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Effect of Centrally Injected Allopregnanolone on Sexual Receptivity, Luteinizing Hormone Release, Hypothalamic Dopamine Turnover, and Release in Female Rats

Myriam R. Laconi and Ricardo J. Cabrera

Laboratorio de Investigaciones Neuroquímicas, Comportamentales y Endócrinas (LINCE), Instituto de Medicina y Biología Experimental de Cuyo (IMBECU-CONICET), Cátedra de Farmacología, Facultad de Ciencias Médicas, Mendoza, Argentina

The effect of intracerebroventricular (icv) injection of allopregnanolone (5α-pregnan-3α-ol-20-one) on the dopaminergic and reproductive function in ovariectomized rats primed with estrogen and progesterone was investigated. Thirty minutes after icv allopregnanolone injection, the sexual receptivity, luteinizing hormone (LH) release, dopamine content, and release in the medial basal hypothalamus (MBH) and preoptic area (POA) were determined. After allopregnanolone injection, LH serum levels were reduced ($p < 0.001$) and lordosis behavior was inhibited ($p < 0.005$). Intracerebroventricular injection of bicuculline (a γ-aminobutyric acid$_A$ [GABA$_A$] antagonist) alone was ineffective. The injection of allopregnanolone plus bicuculline blocked the effects of allopregnanolone on sexual receptivity and on LH serum levels. At the same time, endogenous dopamine concentration in both the MBH and POA was augmented ($p < 0.005$ and $p < 0.006$, respectively) and the turnover rate decreased in both structures. Moreover, in vitro ^{3}H-dopamine release from MBH and POA was lower in rats injected with allopregnanolone in comparison with vehicle-treated rats. These results suggest that allopregnanolone influences the dopaminergic mechanisms in female rats, which may, in turn, be responsible for the reduced reproductive activity. Allopregnanolone may exert its effects on sexual behavior through GABA$_A$ receptor modulation and a decrease in dopaminergic activity in the MBH and POA. These actions could explain the inhibition of LH release.

Key Words: Neurosteroids; allopregnanolone; dopamine; luteinizing hormone; sexual receptivity.

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Author to whom all correspondence and reprint requests should be addressed: Dr. Myriam R. Laconi, Cátedra de Farmacología, Facultad de Medicina, Universidad Nacional de Cuyo, C.C.33, (5500) Mendoza, Argentina.

Introduction

The term neurosteroids applies to those steroids that are both formed and accumulated in the nervous system independently of peripheral steroidogenic gland secretion *(1)*. Neurosteroids that are active on the nervous system mainly include progesterone, pregnenolone, as well as the reduced metabolite of progesterone, 5α-pregnan-3α-ol-20-one, called allopregnanolone *(2)*. These neurosteroids alter neuronal excitability by modulating the activity of several neurotransmitter receptors, and thus can influence behavior. The behavioral effects of progesterone are well known. They block the induction by estradiol of both sexual behavior and the preovulatory surges of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) *(3)*. Among the central neurotransmitters involved in the control of sexual behavior, dopamine is certainly one of the most extensively studied. Much evidence supports the idea that dopamine, through its different neuronal systems, plays important roles in the control of several aspects of sexual behavior; that is, the incerto hypothalamic system plays a major role in the consummation of sexual behavior *(4)*. It is well documented that the dopaminergic neurons with terminals in ventromedial hypothalamus are part of a neuronal pathway mediating the feed back effects of estradiol on GnRH secretion and the LH surge *(5)*. Dopamine can play an inhibitory or a facilitatory role in the proestrous LH surge depending on the hormonal status of the animals and of the brain area in the study *(5,6)*. Further evidence suggests that γ-aminobutyric acid (GABA) containing neurons may inhibit LH release under certain circumstances. The GABAergic system regulates LH release via modulation of catecholaminergic systems that control LHRH secretion *(7,8)*.

Given these data, it is interesting to determine whether centrally injected allopregnanolone in the brain has the same effects as progesterone. Since the central areas of action of allopregnanolone are not completely studied, the first objective of the present study was to investigate whether centrally injected allopregnanolone is able to regulate the LH serum levels and sexual receptivity in ovariectomized (OVX) estrogen and progesterone-primed rats.

Previous studies from our laboratory have demonstrated that functions such as biosynthesis, turnover, and release of dopamine are centrally modified by progesterone in rats under different endocrine conditions *(9,10)*. Progesterone increased dopaminergic activity, induced the release of dopamine, modulated the amphetamine-stimulated release *(11)*, and increased K^+-evoked tritium dopamine release (^{3}H-dopamine) from the striatum slices of estrous rats *(10)*. In the rat, cell bodies of GnRH neurons are located in the preoptic area (POA) of the anterior hypothalamus with axonal projections to the external layer of the median eminence *(12)*. The GABAergic neuronal system is a steroid-receptive neuronal system that mediates luteinizing hormone–releasing hormone (LHRH) neuronal activity. These neurons are found in the POA and in the medial basal hypothalamus (MBH) *(13,14)*. POA and MBH are strictly hormone-dependent brain areas and have been extensively studied. For this reason, both areas represent a good model for exploring the actions of neurosteroids in the central nervous system.

The other aim of our study was to investigate whether allopregnanolone, which usually acts through the GABAa receptor *(15,16)*, can influence both these physiologic parameters (sexual receptivity and LH serum levels) in MBH and POA, and whether this neurosteroid is able to modify the dopamine levels in the same brain areas.

Results

Effect of Allopregnanolone on Sexual Behavior

The intraverebroventricular (icv) injection of allopregnanolone (6 μ*M*) significantly decreased sexual receptivity in female rats ($p < 0.005$). Bicuculline (9.8 μ*M*) did not modify the lordosis/mount quotient compared with the vehicle group, but the bicuculline plus allopregnanolone injection completely abolished the inhibitory effect of allopregnanolone observed on sexual behavior ($p < 0.005$) (Fig. 1).

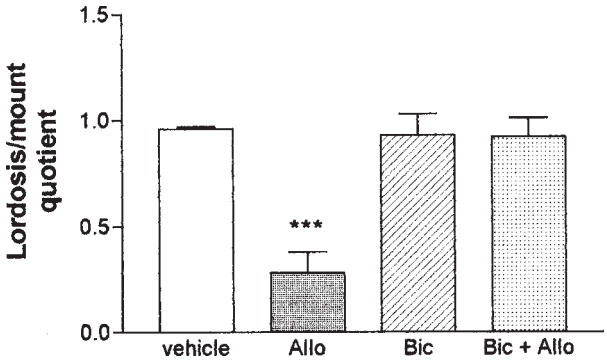

Fig. 1. Lordosis quotient in OVX estrogen- and progesterone-primed rats 30 min after icv allopregnenolone (Allo), bicuculline (B), bicuculline plus allopregnenolone (B + A), or vehicle (V) injection into right lateral ventricle. Data represent the means ± SEM of 10–12 rats. ***$p < 0.001$.

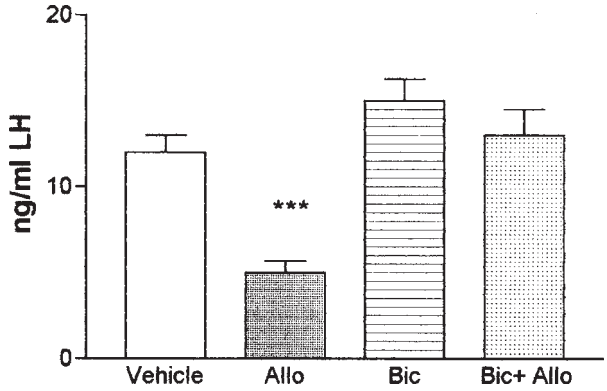

Fig. 2. Serum levels of LH in OVX estrogen- and progesterone-primed rats after allopregnenolone (Allo), bicuculline (B), bicuculline plus allopregnenolone (B + A), or vehicle (V) injection into right lateral ventricle. Data represent the means ± SEM of 10–12 rats. ***$p < 0.001$.

Effect of Allopregnanolone on LH Serum Levels

Allopregnanolone significantly reduced LH serum concentrations in gonadectomized estrogen- and progesterone-primed rats in comparison with the vehicle group ($p < 0.001$) (Fig. 2). Bicuculline had no effect on the LH serum levels, but the effect of allopregnanolone on LH serum levels was reverted when bicuculline was injected prior to allopregnanolone ($p < 0.001$).

Dopamine Turnover Rate in MBH and POA

Allopregnanolone increased dopamine and dihydroxyphenylacetic acid (DOPAC) concentrations in the POA ($p < 0.005$), but the turnover rate was significantly lower than in the vehicle group ($p < 0.05$) (Fig. 3). In the MBH, allopregnanolone increased dopamine ($p < 0.001$) but showed a decrease in DOPAC concentrations in comparison with the vehicle group ($p < 0.001$). The turnover rate in this area was significantly lower than in the vehicle group ($p < 0.001$) (Fig. 4).

Effect of Allopregnanolone on K^+-Evoked ^{3}H-Dopamine Release in POA-MBH Slices

Allopregnanolone induced an inhibitory effect on the K^+-evoked ^{3}H-dopamine release from the MBH and POA slices in gonadectomized, estrogen- and progesterone-primed rats whereas it had no inhibitory effect in the vehicle group ($p < 0.0006$) (Fig. 5).

Discussion

In the present work we have demonstrated that allopregnanolone injected intracerebroventricularly inhibited sexual receptivity and caused a decrease in the LH serum levels in OVX primed rats; both effects could be mediated through GABAa receptor, because they were completely reverted by

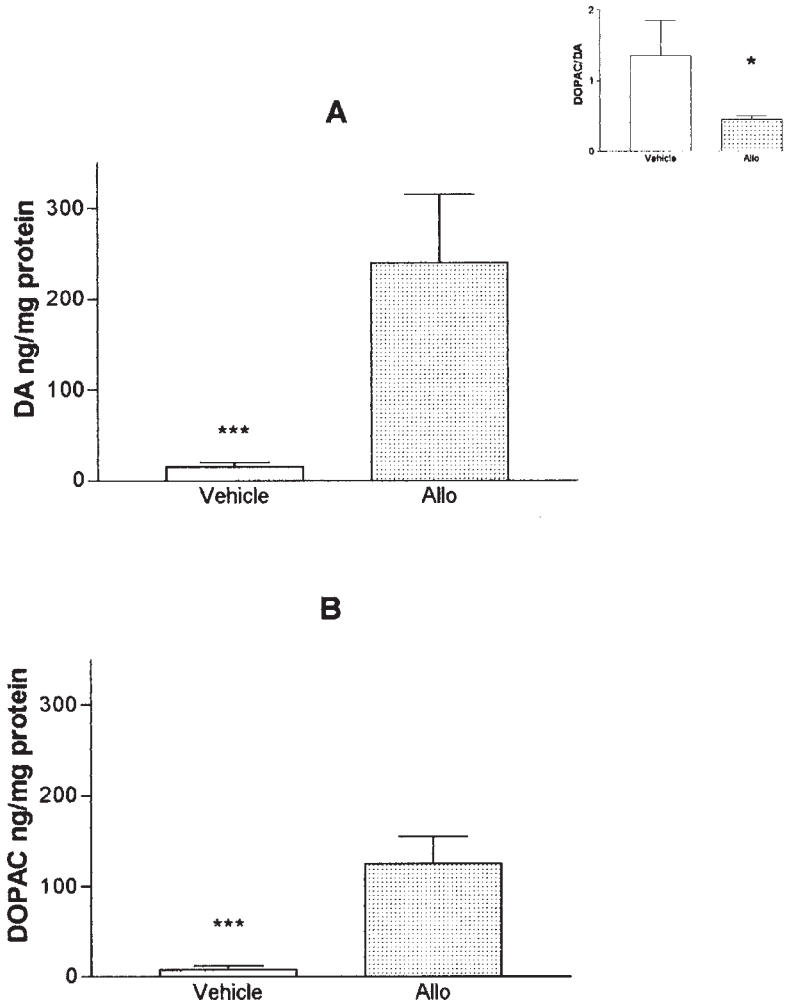

Fig. 3. (**A**) Dopamine and (**B**) DOPAC concentrations in POA of OVX estrogen- and progesterone-primed rats with icv injection of allopregnenolone (Allo) or vehicle (V). Each column represents the mean ± SEM for 10–12 rats. ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$. The inset in (A) shows the DOPAC/dopamine turnover ratio.

bicuculline. In addition, allopregnanolone caused an increase in endogenous dopamine content in both the POA and MBH (high-performance liquid chromatography [HPLC] experiments) with a concomitant decrease in dopamine turnover in the POA and MBH. Allopregnanolone was able to induce a decrease in dopamine release in both POA and MBH slices (superfusion experiments).

The effects of allopregnanolone in sexual receptivity and LH serum levels are similar to those of progesterone injected centrally to OVX estrogen- and progesterone-primed rats. Is well known that lordosis is a steroid-dependent behavior, requiring the presence of estradiol and progesterone and is associated with ovulation in female rats *(17)*. The mechanism regulating receptivity involves an interaction between ovarian steroids and different neurotransmitter pathways, such as GABA and dopamine transmission *(14,18,19)*. Since allopregnanolone has a high affinity for the GABAa receptor, but a very low affinity for the intracellular progesterone receptor (PR) *(15)*, one might hypothesize that the effect of progesterone on receptive behavior is a consequence of progesterone's conversion to the neurosteroid allopregnanolone and its subsequent actions via the GABAa receptor rather than via direct actions at the PR *(18)*.

Is known that allopregnanolone suppresses the hypothalamic GnRH release in vitro, and this effect appears to be mediated by an interaction with the GABA receptor *(20, 21)*. In the present study, both sexual receptivity and LH serum levels were abolished by icv administration of allopregnanolone, and this effect could be a consequence of the interaction with the GABAa receptor because the association with bicuculline reverted this effect *(8,22,23)*.

Changes in allopregnanolone levels by icv administration could modify the GABA secretion and could define the final response of GnRH and, subsequently, the LH levels

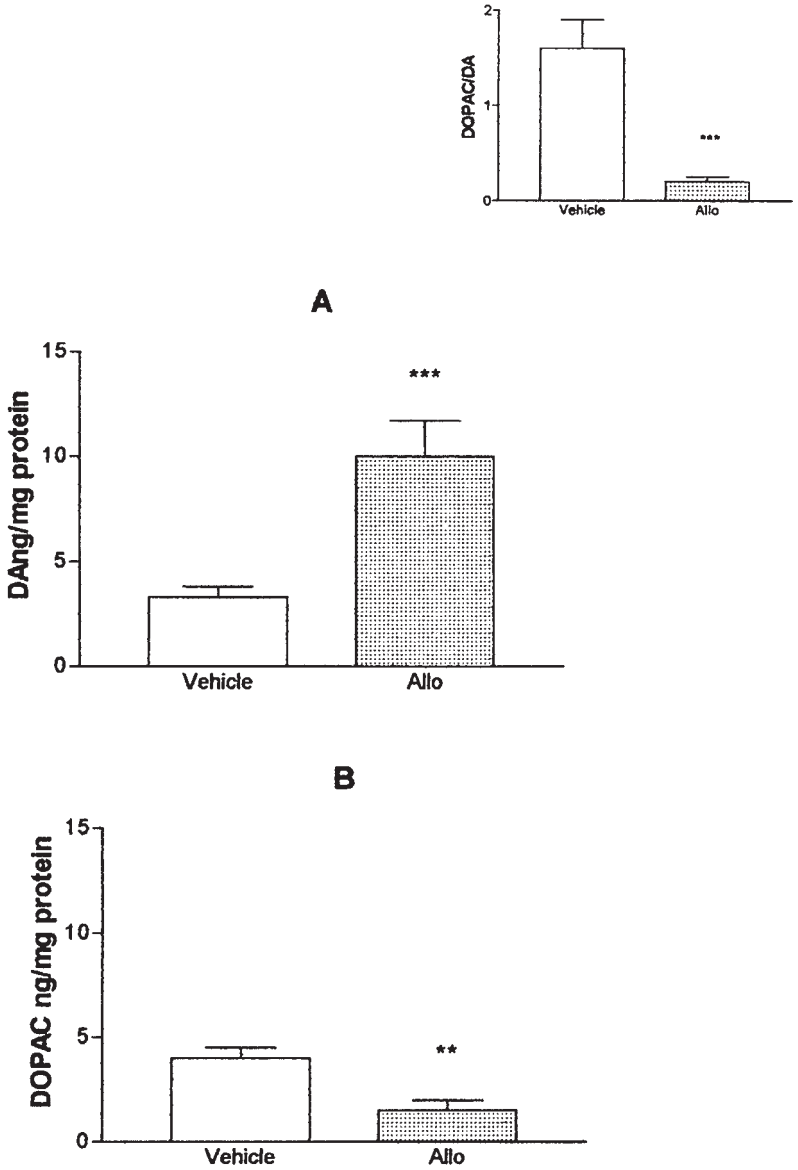

Fig. 4. (**A**) Dopamine and (**B**) DOPAC concentrations in MBH of OVX estrogen- and progesterone-primed rats with icv injection of allopregnenolone (Allo) or vehicle (V). Each column represents the mean ± SEM for 10–12 rats. ***$p < 0.001$; *$p < 0.05$. The inset in (A) shows the DOPAC/dopamine turnover ratio.

and sexual behavior. The inhibitory actions of allopregnanolone on LH release and sexual behavior may take place on the afferent nerve terminals that regulate the GnRH secretion. Our likely candidate is dopamine, as suggested by the synaptic connections between dopaminergic nerve terminals and GABAergic interneurons in the POA. More experiments are needed to provide complementary evidence for the physiologic modulation of dopamine to inhibit or evoke LH release.

The role of monoaminergic neuronal tracts terminating in the hypothalamus on gonadotropin release is well established *(24–26)*. The role of the dopaminergic innervation remains controversial since raising brain dopamine activity can inhibit *(27–29)* or stimulate *(5,30,31)* gonadotropin release. The variation in effect seems to depend on the endocrine state of the experimental model. The stimulatory effects occur in the adult animal in the presence of ovarian steroids *(32,33)*. Our data expands previous findings in several additional ways. First, they indicate that the described effect of the neurosteroid is zonal, restricted to the POA and MBH, which are nearby areas intimately associated with dopaminergic transmission, and that it influences reproductive activity. Frye et al. *(34)* have found that allopregnanolone injected in the ventral tegmental area has an opposite

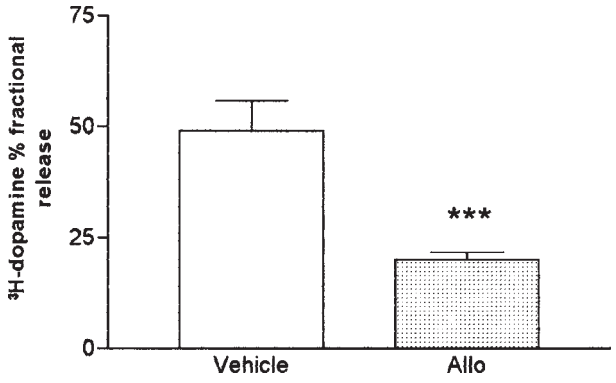

Fig. 5. Effect of allopregnenolone on ^{3}H-dopamine-evoked release from POA and MBH slices of OVX estrogen- and progesterone-primed rats. Each column represents the mean value ± SEM for 10 rats. ***$p < 0.001$.

effect to that described here. We agree with them in the primary mediation of GABAergic and dopaminergic systems on the sexual receptivity, but we are investigating the independent regulation of both systems and are focusing our attention on two specific hypothalamic areas in close relationship with sexual behavior. Moreover, it is well known that the effect of dopamine on sexual behavior and LH release may vary in different brain areas and in each phase of the estrous cycle. It is important to note that in order to function, allopregnanolone needs the previous estrogen-progesterone activation of the brain neural plasticity *(35)*. Other experiments with only estrogen priming have resulted in significantly different results *(17)*.

We have shown previously that progesterone and allopregnanolone exert regulatory effects on the dopaminergic system *(9,10,36)* in proestrous rats, in OVX estrogen- and progesterone-primed rats, and in pregnant rats *(37)*. In this article, we report the central modifications in the dopamine system caused by administration of allopregnanolone that may result in an alteration in LH release.

We have shown for the first time that the effect of allopregnanolone could include a close interaction between the dopaminergic and GABAergic systems in the POA and MBH with the consequent alteration in both LH serum levels and sexual behavior. A previous report from our laboratory shows that an important effect can be obtained by progesterone on the K^+-induced ^{3}H-dopamine release in vitro *(9)*. Given that this effect is observed on newly incorporated ^{3}H-dopamine and is calcium dependent, it probably takes place on the pool related to the exocytotic process of dopamine release *(9)*.

The increase in dopamine and DOPAC levels in the POA and MBH could be interpreted as an increase in the activity of the dopaminergic system as a consequence of neurosteroidal action in the same way as the action of gonadal hormones *(37)*. This supports the view that the accumulation of dopamine in both areas may be owing to increased synthesis of this neurotransmitter. Dopamine is converted to DOPAC by monoamine oxidase (MAO) after it is recaptured by the dopamine nerve terminals, and it may be possible that less MAO activity occurs in these areas with allopregnanoloe injection than in the same areas with vehicle. Taken together, these findings suggest that the large amount of dopamine caused by allopregnanolone injection could be owing to greater dopamine synthesis, but with a reduced turnover and lower dopamine release. The evidence of high dopamine concentration in both studied areas with low turnover and low dopamine release is an index of responsiveness to the putative inhibitory allopregnanolone effect on the hypothalamic dopamine system. This suggests that the inhibition of sexual receptivity and the decrease in LH serum levels may be a consequence of the possible role of hypothalamic dopaminergic systems, mediated or not by GABA, in these brain areas.

We conclude that allopregnanolone induces changes in dopamine and GABA activity in the POA and the MBH. These changes may be involved in the inhibition of sexual receptivity and/or gonadotropin release and highlight the role of the neurosteroids as an active modulator of the reproductive patterns in the female rat.

Materials and Methods

Animals

Adult Sprague-Dawley female rats (200–250 g body wt and 90–120 d old), bred at our laboratory, were used. They were housed in groups of four to five animals/cage. Animals were maintained at a constant temperature (22 ± 1ºC) and under a reversed day-night lighting cycle (lights on 7:00 PM to 7:00 AM) with food pellets and water available ad libitum. Animals for these experiments were kept and handled according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 80, 23; 1978).

Drugs

The drugs used were allopregnanolone (5α-pregnan-3α-ol-20-one), 17-β-estradiol 3-benzoate, progesterone (Sigma, St. Louis, MO), bicuculline methiodide (Research Biochemicals), and ^{3}H-dopamine (New England Nuclear, Boston, MA).

Steroids were dissolved in 0.1 mL of corn oil as a vehicle. Allopregnanolone was initially dissolved in propylenglycol to a concentration of 0.6 m*M*. As a vehicle, injections of Krebs Ringer bicarbonate glucose (KRBG) buffer at pH 7.4 with propylenglycol were used. A 6 μ*M* dose of allopregnanolone was used. This was chosen to mimic its maximal circulating levels during stress *(38)*, and it was used in our previous report of anxiety behavior *(39)*. Drugs were injected intracerebroventricularly in a 0.6-μL injection volume.

Surgical Procedures

Female rats (n = 48) were bilaterally OVX under light ether anesthesia and were thereafter housed singly in Plexiglas cages. Ten to 14 d after ovariectomy, rats were injected subcutaneunsly with 25 μg of estradiol benzoate/rat 48 h before testing and 1 mg of progesterone/rat 4 h before testing. Then they were tested for sexual receptivity responsiveness in training tests *(40)*.

One week before the experiments, only respondent rats with a lordosis/mount (see Sexual Behavior) quotient of ≥0.80 in the training test were implanted with right lateral ventricle guide cannulae. The stainless-steel cannula assembly was stereotaxically implanted under chloral hydrate anesthesia (400 mg/kg intraperitoneally) into the right lateral ventricle. The coordinates of the atlas of Paxinos and Watson *(41)* were used. After surgery, animals were housed individually and maintained undisturbed for recovery for 1 wk. After the end of experiments, the location of the guide cannula into the lateral ventricle was confirmed.

Experimental Procedure

Forty-eight hours before the day of the experiments, the OVX rats were primed again with 25 μg of estradiol benzoate/rat subcutaneously. Four hours before the experiment, 1 mg of progesterone/rat was injected subcutaneously, and the animals were returned to their home cages to avoid endocrine stress responses.

All experimental drugs were injected into the right lateral ventricle (intracerebroventricularly) of freely moving rats in a volume of 0.6 μL for during 1 min. A stainless-steel needle was inserted into the guide cannula and connected by a silicone catheter to a Hamilton microliter syringe. The injection cannula was left placed for an additional minute to avoid reflux.

Thirty minutes after the allopregnanolone injection (6 μ*M*), the receptivity index was evaluated, and then the animals were killed by decapitation. Trunkal blood was collected and rapidly centrifuged, and the serum was separated and stored at –30ºC for LH serum level measurements by radioimmunoassay (RIA). At the same time, the brains were removed and the POA and MBH were extracted. Dopamine and DOPAC concentrations were measured by HPLC. When bicuculline, a competitive GABAa antagonist, was used, rats were injected intracerebroventricularly with 0.6 μL (9.8 μ*M*) of bicuculline 15 min before the allopregnanolone injection. Then, 30 min after this last injection, the animals were killed. Finally, using the same priming model (estrogen and progesterone) and the same drug injection schedule, but in a separate rat group, the release of the ^{3}H-dopamine from medial basal preoptic hypothalamic slices was measured by superfusion experiment.

Sexual Behavior

Behavioral testing started 2 to 3 h after the onset of the colony dark period, 4 h after progesterone injection, and was conducted in a red, dimly illuminated, and sound proof animal room. Mating tests were conducted in a 50 × 50 cm × 30 cm chamber. Animals were tested for lordosis behavior, as a measure of female sexual receptivity, with two stud males (300–400 g). Sexually experienced male rats were introduced into the arena at least 10 min before the female. Later, a female rat was gently dropped into the corner most distant from the males. After every mount, which consisted of the male clasping the female, showing pelvic thrusting, and completely dismounting, the female was scored as to whether or not she had displayed lordosis. Each test consisted in 10 mounts, and the number of lordosis responses was expressed as the lordosis quotient, equivalent to the number of lordosis responses per 10 mounts, expressed as a percentage of the total mounts *(42–44)*.

LH Assay

LH was assayed in the serum samples by RIA, using kits supplied by the National Hormone Pituitary Program. The intra- and interassay coefficients of variation were 9 and 11%, respectively. The data were expressed in nanograms per milliliter of serum in terms of NIAMDD-Rat-RP-1 reference preparation.

Dopamine Assay

MBHs and POAs were dissected from the female brains and placed in Eppendorf tubes containing 200 μL of orthophosphoric acid (0.2 *N*) and frozen at –80ºC until the day of assay. On the day of assay, tissues were thawed, homogenized by sonication, and centrifuged at 12,000*g* for 30 min. Dopamine and its primary metabolite, DOPAC, were measured in the clear supernatants by reverse-phase HPLC with electrochemical detection. The HPLC mobile phase consisted of 45 n*M* sodium phosphate dibasic buffer (pH 3.5) containing 0.43 n*M* sodium octyl sulfhate, 0.34 m*M* EDTA, and 20% acetonitrile. The oxidation potential was set at 0.55 V. Tissue pellets were dissolved in 1.0 *N* NaOH for protein assay *(45)*. The results were expressed as nanograms of compound per milligram of protein.

Superfusion Experiments

To evaluate the effect of allopregnanolone on dopamine release, another group of OVX estrogen- and progesterone-primed rats was used to study the effect of K^+-evoked ^{3}H-dopamine release from slices of MBH and POA. The rats were sacrificed by decapitation 30 min after icv allopregnanolone injection. The brain was rapidly removed and cooled in ice. MBH and POA was dissected out according to the procedures described by Cabrera and Bregonzio *(37)*. In brief, 240-μm-thick coronal slices were obtained with a McIllwain tissue chopper. Eight to 10 slices were incubated in a Dubnoff shaker in 2 mL of KRBG buffer (pH 7.4) for 30 min at 37ºC gassed with a mixture of O_2 (95%) and CO_2 (5%), containing 25 n*M* of ^{3}H-dopamine (specific activity of 57 Ci/mmol, New England Nuclear, Boston, MA). Then, the

slices were transferred to superfusion chambers and superfused at 0.7 mL/min for 40 min with KRBG to wash out the ^{3}H-dopamine not incorporated into the tissue (washing period). After the washing period, the tissues were superfused with KRBG. Five 2.5-min fractions were collected and considered as basal release. Starting at time 12.5 min, the slices were exposed for 7.5 min to ClK (28 m*M*). At the end of the experiments, the slices were homogenized in 3 mL of 0.2 *N* perchloric acid and centrifuged, and 1 mL of the clear supernatant and 1 mL of each fraction collected were mixed with 8 mL of scintillation fluid (Toluene–PPO–POPOP–Triton X-100 cocktail) and radioactivity was counted. Total radioactivity was calculated as the sum of total tritium collected during the superfusion plus the amount remaining in the tissue at the end of the experiment. The results of the effect of allopregnanolone on K^+ -induced ^{3}H-dopamine release were expressed as a ratio of the dopamine loaded by the tissue in percentage over basal (for details, see ref. *34*). ^{3}H-dopamine-evoked release is the percentage increase after stimulation (mean of fractions 7 and 8) with respect to basal release (mean of fractions 4 and 5).

Statistical Analysis

One-way analysis of variance was used in multigroup comparisons followed by a post-hoc Student-Newman-Keuls test. Comparisons among groups were carried out using the unpaired student's *t*-test. Differences between means with a $p < 0.05$ were considered to be significant.

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