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Effects of Naloxone Infusion on Nocturnal Prolactin Secretion and Fos/FRA Expression in Pregnant Rats

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Dopamine (DA) secreted by tuberoinfundibular dopaminergic (TIDA) neurons in the hypothalamus is the major inhibitory factor controlling prolactin (PRL) secretion from the anterior pituitary. Endogenous opioid peptides (EOPs), mainly the neuropeptide β-endorphin, have a stimulatory influence on PRL secretion. During the first half of pregnancy in rats, PRL secretion is characterized by two daily surges, the nocturnal surge and the diurnal surge. We tested the hypothesis that EOPs are critical stimulatory factors in regulating the nocturnal PRL surge, possibly via inhibition of TIDA neuronal activity. Naloxone (NAL) , an opioid antagonist, was continuously infused (0.2 mg/10 μL/min iv) during the expected time of the nocturnal PRL surge in day 8 pregnant rats. Radioimmunoassay (RIA) was used to measure plasma PRL levels, and the immunocytochemical (ICC) staining of Fos/FRA was performed to detect changes in transcriptional activity of neurons in the hypothalamus. ICC of tyrosine hydroxylase (TH), the rate-limiting enzyme for DA synthesis, was performed to visualize TIDA neurons in the arcuate nucleus. The results showed that the nocturnal surge of PRL was markedly delayed and dampened in NAL-treated rats ($p < 0.01$). The numbers of both Fos/FRA and (Fos/FRA)/TH dual-staining neurons increased in the arcuate nucleus following NAL treatment ($p < 0.05$ for both comparisons).

These data indicate that EOPs are critical stimulatory factors for the nocturnal PRL surge and that the actions of EOPs are partially mediated via decreasing TIDA neuronal activity.

Key Words: Endogenous opioid peptides; β-endorphin; tuberoinfundibular dopaminergic; prolactin; naloxone.

Introduction

During early pregnancy in the rat, pituitary prolactin (PRL) secretion is characterized by two daily surges *(1)*. One is the nocturnal PRL surge (0100–0600 h), and the other is the diurnal PRL surge (1700–2100 h). PRL secretion, especially that seen during the nocturnal PRL surge, is very important in stimulating the corpus luteum to secrete progesterone in order to maintain pregnancy in the rat *(2,3)*.

Numerous studies have established that dopamine (DA) secreted from tuberoinfundibular dopaminergic (TIDA) neurons is the major inhibitory factor of PRL secretion *(4)*. The cell bodies of the TIDA neurons are located in the arcuate nucleus, and their terminals project to the median eminence *(5)*. Many experiments also have demonstrated that endogenous opioid peptides (EOPs), particularly β-endorphin, are significant positive regulators of PRL secretion *(6–8)*. The μ receptor is recognized as the predominant receptor of EOPs *(9)*. Administration of β-endorphin stimulates PRL secretion in many circumstances, such as during proestrus *(8)*, stress *(7)*, and lactation *(10)*. Naloxone (NAL) is a potent nonselective opioid receptor antagonist. It lowers both basal *(11)* and stimulated *(8,10)* PRL levels.

The arcuate nucleus is a major source of both β-endorphin *(12)* and TIDA neurons *(13)*. Contacts between β-endorphin axon terminals and TIDA neurons in the arcuate nucleus have been described *(14,15)*. Opioid μ, δ, and κ receptors and/or their mRNA are abundantly distributed in the rat hypothalamus *(16)*. EOPs may exert their stimulatory action on PRL secretion by inhibiting TIDA neuronal activity. Existing data support a role for EOPs in influencing hypothalamic DA neuronal activity and DA synthesis, release, and turnover *(17–19)*.

In our previous study, NAL treatment during the nocturnal surge in pregnant rats suppressed PRL secretion, whereas dihydroxyphenylalamine (DOPA) accumulation in the stalk median eminence of the hypothalamus was shown to increase significantly *(20)*. In this previous experiment, DOPA accumulation in the median eminence was used as an indicator of TIDA neuronal activity in the hypothalamus. DOPA accumulation in the median eminence is a measure of all tyrosine hydroxylase (TH) neurons, which have terminals in that location. Although a measure of TH

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activity is a powerful tool in assessing neuronal activity, it does not present a complete picture. In order to look at subpopulations of TIDA neurons in the arcuate nucleus, dual labeling of Fos/FRA and TH was done to monitor the transcriptional activity of TIDA neurons in pregnant rats treated with NAL.

Some reports have suggested that different regions of the arcuate nucleus may be differentially involved in the regulation of PRL secretion *(21,22)*. We divided the arcuate nucleus into the rostral and caudal portions, and processed the data separately in order to determine whether such regional differences exist.

Result

Changes in Plasma PRL Levels During NAL Treatment (Fig. 1)

In two groups of pregnant rats, NAL or saline (SAL) was infused continuously beginning at 2400 h on d 7 to 1400 h on d 8, and blood was collected once every 2 h. In SAL-treated rats, a large nocturnal PRL surge was observed with basal levels of 50 ng/mL at 2400 h to peak values of approx 500 ng/mL at 0200 h, returning to basal levels by 0600 h. In NAL-treated rats, the nocturnal PRL surge was delayed and dampened. Throughout the NAL treatment, PRL remained relatively low, with a small, but significant peak at 0600 h. This may reflect an escape from the inhibitory effects of NAL.

Expression of Fos/FRA After NAL Treatment (Fig. 2–4)

The arcuate nucleus was divided into a rostral and a caudal sections, based on reports suggesting that there are functional differences regarding PRL regulation within the arcuate nucleus *(21,22)*. Fos/FRA expression was seen in all areas of the arcuate nucleus in both SAL, and NAL-treated animals (Figs. 2 and 3). Over time, Fos/FRA expression in control rats did not change. However, under NAL treatment, Fos/FRA expression increased significantly at 0200 and 0400 h in the rostral and at 0200, 0400, and 0600 h in the caudal arcuate nucleus compared to controls (Fig. 4).

Fos/FRA Positive TH Neurons in the Arcuate Nucleus (Figs. 5 and 6)

TH neurons that show transcriptional activity as measured by Fos/FRA immunocytochemistry (ICC), are concentrated in the dorsal-medial part of the rostral arcuate nucleus, and are upregulated by NAL treatment (Fig. 5). In the caudal arcuate nucleus, there are less TH-positive neurons (photomicrograph not shown). As seen in Fig. 6, the percentage of TH neurons in the rostral arcuate nucleus which are also Fos/FRA positive at 0200 and 0400 h was lower than that at 1400 h ($p < 0.05$) in controls. After NAL treatment , the percentage of Fos/FRA-positive TH neurons increased significantly at 0200 and 0400 h (Fig. 6) (Fisher's protected least-significant-difference test [PLSD]; $p < 0.05$). In the caudal arcuate nucleus of control rats, the percentage of Fos/FRA-positive TH neurons did not vary over time. However, NAL treatment increased the percentage of Fos/FRA-positive TH neurons at both 0200 and 0400 h (Fisher's PLSD; $p < 0.05$).

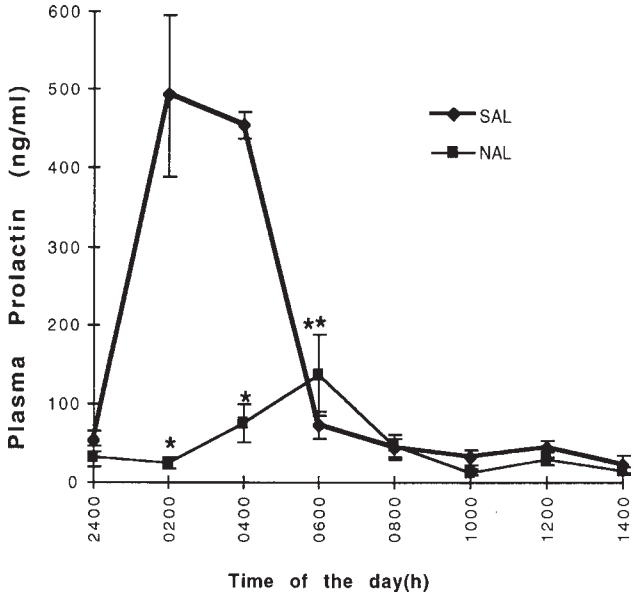

Fig. 1. Pregnant rats received a continuous infusion of SAL or NAL (2 mg/10min) beginning at 2400 h on d 7 to 1400 h on d 8. There was a surge of PRL during the 2400–0600h period in SAL infused rats. PRL secretion was markedly inhibited in NAL-infused rats. ANOVA showed that the changes in PRL levels over time are significant, $p < 0.0001$; the difference between treatments is significant, $p < 0.01$. Vertical lines represent the SE of the mean in this and all subsequent experiments. n = 5–6/group. *Significantly lower than SAL-treated animals (Fisher's PLSD; $p < 0.05$). **Significantly higher than any other time points in NAL-treated animals (Fisher's PLSD; $p < 0.05$) except 0400 h ($p = 0.07$).

Discussion

The regulation of PRL release is a complex process involving various hypothalamic and nonhypothalamic factors *(23–24)*. EOPs are positive regulators of PRL secretion, and opioid administration increases circulating PRL levels *(6–8)*. Among EOPs, β-endorphin is the most potent stimulator of PRL secretion. Even though three major opioid receptors are present within the hypothalamus, the μ receptor is generally recognized to be the predominant one for PRL control *(9,25)*. NAL is a potent μ-opioid receptor antagonist with some antagonism of δ and κ sites at higher concentrations *(26)*. The route and dosage of NAL treatment selected for our experiments were previously shown to block the nocturnal PRL surge effectively *(20)*.

Reports have demonstrated that NAL can lower both basal *(11)* and stimulated PRL *(8,10)* levels. In our experiment, infusion of NAL delayed and dampened plasma PRL levels rapidly(Fig. 1). This result is consistent with our previous report *(20)*, in which the amount of DOPA accu-

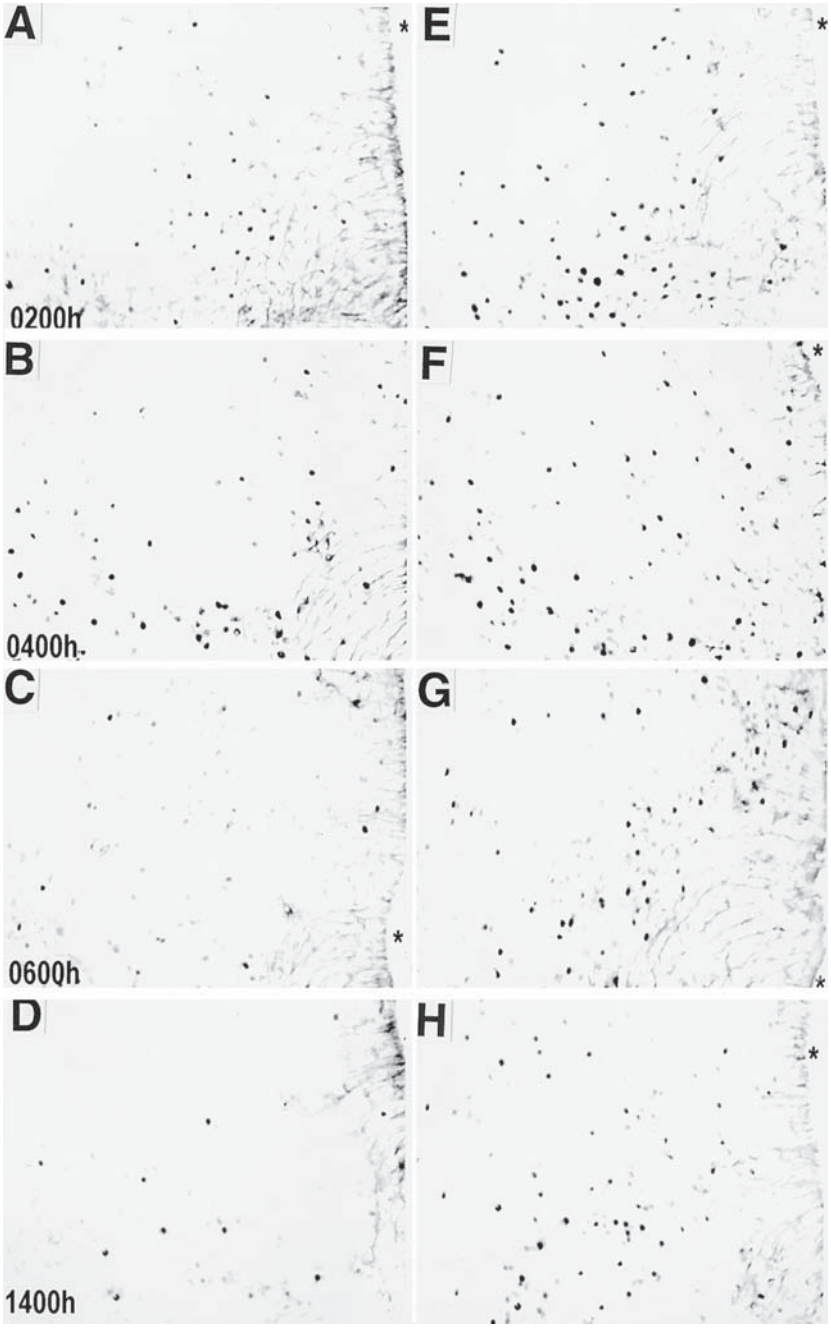

Fig. 2. Pregnant rats were infused with either SAL **(A–D)** or NAL **(E–H)** continuously beginning at 2400 h on d 7 to 0200, 0400, 0600, and 1400 on d 8. Photomicrographs show Fos/FRA expression in the rostral arcuate nucleus increased during NAL infusion. *Third ventricle; magnification, ×100.

mulation in the stalk median eminence after NAL treatment increased, an indication that the activity of the enzyme TH increased. Since DOPA is synthesized by all neurons containing TH, it is not possible to differentiate specific regions of the arcuate nucleus involved in the regulation of PRL. In the present study, the actual percentage of TH neurons that were transcriptionally active was measured in both the rostral and caudal arcuate nucleus.

During the intersurge time between 0600 and 1400 h, PRL levels were low. However, even with continuous NAL infusion, a small but significant increase of PRL secretion at 0600 h was observed (Fig. 1). The mechanisms responsible for this delayed and attenuated PRL surge during the NAL treatment are not known. Additional hypothalamic stimulatory factors or prolactin-releasing factors (PRFs) may be involved in activating the PRL surge during NAL treatment *(27–29)*.

The arcuate nucleus was divided into rostral and caudal regions. Fos/FRA expression (Fig. 4) in SAL-infused rats is relatively constant in both the rostral and caudal regions

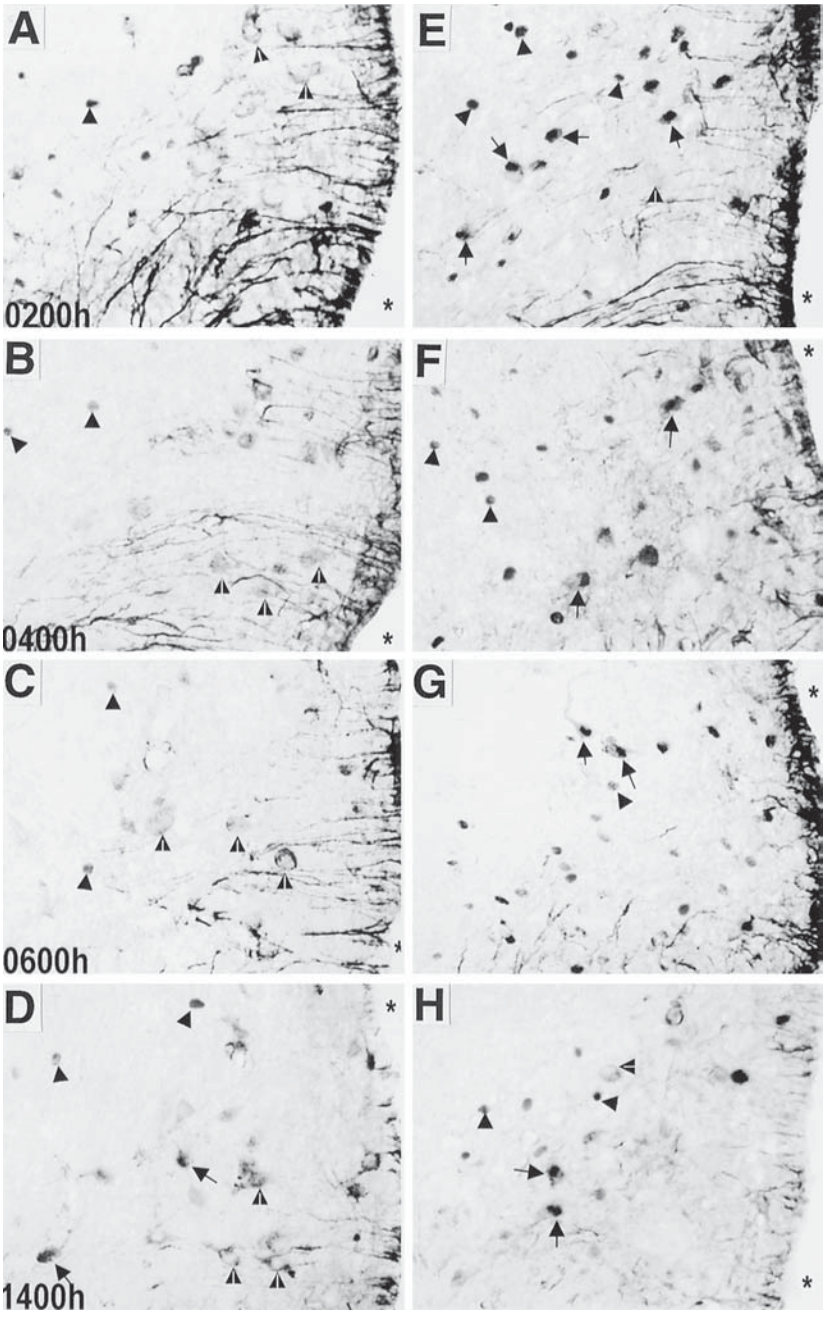

Fig. 3. Pregnant rats were infused with either SAL (**A–D**) or NAL (**E–H**) continuously beginning at 2400 h on d 7 to 0200, 0400, 0600, and 1400 h on d 8. Photomicrographs show Fos/FRA expression in the caudal arcuate nucleus increased with NAL infusion. *Third ventricle; magnification, ×100.

of the arcuate nucleus over time. After NAL treatment, a significant increase in Fos/FRA expression was observed at 0200 and 0400 h in the rostral arcuate nucleus and at 0200, 0400, and 0600 h in the caudal arcuate nucleus. This demonstrates that NAL treatment increases the neuronal transcriptional activity in the arcuate nucleus, especially during the PRL surge time. The rational for dividing the arcuate nucleus into two regions is based on previous reports. For example, during lactation, TH gene expression is significantly suppressed in TIDA neurons in the rostral, but not in the caudal arcuate nucleus *(21)*. In another study, suckling induced Fos expression only in subgroups of hypothalamic POMC neurons located in the rostral part of the arcuate nucleus *(22)*. Although we did not see significant differences between the rostral and caudal arcuate nucleus in terms of the percentages of TIDA neurons, which are also Fos/FRA-positive after NAL infusion, there were many more TIDA neurons in the rostral arcuate nucleus than in the caudal area. The number of TIDA neurons in the caudal arcuate nucleus is only about one-sixth of that in the

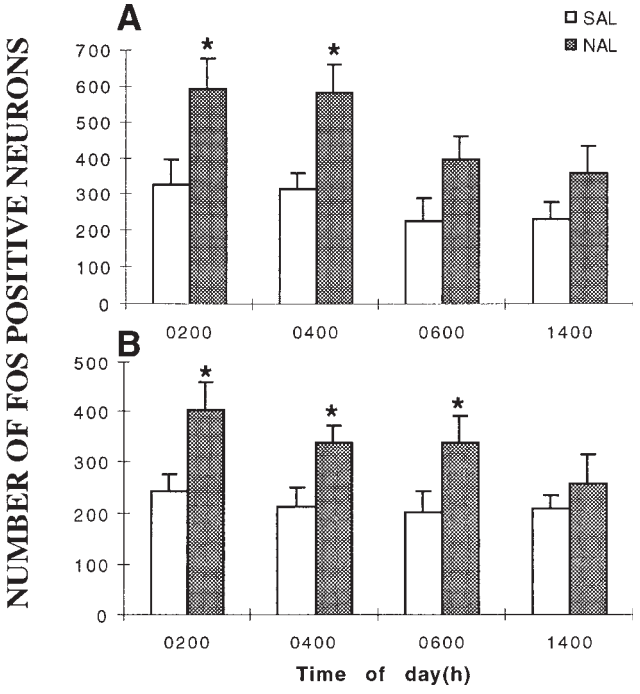

Fig. 4. Pregnant rats received continuous infusion of SAL or NAL beginning at 2400 h on d 7 to 0200, 0400, 0600, and 1400 h on d 8. In the rostral **(A)** and caudal **(B)** arcuate nuclei, respectively, Fos/FRA expression increased at 0200 and 0400 h after NAL treatment compared to SAL treatment, and at 0200, 0400, and 0600 h compared to SAL treatment. *Significantly higher than SAL-treated animals (n = 5–6, Fisher's PLSD; $p < 0.05$). Two-way ANOVA test showed that the difference between treatments is significant ($p < 0.001$).

rostral arcuate nucleus. Even though EOPs may inhibit TH neuronal activity equally well in both areas of the arcuate nucleus, the TIDA neurons in the rostral area could bear more responsibility in mediating EOPs' effects on PRL secretion.

The total number of TH positive neurons (data not shown) in the arcuate nucleus did not change significantly over time or following NAL treatment. Thus, changes in TH neuronal numbers are not likely a mechanism involved in EOPs' regulation of the nocturnal PRL surge. Other approaches, such as the modulation of TH activity, TH mRNA synthesis, dopamine (DA) synthesis, or DA release may be more important *(17–19)*. The differences in the number of TH positive neurons in the rostral and caudal regions of the arcuate nucleus may account for differences in the involvement of these regions in PRL control.

As indicated above, after NAL treatment, Fos/FRA expression increased significantly in the arcuate nucleus. The identity of the activated neuronal populations was determined by dual (Fos/FRA)/TH staining. TIDA neurons in the arcuate nucleus are at the center of PRL regulation. Since it has been postulated that β-endorphin neurons function by inhibiting TIDA neurons, it would be reasonable to look at the change in TIDA neuronal activity after NAL treatment. Consistent with our Fos/FRA expression data (Fig. 4), NAL treatment also resulted in an increase in percentage of Fos/FRA-positive TH neurons in all areas of the arcuate nucleus at 0200 and 0400 h. Thus, NAL treatment during the PRL surge was associated with an increased TIDA neuronal activity in the arcuate nucleus. Even though some studies suggest that EOPs exert their action directly on TIDA neurons *(15)*, this is still controversial. Few of the TIDA perikarya receive direct input from β-endorphin neurons *(14,30)*. Our results demonstrated that there were many more Fos/FRA expressing neurons than Fos/FRA-positive TIDA neurons in the arcuate nucleus, and NAL treatment activated these neurons as well. This suggests there may be additional pathways by which β-endorphin stimulates PRL.

In conclusion, EOPs, especially β-endorphin, play a critical stimulatory role in nocturnal PRL surge regulation, and do this at least in part by inhibiting either directly or indirectly TIDA neuronal activity in the arcuate nucleus. The data also suggest that the rostral region of the arcuate nucleus is more important than the caudal region in regulating the nocturnal PRL surge in pregnant rats.

Materials and Methods

Animals

Adult female Sprague-Dawley rats (Sasco Co., Omaha, NE) weighing 180–200 g were housed in a temperature- (24°C) and light- (0600–1800 h) controlled room. Animals were provided with food and water *ad libitum*. Estrous cycles were monitored by daily vaginal lavage. A female was paired with a male on the day of proestrus for the purpose of mating. The first appearance of sperm in the lavage was designated as day 0 of pregnancy. The right jugular vein was cannulated under ether anesthesia on d 6 of pregnancy. The cannula was exteriorized through the back of the neck and extended outside of the cage. Patency of the cannula was maintained by flushing with 20 IU heparin/mL saline (Hep-Sal) solution.

Preparation of Samples for ICC and RIA

The cannula in the right jugular vein of each rat was connected to a peristaltic pump. NAL (Sigma Chemical Co.) (0.2 mg/10 μL/min) or SAL was infused started at 2400 h on d 7 of pregnancy and continued to either 0200, 0400, 0600, or 1400 h on d 8. Blood samples were collected (0.3 mL/sample) every 2 h beginning at 2400 h. After the last sample was taken, rats were injected with an over-dose of sodium phenobarbital (1 mL/Kg body wt) through the cannula. Immediately following injection, rats were perfused transcardially with 150 mL SAL followed by 200 mL 4% paraformaldehyde (Polysciences Inc., Warrington, PA) in potassium phosphate buffer solution (KPBS, 0.05 *M*, pH 7.6). Fixed brains were removed and then postfixed in the same fixative overnight. Brains were

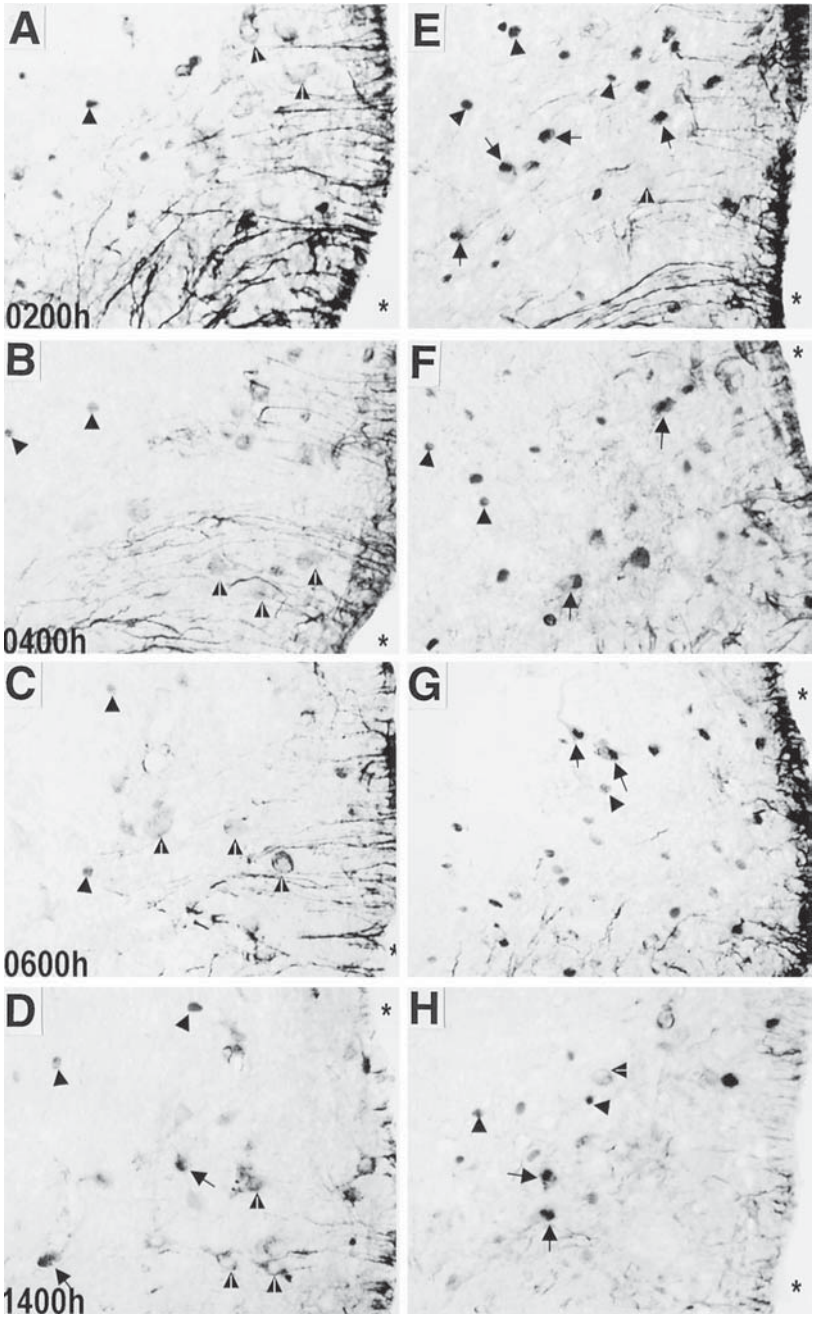

Fig. 5. Pregnant rats were infused with either SAL **(A–D)** or NAL **(E–H)** continuously beginning at 2400 h on d 7 to either 0200, 0400, 0600, or 1400 h on d 8. Photomicrographs show Fos/FRA and TH dual-staining in the rostral arcuate nucleus. *Third ventricle; arrowhead, Fos/FRA-positive neurons; broken arrowhead, TH-positive neurons; arrow with tail, (Fos/FRA)/TH dual-labeled neurons; Magnification, ×200.

immersed into 25% sucrose solution (in 0.05 *M* KPBS, pH 7.6) until they sank to the bottom of the container. Sucrose-saturated brains were quickly frozen in the HistoFreeze™-2000 (Fisher, Pittsburgh, PA) for 10–15 s, wrapped with foil, and kept in a –70°C freezer. Sectioning was done with a cryostat (Reichert-Jung 1800, Germany) at 25 μm. Sections were mounted on slides, stored at –70°C until the ICC was performed.

RIA

Plasma PRL concentrations were determined by RIA methods with materials supplied by the NIDDK and expressed in terms of the rat PRL RP-3 standard. The sensitivities of the PRL RIAs (defined as the amount of unlabeled PRL that caused a 10% displacement of iodinated PRL from antibody) ranged from 0.05 to 0.1 ng/tube. Since 50 μL of serum were routinely used in the assay, the sensi-

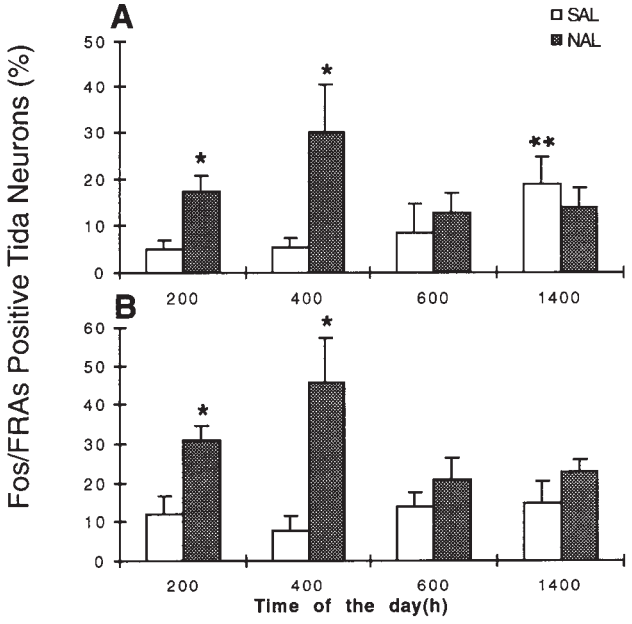

Fig. 6. Pregnant rats received continuous infusion of SAL or NAL beginning at 2400 h on d 7 to either 0200, 0400, 0600, or 1400 h on d 8. In both the rostral **(A)** and caudal **(B)** arcuate nucleus, NAL treatment caused a significant increase of the percentage of (Fos/FRA)/TH dual-labeled neurons in 0200 and 0400 h groups. Two-way ANOVA shows that the difference between two treatments is significant ($p < 0.001$). *Significantly higher than SAL-treated animals ($n = 3–4$, Fisher's PLSD; $p < 0.05$), **Significantly higher than that at 0200 and 0400 h in the SAL-treated animals ($p < 0.05$).

tivity was 1–2 ng/mL serum. The interassay coefficient of variation was 10%, and the intraassay coefficient of variation was 5%.

ICC

Fos/FRA antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). These antibodies were raised in rabbits against a highly conserved domain of p62 Fos of human origin (amino acid 128–152). This region of Fos is identical in the human, mouse, rat, and chicken. According to the data provided by the company, the specificity of the antibody is broad, recognizing c-Fos, FosB, Fra-1, and Fra-2. Every third section from the anterior commissure (Bregma -0.26mm) to the end of the arcuate nucleus (Bregma -4.52mm) was analyzed, according to the rat brain atlas *(31)*.

Brain sections were washed with KPBS (0.05 *M*, pH 7.6) and treated with 0.6% hydrogen peroxide in methanol (Fisher Scientific) to quench endogenous peroxidase activity, and with 1% normal goat serum in KPBS to reduce nonspecific binding. The sections were then incubated with the Fos/FRA antibody diluted at 1:6000 in KPBS containing 2% normal goat serum and 0.01% thimerosal for 2 d at 4°C. Brain sections were incubated consecutively with biotinylated goat antirabbit immunoglobulin (1:600, Vectastain Elite Kit) in KPBS with 0.4% Triton X-100 for 1 h at room temperature, and with avidin DH-biotinylated horseradish peroxidase-H complex (45 μL of each A and B reagents/10 mL KPBS with 0.4% Triton X-100, Elite Kit) for another 1 h at room temperature. The sections were rinsed in 0.175 *M* sodium acetate buffer (pH 6.5) prior to visualization of Fos/FRA immunoreactivity. The brain sections were developed with 3,3'-Diaminobenzidine (0.2 mg/mL), nickel sulfate (25 mg/mL) and hydrogen peroxide (0.83 μL of 3% solution/mL) in the same acetate buffer for 20 min.

After the visualization of Fos/FRA signals, KPBS-rinsed brain sections were treated again with 0.6% hydrogen peroxide in methanol and with 1% normal horse serum in KPBS. Sections were then incubated with a mouse monoclonal TH antibody (Chemicon Inc.) diluted at 1:100,000 in KPBS containing 2% normal horse serum, 0.4% Triton X-100, and 0.01% thimerosal for 2 d at 4°C. We chose this antibody concentration after we tried several others. This dilution appeared to be the best for our purpose. The antibody has a broad species crossreactivity that includes the rat. After incubation with the TH antibody, sections were incubated serially with biotinylated horse antimouse immunoglobulin (1:600, Vectastain Elite Kit; Vector Lab., Burlingame, CA) in KPBS with 0.4% Triton X-100 for 1 h at room temperature, and with avidin DH-biotinylated horseradish peroxidase-H complex (45 μL of each A and B reagents/10 mL KPBS with 0.4% Triton X-100, Elite Kit) for another 1 h at room temperature. At this time, the sections were rinsed in Tris buffer (0.05 *M*, pH 7.25) before visualization of TH immunoreactivity. 3,3'-Diaminobenzidine (0.2 mg/mL) and hydrogen peroxide (0.83 μL of 3% solution/ml) in the same Tris buffer were used to visualize TH signals. Finally, sections were rinsed in KPBS, dehydrated in an ethanol series, cleared in Histoclear (Histo-Clear, National Diagnostics, Atlanta, GA) and cover slipped with DPX.

Fos/FRA-positive signals were confined to the nucleus of the cell. Each area of interest was defined according to the rat brain atlas *(31)*. The number of Fos/FRA, TH, and (Fos/FRA)/TH dual-labeled neurons were counted by visual inspection with the aid of a microscope. The arcuate nucleus was divided into rostral and caudal portions also according to the rat brain atlas *(31)*. The rostral region extends from Bregma –2.12mm to –3.60mm, whereas caudal region extends from Bregma –3.60mm to –4.30mm *(32)*. The appearance of the infundibulum was the demarcation between the two areas.

Statistics

Results are expressed as the mean ± SE. Two-way repeated measures ANOVA was used to analyze plasma PRL levels from rats treated with SAL or NAL. Two-way ANOVA was used to analyze Fos/FRA expression, total TH neurons, and (Fos/FRA)/TH dual-staining. Post-hoc

comparisons were performed with Fisher's PLSD. Unpaired *t*-test was used to test the difference between treated and control animals at each time point. The statistics program, StatView 512+ (Brainpower, Agoura Hills, CA), was used to analyze all data.

Acknowledgments

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