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Prolactin Modulates Hypothalamic Preproenkephalin, but Not Proopiomelanocortin, Gene Expression During Lactation

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The aim of this study was to examine prolactin (PRL) regulation of preproenkephalin and proopiomelanocortin (POMC) gene expression in the hypothalamus during lactation. In the first experiment, lactating rats were deprived of pups for 3, 6, 12, or 24 h. Preproenkephalin mRNA levels were decreased in the arcuate nucleus (ARC) to 60 or 53% of suckled levels and in the ventromedial nucleus to 70% of suckled levels after 12 or 24 h but were unchanged in the striatum. POMC mRNA levels in the ARC and periarcuate area were increased to 165% of suckled levels within 3 h and remained elevated two- to threefold for 24 h. Subcutaneous administration of bromocriptine to suckled dams markedly suppressed circulating PRL levels and decreased preproenkephalin mRNA signal levels to 38 and 50% of control levels in the arcuate and ventromedial nuclei, respectively. Intravenous administration of oPRL completely reversed this effect. By contrast, bromocriptine with or without administration of ovine PRL (oPRL) did not alter POMC mRNA signal levels in the ARC. Administration of oPRL to pup-deprived dams increased preproenkephalin mRNA levels in the arcuate and ventromedial nuclei and reduced POMC mRNA levels in the ARC to levels similar to suckled control levels. In conclusion, POMC neurons in the ARC appear to be refractory to PRL regulation in the presence of a suckling stimulus, and other components of the suckling stimulus may contribute to the suppression of POMC mRNA levels during lactation. By contrast, PRL provides a regulatory influence for the suckling-induced increase in preproenkephalin mRNA signal levels in arcuate and ventromedial nuclei.

Key Words: Enkephalin; proopiomelanocortin; lactation; prolactin; hypothalamus; arcuate nucleus.

Introduction

The endogenous opioid peptides have modulatory roles in multiple lactational processes including reproduction, behavior, and energy homeostasis. The suckling stimulus is transmitted by a neuronal network to the hypothalamus and leads to elevated prolactin (PRL) and oxytocin, essential for full lactational competency. The endogenous opioid peptides contribute to the suckling-induced rise in PRL, as evidenced by the ability of opioid receptor antagonists to attenuate the PRL surge *(1–4)*. The stimulatory effect on PRL release is mediated by the capacity of endogenous opioid peptides to suppress tuberoinfundibular dopaminergic (TIDA) activity *(3,4)*. In many physiologic states, the PRL short feedback loop is operational and elevated PRL levels increase TIDA neuronal activity, and in turn suppress endogenous PRL levels *(5–7)*. However, the responsiveness of these dopaminergic neurons to the stimulatory effects of PRL feedback is diminished during lactation *(8,9)*, and PRL may exert an uncharacteristic inhibitory action on some aspects of dopaminergic neuronal activity during midlactation *(10)*.

Immunoneutralization studies indicate that enkephalin, β-endorphin, and dynorphin peptides are involved in suckling-induced PRL release, although only dynorphin and enkephalin exert an inhibitory action on TIDA neuronal activity *(11,12)*. When compared to diestrous rats, dynorphin mRNA levels in the periventricular nucleus *(13)* and preproenkephalin pmRNA levels in the arcuate nucleus (ARC) *(14)* are elevated. By contrast, proopiomelanocortin (POMC) mRNA levels in the ARC are lower in lactating compared with diestrous females *(15,16)*. Enkephalin expression is induced in the TIDA neurons during lactation *(17)*, and enkephalin peptide or preproenkephalin mRNA is elevated under conditions of hyperprolactinemia *(18)*. However, the role of PRL feedback on enkephalin expression in the hypothalamic nuclei during lactation has not been directly evaluated. Moreover, the effect of PRL on POMC mRNA levels, especially during lactation, is less clear. PRL increases POMC mRNA levels in the ARC of male rats *(19)* and suppresses POMC mRNA levels in intact female rats *(20)*.

The primary objective of the present study was to evaluate the ability of the suckling-induced PRL elevation to regulate preproenkephalin gene expression in the ARC,

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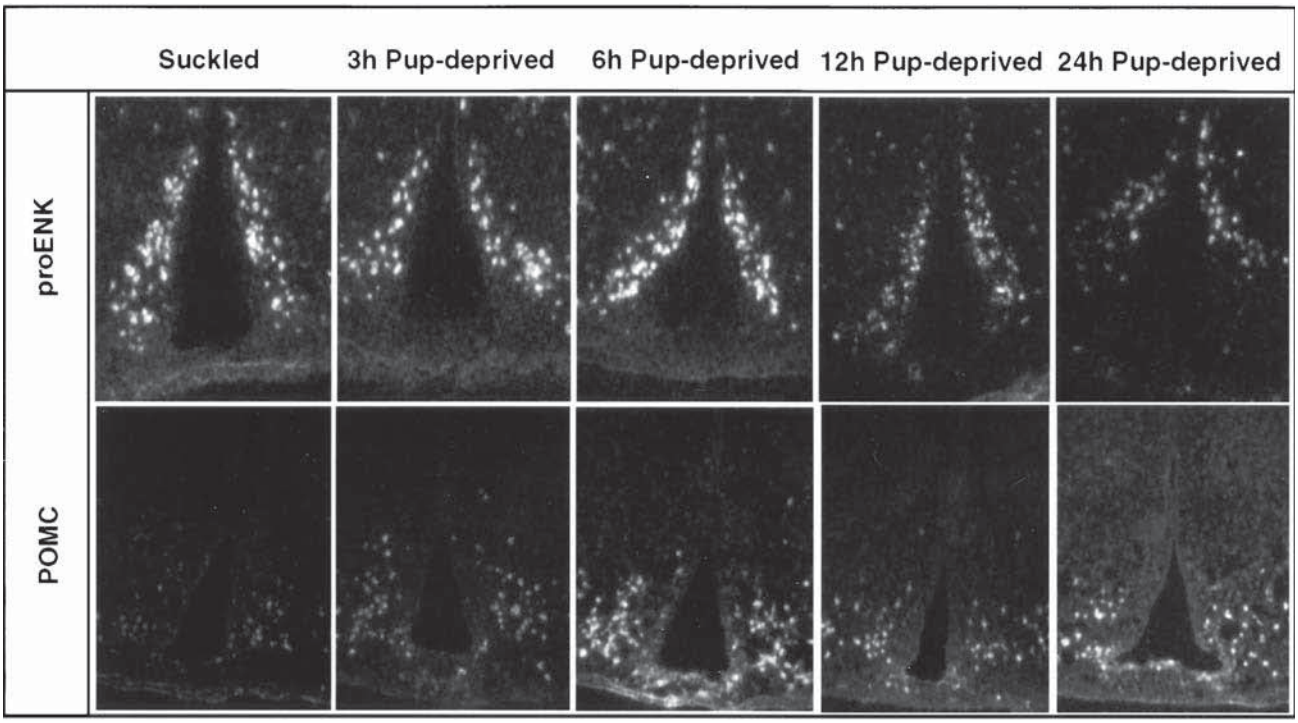

Fig. 1. Preproenkephalin (**top**) and POMC (**bottom**) mRNA–containing cells in ARC of hypothalamus of suckled dams and dams deprived of their pups for 3–24 h. Dark-field photomicrographs show coronal sections at the level of mid–median eminence. Note the presence of cell bodies in the ARC with a dense accumulation of silver grains. Preproenkephalin mRNA signal levels decreased with pup deprivation, whereas POMC mRNA signal levels increased after pups were removed from lactating dams.

ventromedial nucleus (VMN), and striatum and POMC gene expression in the ARC and periarcuate area. The specific aims were (1) to evaluate preproenkephalin and POMC gene expression at specific times after pup deprivation, (2) to examine opioid peptide gene expression after bromocriptine-induced suppression of endogenous rat PRL (rPRL) with and without ovine PRL (oPRL) replacement, and (3) to determine the effect of exogenous PRL administration on opioid peptide gene expression in pup-deprived dams.

Results

Effect of Pup Deprivation on Preproenkephalin and POMC mRNA Signal Levels During Lactation

The first experiment evaluated preproenkephalin and POMC gene expression in hypothalamic nuclei and striatum of lactating rats after pup deprivation for 3–24 h. After 3 and 6 h of pup deprivation, cellular preproenkephalin mRNA signal levels in the ARC were similar to levels in the suckled control rats but were significantly reduced to 60 and 53% of suckled levels after 12 and 24 h, respectively (Fig. 1, top and Fig. 2A). In a similar manner, preproenkephalin expression in the VMN was similar to levels in the suckled control group after 3 and 6 h of pup deprivation but was reduced to 70% of control levels within 12 and 24 h of pup deprivation (Fig. 2A). In contrast to changes in hypothalamic nuclei, preproenkephalin mRNA signal levels in the striatal regions of the same brain sections were unaltered after pup deprivation (Fig. 2A).

Within 3 h of pup deprivation, POMC mRNA signal levels in the ARC and periarcuate area increased to 166% of suckled levels (Fig. 1, bottom and Fig. 2B). POMC mRNA signal levels were further elevated to 260% of suckled levels within 6 h and remained elevated for 24 h after pup deprivation.

Circulating PRL levels were measured in lactating rats with or without their pups. When the suckling stimulus was maintained by the presence of pups with dams, PRL levels were 193 ng/mL (Fig. 2C). Serum PRL levels declined to 34% of suckled levels within 3 h after pup deprivation and were suppressed to very low levels after 6–24 h.

Role of PRL in Regulating Preproenkephalin and POMC mRNA Signal Levels in Suckled Dams

The second experiment evaluated whether hyperprolactinemia induced by the suckling stimulus was responsible for changes in preproenkephalin gene expression in the ARC and VMN of the hypothalamus. A 24-h treatment period was selected because maximal changes in preproenkephalin and POMC mRNA signal levels in hypothalamic nuclei occurred within this period. PRL secretion in constantly suckled dams was suppressed with bromocriptine, a dopamine agonist (Fig. 3C). PRL levels were restored with oPRL

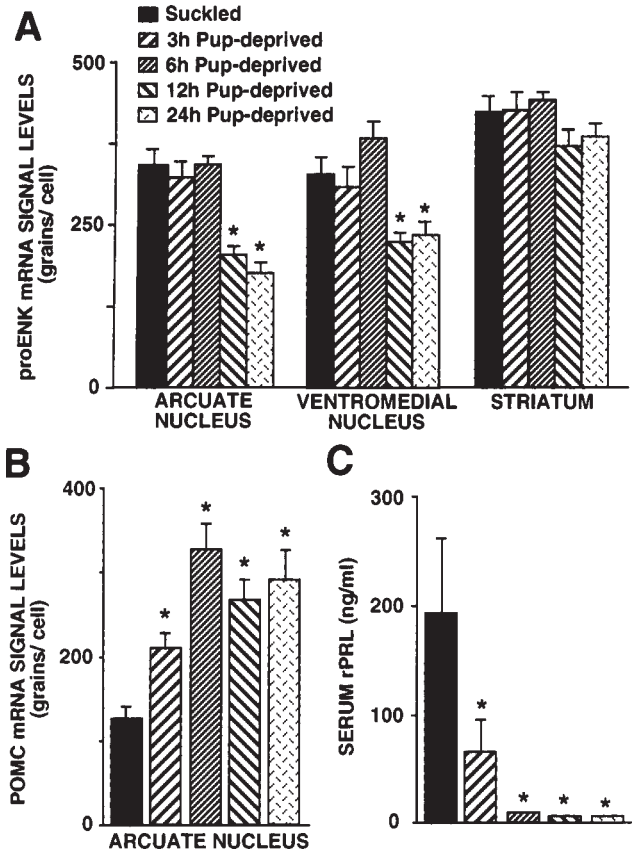

Fig. 2. (**A**) Preproenkephalin mRNA signal levels in ARC and VMN of hypothalamus and in striatum in suckled dams and after 3–24 h of pup deprivation; (**B**) POMC mRNA signal levels in ARC and periarcuate area of hypothalamus in suckled and pup-deprived lactating rats; (**C**) circulating PRL levels in suckled and pup-deprived dams. Lactating rats on d 7 postpartum were maintained with their pups or deprived of pups for 3, 6, 12, or 24 h. Preproenkephalin mRNA levels in the arcuate and ventromedial nuclei were suppressed after 12 and 24 h of pup deprivation, whereas POMC mRNA levels in the ARC and periarcuate area were increased within 3 h of pup deprivation. Circulating rPRL levels rapidly declined after pups were removed from dams. Each value is a mean ± SEM of determinations from six rats/group. *Significantly different ($p < 0.05$) from suckled control group.

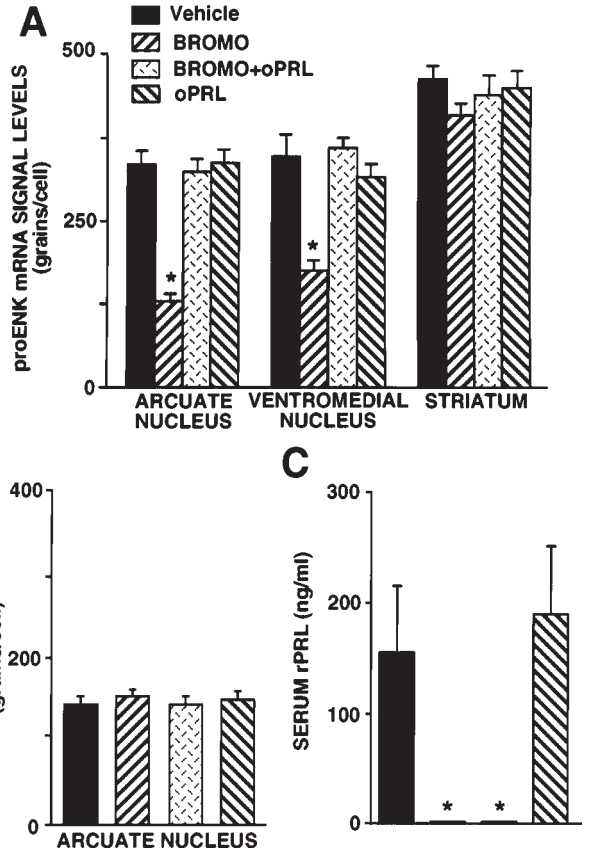

Fig. 3. (**A**) Preproenkephalin mRNA signal levels in ARC, VMN, and striatum of suckled lactating dams treated with vehicle, bromocriptine mesylate (BROMO; 3 mg/kg subcutaneously), oPRL (6.25 μg/h intravenously) or bromocriptine + oPRL for 24 h; (**B**) POMC mRNA signal levels in ARC and periarcuate area of suckled lactating dams treated as in (A); (**C**) circulating rPRL levels in suckled lactating dams on d 7 postpartum after bromocriptine and/or oPRL treatment. Bromocriptine treatment suppressed preproenkephalin mRNA signal levels in the arcuate and ventromedial nuclei of the hypothalamus, and this effect was completely reversed by coadministration of oPRL, whereas neither bromocriptine nor oPRL treatment altered POMC mRNA signal levels in the arcuate and periarcuate area. Each value is a mean ± SEM of determinations from six to eight rats/group. *Significantly different ($p < 0.05$) from vehicle-treated group.

treatment. Serum rPRL levels remained low in rats treated with both bromocriptine and oPRL, although our previous data indicate that circulating PRL levels are elevated to approx 100–125 ng/mL using constant infusion with an osmotic minipump *(6)*. Administration of oPRL alone did not alter circulating rPRL levels in suckled dams, even though this same paradigm of oPRL treatment suppresses endogenous PRL to very low levels in ovariectomized rats *(6)*.

Bromocriptine treatment suppressed preproenkephalin mRNA signal levels in the arcuate and ventromedial nuclei to 38 and 50%, respectively, of suckled levels (Fig. 3A). Coadministration of oPRL completely reversed the inhibitory effect of bromocriptine on preproenkephalin mRNA levels in both the arcuate and ventromedial nuclei. Preproenkephalin mRNA levels in hypothalamic nuclei were not altered above suckled levels when circulating PRL levels were further elevated by infusion of oPRL alone. There were no changes in preproenkephalin mRNA signal levels in the striatum with treatments that altered circulating PRL levels. In contrast to PRL-dependent changes in preproenkephalin mRNA levels in hypothalamic nuclei, POMC mRNA signal levels in the ARC and periarcuate area of suckled dams were unaltered by bromocriptine treatment with or without concomitant oPRL treatment (Fig. 3B).

Litters of eight pups were weighed before bromocriptine treatment was started on d 6 and at the end of the experiment

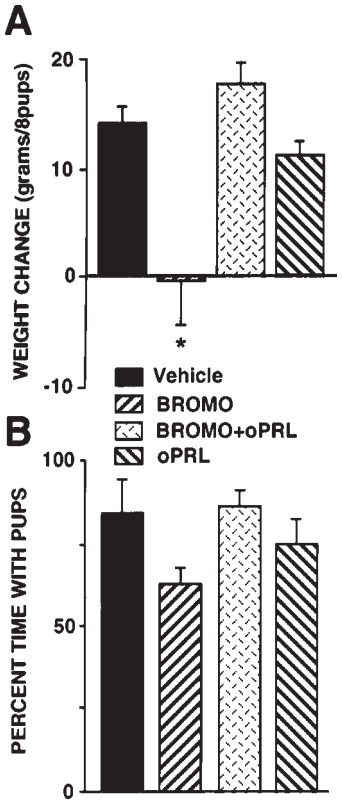

Fig. 4. (**A**) Change in litter (eight pups) weights of suckled dams treated with vehicle, bromocriptine (BROMO; 3 mg/kg subcutaneously), oPRL (6.25 μg/h intravenously) or bromocriptine + oPRL; (**B**) percentage of time dams spent in nest with pups during a 12-h observational period on d 7 postpartum in suckled dams treated as in (A). Pups failed to gain weight in bromocriptine-treated dams, but coadministration of oPRL returned weight gain to control (vehicle) levels. However, these treatments did not alter the amount of time dams spent in the nest with their pups. Each value is a mean ± SEM of determinations from six to eight rats/group. *Significantly different ($p < 0.05$) from vehicle-treated group.

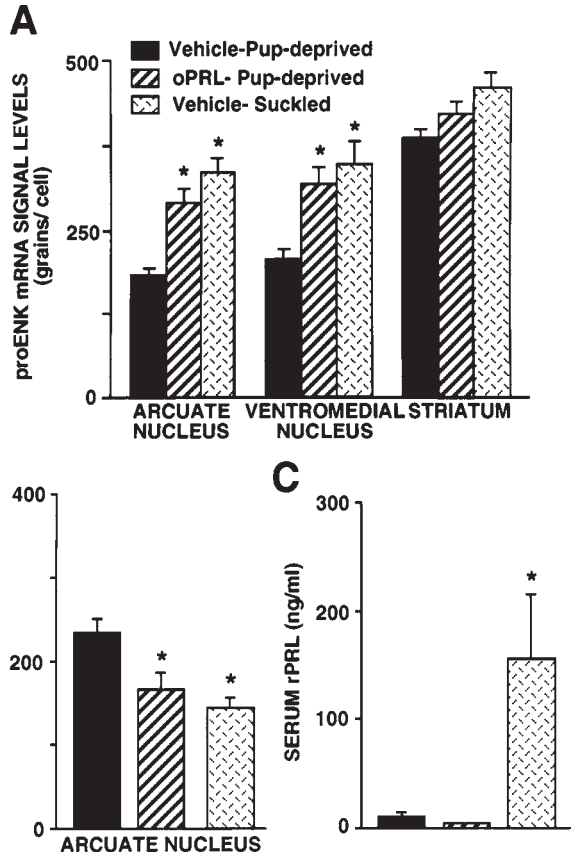

Fig. 5. (**A**) Preproenkephalin mRNA signal levels in ARC, VMN, and striatum of lactating rats deprived of their pups for 24 h and treated with vehicle or oPRL (6.25 μg/h intravenously) or in suckled lactating rats treated with vehicle; (**B**) POMC mRNA signal levels in ARC and periarcuate area in pup-deprived and suckled lactating dams treated as in (A); (**C**) circulating rPRL levels in pup-deprived or suckled lactating rats with or without oPRL infusion. PRL treatment increased preproenkephalin mRNA levels in the arcuate and ventromedial nuclei of pup-deprived rats to levels similar to suckled levels and decreased POMC mRNA levels in the ARC and periarcuate area to suckled levels. Each value is a mean ± SEM of determinations from six rats. *Significantly different ($p < 0.05$) from vehicle-treated pup-deprived group.

on d 7. There was no difference in the litter weights among the treatment groups at the beginning of bromocriptine and/or oPRL treatment. The change in litter weight for the 24-h treatment period was determined. Control litters gained 14 g/8 pups during this 24-h period (Fig. 4A). Bromocriptine completely prevented any weight gain, although litters did not lose weight, as would be expected if pups were separated from dams *(3)*. Coadministration of oPRL restored litter weight gain to control levels, which was similar to litter weight gain after oPRL infusion alone. The percentage of time that dams spent in the nest was estimated by observing the rats at 2-h intervals from 8:00 AM to 8:00 PM on d 7. Dams spent 63–84% of the time with their litters, but there was no significant difference among the groups (Fig. 4B).

Effect of oPRL Treatment on Preproenkephalin and POMC mRNA Signal Levels in Pup-Deprived Dams

The third experiment evaluated the effect of PRL when the suckling stimulus was removed. PRL was retained at elevated levels by oPRL infusion with an osmotic minipump after pup deprivation. PRL treatment of 24-h pup-deprived dams caused a 57 and 54% increase in preproenkephalin mRNA signal levels in arcuate and ventromedial nuclei, respectively, resulting in levels similar to suckled levels (Fig. 5A). Similar to a lack of a suckling or PRL effect in the previous experiments, oPRL had no effect on preproenkephalin mRNA levels in the striatum. In pup-deprived dams, oPRL treatment suppressed POMC mRNA signal levels in the ARC and periarcuate area to 70% of pup-deprived levels

and resulted in levels similar to suckled levels (Fig. 5B). Endogenous rat PRL levels were low in pup-deprived dams with or without oPRL treatment (Fig. 5C).

Discussion

We have reported that preproenkephalin mRNA signal levels in the ARC and VMN of the hypothalamus were elevated during lactation and that the suckling-dependent PRL elevation was primarily responsible for this increased preproenkephalin gene expression. By contrast, POMC mRNA signal levels in the ARC and periarcuate area were suppressed in response to the suckling stimulus, but this inhibitory effect was not owing to elevated PRL levels. In fact, POMC neurons appeared to be refractory to PRL feedback when the suckling stimulus was present, but PRL responsiveness rapidly returned when the suckling stimulus was removed. Other neural or hormonal components of the suckling stimulus likely regulate POMC gene expression during lactation.

These data indicate that the suckling stimulus in lactating rats sustained elevated preproenkephalin gene expression in the ARC and VMN of the hypothalamus. When the suckling stimulus was removed by pup deprivation, cellular preproenkephalin mRNA signal levels in the hypothalamus declined after 12 h. The changes in preproenkephalin gene expression lagged behind changes in circulating PRL levels as preproenkephalin mRNA levels decreased between 6 and 12 h after pup deprivation, whereas PRL levels were significantly suppressed by 3 h and were very low by 6 h. The delay before the changes in preproenkephalin mRNA expression may be owing to the half-life of preproenkephalin mRNA, which is estimated to be 9.6 h in the adrenal medulla *(21)*. These results extend previous observations that preproenkephalin mRNA levels in the ARC are higher in lactating rats on d 3 or 10 postpartum as compared with diestrous rats *(14)*. This indicates that a sustained suckling stimulus is necessary to maintain these elevated levels. Preproenkephalin mRNA levels were also suppressed in the VMN within 12 h after the suckling stimulus was removed, indicating that enkephalinergic neurons in this region may also play a role in lactational events. By contrast, Ottinger et al. *(14)* observed no difference in preproenkephalin mRNA levels in the VMN of lactating and diestrous rats. It is not clear if there are differential regulatory mechanisms as steroid hormone input *(22,23)*, which maintain elevated levels during the estrous cycle as compared to the influence of PRL during lactation. In the present study, preproenkephalin mRNA signal levels were not altered in striatal regions contained in these same brain sections. Thus, the changes in preproenkephalin gene expression appeared to be specific to hypothalamic nuclei.

The elevated PRL levels associated with the suckling stimulus provided the primary regulatory influence for enhanced preproenkephalin gene expression in the arcuate and ventromedial nuclei during lactation. When bromocriptine suppressed circulating PRL to very low levels, preproenkephalin mRNA levels in these hypothalamic nuclei were reduced to levels similar to those observed after pup deprivation. This suppression was completely reversed by coadministration of oPRL, indicating that this suppression was owing to low PRL levels as opposed to the dopamine agonist properties of bromocriptine. Although increasing PRL above the already high levels of PRL in suckled dams had no effect on preproenkephalin mRNA levels in the arcuate and ventromedial nuclei, oPRL treatment of 24-h pup-deprived dams resulted in an increase in preproenkephalin mRNA to levels similar to those in suckled dams. However, alterations in PRL levels had no effect on preproenkephalin mRNA levels in striatal regions on these same brain sections, indicating that PRL regulation of enkephalinergic neurons is specific to the hypothalamic regions. These results indicate that enkephalinergic neurons in the hypothalamus retained their responsiveness to PRL feedback with or without the suckling stimulus. It is not surprising that PRL is the primary influence on enkephalinergic neurons during lactation, since enkephalin expression is increased in the ARC under many conditions of chronically elevated PRL levels *(18,24)*. Indeed, there is a marked induction of enkephalin in the TIDA neurons during lactation *(17,24,25)*, suggesting that these neurons may have an important role in maintaining the elevated PRL levels of lactation. This notion is further supported by immunoneutralization studies showing that enkephalin antiserum can block the suckling-induced PRL surge and prevent the suckling-induced decline in TIDA neuronal activity *(12)*. However, given the high degree of colocalization of enkephalin in dopaminergic neurons, it is puzzling that the responsiveness of tuberoinfundibular dopaminergic neurons to PRL stimulation is diminished *(8,9)*, whereas the present study indicates that enkephalinergic neurons are highly responsive to PRL.

The biologic data indicated that bromocriptine and/or oPRL treatment was effective. Bromocriptine treatment suppressed circulating rPRL to very low levels and this manifested in the inability of pups to gain weight. The lack of weight gain was not owing to a complete failure of maternal behavior. In fact, dams spent an equivalent amount of time in the nest with or without bromocriptine treatment, and the number of pups attached to the nipple and the suckling vigor appeared to be greater in the bromocriptine-treated dams. Restoration of PRL resulted in pup weight gain similar to pups from control dams, indicating that the method of oPRL infusion used in the present study was appropriate for mimicking suckling-induced PRL release.

In contrast to a stimulatory effect of the suckling stimulus on preproenkephalin gene expression in the ARC, the suckling stimulus suppressed POMC mRNA levels in the ARC and periarcuate area. Removal of the suckling stimulus

by pup deprivation resulted in a marked elevation of POMC levels, which was observed as early as 3 h after pup removal and reached maximal levels by 6 h. These data extend those of previous studies demonstrating that POMC mRNA levels in the ARC are lower in lactating dams compared with diestrous rats *(15,16)*. However, Pape and Tramu *(16)* report that removal of the suckling stimulus results in only a modest increase in POMC mRNA levels, whereas in the present study there was a marked 2- to 2.6-fold increase in POMC mRNA levels within 6–24 h after pup deprivation.

The elevated PRL levels during lactation did not appear to have a role in the suckling-dependent decrease in POMC mRNA signal levels in the ARC and periarcuate area. Thus, another hormonal or neuronal component of the suckling stimulus likely provided the primary regulatory influence on POMC neurons during lactation. POMC mRNA levels in suckled dams were not altered when circulating PRL levels were suppressed by bromocriptine treatment. Moreover, restoration of PRL by oPRL infusion also had no effect on POMC mRNA signal levels. These data would suggest that the POMC neurons were refractory to PRL regulation when the suckling stimulus was present. However, PRL responsiveness was restored after only 24 h of pup deprivation in the present study, and PRL infusion during that period resulted in a marked decrease in POMC mRNA levels in the ARC. Although the present and previous studies *(15,16)* indicate inhibition of POMC mRNA levels as the response to prolonged suckling, Pape and Tramu *(16)* report that the early response is a PRL-dependent increase in POMC mRNA levels within 2 h after restoration of pups to pup-deprived dams *(16)*. PRL has both stimulatory and inhibitory effects on POMC neurons *(19,20)*, but it is not clear how different endocrine states modify the responsiveness to PRL.

An alternative explanation for the lack of effect of bromocriptine may be dopaminergic suppression of POMC mRNA levels in the suckled lactating rat, which overrides an effect of PRL. However, this premise seems unlikely. First, TIDA neuronal activity is suppressed during lactation *(8,9,26)*. Second, β-endorphin suppresses dopamine turnover in the median eminence *(27)*, although β-endorphin does not appear to mediate the decrease in dopaminergic neuronal activity during lactation *(11)*. Third, bromocriptine treatment of intact or hypophysectomized female rats results in an increase in POMC mRNA levels *(28)*, whereas POMC mRNA levels were suppressed as a result of the suckling stimulus rather than elevated in the present study. On the other hand, if diminished dopaminergic input resulted in lower suckling-dependent POMC mRNA levels, it would be expected that bromocriptine treatment would restore dopaminergic input and increase POMC mRNA levels, but that PRL restoration would have no effect. This was not the case, which suggests that dopaminergic input did not override a PRL effect on POMC neurons.

Our study provides information about how POMC and enkephalin expression is regulated in hypothalamic neurons during lactation. PRL provided the primary regulatory influence for the suckling-induced increase in preproenkephalin expression in the arcuate and ventromedial nuclei, whereas components of the suckling stimulus other than PRL appeared to drive the suckling-dependent decrease in POMC expression in the ARC and periarcuate area. However, we still do not completely understand how these changes fit with opioid regulation of TIDA neuronal activity and PRL secretion during lactation. Studies with opioid receptor antagonists indicate that increased opioidergic activity mediates the effects on PRL *(1–4)*, as was observed with hypothalamic enkephalinergic neurons. However, μ and κ opioid receptor subtypes are implicated for both the rise in PRL and the inhibition of TIDA neuronal activity *(2,4)* rather than δ receptors, which are more closely associated with enkephalin actions. Indeed, δ opioid receptors increase, rather than decrease, TIDA neuronal activity in male rats *(29)*. Although their role and regulation is not completely understood, the endogenous opioid peptides appear to have a key modulatory influence for many lactational processes and unique regulatory mechanisms.

Materials and Methods

Animals

Female and male Sprague-Dawley rats (Charles River, Raleigh, NC) were maintained in a light- (lights on at 7:00 AM and off at 9:00 PM) and temperature-(23ºC) controlled room and supplied with food and water ad libitum. The estrous cycles of female rats were followed by daily vaginal lavage. Each female was placed with a single male on the day of proestrus for mating purposes. Pregnant females were housed individually from d 16 of gestation. Litters were adjusted to eight pups/dam on the d 2 after delivery (parturition day = d 1). The animal care and use committee at Southern Illinois University approved all procedures.

Experimental Protocols

In experiment 1, lactating rats were divided into five groups. All rats were allowed to nurse their pups continuously until d 6 of lactation. The first group was the suckled control dams, which were allowed to nurse their pups until they were sacrificed. The other four groups of dams were separated from their pups for 3, 6, 12, or 24 h.

In experiment 2, four groups of lactating rats were used and pups were retained with dams throughout the experiment. Each rat was implanted with a jugular cannula on d 5 postpartum. The cannula was connected to an osmotic minipump (Alza, Palo Alto, CA) containing oPRL (NIDDK-oPRL-21; 1.25 mg/200 μL) or vehicle (10 m*M* sodium bicarbonate, pH 8.6; 150 m*M* NaCl; 50 U/mL of heparin) as described previously *(6)*. Beginning on d 6, bromocriptine mesylate (3 mg/kg subcutaneously) or vehicle (30% ethanol) was injected at 24 and 12 h before rats were sacrificed. The first group received vehicle for bromocriptine and vehi-

cle for oPRL. The second group was administered bromocriptine and vehicle for oPRL. The third group received both bromocriptine and oPRL. The fourth group was treated with vehicle for bromocriptine and oPRL. Dams and pups were monitored at 2-h intervals, and gross behavioral changes were estimated as the percentage of time the dams spent in the nest with the pups. Litters of eight pups were weighed at the beginning and end of the experiment.

In experiment 3, three groups of lactating rats were used. On d 5 postpartum, rats were implanted with a jugular cannula connected to an osmotic minipump containing oPRL or vehicle as just described. In two groups, pups were removed from dams 24 h before termination of the experiment. Pups were retained with dams in one group.

All rats were sacrificed at 8:00 PM on d 7 postpartum. The brains were quickly removed, frozen immediately in Histofreeze (Fisher, St. Louis, MO) at –80ºC, and then stored at –80ºC. Trunk blood was collected and centrifuged at 10,000*g* for 5 min. Serum was removed and stored at –20ºC for analysis of circulating PRL levels.

In Situ Hybridization for Preproenkephalin and POMC mRNA

Coronal sections (15 µm) were cut through the ARC, and every third section was saved for determination of preproenkephalin or POMC mRNA levels. Brain sections were stored at –80ºC until *in situ* hybridization. Brain sections were fixed in 4% paraformaldehyde and subjected to an *in situ* hybridization procedure as described previously for tyrosine hydroxylase mRNA *(6)*. After prehybridization steps, the brain sections were hybridized overnight at 45ºC with 2×10^5 dpm of a specific antisense ^{35}S-labeled cRNA probe for either preproenkephalin or POMC with specific activities of 5×10^8 dpm/µg. The probes were synthesized using SP6 RNA polymerase. The cDNA for POMC was an *Eco*RI-*Hind*III insert subcloned into an SP65 vector and contained the entire 707-bp coding sequence, 86 bp 3' of the coding sequence and 108 bp 5' of the coding sequence for mouse POMC *(30,31)*. The nucleotide sequence of the coding sequence for the mouse POMC gene shows considerable sequence homology with the rat POMC gene *(30,32)*. The cDNA for preproenkephalin was an *Eco*RI-*Hind*III insert subcloned into an SP64 vector and contained 91–809 bp of the coding sequence and 304 bp 3' of the coding sequence for rat proenkephalin *(31,33)*. At the end of the hybridization period, the slides were subjected to ribonuclease treatment and a series of posthybridization washes that increased in stringency. Subsequently, slides were dipped in Ilford Emulsion (K-5) diluted with 0.25 g/mL of water. The autoradiograms were exposed for 2 wk, developed by standard photographic methods, and poststained lightly with hematoxylin.

The anatomic locations of tissue sections were determined using the rat brain atlas of Paxinos and Watson *(34)*. The rostral and caudal borders were –2.12 and –3.3 mm from the bregma, respectively. POMC or preproenkephalin mRNA–containing cells were identified under dark-field optics as a cluster of reduced silver grains surrounding an identifiable cell nucleus. Approximately 20 cells/tissue section (400 cells/brain area/rat) were selected from each brain area. The number of grains in individual mRNA-containing cells was measured under X400 dark-field illumination by a computerized image processing system using NIH Image software.

PRL Determination

Serum PRL levels were determined using the rat PRL radioimmunoassay kit provided by Dr. A. F. Parlow and the National Hormone and Pituitary Program. PRL RP-3 was used as a reference preparation and [^{125}I] rat PRL (New England Nuclear, Boston, MA) as the radiolabeled antigen. The limit of sensitivity for the assay was 0.5 ng/mL. The intra- and interassay coefficients of variation were 12.1 and 9.1%, respectively.

Statistical Analyses

Results are expressed as the mean ± SEM. *N* for all experiments refers to the number of experimental animals. For determination of cellular preproenkephalin or POMC mRNA signal levels, the mean grain area per cell in a given anatomic area was calculated for individual animals. The individual means were used to calculate the mean ± SEM of each group. Data were evaluated by analysis of variance, and multiple comparisons were made with the Fisher least significant procedure *(35,36)*. Differences were considered significant at $p < 0.05$.

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References

1. Selmanoff, M. and Gregerson, K. A. (1986). *Neuroendocrinology* **42,** 255–259.
2. Baumann, M. H. and Rabii, J. (1991). *Brain Res.* **567,** 224–230.
3. Arbogast, L. A. and Voogt, J. L. (1998). *Endocrinology* **139,** 2857–2862.
4. Callahan, P., Baumann, M. H., and Rabii, J. (1996). *J. Neuroendocrinol.* **8,** 771–776.
5. Moore, K. E. (1987). *Biol. Reprod.* **36,** 47–58.
6. Arbogast, L. A. and Voogt, J. L. (1991). *Endocrinology* **128,** 997–1005.
7. Selmanoff, M. (1985). *Endocrinology* **116,** 1943–1952.
8. Demarest, K. T., McKay, D. W., Riegle, G. D., and Moore, K. E. (1983). *Neuroendocrinology* **36,** 130–137.

9. Arbogast, L. A. and Voogt, J. L. (1996). *Endocrinology* **137,** 47–54.
10. Li, C., Chen, P., and Smith, M. S. (1999). *Endocrinology* **140,** 118–123.
11. Jaworski, R. P., Callahan, P., and Janik, J. (1997). *Life Sci.* **61,** 1301–1311.
12. Callahan, P., Klosterman, S., Prunty, D., Tompkins, J., and Janik, J. (2000). *Neuroendocrinology* **71,** 268–276.
13. Eriksson, M., Ceccatelli, S., Uvnäs-Moberg, K., Iadarola, M., and Hökfelt, T. (1996). *Neuroendocrinology* **63,** 356–367.
14. Ottinger, M. A., Rosewell, K. L., Weiland, N. G., Margaretten, K. T., and Wise, P. M. (1995). *J. Neuroendocrinol.* **7,** 341–346.
15. Smith, M. S. (1993). *Endocrinology* **133,** 1258–1265.
16. Pape, J.-R. and Tramu, G. (1996). *Neuroendocrinology* **63,** 540–549.
17. Merchenthaler, I. (1993). *Endocrinology* **133,** 2645–2651.
18. Merchenthaler, I., Lennard, D. E., Cianchetta, P., Merchenthaler, A., and Bronstein, D. (1995). *Endocrinology* **136,** 2442–2450.
19. Selmanoff, M., Shu, C., Hartman, R. D., Barraclough, C. A., and Petersen, S. L. (1991). *Mol. Brain Res.* **10,** 277–281.
20. Tong, Y. and Pelletier, G. (1992). *Neuroendocrinology* **56,** 561–565.
21. Zhu, Y. S., Branch, A. D., Robertson, H. D., Huang, T. H., Franklin, S. O., and Inturrisi, C. E. (1992). *Mol. Brain Res.* **12,** 173–180.
22. Hoffman, G. E., Le, W.-W., Abbud, R., Lee, W.-S., and Smith, M. S. (1994). *Brain Res.* **654,** 207–215.
23. Demarest, K. T. and Moore, K. E. (1981). *Neuroendocrinology* **33,** 230–234.
24. Merchenthaler, I. (1994). *Neuroendocrinology* **60,** 185–193.
25. Ciofi, P., Crowley, W. R., Pillez, A., Schmued, L. L., Tramu, G., and Mazzuca, M. (1993). *J. Neuroendocrinol.* **5,** 599–602.
26. Selmanoff, M. and Wise, P. M. (1981). *Brain Res.* **212,** 101–115.
27. Deyo, S. N., Swift, R. M., and Miller, R. J. (1979). *Proc. Natl. Acad. Sci. USA* **76,** 3006–3009.
28. Tong, Y. and Pelletier, G. (1992). *Mol. Brain Res.* **15,** 27–32.
29. Manzanares, J., Durham, R. A., Lookingland, K. J., and Moore, K. E. (1993). *Eur. J. Pharmacol.* **249,** 107–112.
30. Uhler, M. and Herbert, E. (1983). *J. Biol. Chem.* **258,** 257–261.
31. Garrett, J. E., Collard, M. W., and Douglass, J. O. (1989). *Mol. Cell Biol.* **9,** 4381–4389.
32. Drouin, J., Chamberland, M., Charron, J., Jeannotte, L., and Nemer, M. (1985). *FEBS Lett.* **193,** 54–58.
33. Rosen, H., Douglass, J., and Herbert, E. (1984). *J. Biol. Chem.* **259,** 14309–14313.
34. Paxinos, G. and Watson, C. (eds.) (1986). *The rat brain in stereotaxic coordinates*. Academic: New York.
35. Zar, J. H. (eds.) (1984). *Biostatistical analysis*. Prentice-Hall: Englewood Cliffs, NJ.
36. Gerald, K. B. (1990). *Nurse Anesth.* **1,** 162–165.