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ORIGINAL ARTICLE

Allopregnanolone induces LHRH and glutamate release through NMDA receptor modulation

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Abstract LHRH release from hypothalamus is influenced by the neurotransmitter glutamate that acts, among others, on NMDA receptors present in LHRH neurons. On the other hand, the neurosteroid allopregnanolone can modulate the activity of specific neurotransmitter receptors and affect neurotransmitter release. We examined the role of allopregnanolone on in vitro LHRH and glutamate release from mediobasal hypothalamus and anterior preoptic area of ovariectomized rats with estrogen and progesterone replacement. Moreover, we evaluated whether the neurosteroid might act through modulation of NMDA receptors. Allopregnanolone induced an increase in LHRH release. This effect was reversed when the NMDA receptors were blocked by the NMDA antagonist 2-amino-7-phosphonoheptanoic acid (AP-7) indicating that this neurosteroid would interact with NMDA receptors. Moreover allopregnanolone induced an augment in K^+ evoked [^{3}H]-glutamate release from mediobasal hypothalamus-anterior preoptic area explants and this effect was also reversed when NMDA receptors were blocked with AP-7. These results suggest an important physiologic function of allopregnanolone on the regulation of neuroendocrine function in female adult rats. Not only appears to be involved in enhancing LHRH release through modulation of NMDA receptors but also in the release of glutamate which is critical in the control of LHRH release.

Keywords LHRH · NMDA receptor · Allopregnanolone · Neurosteroid · Glutamate · Hypothalamus

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Introduction

LHRH release from LHRH neurons is one of the most important events that regulate the reproduction. The pulsatory secretion of LHRH is required for fertility and drives the synthesis and release of gonadotropins LH and FSH from the pituitary [1], which controls gametogenesis and steroidogenesis. Across the oestrous cycle, ovarian steroids, particularly estrogens and progesterone, exert both positive and negative feedbacks to regulate LHRH release, but the cellular mechanisms have not been yet fully elucidated. One possibility is the direct action of steroids on LHRH neurons [2]. Moreover, they may act on other neuronal systems, afferent to LHRH neurons, like GABAergic or glutamatergic neurons [3].

Among the regulators of hypothalamic LHRH secretion, the excitatory neurotransmitter glutamate plays an important role, because it is involved in the mechanisms of the LHRH pulse generator and the preovulatory LHRH surge [4]. Glutamatergic input to LHRH neuronal system is likely to be mediated by direct synapses at LHRH neurons [3] although another important neurotransmitters like nitric oxide, endocannabinoid system, catecholamines, galanin, and neuropeptide Y, may also participate in glutamatergic-induced LHRH secretion [5, 6].

Neurosteroids–steroids that are both synthesized in the nervous system from cholesterol and accumulate in the nervous system independently of peripheral steroidogenic glands secretion [7, 8]—are other important candidates to exert modulatory actions on reproductive function. Allopregnanolone (3α-hydroxy-5α-pregnan-20-one), a progesterone derivative, is one of the best characterized neurosteroids that regulates the function of LHRH neurons [9, 10]. Its biosynthesis begins with progesterone which is converted to dihydroprogesterone by 5-alpha reductase activity present in both neurons and glial cells**.** Furthermore, 3-alpha hydroxysteroid oxide reductase, present in astrocytes, catalyzes the reduction of dihydrometabolite toward allopregnanolone [11].

Due to the conversion of progesterone to allopregnanolone, several effects of progesterone have been attributed to allopregnanolone. It has been reported that allopregnanolone has anxiolytic [12], hypnotic [13], and anticonvulsant effects [14]. Moreover, allopregnanolone has been implicated in the modulation of reproductive function in female rats and in the LH release [15].

Most of the effects of allopregnanolone are thought to be due to interaction of the neurosteroid with neurotransmitter receptors. Considerable data have been accumulated demonstrating that allopregnanolone acts as a potent enhancer of $GABA_A$ receptor function, facilitating the chloride channel opening by allosteric modulation and increasing the response to the neurotransmitter GABA of postsynaptic cells expressing this subtype of receptor [16]. However, interaction of allopregnanolone with other neurotransmitter receptors, like glutamate receptors, has not been greatly studied. Other neurosteroids have been shown to interact with glutamatergic NMDA receptors activity such as pregnanolone sulfate [17], dehydroepiandrosterone sulfate, and allopregnanolone sulfate [18]. However, non sulfated neurosteroids like allopregnanolone have not been demonstrated to interact with NMDA receptors. It is uncertain whether allopregnanolone effects on LHRH are also due to interaction with NMDA receptors, in addition to extensively described effects of allopregnanolone on $GABA_A$ receptors.

The aims of this study were (1) to determine whether allopregnanolone could affect LHRH release in the medium basal hypothalamus (MBH) and anterior preoptic area (APOA) in female rats and whether this possible effect could be due to modulation of NMDA receptors, and (2) to determine whether allopregnanolone could modify the glutamate release from MBH and APOA and whether the effect could also be due to modulation of NMDA receptors. Using MBH-APOA slices of ovariectomized Sprague–Dawley rats we observed that allopregnanolone increases LHRH and glutamate release in vitro, and that these effects could involve an interaction of neurosteroid with NMDA receptors.

Materials and methods

Animals

Adult Sprague–Dawley female rats (90–120 days old, 250–300 g body weight) from the laboratory colony were used. Animals were maintained at a constant temperature (22 ± 2°C) and lighting (lights on 07.00–19.00 h). They were housed in groups (three animals/cage) with free access to standard rat chow (Digna, Mendoza, Argentina) and tap water. All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health (NIH) USA.

Drugs

Allopregnanolone, the agonist *N*-methyl-D-aspartic acid (NMDA), oestradiol benzoate, and progesterone were purchased from Sigma Chemical Co., St. Louis, MO, USA. The competitive NMDA receptor antagonist, 2-amino-7-phosphonoheptanoic acid (AP7) was purchased from Research Biochemical International Inc., MA, USA. [^{3}H]-Glutamic acid ([^{3}H]-Glu) was purchased from New England Nuclear (Boston, MA, USA).

Surgical procedures

Female adult rats were ovariectomized under 8% chloral hydrate anesthesia (0.1 ml/100 g animal). Then the animals were left to recover for 10–14 days. Forty-eight hours before the experiment, ovariectomized rats were injected s.c. with oil 25 μg of oestradiol benzoate and 5 h before the sacrifice they were injected s.c. with 1 mg progesterone. In the experimental model, we used a "priming" dose of estradiol and progesterone according to [19] to induce a LH surge in the moment of sacrifice. The animals were decapitated and their brains removed. MBH-APOA explants were dissected by making a frontal cut just before optic chiasm, extending dorsally for 1.0 mm; a horizontal cut extended from this point caudally to just behind the pituitary stalk, where another frontal cut was made. Longitudinal cuts were made 1.2 mm lateral to the midline bilaterally.

Experimental design

Drugs preparation

Allopregnanolone was initially dissolved in propylenglycol to a concentration of 0.6 mM. The further 6-μM concentration of allopregnanolone was obtained by dilution in Krebs–Ringer bicarbonate glucose (KRBG) Mg^{2+} free buffer at pH 7.4 (118.6 mM NaCl, 4.75 mM KCl, 2.5 mM

$CaCl_2$, 1.2 mM KH_2P0_4, 1.2 mM Na_2S0_4, 1.2 mM $NaHC0_3$, 5.5 mM dextrose, and 0.06 mM ascorbic acid, saturated with 95% O_2/5% CO_2). The 6-μM concentration of allopregnanolone was chosen to mimic its maximal circulating level during sexual behavior [20].

The 20-mM concentration of NMDA and 100-μM concentration of AP-7 were obtained by dilution in KRBG Mg^{2+} free buffer at pH 7.4 as it has been described by [21]. For control KRBG Mg^{2+} free buffer at pH 7.4 with propylene glycol as vehicle was used.

Effect of NMDA on LHRH release

Each MBH-APOA was sliced front to back at 240 μm with a McIIwain tissue chopper. Each sliced MBH-APOA was pre-incubated with 1 ml of KRBG Mg^{2+} free buffer in a Dubnoff metabolic shaker (50 cycles per min; 95% O_2/5% CO_2) at 37°C (to insure that NMDA receptors are blocked before the treatment with NMDA plus AP-7, in this experimental group the pre-incubation was made with AP-7 100 μM). After 30 min, the supernatants were disposed and replaced with 0.5 ml of KRBG Mg^{2+} free buffer containing either, vehicle, NMDA 20 mM, NMDA 20 mM plus AP-7 100 μM or AP-7 100 μM. The incubation was continued for 30 min, followed by removal of the medium and storage at −20°C until LHRH assay. Four to five animals were used per experimental group.

Effect of allopregnanolone on LHRH release

Each MBH-APOA explant was sliced and pre-incubated as it was described before. In order to insure that NMDA receptors are blocked before the treatment with allopregnanolone plus AP-7, in this experimental group the pre-incubation was made with AP-7 100 μM. After that, the slices were incubated with 0.5 ml of KRBG containing either, vehicle, allopregnanolone 6 μM, AP-7 100 μM plus allopregnanolone 6 μM or AP-7 100 μM. The incubation was continued for 30 min, followed by removal of the medium and storage at −20°C until LHRH assay. Four to 5 animals were used per experimental group.

Effect of allopregnanolone on K^+ evoked [^{3}H] glutamate release

The dissected MBH-APOAs were sliced as was described before. Each sliced MBH-APOA explant was exposed to 2.79 μM [^{3}H]-Glu (specific activity 44 Ci/mmol) in 2 ml of gassed (95% O_2 and 5% CO_2) KRBG Mg^{2+} free buffer for 15 min at 37°C in a Dubnoff metabolic shaker. The slices were transferred to a superfusion chamber and superfused at 0.7 ml/min with KRBG Mg^{2+} free buffer for 30 min (washing period), to washout the [^{3}H]-glutamic acid not incorporated into the tissue (to insure that NMDA receptors are blocked before the superfusion with allopregnanolone plus AP-7, in this experimental group 5 min before starting collecting fractions, the tissues were superfused with AP-7 100 μM). Then, the experiment was started by superfusing KRBG Mg^{2+} free buffer containing either the vehicle (propilenglycol), allopregnanolone 6 μM, allopregnanolone 6 μM plus AP-7 100 μM or AP-7 100 μM (pre-stimulus period). During this period, five fractions of 1.75 ml each one (2.5 min each fraction) were collected and considered as basal release. After that, the slices were superfused with KRBG Mg^{++} free supplemented with KCl 28 mM and three 1.75 ml fractions were collected (evoked release). Then, the same solution than pre-stimulus period was superfused and five 1.75 ml fractions were collected. At the end of the experiments, the slices were homogenized in 2 ml of 0.2 N perchloric acid by sonication, and 0.5 ml aliquots of each fraction and homogenates were taken and mixed with scintillation fluid to measure the radioactivity. The percentage (%) of released [^{3}H]-Glu with respect to the amount of [^{3}H]-Glu remaining in the tissue was calculated. The results obtained in the different groups were expressed as % fractional release that was calculated as the difference between the percents of glutamate released by the tissue during K^+ evoked release (mean fraction 6, 7, and 8) and the percents of basal glutamate release (mean fraction 3, 4, and 5) (for details, see [22]). Five to eight animals were used per experimental group.

LHRH assay

LHRH of media stored at −20°C was measured by RIA by using a highly specific LHRH antibody kindly provided by Dr. W. Wuttke (Department of Clinical and Experimental Endocrinology, University of Gottingen, Gottingen, Germany). The sensitivity of the assay was 0.2 pg/tube, and the curve was linear up to 100 pg of LHRH. The intraassay coefficient of variation of the LHRH RIA ranged from 4 to 7.3% and the interassay coefficient of variation was 8.9%. All samples were measured in duplicate.

Statistics

One way analysis of variance (ANOVA I) was used, followed by a post-hoc Newman–Keuls test for the comparisons among groups. Results were expressed as means ± SEM. (Differences of $P < 0.05$ were considered statistically significant).

Results

Effect of NMDA on LHRH release

Incubation of MBH-APOA slices for 30 min with NMDA (20 mM) significantly increased the basal release of LHRH. This effect was reversed when NMDA (20 mM) was coincubated with the NMDA antagonist AP-7 (100 μM). AP-7 (100 μM) had no effect per se on LHRH release when was incubated alone with the MBH-APOA slices (Fig. 1).

Effect of allopregnanolone on LHRH release

Incubation of MBH-APOA slices for 30 min with allopregnanolone (6 μM) significantly enhanced basal release of LHRH. This effect was decreased when AP-7 antagonist (100 μM) was co-administered with allopregnanolone (6 μM). The antagonist had no effect by itself on LHRH release from MBH-APOA slices (Fig. 2).

Stimulatory effect of allopregnanolone on K^+-evoked [^{3}H]-glutamate release

K^+-evoked [^{3}H]-Glu release from MBH-APOA slices under different superfusion buffer conditions was studied. Exposure of slices to allopregnanolone (6 μM) significantly enhanced K^+-evoked [^{3}H]-Glu release (Fig. 3) compared with vehicle. To evaluate if this effect was NMDA receptor activity dependent the slices were superfused with allopregnanolone (6 μM) plus AP-7 (100 μM) buffer before and after K^+ stimulation. The NMDA antagonist, AP-7, reversed the stimulatory action of allopregnanolone when they were present both at the same time. The antagonist AP-7 (100 μM) had no effect by itself on [^{3}H]-Glu release compared to vehicle (Fig. 3).

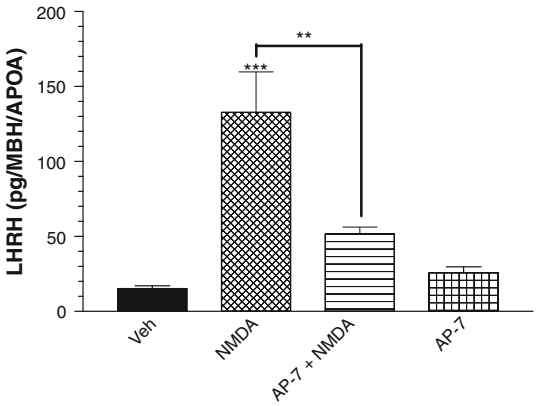

Fig. 1 Effect of NMDA on LHRH release from MBH/APOA slices in vitro. Tissues were incubated with KRBG Mg^{2+} free containing either vehicle (Veh) ($n = 5$), NMDA 20 mM ($n = 5$), NMDA 20 mM plus AP7 100 μM ($n = 4$) or AP-7 100 μM ($n = 5$) for 30 min. Values represent mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$

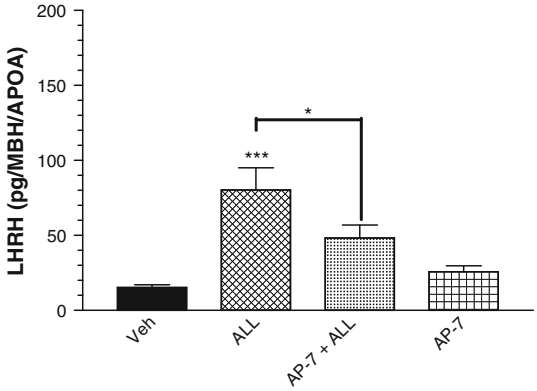

Fig. 2 Effect of allopregnanolone on LHRH release from MBH/APOA slices in vitro. Tissues were incubated in KRBG Mg^{2+} free containing either vehicle (Veh) ($n = 5$), allopregnanolone 6 μM ($n = 5$), allopregnanolone 6 μM plus AP-7 100 μM ($n = 4$) or AP-7 100 μM ($n = 5$) by 30 min. Values represent mean ± SEM. $^{***}P < 0.001$, $^{*}P < 0.05$

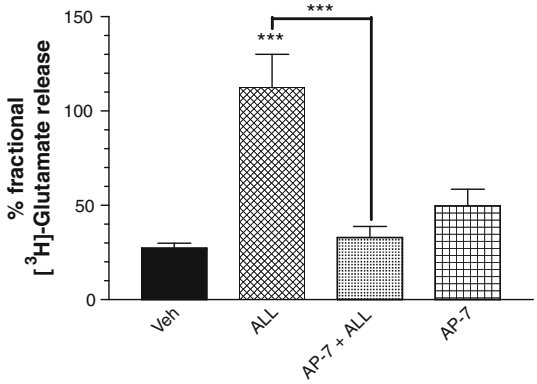

Fig. 3 Effect of allopregnanolone on K^+ evoked [^{3}H]-Glu release in vitro. Tissues were superfused with KRBG Mg^{2+} free containing either vehicle (Veh) ($n = 8$), allopregnanolone 6 μM ($n = 7$), AP-7 100 μM plus allopregnanolone 6 μM ($n = 5$) or AP-7 100 μM ($n = 8$). The values represent mean ± SEM. $^{***}P < 0.001$

Discussion

The objectives were: (1) to determine whether allopregnanolone is able to modify basal LHRH release from MBH-APOA slices through NMDA receptor modulation; and (2) to determine if allopregnanolone can modify the glutamate release through NMDA receptor modulation. When we examined the effect of NMDA on LHRH release, we found that this agonist stimulates basal LHRH release and that the treatment with the NMDA receptor antagonist AP-7 blocks NMDA action. It is clear that excitatory amino acids participate in activation of LHRH neurons through

NMDA and non-NMDA receptors [21, 23]. In this study, however, we observed that LHRH release may be modified by the neurosteroid allopregnanolone. The results in this study indicated that allopregnanolone at 6 μM concentration, strongly stimulates LHRH release (Fig. 2). The modulatory properties of neurosteroids have been proposed as an interesting functional interaction between neurosteroids and neurotransmitter receptors coupled to ionic channels, such as the $GABA_A$ [11] and the glutamic acid receptors [24]. Such interaction could produce fast modifications of neuronal excitability.

Some controversial actions of allopregnanolone on LHRH and LH releases have been reported. This neurosteroid is a potent enhancer of $GABA_A$ receptors function [25]. Because of this modulatory action it would be possible that allopregnanolone negatively influences LHRH by activating $GABA_A$ receptors present in LHRH neurons and enhancing hyperpolarization of neurons. However, it has been shown that LHRH neurons have greater intracellular than extracellular chloride concentration. Therefore, it has been argued that an activation of $GABA_A$ receptors on LHRH neurons provokes an efflux of chloride instead an influx, depolarizing the neuron and stimulating LHRH release [26, 27].

In addition, this results suggest that allopregnanolone might have an important physiologic function due to its possible action on NMDA receptors. Now, we show an acute response to allopregnanolone in an in vitro system on LHRH release. The results suggest that allopregnanolone outcome on LHRH release could be specifically mediated by NMDA receptors since AP-7 co-administration decreased the stimulatory action of the neurosteroid.

Not much is known about the modulatory action of allopregnanolone on the NMDA receptor. It has been demonstrated that some sulfated neurosteroids modulate NMDA receptor activity via a steroid modulatory domain on the NMDA receptor NR2B subunit [18, 28]. The results suggest that the non sulfated neurosteroid allopregnanolone could interact with NMDA receptors modulating the release of LHRH. Accordingly, a recent study using electrophysiological techniques has shown that allopregnanolone modulates GABAergic neurotransmission involving a NMDA receptor-mediated mechanism in the central amygdale [29].

Since we observed an increase in LHRH release mediated by modulation of allopregnanolone on NMDA receptor, we decided to examine the eventual relationship between neurosteroid and the glutamate release using the superfusion model. It is known that a variety of neurosteroids may influence the release of neurotransmitters [30].

K^+ evoked glutamate release from MBH-APOA slices is enhanced by allopregnanolone 6 μM. Interestingly, this action is decreased when the slices were superfused with AP-7 plus allopregnanolone. The reversion observed in the effect of allopregnanolone on glutamate release suggests that this action is also mediated by NMDA receptors. One possible explanation could be the existence of presynaptic NMDA receptors regulating glutamate release in glutamatergic terminals. Thus, allopregnanolone interaction with these putative receptors might enhance excitatory amino acid release. It has been shown that the release of many neurotransmitters is regulated by specific presynaptic receptors in several brain areas. Recently, it has been demonstrated the existence of NMDA presynaptic receptors in glutamatergic terminals in different areas of central nervous system [31–34]. We are tempted to speculate about the possibility of this kind of receptors existing in MBH and POA, being eventually responsible for the neurosteroid modulation shown in this article. Other possibility could be the existence of operating glutamatergic networks with NMDA postsynaptic receptors that have been inhibited when AP-7 is superfused with allopregnanolone.

In this study, we propose a novel mechanism that could involve allopregnanolone on LHRH release. In addition to classical action of allopregnanolone on $GABA_A$ receptors, we suggest: (1) an allopregnanolone enhancing modulatory action on NMDA receptors; and (2) a direct effect of allopregnanolone on LHRH release and the neuronal network afferent to LHRH, involving glutamatergic system.

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