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Mu and Kappa Opioid Receptor Expression in the Mediobasal Hypothalamus and Effectiveness of Selective Antagonists on Prolactin Release During Lactation

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Abstract

Endogenous opioid peptides are involved in prolactin release during lactation, in part by decreasing tuberoinfundibular dopaminergic (TIDA) neuronal activity. Both μ and κ opioid receptors have a role in the suckling-induced prolactin rise after 4–5h pup deprivation. The aim of this study was to investigate effects of μ opioid receptor antagonist, β -funaltrexamine (β -FNA), and κ opioid receptor antagonist, nor-binaltorphimine (nor-BNI), on prolactin secretion and TIDA neuronal activity in lactating rats after 18h pup deprivation. After 4h separation from pups, the suckling-induced prolactin rise was abolished by 16 μ g nor-BNI and 5 μ g β -FNA, coincident with increased dihydroxyphenylacetic acid (DOPAC):dopamine ratio in the stalk-median eminence (SME). However, after 18h pups separation, these same doses of nor-BNI and β -FNA did not alter the prolactin surge or DOPAC:dopamine ratios in the SME. Higher doses of nor-BNI (32 μ g) and β -FNA (10 μ g) were required to inhibit suckling-induced prolactin secretion. β -FNA (10 μ g) increased the DOPAC:dopamine ratio in the SME, whereas nor-BNI (32 μ g) treatment had no effect. The μ and κ opioid receptor mRNA levels in the mediobasal hypothalamus were similar to suckled control rats after 4h pup deprivation, but increased 1.4-fold after 18h pup deprivation. These data support involvement of endogenous opioidergic systems in the suckling-induced prolactin rise after a prolonged (18h) period of pup deprivation, as well as the shorter (4h) pup deprivation period previously reported. Suppression of TIDA neuronal activity likely played a part in μ opioid receptor input to the suckling-induced prolactin rise after both 4h and 18h separation, whereas non-dopaminergic input was implicated with κ opioid receptors after 18h pup deprivation. Increased μ and κ opioid receptors gene expression in the mediobasal hypothalamus may contribute to reduced effectiveness of opioid receptor antagonists to block suckling-induced prolactin release after 18h pup deprivation.

Keywords

lactation; tuberoinfundibular; dopamine; endogenous opioid peptides; median eminence; rat

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Successful reproduction in mammalian species is completed with the transition of a reproductively competent female into a state of pregnancy and then lactation. The physiological changes associated with lactation are complex and include increased secretion of prolactin and oxytocin contributing to milk production and ejection, hyperphagia coupled with negative energy balance, reduced fertility, altered stress response and expression of maternal behavior (Mann and Bridges, 2001; Grattan, 2002; Smith and Grove, 2002; Woodside, 2007; Brunton et al, 2008). A number of neurotransmitter and neuropeptide systems in the brain, particularly in the hypothalamus, play a role in integration of peripheral and central signals and are subjected to adaptive changes during lactation (Grattan, 2002; Smith and Grove, 2002; Crowley et al, 2007).

Prolactin plays an essential role in mammals during lactation. Circulating prolactin levels are greatly enhanced by the suckling stimulus and contribute to milk production. Prolactin secretion is regulated through complex interactions among hypothalamic factors. The primary regulator of prolactin secretion is dopamine from tuberoinfundibular dopaminergic (TIDA) neurons (Freeman et al., 2000; Ben-Jonathan and Hnasko, 2001). TIDA neurons originate in the arcuate nucleus and terminate in the median eminence of the hypothalamus. Dopamine is transported to the anterior pituitary gland via the hypophyseal long portal vessels and activates D₂ dopamine receptors on lactotrophs. Dopamine secretion, synthesis and metabolism are reduced in response to the suckling stimulus (de Greef et al., 1981; Selmánoff and Wise, 1981; Demarest et al., 1983). The control of prolactin secretion during lactation also involves input from prolactin releasing factor(s) (Samson et al., 1986; Murai and Ben-Jonathan, 1987; Hyde et al., 1987; Samson et al., 1989). Other neurotransmitter systems, most notably the endogenous opioid peptides, appear to act at the central nervous system level to modulate dopamine and/or prolactin-releasing factor input to the anterior pituitary gland to control prolactin secretion (Arbogast and Voogt, 1998; Callahan et al., 2000; Ben-Jonathan and Hnasko, 2001).

Studies with opioid receptor antagonists and neutralizing antiserum to endogenous opioid peptides have indicated that endogenous opioid peptides play a critical role in suckling-induced prolactin secretion during lactation. Immunoneutralization of β -endorphin, dynorphin, leu-enkephalin or met-enkephalin abolishes suckling-induced prolactin release (Jaworski et al., 1997; Callahan et al., 2000). Naloxone, an opioid receptor antagonist, suppresses the suckling-induced prolactin surge after a period of pup deprivation and in a continuous suckled model (Selmánoff and Gregerson, 1986; Arbogast and Voogt, 1998). Naloxone suppression of suckling-dependent prolactin release is associated with increased tyrosine hydroxylase activity and tyrosine hydroxylase mRNA levels in TIDA neurons (Arbogast and Voogt, 1998). Three major types of opioid receptors, kappa (κ), mu (μ), and delta (δ), mediate the actions of endogenous opioid peptides, dynorphin, endorphin and enkephalin (Minami and Satoh, 1995). Selective μ and κ , but not δ , opioid receptor antagonists suppress suckling-induced prolactin release, indicating that endogenous opioid peptide input is mediated by μ and κ opioid receptors (Baumann MH and Rabii J, 1991; Andrews ZB and Grattan DR, 2003). β -funaltrexamine (β -FNA), a selective μ opioid receptor antagonist, increases TIDA neuronal activity when assessed by 3,4-dihydroxyphenylacetic acid (DOPAC) levels (Andrews ZB and Grattan DR, 2003) or 3,4 dihydroxyphenylalanine (DOPA) accumulation (Callahan P et al., 1996). Nor-binaltorphimine (nor-BNI), a κ opioid receptor antagonist, results in increased DOPAC levels (Andrews ZB and Grattan DR, 2003), but not DOPA accumulation (Callahan P et al., 1996). These studies using selective opioid receptor antagonists were performed after 4-5h pup deprivation, but little is known about the roles of specific opioid receptors in reinitiating lactation after a longer separation period.

The major aim of this study was to explore opioid input to the suckling-induced prolactin rise and opioid peptide interactions with TIDA neurons after a separation period associated with

adaptive changes in dopaminergic and opioidergic neuronal groups in distinct hypothalamic nuclei. Wang et al. (1993) reported that TH mRNA levels in the arcuate nucleus were lower in lactating rats than in diestrous rats, but were restored to diestrous levels at 6 h after pup removal. However, TH mRNA content appeared to increase approximately 2-fold between 6h and 12h after pup removal, whereas the increase between 12h and 24h was more modest. Nahi and Arbogast (2003) reported time-dependent changes in gene expression of endogenous opioid peptides in distinct hypothalamic nuclei. Preproenkephalin mRNA levels in the arcuate nucleus were similar to suckling levels at 6h after pup deprivation, but were comparably suppressed at 12h and 24h thereafter. The 18h time for pup removal was selected as a time point between 12 and 24h, when both dopaminergic and enkephalinergic neurons in the arcuate nucleus show adaptive changes in gene expression, but when pup return restores a suckling-induced prolactin rise.

The objectives of this study were: 1) to investigate effects of β -FNA and nor-BNI on the suckling-induced prolactin surge after 4h or 18h of pup deprivation, 2) to determine effects of β -FNA and nor-BNI on TIDA neuronal activity during a suckling stimulus initiated after 4h or 18h of pup deprivation and 3) to examine μ and κ opioid receptors mRNA levels in the mediobasal hypothalamus after 4h and 18h pup deprivation.

Experimental procedures

Animals

Female 250-300g Sprague-Dawley rats (Charles River, Raleigh, NC) were housed under controlled temperature and a 14:10 light/dark cycle (lights on at 0700h) with free access to food and water. The estrous cycle was monitored by daily cytological examination of vaginal smears. Proestrous females were mated with males. The presence of sperm in vaginal smears was confirmed on the following day. The day of parturition was designated lactation day 0, litters were normalized to 8 pups/dam on lactation day 2 and experiments were performed between 1200h and 1700h on lactation day 7. All animal experiments were conducted according to NIH Guide for the Care and Use of laboratory Animals and approved by the Institutional Animal Care and Use Committee at Southern Illinois University.

Surgical procedures

Stainless steel guide cannulas (23 gauge, 5 mm long; Plastics One, Inc., Roanoke, VA) were implanted under isoflurane anesthesia into the right lateral cerebral ventricle on day 2 of lactation. The co-ordinates were: 0.9 mm posterior from bregma, 1.3 mm lateral from midline, and 4.0 mm vertical from dura. Cannulas were fixed to the skull with stainless steel screws and dental cement, and an inner dummy cannula inserted. The presence of cerebrospinal fluid in the guide cannula at implantation and the cannula track through brain tissue under a dissection microscope at sacrifice were considered verification of proper placement. For rats used to assess suckling-induced prolactin levels, a right jugular vein cannula was implanted under isoflurane anesthesia on day 6 of lactation.

Drug Treatments

All drug solutions were prepared just before injection. The selective κ receptor antagonist, nor-BNI (16 μ g or 32 μ g), and the irreversible μ receptor antagonist, β -FNA (5 μ g or 10 μ g), were dissolved in a 5 μ l sterile saline. Drug or vehicle was injected intracerebroventricularly (i.c.v.) in 5.0 μ l volumes using a stainless steel injection cannula designed to protrude 0.5 mm beyond the tip of the guide cannula. The injection was completed over 30 second, and the injection cannula was left in place for 30 second after the injection. The guide cannula was then sealed to prevent leaking.

Experimental protocol

Experiment 1 evaluated the effect of opioid receptor antagonists on suckling-induced prolactin levels. Pups were removed from lactating dams for either a 4h or 18h separation period and blood samples were collected at the time of separation from the pups. An extension tubing was connected to the jugular cannula at 1h before reuniting with pups to allow for blood collection from undisturbed freely moving rats. Opioid receptor antagonists or vehicle were administered i.c.v. 15 min before pups return on day 7 of lactation. Lower doses of nor-BNI (16 μ g) or β -FNA (5 μ g) were administered to dams deprived of their pups for 4h or 18h, whereas the higher doses of nor-BNI (32 μ g) or β -FNA (10 μ g) were administered only to dams separated from pups for 18h. Serial blood samples were collected prior to return of pups and at 15 min, 30 min, and 60 min after the onset of suckling. The onset of suckling was defined as the time when at least six pups were attached to nipples. All blood samples were centrifuged and plasma stored at -20°C until prolactin analysis.

Experiment 2 assessed the effect of opioid receptor antagonists on TIDA neuronal activity. A separate group of animals were used for this experiment. Pups were removed from lactating dams for either a 4h or 18h separation period. Opioid receptor antagonists or vehicle were administered i.c.v. 15 min before pup return. Lower doses of nor-BNI (16 μ g) or β -FNA (5 μ g) were administered to dams deprived of their pups for 4h or 18h, whereas the higher doses of nor-BNI (32 μ g) or β -FNA (10 μ g) were administered only to dams separated from pups for 18h. Lactating dams were sacrificed at 60 min after suckling onset. The stalk-median eminence (SME) was dissected with fine scissors using a dissecting microscope and stored at -80°C until catecholamine determination. Trunk blood was collected at the time of sacrifice and serum was stored at -20°C until prolactin analysis.

Experiment 3 examined μ and κ opioid receptors mRNA levels in the mediobasal hypothalamus. Some cycling virgin rats were used on diestrous day 2. Suckled control dams were allowed continuous access to pups. The other two groups of dams were deprived of their pups for either 4h or 18h. Brains were removed and frozen in Friendly Freeze (Fisher Scientific, St. Louis, MO) at -80°C . Brains were equilibrated to -20°C . The mediobasal hypothalamus was punched from the ventral surface of the brain with a 2 mm tissue corer (Fine Science Tools, Foster City, CA) and cut to 1.75 mm depth. The mediobasal hypothalamic fragment contained the arcuate nucleus, median eminence and part of the ventromedial nucleus. Samples were frozen at -80°C until RNA extraction.

Estimation of TIDA neuronal activity

TIDA neuronal activity was assessed using DOPAC:dopamine ratio in the SME. SME samples were homogenized in 400 μ l 0.1N perchloric acid and centrifuged at $13,000 \times g$ for 15 min. The tissue contents of dopamine and DOPAC in the supernatant were determined by HPLC with electrochemical detection (Bioanalytical Systems, Inc., West Lafayette, IN), as described previously (Arbogast and Voogt, 1991). The pellet was solubilized in 0.5N sodium hydroxide and protein content determined using Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA). Dopamine and DOPAC were quantified by comparison with external standards and expressed as pg/ μ g protein.

Radioimmunoassay for prolactin

Plasma and serum prolactin levels were measured by radioimmunoassay using reagents provided by Dr. Albert. F Parlow at the NIDDK National Hormone and Pituitary Program with rat prolactin-RP-3 as the reference preparation. Limit of sensitivity for the assay was 0.25 ng/ml. The intra- and inter-assay coefficients of variation were 11.6% and 7.5%, respectively.

Quantification of opioid receptor mRNA levels

Mediobasal hypothalamic samples were homogenized in 0.8 ml of RNazol™ Bee (Tel-Test Inc, Friendswood, TX) and total RNA isolated according to manufacturer's instructions. Total RNA concentrations were determined spectrophotometrically and samples were stored at -80 °C. First strand cDNA synthesis from 2.5 µg RNA per sample was performed using the Superscript III kit (Invitrogen, Carlsbad, CA).

Specific primers for κ opioid receptor were 5' GGA GTC CCC CAT CCA GAT TTT C 3' and 5' CTG CCA TTG CTG TCC GAT TC 3' corresponding to bases 73 and 192 (NIH accession No. NM017167) and for μ opioid receptor were 5' ACT GGG AGA ACC TGC TCA AAA TC 3' and 5' CGA TAA ATA CAG CCA CGA CCA CC 3' corresponding to bases 749 and 940 (NIH accession No. U35424). GAPDH was used as a reference gene for quantification and primers were forward primer 5' AAC GAC CCC TTC ATT GAC C 3' and reverse primer 5' TCC ACG ACA TAC TCA GCA C 3' corresponding to bases 135 and 325 (NIH accession No. AF106860).

Real-time PCR was performed using the Cepheid Smart Cycler II (Fisher Scientific) and SYBR green jump start mix (Sigma, St. Louis, MO) following the manufacturer's instructions. Samples were run in duplicate. Each reaction contained 1 µl cDNA, 10.5 µl of sterile water, 1 µl primers (0.2 µM each), and 12.5 µl SYBR green jump start mix. The cycling program was: 95° C for 2 min followed by 40 cycles of 95° C for 15 s, 60° C for 30 s and 72° C for 30 s. Simultaneously, samples without template DNA and samples without reverse transcriptase were run as controls and yielded no amplification. Cycle threshold (C_T) versus input concentration was plotted and efficiencies for each primer pair calculated using the equation $E=10^{-1/s}-1$ where s is the slope. Real time PCR efficiency rates for GAPDH, κ and μ genes were 1.86, 1.92, and 1.98, respectively. Melt curve analysis (60° C to 95° C in 0.2° C increments) was performed at the end of the amplification and some samples were subjected to 1.5% agarose gel electrophoresis to examine product purity and verify correct size for the PCR product. Relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Wong and Medrano, 2005). The cycle threshold (C_T) of GAPDH was subtracted from the C_T for opioid receptors for determination of ΔC_T values. The difference between the experimental (pup-deprived lactating and virgin diestrous groups) ΔC_T and control (suckled lactating dams) ΔC_T was determined and the $2^{-\Delta\Delta C_T}$ calculated for each sample. All gene transcription was expressed as an n -fold difference relative to the lactating control dams. Duplicates were averaged and the resulting value used to calculate mean $\pm$ SEM.

Statistical analysis

The effect of opioid receptor antagonists on plasma prolactin levels was analyzed by two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post hoc test. Dopamine concentrations, DOPAC concentrations, DOPAC:dopamine ratios, serum prolactin levels and μ and κ opioid receptor mRNA expression were compared by one-way ANOVA followed by Newman-Keuls post hoc test. Results are expressed as means $\pm$ SEM. $P < 0.05$ was considered statistically significant.

Results

Effects of opioid receptor antagonists on circulating prolactin levels

Circulating prolactin levels were high (150-200 ng/ml) and similar in lactating dams from all treatment groups before separation from their pups (Fig. 1 A-F). Circulating prolactin was reduced to very low (< 5 ng/ml) levels at 4h and 18h after pup removal. Pups were returned to the dams after the 4h or 18h separation period at 15 min after i.c.v. injection of vehicle, β-FNA or nor-BNI.

In saline-treated control dams deprived of pups for 4h, there was a significant effect of time ($F_{4,36}=9.45$, $p<0.001$). Plasma prolactin levels were increased ($p<0.001$) at 15 min to 60 min after pups return and suckling onset, reaching levels of 300 ng/ml after 60 min of exposure to the suckling stimulus (Fig. 1A and 1B). There was a significant treatment effect on prolactin release after β -FNA or nor-BNI treatment in the 4h pup deprivation group ($F_{2,9}=19.45$, $p<0.001$). Suckling-induced prolactin release was inhibited with 5 μ g β -FNA (Fig. 1A) at 15 min ($p<0.05$), 30 min ($p<0.001$) and 60 min ($p<0.001$) or with 16 μ g nor-BNI (Fig. 1B) at 15 min ($p<0.01$), 30 min ($p<0.001$) or 60 min ($p<0.001$) after suckling onset. Pups were observed suckling in all control and opioid receptor antagonist-treated dams.

In saline-treated control dams deprived of pups for 18h, there was a significant effect of time on suckling induced prolactin levels ($F_{4,64}=69.01$, $p<0.001$). Prolactin levels were increased ($p<0.001$) at 15 min to 60 min after pup return and suckling onset, reaching levels of greater than 400 ng/ml within 15 min (Fig. 1C and 1D). In contrast to the 4h pup deprivation studies described above, nor-BNI (16 μ g) and β -FNA (5 μ g) did not block the suckling-induced prolactin release in the 18h pup deprivation group. Plasma prolactin increased within 15 min after suckling onset and remained elevated at 30 min and 60 min in β -FNA-injected (Fig. 1C) and nor-BNI-injected (Fig. 1D) rats. Although treatment effect for low dose opioid receptor antagonists was not significant ($F_{2,16}=3.62$, $p=0.051$) with 18h pup deprivation, the interaction time $\times$ treatment was significant ($F_{8,64}=6.38$, $p<0.01$). Prolactin levels were increased ($p<0.05$) in the β -FNA-treated group at 60 min and peak prolactin levels were delayed ($p<0.05$) at 15 min after nor-BNI treatment. While doses of nor-BNI (16 μ g) and β -FNA (5 μ g) effective with 4h pups separation were not as effective in the 18h pups separation model, higher doses of β -FNA (10 μ g) and nor-BNI (32 μ g) abolished ($F_{2,17}=79.49$, $p<0.001$) the suckling-induced prolactin rise (Fig. 1E and 1F). Prolactin levels were not significantly different from non-suckled levels at 15, 30, and 60 min after suckling onset, but were significantly lower ($p<0.001$) than control rats at all time points in both the nor-BNI- and β -FNA-treated rats.

Effects of opioid receptor antagonists on TIDA neuronal function

TIDA neuronal activity was assessed by the DOPAC:dopamine ratio in the SME at 1h after suckling onset. Following a 4h pups separation period, there was a significant effect of treatment on DOPAC concentrations ($F_{2,21}=3.47$, $p<0.05$) and DOPAC:dopamine ratios ($F_{2,21}=8.61$, $p<0.005$) in the SME. Nor-BNI (16 μ g) and β -FNA (5 μ g) treatment produced a significant increase ($p<0.05$) in DOPAC concentrations (Fig. 2A) with no significant differences in SME dopamine concentrations (Fig. 2B). DOPAC:dopamine ratios (Fig. 2C) in the SME were increased after β -FNA ($p<0.01$) or nor-BNI ($p<0.05$) treatment. TIDA neuronal activity was inversely correlated to circulating prolactin levels. Consistent with results obtained from serial blood sampling in the first set of experiments, there was a treatment effect of serum prolactin levels ($F_{2,11}=6.63$, $p<0.01$). Nor-BNI and β -FNA significantly decreased ($p<0.05$) serum prolactin levels at 1h post-suckling in the 4h pups separated group (Fig. 2D).

In contrast to the 4h group above, nor-BNI (16 μ g) and β -FNA (5 μ g) pretreatment at the same doses did not alter DOPAC concentrations (Fig. 2E), dopamine concentrations (Fig. 2F) or the DOPAC:dopamine ratios (Fig. 2G) after 18h separation from pups. In concordance with the lack of change in DOPAC:dopamine ratio, nor-BNI and β -FNA did not inhibit suckling-induced prolactin release (Fig. 2H).

There was a significant treatment effect on DOPAC concentrations ($F_{2,35}=9.656$, $p<0.001$) and DOPAC:dopamine ratios ($F_{2,35}=10.75$, $p<0.001$) in the SME with a higher dose of β -FNA. β -FNA (10 μ g vs. 5 μ g) increased ($p<0.001$) DOPAC concentrations in SME after an 18h pup deprivation period, whereas a higher dose of nor-BNI (32 μ g vs. 16 μ g) did not alter SME DOPAC concentration (Fig. 2I). There were no significant differences in SME dopamine concentrations in either treatment group (Fig. 2J). Thus, the DOPAC:dopamine ratio in the β -

FNA-treated group was significantly increased ($p<0.001$) compared to saline-treated controls, whereas nor-BNI treatment had no effect on the DOPAC:dopamine ratio (Fig. 2K). Consistent with the serial blood sampling study during 18h separation, a treatment effect was observed on serum prolactin levels ($F_{2,38}=159.5$, $p<0.001$). Prolactin levels were significantly decreased ($p<0.001$) by treatment with 32 μg nor-BNI or 10 μg β -FNA (Fig. 2L).

Opioid receptor mRNA levels in the mediobasal hypothalamus

Given the differential effectiveness of μ and κ opioid receptor antagonists after 4h versus 18h pup deprivation, quantitative real-time PCR technique was used to estimate κ and μ opioid receptor mRNA expression levels in the mediobasal hypothalamus of lactating dams with pups constantly present, lactating dams separated from their pups for 4h or 18h and diestrous day 2 rats. Primer pairs were initially verified by running PCR reactions on a 1.5% agarose gel stained with ethidium bromide. A single band of the expected size was observed for μ opioid receptor (191bp), κ opioid receptor (119 bp) and GAPDH (190 bp). There were no detectable bands in reverse transcriptase-negative samples. The expression levels of μ and κ opioid receptors in the mediobasal hypothalamus of diestrous day 2 rats and lactating rats separated from their pups for 4h was similar to lactating control rats (Fig. 3). After 18h pups separation, there was a significant treatment effect for pup separation on μ and κ opioid receptor mRNA expression ($F_{3,39}=5.17$, $p<0.05$ for μ opioid receptor and $F_{3,40}=4.90$, $p<0.01$ for κ opioid receptor). The mRNA levels for μ and κ opioid receptors in the mediobasal hypothalamus were increased 1.4 fold as compared to control lactating dams ($p<0.05$ for μ opioid receptor and $p<0.01$ for κ opioid receptor). The expression of μ opioid receptor after 18h separation was similar to diestrous levels, whereas κ opioid receptor expression was higher ($p<0.01$) in the 18h pup-deprived lactating rats as compared to diestrous rats.

Discussion

The present study supports the notion that endogenous opioid peptides, acting on both μ and κ opioid receptors participate in suckling-induced prolactin release, in part by suppressing TIDA neuronal activity. These data extend previous findings using 4-5h pup deprivation periods to include the involvement of endogenous opioid peptides acting on μ and κ opioid receptors in reinitiating the suckling-induced prolactin release after a longer (18h) period of pup deprivation. TIDA neurons are a target of μ opioid receptor action after both 4h and 18h pup deprivation. The differential effectiveness of opioid receptor antagonists in preventing suckling-induced prolactin release when reuniting pups with dams after longer periods (18h) of pup deprivation versus shorter periods (4h) of pup deprivation suggests that opioid receptors are regulated during lactation. Indeed, mRNA expression levels in the mediobasal hypothalamus for both μ and κ opioid receptors were increased after 18h pup deprivation, but not after 4h. These data indicate that in addition to regulation of endogenous ligands, the opioidergic systems involved in lactation are also regulated at the level of the receptor.

A 4-5h period of pup deprivation is widely used to remove the suckling stimulus and set circulating prolactin to very low levels. Restoring pups elicits a rapid rise in prolactin levels with onset of a suckling stimulus. Using this paradigm of 4h pup deprivation, our results agree with previous findings that μ and κ opioid receptor antagonists block the suckling-induced prolactin surge (Baumann and Rabii, 1991; Callahan et al., 1996; Andrews and Grattan, 2003). Our data also confirm the finding of Andrews and Grattan (2003) that both μ and κ opioid receptor antagonists cause a marked increase in TIDA neuronal activity using the same 4h pups separation paradigm and identical doses of β -FNA and nor-BNI. On the other hand, Callahan et al. (1996) reported similarly that β -FNA increased DOPA accumulation in pup-deprived and pup-suckling lactating dams, whereas a lower nor-BNI dose did not alter DOPA accumulation under these conditions.

We expanded these initial studies using a 4h pups separation paradigm to an 18h separation paradigm. Transition to a non-lactating state appears to be initiated between the 4h and 18h pup deprivation periods with regard to the opioidergic and dopaminergic systems relevant to this study. Circulating prolactin levels are rapidly reduced after pup removal and are low in both the 4h and 18h separation group (Arbogast and Voogt, 1998). However, the most marked changes in mRNA expression of tyrosine hydroxylase and preproenkephalin in the arcuate nucleus occur only by 12h with modest changes between 12h and 24h (Wang et al., 1993; Nahi and Arbogast, 2003). It is notable that some lactation-induced gene expression changes are reversed within 24h after pup separation such as hypothalamic oxytocin mRNA content on day 4 of lactation (Ghosh and Sladek, 1995) and pituitary gonadotropin releasing hormone receptor (Smith and Reinhart, 1993), whereas other lactation-induced changes are not altered within the 24h time frame, such as neuropeptide Y in a subdivision of the arcuate nucleus (Smith, 1993).

Our data indicate that higher doses of both nor-BNI (32 μ g vs. 16 μ g) and β -FNA (10 μ g vs. 5 μ g) were required to completely suppress suckling-induced prolactin release after 18h as compared to 4h pup deprivation period. Similar to the 4h separation period, DOPAC levels in the SME were higher in β -FNA treated dams after suckling onset as compared to control dams, suggesting that endogenous opioid peptides contribute to the suckling-induced suppression of TIDA neurons activity. There is a common theme irrespective of the pup deprivation time, suggesting that TIDA neurons may be the major target for endogenous opioid peptides acting via the μ opioid receptors and that the μ -receptor-mediated suppression of TIDA neuronal activity contributes to the suckling-induced prolactin rise. It should be noted that these studies do not rule out μ opioid receptor action on prolactin-releasing factor(s).

The role of κ opioid receptors in regulating TIDA neuronal activity during suckling is less clear. Although a higher dose of nor-BNI (32 μ g vs. 16 μ g) was required after 18h as compared to 4h pup deprivation, the κ opioid receptor antagonist prevented the suckling-induced increase in circulating prolactin in both models. However, nor-BNI had differing effects on TIDA neuronal activity. These data suggest that endogenous opioid peptides acting via κ opioid receptors might be exerting their primary action on a prolactin releasing factor(s), rather than TIDA neuronal activity. Indeed, other evidence indicates that decreased TIDA neuronal activity may not completely account for opioid-induced prolactin release and that prolactin-releasing factor(s) may also be involved (Arita and Porter, 1984; Shin, et al., 1988). Data in the current study and data reported by Andrews and Grattan (2003) show increased TIDA neuronal activity and suppressed prolactin with 16 μ g nor-BNI after a 4h separation period. A divergence between prolactin secretion and TIDA neuronal activity was apparent in a study by Callahan et al. (1996) in which 8 μ g nor-BNI did not alter TIDA neuronal activity after 4-5h pup deprivation and in the present study with 32 μ g nor-BNI and 18h pup deprivation. These differences are likely related to the dose of nor-BNI with higher doses of κ opioid antagonist being effective in increasing TIDA neuronal activity. These data provide evidence that a major pathway for κ opioid receptor regulation of prolactin secretion during lactation involves non-dopaminergic inputs, which are more readily blocked by a selective κ opioid receptor antagonist than a reduction in dopaminergic inputs.

Given that only higher doses of μ and κ opioid receptor antagonists were effective after 18h pup deprivation, we examined μ and κ opioid receptor mRNA expression in the mediobasal hypothalamus, where TIDA neurons are located. Moderate to high expression levels for both μ and κ opioid receptors have been reported in the arcuate nucleus-median eminence region. Immunohistochemical localization and mRNA expression indicate moderate levels of μ opioid receptor in the arcuate nucleus (Mansour et al., 1994; Mansour et al., 1995; Ding et al., 1996), whereas the κ opioid receptor is the most prominent opioid receptor subtype expressed in the arcuate nucleus and median eminence (Mansour et al., 1994; Mansour et al., 1995).

Quantification of μ and κ opioid receptors mRNA levels in mediobasal hypothalamus showed a 1.4-fold increase in expression for dams separated 18h from pups, whereas no significant change was observed with 4h separation. These findings indicate that μ and κ opioid receptor expression is regulated during lactation and that the transition to a non-lactating state results in upregulation of these receptors. It is notable that the increased expression of μ opioid receptor was similar to diestrous levels, whereas κ opioid expression was lower on diestrus, suggesting that the 18h separation-induced increase in κ receptor expression may be transient. Although our study did not address a role for steroid hormone, changes in circulating steroid levels after pup removal could contribute to opioid receptor expression. Indeed, progesterone levels are high in rats during mid-lactation and rapidly decrease after litter removal (Taya and Greenwald, 1982; van der Schoot et al., 1978). Progesterone modulates μ opioid receptor mRNA levels in the arcuate nucleus (Petersen and LaFlamme, 1997), but less is known about progesterone regulation of κ opioid receptors in rats. Since number and functional activity of opioid receptors present in the plasma membrane may be modulated by receptor internalization, desensitization, and phosphorylation (Borgland, 2001), it may be important to explore other aspects of opioid receptor regulation and ovarian steroid input in future studies.

It is not clear if changes in opioid receptor expression are subsequent to the down regulation of the endogenous opioid peptide input or if opioid receptor changes are regulated independently of opioidergic neuronal activity. Studies of opioid peptide mRNA expression in different regions of the brain provide some indication of which endogenous opioid peptide systems may be involved in the lactational process. Proenkephalin mRNA levels in the arcuate nucleus and prodynorphin mRNA levels in the paraventricular nucleus are higher during lactation than diestrus (Ottinger et al., 1995; Eriksson et al., 1996). Removal of the suckling stimulus results in a decrease in proenkephalin mRNA levels in the arcuate and ventromedial nuclei of the hypothalamus (Nahi and Arbogast, 2003). Enkephalin peptide immunoreactivity in the arcuate nucleus is increased during lactation and is localized to TIDA neurons (Merchenthaler, 1993). Hyperprolactinemia induces enkephalin expression in the hypothalamus (Merchenthaler et al., 1995) and elevated prolactin levels during lactation are responsible for high proenkephalin mRNA levels during lactation (Nahi and Arbogast, 2003). In contrast, proopiomelanocortin (POMC) mRNA signal levels in the arcuate nucleus and periaqueductal area are less in lactating than diestrous rats (Smith, 1993; Mann et al., 1997). Removal of the suckling stimulus results in an increase in POMC mRNA levels within 3h after pup deprivation (Nahi and Arbogast, 2003).

The recently discovered endogenous opioid peptide, orphanin FQ (OFQ) binds to its own unique receptor, opioid receptor like-1 (ORL-1) (Meunier et al., 1995; Reinscheid et al., 1995). OFQ is a potent stimulus for prolactin secretion and it mediates this effect by rapid inhibition of TIDA neuronal activity (Chesterfield et al., 2006). This effect of OFQ is mediated via actions at ORL-1 receptor subtype and OFQ contributes to suckling-induced prolactin release (Chesterfield et al., 2006). Since moderate to dense OFQ binding, receptor mRNA expression (Neal et al., 1999) and immunoreactivity (Anton et al., 1996) is found in arcuate nucleus, further research is required to determine if ORL-1 expression is changed during lactation.

In conclusion, results from this study indicate μ and κ opioid receptors play an important role in reinstating suckling-induced prolactin release at a time when gene expression changes are beginning to reset hypothalamic opioidergic and dopaminergic neuronal systems to a non-lactational state. These data reinforce that both μ and κ opioid receptors contribute to suckling-induced prolactin release and support the importance of opioid peptide-TIDA interactions during lactation. Taken together with previous studies on opioid peptide gene expression, the changes in μ and κ opioid receptor mRNA expression point to wide spread alterations at the peptide and receptor levels for the hypothalamic opioidergic systems during lactation.

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Abbreviations

TIDA	Tuberoinfundibular dopaminergic
β -FNA	β-funaltrexamine
nor-BNI	norbinaltorphimine
ORL-1	opioid receptor like-1
OFQ	orphanin FQ
DOPAC	3,4 dihydroxyphenylacetic acid
DOPA	3,4-dihydroxyphenylalanine
SME	stalk-median eminence
i.c.v.	intracerebroventricularly

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Figure 1.

Plasma prolactin (ng/ml) profiles from lactating dams deprived of pups for 4h (**A, B**) or 18h (**C, D, E, F**) and injected i.c.v. at 15 min before pup return with β -funaltrexamine (β -FNA) at a dose of 5 μ g/5 μ l (**A, C**) or 10 μ g/5 μ l (**E**) or with nor-binaltorphimine (nor-BNI) at a dose of 16 μ l/5 μ l (**B, D**) or 32 μ g/5 μ l (**F**). Plasma prolactin profiles (open square) of dams deprived of pups for 4h and injected i.c.v. with saline (5 μ l, n=5) are repeated in panels **A** and **B**. Dams deprived of pups for 4h (at -240 min) were injected with (**A**) β -FNA (5 μ g/5 μ l, n=3) or (**B**) nor-BNI (16 μ g/5 μ l, n=4) at 15 min before time 0 min and allowed to suckle for 60 min. Plasma prolactin profiles (open square) of dams deprived of pups for 18h and injected i.c.v. with saline (5 μ l, n=9) are repeated in panels **C, D, E** and **F**. Dams deprived of pups for 18h (at -1080 min) were injected with (**C**) β -FNA (5 μ g/5 μ l, n=5), (**D**) nor-BNI (16 μ g/5 μ l, n=6), (**E**) β -FNA (10 μ g/5 μ l, n=6), or (**F**) nor-BNI (32 μ g/5 μ l, n=6) at 15 min before time 0 min and allowed to suckle for 60 min. Each value represents a mean $\pm$ SEM. Significance level compared to saline-treated controls (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 2.

3,4-dihydroxyphenylacetic acid (DOPAC) content (A, E, I), dopamine content (B, F, J), DOPAC:dopamine ratio (C, G, K) in the SME and serum prolactin concentration (D, H, L) on day 7 of lactation after i.c.v. injection of nor-BNI, β -FNA or saline. Lactating dams deprived of pups for 4h were injected with nor-BNI (16 μ g/5 μ l, n=6), β -FNA (5 μ g/5 μ l, n=6) or saline (5 μ l, n=13) at 15 min before pup return and sacrificed at 60 min after suckling onset (A, B, C, D). Lactating dams deprived of pups for 18h were injected with nor-BNI (16 μ g/5 μ l, n=6), β -FNA (5 μ g/5 μ l, n=6) or saline (5 μ l, n=11) at 15 min before pup return and sacrificed at 60 min after suckling onset (E, F, G, H). Lactating dams deprived of pups for 18h were injected with nor-BNI (32 μ g/5 μ l, n=14), β -FNA (10 μ g/5 μ l, n=14) or saline (5 μ l, n=15) at 15 min before pup return and sacrificed at 60 min after suckling onset (I, J, K, L). Each value represents a mean $\pm$ SEM. Significance level compared to saline-treated controls (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 3.

(**A**) μ opioid receptor and (**B**) κ opioid receptor mRNA expression (normalized to GAPDH) levels in the mediobasal hypothalamus of suckled lactating dams, 4h pup-deprived dams, 18h pup-deprived dams and diestrous rats. Data show fold differences, relative to suckled lactating rats. Each value represents a mean $\pm$ SEM of determinations from six to nine rats for pup-deprived and diestrous rats and 18 rats for suckled lactating dams used as control. Significance level compared to suckled dams (* $p < 0.05$, ** $p < 0.01$).