Prolactin promotes oxytocin and vasopressin release by activating neuronal nitric oxide synthase in the supraoptic and paraventricular nuclei

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Prolactin (PRL) stimulates the secretion of oxytocin (OXT) and arginine AVP as part of the maternal adaptations facilitating parturition and lactation. Both neurohormones are under the regulation of nitric oxide. Here, we investigate whether the activation of neuronal nitric oxide synthase (nNOS) in the hypothalamo-neurohypophyseal system mediates the effect of PRL on OXT and AVP release and whether these effects operate in males. Plasma levels of OXT and AVP were measured in male rats after the intracerebroventricular injection of PRL or after inducing hyperprolactinemia by placing two thalamic extracts by the phosphorylation/inactivation of nNOS at Ser847. Elevated central and systemic PRL correlated with increased thalamic NOS activity in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei by NADPH-diaphorase histochemistry and in hypothalamic extracts by the phosphorylation/inactivation of nNOS at Ser847. Elevated central and systemic PRL correlated with increased NOS activity in the PVN and SON and with higher OXT and AVP circulating levels. Notably, treatment with 7-nitroindazole, a selective inhibitor of nNOS, prevented PRL-induced stimulation of the release of both neurohormones. Also, phosphorylation of nNOS was reduced in hyperprolactinemic rats, and treatment with bromocriptine, an inhibitor of anterior pituitary PRL secretion, suppressed this effect. These findings suggest that PRL enhances nNOS activity in the PVN and SON, thereby contributing to the regulation of OXT and AVP release. This mechanism likely contributes to the regulation of processes beyond those of female reproduction.

hypothalamo-neurohypophyseal system; central prolactin; hyperprolactinemia; nitric oxide

THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM (HNS) secretes the hormones oxytocin (OXT) and AVP that regulate body fluid homeostasis, uterine contractions, reproductive behavior, milk ejection during lactation, and stress-related responses (12, 16). Nitric oxide (NO) is involved in the regulation of OXT and AVP release by the HNS. Neuronal NO synthase (nNOS) expression and NADPH-diaphorase activity, a marker of NOS (25), increase in the paraventricular (PVN) and supraoptic (SON) hypothalamic nuclei when the release of OXT and AVP is enhanced by osmotic stimulation (59, 72), parturition (51, 60), suckling (47), nociceptive stimulation (32), and stress (23, 44). Manipulation of NO production using NO donors, inhibitors, or nNOS deletion in mice, suggests divergent mechanisms of NO action on neurohypophysial hormone secretion. For example, under stress conditions NO can stimulate circulating levels of both OXT and AVP (44), whereas in response to osmotic stimulation, NO inhibits OXT and facilitates AVP release (26, 69). Notably, NO inhibition of OXT release ceases in late pregnancy in preparation of the high OXT levels required for parturition and the milk ejection reflex (26, 60).

The hormone prolactin (PRL) has a wide variety of actions within and beyond reproduction (19). Some of these actions occur in the brain, where PRL serves both as a circulating hormone and as a locally produced neuropeptide (22). For example, the HNS produces and secretes PRL (9, 34, 65), PRL receptors are expressed in OXT and AVP neurons of the PVN and SON (28, 35), and PRL stimulates OXT and AVP release (15, 35, 48). In fact, PRL may be part of the neuroendocrine mechanism controlling NO-mediated secretion of neurohypophysial hormones in females during the reproductive cycle. PRL stimulates the release of OXT in lactating rats (48) and of OXT and AVP in estrogen-treated, ovariectomized animals (15). Furthermore, in steroid-primed rats, reducing PRL circulating levels with the dopamine D2 receptor agonist, bromocriptine, lowers NOS activity and OXT expression in the PVN and SON, and these effects are reversed by PRL (50). Although no such studies have been carried out in males, it is likely that similar neuroendocrine interactions contribute to neurohypophysial hormone secretion. PRL, OXT, and AVP are released in response to stress, osmotic, and behavioral stimuli that upregulate nNOS in the HNS of male rats (12, 16, 19, 45), and all three hormones regulate stress-induced anxiety and neuroendocrine responses, osmotic changes, and male sexual behavior (12, 14, 16, 19, 22, 30, 42, 63).

Here, we investigate whether high levels of brain and circulating PRL activate nNOS in the PVN and SON of male rats, which results in increased concentrations of plasma OXT and AVP.

MATERIALS AND METHODS

Animals. Male Wistar rats (200–250 g body wt) were maintained under standard laboratory conditions (12:12-h light-dark cycle, lights on at 7 AM, 22°C, free access to food and water). All manipulations were conducted in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, (Bethesda, MD), and the Bioethics Committee of the Institute of Neurobiology from the National University of Mexico approved all animal experiments. To avoid stress-induced alterations, animals were handled daily for 7 days before the experiment. All experiments were performed between 9:00 and 12:00 AM. Unless otherwise stated, rats
were rapidly anesthetized in a CO₂-saturated chamber then euthanized by decapitation.

**PRL intracerebroventricular administration.** A stainless-steel guide cannula (21 gauge) was inserted stereotaxically into the right lateral ventricle (0.4 mm behind bregma, 1.4 mm lateral, 3.4 mm below the skull) (49) under 70% ketamine and 30% xylazine anesthesia (1 μl/g body wt) injected intraperitoneally. Animals were housed individually after cannula implantation, and 7 days later, an infusion cannula (25 gauge) was inserted into the guide cannula of conscious, freely moving rats to deliver recombinant rat PRL [National Hormone and Peptide Program (NHPP) and Dr. A. F. Parlow, Torrance, CA]. In the first experiment, 4 μl of vehicle (Ringer solution, pH 7.4) containing 0.0 μg (control), 0.06 μg, 0.125 μg, 0.25 μg, 0.5 μg, or 1 μg of PRL was infused intracerebroventricularly over a 2-min interval. The infusion was left in place for two additional minutes to prevent reflux, and the animals were euthanized 10 min later. In a second experiment, 50 mg/kg body wt of 7-nitroindazole (7-NI; Sigma-Aldrich, St. Louis, MO) in 0.2 ml of 5% DMSO-peanut oil (Sigma) or 5% DMSO-peanut oil alone was injected intraperitoneally 20 min before the intracerebroventricular infusion of PRL (1 μg) or vehicle. In both experiments, trunk blood was collected to evaluate OXT and AVP levels in plasma. In both the central and peripheral nervous system, 7-NI is a selective inhibitor of nNOS (38). The doses and time of administration used in the present study were those previously shown to have a good antioxidant effect in vivo without affecting blood pressure via endothelial NOS inhibition (39). In a third experiment, PRL (1 μg) or vehicle was infused intracerebroventricularly and after 10 min, the animals were anesthetized with inhaled CO₂ and perfused transcardially with 200 ml of 0.9% NaCl followed by 200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and processed for NADPH-diaphorase histochemistry.

**Induced hyperprolactinemia.** Hyperprolactinemia was induced by implanting two female anterior pituitary (AP) glands under the kidney capsule of a male rat as previously described (1). Littermates were used as donors of the AP transplanted to the host rats and the surgery was performed on adult animals (200–250 g body wt). Sham-operated rats were subjected to similar surgery without implantation. All experiments were performed on adult animals (200–250 g body wt). Sham-operated rats were boiled with Laemmli buffer and analyzed by SDS-PAGE and Western blots using 8% gels and a 1:1,000 dilution of a polyclonal anti-p-nNOS Ser847 antibody (ab16650; Abcam, Cambridge, MA). The antigen-antibody complex was detected using an alkaline phosphatase second antibody kit (Bio-Rad Laboratories, Hercules, CA). All Western blots were exposed to polyclonal anti-β-actin antibodies (dilution 1:500; SC-47778; Santa Cruz Biotechnology, Santa Cruz, CA) to normalize for loading variability. Optical density values were determined using the Quantity One, 1-D analysis software (Bio-Rad).

**Statistical analysis.** Data are presented as mean ± SE. Statistical significance between groups was determined by ANOVA followed by the unpaired Student’s t-test or the Tukey test using the SigmaStat 7.0 software (Systat Software, San Jose, CA). The significance level was set at 5%.

**RESULTS**

**Intracerebroventricular PRL increases OXT and AVP circulating levels by activating nNOS in the PVN and SON.** Acute intracerebroventricular infusion of PRL augmented plasma levels of both OXT (Fig. 1A) and AVP (Fig. 1B) in a dose-dependent manner. One microgram of PRL was used in all subsequent experiments. The action of intracerebroventricular PRL on NOS activity was evaluated by NADPH-diaphorase histochemistry. The intracerebroventricular administration of PRL increased NADPH-diaphorase staining by twofold in the PVN (Fig. 2A) and SON (Fig. 2B), compared with the respective vehicle controls. Because nNOS constitutes the predominant source of NO in neurons (77), we used 7-NI, a selective inhibitor of nNOS (38, 39), to investigate whether the stimulation of OXT and AVP release by PRL is dependent on PRL-induced nNOS activity. As shown in Fig. 3A, B, pretreatment with 7-NI prevented the increase of OXT and AVP plasma levels in response to intracerebroventricular PRL, whereas 7-NI alone had no significant effect. Taken together, these data suggest that the central PRL, by activating nNOS in the PVN and SON, stimulates the release of OXT and AVP into the circulation.

**High levels of systemic PRL increase OXT and AVP in the circulation by activating nNOS in the PVN and SON.** To investigate whether circulating OXT and AVP levels can also be regulated by systemic PRL, male rats were rendered hyperprolactinemic by placing two AP grafts under the kidney capsule. In the grafted animals, there was a five-fold increase in serum PRL (Fig. 4A) that was prevented by treatment with bromocriptine, but not with 7-NI. Hyperprolactinemia was associated with significant increases in the circulating levels of OXT (Fig. 4B) and AVP (Fig. 4C), which were inhibited by the treatment with bromocriptine. Notably, the administration of 7-NI blocked the hyperprolactinemia-induced release of both neurohormones. These findings indicate that the increase in the

**Quantification of circulating hormones.** Blood samples were drawn in chilled tubes containing EDTA (1 mg/ml of blood) or no EDTA to obtain plasma or serum, respectively. OXT and AVP in plasma were extracted and measured using specific ELISA kits (Assay Designs, Ann Arbor, MI) following the instructions of the manufacturer. Serum PRL was assayed by RIA using standard procedures and reagents provided by NHPP and Dr. Parlow, with rat PRL RP-3 as the reference preparation. The intra- and inter-assay coefficients of variance were <7% and <12%, respectively. To avoid interassay variations, all samples were measured in the same assay.

**NADPH-diaphorase histochemistry.** Brains were postfixed for 24 h in 4% paraformaldehyde and 30% sucrose at 4°C. Thirty-micrometer-thick, serial coronal sections were cut throughout the PVN and SON on a cryostat and stored at 4°C in cryoprotectant solution [glycerol, ethylene glycol, and 0.1 M phosphate buffer, pH 7.4, 1:1:2]. Free-floating sections were placed in Tris-buffered saline (TBS, pH 7.4) and incubated for 60 min at 37°C in NADPH diaphorase reaction mixture (1 mg/ml β-NADPH, 0.3 mg/ml nitro blue tetrazolium, 0.3% Triton X-100 in TBS). Incubation in the above reaction medium without β-NADPH confirmed the specificity of the reaction. Following incubation, sections were washed three times in ice-cold TBS, mounted on gelatin-coated slides, and cover-slipped with Permount. The slides were scanned and analyzed using the ScanScope Digital Scanner and the Color Deconvolution software (Aperio Technologies, Vista, CA). NADPH-diaphorase-positive areas were evaluated from images captured throughout the rostro-caudal extent of the PVN and SON on three sets of slides, each carrying six hypothalamic sections from a different animal.
The acute intracerebroventricular infusion of prolactin (PRL) increases circulating oxytocin (OXT) and AVP levels. OXT (A) and AVP (B) levels were evaluated by ELISA 10 min after the intracerebroventricular infusion of vehicle only (Veh) or PRL. Values are expressed as means ± SE of seven animals per group. *P ≤ 0.05 vs. Veh-injected animals. **P ≤ 0.05 vs. 0.06 μg PRL-injected animals.

Fig. 2. The acute intracerebroventricular infusion of PRL promotes nitric oxide synthase (NOS) activity in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. Representative micrographs (left) of NADPH-diaphorase-stained PVN (A) and SON (B) sections from rats infused intracerebroventricularly with 1 μg PRL or vehicle (Veh). Scale bar = 100 μm. Quantification of the positive NADPH-diaphorase (NADPH-d) signal (right) evaluated throughout the rostro-caudal extent of the PVN and SON in four different animals. Results are expressed as means ± SE. *P ≤ 0.05 vs. Veh-injected rats.

Fig. 3. Neuronal NOS (nNOS) activity mediates the intracerebroventricular PRL-induced increase of circulating OXT and AVP levels. ELISA was used to assess the circulating concentrations of OXT (A) and AVP (B) 10 min after the intracerebroventricular administration of PRL or vehicle (Veh) in the absence (control) or in the presence of the intraperitoneal injected inhibitor of nNOS, 7-nitroindazole (7-NI). Values are expressed as means ± SE of four animals per group. *P ≤ 0.05 vs. Veh-injected, control rats.

DISCUSSION

The present study demonstrates for the first time that elevating PRL in the brain or in the circulation promotes the activation of nNOS in the HNS leading to the systemic release of OXT and AVP. Moreover, this is the first evidence that PRL affects the secretion of the two neurohormones in males, suggesting that PRL, OXT, and AVP are functionally linked to regulate processes beyond those of female reproduction.

The initial evidence showing that PRL stimulates the secretion of neurohypophysial hormones was obtained 20 years ago in lactating rats, where the intravenous injection of PRL
evoked the increase of OXT levels in the circulation, and the immunodepletion of PRL reduced the suckling-induced OXT release (48). It was proposed that PRL facilitates OXT secretion to maintain lactation (48). Consistent with this proposal, follow-up studies supported a direct effect of PRL on the HNS. Neurons in the PVN and SON were shown to contain PRL receptors (28, 35), preferentially located in OXT neurons (28). In addition, PRL was shown to reduce the firing rate of OXT neurons in the SON (28) and to stimulate HNS explants or incubated neurohypophyseal lobes to release OXT and AVP (35, 48). Moreover, it was reported that the HNS expresses and secretes PRL (9, 34, 65), suggesting that this hormone acts locally to stimulate the release of both neurohormones. Indeed, regulatory mechanisms that induce the secretion of OXT and AVP stimulate the production of PRL by the HNS. For example, suckling during lactation and stressful stimuli in females and males upregulate PRL expression and release within the PVN (64). Estrogens can also stimulate the expression and release of OXT, AVP, and PRL by the HNS (21, 36, 56, 66, 74, 76).

The role of PRL as a regulator of the HNS is further documented by the facts that chronic intracerebroventricular infusion of PRL stimulates expression of OXT mRNA in the SON, increases OXT and AVP levels in the circulation of ovariectomized, estrogen-treated rats (15), and upregulates OXT mRNA in the PVN and SON of ovariectomized, estrogen- and progesterone-primed rats (50). In these studies, steroids were used to mimic hormonal profiles at proestrus and late pregnancy, respectively. Here, we show that an acute intracerebroventricular infusion of PRL induces the release of OXT and AVP in male rats. The effect was dose-dependent, and the doses were equivalent to those used on a chronic basis in the steroid-treated rats (15, 50). The fact that the effect was observed in males suggests that female steroid hormones may not be required for intracerebroventricular PRL to stimulate neurohypophyseal hormone release.

Fig. 4. nNOS activity mediates the hyperprolactinemia-induced increase of circulating OXT and AVP levels. Circulating PRL (A), OXT (B), and AVP (C) were evaluated in nonimplanted (sham) and anterior pituitary-implanted (AP) rats treated or not (control) with bromocriptine (Bromo) or with 7-nitroindazole (7-NI). PRL levels were measured by RIA and OXT and AVP levels by ELISA. Values are expressed as means ± SE of six animals per group. *P ≤ 0.05 vs. sham, control animals.

Fig. 5. Hyperprolactinemia promotes NOS activity in the PVN and SON hypothalamic nuclei. Representative micrographs (left) of NADPH-diaphorase-stained PVN (A) and SON (B) sections obtained from sham and anterior pituitary-implanted (AP) rats. Scale bar = 100 μm. Quantification of the positive NADPH-diaphorase (NADPH-d) signal (right) evaluated throughout the rostro-caudal extent of the PVN and SON in three different animals. Results are expressed as means ± SE. *P ≤ 0.05 vs. sham rats.
Although the intracerebroventricular administration of PRL may mimic the increase in the peptide produced by the brain, it may also reflect the central elevation of PRL derived from the systemic circulation. PRL can enter the cerebrospinal fluid by binding to its receptors in the choroid plexus (33, 73), and the cerebrospinal fluid is a route by which PRL reaches various brain sites to exert different functions (4, 22). In this regard, hyperprolactinemia increases PRL receptor expression in the choroid plexus (20, 33), correlates with increased levels of PRL in the cerebrospinal fluid (18, 33, 43, 55), and has several other effects, including stimulation of maternal and feeding behaviors (6, 22), suppression of fertility (22), attenuation of stress-induced anxiety, and neuroendocrine responses (67), stimulation of neurogenesis (54), and regulation of neurotransmitter and neuropeptide release (4, 22).

Here, we show that the circulating levels of OXT and AVP are higher in hyperprolactinemic rats compared with the normoprolactinemic counterparts. This increase is likely due to hyperprolactinemia favoring the incorporation of PRL into the brain, because lowering systemic PRL with bromocriptine prevented this effect. Notably, a similar molecular mechanism appears to mediate the effects of hyperprolactinemia and the intracerebroventricular administration of PRL. In both cases, there is an increase in NOS activity in the PVN and SON, as revealed by NADPH-diaphorase histochemistry. Furthermore, the blockage of nNOS activity with 7-NI prevented the systemic elevation of the two neurohormones, indicating that the effect of systemic and central PRL is being mediated by NO derived from nNOS. Consistent with this interpretation, our findings also show that hyperprolactinemia reduces NOS phosphorylation at Ser847 and that this effect is prevented by bromocriptine. Phosphorylation is an important mechanism to inhibit nNOS. Constitutively expressed, nNOS activity is calcium-calmodulin dependent, and calcium-calmodulin binding to nNOS is inhibited by the phosphorylation of nNOS at Ser847 catalyzed by calcium/calmodulin-dependent protein kinases (24). Indeed, phosphorylation of nNOS at Ser847 inhibits nNOS-mediated effects in the brain (46).

There is evidence that PRL regulates the expression of inducible NOS in fibroblasts (11), leukocytes (13), and rat C6 glioma cells (53). PRL also modulates endothelial NOS in vascular endothelial cells (37). However, to our knowledge, this is the first report showing that nNOS activity is enhanced by PRL. This observation is consistent with a previous report showing that lowering systemic PRL with bromocriptine inhibits NOS activity in the PVN and SON of ovariectomized, steroid-treated rats, although this effect was attributed to the blockage of PRL-induced OXT expression (50). NO generated by nNOS predominates in the nervous system and mediates many biological functions, making it a likely messenger for the actions of PRL in the brain but also peripherally. For example, PRL interferes with penile erection by directly inhibiting the relaxation of the corpus cavernosum penis (52), and NO, produced within the erectile tissue by nNOS from autonomic innervation, is a major promoter of penile erection. Opposite effects of PRL on nNOS activity are not unexpected since the regulation of this enzyme is cell specific (7, 75). Along this line, the increase in free intracellular calcium (58, 68, 71) and the mobilization of calmodulin (3) by the PRL receptor may activate nNOS. But PRL has also been associated with the regulation of protein phosphatase 2A (8) and calmodulin-dependent protein kinase (2, 5), activities that are both known to downregulate nNOS activity.

The functional link between PRL, OXT, and AVP is strong during reproduction in females, where the positive influence of PRL on neurohypophysial hormone release may contribute to the maternal adaptations balancing osmotic challenges, uterine contractions, milk ejection, and behavioral responses around the time of birth and during lactation (10, 15, 22, 40, 41, 48). The current study suggests that the three hormones are also functionally linked in males. The magnitude of the increase in circulating PRL (50 ng/ml) employed by the study is equivalent to PRL values reported in male rats at circadian peaks (17) or in response to stress (31, 62). The functional implication of the interaction among the three hormones in males remains to be determined, but we hypothesize that this neuroendocrine mechanism operates to regulate stress responses, sodium and water balance, and sexual activity, which are processes where the overlapping release and effects of the three hormones have been described. For example, PRL, OXT, and AVP are released into the circulation in response to different stressors (16, 19), and while PRL acts centrally to inhibit the stress-induced activation of the hypothalamo-pituitary-adrenal (HPA) axis (67), systemic OXT, and AVP stimulate the secretion of adrenocorticotropin and corticosterone into blood (16). By inducing the systemic release of OXT and AVP, PRL could modulate its own central inhibitory effect, helping to adjust the activation of the HPA axis to physiological demands. Likewise, the three hormones are released in response to osmotic stimulation (12, 27). AVP is a major enhancer of water retention and blood pressure, OXT is a potent natriuretic factor, and PRL promotes sodium and water retention, as well as vasoconstriction (12, 19, 37). Accordingly, the PRL-mediated release of neurohormones may influence the cumulative effect on fluid and electrolyte homeostasis. Finally, we propose that the PRL-induced OXT and AVP release contributes to the generation of the postejaculatory refractory period, as PRL and OXT
increase in the male circulation in response to an orgasm (29), hyperprolactinemia is associated with low sexual desire and erectile problems (30, 52), AVP (57) and OXT (61) lengthen the postorgasmic refractory period, and OXT induces postorgasmic penile flaccidity (70).

**Perspectives and Significance**

Our findings demonstrate that increased levels of brain or serum PRL enhance nNOS activity, and thereby stimulate OXT and AVP secretion and that this mechanism operates in males. These observations may help clarify the overlapping functions of all three hormones in physiological states beyond pregnancy and lactation that may include stress responses, water and electrolyte balance, and sexual activity. Furthermore, this study shows that nNOS is a target of the PRL signaling pathway in the brain. Further studies are required to address the functional relevance of the PRL, OXT, and AVP neurohormonal trio in male rats and the actions of PRL involving nNOS regulation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

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