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Opioid modulation of prolactin secretion induced by stress during late pregnancy. Role of ovarian steroids

Susana R. Valdez [a,c,1], Gisela E. Pennacchio [a,1], Dante F. Gamboa [a], Elina G. de Di Nasso [a], Claudia Bregonzio [b], Marta Soaje [a,d,*]

[a] Laboratorio de Reproducción y Lactancia, IMBECU-CONICET, Mendoza, Argentina
[b] Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina
[c] Instituto de Ciencias Básicas, Universidad Nacional de Cuyo, Mendoza, Argentina
[d] Departamento de Morfofisiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina

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ABSTRACT

Background: The opioid system modulates prolactin release during late pregnancy. Its role and the participation of ovarian hormones in this modulation are explored in ether stress-induced prolactin release.

Methods/Results: Estrous, 3-day and 19-day pregnant rats were used. We administered the antagonist mifepristone (Mp) and tamoxifen to evaluate progesterone and estradiol action in naloxone (NAL, opioid antagonist) or saline treated rats. Ether stress had no effect on serum prolactin levels in controls but increased prolactin release in NAL-treated rats. Prolactin response to stress in NAL-treated rats was blocked by L-DOPA administration. Mp treatment on day 18 of pregnancy increased prolactin levels after stress without alterations by NAL. Tamoxifen on days 14 and 15 of pregnancy completely blocked Mp and NAL effects on prolactin release at late pregnancy. In contrast, stress significantly increased prolactin levels in estrous rats and pretreatment with NAL prevented this. On day 3 of pregnancy, at 6.00 p.m., stress and NAL treatment inhibited prolactin levels in saline-treated rat. No effect of stress or NAL administration was detected on day 3 of pregnancy at 9.00 a.m. *icv* administration of specific opioids antagonist, B-Funaltrexamine but not Nor-Binaltorphimine or Naltrindole, caused a significant increase in stress-induced prolactin release.

Conclusions: Opioid system suppression of prolactin stress response during late pregnancy was observed only after progesterone withdrawal, involving a different opioid mechanism from its well-established stimulatory role. This mechanism acts through a mu opioid receptor and requires estrogen participation. The opioid system and progesterone may modulate stress-induced prolactin release, probably involving a putative prolactin-releasing factor.

Introduction

The opioid system modulates prolactin secretion in non-pregnant, pregnant and lactating animals [1–6]. Dual neuromodulatory regulation of prolactin secretion by the opioid system during pregnancy was previously described [7]. After stimulatory action during the first days, a change to inhibitory control was established at the end of pregnancy, starting around day 16 [8]. Thus, both stimulatory and inhibitory actions of opioids, acting through different regulatory pathways, may result in an elevation of prolactin levels [7,9,10].

Administration of the antiprogesterone mifepristone (Mp) facilitates prolactin release by blocking the central inhibitory action of progesterone [7], and the effect of Mp can be enhanced by injecting the opioid antagonist naloxone (NAL). In fact, prolactin secretion during late pregnancy undergoes a paradoxical regulation by the opioid system in which progesterone plays an important role [7,8]. Moreover, Mp inhibits the hypothalamic dopaminergic neuronal system [11], the main inhibitory factor of prolactin secretion in terms of dopaminergic transmission and tyrosine hydroxylase (TH) expression [12,13]. This effect enables a

* Corresponding author.
E-mail address: msoaje@mendoza-conicet.gov.ar (M. Soaje).
[1] S.R. Valdez and G.E. Pennacchio contributed equally to this work.

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significant activation of lactotrophs and primes the pituitary for a subsequent stimulatory action of NAL [14].

Several stressors may affect prolactin secretion [15–17], and endogenous opioid peptides participate in the prolactin response to stress [18–20]. Among the subtypes of opioid receptors (mu, delta and kappa receptors), the activation of mu opioid subtypes plays an important role in stress conditions [21,22]. Furthermore, ether stress induces a rapid increase in plasma prolactin concentrations in female, male, and androgenized rats [23], and ovarian steroids participate in this effect [24,25]. It is known that estradiol has a stimulatory effect on basal as well as on stress-induced prolactin release [26,27] and evidence suggests that progesterone may inhibit prolactin gene expression [28] and prolactin secretion [24] in response to stress. The mechanisms by which endogenous opioids and ovarian steroids may affect stress responsiveness are, however, still unclear.

Interestingly, hyporesponsiveness of the hypothalamus–pituitary–adrenal (HPA) axis to several stressors was described in late pregnancy [29]. Both endogenous opioids and progesterone, more specifically its metabolite allopregnanolone [30], seem to play an important role in the mechanisms involved in this suppressed HPA axis response [24,31].

Several changes occur in the maternal brain to prepare the different neuroendocrine systems involved in mechanisms regulating parturition and lactation [32,33]. Among others, endogenous opioids and progesterone play a role in maternal oxytocin and prolactin system adaptation [31,32].

The primary goal of this study was to examine the participation of the opioid system in the regulation of prolactin secretion in response to ether stress during late pregnancy and to establish a correlation with changes in ovarian steroids. Additionally, the ether stress response in other reproductive situations was evaluated, such as estrus day or day 3 of pregnancy where the stimulatory effect of the opioid system has been clearly established.

Materials and methods

Animals

Virgin female rats, 3 months old (200–220 g), bred in our laboratory and originally from the Wistar strain, were used. They were kept in a light (6.00 a.m. – 8.00 p.m.) and temperature (22 ± 2 °C)-controlled room. Rat chow (Cargill, Argentina) and tap water were available *ad libitum.* Vaginal smears were analyzed daily; virgin rats of 3 months of age showing two or three consecutive 4-day cycles were used on estrus day. Other groups of rats were made pregnant by being caged individually with a fertile male on the night of proestrus. Vaginal smears were checked for the presence of spermatozoa on the following morning; if positive, that day was considered day 0 of pregnancy (normal delivery on day 22 of pregnancy). Animal maintenance and handling were conducted according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication N° 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986). All experimental procedures were approved by the Care and Use of Laboratory Animals Committee (CICUAL) of the Faculty of Medical Sciences, National University of Cuyo, Mendoza, Argentina.

Surgical procedures

In pregnant rats receiving intracerebroventricular (*icv*) injections, stainless-steel guide cannulae were surgically implanted on day 12, 7 days before the experiment. The animals were anesthetized with a combination of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (80 mg/kg) injected *ip* between 9.00 a.m. and 12.00 a.m. Rats were positioned in a stereotaxic frame and the stainless-steel guide cannula was inserted into the right lateral ventricle (M/L 1.5 mm, A/P-0.4 mm relative to bregma, 4.5 mm relative to dura [34]). Cannulae were fixed to the skull using dental acrylic and sealed until the time of drug injection. On the day of the experiment, 5 μl of specific antagonists were injected using a 10 μl Hamilton microsyringe connected to an injection needle that protruded 1 mm beyond the tip of the guide cannula placed in the lateral ventricle. Placement of cannulae was verified histologically at the end of the experiment.

Drugs

The opioid receptor antagonists μ: Beta-Funaltrexamine (B-FNA), κ: Nor-Binaltorphimine (Nor-BNI), δ: Naltrindole (NALT) and the non-specific opioid receptor antagonist: Naloxone (NAL), and the antiprogesterone mifepristone (Mp) (RU-486:17β-hydroxy-11β-[4-dimethyl-amino-phenyl]-17α-propinyl-estra-4,9-dien-3-one); were obtained from Sigma Chemical Co, St Louis, MO, USA. Tamoxifen citrate (T) was provided by Gador S.A., Buenos Aires, Argentina. L-dihydroxyphenylalanine (L-DOPA) was obtained from Roche, Buenos Aires, Argentina.

Exposure to stress

Rats were placed individually in a jar saturated with ether vapor for 2 min [24,35]. Blood samples were obtained by decapitation 3 min after ether exposure. The experiments were conducted at 9.00 a.m. except on day 3 of pregnancy when they were also conducted at 6.00 p.m. (surge prolactin time). Control rats were always included. Several studies suggest that maximum prolactin response is reached between 2 and 5 min after ether exposure [24,25,35,36]. The order of decapitation did not affect circulating hormone levels of the basal or stressed rats groups.

Blood samples were collected without anticoagulant and allowed to clot at room temperature. Serum was separated by centrifugation and stored frozen at −20 °C until subsequent radioimmunoassay.

Experimental procedures

Experiment 1

This experiment was designed to establish the effect of ether stress on prolactin secretion in NAL-treated rats at late pregnancy. We also included, in the present experiment, two other groups of rats on estrous day and day 3 of pregnancy where the stimulatory effect of the opioid system has been clearly established. Animals on day 19 of pregnancy (late pregnancy), day 3 of pregnancy or estrus day received an *ip* injection of NAL (2 mg/kg) or its vehicle (SAL) at 8.30 a.m. and were sacrificed at 9.00 a.m. Five minutes prior to decapitation, the rats were exposed to ether vapors as described above. Another group of rats was sacrificed following the same schedule at 6.00 p.m. on day 3 of pregnancy when serum prolactin levels are elevated.

It is known that in most situations, prolactin release is under an inhibitory dopaminergic tone of hypothalamic origin. Rats on day 19 of pregnancy were treated with saline or the dopamine precursor L-DOPA (25 mg/kg, *ip*) at 8.15 a.m. to prevent any transient decrease of dopaminergic tone, 15 min later with NAL or saline, and following the same stress exposure they were sacrificed at 9.00 a.m. Blood samples were obtained to determine serum prolactin and progesterone concentrations by radioimmunoassay (RIA).

Experiment 2

This experiment was conducted to study the effect of the fluctuations in progesterone and estrogen action occurring during

late pregnancy on the modulation of endogenous opioids on stress-induced prolactin secretion.

Tamoxifen citrate (T, antagonist of estrogen receptor) 500 μg/kg dissolved in 0.14 M NaCl, 0.5% (v/v) Tween 80 or vehicle was orally administered at 4.00 p.m. on days 14 and 15 of pregnancy, when estrogen levels begin to increase until reaching their maximum prior to delivery [37]. On day 18 of pregnancy, rats were treated with mifepristone (Mp, 2 mg/kg, *sc*) or vehicle (oil) at 9.00 p.m. On day 19 of pregnancy, all rats received an *ip* injection of NAL (2 mg/kg) or vehicle (SAL) at 8.30 a.m., and were sacrificed at 9.00 a.m. Five minutes prior to decapitation, they were exposed to ether vapors as above described. Blood samples were obtained for serum prolactin determination by RIA.

Experiment 3

This experiment was conducted to identify opioid receptor subtypes involved in prolactin release regulation in rats exposed to ether stress in late pregnancy. The irreversible mu opioid receptor antagonist B-FNA (5 μg/rat), kappa antagonist Nor-BNI (15 μg/rat), and delta antagonist NALT (5 μg/rat) were dissolved in distilled water immediately before administration. Drugs or vehicle were administered intracerebroventricularly (*icv*) 30 min before sacrifice. Five minutes prior to decapitation, rats were exposed to ether vapors as described. Blood samples were obtained for serum prolactin and progesterone determination by RIA.

Prolactin and progesterone determinations

Serum prolactin concentration was measured by double-antibody radioimmunoassay [8] with materials supplied by Dr. A. F. Parlow (National Hormone and Pituitary Program, USA). Prolactin was radioiodinated using the chloramine T method [38] and purified by passage through Sephadex G-75 and polyacrylamide agarose (ACA 54; LKB, Bromma, Sweden) columns. Assay sensitivity was 1 ng/ml serum, and inter- and intra-assay coefficients of variation were <10%. The prolactin antibody does not cross-react with placental lactogen [39].

Serum progesterone was measured using a commercial kit (DSL-3400 double antibody RIA, Diagnostic Systems Laboratories, Webster, TX, USA). Assay sensitivity was <70 fmol/tube, and inter- and intra-assay coefficients of variation were <10%.

Statistics

The results from experiments 1 and 2 and the inset of Fig. 4 were analyzed by two-way analysis of variance (ANOVA 2) with Bonferroni *post hoc* test. The results from experiments 3 and Table 1 were analyzed using one-way analysis of variance (ANOVA 1) with Dunnett's *post hoc* test.

Results

Experiment 1: serum prolactin and progesterone concentrations in stressed rats: effect of NAL

Ether stress given to saline-treated rats on day 19 of pregnancy had no effect on serum prolactin levels. Also, NAL administration to non-stressed-rats did not modify serum prolactin levels as was previously described [7,8]. However, NAL treatment to rats submitted to stress significantly increased serum prolactin secretion. Two-way ANOVA results of prolactin data shown in Fig. 1a are as follows: Drug factor (SAL, NAL) $F_{(1,32)} = 34.4$ $p < 0.0001$; Treatment factor (Basal, Stress) $F_{(1,32)} = 35.8$ $p < 0.0001$; Interaction (Drug × Treatment) $F_{(1,32)} = 29.5$ $p < 0.0001$. Bonferroni post-test comparisons $t = 8.1$ $p < 0.001$. There were no differences in serum progesterone concentrations in

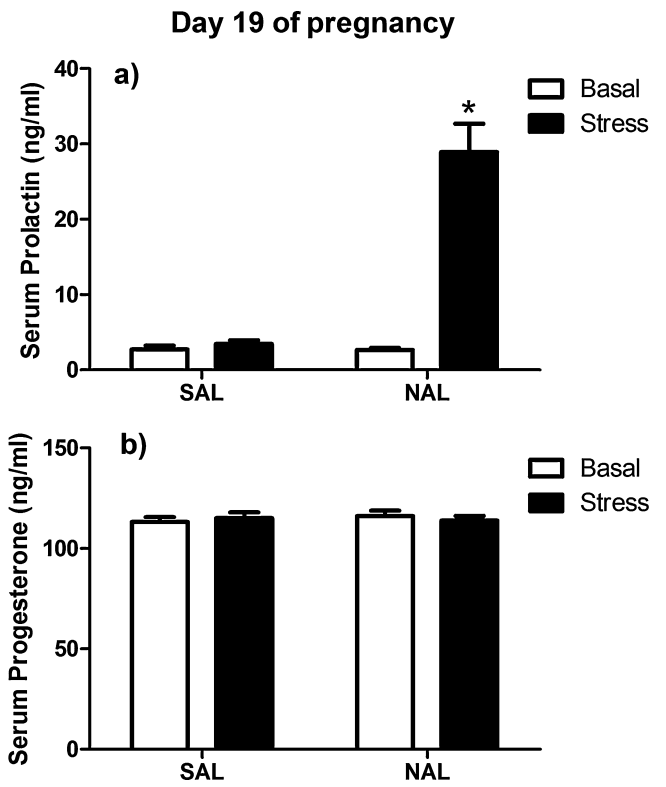

Fig. 1. Effect of ether stress on (a) serum prolactin or (b) serum progesterone concentrations in rats on day 19 of pregnancy pretreated with naloxone (NAL, 2 mg/kg, *ip*) or saline (SAL). Results are means ± SEM of groups of 8–9 animals in each experimental group. $^{*}p < 0.01$ compared to Basal (non-stressed) rats. Two-way (ANOVA 2) followed by Bonferroni *post hoc* test.

ether stressed-rats after saline or NAL administration (Fig. 1b). As dopamine precursor L-DOPA administration prevents any transient decrease in dopamine tone, serum prolactin secretion induced by NAL treatment in rats (day 19 of pregnancy) submitted to ether exposure was blocked by L-DOPA pre-treatment ($p < 0.01$, Table 1). Control animals were used (basal or unstressed rats). Prolactin levels remained unchanged in unstressed animals pretreated with L-DOPA (data not shown). Serum progesterone concentration was not modified by L-DOPA administration in basal (data not shown) or stressed rats treated with saline or NAL (Table 1).

Estrous rats and rats on day 3 of pregnancy were included as controls to show stress-induced prolactin release in different reproductive states with fluctuations in ovarian steroids different to hormonal levels observed during late pregnancy (day 19). Stressed estrous rats showed a significant increase in serum prolactin levels measured at 9.00 a.m., and NAL administration prevented this effect (Fig. 2a). Two-way ANOVA results of data shown in Fig. 2a were as follows: Treatment factor (Basal, Stress)

Table 1
Effect of L-DOPA (L-dihydroxyphenylalanine) administration on serum prolactin and progesterone concentration on day 19 of pregnancy in stressed saline (SAL) or stressed naloxone (NAL) treated rats. Results are means ± SEM of groups of 8–9 animals in each experimental group.

	Serum prolactin (ng/ml)		Serum progesterone (ng/ml)	
	SAL	L-DOPA	SAL	L-DOPA
SAL	3.60 ± 0.71 (9)	4.16 ± 1.16 (8)	114.41 ± 2.41 (8)	119.11 ± 3.57 (8)
NAL	25.46 ± 3.69* (9)	4.02 ± 1.14# (9)	109.09 ± 3.73 (9)	117.80 ± 2.73 (9)

* $p < 0.01$ *vs.* SAL plus SAL-stressed rats.
\# $p < 0.01$ *vs.* SAL plus NAL-stressed rats.

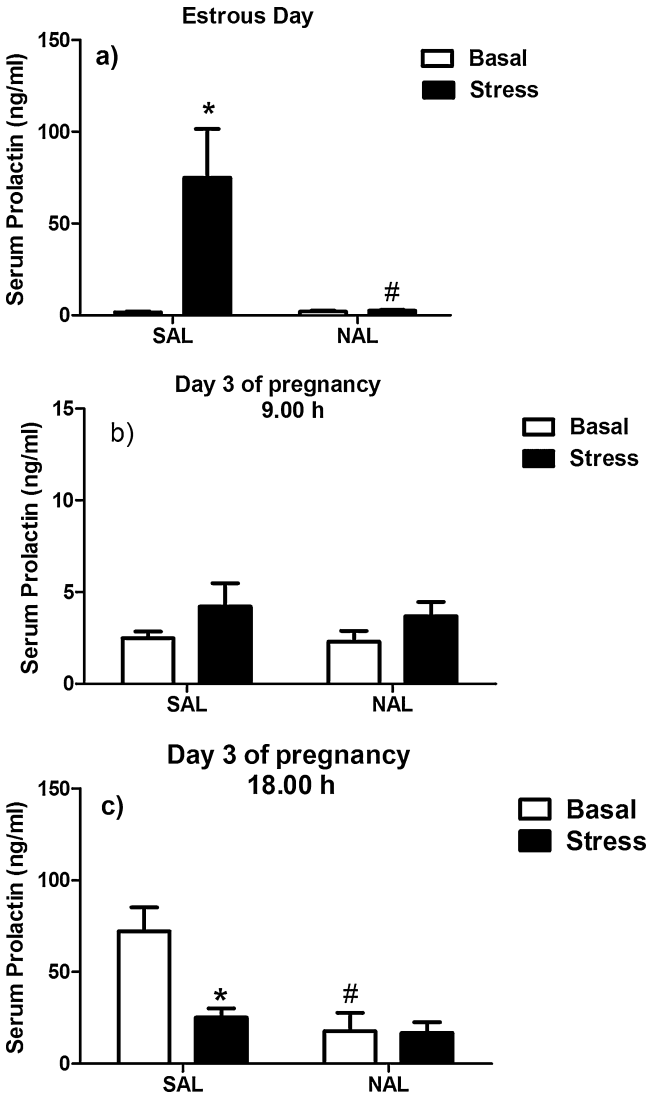

Fig. 2. (a) Effect of ether stress on serum prolactin levels in rats on (a) estrous day (9.00 a.m.) and day 3 of pregnancy at (b) 9.00 a.m. or (c) 6.00 p.m. – pretreated with naloxone (NAL, 2 mg/kg, *ip*) or saline (SAL). Results are means ± SEM of groups of 8–9 animals in each experimental group. $^{*}p < 0.01$ compared to Basal (non-stressed) rats. $^{\#}p < 0.01$ compared to SAL stressed rats.

$F_{(1,34)} = 12.92$ $p < 0.001$, Drug factor $F_{(1,34)} = 13.89$ $p < 0.0007$ and interaction (Drug × Treatment) $F_{(1,34)} = 13.82$ $p < 0.0007$. Bonferroni *post hoc* test comparisons indicated that the groups SAL or NAL (Basal) were different ($p < 0.001$) from SAL (Stress).

On day 3 of pregnancy, the effect of ether stress and NAL treatment was evaluated in the morning (9.00 a.m.) when serum prolactin levels are low (intersurge time) and in the afternoon (6.00 p.m.) at the time when serum prolactin levels are elevated (surge time). In animals exposed to stress on day 3 of pregnancy at 9.00 a.m., no differences were found in serum prolactin levels and NAL administration had no effect on control (Basal) or stressed rats (Fig. 2b). Two-way ANOVA analysis was as follows: Drug factor (SAL, NAL) $F_{(1,20)} = 0.18$ $p = 0.67$, Treatment factor (Basal, Stress) $F_{(1,20)} = 3.54$ $p = 0.07$; Interaction (Drug × Treatment) $F_{(1,20)} = 0.83$ $p = 0.83$ (Fig. 2b). Stress exposure on day 3 of pregnancy at 6.00 p.m. induced a decrease in serum prolactin levels. NAL pretreatment decreased basal serum prolactin levels and did not modify the effect induced by stress on this hormone. Two-way ANOVA analysis was as follows: Drug factor (SAL, NAL) $F_{(1,28)} = 12.02$ $p < 0.01$, Treatment factor (Basal, Stress) $F_{(1,28)} = 6.99$ $p < 0.05$; Interaction (Drug × Treatment) $F_{(1,28)} = 6.45$ $p < 0.05$. Bonferroni post-test comparisons $t = 3.67$ $p < 0.01$ (Fig. 2c).

Experiment 2: effect of progesterone, estrogen and NAL on prolactin secretion in stressed rats

We previously showed that 5 mg/kg [11,14] or 10 mg/kg [7,8] Mp treatment 10 or 12 h before NAL administration induced prolactin secretion, while 2 mg/kg treatment did not. The present study used a 2 mg/kg Mp dose so no changes in prolactin secretion after SAL or NAL administration were expected. Thus, allowing us to perform the study of a prolactin release stimulus such as suckling [37] or ether stress (present work). Mp administration induced a significant increase in serum prolactin levels on saline-treated rats exposed to ether. Tamoxifen (T) administered on days 14 and 15 of pregnancy completely prevented this effect (Fig. 3a). Two-way ANOVA showed a significant effect of Treatment factor $F_{(1,42)} = 42.63$ $p < 0.0001$, Drug factor $F_{(2,42)} = 32.66$ $p < 0.0001$, and interaction between both factors $F_{(2,42)} = 31.27$ $p < 0.0001$. Bonferroni *post hoc* test comparisons indicated significant difference ($p < 0.001$) between Mp-SAL (Basal) and Mp-SAL (Stress). NAL administration to Mp-treated rats significantly increased stress-induced serum prolactin release to similar levels as in the Mp-SAL stressed group (Fig. 3a). T administration on days 14 and 15 of pregnancy prevented stress-induced prolactin secretion in NAL- or Mp-NAL-treated rats on pregnancy day 19 (Fig. 3b). Two-way ANOVA indicated a significant effect of Treatment factor $F_{(1,42)} = 29.34$ $p < 0.0001$, Drug factor $F_{(2,42)} = 39.80$ $p < 0.0001$, and interaction between both factors $F_{(2,42)} = 22.79$ $p < 0.0001$.

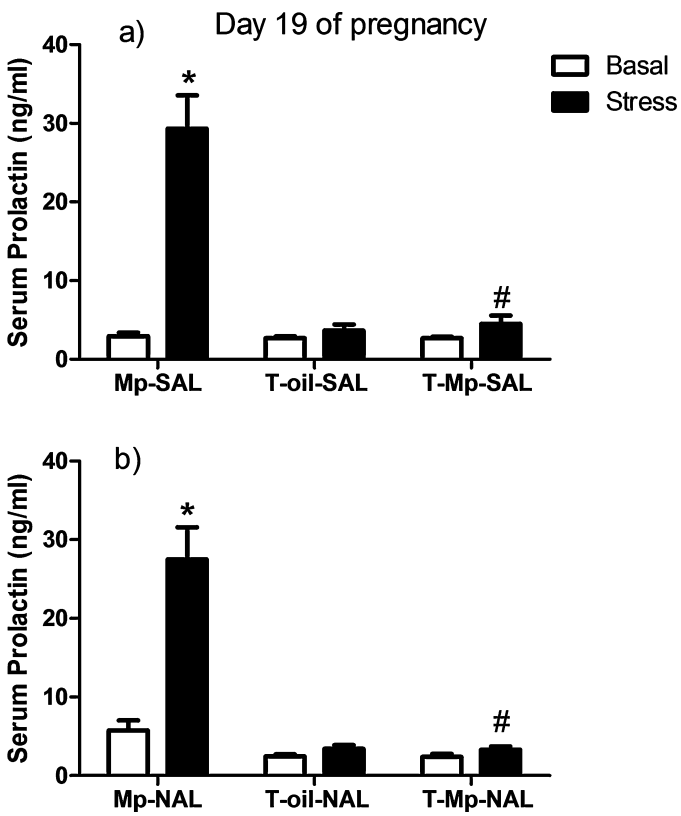

Fig. 3. Effect of tamoxifen (T) (500 μg/kg *per os*) pre-treatment on serum prolactin concentrations on day 19 of pregnancy (9.00 a.m.) in stressed rats, treated with (a) mifepristone (Mp, 2 mg/kg, *sc*) or (b) mifepristone (Mp)-naloxone (NAL, 2 mg/kg *ip*). Results are means ± SEM of groups of 8–9 animals in each experimental group. $^{*}p < 0.01$ compared to Basal (non-stressed) rats. $^{\#}p < 0.01$ compared with Mp-SAL or Mp-NAL stressed rats.

Bonferroni *post hoc* test comparisons indicated that Mp-NAL (Basal) and Mp-NAL (Stress) groups, were significantly different ($p < 0.001$).

Experiment 3: effect of icv administration of different opioid receptor antagonists on prolactin and progesterone secretion in stressed rats

Intracerebroventricular administration of the μ antagonist B-FNA, significantly increased serum prolactin concentration in stressed rats measured at 9.00 a.m. ($p < 0.01$). No differential effect in PRL release by stress was observed after administration of the κ and δ opioid receptor antagonists Nor-BNI and NALT, respectively, compared with saline (Fig. 4a). In basal conditions, *icv* administration of B-FNA, Nor-BNI or NALT did not modify serum prolactin concentration (data not shown). As the inset of Fig. 4a curiously shows, a significant increase in serum prolactin levels was observed in cannulated control rats exposed to ether vapors. Administration of opioid receptor antagonists did not modify serum progesterone levels before or after stress exposure (Fig. 4b). Although the statistical analysis was made with the data expressed as ng/ml, we preferred to show the results of Fig. 4a and b as % of stressed saline-treated rats with the aim to show them in a clearer way.

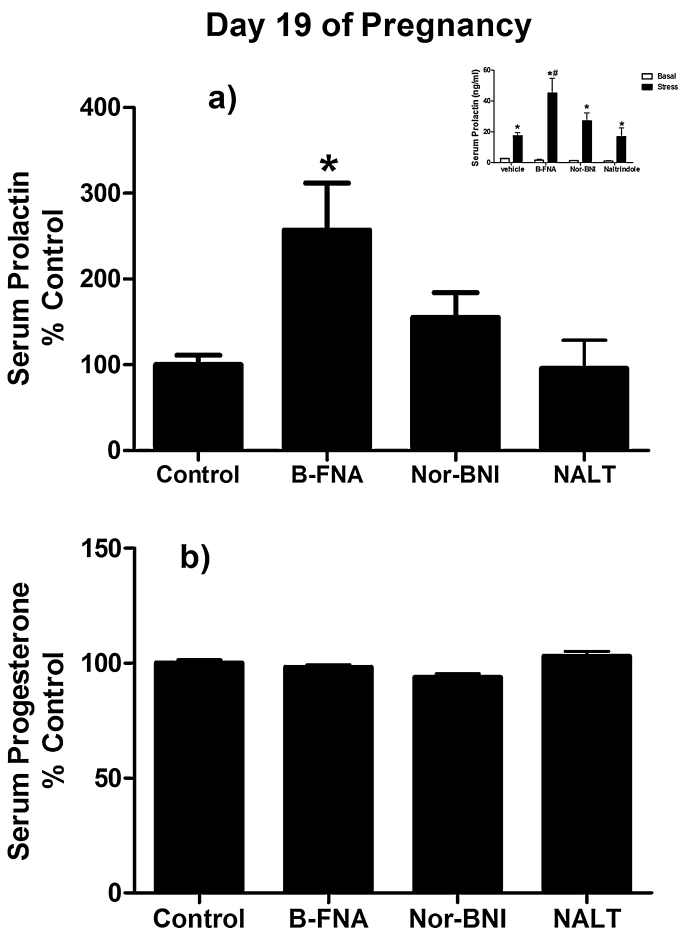

Fig. 4. Effect of the administration of different opioid receptor antagonists on prolactin and progesterone secretion in stressed rats on day 19 of pregnancy: (a) serum prolactin, (b) serum progesterone levels (as % of stressed saline-treated rats). Animals were injected *icv* with either vehicle or B-FNA or Nor-BNI or NALT 30 min before decapitation. Results are means ± SEM of groups of 9–11 animals in each experimental group. $^{*}p < 0.01$ compared to stressed-SAL treated rats. Inset: showing the data as ng/ml; $^{*}p < 0.01$ compared to Basal (non-stressed) rats. $^{\#}p < 0.01$ compared to stressed vehicle treated rats.

Discussion

Progesterone seems to be a key regulator of prolactin secretion during late pregnancy [35,40] by maintaining hypothalamic dopaminergic neuron activity [11]. Moreover, the fall of progesterone action facilitated prolactin secretion and NAL administration potentiated this effect demonstrating that progesterone and endogenous opioids interact to modulate prolactin secretion during late pregnancy [7,8].

Data in this study show that prolactin response to ether stress in non-pregnant rats was abolished during late pregnancy, probably as a consequence of the inhibitory effect of progesterone at this time, without discarding the activation of dopaminergic neurons by placental lactogen [41]. Interestingly, this inhibitory effect was partially reversed when progesterone action was blocked by Mp administration. Reports have shown that progesterone may prevent stress-induced prolactin secretion in male, female [24,42], androgenized [23] and pregnant rats [27]. In addition, opioid tone blockade by NAL significantly increased prolactin secretion in response to ether stress despite elevated serum progesterone levels. Importantly, the response to either Mp or NAL treatment fails to completely reverse the effect in late pregnancy, and prolactin stress-response after these treatments is still much less than the response obtained in non-pregnant animals. Previous studies show that prolactin secretion increases after NAL administration when dopaminergic tone is removed by Mp [11], probably through mechanisms involving the release of a PRF [14].

Moreover, suckling stimulus applied at the end of pregnancy in rats with induced maternal behavior is also subject to inhibitory modulation. [37]. Probably, a decrease of dopaminergic tone induced by suckling facilitates NAL action on prolactin secretion [37]. In fact, the suckling-induced increase in prolactin occurs through a rapid transient decrease in tuberoinfundibular dopaminergic (TIDA) activity [1,43] sensitizing the lactotropes to PRFs [44]. Both Mp administration and suckling stimulus may therefore modify dopaminergic tone and facilitate NAL action. Stressful stimuli induce a decrease in dopaminergic tone, which in turn leads to the release of prolactin by NAL. TIDA neurons are inhibited by afferent neuronal circuits activated by suckling and restraint stress [43,45], supporting the suggestion that TIDA activity suppression is critical for the prolactin stress response [46,47]. The fact that L-DOPA administration blocked NAL-plus stress effect on prolactin release may indicate that an increased and sustained dopaminergic inhibition may prevent any transient decrease of the dopaminergic tone, surpassing any mechanism that may lead to prolactin secretion.

Several hormones and neurotransmitters are involved in the complex response to stress [48,49]. There is firm evidence for adrenergic neuron involvement in afferent regulation of TIDA neurons, suggesting a putative pathway for central adrenergic effects upon prolactin secretion [50]. Similarly, our laboratory has shown that the adrenergic system participates, through α_1 and β_1 receptors, in prolactin release induced by decreased progesterone levels during late pregnancy [51]. A noradrenergic mechanism is activated in response to ether stress, inducing a slight inhibition of dopaminergic tone despite high progesterone levels and facilitating NAL action, probably through a mechanism involving participation of a PRF. However, a direct effect of other neurotransmitters, such as 5-HT [26] or histamine [52], cannot be discarded. Preliminary studies show that oxytocin may participate in the stimulatory action of Mp and NAL on prolactin secretion during late pregnancy [53] suggesting that this hormone may also be involved in prolactin response to stress.

It is interesting to note in this study that prolactin response to ether stress during late pregnancy is quite different from the response observed in early pregnancy or during the estrus day. The

significant increase in serum prolactin response to stress observed in the estrus morning may result from the facilitating effect of estrogen on the response to stress [25]. Estrogen is known to act at pituitary and central nervous system level, stimulating prolactin secretion, and some of these effects are mediated through a modulation of dopaminergic neuronal activity [54]. Moreover, NAL administration prevented increased serum prolactin in stressed rats at estrous day probably mediated by increasing neuronal dopaminergic activity. Interestingly, a differential effect of ether stress was observed on serum prolactin levels during day 3 of pregnancy. Neither stress stimulus nor NAL treatment modified serum prolactin levels in the morning, but either of these prevented serum prolactin in the afternoon [7]. A semicircadian rhythm of hypothalamic dopaminergic neuronal activity has been described which, acting together with PRF action, is responsible for serum prolactin surge in pseudopregnancy [55] or during early pregnancy [56]. So the different prolactin responses to stress and NAL treatment in the morning and in the afternoon of day 3 of pregnancy may result from changes in hypothalamic dopaminergic tone characteristic of the first days of pregnancy. In fact, NAL may inhibit prolactin secretion during the first days of pregnancy [7,10] and this contrasts with the stimulatory effect of a single dose of NAL after Mp treatment, suckling stimulus [37] or stress response (present study) in late pregnancy.

Although there is evidence that ovarian steroids modulate stress response [25], the influence of serum progesterone and estradiol levels on prolactin response to stress remains unclear. In this study, ovarian steroid blockade by Mp or tamoxifen modified prolactin secretion, suggesting an important role of both progesterone and estradiol in prolactin response to stress. Mp administration induced an increase in serum prolactin levels only in stressed rats, suggesting that blocking progesterone alone is not sufficient to induce prolactin secretion, but may facilitate the effect of a stressful stimulus. Similar observations have been reported for suckling stimulus [37] and other prolactin releasers [8]. As Mp can also specifically bind to glucocorticoid receptors [57], a non-selective effect mediated by the lack of glucorticoid action cannot be discarded. Evidence suggests, however, that dexamethasone implantation near the arcuate nuclei does not affect PRL secretion induced by the dopaminergic antagonist, haloperidol [58]. Interestingly, Mp's effect on prolactin release was not modified by NAL, suggesting that the hypophysis can release a moderate amount of prolactin (around 30 ng/ml) after a mild stress, such as exposure to ether. Possibly, the hypophysis is less responsive to stimulation during late pregnancy than in other reproductive states, such as lactation [37] or during estrus. In fact, prolactin secretion in response to ether stress is higher in estrous rats than during late pregnancy ([27] and present study). It is therefore possible that the capacity of the rat's pituitary to release prolactin in response to the same stimulus varies according to the reproductive state.

A noteworthy result of this study is the lack of responsiveness to ether-stress when estrogen action is blocked. Tamoxifen pre-treatment abolished prolactin release induced by ether-stress in rats injected with NAL, Mp, or Mp plus NAL. Based on our previous [7,37] and present results, it can thus be assumed that estrogen action on days 15 and 16 of pregnancy is crucial for opioid system activation, although the involvement of other neurotransmitter systems cannot be excluded. This is supported by the observation that blocking estrogen action at this specific moment of pregnancy induced an increase in TH expression in the medial basal hypothalamus on day 19 [59], preventing any later increase of prolactin. Further experiments are needed to elucidate how estrogen modulates prolactin secretion at this stage of pregnancy.

Opioid receptor subtypes μ and κ are involved in the stimulation of prolactin release [8,19,60,61]. Activation of the μ opioid receptor plays an important role in stress response in adult male rats [21,62]. *Icv* administration of the specific μ, κ and δ opioid antagonists indicated that the μ opioid receptor subtype is involved in the induction of prolactin response to stress in pregnant rats. Interestingly, implanted control rats showed a significant increase in serum prolactin in response to stress compared to non-operated rats, suggesting that the former may be more sensitive to prolactin stress response than intact rats. Possibly, endogenous opioid release during surgery induced a down regulation of μ opioid receptors at hypothalamic level [63,64] and, consequently, a decrease in the inhibitory opioid tone that modulates stress response during late pregnancy. Despite high progesterone levels attenuating HPA axis reactivity to stress [23], the reduced inhibitory opioid tone may thus be facilitating stress-induced prolactin release. However, it is also possible that the protective effect of progesterone may be overridden when animals are subjected to more intense stimuli, such as immobilization or multiple mild stressors.

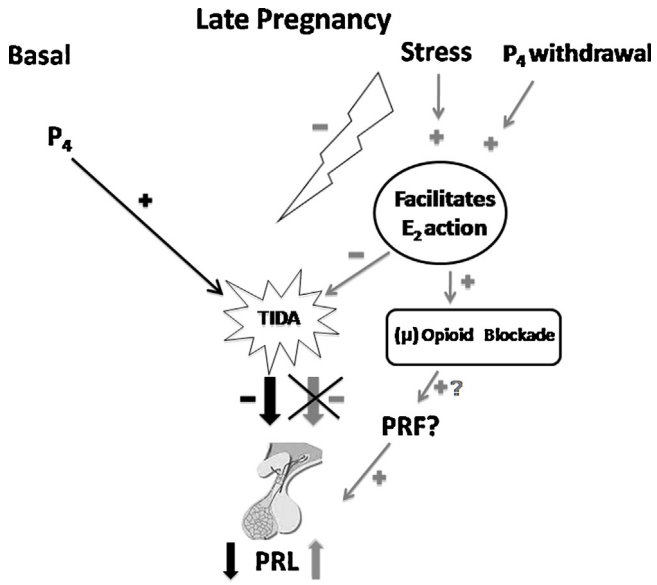

Fig. 5. Scheme depicting a hypothetical mechanism of opioid modulation of PRL secretion on day 19 of pregnancy. In basal conditions P_4 is involved in the stimulation of the TIDA neurons that tonically inhibit PRL release. In addition, the opioid system has an inhibitory action on PRL release (previous and present findings). Under stress or P_4 withdrawal conditions, estrogen action is facilitated and, probably by decreasing TIDA neuron activity, an opioid inhibitory action emerges which is evident when the μ-opioid receptor is blocked. TIDA: tuberoinfundibular dopaminergic neurons; Mp: mifepristone, antiprogesterone; NAL: naloxone, opioid antagonist; PRF: prolactin-releasing factor; E_2: estradiol. P_4: progesterone. PRL: prolactin.

In conclusion, the present findings extend earlier results by confirming the inhibitory action of progesterone and the opioid system on prolactin release during late pregnancy (Fig. 5). This supports the hypothesis that during late pregnancy, progesterone inhibits prolactin release in response to stress through a mechanism involving the opioid system, specifically through activation of a μ opioid receptor. The opioid system seems to play a role in the regulation of stress-induced prolactin release only in presence of estradiol. Its action may be mediated by indirect inhibition of dopaminergic tone and, perhaps, concomitant participation of a putative PRF.

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Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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