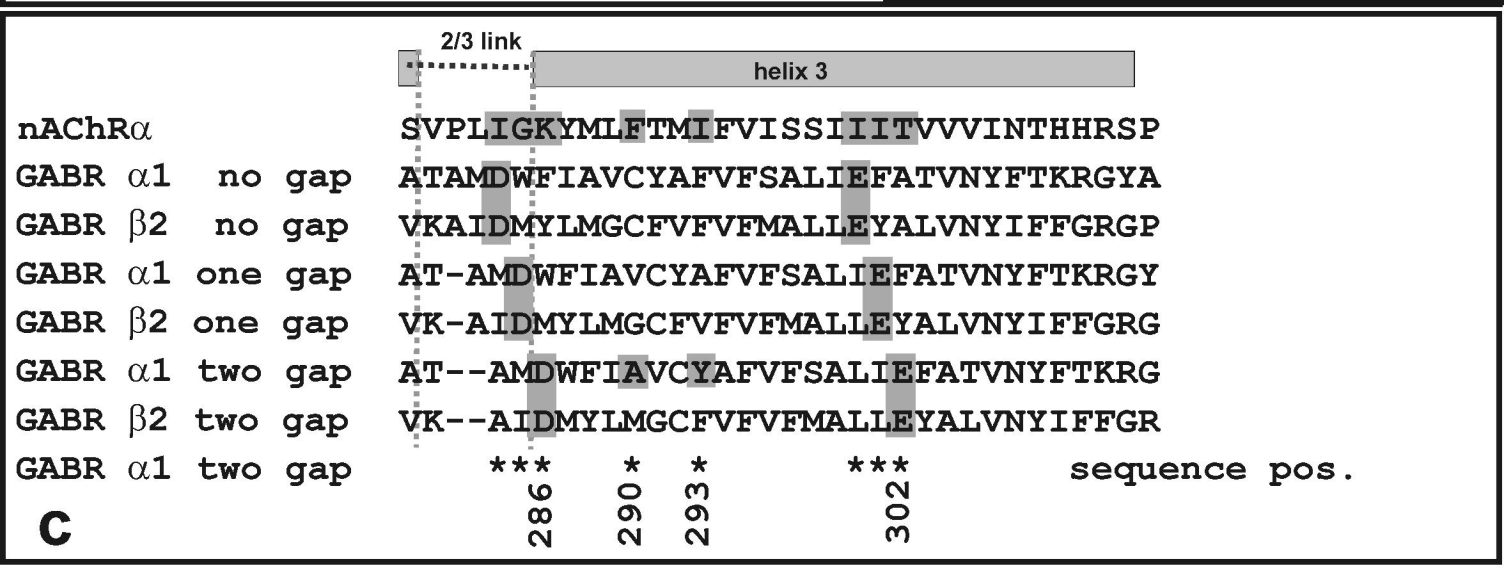
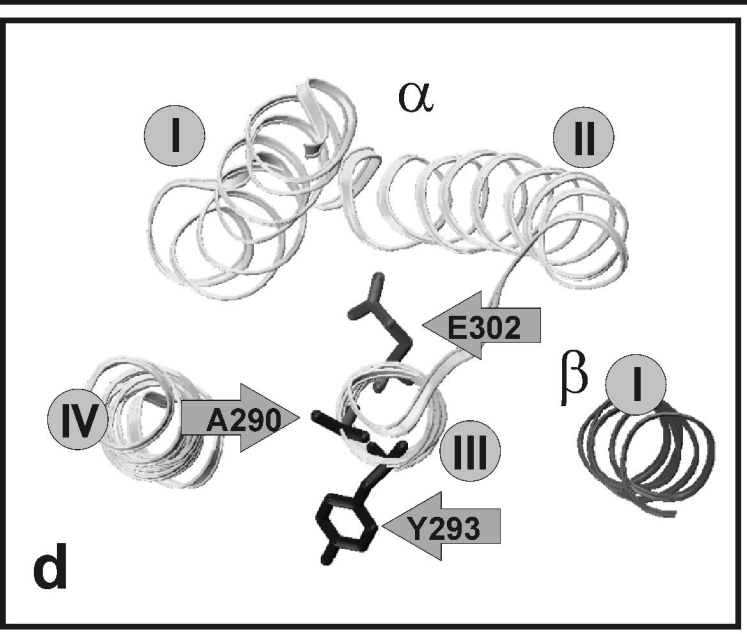
helix 1
helix 2
helix 3
ACHR_β_to QRKPLFYIVYTIIPCILISILAILVFYLPPDAG-EKMSLSISALLAVTVFLLLLADKVPETSLSVPIIIRYLMFIMILVAFSVILSVVVL
ACHR_δ_to RRKPLFYVINFITPCVLISFLASLAFYLPAESG-EKMSTAISVLLAQAVFLLLTSQRLPETALAVPLIGKYLMFIMSLVTGVIVNCGIVL
ACHR_γ_to QRKPLFYIINIIAPCVLISSLVVLVYFLPAQAGGQKCTLSISVLLAQTIFLFLIAQKVPETSLNVPLIGKYLIFVMFVSMLIVMNCVIVL
ACHR_α_to QRIPLYFVVNVIIPCLLFSFLTVLVFYLPTDSG-EKMTLSISVLLSLTVFLLVIVELIPSTSSAVPLIGKYMLFTMIFVISSIIVTVVVI
GABR_α1_mu KRKIGYFVIQTYLPCIMTVILSQVSFWLNRESVPARTVFGVTTVLTMTTLSISARNSLPKVAYAT--AMDWFIAVCYAFVFSALIEFATV
GABR_γ2_mu SRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGITTVLTMTTLSTIARKSLPKVSYVT--AMDLFVSVCFIFVFSALVEYGTL
GABR_β2_mu KRNIGYFILQTYMPSILITILSWVSFWINYDASAARVALGITTVLTMTTINTHLRETLPKIPYVK--AIDMYLMGCFVFVFMALLEYALV
GABR_ρ1_mu RRHIFFFLLQTYFPATLMVMLSWVSFWIDRRAVPARVPLGITTVLTMSTIITGVNASMPRVSYIK--AVDIYLWVSFVFVFLSVLEYAAV
helix 3
helix 4
ACHR_β_to NLHHRSP--AMVADRLFLYVFFVICSIGTFSIFLDASHNVPPDNPFA
ACHR_δ_to NFHFRTP--GQTIDRLSMFIITPVMVLGTIFIFVMGNFNHPPAKPFE
ACHR_γ_to NVSLRTP--GKVIDKACFWIALLLFSIGTLAIFLTGHFNQVPEFPFP
ACHR_α_to NTHHRSP--AMVIDHILLCVFMLICIIGTVSVFAGRLIELSQEG---
GABR_α1_mu NYFTKRGY--VSKIDRLSRIAFPLLFGIFNLVYWATYLNREPQLKAPT
GABR_γ2_mu HYFVSNRK--IAKMDSYARIFFPTAFCLFNLVYWVSYLYL--------
GABR_β2_mu NYIFFGRG--VNAIDRWSRIFFPVVFSFFNIVYWLYYVN---------
GABR_ρ_mu NYLTTVQE--THAIDKYSRIIFPAAYILFNLIYWSIFS----------
a
b
β
α
δ
ACh
γ
α
ACh
nACh R
γ
β
α
GABA
α
β
GABA
GABA$_A$ R


2/3 link
helix 3
nAChRα SVPLIGKYMLFTMIFVISSIIITVVVINTHHRSP
GABR α1 no gap ATAMDWFIAVCYAFVFSALIEFATVNYFTKRGYA
GABR β2 no gap VKAIDMYLMGCFVFVFMALLEYALVNYIFFGRGP
GABR α1 one gap AT-AMDWFIAVCYAFVFSALIEFATVNYFTKRGY
GABR β2 one gap VK-AIDMYLMGCFVFVFMALLEYALVNYIFFGRG
GABR α1 two gap AT--AMDWFIAVCYAFVFSALIEFATVNYFTKRG
GABR β2 two gap VK--AIDMYLMGCFVFVFMALLEYALVNYIFFGR
GABR α1 two gap *** * * *** sequence pos.
286
290
293
302
c


α
I
II
E302
β
I
IV
A290
III
Y293
d

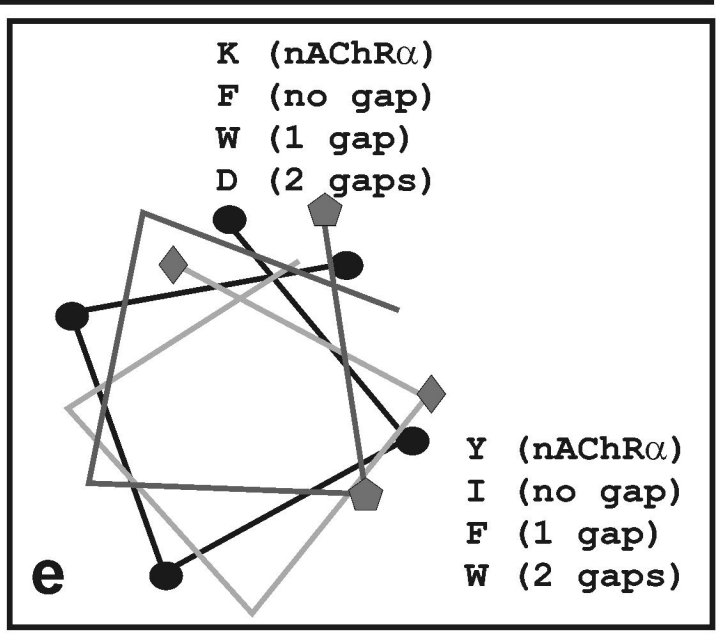

K (nAChRα)
F (no gap)
W (1 gap)
D (2 gaps)
Y (nAChRα)
I (no gap)
F (1 gap)
W (2 gaps)
e

Figure 1

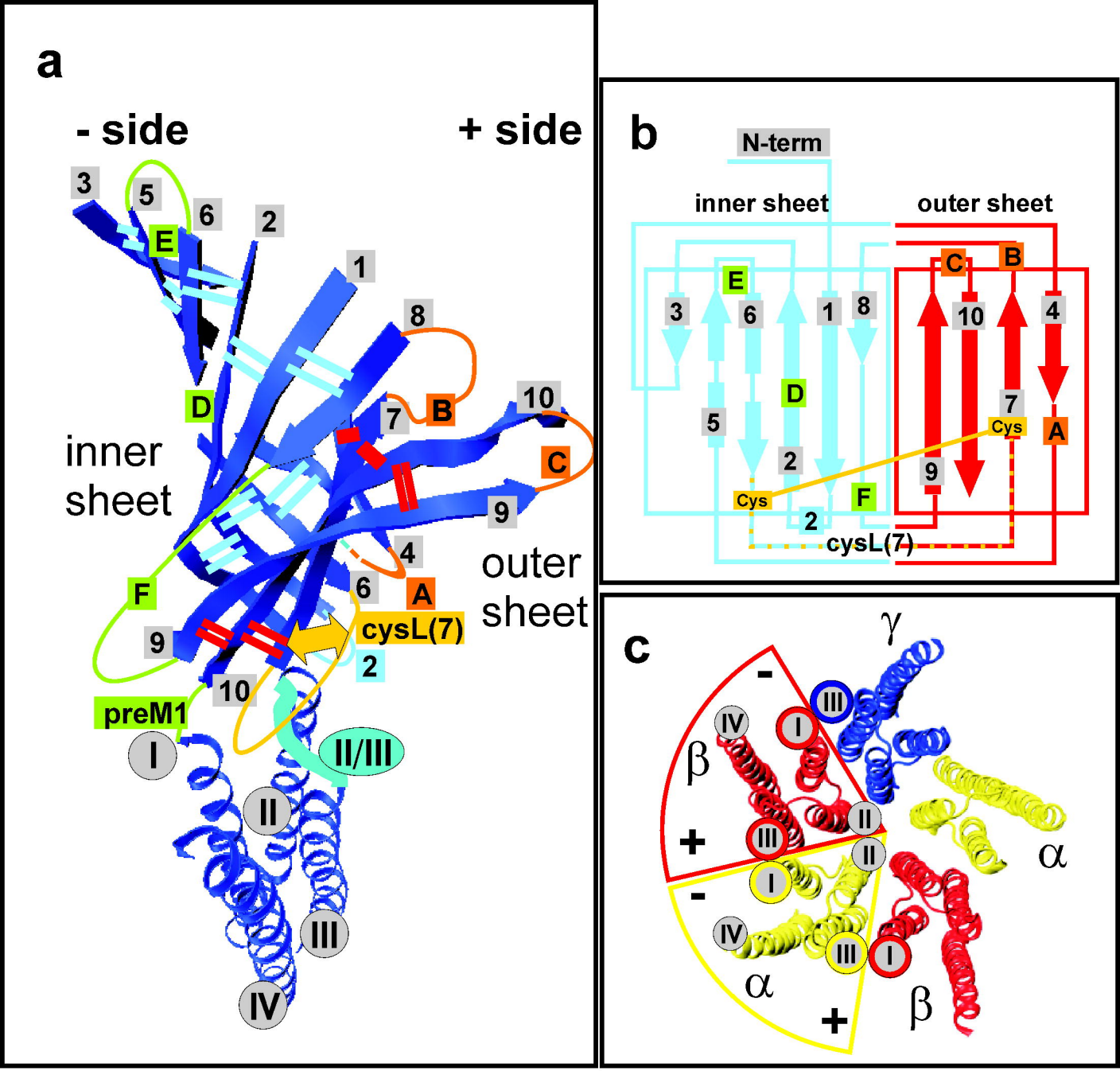

a
- side
+ side
inner sheet
outer sheet
preM1
cysL(7)
II/III
b
N-term
inner sheet
outer sheet
Cys
Cys
cysL(7)
c
γ
β
α
α
β

Figure 2

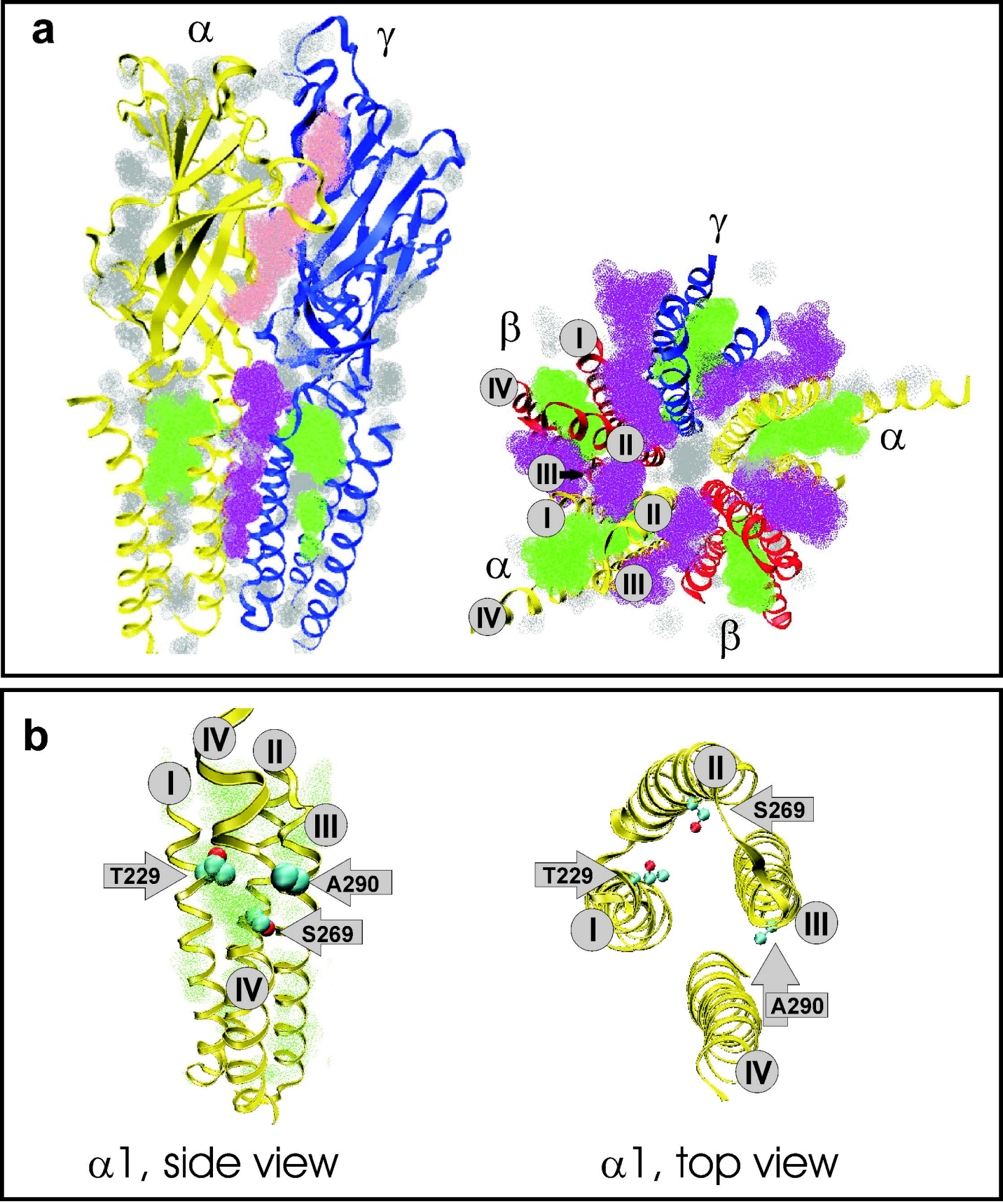

a
α
γ
β
I
IV
II
III
I
II
α
III
IV
γ
α
β
b
IV
II
I
III
T229
A290
S269
IV
II
S269
T229
I
III
A290
IV
α1, side view
α1, top view

Figure 3

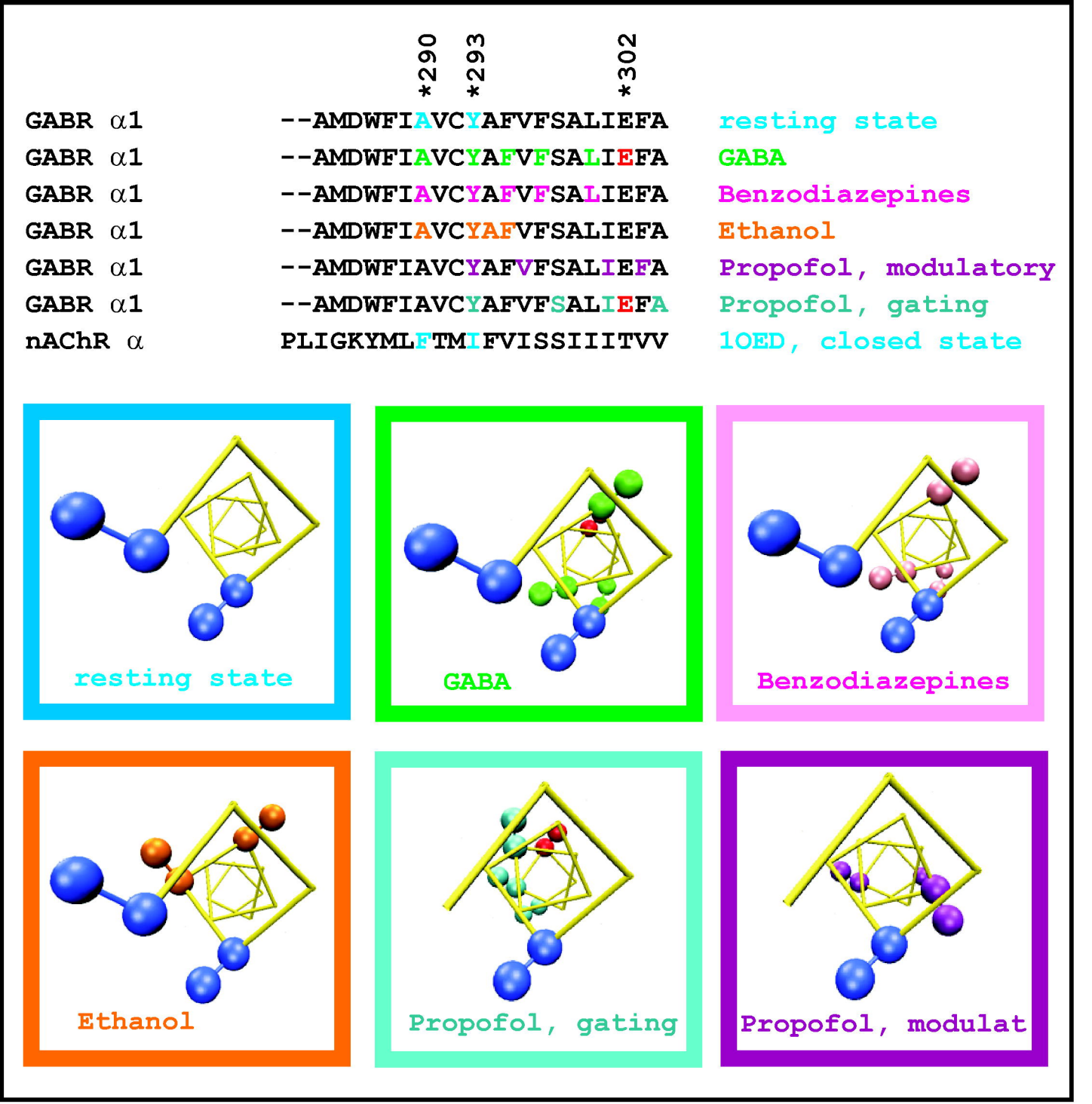

*290
*293
*302
GABR α1 --AMDWFIAVCYAFVFSALIEFA resting state
GABR α1 --AMDWFIAVCYAFVFSALIEFA GABA
GABR α1 --AMDWFIAVCYAFVFSALIEFA Benzodiazepines
GABR α1 --AMDWFIAVCYAFVFSALIEFA Ethanol
GABR α1 --AMDWFIAVCYAFVFSALIEFA Propofol, modulatory
GABR α1 --AMDWFIAVCYAFVFSALIEFA Propofol, gating
nAChR α PLIGKYMLFTMIFVISSIIITVV 1OED, closed state
resting state
GABA
Benzodiazepines
Ethanol
Propofol, gating
Propofol, modulat

Figure 4