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NMDA receptor antagonists block stress-induced prolactin release in female rats at estrus

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Abstract

In order to evaluate the role of glutamate in prolactin secretion, we examined the effects of *N*-methyl-D,L-aspartic acid (NMDA) receptor antagonists on serum prolactin levels at both resting and restraint-stress conditions in female rats at estrus. NMDA increased basal serum prolactin levels. Administration of the selective NMDA receptor antagonist, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755) (5 and 10 mg/kg i.p.), to rats under resting conditions enhanced basal prolactin levels. A low dose of CGS 19755 (3 mg/kg) was unable to modify the hormone serum level. Under stress conditions the pretreatment with CGS 19755 (3 and 5 mg/kg) prevented the increase in serum prolactin levels. This effect was reversed by NMDA (60 mg/kg s.c.). The NMDA receptor antagonist (5 mg/kg) decreased the median eminence concentration of the dopamine metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), without modifying dopamine content. To examine the probable link between serotonin (5-HT) and glutamate in prolactin release, the 5-HT_{2A}/5-HT_{2C} receptor antagonist, ritanserin, was used. Under resting conditions, a dose of 5 mg/kg s.c. blocked the NMDA-induced prolactin release. In rats submitted to restraint, ritanserin decreased the prolactin response and NMDA was unable to correct the stress serum prolactin levels. The 5-HT_{1A} receptor agonist, 8-hidroxypropyl-amino tetralin (8-OH-DPAT) (3 mg/kg s.c.), increased basal serum prolactin levels and restored serum prolactin in stressed animals pretreated with CGS 19755 (5 mg/kg). The present data strongly suggest that the glutamatergic system participates in the regulation of prolactin secretion. A stimulation tone seems to be exerted via the tuberoinfundibular dopaminergic system, and the prolactin release evoked by restraint apparently involves glutamate/NMDA receptors linked to a serotoninergic pathway.

Keywords: Excitatory amino acid; Glutamate; Prolactin release; 5-HT (5-hydroxyptamine, serotonin); Stress

1. Introduction

In contrast to reliable evidence that supports the participation of glutamatergic neurons in gonadotropic regulation (Donoso et al., 1994; Brann and Mahesh, 1994) the literature provides weak and controversial data concerning the role of glutamate in prolactin release. It was shown early, before glutamate was accepted as a neuroendocrine transmitter, that its ionotropic receptor agonist, kainic acid, locally injected within the preoptic/suprachiasmatic area, stimulates both prolactin and luteinizing hormone (LH) release in male rats (Colombo and Ritterman, 1983). Moreover, both systemic or localized brain administration of the glutamate receptor agonist *N*-methyl-D,L-aspartic acid (NMDA), induces a clear-cut increase in prolactin release in male rats (Pohl et al., 1989), adult female and prepuberal male rhesus monkeys (Gay and Plant, 1987), and rams (Kumar et al., 1993). In addition, noncompetitive antagonists of NMDA receptors decrease plasma levels in female rats (Wagner et al., 1993) and attenuate the preovulatory surge of prolactin (Brann and Mahesh, 1991). However, increased rather than decreased prolactin secretion following administration of several competitive NMDA receptor antagonists was also found in male rats (Arslan et al., 1991) and female rams (Kumar et al., 1993).

In the present study, we further examined in female rats at estrus, the role of the glutamate receptor agonist, NMDA, in the control of prolactin release under resting conditions or after exposure to restraint, a potent stress stimulus for prolactin release (Kehoe et al., 1991). The experiments

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were carried out with the selective NMDA receptor antagonist, *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS 19755) (Lehman et al., 1988). Since prolactin is tonically inhibited by dopamine released into hypophysial portal vessels from the tuberoinfundibular dopaminergic system (MacLeod, 1976) and modulated by serotonin (5-HT) from cells of the dorsal raphe nucleus whose terminals project to the hypothalamus (Azmita and Segal, 1970; Van de Kar et al., 1996), in this study we also evaluated their possible association with the glutamatergic system.

2. Materials and methods

2.1. Animals

Adult female Sprague–Dawley rats (200–250 g b.w.) bred at our laboratory were used. They were housed in a room with controlled temperature (22–23°C) and light (14 h light–10 h darkness; lights on at 0600) and with food and water available ad libitum. Estrous cycles were monitored daily by means of vaginal smears. Experiments were performed between 1100–1400 h on the day of estrus. This stage of the estrous cycle is known to be very susceptible to stimuli that elicit prolactin release (Neill, 1970).

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.

2.2. Experimental design

2.2.1. Experiment 1

In this experiment, the effects of competitive NMDA receptor antagonists in rats were examined under resting conditions. The animals received i.p. physiological saline (0.9% NaCl, 0.1 ml/100 g b.w.) or the NMDA receptor antagonist, CGS 19755 (3, 5 and 10 mg/kg). Forty minutes after injection, the animals were killed by decapitation and blood from the trunk was collected. The samples were rapidly centrifuged. Serum was separated and stored at −30°C until prolactin was assayed. In order to measure dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC), the brains were removed from the skulls and the median eminences were dissected with the aid of a stereoscopic microscope. The median eminences were placed in Eppendorf tubes containing 80 μl of 0.2 N phosphoric acid and stored frozen.

2.2.2. Experiment 2

The second experiment was designed to examine the effect of NMDA receptor antagonists on the prolactin response to stress and to verify the action of NMDA itself. Estrous rats were randomly divided into groups of 6–8 rats each. The animals received i.p. CGS 19755 (3 and 5 mg/kg) or saline 25 min before their being placed in a plexiglass restraint chamber for another additional 15 min. In a different group of rats, NMDA (60 mg/kg s.c.) or saline were administered under resting conditions; the animals were killed 30 min later. The selectivity of CGS 19755 was verified in an additional group of rats injected with the antagonist (5 mg/kg i.p.) followed by NMDA at an interval of 5 min. Twenty minutes later, the rats were submitted to restraint for 15 min.

2.2.3. Experiment 3

To establish whether 5-HT participates in responses to both stress and NMDA, the rats were pretreated with the antagonist, (5-HT_{2A}/5-HT_{2C}), ritanserin (5 mg/kg s.c.), and killed 30 min after the injection. One group of these animals was subjected to restraint for 15 min before killing. Another group of rats treated with ritanserin as described above, was additionally injected with NMDA (60 mg/kg s.c.) or saline and killed 30 min later. In the same way these groups of animals were subdivided into animals exposed to restraint or kept in their home cage (unstressed rats).

Another group of animals was pretreated with CGS 19755 (5 mg/kg i.p.) and 25 min later was injected with 8-hidroxydipropyl-amino tetralin (8-OH-DPAT) (1 and 3 mg/kg s.c.), a selective 5-HT_{1A} receptor agonist, and either submitted to restraint for 15 min or left undisturbed.

The animals from all the experiments were killed by decapitation and blood was collected from the trunk.

2.3. Dopamine assay

The median eminences were thawed, homogenized by sonication and centrifuged at $10000 \times g$ for 15 min. Dopamine and its primary metabolite DOPAC, were measured in the clear supernatants by reverse-phase high performance liquid chromatography (HPLC) columns (C18) with electrochemical detection. The HPLC mobile phase consisted of 45 mM sodium phosphate dibasic buffer pH 3.5 containing 0.43 mM sodium octyl sulphate 0.34 mM 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 (Lowry et al., 1951). The results are expressed as nanograms of compound per milligram protein.

2.4. Drugs

The drugs used were NMDA and NMDA receptor antagonists CGS 19755 and 2-amino-7-phosphonoheptanoic acid (AP-7), the 5-HT_{2A}/5-HT_{2C} receptor antagonist, ritanserin, and the 5-HT_{1A} receptor agonist, 8-OH-DPAT (Research Biochemicals, Natick, MA). Drugs were dissolved in physiological saline.

2.5. Prolactin radioimmunoassay

Prolactin was measured in the serum samples by radioimmunoassay, using kits supplied by the National Hormone Pituitary Program, USA. The intra- and interassay coefficients of variation were 9% and 11%, respectively. The data are expressed in nanograms per milliliter of serum in terms of NIAMDD-Rat-RP-3 reference preparation.

2.6. Statistical analysis

Results are expressed as means ± S.E.M. The data were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons; $P < 0.05$ was considered as minimum criterion for assigning statistical significance.

3. Results

3.1. Experiment 1: Effect of the NMDA receptor antagonists at resting conditions

The i.p. injection of CGS 19755, a competitive antagonist for NMDA receptors, at doses of 5 and 10 mg/kg, induced a significant rise of prolactin serum levels, $P < 0.05$ and $P < 0.01$ respectively in the female rats at estrus. A lower dose 3 mg/kg, had no effect on the prolactin basal levels. As demonstrated before, NMDA at a higher dose, 60 mg/kg s.c., produced a similar increase of basal prolactin serum levels ($P < 0.01$, Fig. 1a).

The effective dose of CGS 19755 (5 mg/kg) also caused a significant reduction of the concentration of the metabolite, DOPAC, in the median eminence, without modifying the dopamine levels, indicating an inhibitory effect on the tuberoinfundibular dopaminergic system. According to what was observed with the prolactin response, the lower dose of CGS 19755 (3 mg/kg), was also ineffective on the tuberoinfundibular dopaminergic system (Table 1).

3.2. Experiment 2: Effect of NMDA receptor antagonists on the prolactin release induced by stress

In rats under the same conditions as described above, immobilization stress for 15 min induced a significant rise of serum prolactin levels ($P < 0.01$), and this was prevented by the previous administration of CGS 19755 3 and 5 mg/kg (Fig. 1b). AP-7 (10 nmol i.c.v. dissolved in 3 μl saline), another competitive antagonist, selective for the NMDA site, was able to cause the same effect on the prolactin response to stress: prolactin (ng/ml) 167.5 ± 19.6, $n = 9$ (stress); 24.9 ± 14.7, $n = 6$ (stress + AP-7); and 10.3 ± 1.8, $n = 6$ (sal). The blockade of the stress-induced prolactin secretion by the antagonist, CGS 19755, was reversed with NMDA (60 mg/kg). The agonist per se did not modify the prolactin response to the immobilization stimulus (Fig. 1b) and the tuberoinfundibular dopaminergic system remained unchanged after the stress stimulus (Table 1).

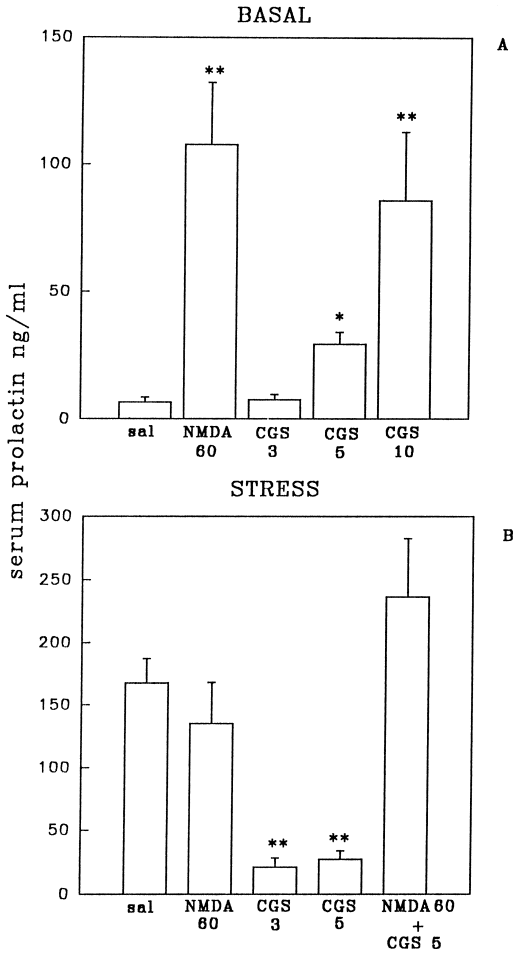

Fig. 1. (A) Serum prolactin levels of female rats in estrus under resting conditions. Effect of treatment with NMDA (60 mg/kg) and the competitive antagonist, CGS 19755, at different doses (3, 5 and 10 mg/kg). (B) Serum prolactin levels of animals submitted to stress, and after the administration of NMDA, the antagonist, CGS 19755, or the combination of both. Doses are indicated in the figure. The data represent the means ± S.E.M. ($n = 6$–10). SAL: saline control; * $P < 0.05$, ** $P < 0.01$ vs. SAL.

Table 1
Dopamine (DA) and its principal metabolite, 3,4-dihidroxyphenylacetic acid (DOPAC), concentrations (ng per mg of protein) in the median eminence of female rats at estrus

Treatment	DA (ng/mg protein)	DOPAC (ng/mg protein)
Saline	214.0 ± 36.0	10.7 ± 1.3 (10)
Saline + stress	168.0 ± 42.0	9.2 ± 1.4 (7)
CGS (5 mg/kg)	163.0 ± 22.0	6.4 ± 0.9 (7)[a]
CGS (3 mg/kg)	225.8 ± 62.4	14.0 ± 2.0 (5)

The data represent the means ± S.E.M. ($n = 6$–10); [a] $P < 0.05$ vs. saline.

3.3. Experiment 3: Interactions with the serotoninergic system

Ritanserin (5 mg/kg), a serotonin antagonist acting on the 5-HT_2 receptors, was able to antagonize the enhancement of serum levels of prolactin induced by NMDA, $P < 0.01$. This drug, at the same dose, did not affect the basal level of the hormone (Fig. 2a).

The previous administration of ritanserin to rats submitted to stress significantly ($P < 0.05$) reduced the prolactin response. This blockade could not be reversed by NMDA (Fig. 2b).

In stressed rats, the blockade of the prolactin secretion by the NMDA receptor antagonist, CGS 19755 (5 mg/kg), was reversed by the serotonin agonist of the 5-HT_{1A} receptors, 8-OH-DPAT (3 mg/kg). At a lower dose of the agonist (1 mg/kg) this effect was not observed. An additional observation was the increase of basal prolactin levels by 8-OH-DPAT (3 mg/kg) alone, and this increase was not additive to the one elicited by CGS 19755 (5 mg/kg) (Table 2).

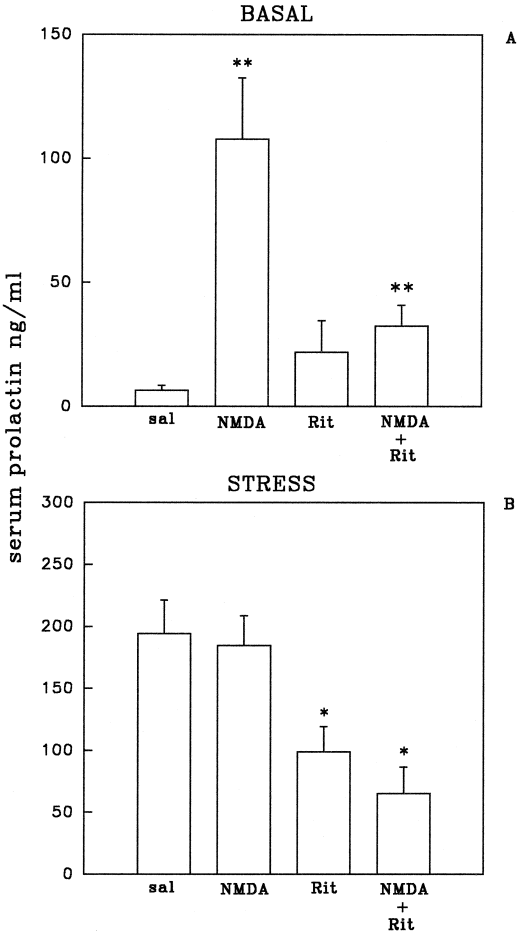

Fig. 2. (A) Serum prolactin levels in estrus rats under resting conditions or (B) submitted to stress. Effect of the previous administration of ritanserin (5 mg/kg) alone or combined with NMDA The data represent the means ± S.E.M. ($n = 5–6$). SAL: saline control, $*P < 0.05$ vs. SAL, $**P < 0.01$ vs. SAL or NMDA.

Table 2
Serum prolactin levels (ng/ml) in female rats at estrus

Drugs	Treatment	
	Resting	Stress
Saline	6.5 ± 1.90	167.5 ± 19.6[a]
CGS (5 mg/kg)	29.3 ± 4.60[b]	27.3 ± 6.4
8OH-DPAT (1 mg/kg)	10.6 ± 5.70	–
8OH-DPAT (3 mg/kg)	58.7 ± 23.10[b]	188.4 ± 48.8
CGS (5 mg/kg) + 8-OH-DPAT (1 mg/kg)	22.5 ± 16.05[b]	25.8 ± 9.7
CGS (5 mg/kg) + 8-OH-DPAT (3 mg/kg)	43.2 ± 18.80[b]	197.2 ± 26.4[c]

The data represent the means ± S.E.M. ($n = 8–10$). [a]$P < 0.001$, [b]$P < 0.05$ vs. saline and [c]$P < 0.01$ vs. CGS + 8-OH-DPAT (1 mg/kg).

4. Discussion

The results of this work confirm a clear participation of the glutamatergic system in the control of prolactin secretion. Under resting conditions, the glutamatergic input seems to be stimulating the TIDA system in a tonic way. This idea is supported by the finding that blockade of the NMDA receptors by CGS 19755, caused a decline in median eminence dopaminergic activity together with a simultaneous rise in serum prolactin levels in female rats at estrus. These results are in agreement with those previously reported by Moore for the inhibition of the tuberoinfundibular dopaminergic system caused by CGS 19755 in female rats at diestrus (Wagner et al., 1993). A similar action of NMDA competitive antagonists on basal prolactin secretion was reported in sexually inactive rams, with CGP 37849 (Kumar et al., 1993), and in rats with 2-amino-5-phosphonoheptanoic acid (AP-5) (Arslan et al., 1991). In contrast, the action of the noncompetitive antagonist dizocilpine (MK-801) was to decrease serum prolactin levels, simultaneously with decreasing median eminence DOPAC concentration (Wagner et al., 1993). This could be explained by the existence of different mechanisms of action at the molecular level (i.e., blockade of the ion channels) or by direct effects of this drug on the pituitary (Login, 1990).

The dopaminergic neurons of the arcuate nucleus are the most likely site of action for the excitatory amino acid regulation of prolactin release. Saitoh et al. (1991) have reported that NMDA induces c-fos immunoreactivity in dopaminergic neurons of the mediobasal hypothalamus. On the other hand, an interaction of glutamate/NMDA input with a prolactin releasing factor (PRF) is more difficult to demonstrate. An early report indicates that thyrotropin releasing hormone (TRH) seems not to be involved in NMDA's effects on prolactin (Wilson and Knobil, 1983).

Under stress conditions, again using the model of the female rats at estrus, we found that the action of NMDA receptor antagonists was to prevent the rise of prolactin serum levels. As shown, competitive blockade of these receptors prevents the prolactin enhancement and accordingly, the levels of this hormone induced by NMDA are the same as those reached after restraint. Hence, the results of this work suggest the participation of glutamate/NMDA receptors in the stress response. In this case, the glutamatergic effect was demonstrated to be independent of the tuberoinfundibular dopaminergic system. Our results indicate that both dopamine and DOPAC concentrations in the median eminence remain stable during stress and after a low dose of CGS 19755, confirming previous results reported by others (Lookingland et al., 1990; Kehoe et al., 1991). A short period of immobilization stress, may therefore involve different neuronal pathways to trigger prolactin secretion (Fig. 3).

One of the purposes of this work was to investigate the involvement of serotoninergic pathways in the stimulatory action of glutamate on prolactin secretion. It is well known that stress-evoked prolactin release is prevented by 5-HT receptor antagonists (Jörgensen et al., 1992). Several events that evoked prolactin release seem to rely at least partially on central serotoninergic activity. In male rats, the effects of restraint and ether exposure on plasma prolactin levels are prevented by pretreatment with methysergide. Other 5-HT receptor antagonists showed the same effects. Ketanserin ($5\text{-HT}_1/5\text{-HT}_2$), 6-methyl-1-(1-methylethyl)-ergoline-8β carboxylic acid-2-hydroxy-1-methyl propyl ester maleate (LY 53 857), ($5\text{-HT}_2/5\text{-HT}_{1C}$), the antagonists of the 5-HT_3 receptor subtype, 3-propanyl-indole-3-carboxylate hydrochloride (ICS 205-930), and 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-4*H*-carbazol-4-one (GR 38032F) were, like methysergide, all able to prevent the prolactin release induced by stress (Kordon et al., 1994).

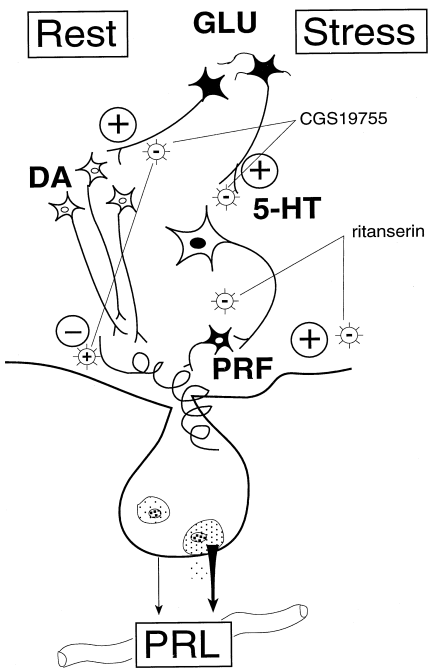

Fig. 3. Hypothesized effects of glutamate on prolactin release in estrus rats. Under resting conditions, glutamate (GLU) exerts, through NMDA receptors, tonic stimulation of tuberoinfundibular dopaminergic neurons (DA) that inhibit prolactin release from the pituitary. Following restraint-stress GLU, by an alternative pathway, interacts with serotonin (5-HT) and prolactin-releasing factor(s) (PRFs) to stimulate prolactin release.

Our experiment with ritanserin, a potent ligand of $5\text{-HT}_{2A}/5\text{-HT}_{2C}$ receptors (Leysen et al., 1985; Apud, 1991), suggests that prolactin liberation by stress is elicited by serotonin through its 5-HT_2 receptors, and in the same fashion, the glutamate stimulatory action via its NMDA receptors is affected by 5-HT_2 receptor antagonists, suggesting a common pathway. Our work also demonstrated that during stress-induced prolactin secretion, an interaction of the 5-HT_{1A} receptor subtype with the glutamatergic input may be conceivable. The evidence was provided by the effect of the agonist, 8-OH-DPAT, to reverse the inhibition induced by NMDA receptor blockade. Moreover, it has been shown previously that this agonist is able to induce a greater prolactin release than the agonists, $5\text{-HT}_{1B}/5\text{-HT}_{1C}$ (Kordon et al., 1994), and that, in addition, 8-OH-DPAT is able to release prolactin in female rats at estrus.

Therefore, it seems that, in order to induce prolactin release, the glutamatergic system needs the activation of the 5-HT_{1A}-mediated serotoninergic input. However, the serotonin antagonist, methiothepine, a drug acting at the 5-HT_1 receptor sites, was unable to hinder the effect of stress on prolactin secretion, and itself elicited prolactin secretion by, both under resting and stress conditions (data not shown). We interpret these results as being caused by the presynaptic action of methiothepine, on 5-HT_1 autoreceptors (Pettibone and Pflueger, 1984), increasing serotonin concentration at the synaptic cleft, and hence prolactin release. We expect that the use of more specific antagonists acting at postsynaptic sites, will clarify the participation of the 5-HT_1 receptors.

The full explanation of an interaction between the glutamatergic and serotoninergic pathways awaits demonstration. Nerve endings of both systems are present in the hypothalamus (Van den Pol et al., 1990; Kordon et al., 1994), so it seems conceivable that there could be either some kind of contact between the systems, or direct receptor–receptor interactions within the same terminal.

It is important to add that we used female rats at estrus for this work because the animals at diestrus 1 show slightly different prolactin responses. At diestrus 1 we observed that CGS 19755 (5 mg/kg) was able to increase prolactin secretion but, in contrast, the hormone response to stress was not blocked. As pointed out by others, diestrus 1 is a stage with higher thresholds for stimulation than other days of the estrous cycle and it is also known

that the hypothalamus is less sensitive to stress challenge during the stage of diestrus (Neill, 1970). Therefore, there seems to be some kind of endocrine conditioning of the prolactin responses. Considering that the response to restraint stress implies massive mobilization of neurotransmitters, several pieces of the mechanism may be affected as well. For example, the concentration and affinity of the pituitary TRH receptors (TRH is a known PRF) fluctuate during the estrous cycle (De Lean et al., 1977).

In relation to responses to NMDA, the prolactin releasing effect in the rat at estrus was known, but it had been demonstrated also that NMDA acts in an inhibitory fashion in lactating rats with higher basal levels of the hormone, (Pohl et al., 1989). A possible explanation for this behavior would be that the glutamatergic/NMDA system exerts simultaneous actions over the multiple mechanism controlling prolactin release, including the dopaminergic inputs and the PRFs. Therefore, the hormonal background of the animal would be the factor that determines the direction of the responses.

In conclusion, we have demonstrated an effective participation of glutamate/NMDA in prolactin regulation. We hypothesize that glutamate effects on prolactin secretion are stimulatory, but either exerted at different sites or mediated by different transmitters according to the animal's hormonal and emotional state.

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