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Pregnenolone sulfate infused in lateral septum of male rats impairs novel object recognition memory

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Abstract:
In the present paper we show for the first time that pregnenolone sulfate (Preg-S) impairs rats' memory for novel object recognition when injected in lateral septum (1.2 μM). The effect of Preg-S is clearly related to the moment the reagent is administered: if administered shortly after the training phase, or prior to the test phase of the experiment, there is no amnesic effect. It is only amnesic when administered 30 min before training. Accordingly, Preg-S does not appear to affect the storage of new memories or their retrieval but rather the acquisition itself. Based on the described afferences and efferences of lateral septum, we suggest a possible stimulatory effect of Preg-S regarding glutamate receptors and/or an inhibitory effect of GABA receptors located in local interneurons or recurrent axon collaterals, both of which have been reported to exist in the aforementioned nucleus.

Key words:
neuroactive steroids, memory, pregnenolone sulfate, lateral septum, novel object recognition, male rats (Sprague-Dawley)

Introduction

Steroid hormones are able to exert their effects *via* at least two well-studied kind of events by: 1) signaling their intracellular receptors, and 2) modulating cell membrane receptors. The second one involves the action of steroid molecules collectively known as neuroactive steroids [17], being the so-called neurosteroids the subclass of neuroactive steroids synthesized strictly in the central nervous system (CNS) independently of any peripheral source [3]. Pregnenolone sulfate (Preg-S) is considered an excitatory neuroactive steroid since it negatively modulates the main inhibitory receptor in the nervous system, the $GABA_A$ receptor and additionally because it positively modulates excitatory glutamatergic NMDA receptors [7].

Neuroactive steroids and neurosteroids among them influence cognitive functions, particularly memory processes [14]. On the one hand, systemic or intracerebral administration of neuroactive steroids, like pregnenolone (Preg) or Preg-S, enhance memory in both young and old rats [15]. Also, several studies performed in rodents have demonstrated the promnesic effect of Preg-S in a passive avoidance test and on spatial memory [13, 16, 27], while allopregnanolone showed opposite effects by deteriorating memory in the Morris water maze when a sort of "human episodic like" memory was evaluated [14, 10]. However, there is an increasing controversy regarding the effect of sulfated steroids on memory, suggesting a more complex modulatory scene. Vallee et al. [26] revised evidence showing that dehydroepiandrosterone and its sulfate derivative are both able of presenting any kind of effect in humans, ranging from memory improvements to memory dysfunctions. In addition, it has been reported that post-training injected Preg-S impairs passive avoidance retention in rats [12]. Lateral septum forms part of the medial temporal system, which is crucial for memory and learning. It connects directly to the hippocampus through the septohippocampal formation, receiving heavy glutamatergic inputs through it [9, 23]. It also receives projections from the entorhinal cortex [11] and other subcortical structures. Injection of β-amyloid peptide into the entorhinal cortex induced selective cognitive deficits in tasks regarding recognition and spatial memory [22]. Little is known about lateral septum complex and the role of neuroactive steroids regarding cognitive functions. Some information related to the presence of Preg-S in the nervous system was considered to some extent controversial [21], although now is accepted that while sulfated steroids are present in the CNS of human beings they would not be natural endogenous modulator in rodents [21]. In this study we have evaluated the pharmacological effects of Preg and Preg-S injected intracerebroventricularly (*icv*) and in lateral septum (LS) on a novel object recognition task. This task is a paradigm based on appetitive dispositions instead of aversive behavioral responses (step through, step down, context conditioning, among others). It is possible that modulation of cognitive-memory processes are differently coordinated when the task to deal with for the subject is an appetitive one instead of its aversive counterpart. This could be particularly relevant for neuroactive steroids, whose subtle modulatory actions – particularly at the low doses utilized in the present study – could be to a great extent different from those attributed to classical neurotransmitters, i.e., modulatory actions quite dependent on context, age, previous state, gender, just to mention a few. We selected the appetitive task in order to avoid a possible confounding factor – the emotional component – by appealing to a more natural behavior, the attraction for novelty in the wild. From all this background we hope to get a better understanding of the role of neuroactive steroids in cognitive processes, in order to eventually attain new insights for future therapeutic applications regarding the important and growing field of neurodegenerative diseases.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (60–70 days old; 280–320 g) were housed in groups of four per cage until surgery. After surgery the rats were housed alone. Room temperature was maintained at 22 ± 1°C with lights on from 7.00 a.m to 7.00 p.m. Food and water were freely available throughout the experiments. Animals for these experiments were kept and handled according to the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Research, Commission on Life Sciences, National Research Council, USA, 1996. All efforts were made to minimize animal suffering.

Reagents

The reagents utilized were Preg-S and Preg (SIGMA, St. Louis, MO, USA), penicillin G benzathine (Richet, Argentina) and chloral hydrate (Anedra, Argentina). Stocks of Preg and Preg-S were initially dissolved in propylene glycol to a concentration of 0.6 mM and 0.4 mM, respectively. The different doses of Preg-S and Preg used in the experiments were obtained by dilution in sterile saline in order to make negligible the final amount of propylene glycol. Notwithstanding, control animals were injected with sterile saline containing propylene glycol in equivalent concentrations to that used in experimental groups.

Novel object recognition test

The apparatus consisted of a wooden box (70 × 45 × 30 cm) with a white acrylic floor. It was located in an isolated testing room that was dimly lit by constant indirect illumination from the main source, a 25 W light bulb suspended over the box. The objects utilized as familiar or unfamiliar (new) objects were three copies of a pink truncated pyramid and a grayish, opaque candlestick of approximately the same size, all of which were heavy enough to prevent displacement by the animals. Since rats are red colorblind, we compared grayscale values for both the pyramids and the candlestick, finding that the grayscale value for the pyramids was a composed red-green-blue (RGB) of 166 (the whole range extending from 0 to 255), while the corresponding value for the candlestick was R165:G169:B161, accounting for an average value of RGB 165. From these values we concluded that the objects were: 1) quite comparable regarding their grayscale values; and 2) the present study dealt mainly with the shape of the objects and not their respective colors.

The novel object recognition test was performed as described elsewhere [5]. For at least a one hour period, the animals were allowed to get used to the experimental room. The day before training, each animal freely explored the apparatus with no objects for 2 min. A training session (T1) was followed by a test session (T2) 24 h later. During training session, animals were placed in the arena containing two identical objects (pink truncated pyramids). In the test session a familiar object was changed for an unfamiliar one (grayish opaque candlestick). Duration of both training and test stages were 3 min each. The position of the objects (familiar and unfamiliar) and the extreme of the box used to place the objects were randomly exchanged for each experimental animal in order to avoid the use of potential confounding spatial clues. Exploration was defined as the orientation of animal's snout towards the object, within a range of 2 cm or less from the object [5]. Running around the object or sitting on it was not recorded as exploration. The objects and floor were carefully cleaned with ethanol (10%) after each individual trial to equate olfactory cues. The experiments were recorded with a camcorder digital camera JVC Everio GZ-MG330 (Japan) using a black and white mode in order to improve the register. The measures in the object recognition test were as follows: 1) time spent by the subject to explore individual objects during T2; 2) e1 and e2: total time spent by the subject exploring both objects during training (T1) and test (T2) sessions, respectively; and 3) discrimination index, the difference between time spent exploring unfamiliar and familiar objects during T2 [20].

For dose-response curve, the subjects received *icv* injections of 5 µl with different doses of Preg-S (12 nM; n = 6, 0.12 µM; n = 6, 1.2 µM; n = 6 and 12 µM; n = 6) and vehicle (n = 6) 30 min before training session started. For experiments dealing with lateral ventricle, the animals were injected *icv* (5 µl) with Preg (1.2 µM; n = 8), Preg-S (1.2 µM; n = 7) or vehicle (n = 6). Finally, regarding lateral septum, animals were injected (1 µl) with Preg (1.2 µM; n = 6), Preg-S (1.2 µM; n = 6) or vehicle (n = 6). Drug administration was performed with a needle (0.5 mm outer diameter), connected to a 10 µl syringe (Hamilton, Reno, Nevada, USA), introduced through the guide cannula until its tip was 1.5 mm below the end of the cannula. The intracerebral needle was removed 60 s after the injection. Thirty minutes after drug injection, animals were put into the arena to perform the training session. Immediately after being tested each animal was placed for 5 min in the Opto-Varimex for an automatic evaluation of locomotor and exploratory behavior and their related variables (see below).

Locomotor and exploratory behavior

A commercial apparatus, a photoelectric device known as Opto-Varimex (OVM; Columbus Instruments, USA), designed to measure photobeam interruptions in individually tracked photocells, was used to assess the locomotor and exploratory activity of the animals once the main object recognition test was finished. The object of this test was to avoid potentially confounding variables affecting the main memory results, i.e., the effect of fear, motivation and limitations regarding locomotor activity. The OVM consisted of a plexiglas transparent cage (30 × 42 × 42 cm) with a homogenous black plastic floor. The walls housed infrared emisors and detectors in order to automatically register several measures: 1) horizontal activity: all movements performed on the horizontal axis; 2) ambulatory activity: all movements detected as displacement; 3) non-ambulatory activity: all movements performed by the animal while remains in the same place; 4) number of movements: number of episodic or consecutive movements performed by the animal; and 5) vertical activity: number of times the

subject rises on their rear feet in the air or against the walls during at least 2 s. Regarding any measure other than discrete ones (for example vertical activity), the measures are referred to as total counts/5 min, scoring a count as an interruption of the photobeam per second.

Surgery

Rats were anesthetized with chloral hydrate (5 ml/kg, *ip*) and fixed in a stereotaxic frame (David Kopf, USA). Guide cannulae were made from stainless steel (0.80 × 38 mm) and were unilaterally implanted in right lateral ventricle or lateral septum. When the cannulae were implanted in LS they were introduced until reaching 1.5 mm above the structure in order to minimize damage. The following coordinates from bregma were used in accordance with the coordinates of Pellegrino's atlas [18]: AP –0.4 mm, L –1.5 mm, DV –4.9 mm (for LV), AP +1.8 mm, L –0.5 mm, DV –3.7 mm (for LS). Cannulae were fixed to the skull with dental cement and a stainless steel screw. At the end of the surgery, cannulae were sealed with a stainless steel wire to protect them from obstruction. To prevent infections, all animals received an intramuscular (*im*) injection of 0.2 ml penicillin G benzathine.

Histology

After the behavioral test, animals were decapitated and injected through the guide cannulae with 1 μl of blue ink to confirm the location of the guide cannulae into LS. The microinjections sites were localized in diagrams of Pellegrino rat brain atlas [18] (Fig. 1). Only animals with microinjections in LS were included in any experimental protocol.

Statistical analysis

For the statistical analysis we utilized the software StatView for Windows (Abacus Concepts, Berkely, CA, USA). The discrimination index for object recognition, as well as locomotor and exploratory activity, were analyzed using a one way analysis of variance (ANOVA), followed by a *post-hoc* Newman-Keuls test. Pairs' comparisons were analyzed using the Student-*t* test. Data are expressed as the mean ± SEM; $p < 0.05$ was considered as minimum criterion for assigning statistical significance.

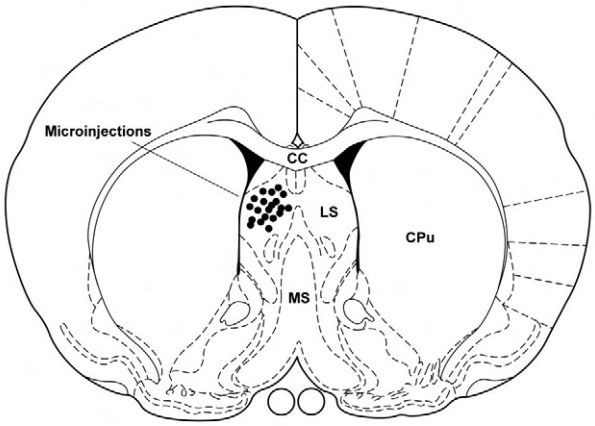

Fig. 1. Schematic representation of a brain coronary section showing lateral septum and the place where microinjections were performed (some points are superimposed). Abbreviations: CC – corpus callosum, CPu – caudate putamen, LS – lateral septum nucleus, MS – medial septal nucleus

Results

Implant localization

Brain examination of canulated rats revealed improper placement of cannulae in eight animals. The cannulae were shown to be obstructed in three additional animals during the experiments. Improper placement or obstructions of cannulae mandated exclusion of those animals from the experiments reported here. A schematic drawing of the brain shows the points of microinjections of most of the subjects included in the present study (Fig. 1).

Locomotor and exploratory activity

Since it is important to exclude the eventual effect of confounding variables in any study dealing with learning and memory, particularly those related to locomotor and exploratory activity of the animals, we used equipment designed to automatically detect changes in the amount and duration of several activities (see Opto-Varimex, Materials and Methods). Statistical analysis by one-way ANOVA showed the following results: ambulatory activity ($F = 3.44$; $p = 0.09$); non-ambulatory activity ($F = 0.54$; $p = 0.6$); number of movements ($F = 1.93$; $p = 0.22$); vertical activity ($F = 1.74$; $p = 0.24$); and horizontal activity ($F = 1.97$;

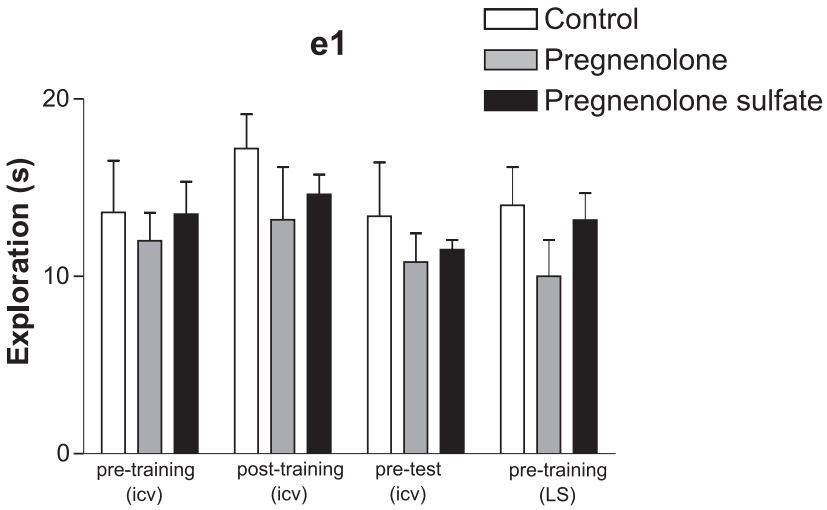

Fig. 2. Total exploratory time of both objects during training (e1) and test (e2) sessions. Treatments were given 30 min before training (pre-training), 10 min after training (post-training), or 30 min before test (test) (for each group n = 6–8). Statistical differences were not detected, either during training session and/or during test session. The results represent the mean time ± SEM expressed in seconds (s). LS – lateral septum, *icv* – intracerebroventricular microinjections

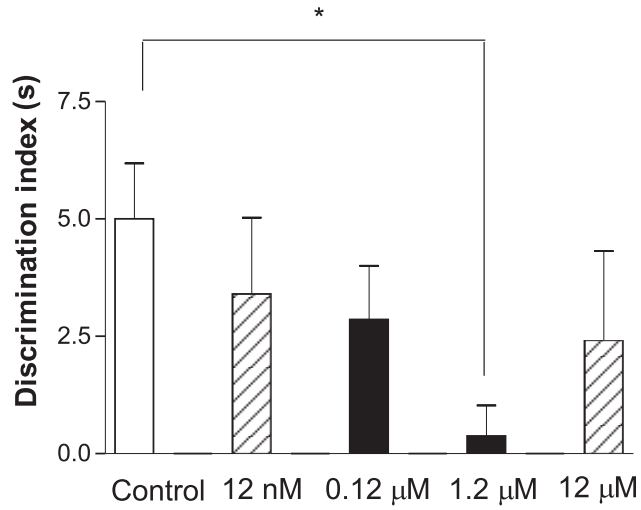

Fig. 3. Novel object recognition test and effects of increasing doses of injected *icv* pregnenolone sulfate. Animals were treated with reagents 30 min prior to training sessions. Tests were performed 24 h later. The results represent the mean ± SEM expressed in seconds during the test session (for each dose n = 6). Discrimination index – difference between the time spent exploring the novel object and the time spent exploring the familiar one. * $p < 0.05$

Tab. 1. *F* values and significance levels for pregnenolone sulfate injected intracerebroventricularly (*icv*) or in lateral septum (LS) on the novel object recognition test. Treatments were made: (**A**) 30 min prior to training sessions (pre-training) (*icv* n = 6–8; LS n = 6); (**B**) 10 min after training sessions (post-training) (n = 6); and (**C**) 30 min before test sessions (test) (n = 6). The pre-training group (LS) was analyzed using the Student *t*-test

Curve dose-response (*icv*)	$F_{1,4} = 2.19$	$p < 0.05$
Pre-Training (*icv*)	$F_{1,3}$ 0 5.79	$p < 0.01$
Post-Training (*icv*)	F = 0.84	NS
Test (*icv*)	F = 0.67	NS
Pre-Training (LS)	t = 2.324 df = 12	$p < 0.05$

$p = 0.21$). Additionally, there were no significant differences between control and treated groups regarding total exploration time, the time spent by the subjects exploring both objects, during training session (e1) and test session (e2) (Fig. 2).

Object recognition test

Excepting the dose 1.2 μM – that was able to clearly impair memory functioning ($F_{1,4} = 2.19$, $p < 0.05$; Tab. 1 and Fig. 3) – treatment with smaller or bigger Preg-S doses administrated *icv* (Fig. 3) did not show a significant effect over memory performance on the object recognition test. Memory impairment was only obtained when Preg-S was administered 30 min prior to training sessions ($F_{1,3} = 5.79$, $p < 0.01$; Tab. 1 and Fig. 4A), being completely unable of showing a simi-

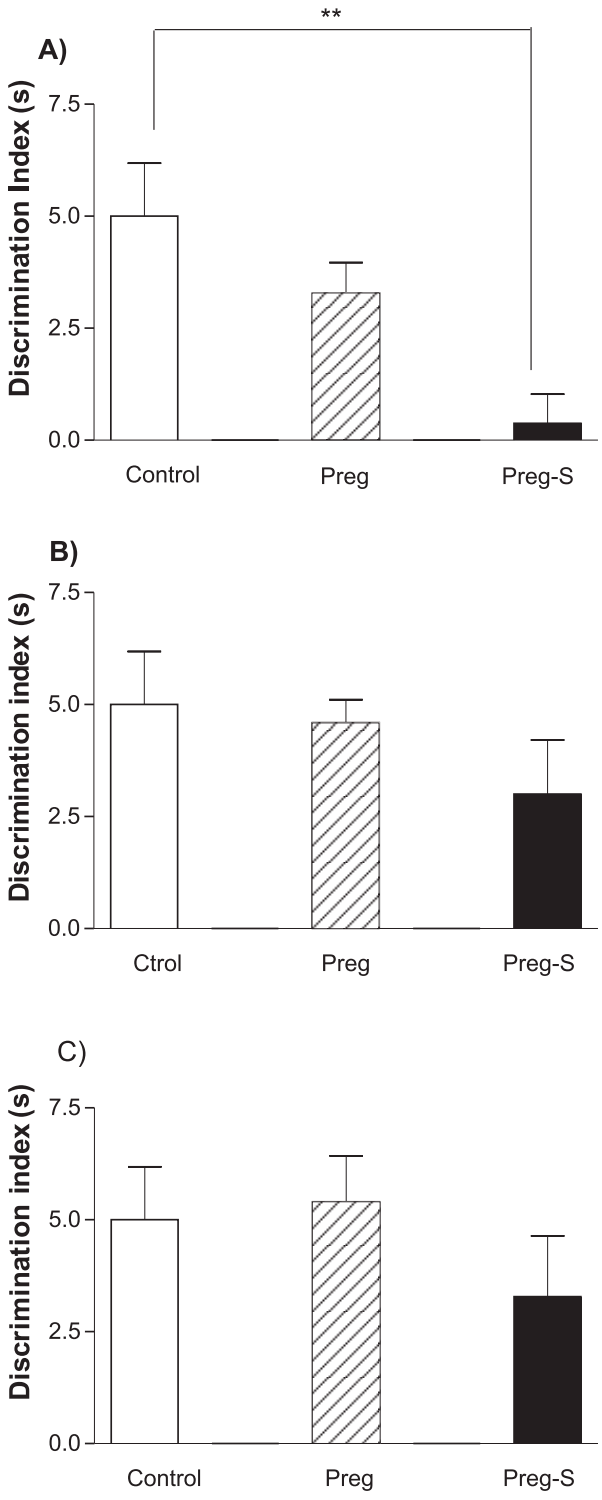

Fig. 4. Effects of *icv* injections of pregnenolone and pregnenolone sulfate on the novel object recognition tests. Treatments were made: (**A**) 30 min prior to training sessions (n = 6–8); (**B**) 10 min after training sessions (n = 6); and (**C**) 30 min before test sessions (n = 6). Tests were always performed 24 h after training. The results represent the mean ± SEM expressed in seconds during the test session. Discrimination index = difference between the time spent exploring the novel object and the time spent exploring the familiar one. ** $p < 0.01$

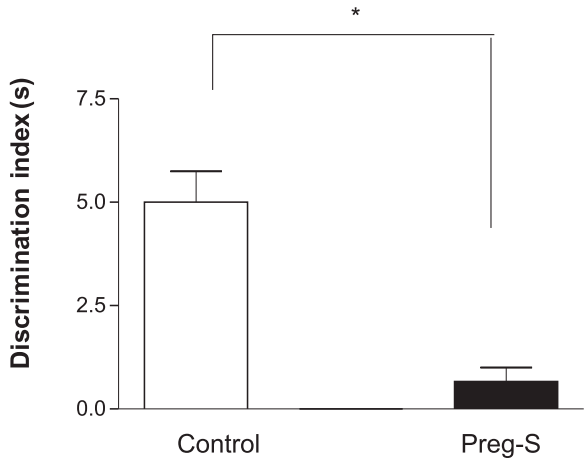

Fig. 5. Effect of pregnenolone sulfate injected in right lateral septum 30 min prior to training sessions on the novel object recognition test. Tests were performed 24 h later. The results represent the mean ± SEM expressed in seconds during the test session (for each group n = 6). Discrimination index – difference between the time spent exploring the novel object and the time spent exploring the familiar one. * $p < 0.05$

lar result if Preg-S was administered shortly after training ($F = 0.84$, $p = 0.44$; Tab. 1 and Fig. 4B) or 30 min prior to test itself ($F = 0.67$, $p = 0.52$; Tab. 1 and Fig. 4C). When the procedure was performed by injecting the LS, it was quite clear that 1.2 μM Preg-S treatment made the animals unable to distinguish between novel and familiar objects ($p < 0.05$; Tab. 1 and Fig. 5).

Discussion

Besides being a "controversial neurosteroid" [21], from our results it is clear that exogenous Preg-S pharmacologically impairs novel object recognition in rats when administered prior to training sessions, but not when it is administered shortly after the training or prior to the test itself. However, Preg alone does not impair neither learning nor memory, what suggests a possible dual role for this neuroactive steroid according to its chemical situation, i.e., sulfated *vs.* non-sulfated derivatives, functioning like a kind of molecular switch, at least in those species where they are known to be present and function as endogenous modulators, i.e., human beings [7]. It is worth mentioning that there is not only an amnesic effect, but

that this effect is also attained with very small doses – 6 pmol when administered *icv* and 1.2 pmol when administered in LS that although not unusual in the literature [12] is clearly suggestive of the potency of these compounds (see below). It is necessary to remember that the aforementioned effects are shown when Preg-S or Preg are injected *icv* – what preclude us of assigning the effect just to the LS but rather a kind of broad action on different structures of the CNS and also into the LS, what suggests a much more delicate regulation. This is not surprising, since neuroactive steroids have been proposed to exert a well tuned balance between excitatory (primarily glutamatergic systems) [8] and inhibitory systems (primarily GABAergic systems) [1, 25], acting in both cases as allosteric regulators of the correspondent receptors [7].

Regarding the effects of Preg-S on memory tasks, it has been reported a better response regarding spatial memory tasks in rats, associated to an increase in hippocampal acetylcholine [4]. In addition, a review dealing with neuroactive steroids and their sulfated variants [27] has summarized information regarding the promnesic effects of Preg-S in aging rats correlated with an increase of the levels of Preg-S in the hippocampus. However, Vallee et al. [26] and Martin-Garcia and Pallares [12] have reported deleterious memory effects for the same neuroactive steroid. In agreement with these reports, we show a clear impairment of memory tasks when dealing with novel object recognition. While hippocampus has been a traditional target of memory studies, the LS has received little or no attention at all, particularly within the framework of the memory task we utilize here (this paradigm involves at least a few components of what is known as episodic memory in human beings). It is possible that neuroactive steroids modulate one or more memory system, and that this modulation could be not unitarily ascribed to one effect or the opposite. According to the place and without changing any basic molecular and cellular mechanism, not to mention the dose utilized, it could be obtained an inhibitory or stimulatory result. In fact, that is what we are precisely showing here, an amnesic effect for a neuroactive steroid that otherwise has been reported to be primarily promnesic [27]. This is something to be taken into account every time we think of possible practical uses of neuroactive steroids regarding different kind of physiological or pathological conditions.

Lateral septum receives afferences from several places, among them glutamatergic afferences from the hippocampus *via* the hippocampus-septal pathway [2]. On the other hand, and notwithstanding efferents from LS to several CNS regions, a particularly important one is its GABAergic output [6]. There is some controversy over whether or not true GABAergic interneurons exist in the LS, or rather recurrent axon collaterals able of releasing GABA [19, 24]. Preliminary results from our laboratory (not shown here) indicate a great possibility of having GABA receptors that heavily influence neurotransmission in LS prior to its recognized GABA output. Since neuroactive steroids, particularly those having sulfated and not sulfated forms, affect both stimulatory and inhibitory neural systems, we are tempted to hypothesize both a stimulatory effect of Preg-S on afferent glutamatergic receptors coming from the hippocampus, and/or an inhibitory effect of local GABA receptors in LS. Of course, we cannot ignore the possibility of a much more complex mechanism involving both kind of effects and the possible putative promnesic effects of Preg alone.

Summarizing, we report here the amnesic effect of Preg-S when injected in LS of male rats, an otherwise promnesic compound when tested in different areas of the CNS. On the other hand, we can not rule out the possibility of Preg being promnesic, but we do not tested this possibility here. Since we tested an appetitive task, a novel object recognition test, we are tempted to speculate about a possible role for emotion, in the present context of Preg-S acting on the LS, in acquiring a mnesic trace. An appetitive task – one that does not require a strong emotional compromise, and so does not require a strong limbic involvement, could be possibly sensitive to a kind of neuroactive steroid modulation, while on the other hand, a strong emotional component (like the one involved in strong aversive tasks) could be related to the opposite. Finally, just a few words to emphasize the role of the LS in the results presented here. While little is known regarding its function, in the present work we present clear evidence regarding its fundamental role at acquiring memory traces after exposing the subjects to an appetitive task. Their afferences from the hippocampus, and their not so well defined afferences to different places of the CNS, to the amygdala among others, could account for this critical role. At present, we are working in our laboratory to contribute to answer these questions.

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