—**Original**—

Two Distinct Populations of Neurons Expressing Nitric Oxide Synthase mRNA in the Female Rat Preoptic Area: Site Specific Changes Induced by Sex Steroids

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Abstract

Non-isotopic *in situ* hybridization histochemistry in the basal forebrain of gonadectomized juvenile female rats visualized neuronal nitric oxide synthase (nNOS) mRNA in two distinct cellular populations, one in the organum vasculosum of the lamina terminals (OVLT) and the other in the rostral preoptic area at the level of the anteroventral periventricular nucleus (rPOA). In the rPOA, digoxigenin-labeled nNOS mRNA positive cells were in close proximity to the cell body of gonadotropin-releasing hormone (GnRH)-immunoreactive neurons. In the OVLT, the labeled cells were in an area rich in GnRH fibers. In the frontal section of the rPOA, the labeled cells were distributed in an inverted V-shaped area over the third ventricle. Combined treatment with estradiol and progesterone caused a significant reduction in the number of nNOS mRNA positive cells in the inverted V-shaped area in the female rat rPOA. The treatment induced a luteinizing hormone surge at the time of sacrifice. In the OVLT, ovarian steroids had no effect on nNOS mRNA expression. The results indicate that nNOS mRNA expression in the rPOA is regulated by ovarian steroids in a site-specific manner. (J Nippon Med Sch 2001; 68: 328—334)

Key words: *in situ* hybridization, juvenile rat, organum vasculosum of the lamina terminalis, preoptic area, nitric oxide synthase

Introduction

Nitric oxide (NO) is involved in many important brain functions[1]. Among the different isoforms of NO synthase (NOS), neuronal NO synthase (nNOS) is the major form in the preoptic area and hypothalamus[2]. nNOS immunostaining is especially dense in the organum vasculosum lamina terminalis (OVLT)[3] and the rostral portion of the preoptic area at the level of the anteroventral periventricular nucleus (rPOA)[4], both of which contain numerous GnRH neurons.

An immature female rat model to demonstrate induction of gonadotropin surge by a combined treatment with estradiol and progesterone has been well established and characterized[5]. Estradiol induces region-specific changes in gonadotropin-releasing hormone (GnRH) mRNA levels in the basal forebrain before the surge[6]. Progesterone then triggers release of a pre-existing GnRH store by altering the synthesis or activity of neurotransmitters involved in GnRH regulation[5], although the role played by progesterone in GnRH synthesis in the prepubertal rat[7] remains controversial. This series of events apparently depends on neural transmission, because there is a rather close consensus on the lack of estrogen receptor in GnRH

neurons[8].

In the female guinea pig preoptic area, certain NOS-immunoreactive cells co-express progesterone receptor after estrogen treatment[9]. The observation that the magnitude of the luteinizing hormone surge following estradiol and progesterone is significantly attenuated after intraventricular administration of nNOS antisense oligonucleotides[10] suggests an intermediary role of NO in modifying gonadotropin secretion. Activation of progesterone receptors in the regions including the anteroventral periventricular nucleus has been shown to be an obligatory event in the estrogen-induced GnRH surges[11].

NO enhances pulsatile secretion of GnRH from immortalized GT 1-7 cells[12]. Levels of nNOS mRNA and protein in the rostral preoptic area increase prior to luteinizing hormone surge[13], but their spatial expression pattern awaits further scrutiny. We tackled this subject in gonadectomized prepubertal rats, because they lack the machinery for spontaneous gonadotropin surge and sexual behavior[14] but respond with a well-defined, typical luteinizing hormone surge when injected with estradiol and progesterone.

To obtain good spatial resolution, NOS mRNA expression was visualized by non-isotopic *in situ* hybridization, and a quantitative evaluation was made in the rPOA and OVLT at a time when the hormonal treatment induced a gonadotropin surge.

Materials and Methods

Animals

Female rats of the Sprague-Dawley strain (Saitama Experimental Animals, Saitama, Japan) were weaned on postnatal day 21 (day 0=the day of birth) and maintained in a controlled environment at 23℃ on an illumination schedule of 14 h light/10 h dark (lights on at 5 a.m.). Free access to laboratory chow and water was allowed at all times. At the time of ovariectomy on day 26, which was carried out under ether anesthesia, the rats weighed approximately 80 g. On days 27 and 28 at 5 p.m., *s.c.* injections of 2 μg of 17 β-estradiol benzoate were given in a 10 μg/ml solution of 20% ethanol-saline, and were followed on day 29 by an *s.c.* injection of 0.8 mg progesterone in a 4 mg/ml solution of 20% ethanol-saline at 9 a.m. Control animals received vehicle on the same schedule.

Tissue preparation

On day 29 at 2-3 p.m., the rats were anesthetized with an overdose of Nembutal and bled from the heart for collection of samples for luteinizing hormone assay. Plasma was immediately separated and kept at −80℃ until rat luteinizing hormone assay with a kit utilizing magnetic separation (Cat. No. RPA 552, Amersham Life Science, Little Chalfont, England),

After the collection of blood samples, the rats were perfused through the heart with 0.1 M phosphate-buffered saline (PBS, pH 7.4), which was followed by ice cold 4% paraformaldehyde fixative in 0.1 M phosphate buffer. The brain was removed and postfixed in the fixative at 4℃ overnight, then transferred into 4℃ 20% sucrose in 0.1 M phosphate buffer, in which it remained for 3 days or until it settled. Serial frontal sections (20 μm thick), which rostrally included the OVLT and extended caudally to the rPOA, were cut with a freezing microtome, rinsed in 0.1 M PBS, and mounted on silan-coated glass slides. Six series of the slides were prepared for each brain sample, and serial sections were distributed to the slides in sequence so that every 6 th section, all 120 μm apart, was mounted on each slide. Excess PBS was removed by blotting and kept overnight at 4℃. The slides were stored at −80℃ until hybridization.

Throughout the experiment care was taken to perform all procedures under fixed conditions. The processing of sections from different experimental groups was done simultaneously in a same batch as far as possible. Otherwise, temperature and other hybridization requirements, and reaction times for visualization were kept constant for all experiments to allow comparison.

cRNA probe for hybridization

599 bp nNOS cDNA that encodes the rat nNOS (2115-2714 nt), a region that encompasses the putative calmodulin-binding site, was ligated into the plasmid pBluescript KSII (−). To prepare a digoxigenin (dig)-labeled nNOS cRNA antisense probe, the rat nNOS cDNA was linearized with EcoR 1 and transcribed with T 7 RNA polymerase in the presence of dig-11-UTP (Roche Molecular Biochemicals, Mannheim,

Germany) as a substrate. The nNOS cDNA linearized with BamH 1 was transcribed with T 3 to prepare a sense probe. The dig-labeled antisense and sense probes were precipitated and purified with 10% 4 M LiCl, 5% tRNA (10 mg/ml) and 2.5 fold absolute ethanol twice. The precipitants were diluted to a final concentration of 20 μg/μl with diethyl pyrocarbonate-treated water. The identity of the nNOS calmodulin binding site is lower than the sequence for the inducible NOS calmodulin binding site according to the data obtained from the BLAST network service.

In situ hybridization

The sections were rinsed twice in 0.1 M PBS and placed in 0.2 N HCl for 15 min. They were passed through 0.1 M PBS twice, each for 5 min, and treated with proteinase K (20 μg/ml, Roche Molecular Biochemicals), washed twice for 10 min with 0.1 M PBS, dehydrated in a series of increasing concentrations of ethanol, and dried at room temperature. Hybridization with the probe at a concentration of 20 ng/100 μl was carried out at 50 (C overnight in hybridization buffer containing 50% formamide, 20 mM Tris-HCl (pH=8.0), 0.3 M NaCl, 2.5 M EDTA (pH=8.0), 10% dextran sulfate (Sigma, St. Louis, MO), E. coli tRNA (0.5 mg/ml, Roche Molecular Biochemicals), and 1 × Denhardt's solution. 5 μl/cm² of the hybridization solution was pipetted onto each slide. Hybridization was carried out in a moist chamber containing 50% formamide in 2 × SSC (1 × SSC=0.15 M NaCl containing 15 mM sodium citrate, pH=7.0) for 16 h. After hybridization, the sections were washed in 2 × SSC/50% formamide at 50℃ and treated with RNAse A (20 μg/ml, in 10 mM Tris-HCl, pH=8.0 containing 0.5 M NaCl; Roche Molecular Biochemicals) at 37℃ for 30 min. They were then rinsed in a series of decreasing concentrations of 1 × SSC containing 50% formamide at 50℃ for 1 h and washed in buffer I (0.1 M Tris-HCl, pH=7.5/ 0.15 M NaCl).

For visualization, the sections were incubated in a 1 %blocking reagent for 2 h at room temperature, reacted with alkaline-phosphatase conjugate of anti-dig-Fab-fragment (1: 500) overnight at room temperature. They were then washed in buffer I for 30 min, 3 times and transferred to buffer III (0.1 M Tris-HCl, pH=8.0/ 0.15 M NaCl/0.05 M MgCl₂), in which chromogen was
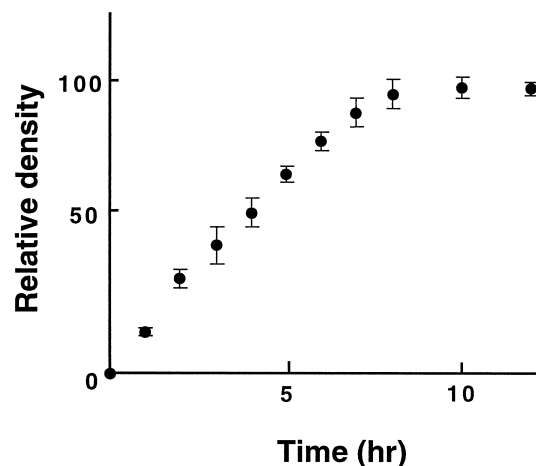

Fig. 1 Changes in the optical density (mean ± SEM) in 3 cells in the organum vasculosum of the lamina terminalis in different sections at 1-hr intervals during the visualization of hybridization signals by immunohistochemistry. A linear regression was obtained between 1 to 8 h.

developed with nitro-blue tetrazolium (NBT), 5-bromo-4-chloro-indolylphosphateptoluidine (BCIP). The reaction was terminated at 4 h with buffer IV (0.1 M Tris-HCl, pH=8.0/0.15 M NaCl/0.01 M EDTA) for 5 min, and the sections were rinsed twice in MilliQ water for 10 min and dried at room temperature. The nNOS mRNA hybridization signals were detected in the cytoplasm as blue/purple stains. Crystal/Mount (Biomeda, Foster City, CA) was applied over the dried sections, and the slide was placed in an oven at 70℃ for 15 min to allow the aqueous mounting medium to settle.

The reaction time was based on prior trials, in which representative dig-labeled cells in the OVLT were photographed (×700) at 1-h intervals up to 12 h during the development of chromogen. An NIH image was used to determine the optical density of the stained cells. The density increased linearly between 1 and 8 h, and eventually reached a plateau (**Fig. 1**). A 4-h reaction time in the present study would therefore suffice to detect any difference between the groups of rats.

Data analysis

Rostro-caudal levels of each frontal section were identified under a microscope, based on anatomical markers that included the third ventricle, anterior
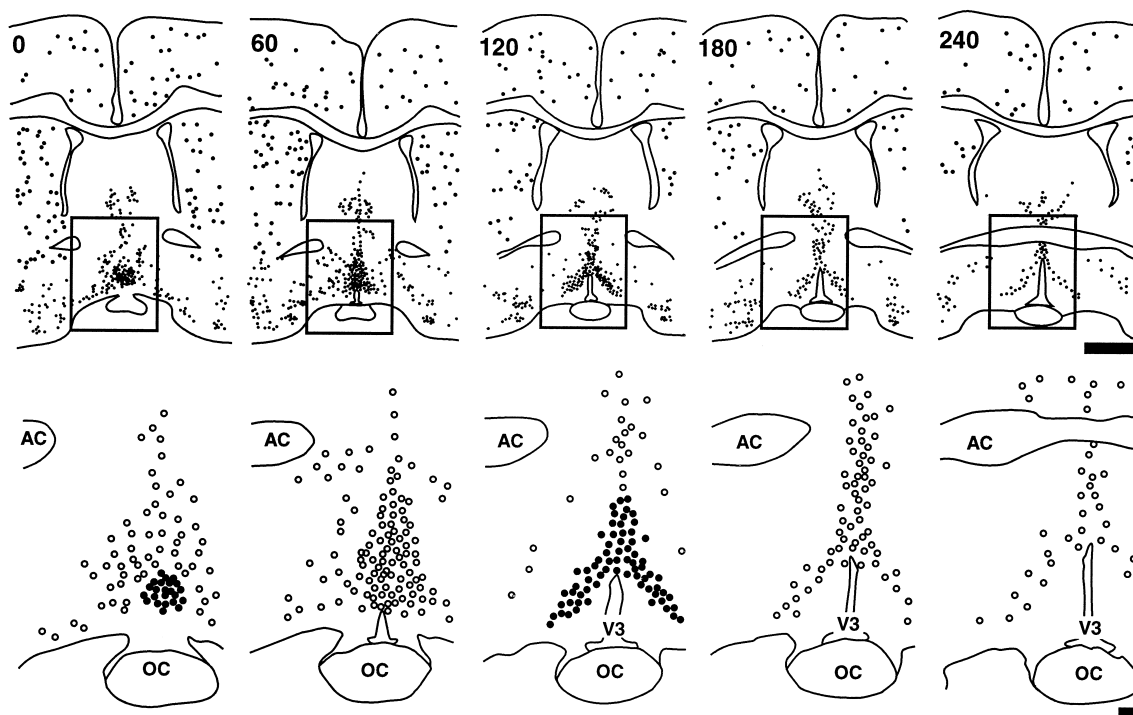
Fig. 2 A scheme showing representative distribution of digoxigenin labeled-nNOS mRNA positive cells in the frontal section of the forebrain of an ovariectomized, non-treated 29 day-old female rat. Numbers in the upper panel: distance from the OVLT in μm. Each circle represents a labeled cell in the upper panel or five labeled cells in the lower panel. Solid circles denote labeled cells in the organum vasculosum of the lamina terminalis (OVLT) and those in the rostral preoptic area (rPOA), on which quantitative analyses have been made. The lower panel is enlarged from the rectangle area in the upper panel (×3). Scale, 100 μm. Abbreviations: AC, anterior commissure; OC, optic chiasm; V 3, third ventricle.

commissure and optic chiasm, among others. Comparisons of matched sections from the rats were made. Photomicrographs of sections were made beginning rostrally at the level of the rostral-most portion of the OVLT, and continued caudally at 60 μm intervals well into the preoptic area (**Fig. 2**). The numbers of cells with the hybridization signal were determined on photomicrographs within two standardized areas in separate sections, one in the OVLT (0 μm in **Fig. 2**) and the other in the inverted V-shaped region in the rPOA (120 μm in **Fig. 2**). The distinctive pattern of cellular distribution allowed ready demarcation of both areas in sections from different rats.

Identification of nNOS-positive cells was based on blue/purple stains in the cytoplasm, with a distinctive boundary toward the background. The nucleus remained clear. Stains without a distinctive boundary, a rare occurrence in the present study, were discarded as non-specific reaction products. The number of la-

beled cells in the microphotography was tallied independently by two experimenters who had no prior knowledge of the treatment of any rat. The two groups of data thus produced closely coincided without significant difference in paired t-test.

In addition to the number of the cells with the hybridization signal, the area in which dig-immunoreactive materials were deposited within the OVLT or the rPOA was quantified by the NIH image. The area darker than a particular threshold, which remained constant across sections, was determined and expressed in μm^2.

Statistics

Both the number of labeled cells and the area occupied by immunoreactive materials were analyzed by using 2×2 analysis of variance (ANOVA) with repeated measure. The criterion level of significance was set at $p < 0.05$.

Results

Localization of nNOS mRNA positive cells

nNOS mRNA positive cells were detected in serial frontal sections that included rostrally the OVLT and extended caudally to the rPOA below the level of the anteroventral periventricular nucleus (**Fig. 2**), over a distance of 240 μm from the rostral OVLT.

There were discrete nNOS mRNA hybridization signals in the OVLT and rPOA. In the rPOA, labeled cells were distributed forming an inverted V-shaped aggregate over the third ventricle at the level of the anteroventral periventricular nucleus (**Fig. 2**).

In the rPOA GnRH neurons were distributed in a comparable pattern to that of nNOS mRNA positive neurons, forming an inverted V-shaped aggregate over the third ventricle at the level of the anteroventral periventricular nucleus. In no cells were the nNOS labeled cell and GnRH immunoreactivity co-localized (data not shown).

nNOS mRNA expression during LH surge

Treatment of gonadectomized females with estrogen on days 27 and 28 caused a well-defined surge in LH at 5 hrs after progesterone supplement on day 29 (**Fig. 3**).

Ovarian steroids altered nNOS mRNA expression specifically in the inverted V-shaped area in the rPOA of gonadectomized females, but the effect was not seen in OVLT. The estrogen and progesterone treatment caused a significant reduction in the number of nNOS mRNA positive cells in the inverted V-shaped area in females compared to the vehicle-treated control (F(1, 16) = 52.98, p<0.001) (**Fig. 4**).

Semi-quantitative evaluation of overall density of the hybridization signal in the OVLT and rPOA of the NIH image was done (**Table 1**).

Discussion

These results provide that nNOS mRNA positive cells were distributed in an inverted V-shaped area in the rPOA over the third ventricle at the level of the anteroventral periventricular nucleus in juvenile female rats. There was a separate group of nNOS mRNA positive cells was in the OVLT. The pattern of cellular distribution similar to that shown earlier by nNOS immunocytochemistry in adult, cycling female rats[4].In the rPOA, the distribution overlapped with
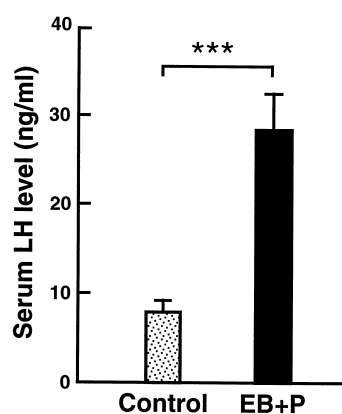

Fig. 3 Serum luteinizing hormone (LH) concentration in ovariectomized 29 day-old female rats after injections of vehicles (stippled columns) or 5 hours after progesterone (P) supplement after injection of estradiol benzoate (EB) on days 27 and 28 (solid columns). Note ovarian steroid-induced typical LH surge in females (***p<0.001).

Table 1 The area deposited with digoxigenin-immunoreactive materials within the organum vasculosum of the lamina terminalis (OVLT) or the rostral preoptic area (rPOA) of ovariectomized, juvenile rats, determined by NIH image (mean ± SEM μm^2). Vehicle, 20% ethanol saline ; EB + P, estradiol and progesterone-treated

Animals	OVLT	rPOA
Female vehicle (n = 5)	9,622 + 292	65,238 + 2,584
Female EB + P (n = 5)	10,017 + 584	40,379 + 1,741***

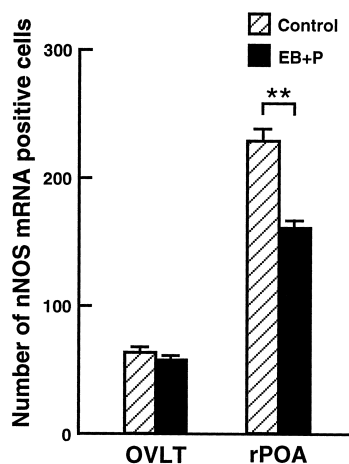Numbers of animals in parentheses. ***p < 0.001 vs ovariectomized, non-treated females.

Fig. 4　Number of nNOS mRNA positive cells in the organum vasculosum of the lamina terminalis (OVLT) and the rostral preoptic area (rPOA) in the ovariectomized female juvenile rats. Bars: SEM. Hatched columns: vehicle injected controls; solid columns: estrogen-and progesterone-treated rats (**p<0.01).

that of GnRH neuronal cell bodies. The OVLT contained numerous GnRH-positive fibers and presumed terminals, but few immunoreactive cell bodies. As in earlier reports[4,15], nNOS mRNA-positive cells in the rPOA were located in close proximity to GnRH neurons, which did not express nNOS mRNA.

It has been well established that progesterone treatment of estrogen-primed immature female rats facilitate a release of GnRH[16]. Estradiol alters GnRH mRNA levels in the rPOA and OVLT before the LH surge[6]. Progesterone then triggers the release of a preexisting GnRH store by altering synthesis or activity of neurotransmitters involved in GnRH regulation[5]. The involvement of progesterone-receptor activation in gonadotropin surge has been demonstrated by the lack of positive feedback effects of estrogen on gonadotropin release in the progesterone-receptor knockout mice[17]. The release of NO from NOS-containing neurons and the subsequent release of GnRH were implicated in the progesterone-dependent enhancement of the lordosis reflex in the ovariectomized, estrogen-primed adult rat[18]. In the guinea pig rPOA and hypothalamus, a considerable number of cells contained both NOS and progesterone receptor immunoreactivity[9]. These earlier results suggest that

progesterone may activate GnRH neurons by stimulating NOS-synthesizing cells.

nNOS mRNA expression was reduced by the treatment with estrogen and progesterone in the rPOA but not in the OVLT. A recent observation describes otherwise: elevations of nNOS mRNA, NOS protein levels and activity have been detected in the rat POA on the proestrus afternoon, prior to the initiation of LH surge[13]. The discrepancy may be due, at least partially, to the fact that in the present study, the brain tissue was sampled for *in situ* hybridization after the termination of LH surge. The observed decrease in mRNA expression may reflect inactivation of NOS by a feedback action of NO at high concentrations that induce GnRH release[19]. **In vitro** experiments employing the hypothalamic cell line GT-1 do not provide many clues to explain the present results, describing either repression of GnRH gene expression[20], or stimulation of GNRH release[21]. Further investigation is needed to examine how NO may regulate the activity of GnRH neurons at the level of the individual cells.

There is also a controversy over the site specificity of NO effect on GnRH release. In brain slices, NO-induced facilitation of GnRH release was obtained from the medial hypothalamic explants rather than that of the POA[22]. It has been suggested that NO alters pulsatile GnRH release in the median eminence[23]. It is therefore possible that NO may be involved differently in the activation of GnRH neurons in the rPOA for the LH surge[13] and in the maintenance of pulsatile secretion in the median eminence[23].

It is yet possible that the current findings suggest an association, not a cause and effect relationship between nNOS mRNA expression and the ovarian steroid-induced LH surge. However, juvenile female rats rarely display lordosis reflex in response to estrogen and progesterone[14]. Although no quantitative evaluation was made, ovariectomized juvenile females in this study also failed to show lordosis reflex in response to manual cutaneous stimulation despite treatments with ovarian steroids (data not shown). Thus, unlike in adult females[18], nNOS activity in the rPOA of juvenile female rats can hardly be associated with behavioral regulation.

In conclusion, ovarian-steroid treatment suppressed nNOS mRNA expression in the rPOA, but not in the

OVLT, of juvenile, ovariectomized rats. The observed decrease in mRNA expression may reflect inactivation of NOS by a feedback action of NO.

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