Journal of Reproduction and Development, Vol. 52, No. 1, 2006

—Full Paper—

Role of Noradrenergic Receptors in the Bed Nucleus of the Stria Terminalis in Regulating Pulsatile Luteinizing Hormone Secretion in Female Rats

Shunji YAMADA[1)], Yoshihisa UENOYAMA[1)], Kei-ichiro MAEDA[1)] and Hiroko TSUKAMURA[1)]

[1)]*Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan*

Abstract. The bed nucleus of the stria terminalis (BNST) is one of the brain areas densely innervated by noradrenergic neurons originating in the brain stem. The present study aims to determine the role of noradrenergic receptors in the BNST in regulating pulsatile luteinizing hormone (LH) secretion in female rats. Ovariectomized (OVX) or estrogen-primed OVX (OVX+E_2) rats received three 1-h-interval injections of 0.05 μmol of noradrenaline (NA), phenylephrine (α_1-adrenergic receptor agonist), clonidine (α_2-agonist), or isoproterenol (β-agonist) into the BNST. Injection of NA or α_1-adrenergic agonist into the BNST strongly suppressed pulsatile LH secretion in OVX+E_2 rats with a significant (P<0.05) decrease in the mean LH level for 3 h and LH pulse frequency, but α_2- and β-agonists did not affect any of the LH pulse parameters. In OVX animals, α_1- and α_2-adrenergic agonists caused a significant change in LH pulse frequency and amplitude, respectively, though the effect was not as apparent as the NA- or α_1-agonist-induced changes in OVX+E_2 animals. These results indicate that NA inputs to the BNST suppress pulsatile LH secretion via α_1-adrenergic receptors and that estrogen enhances this suppression.

Key words: Adrenergic receptor, Bed nucleus of the stria terminalis, Luteinizing hormone, noradrenaline

(**J. Reprod. Dev. 52:** 115–121, 2006)

Brain noradrenergic pathways have been reported to be involved in regulating reproductive functions through controlling gonadotropin secretion. Noradrenergic inputs to the hypothalamic paraventricular nucleus (PVN) would be a good example of their suppressive role in gonadotropin secretion [1], and the inputs mediate fasting- or glucoprivation-induced suppression of pulsatile luteinizing hormone (LH) secretion in female rats [2, 3]. However, release of noradrenaline (NA) into the preoptic area (POA) increases at the onset of the LH surge [4], and expression of dopamine β-hydroxylase (DBH), a synthetic enzyme of NA, mRNA in the A1 and A2 regions also rises during LH surge [5]. Thus, the brain noradrenergic system plays diverse roles in regulating gonadotropin-releasing hormone (GnRH)/LH secretion.

The bed nucleus of the stria terminalis (BNST) is densely innervated by noradrenergic neurons originating mainly in the A1 and A2 regions and partly in the locus coeruleus (LC) in rats [6]. In accordance with the innervation, α-adrenergic receptor mRNA has been found in the BNST by *in situ* hybridization in rats [7]. The BNST has also been considered to be involved in regulating GnRH/LH secretion, since electrochemical stimulation of the BNST stimulates or inhibits the

Accepted for publication: October 31, 2005
Published online: December 12, 2005
Correspondence: H. Tsukamura
(e-mail: htsukamu@nuagr1.agr.nagoya-u.ac.jp)

preovulatory surge of LH in the female rat [8]. In addition, the suckling stimulus increases c-Fos expression in the BNST and brainstem noradrenergic neurons in lactating rats [9], in which the pulsatile LH release is profoundly inhibited [10]. However, there is no direct evidence to show whether the noradrenergic inputs to the BNST plays a role in the regulation of GnRH/LH release in female rats.

The present study aims, therefore, to clarify the role of noradrenergic inputs to the BNST in regulating pulsatile LH secretion. For this purpose, we examined the effect of NA or adrenergic agonist injection into the BNST on LH pulses in female rats. The animals were ovariectomized and treated with or without estrogen to determine the role of the steroid in modulating the action of adrenergic agents on LH secretion because the effect of NA on LH secretion is largely dependent on the estrogen milieu; NA injection into the third ventricle (3V) reduces LH secretion in the absence of estrogen, but stimulates it in the presence of the steroid [11]. It has also been reported that local NA administration into the PVN suppresses pulsatile LH release in an estrogen-dependent manner [1].

Materials and Methods

Animals

Female Wistar-Imamichi strain rats, weighing 205–215 g, were used. They were kept under the following conditions: 14 h light: 10 h darkness (lights on at 0500 h), 22 ± 2 C and free access to food (CE-2, Clea Japan Inc., Japan) and water. Animals having shown two consecutive estrus cycles were bilaterally ovariectomized (OVX), and some of them immediately received a subcutaneous Silastic tubing (1.5 mm i.d.; 3.0 mm o.d.; 25 mm in length; Dow Corning, Midland, MI) containing 17β-estradiol (E_2, Sigma, St. Loius, MO) dissolved in peanut oil at 20 μg/ml (OVX+E_2). The estrogen treatment has been shown to produce a plasma E_2 level at 30 pg/ml and to give a negative feedback effect on LH release [12]. The present study was approved by the Animal Experiment Committee of the Graduate School of Bioagricultural Sciences, Nagoya University.

Brain surgery

One week before blood sampling, the animals were stereotaxically implanted with a stainless-steel guide cannula (23 gauge; Plastics One, Roanoke, VA) for drug injection into the right BNST with the tip end at 0.4 mm posterior and 6.9 mm ventral to the bregma and 1.4 mm lateral to the midline, according to a rat brain atlas [13].

Drug administration and blood sampling

Blood samples (100 μl) were collected every 6 min for 3 h through a silicone cannula (Shin-Etsu Polymer, Tokyo, Japan) inserted into the right atrium through the right jugular vein on the day before blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the atrial cannula after each blood collection. Noradrenaline, phenylephrine (α_1-adrenergic receptor agonist, Sigma), clonidine (α_2-agonist, Sigma), or isoproterenol (β-agonist, Sigma) were dissolved in saline at 0.05 μmol/0.5 μl and unilaterally injected into the BNST of conscious rats at the rate of 0.25 μl/min for 2 min using a microsyringe pump (EICOM, Kyoto, Japan) through an internal cannula (26G; Plastics One) every 1 h for the first 2 h of the 3-h blood sampling period, so that each individual received three injections of 0.05 μmol (totally 0.15 μmol/site) of either NA or an adrenergic agent. Control animals were injected with an equal volume of saline in a similar manner.

At the conclusion of the blood sampling, all animals were injected with 0.5 μl of 2% brilliant blue solution through the brain cannula to confirm the placement of the cannula tip. The animals were perfused with saline followed by 10% formalin. Frozen coronal sections (50 μm) of the brain were made with a cryostat and stained with thionin. Brain cannula placement was determined under microscope. Only the data from the animals with the cannula tip in the BNST were analyzed.

LH assay

LH contents in 50-μl plasma samples were measured by a double antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Pituitary Program (Baltimore, MD). Plasma LH concentrations were expressed in terms of NIDDK rat LH RP-3. The least detectable level was 0.156 ng/ml, and the intra- and interassay coefficients of variation were 4.9% at 1.3 ng/ml and 2.7% at 2.3 ng/ml,

respectively.

Immunohistochemistry for DBH and estrogen receptor α (ERα)

Rats ovariectomized for 2 weeks were deeply anesthetized with sodium pentobarbital and perfused with 4% paraformaldehyde in 0.05 M phosphate buffer (PB). The brain was immediately removed from the skull, postfixed with the same fixative for 6–7 h at 4 C, and then cryoprotected with 30% sucrose in PB for 3–4 days at 4 C. Serial coronal sections (50 μm in thickness) containing the BNST were obtained using cryostat, and then stored at –20 C in a cryoprotectant. Every second section through the BNST taken from each rat was processed for DBH immunohistochemistry with the avidin biotin complex method (Vectastain Elite kit; Vector Laboratories, Burlingame, CA) as described previously [14]. Briefly, tissue sections from each rat were incubated in mouse anti-DBH antibody (1:4000; Chemicon International Inc., Temecula, CA) for 3 days at 4 C, followed by incubation in biotinylated horse anti-mouse IgG (Chemicon) for 90 min and avidin biotin complex for 60 min. DBH immunoreactivities were visualized using 3'3-diaminobenzidine as chromogen.

Every second section through the BNST taken from the other OVX rats was dual-stained with DBH and ERα as previously described [15]. Briefly, tissue sections were incubated for 4 days at 4 C in a mixed solution of mouse anti-DBH and rabbit anti-ERα antibody (1:4000; Upstate, Lake Placid, NY). Subsequently they were incubated in a mixture of Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:800; Molecular Probes, Eugene, OR) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (1:800; Molecular Probes) for 2 h in darkness at room temperature. The sections were mounted with an antifade reagent (FloroGuard; Bio-Rad, Hercules, CA) and observed under a sectioning microscope (ApoTome; Carl Zeiss, Germany).

Statistical analysis

LH pulses were detected by the PURSAR computer program [16]. Mean LH concentration for 3-h sampling period was calculated for each individual and then for each group. Statistical differences were determined by one-way ANOVA followed by the Bonferroni/Dunn test.

Results

The target injection site was the ventral part of the BNST (Fig. 1A), where strong and dense DBH-immunoreactivities were found (Fig. 1B). ERα-immunoreactive cells were often surrounded by or in close apposition with DBH-immunoreactive fibers in the BNST (Fig. 1C). Histological examination showed that noradrenergic agents were injected into the ventral part of the BNST, where many of the noradrenergic fibers were projecting (Figs. 1D–1G). Vehicles were also injected into the same regions as the noradrenergic agents (data not shown).

Frequent and regular LH pulses were observed in vehicle-treated OVX and OVX+E_2 rats (Figs. 2A and 3A). NA injection into the BNST suppressed pulsatile LH secretion throughout the sampling period in OVX+E_2 rats, with a significant decrease in mean LH levels and pulse frequency compared with vehicle-treated controls (Fig. 2B). Likewise, injection of α_1-adrenergic agonist into the BNST suppressed pulsatile LH secretion and significantly reduced mean LH levels and pulse frequency (Figs. 2A and 2B). In contrast, injection of either α_2- or β-adrenergic agonists into the BNST did not affect pulsatile LH secretion without significant effects on any LH pulse parameters compared with vehicle-injected controls in OVX+E_2 rats. In OVX animals, NA did not cause significant changes in any pulse parameters, and injection of α_1-adrenergic agent caused irregular LH pulses, resulting in a significant but slight suppression of pulse frequency compared with that in vehicle-treated controls (Figs. 3A and 3B). Other pulse parameters were comparable to those in vehicle-treated OVX rats except that LH pulse amplitude was significantly higher in α_2-agonist-injected animals than vehicle-injected controls.

Discussion

The present study clearly showed that local NA injections into the BNST suppressed pulsatile LH secretion in OVX+E_2 rats, and the effect of NA was mimicked by an injection of α_1-adrenergic agonist but not by the other agonists. These results suggest that noradrenergic inputs to the BNST have a role in suppressing pulsatile LH secretion, and this inhibition is mediated by α_1-adrenergic receptors.

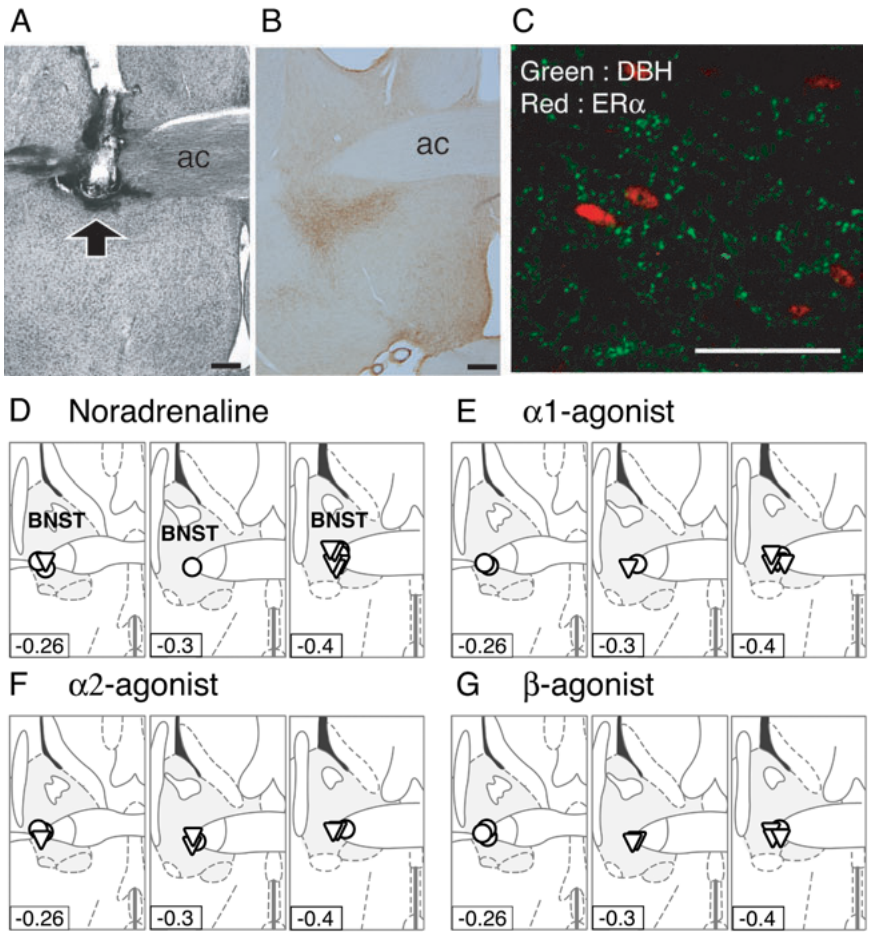

Fig. 1. Photomicrograph and schematic illustration of frontal sections of rat brains including the BNST indicating the injection sites of adrenergic agents. A, A photomicrograph of a frontal section of the brain in a representative animal implanted with a cannula for the injection of adrenergic agents in the BNST. The *arrow* indicates the site of the inner cannula tip placed at the ventral part of the BNST. B, DBH-immunoreactive fibers in the BNST. C, Dual immunohistochemistry of ERα (red) and DBH (green) in the ventral parts of the BNST. D–G, Schematic drawings of the BNST according to Paxinos and Watson [13] illustrating the individual sites of injection with noradrenaline (D), α_1-agonist (E), α_2-agonist (F) or β-agonist (G). *Circles* and *triangles* show the injection sites of OVX and OVX+E_2 rats, respectively. Numbers in each drawing indicate the distance (mm) to the bregma. ac, anterior commissure; BNST, bed nucleus of the stria terminalis. Scale bars, 50 μm

NA has been shown to act on various brain sites and exerts different effects on LH secretion depending on these sites. For example, injection of NA or α_1-adrenergic agonist into the PVN in OVX+E_2 rats suppressed pulsatile LH secretion [1]. On the other hand, NA has been reported to facilitate GnRH synthesis and secretion in the POA to generate the LH surge in proestrous rats [11]. Thus, the present study provides the first direct evidence showing that NA inputs to the BNST have a suppressive role in LH secretion and then reproductive function.

It is possible that the suppression of pulsatile LH secretion induced by the activation of α_1-receptors in the BNST is due to the activation of the hypothalamus-pituitary-adrenal (HPA) axis. NA inputs to the BNST have been known to stimulate adrenocorticotropin (ACTH) release through the

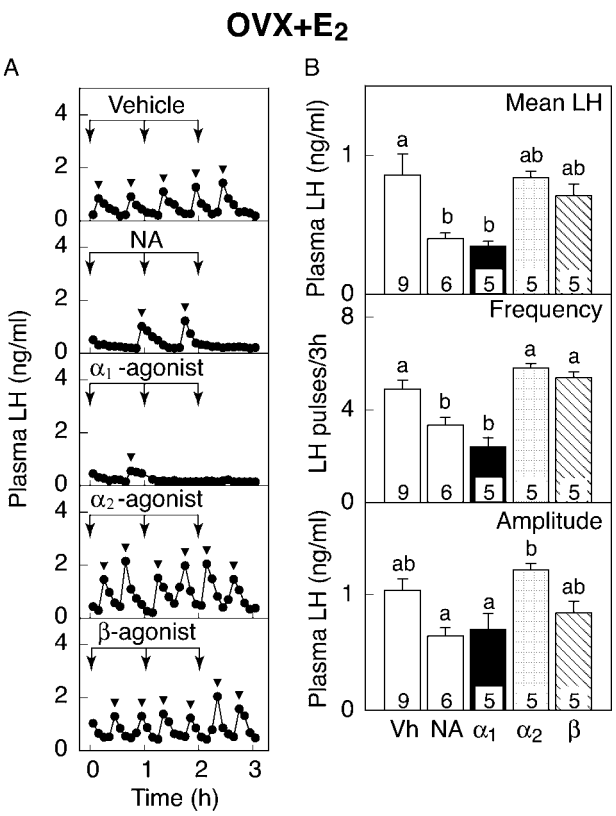

Fig. 2. Plasma LH profiles and LH pulse parameters in OVX+E_2 rats injected with adrenergic agents into the BNST. A, Plasma LH concentrations in representative animals injected with adrenergic agent (0.05 μmol/0.5 μl saline for one injection) into the BNST in OVX+E_2 rats. *Arrows* indicate the timing of drug injection. *Arrowheads* indicate the peaks of LH pulses identified by PURSAR computer analysis. B, Mean plasma LH concentrations and the frequency and amplitude of LH pulses in animals treated with adrenergic agonists. The number in each column indicates the number of animals used. Values are means ± SEM. Values with different letters are significantly (P<0.05) different from each other (Bonferroni/Dunn test). NA, noradrenaline; α_1, α_1-adrenergic; α_2, α_2-adrenergic; β, β-adrenergic receptor agonists; Vh, vehicle.

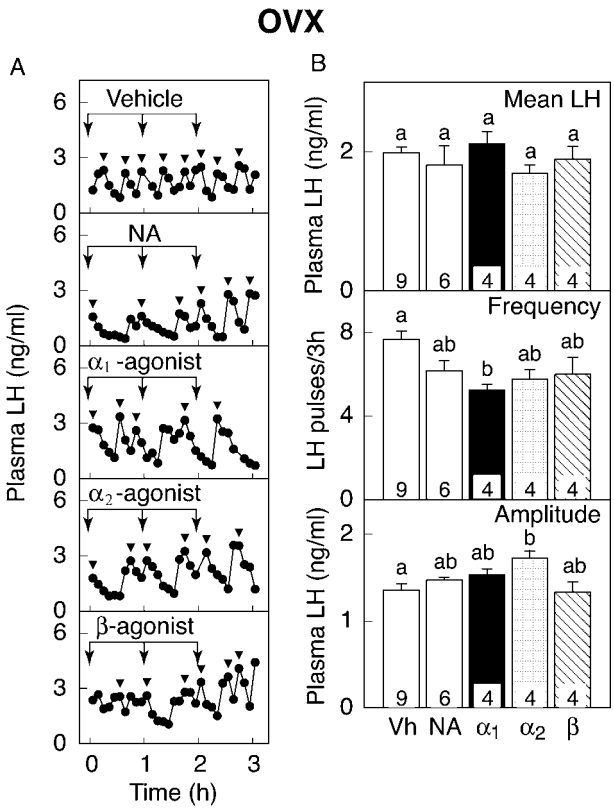

Fig. 3. Plasma LH profiles and LH pulse parameters in OVX rats injected with adrenergic agents into the BNST. A, Plasma LH concentrations in representative animals injected with adrenergic agent (0.05 μmol/0.5 μl saline for one injection) into the BNST in OVX rats. *Arrows* indicate the timing of drug injection. *Arrowheads* indicate the peaks of LH pulses identified by PURSAR computer analysis. B, Mean plasma LH concentrations and the frequency and amplitude of LH pulses in animals treated with adrenergic agonists. See Fig. 2 for details.

activation of α_1-adrenergic receptors in rats [17]. Noradrenergic inputs to the limbic systems have also been known to have modulatory effects on the HPA axis, because injection of α_1-adrenergic receptor antagonist into the medial amygdala (MeA) blocks the acute immobilization-induced increase in ACTH secretion in rats [18]. Morphological studies showed that noradrenergic terminals in the ventral part of the BNST have a direct synaptic contact with the dendrites of corticotropin-releasing hormone (CRH) neurons in the region [19] projecting to the PVN [20]. Furthermore, the BNST provides stimulatory inputs to the PVN CRH neurons, because chemical lesion of the BNST with ibotenic acid decreased the expression of CRH mRNA in the PVN in male rats [21]. Therefore, noradrenergic neurons in the ventral part of the BNST in direct contact with the

CRH neurons may activate the CRH neurons in the PVN to consequently stimulate the HPA axis.

In the present study, the suppression of LH pulses by the BNST α_1-adrenergic receptor activation was more apparent in the presence of estrogen than in its absence, suggesting that estrogen enhances the suppressive effect of noradrenergic inputs to the BNST on LH secretion. The estrogen-induced increase in the suppression of LH secretion in the present study could be partly explained by the increased population of adrenergic receptors in the BNST to enhance sensitivity of the BNST to NA in the presence of estrogen, which has been known to increase the α_1-adrenergic receptor mRNA in the hypothalamus-POA [22]. In addition, ERα-containing cells were found in close apposition to DBH-immunoreactive fibers and/or terminals in the present study (Fig. 1C). The ERα-containing neurons that enhance the sensitivity to NA may include gamma aminobutyric acid (GABA), which is inhibitory to LH secretion, because GABA-immunoreactive cell bodies were found in the ventral parts of the BNST [23].

In conclusion, the present study demonstrates that noradrenergic inputs to the BNST have a role in suppressing reproduction via inhibition of pulsatile LH secretion, and that this LH suppression is mediated by α_1-adrenergic receptors and enhanced by estrogen. The estrogen enhancement of LH suppression evokes the notion that the noradrenergic inputs to the BNST are involved in the suppression of reproductive function under stressful conditions and/or undernutrition, because the stress- or undernutrition-induced suppression of LH secretion is also enhanced by estrogen [12, 24]. Further study is required to clarify the physiological role of the noradrenergic inputs to the BNST in the regulation of reproductive functions.

Acknowledgments

We are grateful to the National Hormone and Pituitary Program for the LH assay kit, Drs. G. R. Merriam and K. W. Wachter for the Pulsar computer program, and Y. Niwa for technical assistance. The RIA and LH pulse analyses were performed at the Nagoya University Radioisotope Center and Information Technology Center, respectively.

References

1. **Tsukamura H, Nagatani S, Cagampang FR, Kawakami S, Maeda KI**. Corticotropin-releasing hormone mediates suppression of pulsatile luteinizing hormone secretion induced by activation of α-adrenergic receptors in the paraventricular nucleus in female rats. *Endocrinology* 1994; 134: 1460–1466.
2. **Maeda KI, Cagampang FR, Coen CW, Tsukamura H**. Involvement of the catecholaminergic input to the paraventricular nucleus and of corticotropin-releasing hormone in the fasting-induced suppression of luteinizing hormone release in female rats. *Endocrinology* 1994; 134: 1718–1722.
3. **Nagatani S, Tsukamura H, Murahashi K, Bucholtz DC, Foster DL, Maeda KI**. Paraventricular norepinephrine release mediates glucoprivic suppression of pulsatile luteinizing hormone secretion. *Endocrinology* 1996; 137: 3183–3186.
4. **Mohankumar PS, Thyagarajan S, Quadri SK**. Correlations of catecholamine release in the medial preoptic area with proestrous surges of luteinizing hormone and prolactin: effects of aging. *Endocrinology* 1994; 135: 119–126.
5. **Temel S, Lin W, Lakhlani S, Jennes L**. Expression of estrogen receptor-α and cFos in norepinephrine and epinephrine neurons of young and middle-aged rats during the steroid-induced luteinizing hormone surge. *Endocrinology* 2002; 143: 3974–3983.
6. **Forray MI, Gysling K, Andres ME, Bustos G, Araneda S**. Medullary noradrenergic neurons projecting to the bed nucleus of the stria terminalis express mRNA for the NMDA-NR1 receptor. *Brain Res Bull* 2000; 52: 163–169.
7. **Day HE, Campeau S, Watson SJ, Jr., Akil H**. Distribution of α_{1a}-, α_{1b}- and α_{1d}-adrenergic receptor mRNA in the rat brain and spinal cord. *J Chem Neuroanat* 1997; 13: 115–139.
8. **Beltramino C, Taleisnik S**. Dual action of electrochemical stimulation of the bed nucleus of the stria terminalis on the release of LH. *Neuroendocrinology* 1980; 30: 238–242.
9. **Li C, Chen P, Smith MS**. Neural populations in the rat forebrain and brainstem activated by the suckling stimulus as demonstrated by cFos

expression. *Neuroscience* 1999; 94: 117–129.

10. **Tsukamura H, Maeda KI**. Non-metabolic and metabolic factors causing lactational anestrus: rat models uncovering the neuroendocrine mechanism underlying the suckling-induced changes in the mother. *Prog Brain Res* 2001; 133: 187–205.
11. **Herbison AE**. Noradrenergic regulation of cyclic GnRH secretion. *Rev Reprod* 1997; 2: 1–6.
12. **Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K**. Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. *J Endocrinol* 1991; 129: 321–328.
13. **Paxinos G, Watoson C**. The Rat Brain. 3rd ed, San Diego: Academic Press, 1997.
14. **Estacio MA, Yamada S, Tsukamura H, Hirunagi K, Maeda KI**. Effect of fasting and immobilization stress on estrogen receptor immunoreactivity in the brain in ovariectomized female rats. *Brain Res* 1996; 717: 55–61.
15. **Reyes BA, Estacio MA, I'Anson H, Tsukamura H, Maeda KI**. Glucoprivation increases estrogen receptor α immunoreactivity in the brain catecholaminergic neurons in ovariectomized rats. *Neurosci Lett* 2001; 299: 109–112.
16. **Merriam GR, Wachter KW**. Algorithms for the study of episodic hormone secretion. *Am J Physiol* 1982; 243: E310–318.
17. **Cecchi M, Khoshbouei H, Javors M, Morilak DA**. Modulatory effects of norepinephrine in the lateral bed nucleus of the stria terminalis on behavioral and neuroendocrine responses to acute stress. *Neuroscience* 2002; 112: 13–21.
18. **Ma S, Morilak DA**. Norepinephrine release in medial amygdala facilitates activation of the hypothalamic-pituitary-adrenal axis in response to acute immobilisation stress. *J Neuroendocrinol* 2005; 17: 22–28.
19. **Phelix CF, Liposits Z, Paull WK**. Catecholamine-CRF synaptic interaction in a septal bed nucleus: afferents of neurons in the bed nucleus of the stria terminalis. *Brain Res Bull* 1994; 33: 109–119.
20. **Champagne D, Beaulieu J, Drolet G**. CRFergic innervation of the paraventricular nucleus of the rat hypothalamus: a tract-tracing study. *J Neuroendocrinol* 1998; 10: 119–131.
21. **Herman JP, Cullinan WE, Watson SJ**. Involvement of the bed nucleus of the stria terminalis in tonic regulation of paraventricular hypothalamic CRH and AVP mRNA expression. *J Neuroendocrinol* 1994; 6: 433–442.
22. **Karkanias GB, Ansonoff MA, Etgen AM**. Estradiol regulation of α_{1b}-adrenoceptor mRNA in female rat hypothalamus-preoptic area. *J Neuroendocrinol* 1996; 8: 449–455.
23. **Lonstein JS, De Vries GJ**. Maternal behaviour in lactating rats stimulates c-fos in glutamate decarboxylase-synthesizing neurons of the medial preoptic area, ventral bed nucleus of the stria terminalis, and ventrocaudal periaqueductal gray. *Neuroscience* 2000; 100: 557–568.
24. **Nagatani S, Bucholtz DC, Murahashi K, Estacio MA, Tsukamura H, Foster DL, Maeda KI**. Reduction of glucose availability suppresses pulsatile luteinizing hormone release in female and male rats. *Endocrinology* 1996; 137: 1166–1170.