Evidence for a role for the neurosteroid allopregnanolone in the modulation of reproductive function in female rats

Andrea R Genazzani, Marco A Palumbo, Antonio A de Micheroux, Paolo G Artini, Mario Criscuolo, Guido Ficarra, Ai-Li Guo, Augusta Benelli, Alfio Bertolini, Felice Petroglia and Robert H Purdy

Institute of Obstetrics and Gynecology, University of Pisa, Pisa, Italy; Department of Gynecological Obstetrics and Pediatric Sciences, University of Modena, Modena, Italy; Institute of Pathological Anatomy, University of Modena, Modena, Italy; Department of Pharmacology, University of Modena, Modena, Italy; Department of Endocrinology, Peking Union Medical College, Beijing, People’s Republic of China; Department of Veterans Affairs, Medical Center, La Jolla, San Diego, CA, USA


The present study investigated the effect of allopregnanolone (5α-pregnan-3α-ol-20-one) or of passive immunoneutralization of brain allopregnanolone, the most potent steroid produced by neurons, on ovulation rate and sexual behavior in female rats. Allopregnanolone was injected intracerebroventricularly in rats on diestrus and proestrus and tests were done on estrus. The intracerebroventricular injection of allopregnanolone significantly decreased the number of oocytes collected on estrus (p < 0.01). To support a physiological involvement, antisera to allopregnanolone was injected centrally to block the activity of the endogenous neurosteroid. When administered on diestrus and proestrus or only on proestrus, the antisera was shown to be correlated with a significant increase (p < 0.01) in oocytes retrieved on estrus. In female rats treated with antisera to allopregnanolone, the lordosis intensity was augmented significantly as compared to controls. Finally, the possible changes of medial basal hypothalamus concentration of allopregnanolone throughout the estrous cycle and at the time of ovulation were investigated. Hypothalamic extracts were eluted on high-pressure liquid chromatography and allopregnanolone concentration was measured by radioimmunoassay. Brain cortex was used as control tissue. Hypothalamic allopregnanolone concentration on proestrus morning and afternoon was found to be significantly lower than in the remaining phases of the estrous cycle (p < 0.01), while no significant changes were observed in brain cortex concentration of allopregnanolone. The present results suggest that hypothalamic allopregnanolone may be involved in the mechanism of ovulation, affecting hormonal and behavioral events.

AR Genazzani, Institute of Obstetrics and Gynecology, University of Pisa, via Roma 67, 56100 Pisa, Italy

Several data indicate that steroid hormones are synthesized in brain neurons. These steroids of central origin have been termed "neurosteroids". Glial cells have the enzymes necessary to synthesize steroids from cholesterol (1–4). One of the most diffused neurosteroids is 5α-pregnan-3α-ol-20-one (allopregnanolone or tetrahydroprogesterone). Several biological actions have been suggested for neurosteroids. Like the steroid hormones, they may act in numerous brain areas, modulating some neurophysiological actions. In particular, a neuromodulatory role for neurosteroids in the central nervous system has been proposed (5–8). In fact, it has been shown that neurosteroids may bind γ-aminobutyric acid (GABA) receptor sites, acting as agonist or antagonist. An involvement of neurosteroids in stress (9), depression and anxiety (10, 11) and cognitive functions (12) has been demonstrated. The exposure of rats to stress increases allopregnanolone concentration in brain (9) and the administration of this neurosteroid disrupts memory (12). In addition, a regional difference in the acute effects of neurosteroids on GABA receptor affinity in rat brain has been shown (13).

In particular, the evidence that the estrous cycle and some sexual behavioral changes are associated with modified neurosteroid activity has suggested an impact of neurosteroid on reproductive functions. Supporting this hypothesis, the suppression of basal GnRH-stimulated FSH release in rat cultured pituitary cells by the neurosteroid 3α-hydroxy-4-pregnan-20-one (3αHP) has been shown (14, 15).

The aim of the present study was to investigate a possible role for allopregnanolone in modulating ovulation and sexual behavior of female rats on estrus. The effect of allopregnanolone or of a passive immunoneutralization of brain allopregnanolone on ovulatory events (ovulation test and sexual behavior) was investigated. Furthermore, hypothalamic and brain
cortex concentrations of allopregnanolone in female rats throughout the estrous cycle were evaluated.

Materials and methods

Animals

All experiments were performed in naive adult female (60–65 days, weighing 180-200g) Wistar rats (Morini, S. Polo d’Enza, Italy). A group of male rats was also used in the experiment on sexual behavior. Animals were maintained according to approved laboratory conditions (four to six per cage) under a 12-h light/dark photoperiod at 19–22°C and were allowed free access to standard rat chow and water. Stage of estrous cycle was determined via daily vaginal smears for 3 weeks. Only female rats exhibiting a minimum of two regular 4-day estrous cycles were used. Rats for experiments on sexual behavior were housed in single plexiglass cages under standard conditions. Changes in sexual behavior were evaluated 2 h after the beginning of the dark period, in a sound-proof room, in a dim red light. For this experiment a rectangular glass arena (60 x 50 x 40 cm) with wood shavings covering the bottom was used.

Experimental protocol

Ovulation test. On proestrus morning rats received a single intracerebroventricular injection (20 µg) of allopregnanolone (Sigma Co., St Louis, MO) or of antiserum (10 µl) raised in rabbits to allopregnanolone. The antiserum was titered according to the capability of binding 50 pg of the labeled steroid. Cross-reactivity with other steroid hormones and metabolites is reported in a previous publication (16). In particular, this polyclonal antibody cross-reacts with a 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 3β-hydroxy-5β-pregn-20-one, 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-pregnen-5-en-20-one, 3β-hydroxy-5α-pregnan-20-one, pregn-4-ene-3,20-dione, 3α-hydroxypregnen-4-en-20-one, 20β-hydroxy-5α-pregnan-3-one and 3α-hydroxy-5β-pregnan-20-one.

A second group of rats was tested with a double dose of steroid (40 µl) or antiserum (20 µl), receiving an injection both on diestrus-II and on proestrus. Rats injected intracerebroventricularly with a single (10 µl) or a double dose (20 µl) of vehicle (ethanol) or normal rabbit serum served as control groups. In the morning of estrus (09.00 h) the rats were sacrificed, their ovaries removed and cleaned free of fat and their oviducts were flushed with saline solution onto a slide; oocytes were counted under a microscope (17).

Sexual behavior. Female rats were injected with antiserum to allopregnanolone (10 µl) or with an equivalent of normal rabbit serum, this serving as the control group. Four hours later, the two groups of female rats were tested for lordosis behavior. A sexually experienced active male was placed in the arena and 10 min later a female rat in natural estrus was gently dropped into the corner most distant from the male. The male was allowed to mount the female ten times; each lordosis response of the female was noted, and a lordosis quotient (LQ) was computed as the percentage of mounts provoking the lordosis reflex. Moreover, each response to a mount was scored on a lordosis intensity (LI) scale: 0, no lordosis; 1, marginal lordosis; 2, normal lordosis; 3, intense lordosis; 4, exaggerated lordosis (18). The following signs of “feminine” behavior were also part of the LI scale: hop (a short leap with the female landing on all four paws, followed by the assumption of a crouching posture); dart (a run of several steps, abruptly terminated by the assumption of a crouching posture); ear wiggling (the ears vibrate rapidly, usually accompanied by an upwards toss of the head) (19, 20).

Brain allopregnanolone concentration. In the morning (09.00 h) of proestrus, estrus, diestrus-I or diestrus-II groups of rats (six each) were sacrificed by decapitation and their brains rapidly removed. Rats on proestrus and diestrus-I were also sacrificed at 18.00 h. Medial basal hypothalamus (MBH) and brain cortex were removed according to the method described previously by Glowinski and Iversen (21). Protein content was measured according to Lowry et al. (22) in aliquots of homogenates.

Radioimmunoassay for allopregnanolone

Medial basal hypothalamus and brain cortex were placed in 2 ml of 0.1 mol/l acetic acid. After homogenization and centrifugation at 3500 rpm for 15 min, supernatants were collected and purified by high-performance liquid chromatography (HPLC). A Waters Instruments (Rochester, MN) apparatus was used, equipped with a reverse-phase C18 Bondapak column (3.9 x 300 mm). The elution was carried out in a convex gradient, starting from an HCl/acetonitrile rate of 30-70% with a 2-ml flux of HCl in 15 min. Fractions were collected at 0.5 min intervals, dried in a speed vacuum specimen and redissolved in 0.5 ml of phosphate buffer. Allopregnanolone concentration was measured in all fractions by radioimmunoassay (RIA). Tracer (3H) was purchased from NEN Products (Boston, MA). Antiserum, tracer and standard or unknown specimen were incubated for 24 h at 4°C. Dextran-coated charcoal was used to separate bound from free allopregnanolone. Inter and intra-assay coefficients of variation were 5.5% and 3.0% respectively. The assay detection limit was 10 pg/ml.

Statistical analysis

Statistical analysis of the results was done using one- or two-way analysis of variance.
Results

Ovulation test

The number of oocytes retrieved in rats injected with a double dose of allopregnanolone was significantly lower than in the corresponding control group (p < 0.01). The administration of a single dose of steroid did not modify significantly the number of oocytes retrieved. The administration of either a single or a double dose of antiserum antiallopregnanolone was associated with an increased number of oocytes retrieved (p < 0.01) (Fig. 1).

Sexual behavior

Female rats injected with antiserum to allopregnanolone showed a lordosis intensity 4 h after treatment that was significantly higher (p < 0.01) than before treatment and in rats injected with normal rabbit serum (Fig. 2). The lordosis quotient was also significantly higher after hours in rats injected with antiserum to allopregnanolone than in the control group (Fig. 3).

Brain allopregnanolone concentration

The measurement of the MBH allopregnanolone concentration did not show a significant difference between rats on diestrus-I or diestrus-II or on estrus. However, rats on proestrus morning and proestrus afternoon showed a hypothalamic concentration of allopregnanolone that was significantly lower than on diestrus or on estrus (p < 0.01) (Fig. 4). No significant circadian difference of hypothalamic allopregnanolone concentration was observed in rats on diestrus (data not shown). Brain cortex concentration of allopregnanolone did not differ among the various phases of the estrous cycle, resulting in the same range of values (data not shown).

Discussion

The present study shows an inhibitory effect of centrally injected allopregnanolone on ovulation in rats, while an increased ovulatory rate and sexual behavior is obtained by blocking the activity of brain allopregnanolone. The evidence of decreasing allopregnanolone concentration in the hypothalamus throughout proestrus day, when ovulation occurs, supports the putative role of a neurosteroid inhibitory pathway on hypothalamus–pituitary control of the ovulatory process.

Previous data have emphasized the wide distribution of neurosteroid in various brain areas and the functional interaction between neurosteroids and GABA receptors (23, 24). The present finding focuses attention on a specific area (the medial basal hypothalamus) and on the neuroendocrine control of ovulation. An endogenous inhibitory GABAergic pathway on GnRH release has clearly been shown (25). However, stimulatory effects on gonadotropin secretion are also seen (26, 27). Such stimulatory effects through the mediation of GABA_A receptors have also been reported after the administration of neurosteroid 3α-hydroxy-5α-pregnan-20-one (28). Because allopregnanolone acts as a GABA receptor agonist compound, it may be hypothesized that this neurosteroid has a central site of action.
action. However, a direct action on pituitary gonadotropin release cannot be ruled out. In fact, FSH release from cultured rat pituitary cells is decreased in the presence of neurosteroid (13, 14). The substance injected in the third ventricle may diffuse to the adjacent hypothalamic areas and, through the median eminence, in part may reach the pituitary gonadotrophs. Therefore, allopregnanolone may act both on the hypothalamus and on pituitary sites of action. However, the evidence that central injection of an antiserum antiallopregnanolone was able to affect the ovulatory rate supports the hypothesis of a brain more than a pituitary site of action. Further support is provided by the influence on sexual behavior and the evidence of a decreasing concentration of hypothalamic allopregnanolone on proestrus.

Fig. 2. Sexual behavior with intracerebroventricular injection of antiserum to allopregnanolone. Lordosis intensity scale: basal and after 4 h from injection ± sd. Female rats injected with antiserum to allopregnanolone showed, after 4 h, a lordosis intensity scale score that was significantly higher (p < 0.01) than rats of the control group injected with normal rabbit serum and rats of both pretreatment groups. as-A: antiserum to allopregnanolone.

Fig. 3. Sexual behavior with intracerebroventricular injection of antiserum to allopregnanolone. Lordosis quotient percentage: basal and after 4 h from injection ± sd. Female rats injected with antiserum to allopregnanolone showed, after 4 h, a lordosis quotient percentage that was significantly higher (p < 0.01) than rats of the control group injected with normal rabbit serum and rats of both pretreatment groups. as-A: antiserum to allopregnanolone.
The impact of neurosteroids on sexual behavior may also be mediated through an effect of GABA receptors. Indeed, GABA inhibits sexual behavior in female rats (29), with particular evidence on lordosis behavior (30, 31). Lordosis is a steroid-dependent behavior that requires the presence of estradiol and progesterone and is associated with ovulation in female rats. The mechanism regulating receptivity involves an interaction between ovarian steroids and GABA transmission (32).

The present data support the putative role of allopregnanolone as a neuromodulator (5–7). This is also suggested by changes of hypothalamic allopregnanolone: a twofold difference on proestrus morning reaches a fivefold lower magnitude at 18.00 h when ovulation occurs, probably reflecting a decreased activity of the inhibitory effect of allopregnanolone. The lack of changes in brain cortex concentration suggests that hypothalamic allopregnanolone is related to the events of ovulation. The evidence that hypothalamic concentration of allopregnanolone does not change on diestrus afternoon in part counteracts the hypothesis of a circadian change of allopregnanolone and supports the functional role of changes occurring on proestrus. The present data also suggest that different brain areas contain neurosteroids acting on different functions (33).

In conclusion, the present study shows an involvement of allopregnanolone in the central mechanisms related to ovulation, probably mediating some steroid-dependent behavioral changes.

Acknowledgments. We thank Daniele Radi and Francesco Sabbatini for their help.

References

gonadotropes of gonadotropin-stimulated follicle stimulating hormone release by the gonadal- and neurosteroid 3α-hydroxy-4-pregnane-20-one involves cytosolic calcium. Endocrinology 1994;134:377–82
15. Wiebe JP, Wood PH. 1987 Selective suppression of follicle-stimulating hormone by 3α-hydroxy-4-pregnane-20-one, a steroid found in Sertoli cells. Endocrinology 1987;120:2259–64
18. Hardy DF, De Bold JF. Effects on mount without intromission upon the behavior of female rats during the onset of estrogen induced heat. Physiol Behav 1971;7:643–5

Received January 23rd, 1995
Accepted April 24th, 1995