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Exploring the molecular basis of neurosteroid binding to the β3 homopentameric $GABA_A$ receptor

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ABSTRACT

Neurosteroids are the principal endogenous modulators of $GABA_A$ receptors ($GABA_A$Rs), which are pentameric membrane-bound proteins that regulate the passage of chloride ions from the extracellular to the intracellular compartment. As consequence of their ability to modify inhibitory functions in the brain, neurosteroids have high physiological and clinical importance and may act as anesthetic, anticonvulsant and anxiolytic drugs. Despite their relevance, essential issues regarding neurosteroid action on $GABA_A$Rs are still unsettled. In particular, residues taking part of the steroid recognition are not definitely identified. Taking as starting point the first reported crystal structure of a human $GABA_A$ receptor (a β3 homopentamer), we have explored through a combination of computational methods (a cavity-detection algorithm, docking and molecular dynamics simulations) the binding mode of two structurally different representative neurosteroids, pregnanolone and allopregnanolone. We have identified a neurosteroid binding site between the TM3 of one subunit and TM1 and TM4 of the adjacent subunit that is consistent with the set of experimental data reported for the action of neurosteroids on β_3 homopentamers. These sites are able to properly accommodate both overall torsioned and flat steroidal structures and they specifically recognize the 3-OH group, explaining the requirement of a 3α-configuration for the activity. We believe that this work provides for first time convincing information about the molecular interaction between neurosteroids and a $GABA_A$R. This information largely increases our understanding of this fundamental ligand-receptor system.

1. Introduction

The major inhibitory neurotransmitter in vertebrate central nervous system (CNS), γ-Aminobutyric acid (GABA), exerts its action primarily by activating the Gamma-Aminobutyric Acid type A Receptors ($GABA_A$Rs) [1]. This neurotransmitter-receptor system is involved in practically all neuronal circuits, modulating functions of critical physiological importance. Central roles in cognition, learning and memory, as well as in anxiety, schizophrenia and epilepsy, among other diseases, have been related to these receptors [2]. Moreover, since a variety of small molecules, such as benzodiazepines, barbiturates, ethanol and neurosteroids, exhibit the ability to modulate their action by binding to different allosteric binding sites, $GABA_A$Rs are of high pharmaceutical relevance [3–5].

The $GABA_A$Rs are pentameric membrane-bound proteins belonging to the Cys-loop superfamily of ligand-gated ion channels [6,7]. They may be assembled from at least 19 subunits belonging to eight different classes (6α, 3β, 3γ, 1δ, 1ε, 1θ, 1π and 3ρ). Each subunit can be subdivided into three domains: the extracellular domain (ECD), the transmembrane domain (TMD) formed by four α-helices (TM1–TM4), and a cytoplasmic loop of variable length. The assembling of subunits creates a central ion conducting pore for the passage of chloride ions from the extracellular to the intracellular compartment. The GABA-binding pocket and sites for allosteric modulators have been located in the ECDs [5,6].

The principal endogenous modulators of GABAergic function in the brain are neurosteroids. They exhibit clear behavioral effects that include anxiolysis, sedation and analgesia [8–10]. Despite the relevance of $GABA_A$R-neurosteroid interaction, and the intense efforts focused to elucidate the molecular mechanism of action, the neurosteroid binding site has not been yet determined. Endogenous steroids, such as 3α-hydroxy-5β-pregnan-20-one (pregnanolone) and its 5α isomer (allopregnanolone) (Fig. 1), and exogenous

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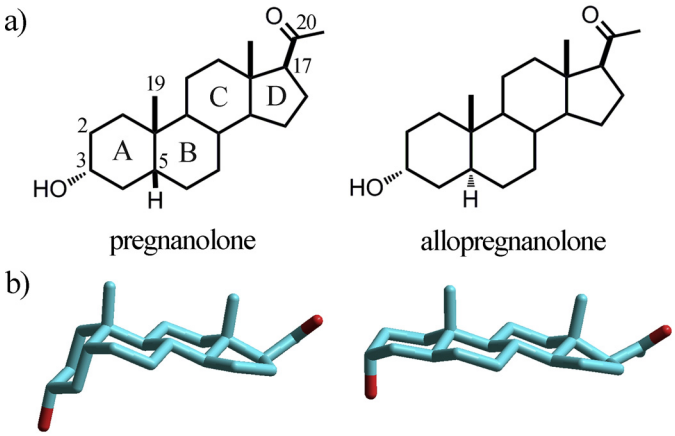

Fig. 1. Neurosteroid modulators of the $GABA_AR$-β_3. (a) Structure of pregnanolone and allopregnanolone; (b) Schematic representations of the optimized geometries at the HF/6-31G** level of theory.

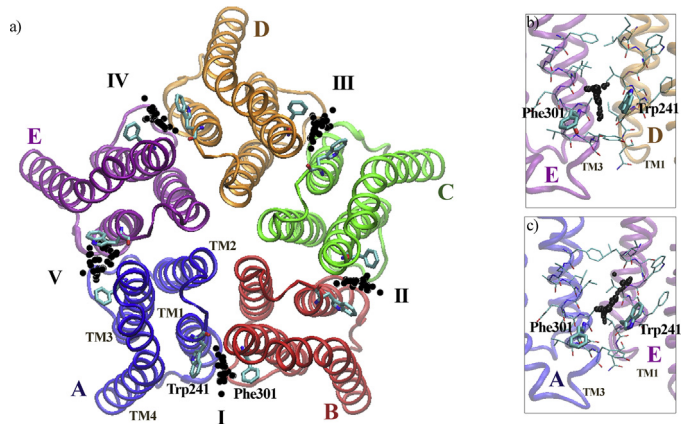

Fig. 2. The $GABA_AR$-β_3 TMD presents five surface cavities which are partially delimited by Phe301 residues. The centers of alpha spheres generated by fpocket are depicted as black points. (a) Overall view of the $GABA_AR$-β_3 TMD structure showing the localization of the subunits (A–E), TM α-helices (TM1–TM4) and cavities detected by fpocket (I–V). Residues Phe301 and Trp241 of each subunit are indicated. (b and c) Detailed view of cavity IV (b) and cavity V (c) showing the conformation of Phe301 and Trp241.

neuroactive steroids can either potentiate the chloride currents elicited by GABA or directly activate the receptor, suggesting that two different binding sites could be present. Previous evidence indicates that these sites should be located in the TMD [5,11,12], but the specific residues involved in the steroid recognition are still not convincingly established. In a pioneer work, Hosie et al combined mutational studies with a homology model of $\alpha_1\beta_2\gamma_2$ receptor constructed from the structure of the nicotinic acetylcholine receptor, to suggest that a site located between αTM1 and αTM4 is the responsible for the potentiation action, while other localized between αTM1 and βTM3 is involved in the direct activation [13,14]. However, recently reported biochemical data and more reliable models based in more conserved templates seem to indicate that these sites would not be operative [12,15–17].

Auspiciously, the X-ray structure of a $GABA_AR$ homopentamer composed by β_3 subunits ($GABA_AR$-β_3), the first structure reported for a mammalian anion channel, was recently solved [18]. Although $GABA_AR$-β_3 has not been identified as discrete populations in the CNS, it can efficiently form functional channels that can be modulated by some general anesthetics, such as barbiturates and neurosteroids [19] and may actually be employed as a simplified model for the study of $GABA_AR$ function. In contrast to other receptors, β_3 homopentamers have one class (rather than two classes) of neurosteroid binding sites [19,20]. Furthermore, employing a photolabeling analogue of pregnanolone (6-azipregnanolone), Chen et al have found that only one residue of $GABA_AR$-β_3 (Phe301 in the TM3) resulted photolabeled [20], suggesting that a specific neurosteroid binding site near this residue might exist.

Computational techniques for modeling protein-ligand binding have emerged during the last decades as an important tool to complement experimental information. In recent years, the increase in the computing power and in the accuracy of the models, made possible to draw biologically relevant conclusions and propose new hypothesis based mainly on computer generated data. In the specific case of steroid receptors, our group and others have obtained valuable information regarding the ligand binding mode and the molecular basis of action [21–28].

In this work we used state of the art computational schemes to explore in detail the neurosteroid binding mode in the recently characterized $GABA_AR$-β_3 receptor. Our results clearly confirm that an open cavity exists in the $GABA_AR$-β_3 surface, partially delimited by Phe301, in which neurosteroid molecules can be stably and specifically recognized.

2. Results and discussion

2.1. Preliminary analysis of the $GABA_AR$-β_3 TMD structure

An intracellular view of $GABA_AR$-β_3 TMD (PDB ID: 4COF) describing the relative disposition of α-helices is shown in Fig. 2a. The ion channel is located along the central symmetry axis, bordered by five TM2 segments, which, in turn, are surrounded by TM1 and TM3 α-helices, shielding TM2 residues from the membrane. TM4 helices are located at the periphery of the protein and are buried in the membrane. The intersubunit contact is formed mainly by residues of TM1 and TM3 α-helices.

As a first approximation to investigate the neurosteroid binding mode, we examined the TMD structure in search of superficial cavities that may be able to accommodate a steroid molecule. With this goal in mind we used the fpocket program [29], a pocket/cavity detection algorithm based on Voronoi tessellation. We found eight superficial and mainly hydrophobic cavities in the membrane exposed area of the $GABA_AR$-β_3 TMD. Remarkably, five of them (termed I to V) are located very close to Phe301 (TM3) of each subunit, the residue that was photolabeled by 6-azipregnanolone [20]. The other three cavities, located very far away from these residues, are not large enough to contain steroidal molecules, and so will not be further considered in this study.

A detailed visual inspection of fpocket results reveals that cavities I–V are mainly delimited by two aromatic residues, Phe301 (TM3) on one subunit and Trp241 (TM1) of the adjacent subunit (Fig. 2a). In these cavities, the relative disposition of Phe301 and Trp241 is very similar (see Fig. 2b as example), which generate holes comparable in volume (V_I = 489 Å^3; V_{II} = 552 Å^3; V_{III} = 591 Å^3 and V_{IV} = 527 Å^3). In contrast, the different conformation of Trp241 observed for the subunit E (Fig. 2c) produces a cavity considerably larger (V_V = 669 Å^3). Hence, a preliminary analysis of the $GABA_AR$-β_3 TMD structure revealed the presence of conserved cavities in the interface between TM1 and TM3 of adjacent subunits, which are large enough to accommodate steroidal molecules.

2.2. Docking of neurosteroids on the $GABA_AR$-β_3 TMD

2.2.1. Pregnanolone

Pregnanolone is an endogenous 5β steroid with an overall torsioned conformation produced by the *cis* junction between A and B rings (Fig. 1b). It is known that this neurosteroid is able to modulate

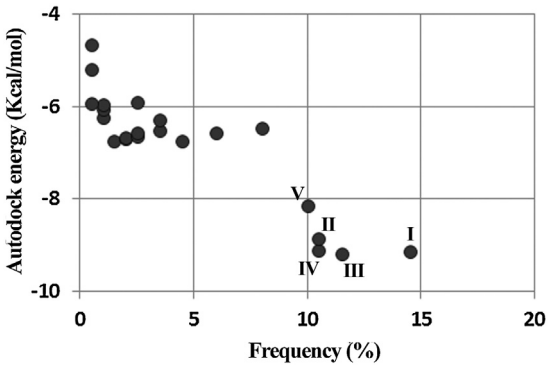

Fig. 3. Docking of pregnanolone in the $GABA_AR$-β_3 TMD reveals that the steroid is preferentially located in the cavities I–V. Cluster distribution for docking of pregnanolone in the $GABA_AR$-β_3 TMD. The *x*-axis represents the frequency observed and the *y*-axis represents the Lowest Binding Autodock Energy. Clusters corresponding to cavities I–V are indicated.

the activity of several classes of $GABA_ARs$, but regarding to effects on β_3 homopentamers, no information has been reported to our knowledge. Nevertheless, Chen et al have recently determined that a photolabeling analog of pregnanolone, 6-azipregnanolone, inhibited [^{35}S]TBPS binding to $GABA_AR$-β_3 in a concentration dependent manner [20]. This strongly suggests that this class of $GABA_AR$ is also able to bind torsioned steroidal molecules. With this in mind, we used the Autodock program [30] to analyze possible binding modes of pregnanolone into the $GABA_AR$-β_3 TMD crystal structure. In the docking process, the ligand was considered flexible while the receptor was assumed to be a rigid. In the first place, we constructed large grids centered in the geometrical center of the TMD using a large point spacing (0.5 Å), in a way to include all receptor residues. 500 runs of genetic algorithm analyzed with a cluster tolerance of 10 Å showed a total of 22 clusters, although only five are certainly relevant, with both low energy and high frequency values (Fig. 3). Consistently with the fpocket results, these five docking clusters correspond to solutions in which the pregnanolone molecule is located in each one of the cavities I–V. The docking analysis performed with 6-azipregnanolone showed very similar results (data not shown), indicating that the small group attached at C-6 position did not alter the binding mode of the steroid.

In order to refine the previous docking study, we constructed finer grids (point spacing equal to 0.2 Å) centered in the geometrical center between Phe301 and Trp241 of each cavity. Fig. 4a shows results obtained through 500 runs of genetic algorithm on each cavity (I–V), analyzed with a cluster tolerance of 2 Å. We found that pregnanolone can exhibit three principal different orientations (pose A, pose B and pose C) inside cavities, with one (pose A) more extensively represented (frequency larger than 45%) for cavities I–IV.

In this pose, the D ring was located pointing toward the Phe301 (TM3) while the A ring was located on the Trp241 (TM1) (Fig. 4b). Other residues from the TM3 (Phe293, Leu297, Ala300 and Tyr304) and from the TM1 (Ile234, Trp237, Val238 and Phe240) and the TM4 (Arg428 and Pro432) of the adjacent subunit are in contact with the steroid. The 3α-OH group of pregnanolone in pose A occupies a position in which two hydrogen bonds with receptor residues are established. On one side, the oxygen atom accepts the hydrogen atom bounded to the NE1 nitrogen atom of Trp241 (TM1). On the other, the hydrogen atom interacts with the oxygen backbone atom of the Arg428 (TM4). No hydrogen bonds are formed between the 20-carbonyl group of pregnanolone and receptor residues. In the case of the less probable pose B, the steroid is positioned in an inverse mode, with the A-ring toward the Phe301 and the D-ring on the Trp241 (Fig. 4c). In this disposition, no hydrogen bonds are formed between the ligand and the receptor. Finally, a much less frequent pose (pose C) was observed, in which the steroid is oriented with the C-18 and C-19 methyl groups pointing to the Trp241 (Fig. 4d).

For cavity V, pose A was less frequent. As consequence, poses A and B have similar frequencies (Fig. 4a). This alteration can be explained taking in consideration that in this cavity the Trp241 presents an alternative conformation. In the pose A, the polar group of the indol is positioned too far away from the ligand 3α-OH group, losing one of the ligand-receptor hydrogen bonds, and therefore disfavoring this binding mode. With the goal to examine if this cavity presents, besides of the Trp241 modification, a more global alteration, we rotated the χ_1 and χ_2 torsion angles of Trp241 in way to obtain a conformation similar to the observed in the other cavities. Then we redocked the pregnanolone molecule, finding now that pose A was extensively more favored, with a frequency superior to 59%, while pose B resulted much less probable (10%). This suggests that the overall shape of cavity V is similar to other cavities, and that the only rotation of Trp241 could reestablish the docking pattern observed in the other cavities.

Taken together the docking results, we can conclude that there is a very favored orientation of pregnanolone, in which specific neurosteroid-$GABA_AR$-β_3 hydrogen bonds are involved.

2.2.2. 3β-pregnanolone

Large accumulated evidence of the structure–activity relation indicates that the configuration of the 3-OH group results determinant to the neurosteroid activity [31]. 3β estereoisomers of 3α-OH neurosteroids lose the capacity to modulate $GABA_AR$ action. In particular, it was found that the $GABA_AR$-β_3 was not photolabeled by 3β-6-azipregnanolone [20], indicating that the inversion of C-3 stereochemistry also affect the binding at this class of $GABA_ARs$. Therefore, in order to investigate the molecular determinants involved in this phenomenon, we docked 3β-pregnanolone into the cavity IV of the $GABA_AR$-β_3 TMD. This cavity was chosen since pregnanolone presented the highest preference for the pose A. A different cluster distribution (Fig. 5a) was obtained for 3β-pregnanolone. Although the pose A was again the more frequent, the pose B resulted more energetically favored. The visual inspection of pose A revealed that the inverse orientation of 3β-OH group impedes the formation of both hydrogen bonds observed with pregnanolone (Fig. 5b). Thus, the lack of these polar interactions has a considerably effect on the Autodock energy, inverting the corresponding values for poses A and B compared to the pregnanolone results.

The docking study performed on the $GABA_AR$-β_3 TMD structure indicated that specific ligand–receptor interactions observed for pregnanolone are not established when the configuration of the 3-OH is changed, which could explain the lack of activity of this 3β estereoisomer. On the other hand, this result also suggests that cholesterol, being a 3β steroid, could not be bound at this GABAAR-β_3 TMD site, which is expected to be specific to neurosteroids.

2.2.3. Allopregnanolone

In allopregnanolone, the 5α isomer of pregnanolone, the *trans* junction between A and B rings produces an overall flat structure (Fig. 1b). Davies et al have showed that allopregnanolone also modulate the [^{35}S]TBPS binding to $GABA_AR$-β_3 [19], suggesting that this receptor has a binding site structurally capable of accommodating both torsioned and flat steroids, and the motivating our interest in the study of how allopregnanolone binds to β3 homopentamers. Again, taking into account the above mentioned results obtained for pregnanolone, only the cavity IV was used. Docking results showed that four main binding modes for allopregnanolone are possible, but again only one (pose A) was much more frequent than the

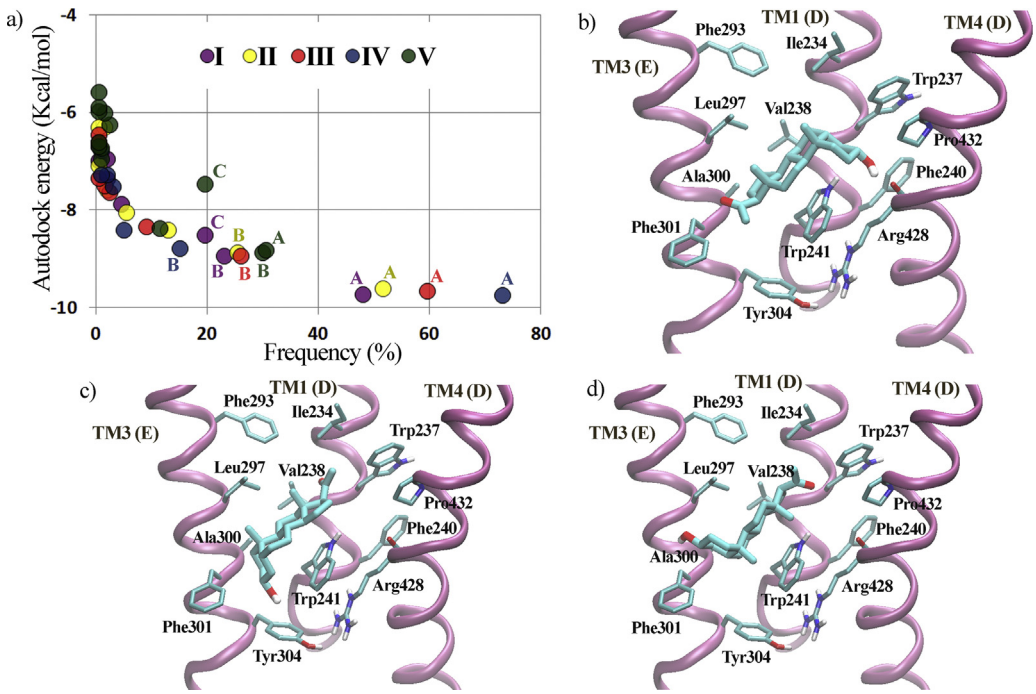

Fig. 4. Docking of pregnanolone in cavities I–IV of the $GABA_A$R-β_3 TMD reveals that the pose A is the more favored orientation. Cluster distribution and detailed view of docking poses are shown. (a) Cluster distribution for docking of pregnanolone in cavities I–V of the $GABA_A$R-β_3 TMD. The *x*-axis represents the frequency observed and the *y*-axis represents the Lowest Binding Autodock Energy; (b, c and d) Poses of pregnanolone in the cavity IV: pose A (b); pose B (c) and pose C (d).

others (Fig. 5a). In this pose, allopregnanolone acquired a similar global orientation than pregnanolone, with hydrogen bonds established between atoms of the ligand 3α-OH group and Trp241 and Lys428 residues (Fig. 5c). The superposition of the best solutions of pregnanolone and allopreganolone, allows comparing the relative location of these molecules in the cavity IV (Fig. 5d). The B rings of steroids occupy practically the same position, with a distance between the C19 atoms of only 0.6 Å. However, C and D rings of allopregnanolone are displaced above of the pregnanolone ones. This modification in the global inclination of the allopregnanolone

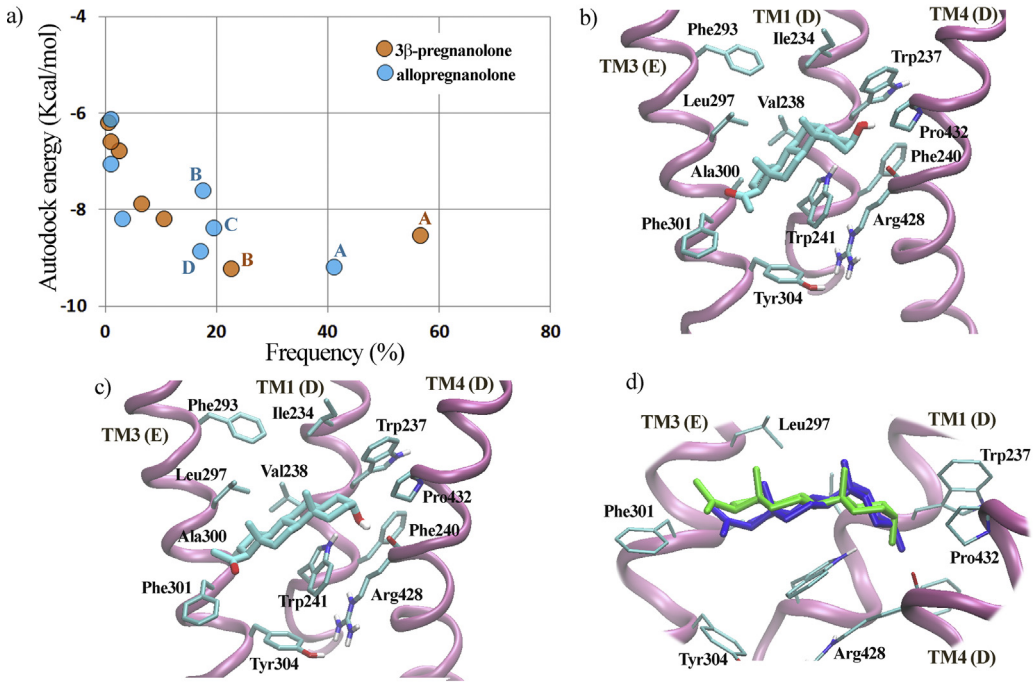

Fig. 5. Docking of 3β-pregnanolone and allopregnanolone in cavity IV reveals different and similar results compared to pregnanolone, respectively. Cluster distribution and detailed view of docking poses are shown. (a) Cluster distribution for docking of 3β-pregnanolone and allopregnanolone in cavity IV of the $GABA_A$R-β_3 TMD. The *x*-axis represents the frequency observed and the *y*-axis represents the Lowest Binding Autodock Energy for each cavity. (b) Pose A of 3β-pregnanolone in the cavity IV. (c) Pose A of allopregnanolone in the cavity IV. (d) Superposition of poses A in cavity IV of pregnanolone (blue) and allopregnanolone (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

carbon skeleton allows that the C-3 atoms of the A ring of both steroid reside in close proximity (distance equal to 0.5 Å), and then the 3-OH group acquire a similar orientation (oxygen atoms are separated by 0.8 Å).

Hence, the neurosteroid binding site of the $GABA_AR$-β_3 also has the ability to accommodate overall flat steroids, which is consistent with the reported activity of allopregnanolone.

2.3. MD simulations of $GABA_AR$-β_3 systems

With the goal of investigating the dynamic behavior of the $GABA_AR$-β_3 TMD, we carried out MD simulations of the receptor immersed in a model of lipidic membrane composed by 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine (POPC) molecules. In the first place, we performed a 100 ns long Amber-MD trajectory starting from the TMD crystal structure (*apo* system). Next, different *holo* complexes were constructed from the docking solutions obtained with pregnanolone and allopregnanole, and then 100 ns of MD trajectories were obtained in each case.

2.3.1. apo $GABA_AR$-β_3 TMD system

The root mean square desviation (RMSD) measured over the CA atoms of TMD α-helices revealed a stable trajectory in which the four α-helices of all subunits practically conserved their original position (Fig. 6a). Regarding to cavities detected by fpocket program, the visual inspection of the trajectory indicates that four of them (II–V) were rapidly occupied by atoms of POPC molecules. As example, Fig. 6b shows a representative snapshot of the cavity IV describing the disposition that a palmitoyl chain of the POPC reached between Phe301 and Trp241. In all cases, an extensive contact between POPC carbon atoms and the planar surface of the Trp241 side chain was observed.

The cavity I (Fig. 6c) was not occupied by lipidic molecules, but a direct aromatic–aromatic interaction between Phe301 and Trp241 was stably formed. In order to further characterize the dynamic behavior of neurosteroid cavities, we deleted all lipidic molecules from the MD trajectory and performed an analysis with mdpocket, a program that applied the fpocket algorithm on extracted snapshots. Calculating the time evolution of the volume between Phe301 and Trp241 generated in this way, we can estimate the cavities behavior along the timescale of the simulation. Results revealed that volumes of cavities II–V are stabilized after 40 ns, achieving values smaller than the ones observed in the initial crystal structure (Fig. 6d). Cavity II resulted the more reduced (with an average volume in the last 60 ns equal to 138 $Å^3$), while the cavity V was the larger (439.0 $Å^3$). Cavities III and IV have similar intermediate dimensions (307 and 341 $Å^3$, respectively). Consistently, mdpocket does no detect any free space in the cavity I, in where Phe301 and Trp241 are directly in contact.

The MD results obtained with the *apo* $GABA_AR$-β_3 system revealed that the neurosteroid cavities are rapidly occupied by lipidic molecules.

2.3.2. $GABA_AR$-β_3/pregnanolone complexes

Docking results have clearly indicated that the pose A is the more favored binding mode of pregnanolone in the $GABA_AR$-β_3. Since this pose was particularly preferred in the cavity IV, we selected the best solution obtained in this cavity and constructed the corresponding $GABA_AR$-β_3 TMD/pregnanolone complex immersed in the POPC membrane model. Then, 100 ns of Amber-MD simulation from these initial coordinates were obtained. The analysis of the MD trajectory clearly revealed a tight pregnanolone binding mode. The RMSD measured over ligand atoms was always less than 2.5 Å, indicating that only minor modification occurred in the position of the steroid (Fig. 7a). Furthermore, pregnanolone always contacted the same residues: Phe293, Leu297, Ala300, Phe301 and Tyr304 of the TM3 (subunit E); Ile234, Trp237, Val238, Phe240 and Trp241 of the TM1 (subunit D); Arg428 and Pro432 of the TM4 (subunit D) (Fig. 8a). On the other hand, several POPC molecules were in close interaction with pregnanolone during the timescale of our simulation.

Regarding the polar interactions, strong hydrogen bonds between the 3α-OH group of pregnanolone and Trp241 or Arg321 were formed (Fig. 7b). This ligand hydroxyl group conserved the same orientation during the whole 100 ns simulation (Fig. 7c), establishing very frequent interactions with these residues (94 and 93% of the total simulation time, respectively). No polar interactions were observed between the 20-carbonyl group of the steroid and receptor residues, showing that the hydrogen bonding between the steroid D-ring and the $GABA_AR$-β_3 residues seems to be not essential for a stable binding. A similar conclusion was formulated for $\alpha_1\beta_{2\gamma2L}$ receptors, in which hydrogen bonding with the 17β-substituent is not a critical requirement for channel potentiation [32]. However, in these receptors, a steroid analogue without a hydroxyl group on C3 also has the ability to modulate their activity [33]. Since receptors composed of different subunits are expected to have non-identical binding sites for neurosteroids, it is not possible to extract general conclusions.

In this way, MD results confirmed that a highly conserved neurosteroid binding mode was established in this system, in which both the non-specific hydrophobic and the specific polar interactions predicted by the docking analysis were actually conserved in the MD study.

In order to investigate what happens when the $GABA_AR$-β_3 TMD/pregnanolone complex is constructed with pregnanolone in the less favored pose B, we also carried out an Amber-MD simulation from these initial coordinates. Notably, a very unstable pregnanolone binding mode was observed, as indicated by the highly fluctuating RMSD values measured over ligand atoms (Fig. 7a). The steroid rapidly left its initial position in the cavity, moving away towards the membrane (Fig. 8b). No hydrogen bonds were formed between the ligand polar groups and the receptor residues. This dynamic behavior clearly showed that the receptor was unable to recognize the pregnanolone in pose B.

With the goal to investigate if the receptor can be adapted in order to properly bind the 3β isomer of pregnanolone, we constructed the $GABA_AR$-β_3 TMD/3β-pregnanolone complex using the best solution of pose A in cavity IV. The visual inspection of the MD trajectory revealed that the steroid is expelled from the cavity during the first nanoseconds and it never recovers its original position (data not shown).

Taking these results together, we confirmed by MD simulations the above mentioned docking findings: while the pose A of pregnanolone is the favored binding mode in the $GABA_AR$-β_3, neither the pose B nor the pose A of its 3β isomer can be appropriately recognized.

2.3.3. $GABA_AR$-β_3/allopregnanolone complex

Finally, we analyzed the dynamic behavior of the $GABA_AR$-β_3 TMD/allopregnanolone complex. The system was constructed with allopregnanolone in cavity IV using the best solution of pose A. Both the visual inspection of the trajectory and the RMSD values measured over ligand atoms (Fig. 7a) revealed that, after a slightly initial readjustment of the steroid position, a stable binding mode was achieved (Fig. 8c). Comparing to the binding mode of pregnanolone, the major difference resides in the behavior of the 3α-OH group. Although the hydrogen bond with the NE1 nitrogen atom of Trp241 (TM1) was stablished (Fig. 7d), it was much less frequent (24%). More markedly, the interaction with the oxygen backbone atom of the Arg428 (TM4) disappeared (Fig. 7d). Instead, the 3α-OH group rotated in a way to form persistent hydrogen bonds (51%) with the oxygen backbone atom of a residue at TM3

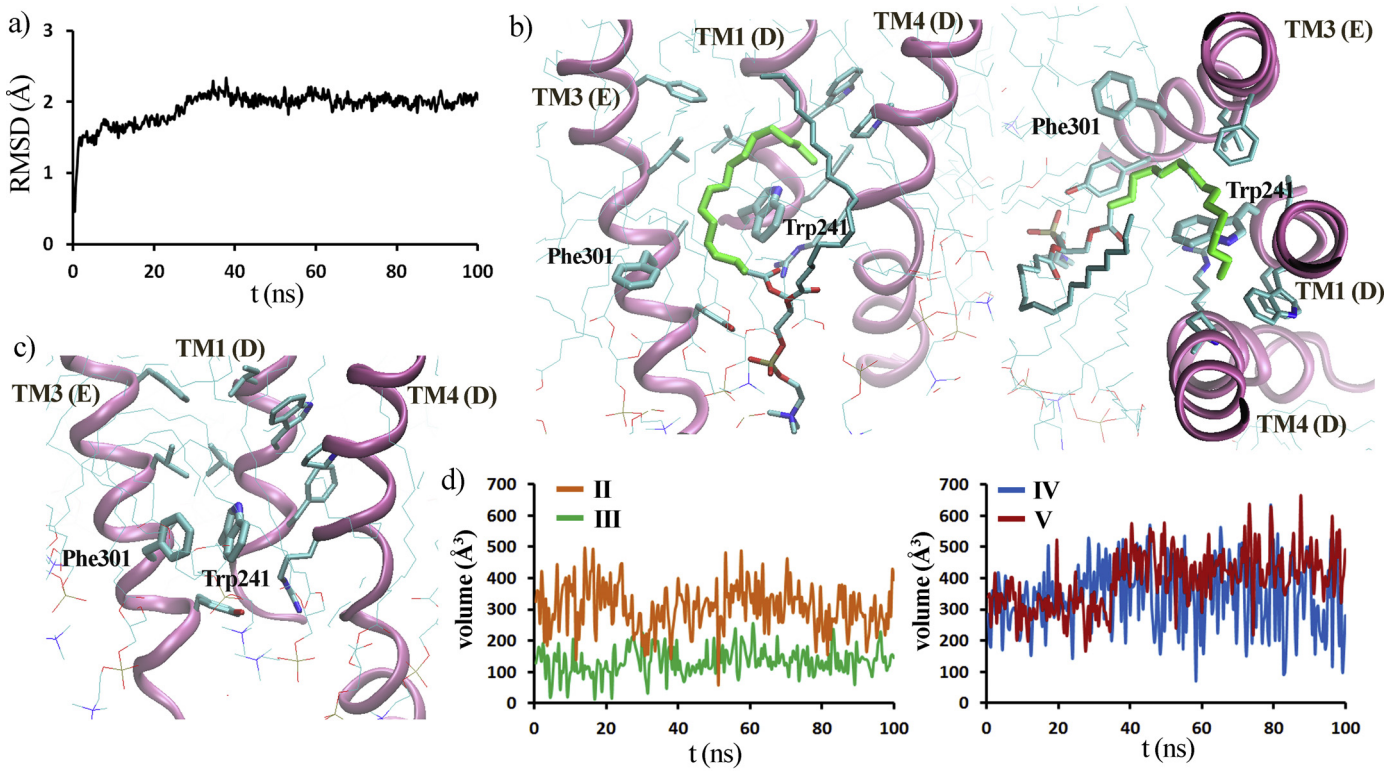

Fig. 6. The MD simulation of the apo $GABA_AR$-β_3 system shows that the neurosteroid cavities II–V are occupied by lipidic molecules. Temporal evolution of the RMSD and representative snapshots of the system are shown. (a) RMSD measured over CA atoms of $GABA_AR$-β_3 TMD. (b) Representative snapshot of the *apo* $GABA_AR$-β_3 TMD system showing the palmitoyl chain (in green) of the POPC lipid inside of the cavity IV (left panel: side view, right panel: top view). (c) Representative snapshot of the *apo* $GABA_AR$-β_3 TMD system showing the direct contact between Phe301 and Trp241 in cavity V. (d) Time evolution of the cavity volume (II in orange, III in green, IV in blue and V in red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

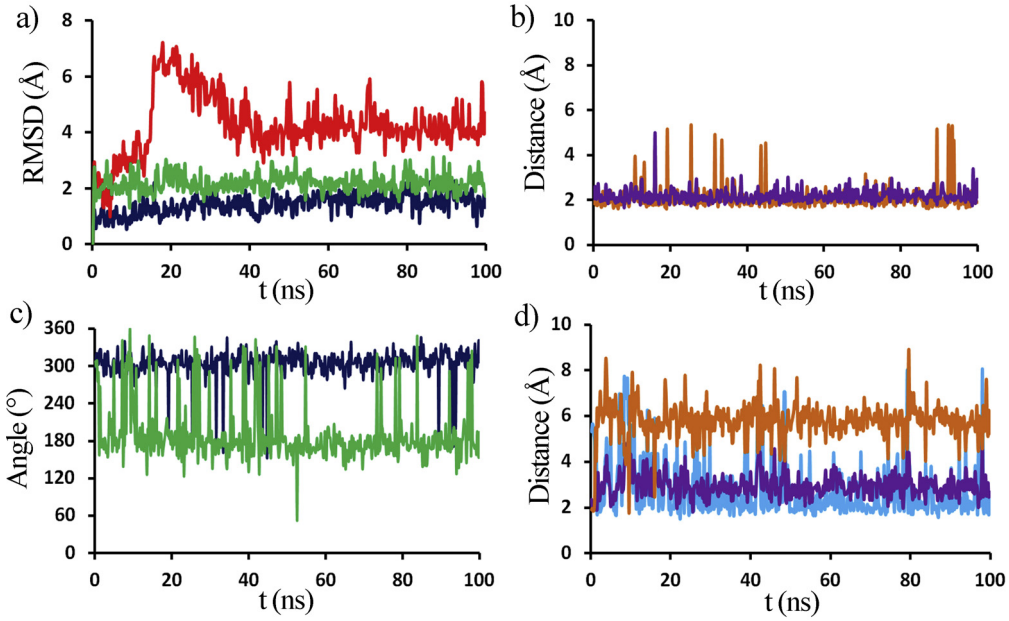

Fig. 7. The MD simulations of the $GABA_AR$-β_3/pregnanolone and $GABA_AR$-β_3/allopregnanolone systems reveal a stable neurosteroid binding. Temporal evolution of the RMSD and temporal evolution of selected distances and torsion angles are shown. (a) RMSD measured over ligand atoms for $GABA_AR$-β_3 TMD/pregnanolone complexes (pose A in blue and pose B in red) and $GABA_AR$-β_3 TMD/allopregnanolone complex (pose A in green). (b) Distance evolution in the $GABA_AR$-β_3/pregnanolone complex (pose A) between the HE1 atom of Trp241 and the oxygen atom of the 3α-OH group (in purple) and between the oxygen backbone atom of Arg428 and the hydrogen atom of the 3α-OH group (in orange). (c) Temporal evolution of the C2-C3-O-H torsion angles of pregnanolone (blue) and allopregnanolone (green) systems. (d) Distance evolution in the $GABA_AR$-β_3 TMD/allopregnanolone complex (pose A) between the HE1 atom of Trp241 and the oxygen atom of the 3α-OH group (in purple), between the oxygen backbone atom of Arg428 and the hydrogen atom of the 3α-OH group (in orange) and between the oxygen backbone atom of Trp237 and the hydrogen atom of the 3α-OH group (in cyan). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

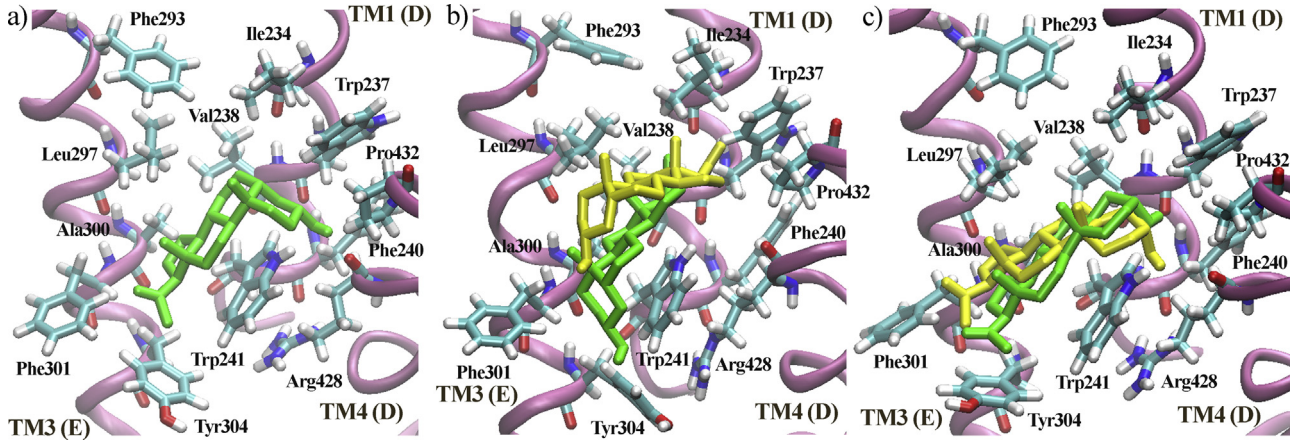

Fig. 8. The MD simulations of the $GABA_A$R-β_3/pregnanolone and $GABA_A$R-β_3/allopregnanolone systems reveal differences in the recognition of the 3α-OH group. Representative snapshots of the systems are shown. (a) Representative snapshot of the $GABA_A$R-β_3/pregnanolone complex (pose A). (b) Representative snapshot of the $GABA_A$R-β_3/pregnanolone complex (pose B), showing the pregnanolone molecule in the initial (yellow) and final position (green). (c) Representative snapshot of the $GABA_A$R-β_3/allopregnanolone complex (pose A), showing the allopregnanolone molecule in the initial (yellow) and final position (green). POPC molecules are omitted for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Trp237) (Fig. 7d). The difference of orientation of the 3α-OH group between neurosteroids can be clearly observed by monitoring the time evolution of the C2-C3-O-H torsion angles (Fig. 7c). Anyway, since allopregnanolone atoms always contacted the same residues than pregnanolone, the global conformation of the binding site was well conserved.

Thus, MD simulations showed that the binding mode determined by docking resulted unstable for allopregnanolone. However, a rapid and small modification of the disposition of the steroid allowed a stable binding mode, in which also two hydrogen bonds were involved. Although these interactions were less efficient than in the pregnanolone system, they are maintained during the timescale of the simulation, suggesting that $GABA_A$R-β_3 also would be able to properly recognize an overall flat structure.

3. Conclusion

In the past, understanding the molecular basis of neurosteroid modulation of $GABA_A$Rs was extremely difficult, mainly due to the lack of structural information. Fortunately, the recent resolution of the crystal structure of the β_3 homopentamer has paved the way to achieve this goal. Due to the heterogeneity of the system, different scenarios should be considered in order to comprehensively decipher the intricate relationship among steroids, protein and membrane, including a possible unspecific action of neurosteroids through the modification of the physical properties of the membrane [12].

In order to shed light on the molecular basis of the direct neurosteroid action, we believe that a mandatory first step resides in the precise identification of receptor residues involved in the binding of these compounds. We have applied a combination of computational methods to examine the $GABA_A$R-β_3 TMD structure and its interaction with neurosteroids, discovering that there are conserved superficial cavities between TM1 and TM3 α-helices of adjacent subunits in which neurosteroids are specifically recognized. These theoretical findings are well supported by the set of experimental data reported for the action of neurosteroids on β_3 homopentamers. First, the residue that was photolabeled (Phe301) by 6-azipregnanolone is actually part of the neurosteroid binding site. Although in this binding mode the distance between the C6 of the steroid and Phe301is too large to directly interact, we speculate that the reaction between the photoreactive group and the aromatic ring could take place during the binding or unbinding processes. Second, both overall torsioned and flat steroids can be properly bound, sustaining the inhibition of [^{35}S]TBPS binding observed with 6-azipregnanolona and allopregnanolone, respectively. Third, the selectivity for the α configuration of the 3-OH group can be certainly explained based on the structural characteristics of this site. Thus, to our knowledge, this constitutes the first neurosteroid binding site identified for a $GABA_A$Rs.

As was previously discussed by Chen et al. [20], it is not trivial to assign this site to the potentiation or the activation functional effects of neurosteroids, basically because these effects have not been characterized in β_3 homopentamers. In HEK293 cells expressing these receptors, allopregnanolone did not activate discernible currents in electrophysiological assays [19], suggesting that the identified neurosteroid site would not be functional. However, this steroid does caused a small enhancement of propofol-activated currents and strongly inhibited the [^{35}S]TBPS binding [19]. As these authors have indicated, the relationship between the electrophysiological actions of neurosteroids and the modulation of $GABA_A$ receptor binding is complex, and correlations between these two experimental approaches are not trivial due to their different time courses.

Nevertheless, we propose that the inhibition of [^{35}S]TBPS binding can be associated with the presence of neurosteroids at the identified site. Since this effect is produced at high neurosteroid concentration, it could be correlated with sites mediating direct activation in heteropentameric $GABA_A$Rs [20]. On the other side, Hosie et al have proposed that the activation site in $\alpha_1\beta_2\gamma_2$ receptor is formed by residues of adjacent subunits, while the potentiation site is formed by residues of the same subunit [13,14], supporting the idea that the site we found at subunit interface is involve in the activation effects. Nevertheless, since the number and class of neurosteroid sites depend on the subunit composition, extrapolations between $GABA_A$Rs could not be in principle possible, and each particular case should be considered. The construction of homology models using the $GABA_A$R-β_3 as template, followed by a computational analysis, could provide a valuable tool to identify neurosteroid binding sites in other, more physiological, receptors. In this context, our finding could be taken as a first step to explore the allosteric mechanism by which these small lipophilic molecules affect the receptor structure and dynamics.

4. Computational methods

4.1. Initial $GABA_A R$-β_3 TMD structure

The initial coordinates of $GABA_A R$-β_3 TMD were obtained from the crystal structure of the full-length $GABA_A R$-β_3 (PDB ID: 4COF) [18] by removal of residues 1–213 of each subunit. In this way, the used $GABA_A R$-β_3 TMD is formed only by residues 214–447. The artificial loops between TM3 and TM4 were conserved as in the crystal structure. The name of subunits (A–E) corresponds to the name of crystalized chains.

4.2. Initial structures of steroids

The structure of 3α-hydroxy-5β-pregnan-20-one (pregnanolone), 3β-hydroxy-5β-pregnan-20-one (3β-pregnanolone), 3α-hydroxy-5β-6-azi-pregnan-20-one (6-azipregnanolone) and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) were optimized with the *ab initio* method HF/6-31G** using the Gaussian 03 program [34].

4.3. Cavity detection

The fpocket method [29] was applied on the initial $GABA_A R$-β_3 TMD structure using the default parameters, except the *-m* values that was adjust to 4 Å in order to analyze more solvent exposed cavities, thus filtering out very buried ones. The mdpocket method was applied using the default parameters over 100 snapshots extracted from the apo $GABA_A R$-β_3 TMD trajectory, in which were previously deleted all lipidic and water molecules. Then cavities II–V were selected with the Pymol program [35] and the time evolution of volumes were calculated.

4.4. Docking

The Autodock 4.2 method [30] was used to dock the optimized structures of steroids in the $GABA_A R$-β_3 TMD. Rotatable bonds of steroids (C3-O3 and C17-C20) were allowed to rotate freely, the receptor was considered as a rigid molecule. Two strategies were used for docking pregnanolone. On one side, a grid of 126x126x64 points with a spacing of 0.5 Å centered in the origin of the $GABA_A R$-β_3 TMD, was calculated and used to obtain 500 runs of the genetic algorithm method. The solutions obtained were analyzed with a cluster tolerance of 10 Å. On the other side, five grids (one for each cavity I–V) of 110x110x110 points with a spacing of 0.2 Å centered in the geometrical center between residues Phe301 and Trp241, were calculated and used to obtain 500 runs of the genetic algorithm method. In this case, solutions were analyzed with a cluster tolerance of 2 Å. The dockings of 3β-pregnanolone and allopregnanolone were performed in the cavity IV using the same calculated grid. For all steroids, the torsion angles defined by the C3-O bond and the C17–C20 bond were considered as rotables.

4.5. Molecular dynamics simulation

Molecular dynamics simulations (MD) were performed with the AMBER 14 software package [36]. The initial coordinates of the $GABA_A R$-β_3 TMD immersed in a 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphatidylcholine (POPC) membrane bilayer (*apo* system) were obtained with the Membrane Builder utility [37] using the replacement method. The first principal axis of the receptor was aligned to the *z*-axis and then the receptor was embedded into a 125 Å × 125 Å POPC membrane fully hydrated with TIP3P water molecules (thickness of 30 Å on top and bottom of the system). Na^+ and Cl^- were added to obtain a final ion concentration of 0.15 M. The resultant system is composed by one $GABA_A R$-β_3 TMD, 418 POPC, 110 Cl^- ions, 85 Na^+ ions, and 35.545 water molecules given a total of over 173.000 atoms.

All *holo* systems were constructed from the initial apo system using the best docking solution of pose A and B of pregnanolone, pose A of 3β-pregnanolone and pose A of allopregnanolone in the cavity IV. No superpositions between steroids and POPC molecules were found in these initial systems. The FF14SB force field parameters were used for all receptor residues and the Lipid14 force field parameters were used for POPC molecules. Steroids parameters were assigned according to the general AMBER force field (GAFF) and the corresponding RESP charges at the HF/6-31G** level using the Antechamber module.

All systems were initially minimized for 10,000 steps and then were heated through two sequential steps of 250 picoseconds. First, systems were heated to 200 K at constant volume. Then temperature was slowly increased at constant pressure to the desired production temperature (300 K). In both steps a restraint (10 kcal/mol/$Å^2$) fixing the backbone protein and lipid atoms was applied. Finally, 250 picoseconds were carried out at 1 atm and 300 K in which the restraint on the protein backbone and lipids was gradually reduced to zero. Starting from these equilibrated structures, MD production runs of 100 ns of *apo* $GABA_A R$-β_3 TMD, $GABA_A R$-β_3 TMD/pregnanolone in pose A and B, $GABA_A R$-β_3 TMD/3β-pregnanolone in pose A, $GABA_A R$-β_3 TMD/allopregnanolone in pose A were carried out. All production simulations were performed at 1 atm and 300 K, maintained with the Berendsen barostat and thermostat, respectively, using periodic boundary conditions and the particle mesh Ewald method (grid spacing of 1 Å) for treating long-range electrostatic interactions with a uniform neutralizing plasma. The SHAKE algorithm was used to keep bonds involving H atoms at their equilibrium length, allowing the use of a 2 fs time step for the integration of Newton's equations. In all systems, in order to maintain the overall position of the truncated N-terminal extreme, a small harmonic restraint (with force constant of 3 kcal/mol/$Å^2$) was applied to CA atoms of residues 214 and 215 of each subunit. A hydrogen bond was defined as present whenever the distance between the hydrogen atom and the heavy atom involved in the interaction was less than 2.5 Å.

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