Journal of Steroid Biochemistry & Molecular Biology 154 (2015) 159–167

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

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

Lautaro D. Alvarez [a,*], Darío A. Estrin [b]

[a] Departamento de Química Orgánica and UMYMFOR (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EGA Ciudad de Buenos Aires, Argentina
[b] Dpto. Química Inorgánica Analítica y Química Física and INQUIMAE (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EGA Ciudad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 27 April 2015
Received in revised form 20 July 2015
Accepted 22 July 2015
Available online 26 July 2015

Keywords:
Neurosteroids
$GABA_A$ receptor
β_3 homopentamers
Binding site
Specific recognition

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.

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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

* Corresponding author. Tel.: +54 11 4576 3385.
E-mail addresses: lalvarez@qo.fcen.uba.ar (L.D. Alvarez), dario@qi.fcen.uba.ar (D.A. Estrin).

http://dx.doi.org/10.1016/j.jsbmb.2015.07.012
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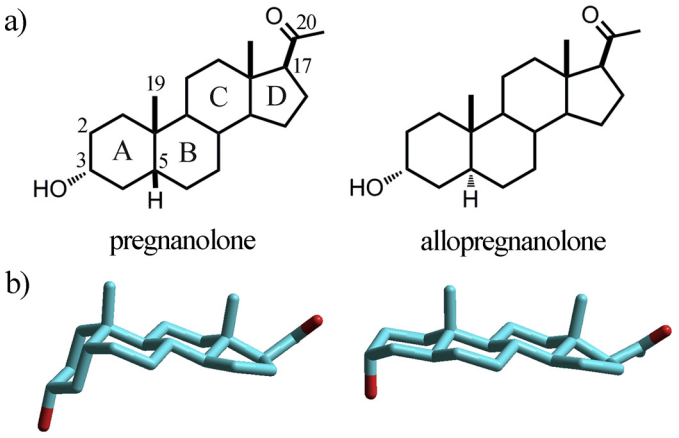

Fig. 1. Neurosteroid modulators of the $GABA_AR$-β_3. (a) Structure of pregnanolone and allopregnanolone; (b) Schematic representations of the optimized geometries at the HF/6-31G** level of theory.

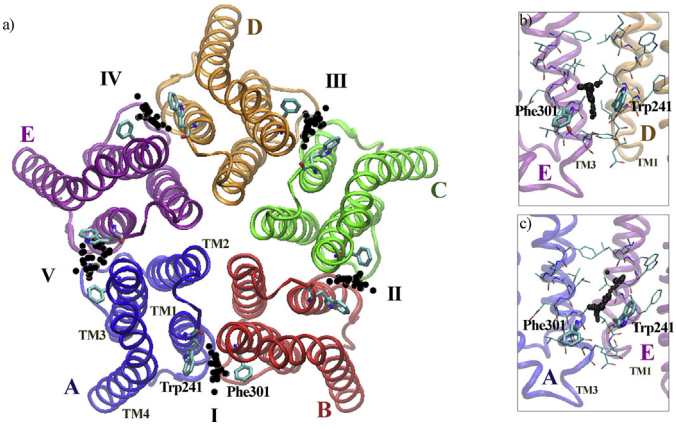

Fig. 2. The $GABA_AR$-β_3 TMD presents five surface cavities which are partially delimited by Phe301 residues. The centers of alpha spheres generated by fpocket are depicted as black points. (a) Overall view of the $GABA_AR$-β_3 TMD structure showing the localization of the subunits (A–E), TM α-helices (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

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