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EFFECT OF SYNTHETIC STEROIDS ON $GABA_A$ RECEPTOR BINDING IN RAT BRAIN

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Abstract—Neuroactive steroids, like allopregnanolone (A) and pregnanolone (P), bind to specifics sites on the $GABA_A$ receptor complex and modulate receptor function. They are capable to inhibit or stimulate the binding of $GABA_A$ receptor-specific ligands, like *t*-butyl-bicyclophosphorothionate, flunitrazepam and muscimol. We have previously characterized a set of oxygen-bridged synthetic steroids (SS) analogs to A or P using synaptosomes. Considering that the subunit composition of the $GABA_A$ receptor throughout the central nervous system affects the magnitude of the modulation of the $GABA_A$ receptor by NAS, we evaluated the action of two selected SS, in brain sections containing the cerebral cortex (CC) and hippocampus (HC) using quantitative receptor autoradiography. Both SS affected the binding of the three ligands in a similar way to A and P, with some differences on certain CC layers according to the ligand used. One of the SS, the 3α-hydroxy-6,19-epoxypregn-4-ene-20-one (compound 5), behaved similarly to the natural neuroactive steroids. However, significant differences with compound 5 were observed on the HC CA2 region, making it steroid suitable for a specific action. Those differences may be related to structural conformation of the SS and the subunits' composition present on the receptor complex.
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Key words: neurosteroids, synthetic steroids, $GABA_A$ receptor, cerebral cortex, hippocampus, autoradiography.

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Abbreviations: CC, cerebral cortex; FLU, flunitrazepam; HC, hippocampus; L-Mol, stratum radiatum lacunosum moleculare layer; MUS, muscimol; SS, synthetic steroids; TBPS, *t*-butyl-bicyclophosphorothionate.

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INTRODUCTION

The $GABA_A$ receptor–ionophore complex includes sites for specific GABA binding, benzodiazepines, barbiturates and convulsant (Akk et al., 2007; Veleiro and Burton, 2009). When two GABA molecules bind to its receptor, conformational changes occur, leading to the opening of the channel and the entry of chloride ions into the neuron. According to the reversal potential of the permeate ions, the postsynaptic GABA response can be excitatory or inhibitory (Akk et al., 2007).

This receptor complex can exist in multiple isoforms with a variety of pharmacological profiles that arise from its heteropentameric structure and diversity of subunits. The distribution of these subunits indicates the existence of heterogeneously constituted $GABA_A$ receptor complexes within the brain. Arrangements with different subunits were described in various cortical or hippocampal subfields where they may exert different physiological or pharmacological actions upon stimulation by GABA or different agonists (Sperk et al., 1997).

Among the endogenous modulators of $GABA_A$ receptor complex are the neurosteroids. These steroids regulate GABA activity on synapses and extrasynaptic sites and cause immediate changes in neuronal excitability (Akk et al., 2007; van Broekhoven and Verkes, 2003; Veleiro and Burton, 2009). Allopregnanolone (**A**; 3α-hydroxy-5α-pregnan-20-one) and its 5β isomer, pregnanolone (**P**; 3α-hydroxy-5β-pregnan-20-one) are well-known endogenous modulators of $GABA_A$ receptor that are locally synthesized by glial cells and neurons (Gasior et al., 1999).

These steroids, 3α-hydroxy-5α/β progesterone metabolites induce sedation and can be used as anesthetic drugs in humans (Carl et al., 1990; Timby et al., 2006). Endogenous neuroactive steroids possess anticonvulsant properties as well as anxiolytic and sedative-hypnotic effects (Akk et al., 2007) applicable to the treatment of several neurological and psychiatric disorders (Schüle et al., 2011). However, their therapeutic potential could be limited to their *in vivo* rapid metabolism. For that reason, synthetic steroids (SS) that exhibit better bioavailability and efficacy have an important therapeutic potential in brain disorders. The effect of neurosteroids on the $GABA_A$-receptor depends on the type of steroid, related to the intrinsic structure of the steroid, the localization of the receptors and the subunit compositions. There are more than 20 distinct $GABA_A$ receptor isoforms widely expressed throughout the central nervous system,

involved on the regulation of neuronal excitability, synaptic integration and network oscillation dynamics. Some receptor isoforms are crucial for many cognitive functions associated to the neocortex and the hippocampus (HC) (Möhler, 2009; Prut et al., 2010; Trincavelli et al., 2012).

Several structure/activity studies indicate that while endogenous neuroactive steroids may accept chemical modification at some carbon atoms, the 3α-hydroxyl configuration is important and required for binding and activity in all assays (Souli et al., 2005; Scaglione et al., 2006; Suñol et al., 2006). On the other hand, the configuration of C5-reduction is important to its potency. Even if steroids with either 5α or 5β conformations are active, spatial difference in this position may affect their pharmacology. These two configurations produce important changes on the A/B steroid rings' angle (Alvarez et al., 2008).

In vitro **A** and **P** inhibit the binding of *t*-butyl-bicyclophosphorothionate (TBPS, Ramanjaneyulu and Ticku, 1984; Majewska et al., 1986; Gee et al., 1988) to the GABA-operated chloride channel, potentiate GABA's effects on chloride uptake, and increase the bindings of muscimol (MUS) and flunitrazepam (FLU) to $GABA_A$ receptor (Belelli and Gee, 1989; McCarthy et al., 1992; Hawkinson et al., 1994; Hamilton, 2002). Based on that information, in a previous work, we evaluated the action of SS (related to **A** and **P)** in synaptosome membranes (Rey et al., 2013). The incorporation of an oxygen bridge was the main feature of these SS. This modification has provided analogs with a conformational restricted A/B angle in the steroid nucleus (Veleiro and Burton, 2009). We have shown that a synthetic **A** analog, 3α-hydroxy-2β, 19-epoxy-5α-pregnan-20-one (compound **3** Fig. 1a) was able to inhibit TBPS and stimulate FLU binding in a similar manner to **A** and **P**, but without effect on MUS binding. Whereas one **P** analog, 3α-hydroxy-6,19-epoxy-pregn-4-ene-20-one (compound **5**; Fig. 1b) was the most efficient SS on the ligands binding (Rey et al., 2013).

The aim of this work was to evaluate the action of these two SS on the $GABA_A$ receptor binding properties, but now on brain tissue sections using quantitative *in vitro* autoradiography. To carry on these observations we tested three specific receptor ligands in sections containing the dorsal HC and cerebral cortex (CC).

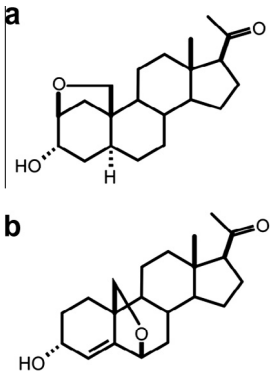

Fig. 1. Planar structure of 3α-hydroxy-2β, 19-epoxy-5α-pregnan-20-one, (compound **3**; a) and 3α-hydroxy-6,19-epoxypregn-4-ene-20-one (compound **5**, b).

EXPERIMENTAL PROCEDURES

Steroids

The SS were divided according to their spatial conformation in: **A** like, the 3α-hydroxy-2β, 19-epoxy-5α-pregnan-20-one (compound **3**, Eduardo et al., 2003; Fig. 1a), and **P** like, the 3α-hydroxy-6,19-epoxypregn-4-ene-20-one (compound **5**, Veleiro et al., 2003; Fig. 1b). The compounds' homogeneity was confirmed by thin layer chromatography (Alvarez et al., 2008). A 5 mM stock solution in dimethylsulfoxide (DMSO, Sigma–Aldrich, St. Louis, MO, USA) was used for all steroids. Steroid concentration (25 nM) was chosen based on our previous results of ligand's inhibition or stimulation binding curves (Rey et al., 2013).

Experimental animals

All procedures concerning animal care and use were carried out according to the European Community Council Directive (86/609/EEC), and the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague–Dawley rats (200–250 g) were housed under standard laboratory conditions with a 12-h light–dark cycle and with food and water *ad libitum*. Animals were rendered unconscious by CO_2 and perfused through the left ventricle with 60 ml of cold saline and then decapitated. Brains were removed, quickly frozen with powdered dry ice and stored at −80 °C until sectioning.

Preparation of coronal slices

Frozen brains were mounted onto cryostat chucks using Tissue-Tek for assembly, and sectioned at −15 °C. Successive coronal sections (16-μm-thick) were taken, from Bregma −2.20 mm to Bregma −3.8 mm, (Plates 27–33 of Paxinos and Watson Atlas (1998)). Sections were mounted on subbed slides (Gelatine 1%, chromium potassium sulfate 0.05%). Slides were dried and stored at −80 °C until use. Sections from different animals were matched prior to the binding assays by Nissl stain.

$GABA_A$ receptor binding patterns with autoradiographic methods

Binding studies were performed using three different ligands: butyl bicyclophosphorothionate, tertiary-[^{35}S] ([^{35}S]-TBPS, 200 Ci/mmol, NEG-049), [^{3}H]-Flunitrazepam ([^{3}H]-FLU, 85.2 Ci/mmol, NET-567) and [^{3}H]-Muscimol ([^{3}H]-MUS, 18 Ci/mmol, NET-574), all purchased from Perkin Elmer. Twelve slides containing two coronal sections were used for each ligand per animal. Slides were air dried for 30 min (min) at room temperature (25 °C), and washed 20 min with the specific ligand buffer. The sections were incubated 5–10 min in the presence of steroids (25 nM) and GABA (50 μM) in TBPS assay at 0–25 °C. Afterward, radioactive ligand was added, with respective

treatments. Incubation conditions for each ligand were: for [^{35}S]-TBPS (10 nM), buffer 50 mM Tris–HCl, 200 mM NaCl pH 7.1, 60 min at 25 °C in the presence of 50 μM GABA (Gee et al., 1988); for [^{3}H]-FLU (10 nM) buffer 50 mM Tris–HCl buffer pH 7.1; 30 min at 4 °C (Gonzalez et al., 1992) and for [^{3}H]-MUS (5 nM) buffer 50 mM Tris–Acetate buffer pH 7.4, 40 min at 4 °C (Schumacher et al., 1989). Nonspecific binding was determined by adding PTX (1 mM), Diazepam (Plidan 10, Roemmers, Argentina; 5 μM) or GABA (10 μM) to the incubation buffer respectively. After incubation, slides were rinsed 60 s in corresponding cold buffer, immediately dipped in deionized cold water and rapidly dried at room temperature. Slides containing incubated sections were exposed to tritium sensitive film: 10 days for [^{35}S]-TBPS, 15 days for [^{3}H]-FLU, and 180 days for [^{3}H]-MUS. Film exposure time was selected so that the optical densities in the regions measured lay in the linear response range of the film. Slides were exposed along with ^{14}C standards for TBPS or tritium standards ([^{3}H]-microscales, Amersham, Pittsburgh, PA, USA) for the other two ligands.

The generated autoradiograms were analyzed for quantitative densitometry using a video camera (CCD Sony-XC77), coupled to a Macintosh computer equipped with a video card (Data Translation) and a computerized image processing system NIH-Image software (developed by Wayne Rasband, 1995, NIH, Research Services Branch, NIMH, Bethesda, MD, USA).

Two sections were evaluated per animal for each case and optical density measurements were made bilaterally. Values were automatically converted to femtomol or picomol of ligand bound per milligram of wet weight tissue (fmol/mg wwt or pmol/mg wwt) using the curve generated with the co-exposed standards. Specific binding (SB) was obtained as the difference between total binding values and nonspecific binding.

Statistical analysis

Statistical analyses were performed with commercial softwares GraphPad Prism (GraphPad Software Inc., v.4) or Statview (SAS Institute Inc. v5.0.1). Steroid effects were analyzed by a one-way ANOVA and comparisons among the five groups were made by Fisher's PLSD test. Differences were considered significant when $p < 0.05$.

RESULTS

The effects of the two SS on the $GABA_A$ receptor complex were evaluated by *in vitro* quantitative autoradiography in coronal brain slices from adult male rats. The effects of each SS were compared to the **A** and **P** effects as an indirect assay for the impact of each steroid on the receptor function (Gasior et al., 1999).

[^{35}S]-TBPS binding

The convulsant TBPS binds to the PTX site in the $GABA_A$ receptor complex, and NAS can allosterically modulate its binding (Majewska et al., 1986). In the presence of GABA, these metabolites have a significantly increased binding affinity, and under this condition it is possible to reflect the functional state of $GABA_A$ receptors (Majewska et al., 1986; Hawkinson et al., 1994).

In the CC, all the steroids inhibited [^{35}S]-TBPS binding (Fig. 2b–d). However, steroid inhibition showed differences among layers (Table 1). Despite compounds **3** and **5** inhibited this ligand binding; the effect was reduced compared to their natural analogs. In cortical layers 1–3, natural NAS produced 84% of reduction on [^{35}S]-TBPS binding, whereas compound **3** and compound **5** inhibited 66% and 74%, respectively ($p < 0.05$). In layer 4, compound **5** showed significant differences compared to **P** ($p < 0.05$). In layers 5–6, **A** and **P** reduced 77% of the binding, whereas compounds **3** and **5** 60% and 73%, respectively.

In the HC, all steroids inhibited [^{35}S]-TBPS binding significantly (Fig. 2b, c), however compound **3** was less potent than the other steroids ($p < 0.05$; Fig. 2e). In the hippocampal regions all the steroids produced a binding reduction. In the oriens layer (Ors) of the CA1 and CA3 regions, compound **5** and **A** showed similar effects ($p < 0.05$), but in the CA2 region the NAS strongly inhibited the binding, especially **P** (93%; Table 1). In the stratum radiatum lacunosum moleculare layer (L-Mol) significant differences were observed in the CA1 region, where compounds **3** and **5** showed lower effects than **A** and **P** ($p < 0.05$). In CA2 and CA3 regions, the steroid effects followed the same pattern of [^{35}S]-TBPS binding inhibition (compound **3** < compound **5** = **A** < **P**; $p < 0.05$).

[^{3}H]-FLU binding

FLU binds to the BZDs site in the $GABA_A$ receptor, and NAS are capable to stimulate its binding (Majewska et al., 1986; Hawkinson et al., 1994). Therefore, we evaluated the effects of the SS treatments. In the CC, all steroids enhanced [^{3}H]-FLU binding (Fig. 3b, c). Compound **3** and **5** increased this binding similarly to their natural analogs (Fig. 4a). In cortical layers 1–4, **P** produced a higher stimulation than **A** ($p < 0.05$), and compound **3** significantly increased the binding in comparison to its analog in cortical layers 1–3 (Table 2). Compound **5** stimulated [^{3}H]-FLU binding like **A** in all CC layers.

In HC all the steroids also stimulated binding of this ligand to its site (Fig. 3b, c). Compound **5** showed similar effects as **A** and **P**, and compound **3** was the least potent ($p < 0.05$; Fig. 4b). In hippocampal regions, some differences on the binding stimulation were found (Table 2). In the CA1 Ors, both SS and **P** produced a lower increase than **A**. In contrast, the stimulation of [^{3}H]-FLU binding in the CA2 region by compound **5** was significantly higher than the other three steroids. In the CA3 region both SS showed a lower stimulation than **A** and **P** (27–28% vs. 166% and 133%, respectively). In CA1 and CA3 L-Mol, compound **5** stimulated the ligand binding 35% more than **A** or **P**. Nevertheless, it was much less effective that those steroids in the CA2 region ($p < 0.05$). Compound **3** produced the lowest increase on [^{3}H]-FLU binding in the hippocampal layers analyzed, except on L-Mol CA2.

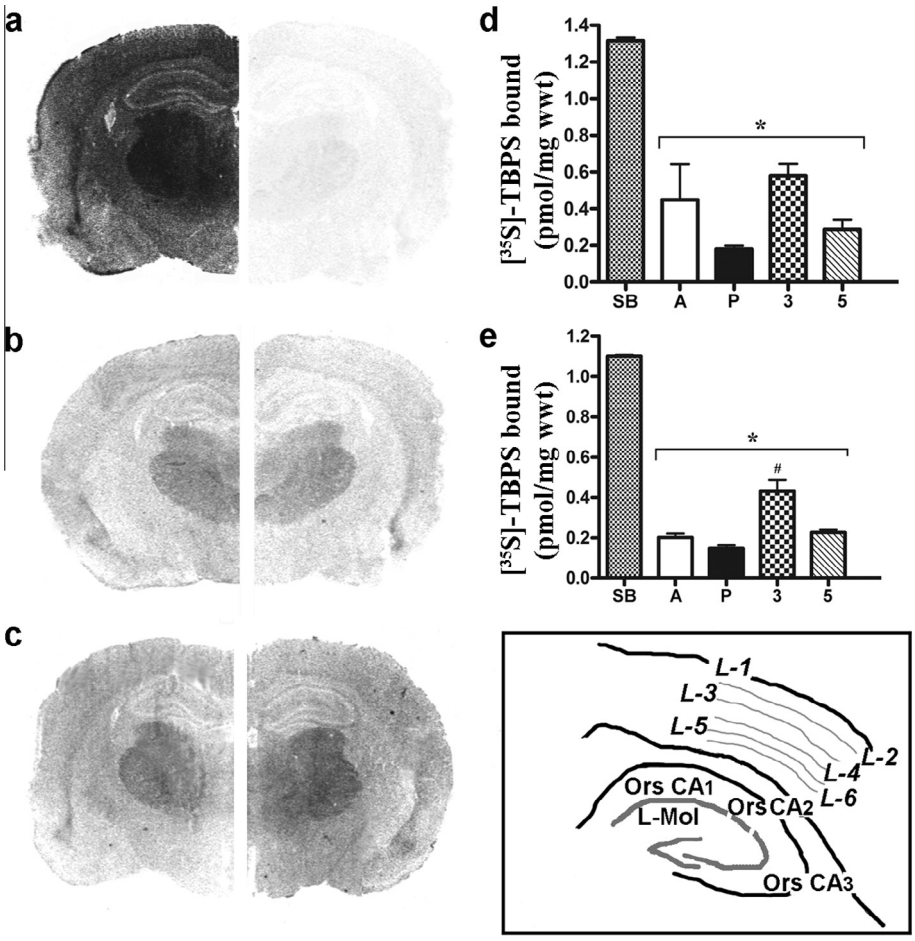

Fig. 2. Representative autoradiograms of [^{35}S]-TBPS bound (pmol/mg wwt) to rat brain coronal slices, for the different treatments: total binding (a, *left*), nonspecific binding (a, *right*), allopregnanolone (b, *left*), pregnanolone (b, *right*), compound **3** (c, *left*) compound **5** (c, *right*). ^{35}S-TBPS specific binding (SB) and in the presence of allopregnanolone (**A**), pregnanolone (**P**), compound **3** (**3**) and compound **5** (**5**) in cerebral cortex (d) and hippocampus (e). Results were expressed as mean ± standard error from two different assays, with n = 4–5 animals/group (two sections per animal). Significant effect of all steroids compared to SB and **A** were determined by a one-way ANOVA ($*^{\#}p < 0.05$). In cerebral cortex: $F(5,19) = 20.9$ and in hippocampus: $F(5,21) = 95.1$; $p < 0.001$. Bottom right: schematic diagram for the areas measured in cerebral cortex and hippocampus presented in Tables 1–3. L1–6 = cortical layers 1–6, Ors CA1–3 = hippocampal oriens layers, L-Mol = stratum radiatum and lacunosum moleculare layers.

[^{3}H]-MUS binding

MUS is a specific agonist for the $GABA_A$ high-affinity binding site. NAS increases the apparent affinity of this agonist in rat synaptosomes, in a dose-dependent manner (Harrison and Simmonds, 1984; Rey et al., 2013). In the CC, all steroids enhanced [^{3}H]-MUS binding (Figs. 3e, f and 4c). Analysis of the CC showed that **A** produced less stimulation than **P** and both SS, in layers 1–3 ($p < 0.05$). No differences were observed between compound **3** and **A** or compound **5** and **P** in layer 4. However, both SS showed lower stimulation than **A** or **P** in layers 5 and 6 ($p < 0.05$; Table 3).

In HC, all steroids increased [^{3}H]-MUS binding (Fig. 3e, f), but compound **5** stimulation was lower than the other three steroids ($p < 0.05$; Fig. 4d). In hippocampal regions, significant differences in CA1 Ors among **A** and the SS were observed ($p < 0.05$, Table 3). SS were less effective in CA2 Ors, and compound **5** produced a minor increase in CA3 Ors. In the L-Mol, **A** produced the highest binding stimulation in the CA2 region (87%). No differences were observed among SS or natural NAS in CA1, but in CA3 the presence of compound **5** produced the lowest [^{3}H]-MUS binding stimulation (39%; $p < 0.05$; Table 3).

DISCUSSION

Neurosteroids are synthesized within the brain and modulate neuronal excitability by rapid non-genomic action (Zheng, 2009). In particular, steroids derived from progesterone are highly selective and potent modulators of $GABA_A$ receptor-mediated neurotransmission (Gasior et al., 1999). They can act as paracrine messengers to locally influence neuronal activity (Gago et al., 2004) and play a different role in the regulation of behavior in humans and in several rodent models (Patchev et al., 1997). On the other hand, it is well known that the hippocampal formation is important for the acquisition of short-term memory (Bliss and Collingridge, 1993). Some of its structures are especially prone to neuronal damage in

Table 1. [^{35}S]-TBPS-specific binding in cortical layers and hippocampal areas, for the different conditions assessed. Results were expressed as mean ± standard error from two different assays, with n = 4–5 animals/group (two sections per animal). Significant effect of all steroids compared to SB (*) and differences to **A** (^{a}) and **P** (^{b}) were determined by a one-way ANOVA ($^{*,a,b}p < 0.05$). In cerebral cortex: $F_{CL1\text{-}3}(5,20) = 1371.1$, $p < 0.001$; $F_{CL4}(5,20) = 268.1$, $p < 0.001$; $F_{CL5}(5,20) = 184.8$, $F_{CL6}(5,20) = 193.5$, $p < 0.001$ and in hippocampus: $F_{OrsCA1}(5,19) = 222.4$, $p < 0.001$; $F_{OrsCA2}(5,19) = 139.2$, $p < 0.001$; $F_{OrsCA3}(5,20) = 136.5$, $F_{L\text{-}MolCA1}(5,19) = 300.1$, $p < 0.001$; $F_{L\text{-}MolCA2}(5,19) = 186.2$, $p < 0.001$; $F_{L\text{-}MolCA3}(5,19) = 85.6$, $p < 0.001$. Sub index in each F indicates the region analyzed.

	Specific binding	Allopregnanolone	Pregnanolone	Compound **3**	Compound **5**
Cerebral cortex					
Cortical layers:					
1–3	1.33 ± 0.01	0.21 ± 0.01*	0.20 ± 0.01*	0.45 ± 0.01^{*,a,b}	0.34 ± 0.01^{*,a,b}
4	1.37 ± 0.01	0.32 ± 0.02*	0.27 ± 0.01*	0.50 ± 0.02^{*,a,b}	0.36 ± 0.01^{*,b}
5	1.31 ± 0.01	0.32 ± 0.03*	0.29 ± 0.02*	0.51 ± 0.05^{*,a,b}	0.38 ± 0.03^{*,a,b}
6	1.33 ± 0.01	0.27 ± 0.02*	0.32 ± 0.02*	0.60 ± 0.04^{*,a,b}	0.34 ± 0.02^{*,a,b}
Hippocampus					
Oriens layers:					
CA1	1.18 ± 0.02	0.20 ± 0.03*	0.13 ± 0.01^{*,a}	0.37 ± 0.02^{*,a,b}	0.22 ± 0.01^{*,b}
CA2	1.17 ± 0.02	0.15 ± 0.02*	0.08 ± 0.01^{*,a}	0.32 ± 0.04^{*,a,b}	0.34 ± 0.04^{*,a,b}
CA3	1.14 ± 0.03	0.22 ± 0.03*	0.13 ± 0.02^{*,a}	0.43 ± 0.04^{*,a,b}	0.20 ± 0.01^{*,b}
Stratum radiatum					
Lacunosum moleculare layers					
CA1	1.12 ± 0.02	0.16 ± 0.01*	0.17 ± 0.03*	0.50 ± 0.04^{*,a,b}	0.26 ± 0.01^{*,a,b}
CA2	1.12 ± 0.01	0.22 ± 0.01*	0.10 ± 0.01^{*,a}	0.50 ± 0.04^{*,a,b}	0.19 ± 0.03^{*,b}
CA3	1.13 ± 0.02	0.18 ± 0.01*	0.13 ± 0.02^{*,a}	0.44 ± 0.07^{*,a,b}	0.22 ± 0.02^{*,b}

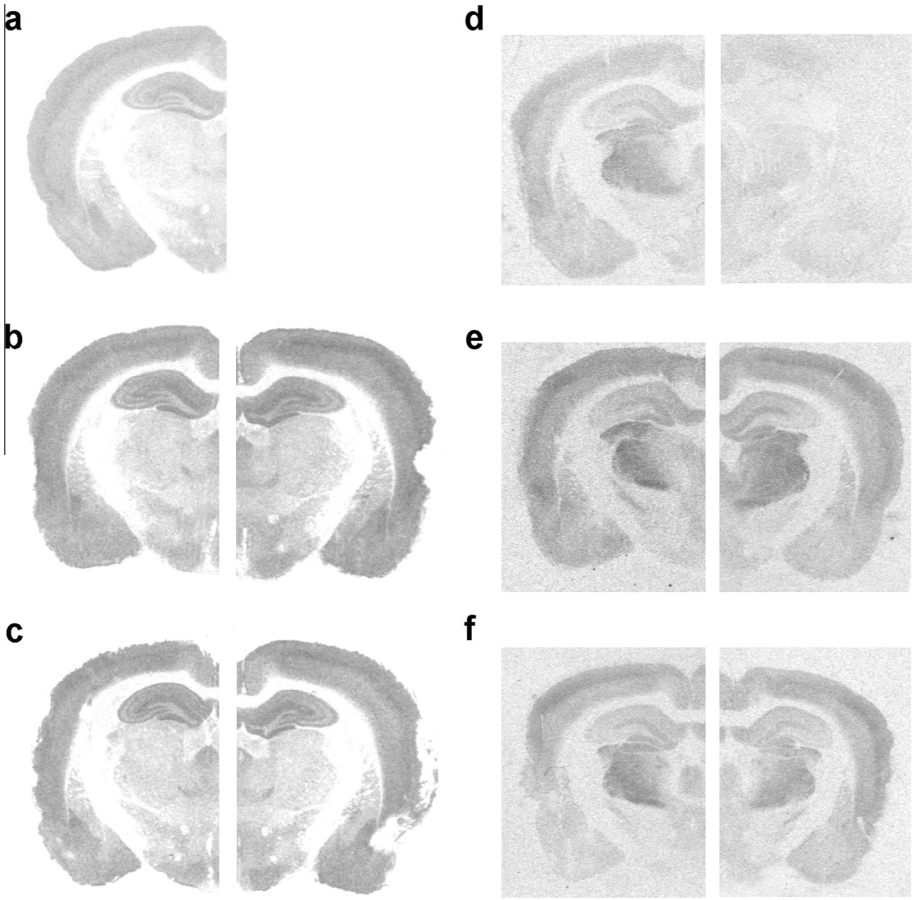

Fig. 3. Representative autoradiograms of [^{3}H]-FLU (a–c) and [^{3}H]-MUS (d–f) bound (fmol/mg wwt) to rat brain coronal slices for the different treatments: total binding (a, d, *left*), nonspecific binding (a, d, *right*), allopregnanolone (b, e, *left*), pregnanolone (b, e, *right*), compound **3** (c, f, *left*) and compound **5** (c, f, *right*).

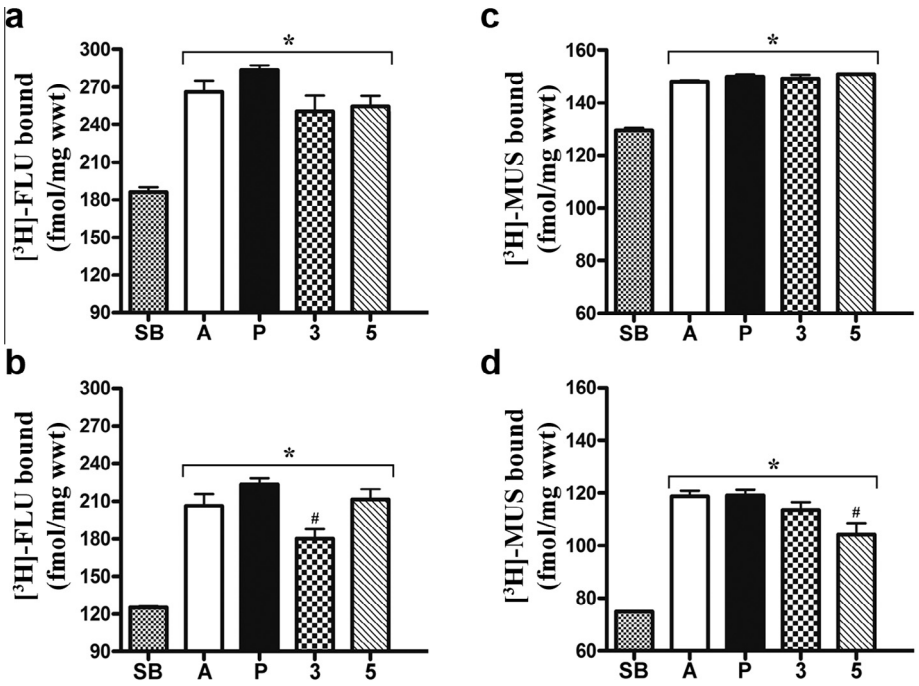

Fig. 4. ^{3}H-FLU (a, b) and [^{3}H]-MUS (c, d) specific binding (SB), in the presence of allopregnanolone (**A**), pregnanolone (**P**), compound **3** (**3**) and compound **5** (**5**) in cerebral cortex (a, c) and hippocampus (b, d). Results were expressed as mean ± standard error from two different assays, with n = 4–5 animals/group (two sections per animal). Significant effect of all steroids compared to SB and differences to **A** were determined by a one-way ANOVA ($*,^{\#}p < 0.05$). In cerebral cortex: $F_{^{3}\text{H-FLU}}(5,19) = 17.5$; $p < 0.001$; $F_{^{3}\text{H-MUS}}(5,28) = 67.4$; $p < 0.001$ and in hippocampus: $F_{^{3}\text{H-FLU}}(5,21) = 21.8$; $p < 0.001$; $F_{^{3}\text{H-MUS}}(5,26) = 22.4$; $p < 0.001$. Sub index in each F indicates the ligand used.

Table 2. [^{3}H]-FLU-specific binding in cortical layers and hippocampal areas, for the different conditions assessed. Results were expressed as mean ± standard error from two different assays, with n = 4–5 animals/group (two sections per animal). Significant effect of all steroids compared to SB (*) and differences to **A** (^{a}) and **P** (^{b}) were determined by a one-way ANOVA ($*,^{a,b}p < 0.05$). In cerebral cortex: $F_{CL1\text{-}3}(5,20) = 17.6$, $p < 0.001$; $F_{CL4}(5,20) = 30.9$, $p < 0.001$; $F_{CL5}(5,23) = 24.9$, $F_{CL6}(5,20) = 10.4$, $p < 0.001$ and in hippocampus: $F_{OrsCA1}(5,20) = 76.6$, $p < 0.001$; $F_{OrsCA2}(5,22) = 50.4$, $p < 0.001$; $F_{OrsCA3}(5,20) = 91.4$, $F_{L\text{-}MolCA1}(5,24) = 16.9$, $p < 0.001$; $F_{L\text{-}MolCA2}(5,21) = 56.7$, $p < 0.001$; $F_{L\text{-}MolCA3}(5,18) = 46.1$, $p < 0.001$. Sub index in each F indicates the region analyzed.

	Specific binding	Allopregnanolone	Pregnanolone	Compound **3**	Compound **5**
Cerebral cortex					
Cortical layers:					
1–3	205.02 ± 6.45	280.23 ± 4.44*	335.56 ± 17.48^{*,a}	333.37 ± 15.38^{*,a}	303.87 ± 13.09*
4	199.33 ± 13.36	334.34 ± 3.74*	432.56 ± 27.95^{*,a}	315.95 ± 15.00*	350.87 ± 18.48^{*,b}
5	161.52 ± 3.56	271.33 ± 13.85*	244.84 ± 9.03*	232.46 ± 5.96^{*,a}	285.61 ± 14.77^{*,b}
6	158.69 ± 2.52	218.06 ± 12.47*	201.27 ± 13.81*	213.82 ± 8.69*	210.91 ± 4.98*
Hippocampus					
Oriens layers:					
CA1	57.62 ± 1.51	162.55 ± 6.69*	127.28 ± 7.63^{*,a}	114.27 ± 6.54^{*,a}	128.48 ± 5.79^{*,a}
CA2	12.12 ± 0.44	38.60 ± 1.27*	39.82 ± 3.98*	22.02 ± 2.62^{*,a,b}	42.29 ± 2.65*
CA3	47.78 ± 0.60	103.02 ± 5.30*	111.30 ± 3.19*	57.39 ± 1.92^{*,a,b}	65.33 ± 1.63^{*,a,b}
Stratum radiatum					
Lacunosum moleculare layers:					
CA1	122.85 ± 7.98	227.96 ± 15.21*	235.94 ± 12.18*	218.76 ± 13.75*	271.48 ± 5.79^{*,a,b}
CA2	63.84 ± 1.57	154.85 ± 7.35*	149.11 ± 8.25*	114.55 ± 2.53^{*,a,b}	90.40 ± 3.19^{*,a,b}
CA3	75.55 ± 2.96	205.76 ± 13.87*	209.48 ± 16.81*	200.36 ± 5.73*	233.50 ± 4.04^{*,a,b}

chronic epilepsy and ischemia. Benzodiazepines and barbiturates are the treatment of choice in severe status epilepticus, although they may induce mild retrograde amnesia (Woods et al., 1992). These actions may be mediated, in part, by $GABA_A$ receptor located within the hippocampal formation (Sperk et al., 1997). Whereas this steroid may have therapeutic potential in anxiety, epilepsy and other brain disorders, bioavailability and *in vivo* efficacy should be tested.

In this study, we evaluated the capacity of two SS, **A**-like (compound **3**) and **P**-like (compound **5**), to modulate the $GABA_A$ receptor in rat coronal brain slices, taking

Table 3. ^{3}H-MUS-specific binding in cortical layers and hippocampal areas, for the different condition assessed. Results were expressed as mean ± standard error from two different assays, with n = 4–5 animals/group (two sections per animal). Significant effect of all steroids compared to SB (*) and differences to **A** ([a]) and **P** ([b]) were determined by a one-way ANOVA ($*^{,a,b}p < 0.05$). In cerebral cortex: $F_{CL1\text{-}3}(5,29) = 195.9$, $p < 0.001$; $F_{CL4}(5,26) = 102.7$, $p < 0.001$; $F_{CL5}(5,26) = 181.4$, $F_{CL6}(5,26) = 108.2$, $p < 0.001$ and in hippocampus: $F_{OrsCA1}(5,26) = 51.3$, $p < 0.001$; $F_{OrsCA2}(5,22) = 90.8$, $p < 0.001$; $F_{OrsCA3}(5,24) = 21.4$, $F_{L\text{-}MolCA1}(5,24) = 89.5$, $p < 0.001$; $F_{L\text{-}MolCA2}(5,26) = 75.9$, $p < 0.001$; $F_{L\text{-}MolCA3}(5,26) = 38.1$, $p < 0.001$. Sub index in each F indicates the region analyzed.

	Specific binding	Allopregnanolone	Pregnanolone	Compound **3**	Compound **5**
Cerebral cortex					
Cortical layers:					
1–3	125.07 ± 1.39	147.25 ± 0.83*	152.26 ± 0.78*,a	152.59 ± 0.65*,a	151.42 ± 0.46*,a
4	130.05 ± 2.13	150.47 ± 0.45*	154.32 ± 0.53*,a	152.47 ± 0.61*	153.86 ± 0.96*,a
5	121.99 ± 0.82	150.86 ± 0.64*	149.99 ± 0.79*	146.20 ± 0.84*,a,b	141.79 ± 0.70*,a,b
6	111.73 ± 1.00	141.84 ± 0.82*	143.09 ± 0.64*	138.12 ± 2.64*,a,b	137.80 ± 1.07*,a,b
Hippocampus					
Oriens layers:					
CA1	87.31 ± 3.73	133.17 ± 0.53*	126.19 ± 2.29*	124.34 ± 2.00*,a	113.55 ± 2.61*,a
CA2	33.66 ± 1.32	83.70 ± 2.41*	84.27 ± 2.66*	69.71 ± 3.21*,a,b	73.15 ± 1.66*,a,b
CA3	34.79 ± 0.99	68.15 ± 2.93*	69.64 ± 3.37*	65.92 ± 3.21*	57.10 ± 3.52*,a,b
Stratum radiatum					
Lacunosum moleculare layers					
CA1	77.60 ± 1.40	116.19 ± 1.09*	116.44 ± 0.94*	110.76 ± 2.55*	110.69 ± 2.60*
CA2	39.88 ± 1.12	74.48 ± 0.48*	64.11 ± 2.01*,a	62.82 ± 1.51*,a	67.43 ± 0.39*,a
CA3	42.15 ± 1.51	73.77 ± 1.22*	79.62 ± 2.55*,a	73.56 ± 3.04*	58.59 ± 2.62*,a,b

into account our previous results on synaptosome membranes (Rey et al., 2013). There are some structural requirements for neurosteroid modulation of $GABA_A$ receptors: a 3α-hydroxy group on the steroid A-ring and a hydrogen bond accepting group on the D ring at the C20 of the pregnane steroid side chain. Both are critical for positive activity (Purdy et al., 1990; Lambert et al., 2003). On the other hand, the orientation of the C5 hydrogen group appears to be essential for increased potency but less critical for activity (Morrow et al., 1990; Kokate et al., 1994; Xue et al., 1997). Both requirements are fulfilled on the SS tested, but in addition both analogs present an A/B restricted angle due to the oxygen bridge incorporation. To our knowledge, this is the first study in which the effects of different steroids on $GABA_A$ receptor activation were compared considering their structural differences.

Compound **3**, that is analog to **A**, was able to stimulate [^{3}H]-FLU, [^{3}H]-MUS and inhibit [^{35}S]-TBPS binding in the CC and HC more weakly than the other steroids. However, compound **5** showed a similar effect to those of the natural NAS. This **P**-like analog, was more effective than compound **3** inhibiting [^{35}S]-TBPS binding and stimulating [^{3}H]-FLU and [^{3}H]-MUS binding. These results are in accordance to the proposed existence of at least two specific sites for steroid interaction in the $GABA_A$ receptor, one involved in the opening process and the other related to potentiation (Akk et al., 2007). The different responses observed with the steroid used among the different layers of the CC and HC may be related not only to the spatial conformation of the steroid but also to the existence of different $GABA_A$ receptor populations (Lambert et al., 2001).

Cortical neurons express a great diversity of $GABA_A$ receptor-subtypes. Analyses of the $GABA_A$ receptor based on the visualization of α-subunit variants revealed a regional distribution pattern. The α1 and α2 subunit expressions are preferentially localized on layers 1–6 and 1–4 respectively, meanwhile the α3 subunit is preferentially expressed on layers 5–6 (Fritschy and Brünig, 2003). In the HC, the subunit composition of the $GABA_A$ receptor could be cell-specific (Weiland and Orchinik, 1995; Sperk et al., 1997). The α1 subunit, which preferentially associates with the β subunit, appears to be expressed in interneurons (Gao and Fritschy, 1994). However, neurosteroid modulation of GABA-induced chloride current is not dependent on the β subunit present in the $GABA_A$ receptor complex (Haddingham et al., 1993) but, the γ subunit isoform seems to play an important role determining their efficiency.

Moreover, regional differences in responsiveness of $GABA_A$ receptors to neurosteroids may occur, and the modulation would be dependent on the type of α and/or γ subunits expressed in a particular region (Garrett et al., 1997).

On the other hand, we have previously demonstrated that both SS affect the 3β-hydroxysteroid dehydrogenase enzyme activity in the CC and HC, quantified indirectly as NADH formation. In those assays, we could observe that compound **5** caused a similar impact as **A** and **P** on the enzyme activity, whereas compound **3** produced an important negative effect (Rey et al., 2013). Moreover, in the same work, a neuroprotective **A**-like effect in a hypoxic event (Kruse et al., 2009) was observed with compound **5** (Rey et al., 2013). Those results as well as the presented here, indicate that compound **5** is an interesting steroid for further trials in other systems.

Summarizing, the effects of two SS, with a decreased flexibility between A/B ring that confers a more favorable spatial arrangement for the steroid binding site, were evaluated using three different ligands for $GABA_A$ receptor, comparing their performance with **A** and **P** in

the CC and HC. At least one of them (compound **5)** showed to be a suitable steroid to be used as an **A** agonist.

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