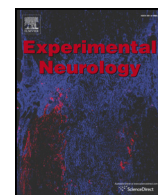
Experimental Neurology 249 (2013) 49–58

Contents lists available at ScienceDirect

Experimental Neurology

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



Neuroprotective action of synthetic steroids with oxygen bridge. Activity on $GABA_A$ receptor

Mariana Rey [a], María Sol Kruse [a], Lautaro D. Alvarez [b], Alberto A. Ghini [b], Adriana S. Veleiro [b], Gerardo Burton [b], Héctor Coirini [a,c,*]

[a] Laboratorio de Neurobiología, Instituto de Biología y Medicina Experimental (IBYME-CONICET), Vuelta de Obligado 2490, C1428ADN Ciudad Autónoma de Buenos Aires, Argentina
[b] Departamento de Química Orgánica, Unidad de Microanálisis y Métodos Físicos aplicados a Química Orgánica (UMYMFOR-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (FCEN-UBA), Pabellón 2, Ciudad Universitaria, Intendente Güiraldes 2160, C1428EHA Ciudad Autónoma de Buenos Aires, Argentina
[c] Departamento de Bioquímica Humana, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 5to Piso, C1121ABG Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 26 April 2013
Revised 24 July 2013
Accepted 27 July 2013
Available online 5 August 2013

Keywords:
Neurosteroids
3β-HSD
Hypoxia
Hippocampus
Hypothalamus
Cerebellum

ABSTRACT

Allopregnanolone (**A**) and pregnanolone (**P**) are able to modify neural activities acting through the $GABA_A$ receptor complex. This capacity makes them useful as anticonvulsant, anxiolytic, or anti-stress compounds. In this study, the performance of seven synthetic steroids (SS) analogous of **A** or **P** containing an intramolecular oxygen bridge was evaluated using different assays. Competition assays showed that compounds **1**, **5**, **6** and **7** affected the binding of specific ligands for the $GABA_A$ receptor in a way similar to that of **A** and **P**, whereas compounds **3** and **4** stimulated [^{3}H]-flunitrazepam and reduced [^{35}S]-TBPS binding. The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) produces the precursor for **A** and **P**, and its activity is regulated by steroids. The action of several SS on 3β-HSD activity was tested in different tissues. All SS analyzed inhibit its activity, but compound **5** was the least effective. Finally, the neuroprotective role of two SS was evaluated in cerebral cortex and hippocampus cultures subjected to hypoxia. Glial fibrillary acidic protein (GFAP) increase was prevented by **A**, **P**, and compounds **3** and **5**. Only **A**, **P** and compound **5** prevented neurofilament (NF160/200) decrease in hippocampus cultures, whereas **A** and compound **5** partially prevented NF200 and NF160 decreases respectively in cerebral cortex cultures. **A** prevented microtubule associated protein (MAP 2b) decrease in cerebral cortex cultures, while in hippocampus cultures only compounds **3** and **5** had effect. All steroids prevented MAP 2c decrease in both brain regions.

© 2013 Elsevier Inc. All rights reserved.

Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). Changes in GABA levels or its metabolism induce profound physiological and behavioral changes. The $GABA_A$ receptor–ionophore complex can exist in multiple isoforms with a variety of pharmacological profiles that arise from its heteropentameric structure and diversity of subunits. These isoforms are formed from the assembly of two α subunits, two β subunits and one additional γ subunit, but the existence of multiple subtypes of subunits (δ, ε, π, θ) creates a vast pool of possible isoforms, resulting in receptors with strikingly different pharmacological and biophysical properties. In the mammalian brain, there is limited combinatorial possibilities where the α1 β2 γ2 isoform is the most abundant. The $GABA_A$ receptor contains many distinct binding sites for a variety of ligands, including GABA, benzodiazepines (BZDs), barbiturates and convulsant channel antagonists like picrotoxin (PTX, Akk et al., 2007; Veleiro and Burton, 2009). Binding of GABA to its receptor gates an intrinsic anion-selective channel, and, depending on the reversal potential of the permeate ions the postsynaptic GABA response can be excitatory or inhibitory (Akk et al., 2007). The inhibition of GABA synthesis or its binding to specific receptors may evoke seizures in a variety of animal models.

The principal modulators of GABAergic function in the brain are neurosteroids. Steroids are able to modify neural activities causing immediate changes in neuronal excitability (non-genomic effects, Akk et al., 2007; Veleiro and Burton, 2009; van Broekhoven and Verkes, 2003). Interaction of neuroactive steroids (NAS) with the $GABA_A$ receptor can result in three types of effects: potentiation of currents elicited by GABA or another activator, inhibition of these currents, or direct activation of the ion channel. The typical EC_{50} values for $GABA_A$ receptor potentiation by NAS are in the high nanomolar range (Akk et al., 2007). Endogenous NAS like 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, **A**) and its 5β isomer (pregnanolone, **P**) are synthesized in neural tissues, from cholesterol *via* progesterone (P4) by 5α

* Corresponding author at: Laboratorio de Neurobiología, Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, C1428ADN Ciudad Autónoma de Buenos Aires, Argentina. Fax: 54-11-4786-2564.
E-mail address: hcoirini@ibyme.conicet.gov.ar (H. Coirini).

0014-4886/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.expneurol.2013.07.020

or 5β-reductases and 3α-hydroxysteroid dehydrogenase (Agis-Balboa et al., 2006; Akk et al., 2007) and are among the most potent positive allosteric modulators of the $GABA_A$ receptor–ion-channel complex (Gasior et al., 1999). The current interest in their therapeutic properties results from their potential activity as anticonvulsants, anesthetics, anxiolytic and sedative-hypnotic agents (Akk et al., 2007; Veleiro and Burton, 2009) applicable to the treatment of several neurological and psychiatric disorders, like epilepsy, anxiety, insomnia, migraine and drug dependence, among others (Gasior et al., 1999). In addition, it has been shown that changes in **A** and **P** levels are associated with various physiological and pathophysiological conditions (Akk et al., 2007; Hamilton, 2002).

Natural NAS inhibit the binding to t-butyl-bicyclophosphorothionate (TBPS, Gee et al., 1988; Majewska et al., 1986; Ramanjaneyulu and Ticku, 1984) and stimulate the binding to $GABA_A$ receptor ligands such as muscimol or flunitrazepam (Hawkinson et al., 1994). These NAS are synthesized from cholesterol, which is transformed into pregnenolone (P5) by cytochrome P450scc (Iwahashi et al., 1990; Jung-Testas et al., 1989; Le Goascogne et al., 1987, 1989). In turn, P5 can be oxidized to active P4 by the 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD, Cascio et al., 1998; Labrie et al., 1992; Zwain and Yen, 1999), this conversion being an essential step in the biosynthesis of all steroid hormones. Then, P4 can be converted into 5α/β-dihydroprogesterone (5α/β-DH PROG) by 5α/β-reductase enzymes. Further reduction of these at the C3 position by the 3α-hydroxysteroid oxidoreductase enzyme (3α-HSOR) gives 3α-hydroxy-5α/β-pregnan-20-one (**A**/**P**, Plassart-Schiess and Baulieu, 2001). The 3β-HSD enzyme is mainly expressed in the adrenal gland and gonads (Luu-The et al., 1989; Rheaume et al., 1991; Zhao et al., 1991). The expression of 3β-HSD mRNA has also been demonstrated in the CNS by *in situ* hybridization (Coirini et al., 2003; Guennoun et al., 1995). Moreover, the conversion of P5 into P4 has been demonstrated in homogenates of amygdala and septum of rats (Weinfeld et al., 1980). It is interesting to note that 3β-HSD gene expression in the brain follows a pattern similar to that of the $GABA_A$ receptor subunits in the hypothalamus, cerebral cortex, cerebellum and hippocampus (Laurie et al., 1992; Wisden et al., 1992). The coexpression of 3β-HSD and $GABA_A$ receptor in the same brain areas gives anatomo-functional support for the *in situ* production of P4 and the autocrine and/or paracrine modulation of $GABA_A$ receptors directly or through its 3α-hydroxymetabolites (Guennoun et al., 1995). The capacity of steroids to negatively modulate 3β-HSD activity in different steroidogenic endocrine glands and peripheral nervous system is well known (Coirini et al., 2003; Guennoun et al., 1995).

On the other hand, cumulative evidence indicates neuroprotective actions of NAS in a variety of experimental paradigms (Schumacher et al., 2004). **A** protects NMDA-induced excitotoxicity and apoptosis in neurons *in vitro* (Frank and Sagratella, 2000; Lockhart et al., 2002; Xilouri and Papazafiri, 2006) and has positive effects on brain injury *in vivo* (di Michele et al., 2000; Djebaili et al., 2005). Neuroprotective actions of **A** have also been shown in hypoxia-induced brain injury. In pregnant sheep, **A** levels increase in response to acute hypoxic stress as a protective mechanism to reduce excitotoxicity (Hirst et al., 2006). We have reported a protective effect of **A** to a hypoxic event using organotypic cultures from cerebral cortex and hippocampus, reflected in a decrease in the glial fibrillary acidic protein (GFAP, Kruse et al., 2009), an increase in neurofilaments (NF200, Kruse et al., 2010).

Several structure/activity studies indicate that while endogenous NAS may accept chemical modification, the 3α-hydroxyl configuration is required for binding and activity (Scaglione et al., 2006; Souli et al., 2005; Suñol et al., 2006). However modifications of the steroid nucleus may emphasize different pharmacophores. Changes at the A, B, C and D rings, like incorporation of intramolecular oxygen bridges provide conformationally restricted analogs on the A/B angle of the steroid nucleus in a controlled way (Veleiro and Burton, 2009). To potentiate **A** and **P** effects and properties, a group of synthetic steroid (SS) analogs of these natural NAS was developed (Veleiro and Burton, 2009). Four of these SS were similar to **A**, with a planar conformation and three similar to **P**, with an angular molecular disposition.

The aim of the present work was to evaluate the performance of these SS, to characterize their functionality and capacity. First, we evaluated the effect of seven SS on the $GABA_A$ receptor function, using displacement-inhibition or binding stimulation of various receptor ligands. Then, we tested the ability of four of the compounds to modulate the activity of 3β-HSD in different tissues. Finally, we evaluated the neuroprotective role of two SS in brain cultures subjected to hypoxia.

Methods

Steroids

The SS were synthesized as described previously. They were divided into two groups: analogs with spatial conformation like **A**: 3α-hydroxy-1β,11α-epoxy-5α-pregnan-20-one (compound **1**, Alvarez et al., 2008), 3α-hydroxy-11β,19-epoxypregn-4-ene-20-one (compound **2**, Alvarez et al., 2008), 3α-hydroxy-2β,19-epoxy-5α-pregnan-20-one (compound **3**, Eduardo et al., 2003) and 3α-hydroxy-4β,19-epoxy-5α-pregnan-20-one (compound **4**, Eduardo et al., 2003), and analogs with spatial conformation like **P**: 3α-hydroxy-6,19-epoxypregn-4-ene-20-one (compound **5**, Veleiro et al., 2003), 3α-hydroxy-1α,11α-epoxy-5β-pregnan-20-one (compound **6**, Alvarez et al., 2008), and 3α-hydroxy-1α,11α-epoxypregn-4-ene-20-one (compound **7**, Alvarez L. doctoral thesis; Fig. 2). The homogeneity of all compounds was confirmed by thin layer chromatography (Alvarez et al., 2008). A 5 mM stock solution in dimethylsulfoxide (DMSO, Sigma-Aldrich) was used for all steroids in the *in vitro* studies. The SS were highly soluble in DMSO; their effects were observed at dilutions of 0.1% DMSO in buffer (used for competition studies). Related to the stability, these steroids seem to be stable for at least three months in DMSO. This was the longest time of storage for the primary solutions.

Experimental animals

Male Sprague–Dawley rats 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 immediately killed by decapitation between 10:00 h and 15:00 h. 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.

$GABA_A$ receptor binding studies

Preparation of synaptosomal membranes

Fifty non-treated adult male rats (200–250 g) were used. The cerebral cortex and cerebellum were dissected (Coirini et al., 1983) and homogenized with ice-cold 0.32 M sucrose 1:5 (w/v), using a Teflon-glass homogenizer (Gee et al., 1987). The homogenate was centrifuged at 3500 rpm for 10 min at 4 °C. The supernatant was carefully separated and centrifuged at 12,500 rpm for 15 min at 4 °C. The pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4, followed by centrifugation at 12,500 rpm for 15 min at 4 °C. The final pellet (synaptosomes) was washed 10 times with the same buffer and stored at −20 °C (Gonzalez et al., 1992). On the day of the assay, synaptosomes were thawed, washed twice with the buffer corresponding to each ligand and diluted to 1 mg protein/ml solution.

$GABA_A$ receptor binding patterns

Binding studies were performed using three different ligands: Butyl bicyclophosphorothionate, tertiary-[^{35}S] (TBPS, 200 Ci/mmol, NEG-049), [^{3}H]-flunitrazepam (FLU, 85.2 Ci/mmol, NET-567) and [^{3}H]-muscimol (MUS, 18 Ci/mmol, NET-574), all purchased from Perkin

Elmer. Synaptosomes were incubated in the presence or in the absence of increasing concentrations of steroids (5–1000 nM) in a 96-well culture dish for 10 min and then the radioactive ligands were added. The incubation temperatures were 25 °C for TBPS and 4 °C for FLU and MUS. TBPS binding was determined in the presence of 50 μM GABA. Total and non-specific binding was determined using the reaction buffer alone and specific inhibitors respectively. We used 50 mM Tris–HCl, 200 mM NaCl pH 7.1 and PTX (1 mM) for TBPS (2 nM), 50 mM Tris–HCl buffer pH 7.1 and diazepam (Plidan 10, Roemmers, Argentina; 1 μM) for FLU (1 nM) and 50 mM Tris-Acetate buffer pH 7.4 and GABA (10 μM) for MUS (10 nM). The incubation times were 90 min for TBPS and FLU and 60 min for MUS. Incubation was stopped by rapid filtration through glass micro-fiber filters (GF/B, Whatman) in an automatic cell harvester (Micro 96 Harvester, Molecular Devices). Filterbound radioactivity was quantified by liquid scintillation counting (Optiphase "Hisafe" 3, Perkin Elmer). The values obtained were referred to total binding for each ligand and IC_{50}/EC_{50} data were obtained from binding curves.

Effect of steroids on 3β-HSD activity

Preparations of microsomes

Ten non-treated 2-month-old male Sprague–Dawley rats (200–250 g) were used. The adrenal gland, cerebral cortex, hippocampus and cerebellum were dissected (Coirini et al., 1983). Tissues were homogenized by sonication (Sonifier Cell Disruptor, Heat System-Ultrasonics Inc.) in ice-cold 100 mM Tris–HCl/1 mM EDTA buffer pH 7.5. Homogenates were centrifuged at 15,000 rpm for 45 min at 4 °C. Supernatants were discarded and pellets were resuspended in ice-cold 0.25 M sucrose, and centrifuged at 5000 rpm for 5 min at 4 °C. The supernatants containing the microsomes were used to test the enzyme activity. Protein concentration was adjusted to 1.5 mg/ml.

3β-HSD activity

Fifteen micrograms of the microsomal fraction, 70 μM P5 (substrate) and DMSO or steroids (10, 50 and 100 μM concentrations) were added over 100 μl of buffer containing 35 mM glycine, 15 mM BSA, and 0.25 mM NAD^+ pH 9.4 at 37 °C. Absorbance measurements were quickly determined with a spectrophotometer (Biospec-1621, Shimadzu Biotechnologies) at 330–360 nm every 15 s for 5 min (Kawano et al., 1988; Kuhn and Briley, 1970). Absorbance was referred as percentage to the first value measured within each treatment for each assay and then compared to the control (with DMSO only). Values were expressed as percentage of NADH formation. At least ten similar but separate experiments of each concentration in each tissue were evaluated, and in all cases samples came from 10 different animals.

Organotypic cultures and hypoxia

Tissue cultures

Ten non-treated male 3–5 day-old Sprague Dawley rats were used for each assay. The cerebral cortex and hippocampus were dissected (Coirini et al., 1983), divided according to the condition, placed on a cell culture insert (PICMORG50 Millipore Millicell, USA) in a culture dish containing 1 ml of serum-based medium and placed in a culture oven at 37 C/5% CO_2. The plating medium contained: 50% Basal Medium Eagle (BME, Sigma-Aldrich), 25% Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich), 25% Horse Serum (HS, Sigma-Aldrich), 25 mM HEPES (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich) for the first 3 days. Then, inserts were placed in a fresh dish with 1 ml of medium as above but containing 70% BME, 25% EBSS, 10% HS, 25 mM HEPES, and 100 μg/ml streptomycin and back to the culture oven (Kruse et al., 2009). At least six similar but separate experiments of each tissue were evaluated, and in all cases samples came from different animals.

Steroid treatment and hypoxia

After 6 days *in vitro*, the medium was replaced and cultures were exposed to steroids (5 μM) or vehicle (DMSO) for 24 h. Then, the culture medium was replaced with fresh medium (normoxia group) or N_2-pre-saturated medium (100% N_2, hypoxia group), both containing either steroids or vehicle. Hypoxia was performed in the second group using an anaerobic chamber (Billups-Rothenberg, Inc.) for 1 h, as previously described (Kruse et al., 2009). All media were then replaced with fresh oxygenated medium and plates were placed at 37 °C/5% CO_2 for additional 24 h. In each experiment, control slice cultures came from the same animals.

Western blotting

Culture tissue sections collected at 8 days *in vitro* were rapidly frozen on dry ice and stored at −80 °C. Frozen tissue fragments were processed as described previously (Kruse et al., 2009). Briefly, proteins from the samples were separated on 6 or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine-SDS electrophoresis buffer at 120 V for 90 min in a Bio-Rad Mini-Protein II apparatus (Bio-Rad). Proteins were transferred onto PVDF membranes (Bio-Rad) in Tris-MetOH-glycine transfer buffer at 100 V for 90 min or 30 V overnight. Membranes were blocked with TBS-T 0.1% (20 mmol/l Tris, 150 mmol/l NaCl and 0.1% Tween-20, pH 7.6) containing 5% of fat-free milk for 1 h at 25 °C on a shaker. Blocked membranes were incubated with the primary antibody in TBS-T 0.1% containing 5% fat-free milk overnight at 4 °C on a shaker. The primary antibodies used were: GFAP (1:600; lane 46 kDa), as injury marker for glia, NF200 (1:800; lanes 160 and 200 kDa) and MAP 2 (1:1000; lanes 72 and 280 kDa) as injury markers for neurons. β-Actin (1:3000; lane 42 kDa) was used as protein loading control. All antibodies were purchased from Sigma-Aldrich. A pre-stained molecular ladder (Thermo Fisher Scientific) was used to determine the molecular weights. The immunoblots were then washed with TBS-T 0.1% three times and incubated for 1 h with the respective HRP-conjugated rabbit and mouse secondary antibodies (1:5000, GE Healthcare Life Sciences) at 25 °C on a shaker. Chemiluminescence was detected with the ECL system and exposed to hyperfilm (GE Healthcare Life Sciences). Signals in the immunoblots were scanned and optical density (OD) was determined using the Scion Image software. For each tissue and injury marker used, the OD level was referred as percentage of the control (vehicle and normoxia). OD was previously corrected for the loading control (β-actin).

Protein determination

The protein concentrations of synaptosomes, microsomes and homogenates from organotypic cultures were determined by Bradford (Bradford, 1976) using BSA (Sigma-Aldrich) as standard.

Statistical analysis

The commercial software GraphPad Prism (GraphPad Software Inc., v.4) or Statview (SAS Institute Inc. v5.0.1) were used for statistical analysis. The EC_{50}/IC_{50} values and their standard deviation for the binding curves of the different $GABA_A$ ligands were obtained from plotting and analysis using the Prism software. Significance between steroid treatments (**A**, **P**, and compounds **2**, **3**, **5** and **6**) and concentrations (10, 50 and 100 μM) in each microsomal fraction for NADH formation as well as significances between condition (normoxic, hypoxic) and steroid treatments (**A**, **P**, and compounds **3**, **5**) for organotypic cultures in each tissue were determined by two-way analysis of variance (ANOVA). Differences between steroid concentrations and control for NADH formation and differences between steroid treatment and control in the organotypic cultures were determined by one-way ANOVA. Further analysis within each treatment was evaluated by Newman–Keuls' test or unpaired "t" test for two-group comparisons using Statview software. In all cases, differences were considered significant at $p < 0.05$.

Results

Effects of steroid binding to the $GABA_A$ receptor

The effects of the SS on the $GABA_A$ receptor complex were evaluated using ligand binding displacement-inhibition or stimulation in synaptosomes from adult male rats (Gee et al., 1987) as an indirect assay for the impact of each steroid on $GABA_A$ receptor function. Each SS group was compared with the typical effects of **A** and **P** taken as positive controls (Gasior et al., 1999).

TBPS binding

The convulsant TBPS binds to the PTX site of the $GABA_A$ receptor complex, and NAS are known to 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 (Hawkinson et al., 1994; Majewska et al., 1986). Both **A** and **P** as well as both SS groups inhibited the binding of TBPS in a dose-dependent manner (Fig. 1). It is interesting to note that compound **5**, which is a **P** analog, showed a similar curve than **A** (Fig. 1b). The different [^{35}S]-TBPS-IC_{50} values for each SS and their 3D structures are shown in Fig. 2.

FLU binding

Flunitrazepam is a ligand for the BZD binding site of the $GABA_A$ receptor. Because the binding of this ligand is stimulated by NAS, we used an *in vitro* assay to evaluate the action of the SS (Hawkinson et al., 1994; Majewska et al., 1986). As expected, **A** and **P** enhanced FLU binding in a dose-dependent manner. Most of the SS stimulated this binding (Fig. 3a), but with different EC_{50} values (Table 1). Compound **3** showed activity similar to that of **A**, while compound **2** was unable to stimulate FLU binding. On the other hand, **P** analogs stimulated binding with different responses, except compound **5**, which showed a curve similar to that of **A** (Fig. 3b).

MUS binding

Muscimol is a specific agonist for the $GABA_A$ high affinity binding site. NAS increase the apparent affinity of this agonist to rat synaptosomes and its effect can also be used to evaluate SS (Harrison and Simmonds, 1984). **A** and **P** enhanced MUS binding in a dose-dependent manner. As shown in Fig. 3b, four of the SS tested showed a dose-dependent enhancement of the binding of this ligand, with large differences in their EC_{50} values. Only one of **A** analogs, was able to stimulate binding, whereas all **P** analogs showed this effect (Table 1). Compound **5** and **A** had similar EC_{50} values.

Steroid effects on NADH formation

The 3β-hydroxysteroid dehydrogenase enzyme (3β-HSD) converts P5 to P4 by an oxidation reaction, reducing the cofactor NAD^+ (nicotinamide adenine dinucleotide) to NADH (nicotinamide adenine dinucleotide hydrate) (Cascio et al., 1998; Labrie et al., 1992; Zwain and Yen, 1999; Coirini et al., 2003). Steroids are able to inhibit the activity of this enzyme in steroidogenic glands and sciatic nerve (Coirini et al., 2003). Taking these effects into account, we evaluated different concentrations of **A**, **P** and SS on 3β-HSD activity in the adrenal gland (AG), cerebral cortex (CC), hippocampus (HC) and cerebellum (Cb). Although little is known on the regulation of steroid synthesis in the CNS, the presence of 3β-HSD has been described in those regions. Thus, we further evaluated the steroid effects on the same brain tissues. Possible differential SS effects on these brain tissues will allow assessing the existence of specific tissue sensitivity. Microsomal fractions containing 3β-HSD were used for the studies. The enzyme activity was quantified measuring the NADH formation and natural NAS were used as internal control. Previously, we confirm the presence of this enzyme, in microsomal fractions, using a specific competitive inhibitor (trilostane, data not shown). Only two analogs of each group (compounds **2** and **3** and compounds **5** and **6**) were chosen for these assays, based on their similar effect to the natural NAS in the binding curves described above. Three different concentrations: 10 μM (low), 50 μM (medium) and 100 μM (high) were used. No effects were observed with lower concentrations than 10 μM (data not shown). Results were first analyzed by a two-way ANOVA with steroid treatment (**A**, **P**, compounds **2**, **3**, **5** and **6**) and concentration (10 μM, 50 μM and 100 μM) as factors. Fig. 4 shows the steroid action on AG microsomes. The effects of all factors were significant (treatment $F(5,122) = 32.2$; $p < 0.001$, concentration $F(3,122) = 804.2$; $p < 0.001$ and interaction $F(15,122) = 20.5$; $p < 0.001$). The one-way ANOVA showed a significant action for each steroid (see figure legend for statistical data). **A** and **P** caused a significant decrease in NADH formation at the higher concentrations (50 μM and 100 μM; $p < 0.01$), whereas the four SS caused significant decrease in NAD^+ at all the concentrations tested ($p < 0.01$). Compound **2** showed a more gradual inhibition than compound **3**, which showed the same effect at the two higher concentrations. Compounds **5** and **6** were similar to **P**, but compound **5** was the least effective at the higher concentrations ($p < 0.01$). The **P** analogs caused less inhibition than **A** analogs at each concentration. The two-way ANOVA for CNS tissues showed significant effects for all analysis factors (CC: treatment $F(5,130) = 225.8$; $p < 0.001$, concentration $F(3,130) = 3172.4$; $p < 0.001$ and interaction $F(15,130) = 71.6$; $p < 0.001$, HC: treatment $F(5,124) = 845.3$; $p < 0.001$, concentration $F(3,124) = 3534.8$; $p < 0.001$ and interaction $F(15,124) = 237$; $p < 0.001$, Cb: treatment $F(5,120) = 190$; $p < 0.001$, concentration $F(3,120) = 4879.7$; $p < 0.001$ and interaction $F(15,120) = 127.1$; $p < 0.001$).

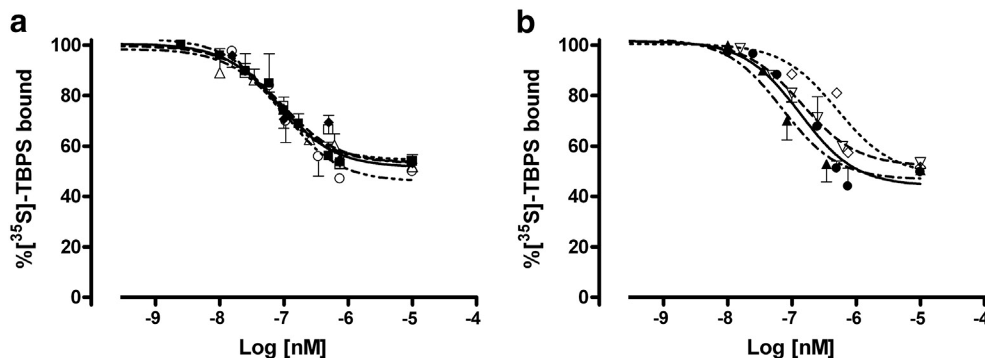


Fig. 1. [^{35}S]-TBPS binding inhibition in male rat synaptosome membranes by (a) allopregnanolone (**A** ■) and its structural analogs (compounds **1** □, **2** ◆, **3** △ and **4** ○) and (b) pregnanolone (**P** ●) and its structural analogs (compounds **5** ▽, **6** and **7** ▲). Membrane preparations were incubated with 2 nM [^{35}S]-TBPS in the absence (total binding) or presence of increasing concentrations of the steroids (5–1000 nM). PTX (1 mM) was used to determine non-specific binding. Assays were carried out at 25 °C for 90 min in the presence of 50 μM GABA.

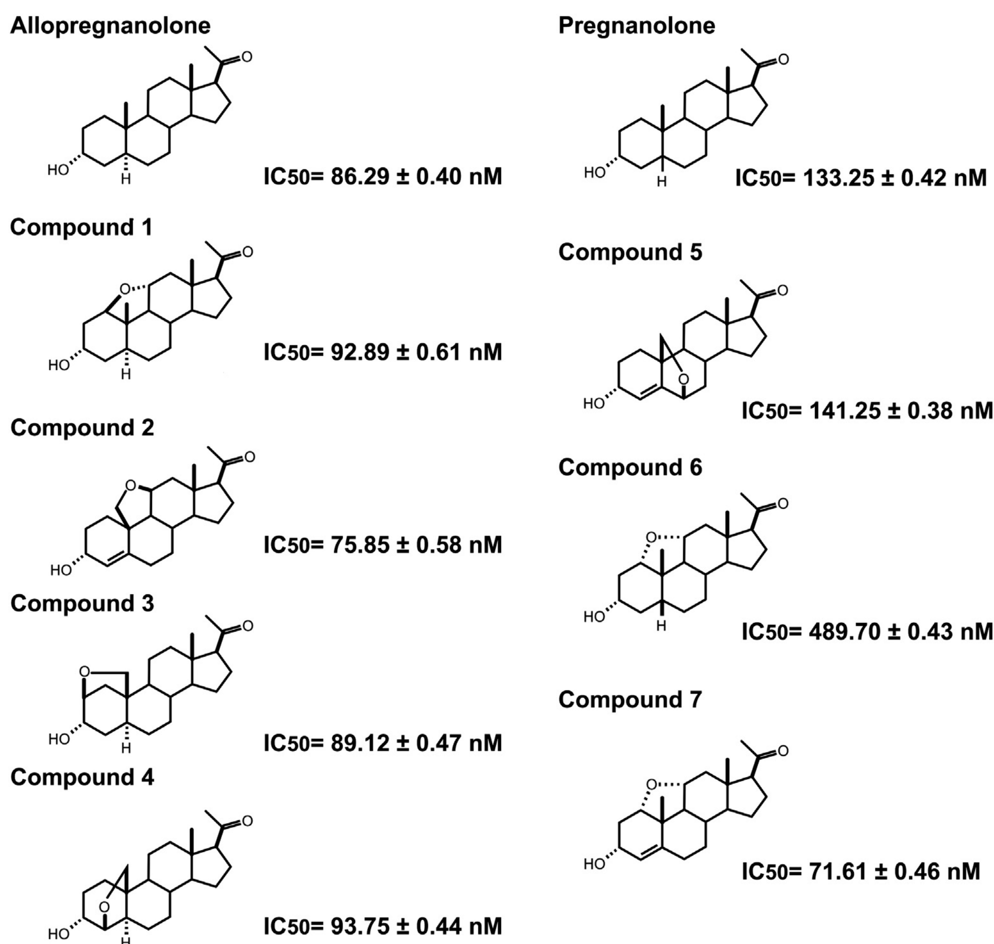


Fig. 2. Steroid 3D structures and IC_{50} inhibition values of [^{35}S]-TBPS binding to male rat synaptosomes by allopregnanolone (**A**) and its analogs (compounds **1**, **2**, **3** and **4**) and pregnanolone (**P**) and its analogs (compounds **5**, **6** and **7**). Values were obtained using the Graph Pad Program.

The results obtained with microsomes from the three brain regions are presented in Fig. 5 (one-way ANOVA data are shown in the figure legend). In CC all the steroids inhibit the NAD^+ reduction, with a different pattern (Fig. 5a). **A** and **P** had similar performance, but **P** was less powerful. Compounds **2** and **3** caused similar effects at the three concentrations used, but compound **3** was more efficient (around 45% inhibition; $p < 0.01$).

Compounds **5** and **6** and **P** have similar effects, but compound **5** was less effective. In the HC **A** caused a small inhibition of NADH formation at the two lower concentrations (Fig. 5b; 7%; $p < 0.01$) and an important effect at the highest concentration (60%; $p < 0.01$), whereas **P** caused a lower impact (18%; $p < 0.01$) than **A** at this concentration and no effect at 10 μM. The action of compounds **2** and **3** was similar to that of **A**, showing a reduction in NADH formation at all concentrations, where compound **3** was the most efficient (45% inhibition at high concentrations; $p < 0.01$). Compound **5** like **P** cause a low effect on NAD^+ reduction and compound **6** was the most potent of all the SS tested (40% inhibition; $p < 0.01$). Finally, in Cb (Fig. 5c), **A** and **P** showed similar results on the cofactor reduction, with a more significant effect of **P** at the highest concentration (60% inhibition; $p < 0.01$). The SS caused a dose-dependent inhibition, with the largest decrease in NADH formation caused by compound **2** at 10 μM (27%; $p < 0.01$) and compound **3** at 100 μM (63%; $p < 0.01$). Interestingly, compound **5** did not cause significant differences with respect to the control at the lowest concentration, whereas compound **6** showed the minimal inhibition at 100 μM (30%; $p < 0.01$).

SS and neuroprotective role against hypoxia injury in organotypic cultures

Previous studies from our laboratory using tissue cultures from rat pup brain subjected to hypoxia for 1 h showed an increase in GFAP signal (Kruse et al., 2009) and a decrease in NF160/200 (Kruse et al., 2010). We have also previously described that treatment with **A** (5 μM) significantly decreased hypoxia-induced astrogliosis in the cerebral cortex and the hippocampus (Kruse et al., 2009). Because some of the SS had shown properties similar to **A**, we evaluated the neuroprotective effects of compounds **3** and **5** under the same paradigm. These were selected by their actions in the above studies. Injury marker levels in the CC and HC were quantified by western blot and were represented as percentage of normoxic control. Results were first analyzed by a two-way ANOVA with condition (normoxic, hypoxic) and treatment (**A**, **P**, compounds **3** and **5**) as factors. Significant differences were observed in CC cultures with the GFAP marker with all factors evaluated (condition: $F(1,42) = 4.4$; $p < 0.05$; treatment: $F(4,42) = 7.1$; $p < 0.001$ and interaction: $F(4,42) = 8.4$; $p < 0.001$). The statistical analysis showed a significant increase in GFAP values in the hypoxic control compared to normoxic control by 105.77% ($p < 0.05$). Under hypoxic conditions, all steroids prevented the increase in GFAP level ($F(4,19) = 6.6$; $p < 0.005$) and the values were similar to the normoxic control (except for **P**; $p < 0.05$). The steroids under normoxic conditions caused no effects, except for **P**, which increased GFAP level by 30% ($F(4,23) = 5.8$; $p < 0.005$, Fig. 6a). In HC cultures, significant differences were observed in all factors (condition $F(1,27) = 25.6$; $p < 0.001$; treatment

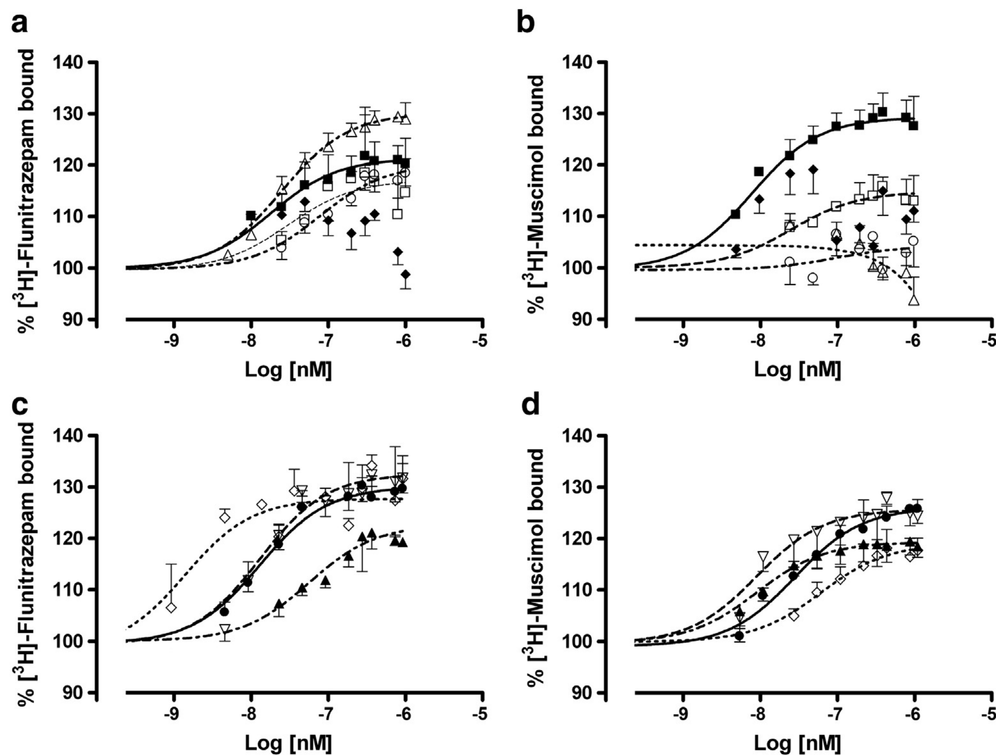


Fig. 3. [^{3}H]-FLU (a, c) and [^{3}H]-MUS binding stimulation (b, d) in male rat synaptosome membranes by (a, b) allopregnanolone (**A** ■) and its analogs (compounds **1** □, **2** ◆, **3** △ and **4** ○) and (b) pregnanolone (**P** ●) and its analogs (compounds **5** ▽, **6** and **7** ▲). Membrane preparations were incubated with 1 nM [^{3}H]-FLU or 10 nM [^{3}H]-MUS in the absence (total binding) or presence of increasing concentrations of the steroids (5–1000 nM). Diazepam (1 μM) or GABA (10 μM) was used to determine non-specific binding. Assays were carried out at 4 °C for 90 or 60 min respectively.

$F(4,27) = 20.7$; $p < 0.001$ and interaction $F(4,27) = 11.9$; $p < 0.001$). GFAP was increased in the hypoxic control compared to normoxic control by 86.25% ($p < 0.05$). Under hypoxic conditions, as shown in CC cultures, all steroids prevented an increase in GFAP ($p < 0.05$) and its levels were similar to the normoxic control ($p < 0.05$). There were no effects on GFAP expression in normoxia by any of the steroids used (Fig. 6b). The impact on neurons was evaluated using two antibodies against neurofilaments and microtubule protein. In CC cultures, NF expression showed significant differences by two-way ANOVA (NF200: condition $F(1,36) = 16.7$; $p < 0.001$, treatment $F(4,36) = 3.4$; $p < 0.05$ and interaction $F(4,36) = 3.2$; $p < 0.05$; NF160: condition $F(1,27) = 21.7$; $p < 0.001$, treatment $F(4,27) = 5.7$; $p < 0.01$ and interaction $F(4,27) = 11.9$; $p < 0.001$). Further analysis showed a significant hypoxic decrease in NF200 by 16.79% compared to the normoxic control ($p < 0.05$). Only **A** treatment partially prevented this decrease by hypoxia ($p < 0.05$). No changes in NF200 expression were observed in normoxia (Fig. 7a). An important decrease was observed in a second lane labeled with the same antibody (NF160) under hypoxic conditions (34.82%; $p < 0.05$). In addition, treatment with compound **5** prevented this decrease whereas the other steroids produced a partial effect ($p < 0.05$). Steroids did not significantly affect the NF160 expression in normoxia (Fig. 7c). When NF200 and NF160 levels were evaluated in HC cultures both showed significant effects (NF200: condition $F(1,37) = 4.7$; $p < 0.05$, treatment $F(4,37) = 3.2$; $p < 0.05$ and interaction $F(4,37) = 7.2$; $p < 0.001$; NF160: condition $F(1,31) = 13.6$; $p < 0.001$, treatment $F(4,31) = 4.5$; $p < 0.01$), except for the interaction ($F(4,31) = 2.5$; $p = 0.06$, two-way ANOVA). A significant 21.46% decrease in NF200 expression was found in the tissue subjected to hypoxia ($p < 0.05$). Under this condition, **A** and compound **5**

Table 1
EC_{50} stimulation values of [^{3}H]-FLU and [^{3}H]-MUS binding to male rat synaptosomes by allopregnanolone (**A**) and its analogs (compounds **1**, **2**, **3** and **4**) and pregnanolone (**P**) and its analogs (compounds **5**, **6** and **7**). Values were obtained using the GraphPad Program. N.D. means not determinable value.

Steroid	[^{3}H]-FLU EC_{50} (nM)	[^{3}H]-MUS EC_{50} (nM)
Allopregnanolone	17.98 ± 0.82	7.94 ± 0.61
Pregnanolone	14.90 ± 0.65	26.90 ± 0.53
Compound **1**	29.51 ± 0.92	25.10 ± 0.70
Compound **2**	N.D.	N.D.
Compound **3**	24.50 ± 0.54	N.D.
Compound **4**	76.73 ± 0.69	N.D.
Compound **5**	18.19 ± 0.54	8.28 ± 0.66
Compound **6**	1.05 ± 0.65	55.71 ± 0.65
Compound **7**	61.37 ± 0.70	8.30 ± 0.72

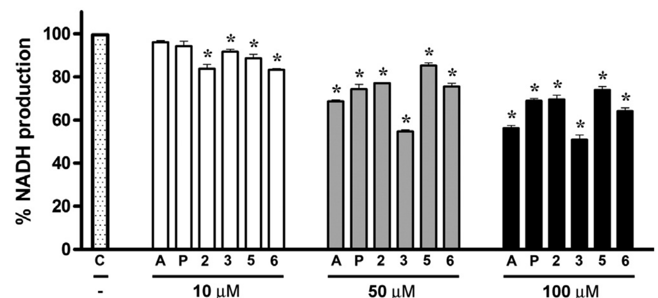


Fig. 4. NADH formation in microsome fraction from male rat adrenal gland by allopregnanolone (**A**), its analogs (compounds **2** and **3**), pregnanolone (**P**) and its analogs (compounds **5** and **6**). Pregnenolone (P5) and DMSO (control, C) or 10 μM, 50 μM, and 100 μM steroid concentrations were added. Absorbance measurements were determined with a spectrophotometer at 330–360 nm every 15 s for 5 min. Absorbance was referred as percentage to the first measured value within each treatment, for each assay and then compared to the control (only with DMSO). Values were expressed as percentage of NADH formation. At least ten similar but separate experiments of each concentration in each tissue were evaluated, and in all cases samples came from 10 different animals. Significant effect of all steroid were found by one way ANOVA ($F_A(3,25) = 360.2$; $p < 0.001$, $F_2(3,20) = 85.5$; $p < 0.001$, $F_3(3,18) = 295.7$; $p < 0.001$, $F_P(3,19) = 79$; $p < 0.001$, $F_5(3,20) = 54.5$; $p < 0.001$, $F_6(3,20) = 130$; $p < 0.001$, sub index in each F indicate the steroid analyzed. *$p < 0.01$ Newman Keuls post hoc test).

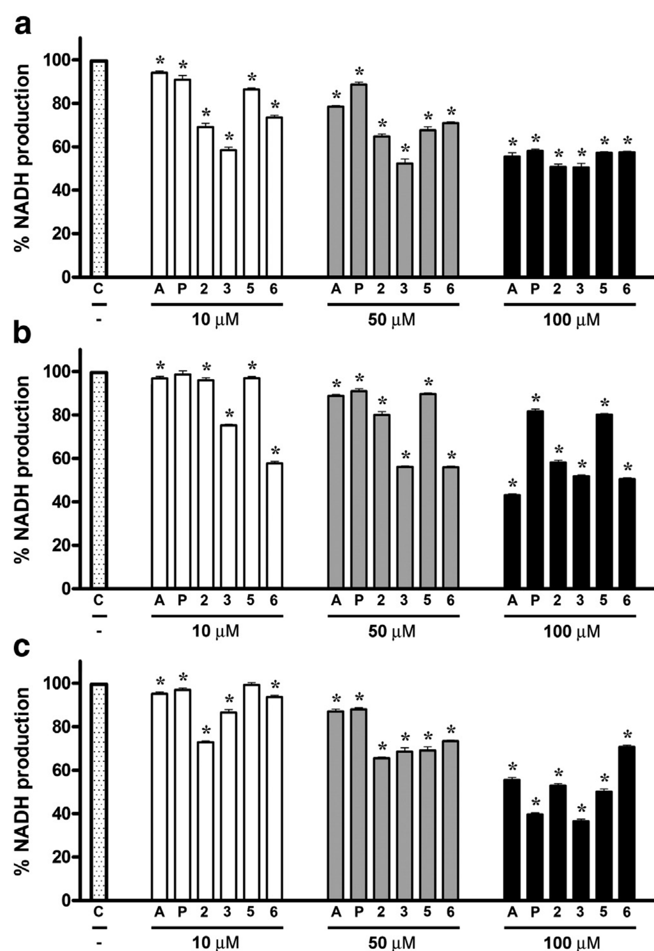


Fig. 5. NADH formation in microsome fraction from male rat (a) cerebral cortex (CC), (b) hippocampus (HC) and (c) cerebellum (Cb) by allopregnanolone (**A**), its analogs (compounds **2** and **3**), pregnanolone (**P**) and its analog group (compounds **5** and **6**). C (control DMSO) or 10 μM, 50 μM or 100 μM of the different steroids were added to microsomes (see Methods). Absorbance values were referred as percentage to the first measured value within each treatment and then compared to the control. All data were expressed as percentage of NADH formation. At least ten similar but separate experiments for each concentration in each tissue were made, tissue samples came from 10 different animals. Significant effect of all steroid were found by one way ANOVA in CC: $F_{\mathbf{A}}(3,24) = 551.9$; $p < 0.001$, $F_{\mathbf{2}}(3,21) = 533.8$; $p < 0.001$, $F_{\mathbf{3}}(3,21) = 507$; $p < 0.001$, $F_{\mathbf{P}}(3,20) = 341.3$; $p < 0.001$, $F_{\mathbf{5}}(3,24) = 976.5$; $p < 0.001$, $F_{\mathbf{6}}(3,20) = 1257.5$; $p < 0.001$. In the HC: $F_{\mathbf{A}}(3,21) = 2323.1$; $p < 0.001$, $F_{\mathbf{2}}(3,20) = 393.2$; $p < 0.001$, $F_{\mathbf{3}}(3,22) = 2271.7$; $p < 0.001$, $F_{\mathbf{P}}(3,21) = 77.2$; $p < 0.001$, $F_{\mathbf{5}}(3,18) = 299.5$; $p < 0.001$, $F_{\mathbf{6}}(3,22) = 1683.5$; $p < 0.001$. And in Cb: $F_{\mathbf{A}}(3,24) = 667$; $p < 0.001$, $F_{\mathbf{2}}(3,22) = 1385.7$; $p < 0.001$, $F_{\mathbf{3}}(3,17) = 775$; $p < 0.001$, $F_{\mathbf{P}}(3,19) = 2172.2$; $p < 0.001$, $F_{\mathbf{5}}(3,19) = 677.6$; $p < 0.001$, $F_{\mathbf{6}}(3,19) = 734.62$; $p < 0.001$, sub index in each F indicate the steroid analyzed. *$p < 0.01$ Newman Keuls post hoc test.

prevented this decrease, while **P** showed a partial action ($p < 0.05$). Compound **3** was the only SS that showed a significant effect in normoxia ($p < 0.05$) and none in hypoxia (Fig. 7b). Moreover, NF160 exhibited a similar decrease in expression in hypoxia (21.1%; $p < 0.05$) and, as in the case of NF200, **A**, **P** and compound **5** prevented this decrease ($p < 0.05$). Steroids had no effect on NF160 expression in normoxia (Fig. 7d).

No significant differences were observed in CC cultures with the MAP 2 by two way ANOVA (condition MAP 2b: $F(1,30) = 0.02$; $p = 0.88$; MAP 2c: $F(1,26) = 3.6$; $p = 0.07$) in contrast, by treatment and the interaction between factors showed significant differences (MAP 2b: $F(4,30) = 3.5$; $p < 0.05$; $F(4,30) = 6.88$; $p < 0.001$; MAP 2c: $F(4,26) = 16.1$; $p < 0.0001$; $F(4,26) = 17.2$; $p < 0.0001$, respectively). Further analysis showed a significant hypoxic decrease in MAP 2b by 14.10% and MAP 2c by 13%, compared to the normoxic control ($p < 0.05$). Only **A** treatment prevented MAP 2b decrease by hypoxia ($p < 0.05$) whereas all treatments prevented MAP 2c decrease ($p < 0.05$) No changes in MAP 2b or MAP 2C expression were observed in normoxia (Figs. 8a and c). When MAP 2b levels were evaluated in HC cultures, no significant differences were observed in all factors (condition $F(1,23) = 3.3$; $p = 0.08$, treatment $F(4,23) = 2.2$; $p = 0.10$ and interaction $F(4,23) = 5$; $p < 0.005$). However MAP 2c levels showed, significant differences in all factors (condition ($F(1,26) = 15.8$; $p < 0.001$), treatment ($F(4,26) = 11.2$; $p < 0.0001$, and interaction $F(4,26) = 24.2$; $p < 0.0001$, two-way ANOVA). A significant 14.80% decrease in MAP 2b expression was found in the tissue subjected to hypoxia ($p < 0.05$). Under this condition, only compounds **3** and **5** prevented this decrease ($p < 0.05$). Moreover, MAP 2c exhibited a similar expression decrease in hypoxia (12%; $p < 0.05$) and all steroids were effective ($p < 0.05$). Steroids had no effect on MAP 2c expression in normoxia (Figs. 8b and d).

Discussion

Neurosteroids are synthesized within the brain and modulate neuronal excitability by rapid non-genomic actions (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. For example, **A** has been shown to be protective against adverse early life events when administered to neonatal rats (Patchev et al., 1997). On the other hand, NAS possess certain activities as anticonvulsant, anxiolytic and sedative-hypnotic agents (Akk et al., 2007) applicable to the treatment of several neurological and psychiatric disorders (Schüle et al., 2011). Thus, neurosteroid treatment may be useful to prevent disorders such as major depression. In this study, we used seven SS, where the incorporation of intramolecular oxygen bridges provides conformationally restricted **A** or **P** analogs to test their ability to respond like natural NAS. The SS were divided into two groups: **A**-like (compounds **1**, **2**, **3** and **4**) and **P**-like (compounds **5**, **6** and **7**). First, we evaluated the capacity of SS to modulate the $GABA_A$ receptor. Different binding assays involving specific $GABA_A$ receptor ligands (TBPS, FLU and MUS) were used. We found that compounds **3** and **4** (**A** analogs) were able to stimulate FLU and displace TBPS binding and that compound **2** was only able to displace TBPS binding. On the other hand, all **P** analogs showed effects similar to those of the natural NAS. Compound **5** was the most effective SS and showed a similar pattern to natural NAS, inhibiting TBPS binding and stimulating FLU and MUS binding.

Natural NAS are synthesized from cholesterol in CNS tissues (Coirini et al., 2003; Guennoun et al., 1995). Because 3β-HSD activity is modulated by steroids, we evaluated the effect of some of the SS in two kinds of tissues: a classical steroidogenic one like the adrenal gland and others from three different brain regions. All steroids began to affect the enzyme activity in the adrenal gland at concentration of 10 μM. Compound **5** caused a lower impact than the other SS on NADH formation (especially at high concentrations), whereas compound **3** caused the largest decrease. The **P** analogs were less efficient inhibiting enzyme activity. In the CNS, different effects were observed depending on the region studied. In cerebral cortex, compound **3** caused the largest impact on the enzyme reducing NADH formation by 50%. As seen in the adrenal gland, **P** analogs, like compound **5**, marginally inhibited NAD^+ reduction. In the hippocampus, compounds **2** and **3** effects were similar to **A**, whereas compound **6** cause the major effect in this region. Compound **5** effect was lower than that observed in the cerebral cortex. In cerebellum, all SS caused a dose-dependent effect, with inhibition values higher than in the other two CNS tissues, except compound **3**, which caused the largest decrease at high concentrations. Compound **5** did not affect the enzyme activity at the lowest concentration, although the other concentrations showed a 40% inhibition. Considering the results obtained, compound **5** was one of the SS that least altered

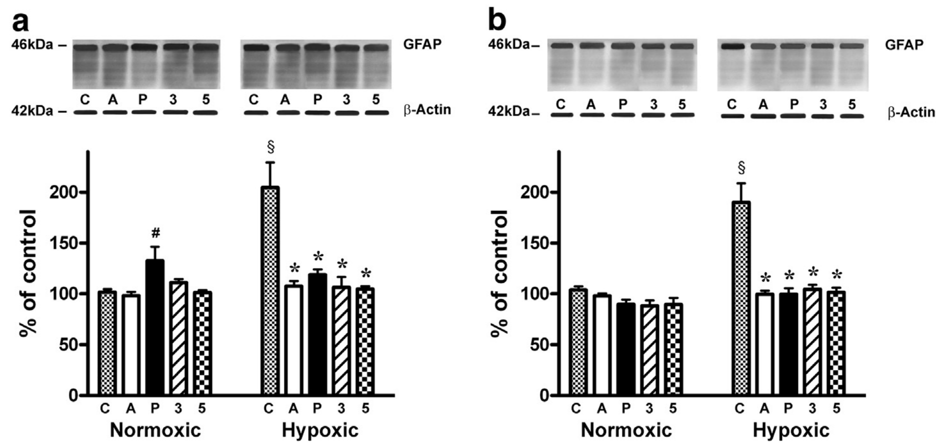


Fig. 6. Effect of pretreatment with allopregnanolone (**A**), its structural analog (compound **3**), pregnanolone (**P**) and its structural analog (compound **5**) on the astrogliosis induced by hypoxia in the (a) cerebral cortex (CC) and (b) hippocampus (HC). The GFAP (injury marker) level was quantified by western blot and represented as percentage of normoxic control. Representative western blots are showed on the top of the figure. Normoxic and hypoxic controls DMSO (C), allopregnanolone (**A**), pregnanolone (**P**), Compound **3** (**3**) and Compound **5** (**5**). At least six similar but separate experiments of each tissue were evaluated, and in all cases samples came from different animals. Steroid effect in normoxia $^{\#}p < 0.05$ and in hypoxia $^{*}p < 0.05$ one way ANOVA Newman Keuls post hoc test. $^{\S}p < 0.05$ unpaired "t" test.

the enzyme activity, while compound **3** caused the largest inhibition. Moreover, the differences observed among the brain regions studied suggest the existence of specific tissue sensitivity. This hypothesis needs further analysis after a better characterization of steroid synthesis regulation in these tissues.

On the other hand, the evidence indicating neuroprotective actions of NAS in a variety of experimental paradigms is overwhelming. **A** protects NMDA-induced excitotoxicity and apoptosis in neurons *in vitro* (Lockhart et al., 2002; Majewska et al., 1986; Zheng, 2009) and has positive effects on brain injury *in vivo* (di Michele et al., 2000; Djebaili et al., 2005). The neuroprotective actions of **A** have been shown in hypoxia-induced brain injury, as well as in a hypoxic event using organotypic cultures (Kruse et al., 2009, 2010). In those experiments we showed that doses of **A** lower than 5 μM had no neuroprotective effects. Compounds **3** and **5** were equally effective as neuroprotective agents against the hypoxic event when GFAP was used as injury marker. However, this action was not observed when the neuronal injury markers NF200 and MAP 2 were analyzed. In cerebral cortex, only **A**

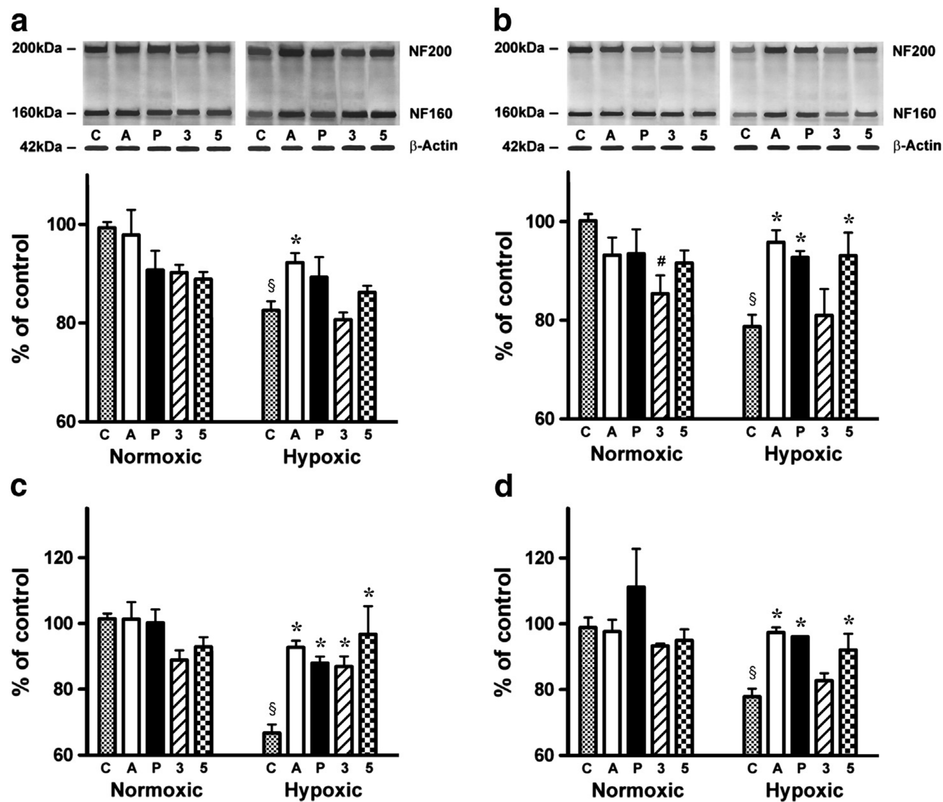


Fig. 7. Effect of pretreatment with allopregnanolone (**A**), its structural analog (compound **3**), pregnanolone (**P**) and its structural analog (compound **5**) on the astrogliosis induced by hypoxia in the (a, c) cerebral cortex (CC) and (b, d) hippocampus (HC). The NF200 (a, b) and NF160 (c, d) (injury marker) levels were quantified by western blot and represented as percentage of normoxic control. Representative western blots are showed on the top of the figure. Normoxic and hypoxic controls DMSO (C), allopregnanolone (**A**), pregnanolone (**P**), compound **3** (**3**) and compound **5** (**5**). At least six similar but separate experiments of each tissue were evaluated, and in all cases samples came from different animals. Steroid effect in normoxia $^{\#}p < 0.05$ and in hypoxia $^{*}p < 0.05$ one way ANOVA Newman Keuls post hoc test. $^{\S}p < 0.05$ unpaired "t" test.

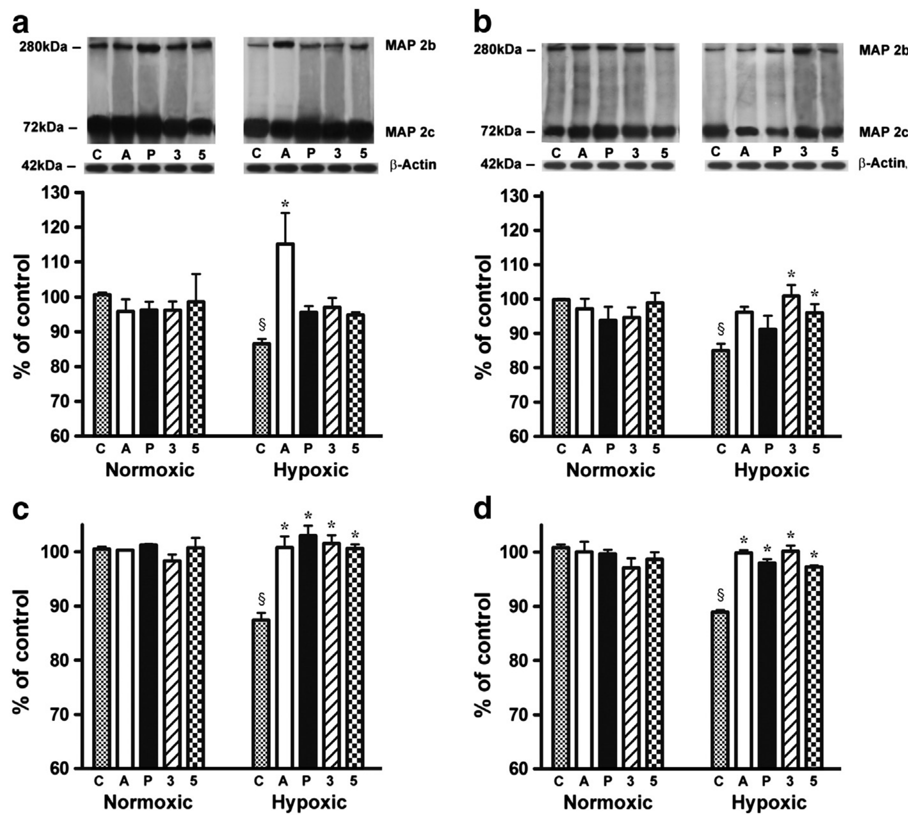


Fig. 8. Effect of pretreatment with allopregnanolone (**A**), its structural analog (compound **3**), pregnanolone (**P**) and its structural analog (compound **5**) on the astrogliosis induced by hypoxia in the (a, c) cerebral cortex (CC) and (b, d) hippocampus (HC). The MAP 2b (a, b) and MAP 2c (c, d) (injury marker) levels were quantified by western blot and represented as percentage of normoxic control. Representative western blots are showed on the top of the figure. Normoxic and hypoxic controls DMSO (C), allopregnanolone (**A**), pregnanolone (**P**), compound **3** (**3**) and compound **5** (**5**). At least six similar but separate experiments of each tissue were evaluated, and in all cases samples came from different animals. Steroid effect in normoxia $^{\#}p < 0.05$ and in hypoxia $^{*}p < 0.05$ one way ANOVA Newman Keuls post hoc test. $^{§}p < 0.05$ unpaired "t" test.

prevented the decreases in NF200 and in MAP 2b. Meanwhile, in NF160, compound **5** was able to prevent this decrease more efficiently than the other steroids, and all treatments were effective in MAP 2c.

Different effects were observed in the hippocampus cultures. The expression of both kinds of NF was altered by **A**, **P** and compound **5**, which prevented the decrease by hypoxia. All steroids prevented MAP 2c decrease whereas only compound **3** and compound **5** were able to prevent it on MAP 2b.

These results suggest that cultures of cerebral cortex are more sensitive than those of hippocampus, and that the neuroprotective roles of steroids are more efficient in the latter, especially when NF200 expression was analyzed.

The neuroprotective action of the SS used was probably mainly due to their interaction with the ionotropic receptors. Both oxysteroids have been designed as analogs of **A** and **P**, considering possible interactions with $GABA_A$ binding pocket (Veleiro and Burton, 2009). Because the interaction of these steroids to cytosolic or membrane progesterone receptor (PR, mPR) has not been explored, we cannot rule out some effect due to those receptors. The time that cultures were exposed to steroids was long enough to involve a genomic effect for the natural NAS. **A** can be bioconverted to 5α-DHP, which in fact may activate gene transcription *via* PR. On the other hand **A** has shown a neuroprotective action *via* mPRδ (Thomas and Pang, 2012) and this could be another possible mechanism involved. However both mechanisms remain to be proved for the SS.

There are comments to be made on the models which have been used. Neurosteroid action on $GABA_A$ receptor functions are usually observed at micromolar concentrations, those were the concentrations used for enzyme and neuroprotection studies. However in binding competition or stimulations studies using synaptosomes, the SS showed similar IC_{50} than the natural NAS, which was in the nanomolar range for the three kinds of ligands used. This conflicting data could be partially explained by possible differences in the kind of interaction of the steroids with the proteins with enzymatic activity or the presence of another endogenous ligand as in the case of organotypic cultures. Moreover, this last assay was performed based on previous studies where we found neuroprotective effect of **A** with 5 μM but not with 0.5 or 50 nM (Kruse et al., 2009). The affectivity of the used doses should be viewed in the context of the different experimental models used for assessing SS effects. The concentrations needed to explore steroid action on a highly sensitive protein in an isolated system (synaptosomes studies) are usually much lower than those used for enzymatic modulation or the required for SS effect on tissues.

Summarizing, in this work we presented a group of synthetic analogs of **A** and **P** with decreased flexibility between A/B ring that confer a more favorable spatial arrangement for the steroid binding site, and their descriptions in different assays. Our study was not limited to describe their action on the $GABA_A$ receptor binding, but also included modulation of a steroidogenesis key enzyme and their possible neuroprotective roles. In this manner, we were able to evaluate not only SS binding action, but also information on specific tissue sensitivity for some of SS studied. We found that two of the oxygen-bridged SS (compound **3** and compound **5**) are suitable to be used as potential therapeutic substances, mainly compound **5**, which, being a **P** analog, showed similar or better results than **A**. Although further studies are necessary for pharmacological validation, the results presented here provide an approach on the functionality and possible applications of these modified steroids.

References

Agis-Balboa, R.C., Pinna, G., Zhubi, A., Maloku, E., Veldic, M., Costa, E., Guidotti, A., 2006. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. Proc. Natl. Acad. Sci. U. S. A. 103, 14602–14607.

Akk, G., Covey, D.F., Evers, A.S., Henry Steinbach, J., Zorumski, C.F., Mennerick, S., 2007. Mechanisms of neurosteroid interactions with $GABA_A$ receptors. Pharmacol. Ther. 116, 35–37.

Alvarez, L.D., Veleiro, A.S., Baggio, R.F., Garland, M.T., Edelsztein, V.C., Coirini, H., Burton, G., 2008. Synthesis and $GABA_A$ receptor activity of oxygen-bridged neurosteroid analogs. Bioorg. Med. Chem. 16, 3831–3838.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cascio, C., Prasad, V.V.K., Lin, Y.Y., Lieberman, S., Papadopoulos, V., 1998. Detection of P450c17-independent pathways for dehydroepiandrosterone (DHEA) biosynthesis in brain glial tumor cells. Proc. Natl. Acad. Sci. U. S. A. 95, 2862–2867.

Coirini, H., Marusic, E.T., De Nicola, A.F., Rainbow, T.C., McEwen, B.S., 1983. Identification of mineralocorticoid binding sites in rat brain by competition studies and density gradient centrifugation. Neuroendocrinology 37, 354–360.

Coirini, H., Gouézou, M., Delespierre, B., Liere, P., Pianos, A., Eychenne, B., Schumacher, M., Guennoun, R., 2003. Characterization and regulation of the 3β-hydroxysteroid dehydrogenase isomerase enzyme in the rat sciatic nerve. J. Neurochem. 84, 119–126.

di Michele, F., Lekieffre, D., Pasini, A., Bernardi, G., Benavides, J., Romeo, E., 2000. Increased neurosteroids synthesis after brain and spinal cord injury in rats. Neurosci. Lett. 284 (1–2), 65–68.

Djebaili, M., Guo, Q., Pettus, E.H., Hoffman, S.W., Stein, D.G., 2005. The neurosteroids progesterone and allopregnanolone reduce cell death, gliosis, and functional deficits after traumatic brain injury in rats. J. Neurotrauma 22 (1), 106–118.

Eduardo, S.L., Ghini, A.A., Burton, G., 2003. Oxido-bridged neurosteroid analogues. Synthesis of 2,19-oxido-allopregnanolone. ARKIVOC X, 468–476.

Frank, C., Sagratella, S., 2000. Neuroprotective effects of allopregnenolone on hippocampal irreversible neurotoxicity *in vitro*. Prog. Neuropsychopharmacol. Biol. Psychiatry 24 (7), 1117–1126.

Gago, N., El-Etr, M., Sananès, N., Cadepond, F., Samuel, D., Avellana-Adalid, V., Baron-Van Evercooren, A., Schumacher, M., 2004. 3Alpha;5alpha-tetrahydroprogesterone (allopregnanolone) and gamma-aminobutyric acid: autocrine/paracrine interactions in the control of neonatal PSA-NCAM + progenitor proliferation. J. Neurosci. Res. 78, 770–783.

Gasior, M., Carter, R.B., Witkin, J.M., 1999. Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders. Trends Pharmacol. Sci. 20, 107–111.

Gee, K.W., Brinton, R.E., Chang, W.C., McEwen, B.S., 1987. Gamma-aminobutyric acid-dependent modulation of the chloride ionophore by steroids in rat brain. Eur. J. Pharmacol. 136, 419–423.

Gee, K.W., Bolger, M.B., Brinton, R.E., Coirini, H., McEwen, B.S., 1988. Steroid modulation of the chloride ionophore in rat brain: structure–activity requirements; regional dependence and mechanism of action. J. Pharmacol. Exp. Ther. 246, 803–812.

Gonzalez, S.L., Ferrini, M., Coirini, H., De Nicola, A.F., 1992. Regulation of flunitrazepam binding in the dorsal horn of the spinal cord. Brain Res. 589, 97–101.

Guennoun, R., Fiddes, R.J., Gouézou, M., Lombès, M., Baulieu, E.-E., 1995. A key enzyme in the biosynthesis of neurosteroids; 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase (3β-HSD) is expressed in rat brain. Brain Res. Mol. Brain Res. 30, 287–300.

Hamilton, N.M., 2002. Interaction of steroids with GABAA receptor. Curr. Top. Med. Chem. 2, 887–902.

Harrison, N.L., Simmonds, M.A., 1984. Modulation of the GABA receptor complex by a steroid anaesthetic. Brain Res. 323 (2), 287–292.

Hawkinson, J.E., Kimbrough, C.L., Belelli, D., Lambert, J.J., Prurdy, R.H., Lan, N.C., 1994. Correlation of neuroactive steroid modulation of [^{35}S]t-butylbicyclophosphorothionate and [^{3}H]-flunitrazepam binding and gamma-aminobutyric acid receptor function. Mol. Pharmacol. 46 (5), 977–985.

Hirst, J.J., Yawno, T., Nguyen, P., Walker, D.W., 2006. Stress in pregnancy activates neurosteroid production in the fetal brain. Neuroendocrinology 84 (4), 264–274.

Iwahashi, K., Ozaki, H.S., Tsubaki, M., Ohniski, J., Taheuchi, Y., Ichikawa, Y., 1990. Studies of the immunohistochemical and biochemical localization of the cytochrome P-450-scc-linked monooxygenase system in the adult rat brain. Biochem. Biophys. Acta 1035, 182–189.

Jung-Testas, I., Hu, Z.Y., Baulieu, E.-E., Robel, P., 1989. Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. Endocrinology 125, 2083–2091.

Kawano, T., Okamura, H., Tajima, C., Fukuma, K., Katabuchi, H., 1988. Effect of RU 486 on luteal function in the early pregnant rat. J. Reprod. Fertil. 83, 279–285.

Kruse, M.S., Rey, M., Barutta, J., Coirini, H., 2009. Allopregnanolone effects on astrogliosis induced by hypoxia in organotypic cultures of striatum, hippocampus and neocortex. Brain Res. 1303, 1–7.

Kruse, M.S., Rey, M., Veleiro, A.S., Burton, G., Coirini, H., 2010. Hypoxia impairs the morphology of neurons in cortex and hippocampus organotypic cultures. Biocell 34 (2), A97.

Kuhn, N.J., Briley, M.S., 1970. The roles of pregn-5-ene-3β,20α-diol and 20α-hydroxy steroid dehydrogenase in the control of progesterone synthesis preceding parturition and lactogenesis in the rat. Biochem. J. 117, 193–201.

Labrie, F., Simard, J., Luu-The, V., Pelletier, G., Belanger, A., Lachance, Y., Zhao, H.F., Labrie, C., Breton, N., de Launoit, Y., Dumont, M., Dupont, E., Rhéaume, E., Martel, C., Couët, J., Trudel, C., 1992. Structure and tissue-specific expression of 3 beta-hydroxysteroid dehydrogenase/5-ene-4-ene isomerase genes in human and rat classical and peripheral steroidogenic tissues. J. Steroid Biochem. Mol. Biol. 41, 421–435.

Laurie, D.J., Wisden, W., Seeburg, P.H., 1992. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J. Neurosci. 11, 4151–4172.

Le Goascogne, C., Robel, P., Gouezou, M., Sananes, N., Baulieu, E.-E., Waterman, M.R., 1987. Neurosteroids; cytochrome $P450_{scc}$ in rat brain. Science 237, 1212–1215.

Le Goascogne, C., Gouezou, M., Robel, P., Defaye, G., Chambaz, E., Waterman, M.R., Baulieu, E.-E., 1989. The cholesterol side-chain cleavage complex in human brain white matter. J. Neuroendocrinol. 1, 153–156.

Lockhart, E.M., Warner, D.S., Pearlstein, R.D., Penning, D.H., Mehrabani, S., Boustany, R.M., 2002. Allopregnanolone attenuates N-methyl-D-aspartate-induced excitotoxicity and apoptosis in the human NT2 cell line in culture. Neurosci. Lett. 328 (1), 33–36.

Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J.L., Strickler, R.C., Labrie, F., 1989. Full length cDNA structure and deduced amino acid sequence of human 3 beta-hydroxy-5-ene steroid dehydrogenase. Mol. Endocrinol. 3, 1310–1312.

Majewska, M.D., Harrison, N.L., Schwartz, R.D., Barker, J.L., Paul, S.M., 1986. Steroid hormone metabolites are barbiturate-like modulators of the $GABA_A$ receptor. Science 232, 1004–1007.

Patchev, V.K., Montkowski, A., Rouskova, D., Koranyi, L., Holsboer, F., Almeida, O.F., 1997. Neonatal treatment of rats with the neuroactive steroid tetrahydrodeoxycorticosterone (THDOC) abolishes the behavioral and neuroendocrine consequences of adverse early life events. J. Clin. Invest. 99, 962–966.

Plassart-Schiess, E., Baulieu, E.-E., 2001. Neurosteroids: recent findings. Brain Res. Brain Res. Rev. 37, 133–140.

Ramanjaneyulu, R., Ticku, M.K., 1984. Binding characteristics and interactions of depressant drugs with [^{35}S]t-butylbicyclophosphorothionate; a ligand that binds to the picrotoxin site. J. Neurochem. 42, 221–229.

Rheaume, E., Lachance, Y., Zhao, H.F., Breton, N., Dumont, M., de Launoit, Y., Trudel, C., Luu-The, V., Simard, J., Labrie, F., 1991. Structure and expression of a new complementary DNA enconding the almost exclusive 3 beta-hydroxysteroid dehydrogenase/delta 5-delta-4-isomerase in human adrenals and gonads. Mol. Endocrinol. 5, 1147–1157.

Scaglione, J.B., Manion, B.D., Benz, A., Taylor, A., DeKoster, G.T., Rath, N.P., Ever, A.S., Zorumski, C.F., Mennerick, S., Covey, D.F., 2006. Neurosteroid analogues. 11. Alternative ring system scaffolds: gamma-aminobutyric acid receptor modulation and anesthetic actions of benz[f]indenes. J. Med. Chem. 49, 4595.

Schumacher, M., Guennoun, R., Robert, F., Carelli, C., Gago, N., Ghoumari, A., Gonzalez Deniselle, M.C., Gonzalez, S.L., Ibanez, C., Labombarda, F., Coirini, H., Baulieu, E.-E., De Nicola, A.F., 2004. Local synthesis and dual actions of progesterone in the nervous system: neuroprotection and myelination. Growth Horm. IGF Res. 14, S18–S33.

Schüle, C., Eser, D., Baghai, T.C., Nothdurfter, C., Kessler, J.S., Rupprecht, R., 2011. Neuroactive steroids in affective disorders: target for novel antidepressant or anxiolytic drugs? Neuroscience 191, 55–77.

Souli, Ch., Avlonitis, N., Calogeropoulou, T., Tsotonis, A., Maksay, G., Biró, T., Politi, A., Mavromoustakos, T., Makriyannis, A., Reis, H., Papadopoulos, M., 2005. Novel 17beta-substituted conformationally constrained neurosteroids that modulate $GABA_A$ receptors. J. Med. Chem. 48, 5203.

Suñol, C., García, D.A., Bujons, J., Kristofíková, Z., Matyás, L., Babot, Z., Kasal, A., 2006. Activity of B-nor analogues of neurosteroids on the $GABA_A$ receptor in primary neuronal cultures. J. Med. Chem. 49, 3225.

Thomas, P., Pang, Y., 2012. Membrane progesterone receptors: evidence for neuroprotective, neurosteroid signaling and neuroendocrine functions in neuronal cells. Neuroendocrinology 96, 162.

van Broekhoven, F., Verkes, R.J., 2003. Neurosteroids in depression: a review. Psychopharmacology 165, 97–110.

Veleiro, A.S., Burton, G., 2009. Structure–activity relationships of neuroactive steroids acting on the $GABA_A$ receptor. Curr. Med. Chem. 16, 1–18.

Veleiro, A.S., Rosenstein, R.E., Jaliffa, C.O., Grilli, M.L., Speroni, F., Burton, G., 2003. Synthesis and $GABA_A$ receptor activity of a 6,19-oxido analogue of pregnanolone. Bioorg. Med. Chem. Lett. 13, 343–346.

Weinfeld, J., Siegel, R.A., Chowers, I., 1980. *In vitro* conversion of pregnenolone to progesterone by discrete brain areas of the male rat. J. Steroid Biochem. 13, 961–963.

Wisden, W., Laurie, D.J., Monyer, H., Seeburg, P.H., 1992. The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J. Neurosci. 3, 1040–1062.

Xilouri, M., Papazafiri, P., 2006. Anti-apoptotic effects of allopregnanolone on P19 neurons. Eur. J. Neurosci. 23 (1), 43–54.

Zhao, H.F., Labrie, C., Simard, J., de Launoit, Y., Trudel, C., Martel, C., Rheaume, E., Dupont, E., Luu-The, V., Pelletier, G., 1991. Characterization of rat 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase cDNAs and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues. J. Biol. Chem. 266, 583–593.

Zheng, P., 2009. Neuroactive steroid regulation of neurotransmitter release in the CNS: action, mechanism and possible significance. Prog. Neurobiol. 89, 134–152.

Zwain, I.H., Yen, S.S.C., 1999. Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology 140, 3843–3852.