

Timing of progesterone and allopregnanolone effects in a serial forced swim test

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Artículo original

SUMMARY

The forced swim test (FST) is commonly employed to test the potency of drugs to reduce immobility as an indicator of anti-despair. Certainly, antidepressant drugs reduce the total time of immobility and enlarge the latency to the first immobility period. FST is preceded by the open field test (OFT) to discard any influence of changes in general motor activity that could interfere with immobility in the FST. Albeit progesterone and its α -reduced metabolite allopregnanolone produce antidepressant-like effects in the FST, the timing of actions is unknown. We hypothesized that the latency and duration of effects produced by progesterone and allopregnanolone may be characterized by repeated FST sessions; we therefore devised a serial-FST experimental design to evaluate the timing effects of these steroids on immobility, locomotion in the open field test, and grooming in the later as an indicator of response to stress.

We included fifty-one ovariectomized adult Wistar rats weighing 200-250 g at the beginning of the experiments. They were ovariectomized by abdominal approach under anesthesia. Rats were housed six per cage, at room temperature ($25 \pm 1^\circ\text{C}$) under a 12 h/12 h light/dark cycle (lights ON at 7:00 a.m.) with *ad libitum* access to purified water and food. All of the experimental procedures followed National Institutes of Health Guidelines. The local Ethics Committee (Biomedical Research Institute, Universidad Nacional Autónoma de México) approved the experimental protocol.

A first group received vehicle (2-hidroxypropyl- γ -cyclodextrin dissolved in injectable sterilized water to obtain a 35% solution, control group $n=17$), the second group progesterone (1.0 mg/kg, $n=17$), and the third group allopregnanolone (1.0 mg/kg, $n=17$). All single injections were applied by intraperitoneal route at a volume of 0.8 ml/kg.

The effects of treatments were evaluated in the serial-FST at 0.25, 0.5, 1, 2, 4, 6, and 24 h after injection, in a rectangular pool (height, 60 cm; length, 30 cm; width, 50 cm), with 24 cm deep water ($25 \pm 1^\circ\text{C}$). We evaluated the total time of immobility, during 5 min, considered as the principal indicator of an anti-despair effect. Before each session of serial-FST, locomotion was evaluated in the OFT during 5 minutes. The apparatus consisted on an acrylic box (height, 20 cm; length, 44 cm; width, 33 cm), with twelve squares delineated on the floor (11×11 cm). In the same OFT sessions, grooming was evaluated as an indicator of response to stress. Statistical analysis consisted in two-way analysis of variance (ANOVA) and Student-Newman-Keuls as *post hoc* test.

Total time in immobility was the highest and remained at similar levels only in the control group throughout the seven sessions of the serial-FST. In the allopregnanolone group a reduction in immobility was observed beginning 0.5 h after injection and lasted approximately 1.5 h. Similarly, progesterone reduced immobility beginning 1.0 h after injection, and the reduction lasted for approximately 5.0 h. In all groups, locomotion in the OFT was reduced after the first serial-FST session and remained at similar low levels during the serial-FST. In the control group, grooming was reduced after the first serial-FST session and lasted 24 h, but grooming did not change in the progesterone-or allopregnanolone-treated rats.

From a serial-FST design, we conclude that progesterone and allopregnanolone exert short time-dependent reductions in immobility and anti-stress-like effects no longer than 24 hrs, and seemingly a reduction in the response to stress, which may have some clinical applications.

Key words: Allopregnanolone, antidepressant, anti-stress, grooming, progesterone, serial-forced swim test.

RESUMEN

Introducción

La progesterona y su metabolito activo allopregnanolona se han estudiado ampliamente en modelos experimentales de ansiedad y depresión, y por su propiedad de ser sintetizadas en el cerebro se les considera como neuroesteroides. Entre las pruebas que permiten determinar la potencia antidepressiva de ciertos fármacos se encuentra la prueba de nado forzado, la cual se diseñó originalmente para detectar la potencia de sustancias con propiedades antidepressivas. Estas sustancias reducen el tiempo de inmovilidad y alargan la latencia al primer periodo de inmovilidad, lo cual es considerado como un efecto antidepressivo. Usualmente, la prueba de nado forzado se aplica dos veces, una sesión de preprueba que dura 15 minutos, en la cual la rata o ratón desarrolla el estado de desesperanza. La preprueba es seguida de la sesión de prueba que se realiza 24 horas después durante 5 minutos. En ella se evalúa el efecto de las sustancias con propiedades antidepressivas. Además, la prueba de nado forzado es precedida por la prueba de campo abierto con la finalidad de identificar cambios en la actividad motora general (hipoactividad o hiperactividad) que pudiera interferir con la interpretación de las variables evaluadas en la prueba de nado forzado.

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Algunos esteroides, como la progesterona y alopregnanolona, reducen la inmovilidad y alargan la latencia a la primera inmovilidad en la prueba de nado forzado, lo que indica su efecto tipo-antidepresivo. Sin embargo, la latencia y la duración de los efectos farmacológicos son desconocidas. La hipótesis de este trabajo fue que, si utilizáramos la prueba de nado forzado de forma repetida, podríamos identificar el tiempo de duración de los efectos de estos esteroides. Por lo tanto, diseñamos un experimento con la prueba de nado forzado seriada para evaluar el tiempo de permanencia de los efectos de progesterona y alopregnanolona en esta prueba conductual.

Materiales y métodos

Sujetos: En este estudio se incluyeron 51 ratas adultas ovariectomizadas de la cepa Wistar, con peso entre 200 y 250 g al inicio de los experimentos. Las ratas fueron anestesiadas y ovariectomizadas por aproximación ventral y fueron alojadas en cajas de acrílico transparente ($n=6$), con una temperatura ambiente de $25 \pm 1^\circ\text{C}$ y con un ciclo de luz-oscuridad de 12 \times 12 h (la luz se encendió a las 7:00 am). Las ratas tuvieron libre acceso al agua purificada y al alimento (Purina). Todos los procedimientos realizados en este estudio fueron de acuerdo con las normas éticas en el uso de animales de experimentación, basándonos en la Guía del National Institute of Health, y el protocolo fue aprobado por el Comité de Ética del Instituto de Investigaciones Biomédicas de la Universidad Nacional Autónoma de México.

Grupos y tratamientos: Las ratas del grupo control recibieron el vehículo (solución al 35% de 2-hidroxipropil- β -ciclodextrina), el segundo grupo recibió progesterona (1.0 mg/kg) y el tercero recibió alopregnanolona (1.0 mg/kg) por vía intraperitoneal, en un volumen de 0.8 ml/kg.

Pruebas conductuales: El efecto de los tratamientos fue evaluado en la prueba de nado forzado a las 0.25, 0.5, 1, 2, 4, 6 y 24 horas después de la administración. Utilizamos un estanque rectangular (base 50 \times 34 cm, altura 60 cm), con agua a 25°C y una altura de 24 cm. Sólo se evaluó el tiempo total de inmovilidad, considerando que es el principal indicador de un efecto antidesesperanza. Antes de cada sesión de nado forzado se evaluó la actividad motora (cuadros deambulados) y el acicalamiento en campo abierto. Esta prueba consistió en colocar

a la rata en una caja de acrílico (base 33 \times 44 cm, altura 20 cm) con el piso dividido en 12 cuadros de 11 \times 11 cm. Los resultados obtenidos de ambas pruebas fueron evaluados por medio de una ANOVA de dos vías y como prueba *post hoc* se aplicó Student-Newman-Keuls.

Resultados

La prueba de nado forzado aplicada de forma repetida resultó ser útil para evaluar los efectos temporales producidos por dos esteroides con potencia antidepresiva. Las ratas del grupo control mostraron los valores más altos de inmovilidad en la prueba de nado forzado, los cuales se mantuvieron así durante las sesiones de prueba. En los grupos tratados con progesterona o alopregnanolona hubo una reducción de la inmovilidad, gradual y temporal. Los animales tratados con alopregnanolona redujeron la inmovilidad a partir de las 0.5 horas después de la administración, efecto que se mantuvo por un periodo de 1.5 h. Los animales tratados con progesterona redujeron la inmovilidad a partir de 1.0 hora después de la administración, efecto que se mantuvo por un periodo de 5.0h. En campo abierto, independientemente del tratamiento, hubo una reducción del número de cuadros cruzados después de la primera sesión de nado forzado, efecto que permaneció hasta las 24h. En el acicalamiento, se observó que sólo los animales del grupo control redujeron significativamente el tiempo empleado en esta conducta, mientras que los animales inyectados con progesterona o alopregnanolona no modificaron esta variable. Es decir, mantuvieron niveles semejantes durante todas las sesiones de prueba y estuvieron por arriba de los valores encontrados en los animales control.

Conclusión

La progesterona y la alopregnanolona ejercen un efecto antidesesperanza de breve latencia, no mayor a 24 horas. Este hallazgo podría tener implicaciones clínicas en pacientes con depresión refractaria al tratamiento convencional.

Palabras clave: Acicalamiento, alopregnanolona, antidepresivo, antiestrés, progesterona, FST seriada.

INTRODUCTION

Progesterone and its metabolite allopregnanolone are gonadal steroids for which anxiolytic^{1,2} and antidepressant-like^{3,4} effects have been demonstrated in experimental animal models.⁵ Progesterone is a drug frequently used in gynecology,⁶ while allopregnanolone has been tested in some clinical approaches.⁷ Because of their actions in Central Nervous System and behavior⁸ and their synthesis *in situ*, both steroids are classified as neurosteroids,⁹ but the timing of their possible anti-despair actions is unknown.

An accepted model for testing antidepressant drugs is the forced swim test (FST). At the beginning of the FST, the rat swims vigorously, apparently seeking an exit. Later, the animal becomes almost immobile while exhibiting minimal movements to maintain its head above the water's surface. The observation that immobility is reversed by systemic administration of a wide variety of clinically effective antidepressant drugs supports the assumption that immobility reflects despair.¹⁰

In experimental FST single high-doses are commonly used (surrounding 10 mg/kg)¹¹ or higher.¹² Notwithstanding, we¹³ and other research groups¹⁴ have repeatedly demonstrated that low doses (about 1 mg/kg) of antidepressants significantly reduces immobility in FST, and increases the neuronal firing rate of forebrain structures related with hedonism, such as lateral septal nucleus, only after several weeks of treatment, resembling with more precision clinical observations.¹⁵

Although the number of FST sessions is controversial,¹⁶ we propose that a serial-FST (i.e., in which the FST is conducted with repeated sessions within a short period of time) may be useful for measuring the time-dependent effects of anti-despair or antidepressant-like drugs. In any case, the influence of locomotor activity on immobility in the FST must be discarded by subjecting the animal to the open field test (OFT).¹⁷ In OFT, some additional indicators of stress may also be incorporated, such as a self-directed behavior, i.e., grooming. This behavior may increase or decrease depending on the severity of stress. Mild stress

(restraint during a short time) increases grooming,¹⁸ while chronic high-stress (footshocks), decreases grooming.¹⁹ Noticeably, antidepressant drugs reverse the effect of mild stress.^{18,20} Herein, FST may be considered as a stressful situation, but some differences may be expected depending on the length of the test.

We, therefore, hypothesized that a serial-FST experimental design may be useful for determining the onset and duration of action of a given treatment (e.g. progesterone and allopregnanolone). In a longitudinal serial-FST design we evaluated anti-despair-like behavior produced by progesterone or allopregnanolone, and compared the results with a vehicle-treated group. The OFT assessed any influence of changes in locomotor activity on immobility time, and grooming to explore the response to stress.

MATERIAL AND METHODS

Animals

Fifty-one adult female Wistar rats were used, weighing 200-250 g at the beginning of the experiments. They were housed in acrylic boxes (six rats per cage) at room temperature ($25 \pm 1^\circ\text{C}$) under a 12 h/12 h light/dark cycle (lights on at 7:00 a.m.) with *ad libitum* access to purified water and food. All of the experimental procedures followed National Institutes of Health Guidelines.²¹ The local Ethics Committee (Biomedical Research Institute, Universidad Nacional Autónoma de México) approved the experimental protocol.

Ovariectomy

Rats were ovariectomized 14 days before the behavioral procedures to control changes in immobility in the FST associated with hormonal oscillations during the estrous cycle.²² Under diethyl ether anesthesia (J.T. Baker, Mexico), the ovaries were removed through an abdominal approach. Hormone levels were allowed to stabilize for 2 weeks before the tests.²³ The rats were then randomly assigned to the experimental groups.

Experimental groups and treatments

A longitudinal study was performed in three independent groups of rats receiving a single injection of different drugs. The control group ($n=17$) received vehicle (2-hydroxypropyl- γ -cyclodextrin dissolved in injectable sterilized water to obtain a 35% solution). The progesterone group ($n=17$) received 1.0 mg/kg progesterone. The allopregnanolone group ($n=17$) received 1.0 mg/kg allopregnanolone. Progesterone, allopregnanolone, and vehicle were injected 15 min (0.25 h) before the behavioral tests (volume injected:

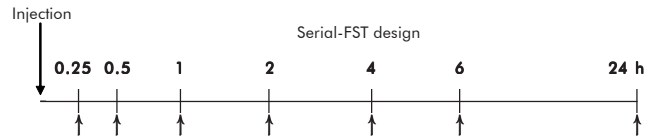


Figure 1. Rats underwent the forced swim test seven times in 24 h, each indicated by arrows (↑). Five minutes before each swimming session, grooming and crossings were evaluated in the open field test.

0.8 ml/kg, i.p.). Steroid doses were chosen from previous studies.^{3,4} All chemical compounds were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Forced swim test

We did not use any pretest session. In this longitudinal design, the behavioral effects of hormones were evaluated 0.25, 0.5, 1, 2, 4, 6, and 24 h after single injections (figure 1). In each of the seven sessions, the rats were placed individually for 5 min in a rectangular pool (height, 60 cm; length, 30 cm; width, 50 cm) with 24 cm deep water ($25 \pm 1^\circ\text{C}$). Immobility was assumed either when the rat floated without making vigorous movements leading to displacements and only maintained its head above the water surface or when the rat touched the bottom of the pool with at least two points of contact (e.g., one or both hind paws and the tail) for more than 2 s.

Open field test

Before each session in the FST, the rats were evaluated in the OFT. Rats were placed individually in an acrylic box (height, 20 cm; length, 44 cm; width, 33 cm), with twelve squares delineated on the floor (11×11 cm) to evaluate the number of squares crossed (i.e., crossings) and the cumulative grooming time during the 5 min test. Crossings were considered an indication of mobility, and grooming was considered an indicator of «emotionality» in response to stress. Crossings were counted when the rat passed from one square to another with all four paws. Grooming included²⁴ paw licking, nose/face grooming (strokes along the snout), head washes (semicircular movements over the top of the head and behind the ears), body grooming/scratching (body fur licking and scratching the body with the hind paws), leg licking, and tail/genital grooming (licking of the legs, genital area, and tail).

After each experimental session, the open field box was carefully cleaned with a cleaning solution (0.5% ammonia, 15% ethanol, 10% extran, 5% isopropyl alcohol, 19% Pinol, and 50.5% water) and immediately dried with paper towels. Five minutes elapsed after cleaning the box, allowing the scent of the substances to disperse. All tests were performed during the light period. The testing room was illuminated with white light (40 lux) by a tungsten lamp placed 2 m above the OFT and FST devices.

All sessions in the FST and OFT were videotaped and subsequently reviewed until reaching 100% agreement by trained observers who were blind to the experimental conditions.

Statistical analysis

We used two-way analysis of variance (ANOVA), with treatment (vehicle, progesterone and allopregnanolone) as the first factor and session (0.25, 0.5, 1, 2, 4, 6, and 24 h) as the second factor. Values of $p < 0.05$ were considered statistically significant. Significant effects in the ANOVA were followed by the Student-Newman-Keuls (SNK) *post hoc* test. Data are expressed as mean \pm standard error.

RESULTS

Serial Forced Swim Test

The two-way ANOVA showed significant effects of treatment ($F_{2,335} = 27.09$, $p < 0.0001$) and session ($F_{6,335} = 16.64$, $p < 0.0001$) and a significant treatment \times session interaction ($F_{12,335} = 4.67$, $p < 0.0001$). The SNK *post hoc* test showed that rats from the control group displayed the highest immobility time (45.5 ± 1.2 s) compared with the progesterone (31.1 ± 1.3 s) and allopregnanolone groups (32.9 ± 1.2 s), which displayed similar low values. The allopregnanolone group exhibited reduced immobility beginning 0.5h after injection, whereas the progesterone group exhibited reduced immobility 1h after injection (figure 2). Additionally, the low immobility time

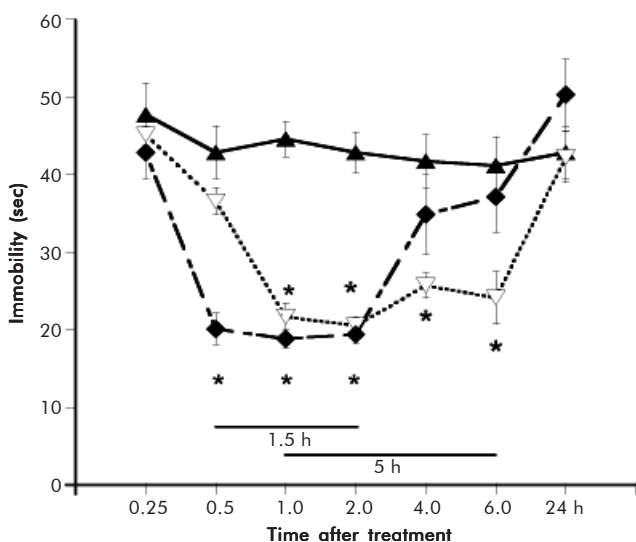


Figure 2. Total immobility time in the serial-FST. The reduction in immobility was significant beginning 0.5 h after allopregnanolone injection (black diamonds) and 1 h after progesterone injection (white triangles). The anti-despair-like effect (dashed line) of allopregnanolone was significantly shorter (1.5 h) than progesterone (5 h). * $p < 0.05$ (SNK *post hoc*), compared with first swim test session and compared with their respective control group session (black triangles).

detected in the allopregnanolone group persisted for a shorter period of time (1.5h) than in the progesterone group (5h).

Open field test

The two-way ANOVA showed no significant effect of treatment ($F_{2,335} = 2.95$, $p = 0.07$) on the crossings but a significant effect of session ($F_{6,335} = 56.38$, $p < 0.0001$). The treatment \times session interaction was not significant ($F_{12,335} = 1.05$, $p = 0.40$). The SNK *post hoc* test showed that during six sessions the crossings were reduced significantly ($p < 0.05$) after the first session of the serial-FST, an effect that was independent of treatment (figure 3).

The two-way ANOVA showed significant effects of treatment ($F_{2,335} = 137.34$, $p < 0.0001$) and session ($F_{6,335} = 5.35$, $p < 0.0001$) on grooming behavior and a significant treatment \times session interaction ($F_{12,335} = 3.93$, $p < 0.0001$). The SNK *post hoc* test showed that all groups displayed similar grooming values in the first OFT session. Afterward, the control group significantly reduced grooming ($p < 0.05$), and this reduction lasted for at least 24h. However, in the progesterone and allopregnanolone groups, the grooming values remained at similar amounts throughout the seven sessions of the serial-FST (figure 4).

DISCUSSION

The present study used a serial-FST to determine the time-dependent effects of two neuroactive steroids, with the OFT used to exclude the possible influence of locomotor activity

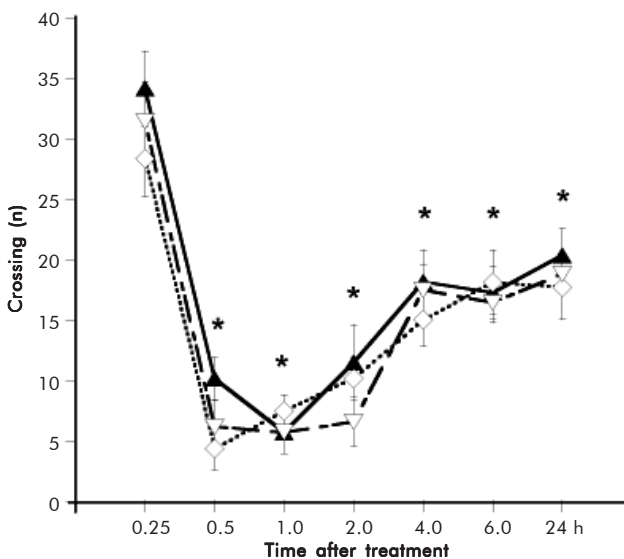


Figure 3. Open field test. Independently of treatments, crossings significantly reduced after the first swim session and it remained during 24 hours. Black triangles, control group; white diamonds, progesterone group; white triangles, allopregnanolone. * $p < 0.05$ (SNK *post hoc*), compared with the first session.

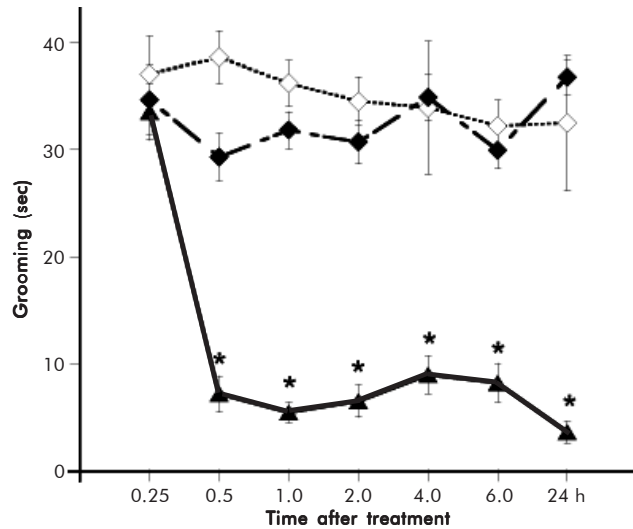


Figure 4. Grooming was significantly reduced in the control group (black triangles) after the first FST session (i.e., at 0.5 h). This reduction was not observed in the allopregnanolone group (black diamonds) or progesterone group (white diamonds). * $p < 0.05$ (SNK post hoc) compared with first session.

on swimming behavior and grooming as an indicator of stress. The control group reduced crossings and grooming from the second test onward. No changes in total immobility time were observed in the control group, but the steroid effects became more pronounced with time. Progesterone and allopregnanolone both reduced immobility time in the FST. Although the effects of allopregnanolone in the FST were observed at an earlier time point than progesterone, this effect endured for less time than that produced by its precursor, without changes in locomotion and grooming in the OFT. Progesterone and allopregnanolone produced effects in FST similar to those produced by antidepressant drugs. However, the steroids produced rapid onset of short-duration anti-immobility effects with a single, low dose.

Both one session of the FST²⁵ and acute stress²⁶ increase allopregnanolone content in brain and plasma of male and female rats, which appears to be an adaptive process given that high plasma levels of this steroid reduce immobility in the FST. Progesterone also exerts anti-stress-like effects.²⁷ The main contribution of the present study is the observation of the time-dependent effects of these steroids. The reduction in immobility appeared earlier with allopregnanolone than progesterone, but the duration of allopregnanolone effects was shorter. These results indicate that some of the anxiolytic- or anti-despair-like effects of progesterone may be attributable to biotransformation to its α -reduced metabolite, allopregnanolone.²⁸ This fact could partially explain the differences in the observed latency and duration of effects. Allopregnanolone is a metabolite of progesterone, which is hydroxylated and sulfated²⁹ and could be more rapidly eliminated, as observed after its infusion in rats,³⁰ thus causing a brief

duration of its anti-despair-like effects. But also, the metabolism of progesterone to allopregnanolone may sustain the longer anti-despair-like effects of progesterone.

Progesterone and allopregnanolone exert anxiolytic-like effects through GABA_A receptor, so it may be a constraint how they produce anti-depressant like effects. The reason is that progesterone exerts anti-stress-like effects and, in addition, allopregnanolone produces anti-despair-like effects in rats parallel to an increase in γ -aminobutyric acid-A (GABA_A) γ 2 subunit mRNA activity.³¹ Furthermore, the brain structures involved in the regulation of despair and anxiety, like the hippocampus and lateral septum, contain a high density of GABA_A receptors in which allopregnanolone may act reducing the immobility time in the FST³² when is microinjected in both brain structures.

Both progesterone and allopregnanolone reduce crossings in the OFT (i.e., less locomotor activity) and decrease immobility in the FST (i.e., more swimming activity), demonstrating that the change in immobility was not related to a change in locomotion. Additionally, the control group did not exhibit altered immobility during the seven sessions. Therefore, the present results obtained using these two steroids may not be attributable to fatigue, which is also supported by the fact that the effects of progesterone and allopregnanolone in the serial-FST showed a U-shaped distribution over time, allowing the detection of differences related to the onset and duration of the effects.

In female rats, grooming is influenced by hormonal oscillations during the estrous cycle. The young and adult rats display the highest values of grooming during the estrus phase, indicating that progesterone and estrogens are involved in the expression of this behavior.³³ In our study, rats were ovariectomized; consequently, the hormonal influence from gonadal source in results may be discarded. However, we found that the control group exhibited nearly no grooming after the first session of the serial-FST, suggesting some stress produced by being forced to swim. Herein, an anxiolytic (muscimol) increases grooming, while an anxiogenic (bicuculline) inhibits grooming in rats.³⁴ We found that the progesterone and allopregnanolone groups displayed this self-directed behavior during the six sessions at similar values than the first session, suggesting a protective effect against the stress induced by repeated FST, supporting the assumption of anti-stress-like effects of both steroids.

From our results, both steroids produced behavioral effects during several hours, an intriguing fact, due to its short half life. While in the rat the plasma half life of progesterone surrounds 1h,³⁵ in mice the half life of allopregnanolone is about 30min.³⁶ However, both steroids accumulate in brain tissue³⁶ and the initial effect produced by progesterone is strengthened by its α -reduction to allopregnanolone.²²

CONCLUSION

We confirm previous observations of an anti-despair-like effect of progesterone and allopregnanolone, in both cases the effects occur with a considerable short latency and effects do not last more than 24 hours, which merits to be considered in clinical studies dealing with some depressive cases, namely those for whom it is needed a brief-short antidepressant action. Lastly, these results show that the serial-FST may be a useful tool for detecting time-dependent anti-despair-like effects of drugs with antidepressant potency.

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Artículo sin conflicto de intereses