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Visualization of Kisspeptin Binding to Rat Hypothalamic Neurons

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The neuropeptide kisspeptin plays an important role in fertility and the onset of puberty, stimulating gonadotropin-releasing hormone (GnRH) neurons to activate the hypothalamic–pituitary–gonadal axis. Several studies have demonstrated a morphological interaction between kisspeptin- and GnRH-expressing neurons; however, few have addressed the interaction of kisspeptin with other neuronal subtypes. We recently showed that fibers immunoreactive for kisspeptin were densely distributed in the dorsal part of the arcuate nucleus. These fibers were found in close proximity to GnRH and tuberoinfundibular dopamine (TIDA) neurons. In the present study, using biotinylated kisspeptin, we established a visualization method for identifying kisspeptin binding sites on TIDA neurons. Biotinylated kisspeptin bound to the cell bodies of TIDA neurons and surrounding fibers, suggesting that TIDA neurons express sites of action for kisspeptin. Our assay also detected biotinylation signals from kisspeptin binding to GnRH fibers in the median eminence, but not to cell bodies of GnRH neurons in the medial preoptic area. Positive signals were completely eliminated by addition of excess non-labeled kisspeptin. This method enabled us to detect kisspeptin binding sites on specific neural structures and neuronal fibers.

Key words: kisspeptin, tuberoinfundibular dopaminergic neuron, binding assay, dopamine, GnRH neuron

I. Introduction

The functional role of the neuropeptide kisspeptin has been thoroughly investigated over the last decade. Studies in kisspeptin receptor (*Gpr54*) knockout mice confirm that the kisspeptin–GPR54 system is essential in regulating the secretion of luteinizing hormone from the anterior pituitary via activation of gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus [5]. Kisspeptin also plays an important role in the initiation of puberty, sexual differentiation of the brain and behavior [5, 14, 15, 22, 29], and fertility and the seasonal control of reproduction via the hypothalamic–pituitary–gonadal (HPG) axis [28]. Furthermore, inhibition of kisspeptin neurons during lactation results in the inhibition of the entire HPG axis [34, 35]. Stress also suppresses kisspeptin–GPR54 signaling [16]. However, the morphological relationship between kisspeptin fibers and GnRH neurons remains unclear. In rats, only a small number of kisspeptin fibers have been observed around GnRH neuronal cell bodies in the medial preoptic region [12]. Furthermore, despite reports of direct contact between kisspeptin and GnRH fibers, there is no evidence of synaptic connectivity [32], suggesting that a proportion of transmission occurring between these two fibers is extrasynaptic. Evidence that GPR54 is located on the GnRH fiber is lacking. However, it is highly likely that kisspeptin acts on other target neurons via synaptic transmission [26, 27]. We therefore hypothesized that synaptic transmission of kisspeptin occurs in regions containing dense kisspeptin fibers.

It was recently shown that kisspeptin stimulates the

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secretion of prolactin from the anterior pituitary [24, 31]. Prolactin-producing lactotrophs are negatively regulated by dopamine secreted by tuberoinfundibular dopaminergic (TIDA) neurons located within the dorsal part of the arcuate nucleus (ArcD) [1, 2, 11]. We previously reported that TIDA neurons are another neuronal target of kisspeptin [26, 27]. In that report, we revealed that kisspeptin and neurokinin B fibers emanating from kisspeptin/neurokinin B/dynorphin neurons in the arcuate nucleus (Arc) are densely distributed around TIDA neurons. We also demonstrated that kisspeptin was coexpressed with synaptophysin on tyrosine hydroxylase (TH)-positive cell bodies, forming synapse-like structures between kisspeptin fibers and TIDA neurons.

In the present study, to acquire morphological evidence of kisspeptin receptors located on individual neurons, we developed a new binding assay using a biotinylated form of kisspeptin-10. Our assay revealed kisspeptin-binding sites on TIDA neurons, which we concluded are the input from kisspeptin fibers to these neurons. We also examined the differences between kisspeptin-binding sites on the GnRH cell body and neuronal fibers around terminals.

II. Materials and Methods

Animals

Female Wistar rats were purchased from Saitama Experimental Animal Supply Co., Ltd (Saitama, Japan). The animals were given food and water *ad libitum* and kept under controlled lighting conditions (14 hr light: 10 hr dark) prior to use. Rats demonstrating at least two consecutive 4- or 5-day estrus cycles were used in experiments, with the stage of estrus cycle established by daily vaginal smear. All experiments were carried out according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals, and with approval from the Committee for Animal Research in Nippon Medical School (approval number: 25-107, 26-060, 27-061).

Immunohistochemistry for kisspeptin and tyrosine hydroxylase (TH)

Diestrus rats (9–10 weeks old, n=5) were anesthetized with sodium pentobarbital (70 mg/kg) and perfused transcardially with physiological saline followed by 150 ml of 0.1 M phosphate buffer (PB; pH 7.4) containing 4% paraformaldehyde. Whole brains were immediately removed and immersed in the same fixative at 4°C overnight, then cryoprotected by immersing in 0.1 M PB containing 30% sucrose for 3 days. To prepare for histology, brains were frozen rapidly with powdered dry ice and cut frontally into sections 30 μm thick using a cryostat (Leica 3050; Leica Microsystems GmbH, Watzlar, Germany). To eliminate cross-reactivity with RFamide related peptides, anti-kisspeptin antibody was absorbed with neuropeptide FF (NPFF). Immunofluorescent double-labeling was carried out using 0.45 μg/ml rabbit anti-kisspeptin antibody pre-absorbed with 10 μg/mL NPFF [12] in combination with monoclonal anti-TH antibody (1:10,000; Sigma-Aldrich, St. Louis, MO, USA), a marker for dopaminergic neurons. Sections were incubated with antibodies diluted in 0.1 M PB saline (PBS) containing 0.3% Triton X-100 (PBST) at 4°C for 2 days, and washed several times in PBST. Alexa Fluor 488-conjugated anti-mouse IgG antibody (1:1,000; Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 568-conjugated anti-rabbit IgG antibody (1:1,000; Life Technologies), both in PBST, were applied for 2 hr to sections as secondary antibodies for TH and kisspeptin, respectively. Sections were then mounted onto glass slides and examined under a confocal laser scanning microscope (LSM710, Carl Zeiss, Oberkochen, Germany). Scanning was performed separately for each secondary wavelength emission to minimize cross-interference. Optical sections were taken at 0.375 μm intervals. Images were deconvoluted using Huygens Essential (Scientific Volume Imaging B.V., Hilversum, Netherlands). Finally, the sections were stained with cresyl violet.

Binding assay for kisspeptin-10 in TIDA or GnRH neurons

Section preparation was performed as described above. Sections (10 μm) were placed on positively charged SuperFrost slides (Matsumani Glass Ind., Ltd., Osaka, Japan), and blocked with 1% bovine serum albumin (Sigma-Aldrich) in Elix water (Merck Millipore, Darmstadt, Germany) for 1 hr. After washing in Elix water, 30 or 50 μg/ml of biotinylated human kisspeptin-10 (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), dissolved in 30% dimethyl sulfoxide (DMSO; Sigma-Aldrich), was added to the slides for 2 hr at room temperature. DMSO was used to dissolve biotinylated kisspeptin-10 because it is insoluble in water. As a negative control, 405 μg/ml of non-labeled human kisspeptin-10 (Peptide Institute, Osaka, Japan) and biotinylated kisspeptin-10 were simultaneously applied to the sections. The slides were washed in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST; pH 7.4) and immediately fixed with Bouin solution (71.5% saturated aqueous picric acid solution, 9.5% formalin, and 19% acetic acid) for 20 min at 4°C. Kisspeptin-10 binding was detected using the Vectastain Elite standard ABC kit (Vector Laboratories, Burlingame, CA, USA) and TSA™ Plus Cyanine 3 System (PerkinElmer, Waltham, MA, USA). After several washes in TBST, slides were incubated with anti-TH antibody (Sigma-Aldrich; 1:10,000 in TBST) or anti-GnRH monoclonal antibody (LRH13, kindly provided by Dr. Park Min Kyun, University of Tokyo; 1:1,000 in TBST) [23] at 4°C overnight. After several washes in TBST, the secondary antibody, Alexa Fluor 488-conjugated anti-mouse IgG (Life Technologies; 1:1,000 in TBST), was applied to the slides for 2 hr. Sections were examined under a confocal laser scanning microscope.

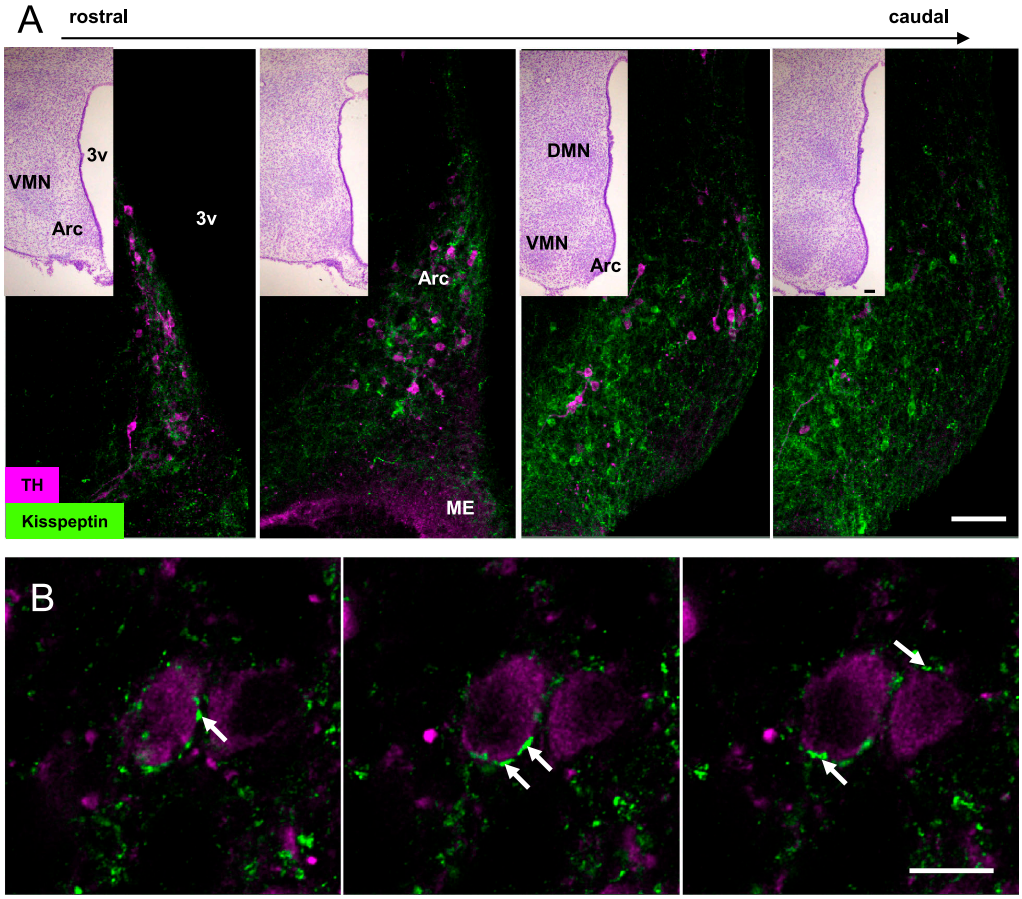

Fig. 1. Double immunofluorescence for TH and kisspeptin in the arcuate nucleus (Arc) of female rats. (**A**) Images of sections arranged rostrocaudally (n=1), showing numerous kisspeptin-immunoreactive fibers (green) in close apposition to TIDA neurons (magenta). Inset, cresyl violet stain. Bars= 100 μm. (**B**) Serial optical sections of a TIDA neuron. Arrows: kisspeptin-immunoreactive fiber surrounding a TIDA neuron. 3v: third ventricle; Arc: arcuate nucleus, ME: median eminence.

III. Results

Morphological interaction between kisspeptin fibers and TIDA neurons

Comparison of immunofluorescent images and cresyl violet staining indicated that the distribution of kisspeptin fibers was restricted to the Arc along the rostrocaudal axis. Fibers with intense kisspeptin immunoreactivity were primarily observed in the ArcD, an area containing TH-positive TIDA neurons. Notably, all TIDA neurons were surrounded by intense kisspeptin immunoreactivity (Fig. 1A). Proximity was further confirmed using serial optical sections at higher magnification, which revealed close apposition of kisspeptin fibers with TIDA neurons (Fig. 1B), with some fibers wound around these cells.

Biotinylated kisspeptin binding assay in TIDA and GnRH neurons

Kisspeptin-10 binding was detected as irregularly-shaped forms on cell bodies and fibers of TIDA neurons located within the ArcD (Fig. 2A). These signals were eliminated by excess application of non-labeled kisspeptin with labeled kisspeptin (Fig. 2B). When this binding assay was applied to GnRH neurons, biotinylated kisspeptin-10 was detected on GnRH fibers in the median eminence (ME) (Fig. 3A, B) and organum vasculosum of the lamina terminalis (Fig. 3D, E), but not on cell bodies in the medial preoptic nucleus (MPO) (Fig. 3F). Specificity of binding signals was confirmed in the ME using excess non-labeled kisspeptin similar to the assay for TIDA neurons (Fig. 3C).

IV. Discussion

In the present study, we examined whether TIDA neurons receive direct kisspeptin input. Our results demonstrate that kisspeptin fibers wind around TIDA neurons and that biotinylated kisspeptin is capable of binding to these cells. Kisspeptin binding signals manifested as small, irregular shapes in the binding assay, clearly distinguishable from the binding signals of the biotinylated forms of ghrelin or gastrin-releasing peptide [4, 25]. We considered that biotinylated kisspeptin-10 formed aggregates in 30% DMSO. Excess non-labeled kisspeptin-10 eliminated these binding signals, indicating that our results are specific for kisspeptin binding sites and suggesting that kisspeptin receptors are expressed on TIDA neurons. Part of the bind-

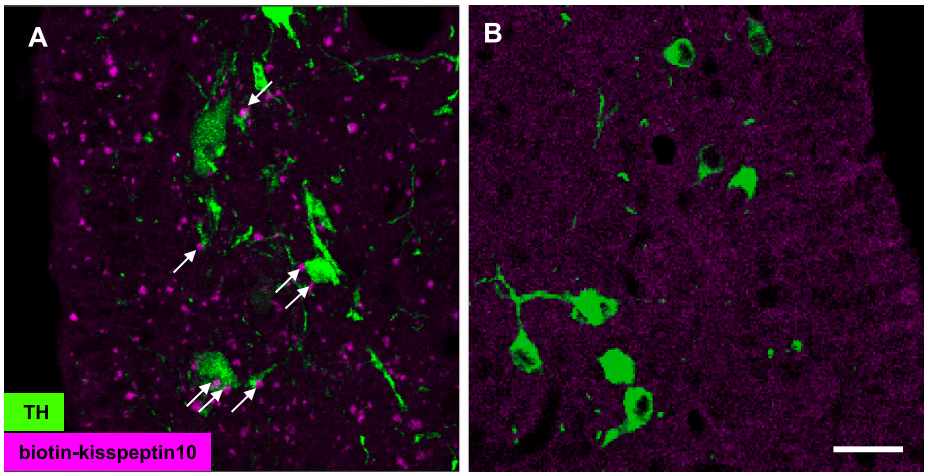

Fig. 2. Biotinylated kisspeptin-10 binding signals on TIDA neurons. (**A**) Incubation with biotinylated kisspeptin-10 yields an accumulation of signal (magenta) on the cell bodies (double-arrows) and fibers (single-arrow) of TIDA neurons (green). (**B**) Incubation with biotinylated kisspeptin-10 and excess non-labeled kisspeptin-10 reveals no binding signal. Bar=20 μm.

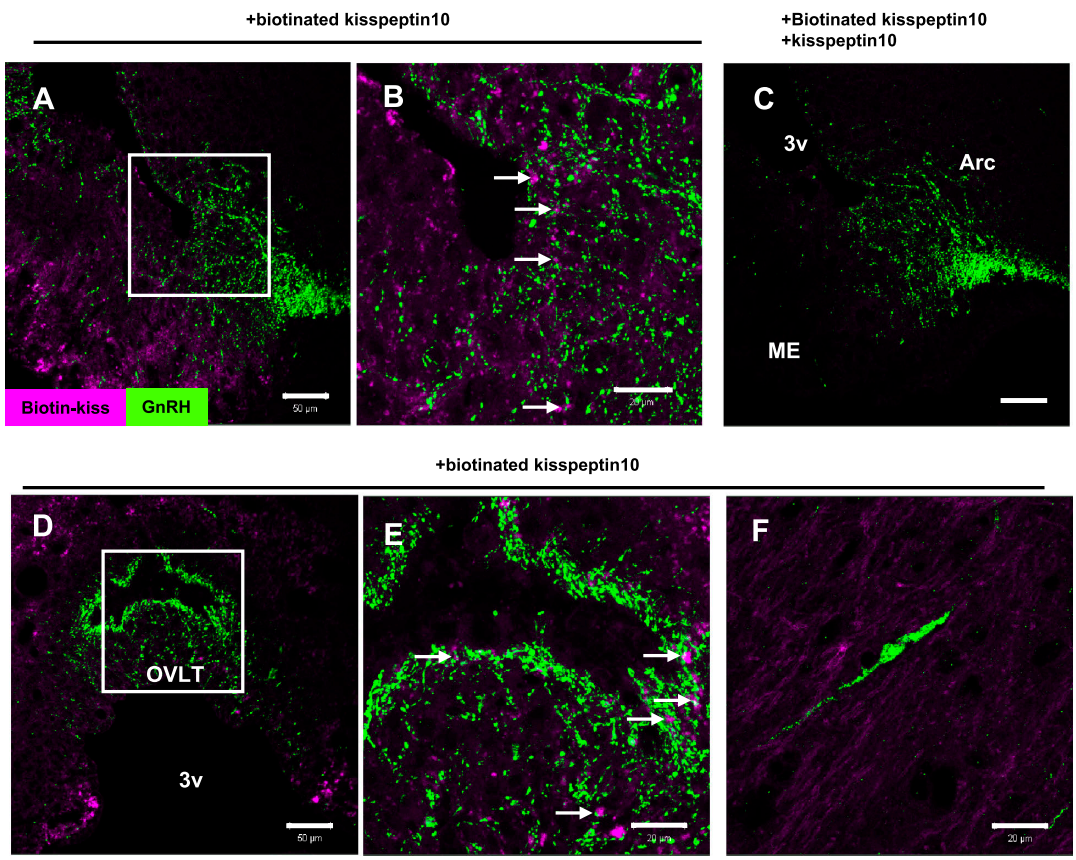

Fig. 3. Biotinylated kisspeptin-10 binding signals on GnRH neurons. Signals in the median eminence (**A–C**), organum vasculosum of the lamina terminalis (**D, E**), and GnRH cell body in the medial preoptic area (**F**). Incubation with biotinylated kisspeptin-10 and excess non-labeled kisspeptin-10 yields no signal (**C**). The sections yield an accumulation of kisspeptin signals (magenta) on GnRH (green) fibers (**A, B, D, E**) but not on the cell body (**F**). Higher magnification views of (**A**) and (**D**) are shown in (**B**) and (**E**), respectively. Bars=50 μm (**A, C, D**); 20 μm (**B, E, F**).

ing signal on the TIDA neurons may represent synaptic contacts between kisspeptin fibers and TIDA neurons. However, the possibility that extrasynaptic binding sites are also expressed on TIDA neurons cannot be excluded.

Similar to other RFamide peptides demonstrating cross-reactivity with various receptors, kisspeptin binds two receptor types, GPR54 and NPFFR2. NPFFR2 is one of two G-protein-coupled receptors for NPFF [3, 6]. As specific antibodies are not yet available, the distribution of GPR54 and NPFFR2 in the brain remains uncertain.

mRNA for both *Grp54* and *NPFFR2* has been detected in the Arc [7, 10, 18], but its expression in TIDA neurons has yet to be examined. Thus, biotin signals detected in the present study suggest localization of at least one of two distinct kisspeptin binding receptors. Additional studies detailing the expression patterns of *Grp54* and *NPFFR2* mRNA in TIDA neurons will further elucidate whether there is direct kisspeptin input to TIDA neurons.

Intracerebroventricular injection of kisspeptin-10 reportedly increases plasma levels of prolactin while decreasing 3,4-dihydroxyphenylacetic acid, a degradation product of dopamine, in adult male and proestrus female rats [24, 31]. Prolactin-producing lactotrophs are known to be controlled by dopamine released from TIDA neurons and transported via the portal capillaries of the ME to the pituitary [1, 2, 11]. Kisspeptin may suppress axonal transport or release of dopamine by TIDA neurons. The specific binding sites detected on TIDA neurons in the present study suggest that kisspeptin is capable of acting directly upon these neurons to suppress the release of dopamine. GPR54 couples primarily with the $G_{\alpha q/11}$-mediated signaling pathway to produce excitatory effects of GnRH neurons, in addition to activating several other second messenger systems [17, 20, 21, 30]. In contrast, NPFFR2 couples with G_{i2}, $G_{\alpha i3}$, $G_{\alpha o}$, and $G_{\alpha s}$ [8]. Excitatory neuromodulator was also reported to reduce dopamine release enhancing prolactin secretion [33]. The types of kisspeptin receptor and G protein that play roles in the suppression of dopamine release from TIDA neurons have yet to be elucidated.

Our present results also show that kisspeptin-10 binds to GnRH fibers in the ME, but not to cell bodies of GnRH neurons. While evidence suggests that GnRH neurons express *Gpr54* mRNA [9, 13], localization of kisspeptin receptors within these neurons remains to be determined. We previously showed that kisspeptin fibers were distributed amongst GnRH fibers in the Arc, and that only a few surrounded the cell bodies of GnRH neurons in the MPO [12]. Uenoyama *et al.* [32] demonstrated the morphological and physiological interactions of kisspeptin with GnRH fibers in the ME. Together, these results suggest that kisspeptin acts mainly on GnRH fibers in the Arc or ME but not on cell bodies located in the MPO. Although GPR54 is widely expressed in the brain, few or no kisspeptin fibers are found in GPR54-expressing regions [10, 18, 19]. Therefore, synaptic contact between kisspeptin fibers and target neurons is not expected in these areas; kisspeptin may act via non-synaptic transmission. Conversely, ArcD contains a dense population of kisspeptin fibers. In this area, kisspeptin can function as neurotransmitter through synaptic connections.

Our method has enabled the visualization of kisspeptin binding sites, which were expected to be involved in both non-synaptic and synaptic transmission. As a further investigation, a new method to distinguish between binding sites of these two kinds of transmission should be developed.

V. Abbreviations

Arc, arcuate nucleus; ArcD, dorsal part of arcuate nucleus; GnRH, gonadotropin-releasing hormone; IR, immunoreactive; ME, median eminence; MPO, medial preoptic nucleus; NPFF, neuropeptide FF; TIDA, tuberoinfundibular dopamine; TH, tyrosine hydroxylase.

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