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Synthetic Epoxy-Pregnan Steroids: Effects on Anxiety Behavior in Rats

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Abstract

Neurosteroids like 3α-OH-5α-pregnan-20-one (allopregnanolone) and 3α-OH-5β-pregnan-20-one (pregnanolone) modulate the γ-aminobutyric acid-A ($GABA_A$) receptor function and produce several effects that can be considered for therapeutical pruposes like antidepressant and anxiolyticts, in a similar way to benzodiazepines. However, their rapid metabolism is a great disadvantage for medicinal treatments consideration. Synthetic steroid analogues with more bioavailability and stability arise like a solution to overcome these limitations. In previous studies, we evaluated the performance of a synthetic steroid group (*Epoxies*, similar to allopregnanolone and pregnanolone) throughout different assays in rat cerebral cortex and hippocampus, including neuroprotection and $GABA_A$ receptor modulation obtaining promising results. Taking into account the anxiolytic effect of allopregnanolone and pregnanolone, in this work we evaluated the effect of an intracranial administration (in dorsal hippocampus) of two synthetic steroids, *Epoxy 1* and *Epoxy 2*, in an anxiety animal model to provide knowledge about the possible *in vivo* effects of these steroids. Allopregnanolone and pregnanolone produced increases in vertical and horizontal exploration behaviors, interpreted as anxiolytic-like effects. However, the two *Epoxies* capable to modulate $GABA_A$ receptor binding have no effect on anxiety at the dose evaluated. These features make them suitable for other therapeutical purposes where the anxiogenic behaviors are not involved.

Keywords: Neurosteroids; Synthetic analogues; Allopregnanolone; Pregnanolone; Open field

Introduction

Inside the central nervous system, the *in situ* synthesized steroids (neurosteroids, NS) are capable of modify the brain excitability [1,2]. Due to their chemical structure, the steroids may produce positive or negative modulations over receptors, like the γ-aminobutyric acid-A ($GABA_A$) receptor [3,4]. Among the positive modulators of this receptor are two progesterone's metabolites: the 5α-pregnane-3α-ol-20-one (allopregnanolone, **A**) and its isomer 5α-pregnane-3β-ol-20-one (pregnanolone, **P**) [5]. In the last decades, the relevance of NS, like **A** and **P**, also has been increasing due to their multiple roles in normal and pathological behavior, ageing processes, damaged tissue regeneration and neuroprotection [6-9].

The interest on these steroids arises specifically from their potential activity as anticonvulsants, anesthetics, anxiolytic or sedative-hypnotic agents [10] useful for the treatment of several neurological and psychiatric disorders [5]. But, the main limitation for the therapeutical administration of these NS is their rapid *in vivo* biotransformation to other metabolites. Therefore, synthetic steroids (SS) analogues with better bioavailability and efficacy have an important therapeutic potential in brain disorders, becoming an alternative approach for different pathologies [11,12].

In the last decades, a significant increase around NS physiology and synthetic analogues development has been observed. The medicinal chemistry of neuroactive steroids (NAS) has been focused in the development of SS analogues preserving the absolute configuration of naturally occurring steroids. Structure/activity studies of the progesterone metabolites, indicate that the 3α-hydroxyl configuration is required for binding and activity maintenance [13], while modifications in some carbon atoms do not affect their functions [14-16]. In previous works we evaluated the performance of a SS group *Epoxies* (similar to **A** and **P**) through different assays as well as neuroprotection and inhibition/stimulation binding of specific ligands of the $GABA_A$ receptor in rat cerebral cortex and hippocampus [17,18]. The main feature of these *Epoxies* is the presence of an oxygen bridge that holds the A/B angle of steroidal nucleus in a controlled way, conferring conformational restricted analogues [19]. In those studies, we demonstrated that, of all the *Epoxies* tested, the *Epoxy 1* (3α-hydroxy-2β,19-epoxy-5α-pregnan-20-one; **A** analogue) and the *Epoxy 2* (3α-hydroxy-6,19-epoxypregn-4-ene-20-one; **P** analogue) produced the most similar effects to **A** and **P** [17,18].

On the other hand, severe psychiatric conditions like anxiety and stress-related disorders affect daily performance in tasks and represent a high cost to public health. Thus, the search of new and improve therapeutical alternatives remains currently. Also, the fact that neurosteroidogenic agents lack benzodiazepine-like side effects shows promise in the treatment of anxiety and depression [11]. Administration of **A** or **P** produces anxiolytic, ataxic, hypnotic-anesthetic and anticonvulsant effects as well as locomotor stimulation [5,20-30], being able to mimic the actions of benzodiazepines. Taking into account their effects as modulators of the $GABA_A$ receptor-ion-channel complex [5] mentioned before, the mechanism underlying their anxiolytic properties appears to be due to a potentiation of neural inhibition via this receptor [31-34]. Previous studies suggest that **A**

increases the channel conductance of $GABA_A$ receptor-gated Cl^- in hippocampal neurons, promoting anxiolytic-like effects [35-37]. This supports the hypothesis about the role that the dorsal (CA1) hippocampus area plays in emotional processes and NS modulation of anxiety and exploration. It has also been postulated that the dorsal region of this brain structure is involved in memory-related functions, whereas the ventral region mediates fear and anxiety responses[38]. In contrast to these anatomical and functional considerations, the recent results of Mòdol *et al.* [39] demonstrate that the dorsal (CA1) area of the hippocampus is also implicated in the modulation of anxiety-related behaviors. Their studies indicate that the hippocampal administration of **A** produces an anxiolytic-like profile and also increases exploratory behavior, observed trough the enhancement of the number of entries into the open arms in the elevated plus-maze device. This suggests that CA1 region together with other brain structures (such as the amygdala or the medial septum) could be an important target for explaining the NS effects on exploration, anxiety, learning and memory [39]. **P** administration in the dorsal hippocampus or in the lateral septum also produces dose-dependent anxiolytic-like effects in several animal models [36]. Moreover, both steroids have shown a comparable potency eliciting anxiolytic effects [24,27,31]. In addition, the ability of these and other NS to decrease anxiety-induced behavior has been demonstrated in several tests such as light/dark transition, rearing events and open-field [22,34,40]. In fact, there is evidence that the anxiolytic action of progesterone is mediated by its reduced metabolites, **A** and **P** [41,42]. Based in the previous results obtained with the *Epoxies* in the $GABA_A$ receptor modulation and the neuroprotective actions [17,18], and the anxiolytic-like effects mediated by **A** and **P**, the aim of this work was to evaluate the effects of two of these SS (*Epoxy 1* and *Epoxy 2*) administration over the animal behavior. These analysis were performed in an anxiety model (open-field test), assessing horizontal and vertical exploration and the anxiolytic-like effects; comparing results between synthetic and natural steroids.

Materials and Methods

Steroids

Two natural steroids (**A** and **P**) and two synthetic analogues, 3α-hydroxy-1β,11α-epoxy-5α-pregnan-20-one (*Epoxy 1*, **A** analogue) [43] and 3α-hydroxy-6,19-epoxypregn-4-ene-20-one (*Epoxy 2*, **P** analogue) [44] were used. Steroid concentration used for microinfusion (6.7 μg/μl) was selected according to our previous results [17,18]. The solutions were prepared using cyclodextrin 30% in artificial cerebrospinal fluid (aCSF) as vehicle one hour before the administration.

Experimental animals

Ninety day old adult male Sprague Dawley rats (400 g; n=48) were housed under standard laboratory conditions with a 12-h light-dark cycle, with food and water *ad libitum* on a temperature (21°C) and humidity (40-70%) controlled vivarium. The animal surgical procedures and treatments were evaluated by the Ethics Committee of the Instituto de Biología y Medicina Experimental (IBYME), CABA, Argentine, and approved according to the described on the protocol CE 040-Jun/2014, in accordance with guidelines defined by the European Community Council Directive (86/609/EEC) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Male rats were anesthetized with ketamine-xylazine (40:4 mg/Kg i.p.; Holliday, Richmond, Argentina) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga CA, USA). Handmade 23-gauge stainless steel cannulae with 30-gauge removable inserts (dummy cannulae) were unilaterally implanted on the dorsal hippocampus using flat skull coordinates (anteroposterior, -2.3 mm; mediolateral, ± 1.6 mm; dorsoventral, 2.8 mm; from Bregma) according to the atlas Paxinos and Watson [45]. Three stainless steel screws and dental cement were used to fix the cannulae to the skull surface. After the surgery, animals were subcutaneously injected with a nonsteroidal anti-inflammatory (Meloxican 2 mg/Kg body weight, Boehringer Ingelheim, Argentina). A painkiller (Tramadol chlorhydrate 0.15 g/L; Finadiet SACIFI, Argentina) and an antibiotic (Enrofloxaxine 5 mg/L; Afford SA, Argentina) were administered in the drinking water for the next 3 days.

Bilateral implants in some brain areas have been widely used to explore the anxiolytic and/or antidepressant like-effects [46-48]. Although, the choice of unilateral cannulation (with left/right alternations) performed in this work, was based in previous reports that described anxiolytic and antidepressant effects with unilateral microinjections of **A** [49] or progesterone [50], without behavioral differences between brain sides.

Microinfusion procedure

Animals were allowed to recover for 10 days before performing the behavioral trials. Vehicle (cyclodextrin 30% in aCSF) or steroids (**A**, **P**, *Epoxy 1* and *Epoxy 2*; 2 μg) were administered in 0.3 μl using a 30-gauge injection needle connected by PC-40 tubing (Rivero SA, Argentina) to a 10 μl glass syringe (Hamilton, Reno NV, USA; 0.1 μl/min). After the 3 minutes of microinfusion, the injection needle was kept in place for 1 additional minute to allow diffusion away from the cannula tip before the dummy cannula reposition. According to the condition, 5 groups of animals (n=8) were established: Vehicle (cyclodextrin 30% in aCSF treatment), **A** (**A** treatment), **P** (**P** treatment), *Epoxy 1* (*Epoxy 1* treatment) and *Epoxy 2* (*Epoxy 2* treatment). Animals were hand restrained during all microinfusion procedure.

Behavioral testing

The open-field task [51,52] was used in accordance with the methods described by McCarthy *et al.* [53]. Behavioral testing occurred between 10:00 and 15:00h. The open-field device (housed in a dimly lit room with controlled temperature 21°C and humidity 40-70%) consisted of 75 cm side squared box divided in 25 quadrants, with 30 cm height walls and an open roof. All animals were allowed to explore the device for 15 minutes (precondition test) one day before steroids microinfusion. At the next day, one minute after the dummy cannula relocation, animals were placed in the center of the device and were allowed to explore it for 300 seconds. The animal activity was videotaped with a digital camera suspended above the test apparatus. The behavioral parameters evaluated were: *Freezing* (duration of the animal in a completely stationary state), *Active time* (determined as total time test minus *Freezing* time), *Line Crossing* (number of grid lines crossed with all four paws), the frequency and duration of *Grooming* (when the animal licks and/or scratches itself while it stays still) and *Rearing* (when the animal stands up on its hind legs). After each trial, the apparatus was thoroughly cleaned with damp and dry towels to prevent olfactory cues. Behavioral videos were scored by two observers who were blind to the rat's treatment condition.

Histology

At the end of the behavioral testing period, animals were rendered unconscious by CO_2 and killed by decapitation. The brains were removed, frozen and stored at -80ºC. Coronal sections (40 µm thick) obtained with a cryostat were mounted on subbed slides and stained with cresyl violet [54] for microscopic visualization of cannulae placements. The results from animals with accurately placed cannulae were used for the statistical analyses.

Statistical analysis

Statistical analyses were performed with commercial softwares GraphPad Prism (Graphpad Software Inc., v.4) and Statview (SAS Institute Inc. v5.0.1). The effects of intracranial microinfusions on the behavioral parameters were analyzed by a one-way ANOVA and comparisons between each steroid and vehicle were made by Newman-Keuls post hoc test. Differences were considered significant when $p<0.05$.

Results

Histological analysis

Cannulae tip localizations in the brain from those animals used for statistical analyses are schematically represented in Figure 1a. They were mainly placed on the dorsal (CA1) area of the hippocampus throughout the rostral-caudal extent. Cresyl violet staining was used for the visualization (Figure 1b). Cannulae distribution was more frequently positioned in -2.40 mm anteroposterior; 1.3 and 2.4 mm mediolateral (left or right sides) and 2.6 mm dorsoventral from Bregma (Figure 1c; Paxinos and Watson [45]). Animals with bad placed cannulae (n=8) were discarded for statistical analyses.

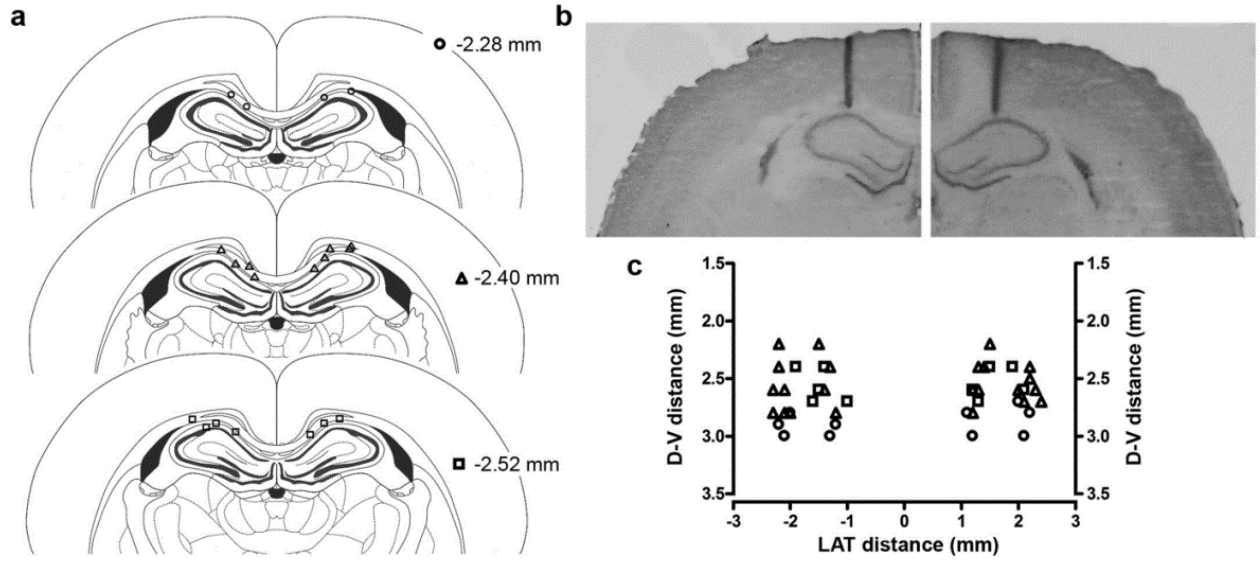

Figure 1: a Schematic representation of the well placed point of injection illustrating cannulae tip localizations throughout the rostral-caudal extent of the CA1 area of the hippocampus (anteroposterior, -2.3 mm; mediolateral, ± 1.6 mm; dorsoventral, 2.8 mm; from Bregma). Each symbol refers to different anteroposterior distance from Bregma according to Paxinos and Watson Atlas (1998; o:n=11; Δ:n=19; □:n=10). **b** Representative photomicrographs of half coronal section, cresyl violet-stained, showing unilateral cannulae tip placements (left or right; magnification x2). **c** Cannulae distributions according to dorsoventral (D-V) and mediolateral (LAT) distance in mm.

Active time

The amount of time in which animals were in activity was determined as the total time of the test (300 sec) minus the *Freezing* time (when the animals remain completely immobile). A significant increase on *Active time* was observed with **A** and **P** treatments compared to control (143% and 147% respectively; $p<0.05$). These increases can be translated as an augmentation in total locomotor activity and horizontal and vertical explorations (Figure 2a). Although, no effects were observed with *Epoxy 1* and *Epoxy 2* treatments.

Line crossing

Ambulation was quantified as the number of grid lines crossed with all four paws within the 300 sec of testing. **A** and **P** administrations produced significant increases of this parameter compared to vehicle (114% and 136% respectively; $p<0.05$; Figure 2b). That can be interpreted as an increase in horizontal exploration activity, although no effects were observed with *Epoxy 1* and *Epoxy 2* administrations.

Grooming and grooming ratio

The frequency and duration of each occurrence (when the animal licks and scratches itself) was measured throughout the 300 sec of testing. A significant increase in *Grooming* duration was observed only with **P** microinfusion (428% compared to control; $p<0.05$; Figure 2c). However, no significant effects were observed on the duration of each *Grooming* event indicated by the *"Grooming ratio"* (defined like *Grooming* duration/*Grooming* frequency; Figure 2d). No effects were observed with the other three steroids in any of both variables.

Rearing and rearing ratio

The frequency and duration of each occurrence (when the animal stands up on its hind legs) was measured in the 300 sec of testing. Significant increases in total *Rearing* duration were observed with **A** and **P** treatments compared to vehicle (265% and 271% respectively; $p<0.05$; Figure 2e). The increase of this parameter can be interpreted as an augmentation of vertical exploration activity, although no effects were observed on the duration of each *Rearing* event indicated by the *"Rearing ratio"* (defined like Rearing duration/*Rearing* frequency; Figure 2f). No effects were observed with *Epoxy 1* and *Epoxy 2* administrations in any of both parameters.

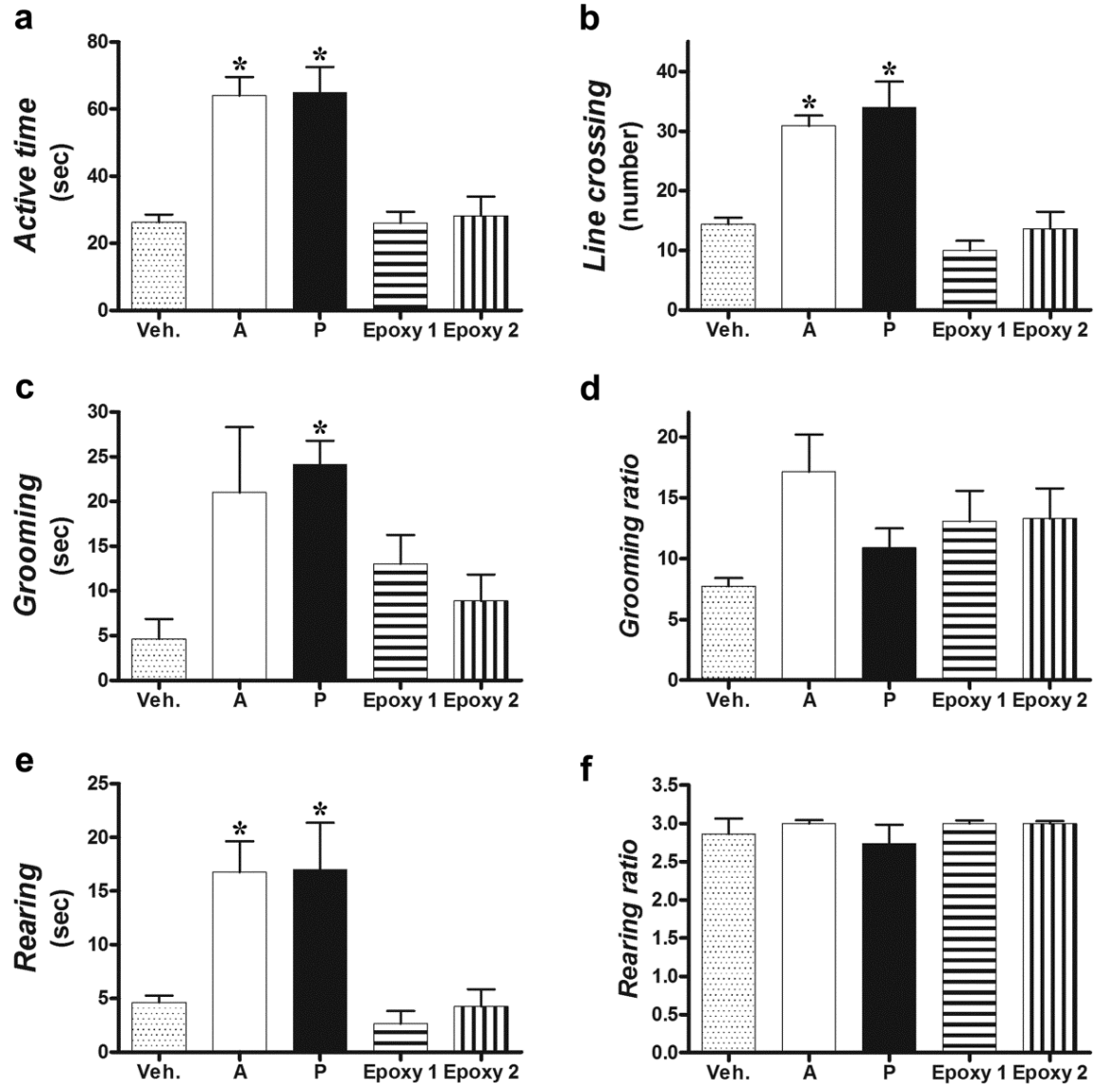

Figure 2: Unilateral microinfusion (0.3 µl) on dorsal hippocampus of vehicle (Veh., cylcodextrin 30% in aCSF) or steroid (**A**, **P**, *Epoxy 1* and *Epoxy 2*; 2 µg) and changes in open-field behavioral parameters: **a** *Active time* (difference between total time test and freezing in sec) **b** *Line crossing* (number of grid lines crossed with all four paws), **c** *Grooming* (when the animal licks or scratches itself while stays still in sec) and **d** ratio (duration/frequency) and **e** *Rearing* (when the animal stands up on its hind legs in sec), and f ratio (duration/frequency). Significant effect was determined by one-way ANOVA: $F_{Active\ time}(4,35)=15.3$; $p<0.001$, $F_{Line\ crossing}(4,35)=18.2$; $p<0.001$, $F_{Grooming}(4,35)=2.8$; $p<0.05$, $F_{Grooming\ ratio}(4,24)=1.1$; $p=0.12$, $F_{Rearing}(4,35)=13.3$; $p<0.001$ and $F_{Rearing\ ratio}(4,28)=0.5$; $p=0.75$, sub index in each F indicates the variable analysed. *$p<0.05$ Newman-Keuls post hoc test.

Correlation between Active time and Line crossing

Analysis of *Line crossing* variable based on *Active time*, showed a positive correlation factor of 0.74, when all the treatments were considered toghether (Figure 3 **C**). When this correlation was performed for each treatment, significant effects of **P** and its analogue, *Epoxy 2* (r2=0.77 and r2=0.69 respectively; Figure 3 **P** and Figure 3 *Epoxy 2*) were observed, but with lower *Active time* for the *Epoxy 2* than **P**. On the other hand, the *Epoxy 1* showed similar pattern than its natural analogue **A** and vehicle, with no significant ANOVA p values (Figure 3).

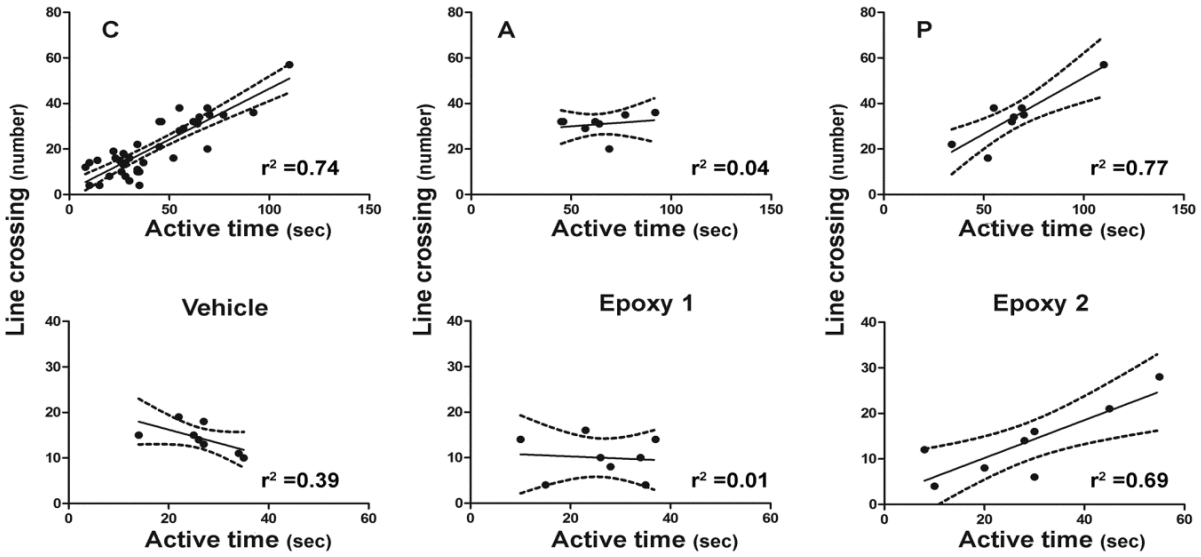

Figure 3: Correlation between *Line crossing* and *Active time* behavioral parameters, under all conditions together (**C**) or for each microinfusion treatment (Vehicle, **A**: allopregnanolone, **P**: pregnanolone, *Epoxy 1* and *Epoxy 2*). Each point represents a value corresponding to an individual animal test. Correlation coefficients are showed in each panel. Significant effects were determined by one-way ANOVA: $F_C(1,40)=108.2$; $p<0.0001$, $F_{Vehicle}(1,6)=3.9$; $p=0.09$, $F_A(1,6)=0.3$; $p=0.62$, $F_P(1,6)=20.5$; $p=0.004$, $F_{Epoxy\ 1}(1,6)=0.1$; $p=0.82$ and $F_{Epoxy\ 2}(1,6)=19.6$; $p=0.01$, sub index in each F indicates the treatment analyzed. Dotted lines indicate the 95% confidence intervals.

Discussion

Anxiety is an unpleasant nervous state of inner restlessness. Although benzodiazepines and barbiturates are among the treatments of choice for this kind of pathologies, they may induce various side effects [55]. Their actions may be mediated by the $GABA_A$ receptor located within the hippocampal formation, which mediates postsynaptic inhibition [56].

Progesterone and its reduced metabolites, **A** and **P**, were found to produce anxiolytic-like effects similar to benzodiazepines [36,57,58]. In the brain, *in situ* synthetized NS are capable of modulating neuronal excitability by rapid non-genomic actions [59] acting as potent modulators of $GABA_A$ receptor-mediated neurotransmission [5]. They can locally influence neuronal activity through their effects as paracrine messengers [60]. Thus, NS may have important effects on the regulation of the behavior in humans and in different murine models.

On the other hand, the hippocampal formation is important for the acquisition of short-term memory [61] and it has been also related to anxiety behaviors. Recently, reports described an association between dorsal hippocampus and the modulation of anxiety behavior [39]. Moreover, other studies have shown a significant correlation between low-anxiety or increased exploratory behaviors and **A** concentration in the hippocampus [52]. Several studies developed in rodents show that **A** and **P** reduce stress and anxiety-like behavior [1,36,62-67]. In fact, the blockade of progesterone's conversion to its metabolites impairs social behavior and induces anxiety-related behavior in rats [68].

The SS arise as a promising solution to the poor bioavailability and rapid metabolism of natural NS administration [12], but it is necessary to compare their biological effects in similar targets and doses of the NS.

In this work, an anxiety model developed to identify the pharmacological mechanisms and potential clinical effects of synthetic and natural steroids was used. Usually, this kind of model based on conflict situations can generate opposite motivational states induced by approach-avoidance situations, however it does not attempt to replicate all features and symptoms of a specific anxiety disorder. Rather, it tries to generate a state of anxiety that could be related to these disorders. Another important methodological consideration that needs to be taken into account is our choice to cannulate animals in a unilateral way (with left/right alternations). Although bilateral cannulae have been widely used to explore the anxiolytic and antidepressant-like effects [46-48], we relied in reports that have not found behavioral differences describing these effects between the brain sides with unilateral progesterone or **A** microinfusions [49,50]. In

agreement with this information, we do not observe lateral influence on the horizontal and vertical exploratory activities either.

Here, we evaluated the anxiolytic properties of an equal dose of **A**, **P** or two synthetic steroid analogues (*Epoxy 1* and *Epoxy 2*) by microinfusions in dorsal hippocampus on the open-field test, quantifying horizontal and vertical exploration. The changes observed on the behavioral parameters by **A** and **P** can be interpreted as anxiolytic-like effects. Both natural NS enhanced the exploratory activity observed trough the *Line crossing*, *Grooming* and *Rearing* increases and *Freezing* decrease. However, in some cases **P** treatment produced the most potent anxiolytic-like effects. Although anxiolytic effects of **A** are widely described in literature [69-72], only few reports have shown that **P** administration is able to produce similar effects [28,36,57] with an improved efficacy in some cases [27].

Therefore, the results described in this work support these last findings and add more evidence to this topic. Moreover, none of the treatment affected the duration of *Grooming* and *Rearing* events observed through the absence of significant differences in their ratios.

Although **A** and **P** treatments produce a significant increase in the *Active time* and *Line crossing*, correlations analyses of *Line crossing* based *Active time* were not equal. Only a positive correlation of these parameters was observed with microinfusions of **P** or its analogue *Epoxy 2*. These differences may probably due to the molecular features of each steroid. These analyses allow to describe similar behavioral effects for steroids with similar A/B rings conformations and suggest that the SS and probably **A** doses should be administered in higher concentrations to probably produce anxiolytic-like effects. Although, these SS had a limited solubility in aqueous medium.

Previously, we described similar responses to **A** and **P** with these SS administrations in a variety of assays, including binding modulation of specific ligands to the $GABA_A$ receptor, the 3β-hydroxysteroid dehydrogenase activity alteration and neuroprotection in cerebral cortex and hippocampus cultures against a hypoxic event [17,18]. However, no effects were observed in these behavioral assays, we do not discard these SS for further *in vivo* evaluations that include another doses and more behavioral assays.

Summarizing, in this work we evaluated, in an open-field test, the *in vivo* anxiolytic-like effects of two synthetic analogues of **A** and **P** with a decreased molecular flexibility (that confers a favorable spatial arrangement for the steroid binding site). Only an increase in animal exploratory activities interpreted as anxiolytic-like effects were observed with **A** and **P** microinfusions. The lack of anxiolytic-like effect at the dose evaluated, and the previously reported modulation on the $GABA_A$ receptor [17,18] may these SS suitable for therapeutical treatments in other mood disorders where the GABAergic function is involved like early chronic psychosocial stress or depression. Nevertheless the action of these *Epoxies* should be further evaluated for these conditions.

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