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Neurokinin B- and kisspeptin-positive fibers as well as tuberoinfundibular dopaminergic neurons directly innervate periventricular hypophyseal dopaminergic neurons in rats and mice

Nobuhiko Sawai[a,*], Norio Iijima[b], Hitoshi Ozawa[b], Toshiyuki Matsuzaki[a]

[a] Department of Anatomy and Cell Biology, Gunma University Graduate School of Medicine, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan
[b] Department of Anatomy and Neurobiology, Graduate School of Medicine, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

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ABSTRACT

Neurons co-expressing kisspeptin, neurokinin B (NKB), and dynorphin in the hypothalamic arcuate nucleus, named KNDy neurons, are directly affected by sex hormones, and are well known for regulating the secretion of gonadotropin-releasing hormone. However, recent studies have shown that KNDy neurons also project and terminate to tuberoinfundibular dopaminergic (TIDA) neurons, suggesting a role in prolactin secretion. Moreover, there is a possibility that other neurosecretory dopaminergic neurons regulating prolactin secretion, such as periventricular hypophyseal dopaminergic (PHDA) neurons, may also be innervated by KNDy neurons. In the present study, by means of double immunohistochemistry and retrograde neural tracer, we examined whether KNDy neurons project directly to PHDA neurons that project to blood vessels, as well as to TIDA neurons. The results revealed that KNDy neurons are widely projecting to neurosecretory dopaminergic neurons of the PHDA and TIDA neurons in rats and mice. Secondary, presence of a major receptor for NKB, neurokinin-3 receptor (NK3R), in PHDA and TIDA neurons was examined and it appeared that most TIDA and PHDA neurons possess NK3R. These findings indicate that, in rodents, KNDy neurons widely project to neurosecretory dopaminergic neurons distributed in the hypothalamus, and may affect them via the NKB-NK3R signaling pathway.

1. Introduction

Tuberoinfundibular dopamine (TIDA) neurons are one of the major neural populations in the hypothalamus that contribute to the tonic inhibition of prolactin secretion (Leong et al., 1983; Ben-Jonathan, 1985). Kisspeptin is a ligand for a G-protein coupled receptor, GPCR54 (Lee et al., 1999; Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). Accumulating evidence indicates a role for kisspeptin in the regulation of gonadotropin-releasing hormone (GnRH) secretion (Gottsch et al., 2004; Irwig et al., 2004; Matsui et al., 2004; Navarro et al., 2004; Messager et al., 2005). However, recent reports have shown the dense distribution of immunoreactive fibers for kisspeptin-10, a polypeptide derived from prepro-kisspeptin, adjacent to TIDA neurons in female rats (Szawka et al., 2010; Sawai et al., 2012). An ultrastructural study has further revealed synaptic connections between these fibers and TIDA neurons (Sawai et al., 2012).

Two populations of kisspeptin neurons exist in rats and mice (Gottsch et al., 2004; Irwig et al., 2004). A subgroup of these neurons in the hypothalamic arcuate nucleus (ARC) co-express kisspeptin, neurokinin B (NKB), and dynorphin A, therefore called KNDy neurons (Lehman et al., 2010). These peptides are also important for maintaining both gonadotropin and sex steroid hormone release (Topaloglu et al., 2009; Guran et al., 2009; Goodman et al., 2004; Foradori et al., 2005). TIDA neurons are considered to receive projections from KNDy neurons because NKB-positive fibers also project to TIDA neurons (Ciofi et al., 2006, Sawai et al., 2012) and most kisspeptin-positive fibers in the ARC co-express NKB (True et al., 2011; Sawai et al., 2012). An anatomical relationship between the subpopulations of KNDy neurons projecting to GnRH neurons and TIDA neurons remains unknown. However, KNDy neurons may play an important role in coordinating the sex cycle and the secretion of prolactin under physiological conditions (e.g. during lactation).

TIDA neurons project fibers to the median eminence, and release dopamine into the portal blood vessels, which is transported to the

* Corresponding author. Tel.: +81 27 220 7906; fax: +81 27 220 7906.
E-mail addresses: nsawai@gunma-u.ac.jp (N. Sawai), niijima@nms.ac.jp (N. Iijima), hozawa@nms.ac.jp (H. Ozawa), matoshi@gunma-u.ac.jp (T. Matsuzaki).

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anterior lobe of the pituitary gland to inhibit prolactin secretion from this region (Kawano and Daikoku, 1987; Higuchi et al., 1992). Besides TIDA neurons, periventricular-hypophyseal and tuberohypophyseal dopaminergic (PHDA and THDA, respectively) neurons of the hypothalamus have also been suggested to affect prolactin release (Freeman et al., 2000). PHDA neurons project to the intermediate lobe (IL) of the pituitary gland (Goudreau et al., 1995), and THDA neurons project to the IL and the neural lobe of this gland (Holzbauer and Racke, 1985). In the IL, dopamine regulates the secretion of α-melanocyte stimulating hormone (α-MSH) that in turn modulates the release of prolactin from the anterior lobe (Lindley et al., 1988; Goudreau et al., 1994). However, whether dopaminergic neurons projecting to the IL also receive direct projection of NKB and kisspeptin neurons (similarly to TIDA neurons) is unknown. Moreover, whether the dopaminergic neurons possess receptors for receiving signals from any neuropeptides of KNDy neurons remains to be elucidated.

Therefore, in the present study, we used a retrograde neural tracer to identify the neurosecretory neurons that project to blood vessels, and further investigated whether kisspeptin/NKB fibers directly attach to PHDA and TIDA neurons. Finally, we explored the presence of neurokinin-3 receptor protein in prolactin-regulating dopaminergic neurons of the hypothalamus. THDA neurons were excluded from this study because they are not clearly distinguishable from TIDA neurons in the ARC of which both are located.

2. Methods and materials

2.1. Animals

Adult female Wistar rats (8–9 weeks old) ($n = 9$) and female BALB/cCr Slc mice ($n = 3$) were obtained from Japan SLC (Shizuoka, Japan), housed at 23 ± 1 °C under a 12:12-h light:dark cycle (lights on at 0600 h), with *ad libitum* access to food and water. The estrous cycle was monitored in virgin female animals by daily vaginal smear cytology, and only those showing at least two consecutive estrous cycles were subsequently used. All animal experiments were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus, Japan.

2.2. Retrograde labeling of neurosecretory neurons

Retrograde labeling for neurosecretory neurons projecting to blood vessels in rats was performed by intravenous administration of fluorogold (FG; Fluorochrome, Denver, CO, USA). Animals were anesthetized with intraperitoneal (i.p.) injection of ketamine (90 mg/kg) and xylazine (10 mg/kg) followed by fluorogold injection (0.2%, w/v, in 0.8 cc saline) via the jugular vein. Rats were then housed for 4 days to allow for tracer transport and accumulation in neuronal cell bodies.

2.3. Brain tissue preparation

Animals were deeply anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), and then perfused transcardially with physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The perfused brains were quickly removed and post-fixed in the same fixative buffer at 4 °C for 12 h. Brains were cryoprotected by their immersion in 30% sucrose in 0.1 M PB at 4 °C for 3 days. Brains were then quickly frozen at −60 °C in *n*-hexane, and then cut into 25-μm thick frontal sections on a cryostat (CM1900, Leica Microsystems, Wetzlar, Germany).

2.4. Double immunofluorescence for tyrosine hydroxylase (TH) and kisspeptin or neurokinin B (NKB)

Immunohistochemistry was performed on brain sections from rats that received FG injection. One section from each three serial sections was used for immunostaining. After sections were rinsed with 0.1 M PB saline (PBS) containing 0.3% Triton X-100 (PBST), they were incubated in 10% normal goat serum for 30 min at 25 °C. The sections were reacted with a cocktail of mouse anti-TH monoclonal antibody (Sigma–Aldrich, St. Louis, MO, USA) (1:5000) and either rabbit anti-NKB polyclonal antibody (Novus Biological, Littleton, CO, USA) (1:1000) or rabbit anti-kisspeptin-10 polyclonal antibody (1:2000) (in which cross-reactivity to neuropeptide FF and RF-amide-related peptide were pre-absorbed according to Iijima et al., 2011). The sections were then rinsed in PBST and incubated with the secondary antibody cocktail of Rhodamine Red-X conjugated donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA) (1:200) and Alexa-488 conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) (1:200) for 24 h at 4 °C. This procedure was also used in brain sections of mice.

2.5. Double immunofluorescence for kisspeptin and NKB

Brain sections were rinsed with 0.1 M PBST, incubated in 10% normal goat serum for 30 min at 25 °C, then exposed to the first primary antibody, rabbit anti-kisspeptin-10 polyclonal antibody (1:2000), for 24 h at 4 °C. Sections were rinsed with PBS and then incubated with Alexa-488 conjugated donkey anti-rabbit IgG (Molecular Probes) (1:200) for 2 h at room temperature. After one wash (in PBS), sections were incubated with the second primary antibody, rabbit anti-NKB antibody (Novus Biologicals), which was conjugated with Alexa-568 using the Zenon labeling kit (Molecular Probes, OR, USA), for 24 h at 4 °C.

2.6. Double immunofluorescence for TH and neurokinin-3 receptor (NK3R)

Rat brain sections were incubated with the primary antibody cocktail of mouse anti-TH monoclonal antibody (Sigma–Aldrich) (1:5000) and rabbit anti-NK3R polyclonal antibody (Novus Biologicals) (1:5000) for 2 days at 4 °C. The sections were then rinsed in PBST and incubated with the secondary antibody cocktail of Rhodamine Red-X conjugated donkey anti-mouse IgG (Jackson Immunoresearch) (1:200), and Alexa-488 conjugated donkey anti-rabbit IgG (Molecular Probes) (1:200) for 24 h at 4 °C. Secondary labeling was also performed with another set of secondary antibodies, Alexa-488 conjugated donkey anti-mouse IgG (Molecular Probes) and Rhodamine Red-X conjugated donkey anti-rabbit IgG (Jackson Immunoresearch), to determine the cross reactivity of the secondary antibodies. Sections incubated without anti-NK3R antibody served as the control.

2.7. Image acquisition and analysis

After immunofluorescence labeling, all sections were rinsed with 0.1 M PB, mounted on glass slides, and coverslips placed on top. Confocal images were obtained using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Tokyo, Japan). The stocks of images were subjected to three-dimensional analysis by NIH ImageJ software. The number of positively stained cells was counted carefully to avoid double counting of the cells between sections.

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