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The Oxytocinergic Neurons in Hypothamo-hypophysial Tract Contributes to CNS Pathway Innervating Ovary in Rat

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시상하부-뇌하수체로 Oxytocin신경세포의 난소로 투사하는 중추신경로에 관한 연구

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ABSTRACT

The mammalian ovary is innervated by sympathetic and sensory neurons which contribute to regulating several aspects of ovarian function, including blood flow, steroidogenesis and follicular development. The existence of a neural connection between central neurons and the ovary has been rarely reported, but the mechanism underlying integration of ovarian activity to broader neuroendocrine responses has not been reported. We have now used a viral transneuronal tracing technique combined with a conventional retrograde labeling procedure of CT-HRP to demonstrate that oxytocin-producing neurons of the hypothalamus are synaptically connected to the ovary. Since ovarian activity is suppressed but the activity of oxytocin neurons is increased during breast feeding. Our finding that the oxytocinergic neural connection is likely to provide a direct transsynaptic mechanism by which the central nervous system maintains the state of infertility that accompanies lactation in mammals.

Keywords : Ovary, Pituitary, Oxytocinergic neurons, CNS pathway, Pseudorabies virus

INTRODUCTION

It has been known for many years that the mammalian ovary is innervated by neurons of the peripheral sympathetic system (Marshall, 1970; Burden, 1978; Baljet & Drukker, 1979; Delgado et al., 2006, 2010; Madekurozwa, 2008; Ricu et al., 2008; Toth et al., 2008). More recent studies have demonstrated that this extrinsic innervation not only regulates blood flow, but perhaps more importantly, contributes to maintaining steroidogenesis and follicular development via the activation of specific neurotransmitter receptors, such as those that recognize norepinephrine and vasoactive intestinal polypeptide (Hsueh et al., 1984; Ojeda & Aguado, 1985; Ojeda & Lara, 1989; Dees et al., 2006; Morales et al., 2007)

Although the neuronal circuitry controlling ovarian func-

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tion may be limited to the peripheral nervous system, evidence exists suggesting the participation of central neurons in this regulatory process. Thus, electrical stimulation of the anterior hypothalamic area led to an increase in ovarian steroid release in the absence of the pituitary gland (Kawakami et al., 1981; Safarinejad, 2010), and unilateral lesions of the same area resulted in ipsilateral changes in the concentration of ovarian peptidergic receptors. Neuroanatomical examination of the hypothalamic projections to the peripheral nervous system demonstrated that certain hypothalamic neurons within the paraventricular nucleus send direct projections to preganglionic nuclei of sympathetic and parasympathetic pathways located in the intermediolateral cell column of the lower thoracic cord (Saper et al., 1976). On the other hand, retrograde tracing studies to identify the first-order neurons that project to the ovary showed that a significant number of these postganglionic neurons are located in both the thoracolumbar paravertebral ganglia and perivertebral ganglia (Gerendai et al., 1998, 2009; Tóth et al., 2007). Thus, a descending hypothalamic pathway may be functionally connected to the ovary via neurons in the intermediolateral cell column synaptically connected to postganglionic neurons of both the para and perivertebral ganglia.

Identification of centrally located neurons that may function as nodal points for the coordination of neuroendocrine activity is important for the understanding of multiple responses associated to single activating events. For instance, stress activates a range of visceral responses, recently shown to be coordinated by a common set of central command neurons (Jansen et al., 1995; Kalantaridou et al., 2010; Sirotkin, 2010). Lactation triggers a number of neuroendocrine responses, which include activation of oxytocin release to ensure milk ejection, inhibition of pituitary gonadotropin secretion, increased prolactin release, and inhibition of ovarian secretory activity (McNeilly, 1994; Wakerley & Clarke, 1994). But there were no morphological evidence to show that hypothalamic connection to ovarian innervations due to transsynatic tracer. Here we report for the first time that the oxytocinergic neurons in hypothamo-hypophysial tract contribute directly to CNS pathway innervating ovary in rat.

MATERIALS AND METHODS

1. PRV-Ba injection to rat ovary

Twenty five adult Sprague-Dawley rats (230～350 gm, Charles River) were used in this study. The animals were anesthetized with ketamine HCl (0.75 mg/kg) and xylazine (1 mg/kg) administered i.p. prior to the surgical procedure. We have now used the viral transneuronal labeling method (Card et al., 1993; Loewy, 1995), and conventional retrograde tracing, to identify the neurons in the hypothalamus that project to the ovary. A Bartha strain (Bartha K) of pseudorabies virus (PRV) was used as the transneuronal tracer for injection into the ovary. Injection of pseudorabies virus (PRV-Ba, Bartha's K strain, 1×10^8 pfu/mL) was made into the ovary with the aid of a surgical microscope. The ovaries were exposed via a transabdominal approach, isolated from adjacent tissues with fresh gauze, and the bursa was nicked with ophthalmic scissors before injecting the virus (1～2 μL) into the ventral and dorsal aspect of the gland under the capsule. After a saline rinsing, the gauze was removed and the ovary was returned to the abdominal cavity.

2. CT-HRP injection to posterior pituitary

Four to five microliters of the retrograde tracer cholera toxin (subunit-peroxidase conjugated, Sigma Chemicals, St Louis, MO) were stereotaxically injected into the neurohypophysis (7.0 mm posterior from Bregma) while the animals were anesthetized with ketamine/xylazine. The injection was made with a 30-gauge needle attached to a Hamilton microsyringe during 5 min period at a site (2～3 mm from the base of the skull) previously determined to target the neurohypophysis.

3. Tissue processing

Four to five days after the intraovarian injection of the viral tracer, the rats were reanesthetized and perfused transcardially with 100 to 200 mL of heparinized saline (18˚C) followed by 400 mL of 4% paraformaldehyde-lysine periodate in 0.1 M sodium phosphate buffer (pH=7.4). The brains were removed, placed in the same fixative for 4 h at 4˚C, and then transferred to 0.1 M phosphate buffer solution containing 20% sucrose where they were kept overnight at 4˚C.

The brains were sectioned in the transverse plane at 30 μm on a cryomicrotome. The sections were then processed for HRP histochemistry with tetramethylbenzidine as the chromogen. Upon completion of the reaction, the sections were mounted on glass slides, coverslipped using an aqueous mounting medium, and photographed. Thereafter, they were returned to the staining jars for removal of the color reaction.

The sections were then subjected to a doubb immunofluorescence procedure using polyclonal primary antibodies to PRV-Ba, oxytocin or CT-HRP, and fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (TRITC)-labeled secondary antibodies raised in a third species. One every six sections was incubated ovemight at room temperature with a mixture of goat anti-PRV-Ba (1 : 200) and rabbit anti-oxytocin serum (1 : 100, Chemicon Co., diluted in 0.1 M sodium phosphate buffer containing 1% normal donkey serum and 0.3% Triton X-100). The sections were reacted for 2 h with a cocktail of FlTC-labeled donkey anti-goat IgG (1 : 50, Jackson Immunoresearch Lab), and TRlTC-labeled donkey anti-rabbit IgG (1 : 50, Jackson Immunoresearch Lab). Thereafter, the sections were washed, mounted onto gelatin coated slides and coverslipped with glycero UPBS (29 : 1). Examination of the immunohistochemical reactions was carried out using an Olympus fluorescent microscope equipped with a BP 450～490 excitation filter to visualize FITC and a BP 546/12 excitation filter for visualization of rhodamine. Cells were counted as positive if a reasonable portion of the cell body was visible in the section.

RESULTS

Ninety six hours after the intraovarian injection of PRV-Ba, a number of transneuronally labeled neurons were detected in several brain regions (Fig. 1). Although several neurons within nuclei of the medulia oblongata (area postrema, C1 area, paragigantocellular reticular nucleus, caudal raphe nucleus (dorsalis, magnus, obsculus, pallidus) and pons (C5 area) were moderately labeled, the greatest number of PRV-Ba positive neurons was observed in diencephalic nuclei. Among these, the bed nucleus of the stria termi-

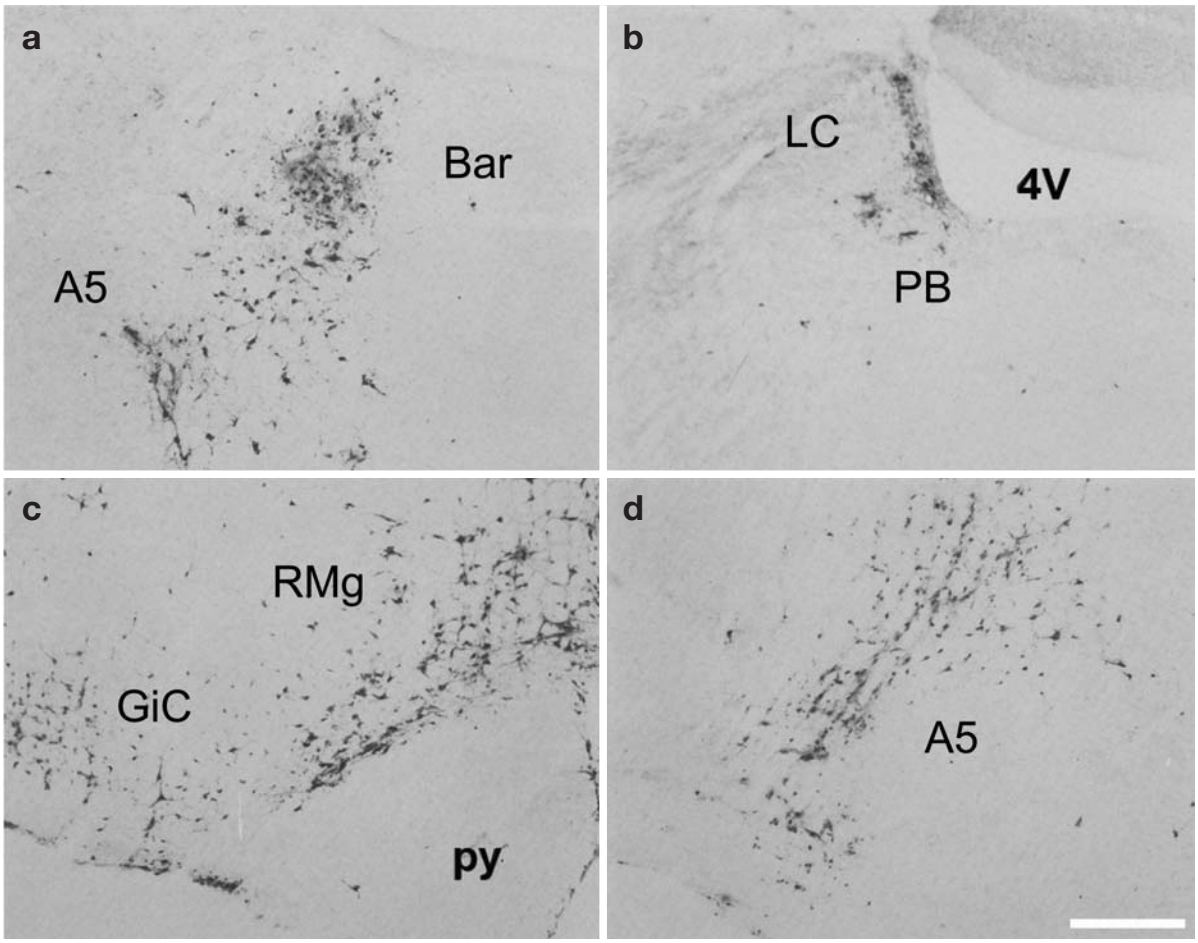

Fig. 1. Regions in the CNS exhibiting PRV-Ba immunoreactive neurons following the injection of the viral transneuronal tracer into the ovary of adult rats. a. Barington's nucleus (Bar) and A5 noradrenergic cells (A5), b. locus ceruleus (LC) and parabrachial nucleus (PB), c. Raphe magnus nucleus (TMg) and gigantocellular nucleus (GiC), d. A5 adrenergic cells (A5), py=pyramidal tract, ×40, bar=100 μm.

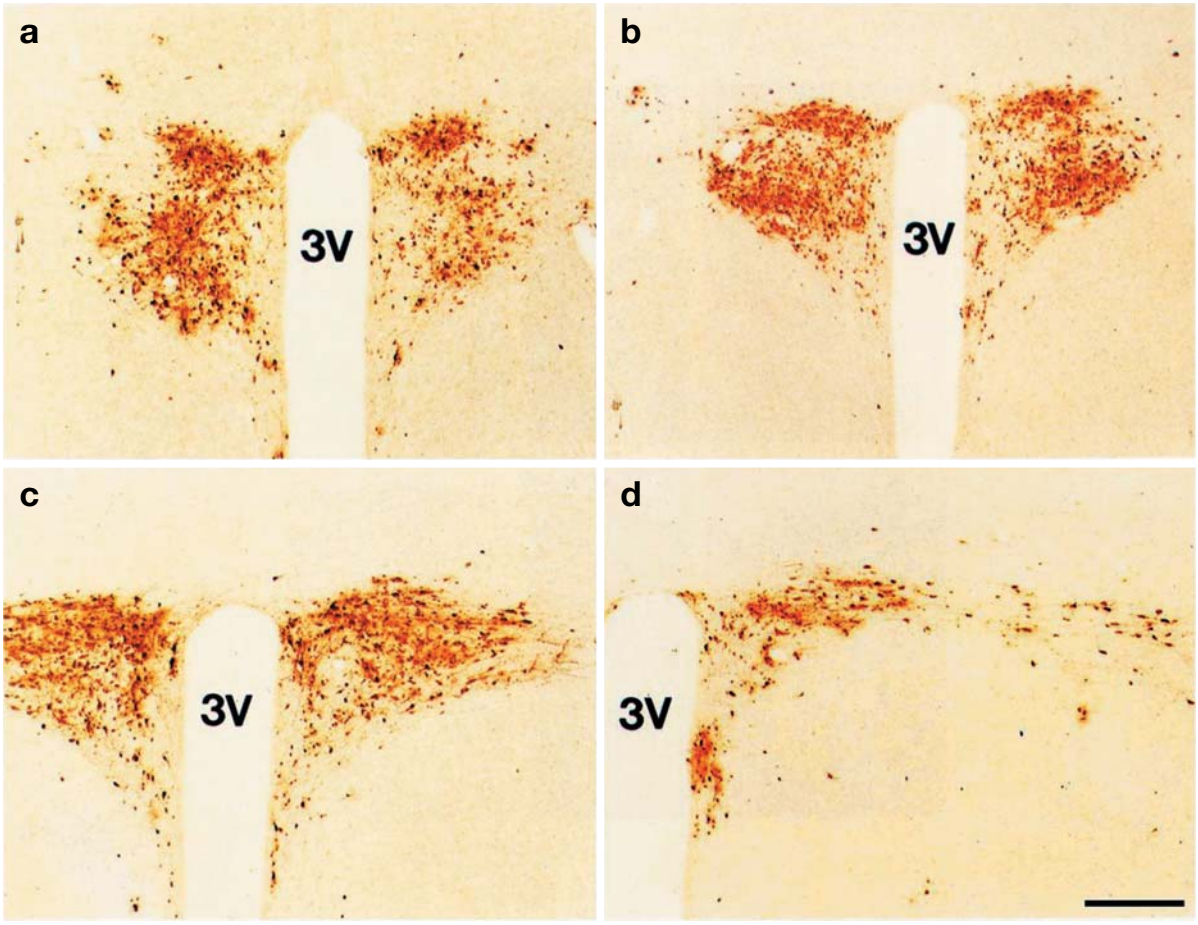

Fig. 2. Transneuronal labeling of the paraventricular nucleus (PVN) at four days after the injection of PRV-Ba into the ovary of the adult rat. Each panel represent a different antero-posterior plane of the PVN, with (a) being the most anterior portion of the nucleus, and (d) the most caudal portion of the PVN respectively. 3V=third ventricle. ×40, bar=100 μm.

nalis, and the lateral and medial preoptic area contained the greatest number of immunopositive neurons. The nucleus most intensively labeled was, however, the paraventricular nucleus (Fig. 2). Interestingly, although only one ovary was injected with the virus, a clear-cut bilateral labeling of the PVN was observed (Fig. 2). Four days after the intraovarian injection of PRV-Ba, the parvicellular region of the PVN was most prominently labeled (Fig. 3). However, 24 h later most of the labeled neurons were found in the magnocellular region (Fig. 3, right panel), a region that contains many of the oxytocin-secreting neurons that project to the neurohypophysis. Identification of these neurons by their content of retrogradely transported CT-HRP, and the simultaneous identification of oxytocin and PRV-Ba double labeled immunoreactive neurons in the same sections demonstrated that after 96 h transit period only a fraction of oxytocin neurons were transneuronally labeled (Fig. 3a-c). Twenty-four hours later, the number of transneuronally labeled oxytocin neurons had increased substantially, as demonstrated by the presence of PRV-Ba immunoreactivity in oxytocin-immunoreactive, CT-HRP labeled neurons (Fig. 3d-f).

Within the hypothalamus, some cells of the suprachiasmatic nucleus were also transsynapticalty labeled, as well as cells in the perisupraoptic nucieus. In contrast to the PVN, most of the labeled neurons in the SCN were found in the nucleus ipsilateral to the injected ovary, suggesting that the hypothalamic descending pathway that influences ovarian function has both uni- and bi-lateral components. All the nuclei are illustrated as a rate of abundance in Fig. 4.

DISCUSSION

PRV-Ba has been shown to produce highly selective infec-

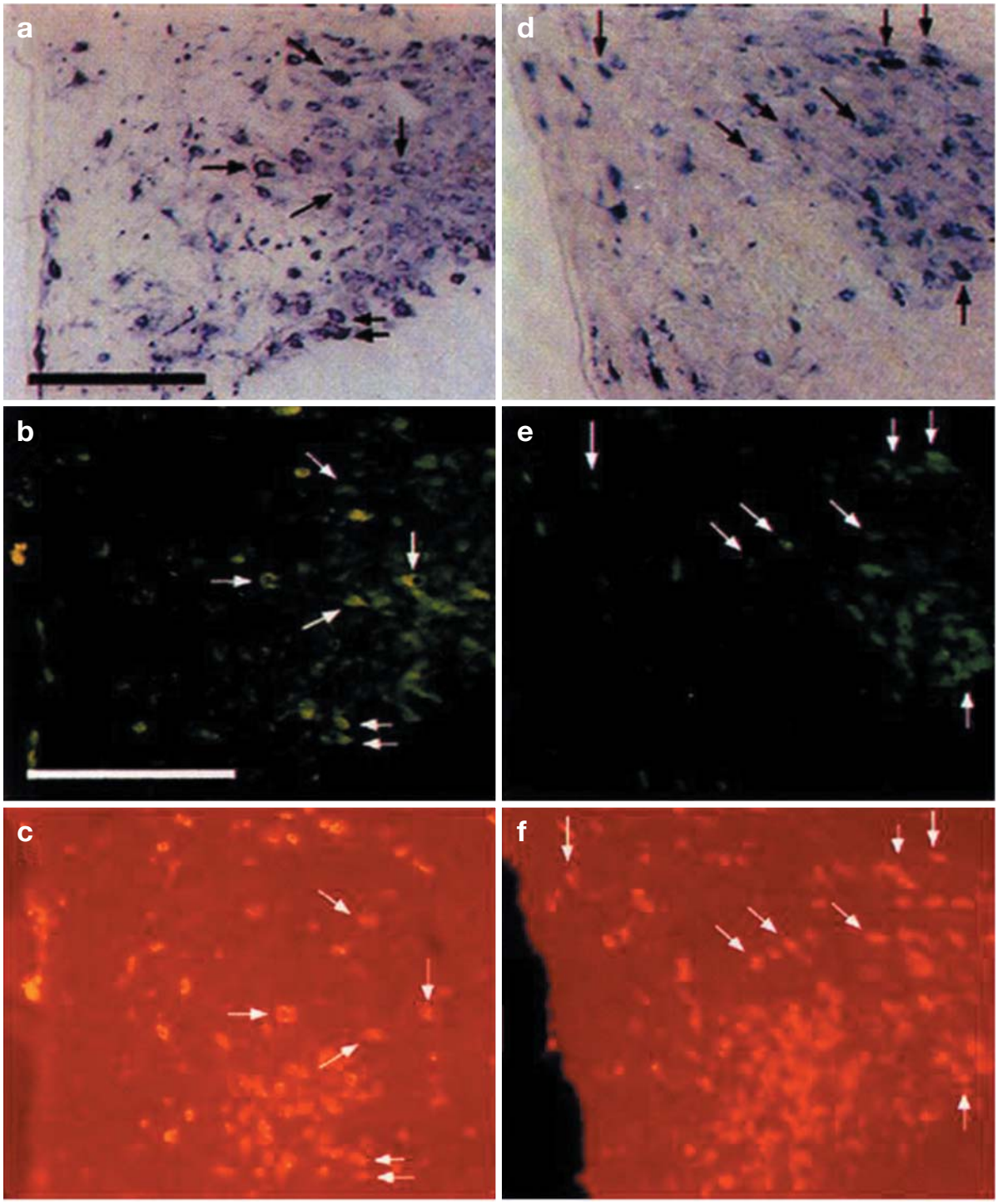

Fig. 3. Triple labeling of the PVN with CT-HRP, PRV or oxytocin in the same tissue of the rat. Triple labeling of oxytocinergic neurons of the PVN projecting to the neurohypophysis as determined by retrograde neuronal tracer (CT-HRP) injected into the neurohypophysis, oxytocin (OXT) and antibodies against PRV-Ba (PRV) after the intraovarian injection of PRV-Ba. (a)-(c), 96 h post-ovarian injection; (d)-(f), 120 h post-injection. Notice the triple colocalization of OXT, PRV-Ba or CT-HRP in the same neurons (arrows), and triple labeling become more abundant in 120 h group (d-f). ×40, bar=100 μm.

tions that spread exclusively via a transsynaptic pathway, in a retrograde fashion, with minimal lytic activity, and negligible lateral viral spread (Card et al., 1993; Loewy, 1995). For conventional retrograde tracing, cholera toxin conjugat-

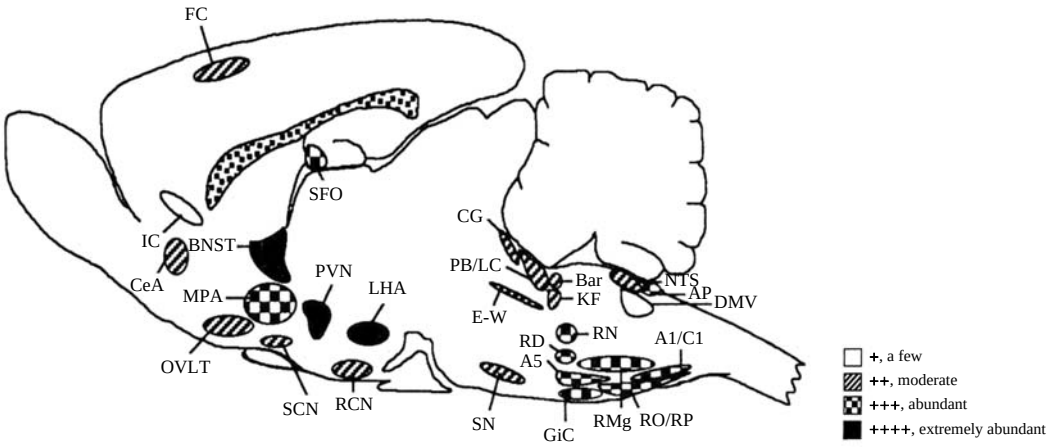

Fig. 4. Schematic drawings illustrating all the CNS nuclei innervating the ovary after PRV-Ba injection into the ovary of the adult rat. 2 nuclei including IC and DMV showed a few labelling, 12 nuclei including FC, CeA, OVLT, SCN, RCN, SN, E-W, CG, PB/LC, Bar, KF, and NTS revealed as moderate labeled nuclei, 9 nuclei including MPA, RN, RD, RMg, RO/RP, A5, GiC, A1/C1 and AP showed abundant labeling and 3 nuclei of BNST, PVN and LHA showed the most abundant labeling.

ed to horseradish peroxidase (CT-HRP) was stereotaxically injected into the neurohypophysis two days after intraovarian injection of the viral tracer. Four and five days after the intraovarian injection, the animals were anesthetized for transcardiac perfusion of fixative, and their brains were processed for immunohistochemical detection of the BRV-Ba 9282 antigen, oxytocin and the CT-HRP retrograde tracer. Previous studies have shown that PRV-Ba produces highly specific infections within a given group of synaptically connected neurons without spreading to adjacent, functionally unrelated neuronal networks (Card et al., 1993; Jansen et al., 1995). In as much as we have used this genetically attenuated strain of the vinus, and not the wild type forms which can produce non-specific labeling (Loewy, 1995), our results provide evidence for the longheld, but hitherto unsubstantiatod, belief that certain subsets of central neurons are able to influence gonadal function via a neurally mediated rnechanism.

The location of the transsynaptically labeled neurons is, in general, consistent with earlier studies showing that lesions or stimulation of some diencephalic and myelencephalic regions affects ovarian function independent of the pituitary gland (Jansen et al., 1995; Dees et al., 2006; Toth et al., 2008). Thus, our results identify for the first time the neuronal subsets that may contribute to the central, transsynaptically mediated regulation of gonadal activity. Of all the regions found to contain retrogradely labeled neurons, the PVN showed the greatest density of positive cells indicating that this nucleus plays a major regulatory influence on ovarian function. Earlier studies suggested the involvement of the parvicellular region of the PVN in the regulation of autonomic functions. Neurons in this region project to the medulla oblongata (Toth et al., 2008), and to the stellate and adrenal sympathetic preganglionic outflow systems (Jansen et al., 1995). These are connections that implicates them in the central coordination of neural and endocrine response to stress. The present results identify the ovary as another endocrine gland affected by sympathetic inputs activated by these central command neurons, Surprisingly, a substantial number of oxytocin neurons in the PVN were transsynaptically labeled with PRV-Ba suggesting that these neurosecretory neurons have the capability of influencing ovarian functjon via a neural pathway that operates independently of hormonally mediated regulatory inputs (Delgado et al., 2010). Whether this pathway is functionally connected to the projection of oxytocin neurons to autonomic nuclei of the medulla oblongata is currently unknown.

One of the most remarkable events associated with lactation is a state of infertility characterized by low plasma gonadotropin levels and suppressed follicular growth (McNeilly, 1994). While a deficiency in gonadotropin secretion appears to be the primary cause of lactational amenorrhea, the existence of a neural link between oxytocin neurons and the ovary strongly suggests that the suckling induced activation of these neurons leads to neutrally mediated changes in ovarian function. It is thus plausible that the relative refractoriness of the ovary to gonadotropins observed during early lactation is a consequence of the activation

of this descending oxitocinergic pathway.

During lactation, oxytocin neurons respond to breast-derived somatosensory inputs with massive increases in firing rate (Wakerely & Clarke, 1994). In addition to stimulating milk ejection (via oxytocin release to the blood stream), this activation is potentially able to affect both the autonomic outflow to nonreproductive organs (via projections to the pertinent CNS neuronal subsets, and ovarian function (through a direct neural pathway). The multiple effects oxytocin neurons of the PVN appear to act as a nodal point in the central coordination of neural and endocrine responses to lactation and could be a good target for drug discovery regarding ovarian-endocrine disease.

ABBREVIATION

3V	3rd ventricle
4V	4th ventricle
A1	A1 noradrenaline cell group
A5	A5 noradrenaline cell group
AP	Area postrema
Bar	Barington's nucleus
BNST	Bed nucleus of stria terminalis
C1	C1 adrenaline cell group
cc	Corpus callosum
CeA	Central nucleus of Amygdala
CG	Central gray matter
DMV	Dorsal motor nucleus of vagus nerve
f	Fornix
FC	Frontal cortex
GiC	Gigantocellular nucleus
IC	Insular cortex
KF	Kolliker-Fuse nucleus
LC	Locus ceruleus
LHA	Lateral hypothalamic area
MPA	Medial preoptic area
NTS	Nucleus tractus solitaries
OXT	Oxytocin
OVLT	Organum vasculosum of lamina termicalis
PB	Parabrachial nucleus
PVN	Paraventricular nucleus
py	Pyramidal tract
RCN	Retrochiasmatic nucleus
RD	Raphe dorsalis nucleus
RN	Red nucleus
RMg	Raphe magus nucleus
RO	Raphe obsculus nucleus
RP	Raphe pallidus nucleus
SCN	Suparchiasmatic nucleus
SFO	Subfornical organ
SNc	Compact zone of substantia nigra

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＜국문초록＞

포유동물의 난소는 호르몬과 감각신경 및 교감신경의 지배를 받아 난소 내 혈류의 조절, 스테로이드 호르몬 생성 및 난포의 발달과 관계된 난소의 고유기능이 조절되고 있다. 본 실험에서는 통상적인 추적자인 CT-HRP와 Pseudorabies바이러스(PRV)의 Bartha strain을 신경추적자로 이용하여 뇌하수체 후엽 및 난소와 연결된 중추신경부위를 밝히고자 하였다. 또 oxytocin을 난소 지배 신경축속에서 동정함으로써 신경축내 oxytocinergic neuron들의 존재를 확인하고, 이들이 배란을 중심으로 한 난소생식주기에 따라 보이는 중추 내 oxytocin신경세포의 변화를 조사하고자 하였다. Sprague-Dawley 흰쥐를 대상으로 난소 내에 PRV를 주사하고 48시간 후 실험동물들은 4% paraformaldehyde-lysine periodae로 고정하였으며, 뇌를 적출하여 30㎛ 두께의 관상연속 절편을 만들어 CT-HRP, PRV 및 oxytocin에 대한 삼중염색을 시행하였다. 본 실험 결과 후뇌에서부터 전뇌에 이르기까지 PRV에 양성반응을 보인 신경핵들이 관찰되어 난소를 지배하는 신경축을 구성할 수 있었다. 또 시상하부의 뇌실옆핵에서 oxytocin, PRV와 CT-HRP에 삼중으로 염색된 세포가 관찰됨으로써 신경내분비축과 자율신경축이 공동으로 기원하고 있다는 것을 형태학적으로 보여주었다. 따라서 oxytocin은 이 두 계통 내에서 호르몬의 역할과 신경전달물질의 역할을 겸할 것이라는 것을 추측할 수 있었다.