Pup removal suppresses estrogen-induced surges of LH secretion and activation of GnRH neurons in lactating rats

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Abstract

During lactation, the suckling stimulus exerts profound influences on neuroendocrine regulation in nursing rats. We examined the acute effect of pup removal on the estrogen-induced surge of LH secretion in ovariectomized lactating rats. Lactating and nonlactating cyclic female rats were given an estradiol-containing capsule after ovariectomy, and blood samples were collected through an indwelling catheter for serum LH determinations. In lactating, freely suckled ovariectomized rats, estrogen treatment induced an afternoon LH surge with a magnitude and timing comparable to those seen in nonlactating rats. Removal of pups from the lactating rats at 0900, 1100, or 1300 h, but not at 1500 h, suppressed the estrogen-induced surge that normally occurs in the afternoon of the same day. The suppressive effect of pup removal at

0900 h was completely abolished when the pups were returned by 1400 h. In contrast, pup removal was ineffective in abolishing the stimulatory effect of progesterone on LH surges. Double immunohistochemical staining for gonadotropin-releasing hormone (GnRH) and c-Fos, a marker for neuronal activation, revealed a decrease, concomitantly with the suppression of LH surges, in the number of c-Fos-immunoreactive GnRH neurons in the preoptic regions of nonsuckled rats. An LH surge was restored in nonsuckled rats when 0.1 µg oxytocin was injected into the third ventricle three times at 1-h intervals during pup removal. These results suggest that the GnRH surge generator of lactating rats requires the suckling stimulus that is not involved in nonlactating cyclic female rats. *Journal of Endocrinology* (2006) **191**, 339–348

Introduction

The preovulatory surge of luteinizing hormone (LH) secretion in cyclic rats and the daily surge in ovariectomized, estrogen-treated rats are caused by the central action of estrogen on the gonadotropin-releasing hormone (GnRH) surge generator in the preoptic region, and by its direct sensitization of pituitary gonadotrophs to GnRH (Goodman & Knobil 1981). The activity of the GnRH surge generator is controlled by the integration of a variety of neural and humoral inputs converging upon GnRH neurons (Freeman 1994). The first requirement for the GnRH and subsequent LH surges is the ovarian steroid hormone estrogen (Neill 1972, Legan *et al.* 1975), which acts on estrogen-responsive neurons existing in the limbic–preoptic–hypothalamic regions (Goodman & Knobil 1981). The LH surge induced by estrogen is stimulated and thereafter inhibited when progesterone, another ovarian hormone, acts after estrogen priming (Brann & Mahesh 1991). Secondly, the GnRH surge generator requires information regarding the light–darkness environment (Freeman 1994); a circadian rhythm generated in the hypothalamic suprachiasmatic nucleus in response to light–darkness cycles entrains the GnRH surge generator to produce the GnRH surge at a specific time. Thirdly, the hypothalamic structures regulating LH secretion should be

sexually differentiated to the female type. The LH surge is not induced by estrogen treatment in males or in perinatally androgen-treated females, in both of which the GnRH surge generator undergoes male-oriented sexual differentiation (Gorski 1979). Fourthly, stimulatory and inhibitory neurotransmitters are involved in the neural regulation of the GnRH surge; the noradrenergic system ascending from the brain stem plays a key role in maintaining the activity of GnRH neurons (Ramirez *et al.* 1984), whereas tonic inhibition by the γ -aminobutyric acid (Kimura & Jinnai 1994, Mitsushima *et al.* 2002) and opioidergic neural systems should be removed before the GnRH surge is initiated (Allen & Kalra 1986, Kerdelhue *et al.* 1988).

During lactation, the suckling stimulus exerts profound influences on the hypothalamic neuroendocrine functions (Russell *et al.* 2001). The suckling stimulus, which elicits robust secretion of oxytocin and prolactin responsible for milk ejection and production respectively, is also known to modify gonadotropin secretion. It is generally accepted that the effects of the suckling stimulus on gonadotropin secretion are inhibitory: the postcastration rise in serum and pituitary LH is markedly inhibited in ovariectomized lactating rats when compared with nonlactating ovariectomized rats (Hammons *et al.* 1973, Smith & Neill 1977, Smith 1978a, Maeda *et al.* 1989). During early lactation, this inhibition is mainly due to

the suckling stimulus itself rather than the high prolactin levels caused by the suckling stimulus (Lu *et al.* 1976, Smith 1978b). Furthermore, even though estrogen treatment can induce daily LH surges in lactating ovariectomized rats (Smith 1978b, Coppings & McCann 1979), the magnitudes of the daily surges in lactating rats have been found to decline more rapidly than in nonlactating rats (Coppings & McCann 1979, Tsukamura *et al.* 1988).

In the present study, we readdressed the role of the suckling stimulus in the estrogen-induced LH surge in lactating ovariectomized rats. Surprisingly, we found that pup removal from lactating rats acutely blocked the estrogen induction of LH surges and activation of GnRH neurons, and intracerebroventricular (i.c.v.) injections of oxytocin restored LH surges in the pup-removed rats.

Materials and Methods

Animals

Female rats of the Wistar strain (Japan SLC, Shizuoka, Japan) were housed under lighting conditions of 12 h light:12 h darkness (lights on at 0600 h). Experiments were carried out in accordance with the Guiding Principles on Animal Experimentation of the University of Yamanashi. In studies using nonlactating cyclic rats, vaginal smears were taken each morning, and only rats with regular 4-day estrous cycles were used. Pregnancy was induced by mating the females with males in the evening of proestrus. The day of parturition was designated day 0 of lactation. Litter sizes were adjusted to ten pups per litter on day 1, and lactating rats were bilaterally ovariectomized under ether anesthesia on day 2. A silicone capsule containing 17 β -estradiol (Sigma), which was prepared to maintain the estradiol concentration comparable to that during proestrus as described previously (Hashi *et al.* 1996), was implanted subcutaneously at 1200 h on day 8, 9, or 10 of lactation in lactating ovariectomized rats and 1 week after ovariectomy in nonlactating rats. For analysis of serum LH concentrations, an intraatrial indwelling silicone catheter (Medical Tubing A-1, Kaneka Medix Corp., Osaka, Japan) was inserted into the right jugular vein under ether anesthesia at 1900–2100 h on the day after estradiol treatment by the method of Harms & Ojeda (1974). A blood sample (300–400 μ l) was collected through the indwelling catheter at 2-h intervals at 1300, 1500, 1700, and 1900 h, 2 days after estradiol treatment. The sera were stored at -30°C until they were assayed for LH. Pups were removed from mothers at various times on the day of blood collection and, if the lactating rats were to be resuckled by their pups, the removed pups were maintained in a separate room. In experiments in which the effect of pup removal on the progesterone-enhanced LH surge was examined, the estradiol-treated ovariectomized rats were injected subcutaneously with 0.5 or 2.0 mg progesterone (Progehormone, Mochida Pharmaceutical, Tokyo, Japan) dissolved in sesame oil at 1200 h on the day of blood collection.

For the determination of serum corticosterone concentrations, rats were decapitated within 20 sec after removal from their home cages and bled into centrifuge tubes fitted with funnels. Corticosterone concentrations were determined using the Rat Corticosterone Biotrak Assay System (Amersham). The minimum detectable concentration for the corticosterone assay was 53 pg/assay tube.

Intracerebroventricular administration of oxytocin

In some experiments, on day 1 of lactation, ovariectomized rats were stereotactically implanted with a guide cannula for i.c.v. administration of oxytocin. A cannula, made of a stainless steel tube with an outer diameter of 0.70 mm, was stereotactically inserted into the third ventricle, when rats were under ether anesthesia, according to the atlas of Albe-Fessard *et al.* (1966) (stereotaxic coordinates: $A=6.5$, $V=1.6$, and $L=0.0$) and fixed to the skull with screws and dental cement. On the day of blood collection, i.c.v. administration was performed through an injection cannula, with an outer diameter of 0.35 mm, that was inserted into the third ventricle by way of the guide cannula immediately before administration. Two microliters of solution containing synthetic oxytocin (Peptide Institute, Osaka, Japan) or normal saline vehicle were injected for 60–90 s at 1-h intervals at 1400, 1500, and 1600 h.

Radioimmunoassays for LH

Serum concentrations of LH were determined by radioimmunoassays with reagents provided by Dr A F Parlow and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). LH values are expressed in terms of NIDDK-rLH-RP-3. The minimum detectable concentration for the LH assay was 18 pg/assay tube. The intraassay coefficient of variation was 5.7%.

Double immunostaining for GnRH and c-Fos

Ovariectomized estrogen-treated lactating rats were either freely suckled or separated from pups at 0900 h, 2 days after estrogen treatment. They were anesthetized with sodium pentobarbital (70 mg/kg, intraperitoneally) at 1100 and 1700 h and then perfused via the ascending aorta with 120 ml normal saline containing 20 U/ml heparin followed by 360 ml 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.0). Brains were removed, blocked, and immersed in 20% sucrose in PBS for 48 h. The brains were then stored at -30°C until sectioning. With a freezing microtome, each brain was cut to obtain 12 serial coronal 40 μ m thick sections containing preoptic tissues in which the majority of GnRH neurons exist. The sections were stored at -30°C in a cryoprotectant solution (Watson *et al.* 1986) until they were immunostained for GnRH and c-Fos. Every fourth section of the 12 serial sections of each brain was subjected to

immunofluorescence/immunoperoxidase staining for GnRH and c-Fos. The sections were rinsed with PBS for 20 min and immersed in 50% ethanol for 30 min to diminish background staining. After rinsing with PBS three times, for 10 min each, the sections were treated with 3% H₂O₂ in PBS for 10 min to inactivate endogenous peroxidase and then blocked with 10% normal horse serum in Tris-buffered saline (TBS) for 1 h. Thereafter, the sections were incubated at 4 °C for 48 h with a mixture of mouse monoclonal antibody against GnRH (LRH13, gift from Dr K Wakabayashi, University of Gunma, Maebashi, Japan; Park & Wakabayashi 1986) at a dilution of 1:500 and rabbit antibody against c-Fos (Ab-5, Calbiochem, Darmstadt, Germany) at a dilution of 1:5000. After 48-h incubation with the primary antibodies, the sections were incubated with a mixture of Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) at a dilution of 1:100 and biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:100 for 1 h at room temperature. The sections were then incubated with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector) for 1 h. The peroxidase–substrate reaction was performed for 5 min using a 3,3'-diaminobenzidine peroxidase substrate tablet set (Sigma) in the presence of 1% nickel ammonium sulfate. The immunological reagents used were diluted with 10% horse serum, and after incubation with the reagents, the sections were rinsed with TBS containing 0.3% Tween 20 four times, for 8 min each. The immunostained sections were mounted on glass microscope slides and covered with PermaFluor (Immunon, Pittsburgh, PA, USA). The specificities of GnRH and c-Fos double immunostaining were validated by omission of either GnRH or c-Fos antibody.

The immunostained sections were observed with a fluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan) equipped with a mirror unit for fluorescein isothiocyanate. All GnRH-immunoreactive neurons found in three sections were analyzed for each animal. The investigator counting immunoreactive neurons was blind to the treatment of the experimental groups. Every GnRH-immunoreactive neuron, identified by green fluorescence labeling in the cytoplasm, was counted with fluorescence microscopy and then scored in bright field microscopy for c-Fos immunoreactivity, identified by dark brown products in the nucleus at a magnification of $\times 400$. The data were expressed as the percentage of GnRH-immunoreactive neurons that were c-Fos-immunoreactive.

Statistical analysis

The minimal number of rats used was 5–8 per group depending upon experiments. Differences between groups were statistically analyzed using one-way ANOVA followed by Fisher's protected least significant difference test. Differences with a *P* value < 0.05 were considered statistically significant.

Results

Requirement of the suckling stimulus for estrogen-induced surges of LH secretion in lactating rats

Serum LH levels remained consistently low in lactating ovariectomized rats without estrogen treatment (Fig. 1, right panel). Treatment of these rats with estradiol induced an LH surge peaking at 1700 h, 2 days after treatment ($P < 0.01$), which was comparable in magnitude to that in nonlactating ovariectomized rats but occurred at a slightly earlier time (Fig. 1, left panel). When pups were removed from the lactating rats at 0900 h in the morning and not returned until after blood collection, i.e., at 1900 h, the LH surge seen in the afternoon in the freely suckled rats was completely suppressed (for freely suckled rats at 1700 h, 6.7 ± 1.7 ng/ml (mean $\pm$ S.E.M.), $n = 8$; for nonsuckled rats at the same time, 0.5 ± 0.1 ng/ml, $n = 6$, $P < 0.01$).

To determine at which time pup removal is effective in suppressing LH surges, the effects of changes of initiation and termination times of pup removal were examined. Pups were removed from lactating rats at various times and not returned until after blood collection. Pup removal at 0900 h was highly effective in suppressing LH surges that were expected in the afternoon, confirming the results mentioned earlier (Fig. 2, left panel). When pups were removed at 1100 or 1300 h, complete suppression of the LH surges was still observed (Fig. 2, middle panel). However, pup removal at 1500 h did not suppress LH surges, and the peak LH levels of these rats were not significantly different from those of freely suckled control rats ($P > 0.05$). When pups were removed at 0900 h and returned at 1000 or 1400 h, an LH surge comparable to that in the freely suckled control rats occurred (Fig. 2, right panel). Pup return immediately before the blood collection at 1700 h in rats that had been separated from pups at 0900 h induced a delayed LH surge that peaked at 1900 h, with a much higher level than that seen in the freely suckled control rats (for rats for which pups were returned at 1700 h, 18.6 ± 3.8 ng/ml, $n = 6$; for freely suckled rats, 6.4 ± 2.0 ng/ml, $n = 7$, $P < 0.05$).

Effect of pup removal on progesterone-induced LH surges in lactating rats

Treatment of lactating rats with progesterone enhanced estrogen-induced LH surges. Peak levels of estrogen-induced LH surges seen at 1700 h in freely suckled control rats were increased approximately 2.1- and 3.9-fold by treatment at 1200 h with 0.5 and 2.0 mg doses of progesterone respectively ($P < 0.05$; Fig. 3, left, middle, and right panels). In rats that were separated from pups at 0900 h, there was no estrogen-induced LH surge as observed earlier. Treatment with progesterone at both doses was highly effective in inducing an LH surge in these nonsuckled rats. Peak levels of the progesterone-induced LH surge at 1700 h in the

Figure 1 The estrogen-induced surge of LH secretion in nonlactating and lactating ovariectomized rats with or without pups. Nonlactating cyclic (left panel) and lactating female rats (right panel) were ovariectomized and given an estradiol-containing capsule subcutaneously. Blood samples were collected in the afternoon, 2 days after estradiol treatment. Lactating ovariectomized rats were assigned to one of the three groups: those not treated with estradiol and freely suckled (Δ); those treated with estradiol and freely suckled ($\circ$); and those treated with estradiol and separated from pups between 0900 and 1900 h ($\bullet$). Data are the mean $\pm$ S.E.M. based on 6–8 animals. * $P < 0.05$ vs estradiol-treated and freely suckled rats. Pups(+), with pups; pups(-), without pups.

nonsuckled rats did not differ from the peak levels in the freely suckled control rats ($P > 0.05$).

Effect of pup removal on estrogen-induced activation of the GnRH surge generator in lactating rats

The immediately early gene *c-Fos*, a parameter for neuronal activation, is expressed concomitantly in GnRH-containing preoptic neurons when the LH surge occurs in the afternoon at proestrus in cyclic rats (Lee *et al.* 1990a) or in response to ovarian steroid treatment in ovariectomized rats (Lee *et al.* 1990b). We investigated whether pup removal suppresses estrogen-induced LH surges in lactating rats via suppression of neuronal activation of the GnRH surge generator. The average number of GnRH-immunoreactive cells identified per section was 40.1 ± 1.5 ($n = 22$). Double immunostaining for GnRH and *c-Fos* revealed that some GnRH-immunoreactive cells as shown by green fluorescence in the cytoplasm and neurites under fluorescence microscopic field were also immunoreactive for *c-Fos* as shown by dark-colored deposits in their nuclei under light microscopic field (Fig. 4). There were few *c-Fos*-positive GnRH neurons at 1100 h, before the initiation of the LH surge, in both freely suckled and nonsuckled estrogen-treated rats (Fig. 5). The percentage of *c-Fos*-positive GnRH neurons was markedly increased at 1700 h in the freely suckled rats ($P < 0.01$) but not in the nonsuckled rats compared with the percentages at 1100 h ($P > 0.05$). The percentage of *c-Fos*-positive GnRH neurons at 1700 h was significantly less in the nonsuckled rats than in

the freely suckled rats ($9.5 \pm 3.6\%$, $n = 6$ vs $26.5 \pm 4.9\%$, $n = 6$, $P < 0.05$).

Effect of i.c.v. injections of oxytocin on pup removal-induced suppression of LH surges

To elucidate the neural mechanism by which pup removal suppresses estrogen-induced LH surges in lactating rats, we tested the hypothesis that the central action of oxytocin released in response to the suckling stimulus is required for the estrogen-induced LH surge and GnRH activation in lactating rats. This hypothesis is based on the finding that suckling induces oxytocin release and oxytocin regulates LH secretion by acting at the hypothalamic level (Rettori *et al.* 1994, Selvage & Johnston 2001). To mimic oxytocin release in response to the suckling stimulus, oxytocin was intracerebroventricularly injected into nonsuckled and, therefore, LH surge-suppressed rats. Oxytocin injection times of 1400, 1500, and 1600 h were chosen on the basis of the results obtained by the experiments in which the times of initiation and termination of pup removal were varied. Saline i.c.v. injections into nonsuckled rats had no effect on serum LH, which remained at low levels (Fig. 6, left panel). However, when oxytocin was intracerebroventricularly injected at a dose of $0.1 \mu\text{g}$ into these rats, an LH surge was seen with a peak time and magnitude comparable to those seen in freely suckled rats ($P < 0.05$). Injections of a smaller dose of oxytocin ($0.01 \mu\text{g}$) did not significantly change serum LH levels relative to the levels observed with saline injections

Figure 2 Effect of varying time schedules of pup removal on estrogen-induced LH surges in lactating rats. Lactating rats were ovariectomized and given an estradiol-containing capsule subcutaneously. Blood samples were collected in the afternoon, 2 days after estradiol treatment. Lactating rats were freely suckled throughout the day of blood collection (control group; left panel, ○) or separated from their pups during various periods beginning and ending at the times indicated in the insets. Data are the mean $\pm$ S.E.M. based on 6–7 animals. * $P < 0.05$ vs freely suckled control rats.

($P > 0.05$). It was confirmed that i.c.v. injections of $0.1 \mu\text{g}$ oxytocin had no effect on LH surges induced in freely suckled rats (Fig. 6, right panel; $P > 0.05$).

Since pup removal is a likely stressor, we investigated whether pup removal activates the hypothalamo–pituitary–adrenal axis by determining serum corticosterone levels. Freely suckled lactating rats revealed a corticosterone level comparable to nonlactating female rats when they were decapitated at 1500 h. Pup removal did not increase serum corticosterone concentrations in the lactating rats but rather significantly decreased them by 65% ($P < 0.05$; Fig. 7).

Discussion

The present study demonstrates that pup removal at least between 1300 and 1900 h suppresses estrogen-induced LH surges and activation of the GnRH surge generator. In contrast, the stimulatory action of progesterone on LH surges was not suppressed by pup removal. The suppressive effect of pup removal was overcome by oxytocin, intracerebroventricularly

injected during the period of pup removal. Taken together, these results suggest that, to induce the LH surge, the GnRH surge generator in lactating rats requires the suckling stimulus (an additional factor/stimulus that is not involved in the regulation of the estrogen-induced LH surge in cyclic rats), and that central release of oxytocin might mediate the action of the suckling stimulus on LH surges.

The essential role of the suckling stimulus in the estrogen-induced LH surge in lactating rats contrasts surprisingly with its inhibitory effect on pulsatile LH secretion (Hammons *et al.* 1973, Smith & Neill 1977, Smith 1978a, Maeda *et al.* 1989) and the estrogen-induced LH surge in ovariectomized lactating rats (Coppings & McCann 1979, Tsukamura *et al.* 1988) as shown by previous studies. Experiments in which the times of initiation and termination of pup removal were varied indicate that the LH surge is blocked if pups are removed during a period starting immediately before the initiation of the estrogen-induced LH surge, and continuing throughout the occurrence of the surge. The concurrence of the estrogen-induced LH surge and the effective time of pup removal for blocking the LH surge suggest that the effect of

Figure 3 Effect of pup removal on progesterone-induced LH surges in lactating rats. Lactating rats were ovariectomized and given an estradiol-containing capsule subcutaneously. Blood samples were collected in the afternoon, 2 days after estradiol treatment. The estradiol-primed lactating rats were either freely suckled (○) or separated from pups between 0900 and 1900 h (●) and treated subcutaneously at 1200 h with vehicle (left panel), or 0.5 (middle panel) or 2 mg progesterone (P; right panel). Data are the mean $\pm$ S.E.M. based on 6–7 animals. * $P < 0.05$ vs freely suckled rats. E2, estradiol.

pup removal is rapid and probably neural event-mediated, but not a result of secondary changes in hormone secretion caused by pup removal.

Pup removal that was effective in blocking estrogen-induced LH surges failed to block LH surges after the administration of progesterone at any dose, suggesting differential involvements of the suckling stimulus in the actions of estrogen and progesterone on LH surges in lactating rats. In contrast to its indispensable role in the estrogen-induced LH surge, the suckling stimulus may not be required for the stimulatory action of progesterone. Similar differential regulation of LH surges by estrogen and progesterone has been reported in suprachiasmatic nucleus-lesioned rats (Wiegand *et al.* 1978, Kawakami & Arita 1981a,b, Wiegand & Terasawa 1982). However, the action of progesterone to stimulate LH surges occurs only in the presence of the priming action of estrogen (Brann & Mahesh 1991), and progesterone seems to act by enhancing the estrogen-induced LH surge rather than by inducing an LH surge itself (Wise *et al.* 1981). In light of this mode of action of progesterone, it is possible that in pup-removed lactating rats estrogen might

have induced an LH surge with a small magnitude, which is difficult to consistently detect but sufficient for allowing the enhancing action of progesterone.

The requirements known to date for LH surges in cyclic rats are estrogen (Goodman & Knobil 1981), circadian rhythm (Freeman 1994), female-phenotypic hypothalamus (Gorski 1979), and the stimulatory and inhibitory neurotransmitters that are involved in the integration and mediation of estrogen and circadian rhythm in the GnRH surge generator (Ramirez *et al.* 1984). The present study demonstrates that estrogen-induced LH surges do not occur in lactating rats when the suckling stimulus is absent, making the suckling stimulus a new addition to the list of requirements for estrogen-induced LH surges in lactating rats. It has been shown that stimuli that are not ordinarily required become necessary for estrogen-induced LH surges under specific physiological conditions. Fox & Smith (1984) found that LH surges that normally occur in the afternoon on the day of parturition were blocked when pups were delivered by cesarean section, and that uterine cervical stimulation in the morning restored the LH surges in these cesarean-sectioned rats. They suggested that the *post*

Figure 4 Photomicrographs showing c-Fos-positive GnRH neurons in lactating rats. Lactating rats were ovariectomized and given an estradiol-containing capsule subcutaneously. Lactating rats were either freely suckled or separated from pups at 0900 h and perfused with paraformaldehyde solution at 1100 or 1700 h, 2 days after estradiol treatment. The rats' brains were sectioned and subjected to double immunostaining for GnRH and c-Fos. GnRH-immunoreactive cells have green fluorescence-labeled cytoplasm and neurites with no labeling in the nucleus under fluorescence microscopy (upper panels), and c-Fos-immunoreactive cells have brown-colored nuclei under bright field microscopy (lower panels). Left panels are from a freely suckled rat and right panels are from a nonsuckled rat at 1700 h. The arrows indicate c-Fos-positive GnRH neurons. Scale bar, 20 μ m.

Figure 5 Effect of pup removal on the percentage of c-Fos-positive GnRH neurons in the preoptic region of estrogen-treated ovariectomized lactating rats. See the legend to Fig. 4 for details. The percentage of c-Fos-positive GnRH neurons is the proportion of both c-Fos- and GnRH-immunoreactive cells in GnRH-immunoreactive cells found in the preoptic region. Data are the mean $\pm$ S.E.M. based on 5–6 animals. * $P < 0.05$ vs 1100 h; # $P < 0.05$ vs 1700 h in freely suckled rats. Pups (+), with pups; pups (-), without pups.

partum preovulatory LH surge requires the cervical stimulation of the labor process. On the other hand, when one of the requirements for the estrogen-induced LH surge is eliminated under nonphysiological conditions, (1) other new stimuli may trigger an LH surge instead of the eliminated requirement (Dempsey & Selaes 1943, Everett 1967, Zarrow & Clark 1968, Brown-Grant *et al.* 1973), or (2) an LH surge may be resumed without the eliminated requirement if the condition lasts for a long time (Clifton & Sawyer 1980). Taken together, these findings suggest that the estrogen-induced LH surge, which plays a central role in female reproductive functions and is therefore considered to be tightly regulated, loosens the intransigency of its regulation under certain conditions, showing a flexibility that is probably based on context-activated neural plasticity in the GnRH surge generator.

Estrogen-induced LH surges were restored in pup-removed lactating rats when oxytocin was given by the i.c.v. route, suggesting that the intracerebral release of oxytocin in response to the suckling stimulus is involved in the occurrence of the LH surge. There is strong evidence indicating that oxytocin plays a role in the regulation of LH secretion at both the anterior pituitary and hypothalamic levels. Oxytocin stimulates basal pituitary secretion of LH (Evans *et al.* 1989) and augments the response of LH secretion to GnRH (Evans *et al.* 1995). Furthermore, peripheral administration of oxytocin antagonists prevents

the estrogen-induced LH surge normally observed on the afternoon of proestrus in cyclic rats (Johnston & Negro-Vilar 1988, Robinson & Evans 1990). Based on these findings, oxytocin that originates from an unidentified source and acts on pituitary gonadotrophs has been implicated in the regulation of the estrogen-induced LH surge in cyclic female rats. It can be argued that restoration of LH surges by i.c.v. administration of oxytocin in pup-removed rats is not due to the central action of oxytocin, but rather to the pituitary action of oxytocin that leaked from the third ventricle to the anterior pituitary gland. We did not examine the involvement of oxytocin acting directly on the pituitary in the regulation of LH surges in lactating rats. However, at least, suppression of LH surges in pup-removed lactating rats is not attributable to blockade of oxytocin action at the pituitary level, because the primary cause of the suppression of LH surges by pup removal is at the hypothalamic level, i.e., it is attributable to the failure of activation of the GnRH surge generator in the hypothalamus as evidenced by the GnRH/c-Fos immunohistochemical studies. On the other hand, several studies have demonstrated the central action of oxytocin on GnRH release (Rettori *et al.* 1994, Selvage & Johnston 2001). These findings may support our conclusion regarding the involvement of

Figure 6 Effect of i.c.v. injection of oxytocin on suppression by pup removal of estrogen-induced LH surges in lactating rats. Lactating rats were ovariectomized and given an estradiol-containing capsule subcutaneously. Blood samples were collected in the afternoon, 2 days after estradiol treatment. Lactating rats were either freely suckled (right panel) or separated from pups at 0900 h (left panel) and intracerebroventricularly injected with normal saline or 0.01 or 0.1 µg oxytocin at 1400, 1500, and 1600 h. Data are the mean $\pm$ S.E.M. based on 8–9 animals. * $P < 0.05$ vs saline injections.

central oxytocin in the regulation of the estrogen-induced LH surge and activation of GnRH neurons in lactating rats.

Recent studies have shown that oxytocin possesses potent anxiolytic properties and suppresses stress-induced hypothalamo–pituitary–adrenal activity (Windle *et al.* 1997, Neumann 2002). It was therefore possible that pup removal acts as a stressor to block LH surges and that the restoration of LH surges by i.c.v. oxytocin injections is due to the ability of oxytocin to reduce anxiety responses. However, unexpectedly, serum corticosterone concentrations were not increased but rather decreased by pup removal in lactating rats, excluding the possibility of pup removal as a stressor.

LH surges never occur during lactation because follicular development and estrogen secretion are profoundly suppressed by the suckling stimulus through inhibiting pulsatile LH secretion. Thus, the neural regulation of LH surges is not very important during lactation in suppressing estrus cyclicity. The physiological significance of the role of the suckling stimulus in estrogen-induced LH surges shown in the present study remains to be clarified. A variety of physiological adaptations of hypothalamic functions occur dynamically during lactation. Several lines of evidence suggest that neural reorganization and plasticity induced or mediated by lactation, the suckling stimulus, and oxytocin may underlie these adaptations. Structural changes of synaptic contacts and glial relations of magnocellular oxytocin cells (Theodosis *et al.* 1981) and an increase in gap junctions between oxytocin cells (Hatton *et al.* 1987) occur during lactation. Furthermore, oxytocin has been shown to change hippocampal synaptic

plasticity, leading to improved long-lasting spatial memory during lactation (Tomizawa *et al.* 2003). Thus, lactating rats in which there is a new requirement for operation of the GnRH surge generator may be used as a good model for studying the oxytocin-dependent neural plasticity occurring during lactation.

Figure 7 Serum corticosterone concentrations in nonlactating and lactating rats with (pups +) or without (pups -) pups. Nonlactating cyclic and lactating female rats were ovariectomized and given an estradiol-containing capsule subcutaneously. Lactating rats were either freely suckled or separated from pups at 0900 h, 2 days after estradiol treatment. Blood samples were collected at 1500 h by rapid decapitation. Data are the mean $\pm$ S.E.M. based on 7–8 animals. * $P < 0.05$ vs freely suckled lactating rats.

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