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Efferent and afferent connections of the ventromedial hypothalamic nucleus determined by neural tracer analysis: Implications for lordosis regulation in female rats

Yuji Shimogawa[a], Yasuo Sakuma[b], Korehito Yamanouchi[a,*]

[a] Neuroendocrinology, Department of Human Behavior and Environment Sciences, School of Human Sciences, Waseda University, 2-579-15, Mikajima, Tokorozawa, Saitama 359-1192, Japan
[b] University of Tokyo Health Science, 4-11, Ochiai, Tama, Tokyo 206-0033, Japan

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ABSTRACT

Neural connections of the ventromedial hypothalamic nucleus (VMN) to and from forebrain and midbrain structures, which are involved in the neuroendocrine regulation of reproduction, were investigated. A retrograde (fluoro-gold [FG]) or an anterograde neural tracer (phaseolus vulgaris-leucoagglutinin [PHA-L]) was injected into the left side of the VMN in ovariectomized rats. Six days after injection with FG or 11 days after injection with PHA-L, brains were fixed and sectioned. After immunohistochemistry, digital images of FG-labeled neural cell bodies (FG-cells) or PHA-L-labeled fibers (PHA-L-fibers) were analyzed. Injection sites of FG and PHA-L were mainly in the ventrolateral VMN. Considerable numbers of FG-cells and PHA-L-fibers were present in the left side of the medial amygdala, ventral lateral septum, preoptic area, bed nucleus of stria terminalis, dorsomedial hypothalamic nucleus, arcuate nucleus, periventricular nucleus of thalamus, and midbrain central gray. The lateral dorsal raphe nuclei contained many PHA-L-fibers but few FG-cells. By contrast, both sides of the median raphe nucleus contained many FG-cells but few PHA-L-fibers. Reciprocal direct neural connection between the right and left side of the VMN were observed. The present results provide an anatomical basis for functional relationships between the VMN and these nuclei.

1. Introduction

The ventromedial hypothalamic nucleus (VMN) consists of dorsomedial, central, and ventrolateral subdivisions. It is located just lateral to the arcuate nucleus from the middle to caudal level of the hypothalamus (Paxinos and Watson, 2007). The VMN is surrounded by an area containing dendrites of VMN neurons and afferent projections but is lacking in cells (Millhouse, 1973). Projections of the rat VMN have been reported using autoradiographic method (Saper et al., 1976) or using phaseolus vulgaris-leucoagglutinin (PHA-L) tracing (Canteras et al., 1994). Subdivisions of the VMN exhibit different projection patterns (for review, see Bleier and Byne, 1985). A previous electrophysiological study has shown that neural connections between the VMN and brain regions such as the medial preoptic area (POA), stria terminalis, amygdale (AMG), anterior hypothalamic nucleus (AH), midbrain central gray (MCG), and median eminence are important for neuroendocrine functions of rats (Renaud and Martin, 1975). The VMN plays important roles in reproductive behaviors, such as female sexual behavior, male sexual behavior and aggressive behavior. The VMN is a key nucleus in the regulation of female sexual behavior, lordosis (Pfaff et al., 2006). The VMN plays a facilitative role in regulating lordosis because destruction of this nucleus inhibits lordosis; conversely, electrical stimulation facilitates it (Pfaff and Sakuma, 1979a, b). The ventrolateral (vl) VMN shows high expression of estrogen receptor (ER) mRNA (Simerly et al., 1990) and has many ERα-positive neurons (Yamada et al., 2009). Direct application of estrogen into the VMN facilitates estrous behavior in ovariectomized rats (Barfield and Chen, 1977; Rajendren et al., 1991). Thus, the VMN is an important nucleus for induction of lordosis, i.e., induction of estrous states by estrogen in female rats.

The neural systems that regulate lordosis are complicated and involve many neural substrates. The VMN is thought to facilitate lordosis behavior through the function of the MCG, a center for lordosis regulation, because destruction of the MCG prevents the

* Corresponding author. Tel.: +81 4 2947 6727; fax: +81 4 2947 6727.
E-mail address: hedgehog@waseda.jp (K. Yamanouchi).
URL: http://www.f.waseda.jp/hedgehog/ (K. Yamanouchi).

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lordosis facilitating effects of electrical stimulation of the VMN (Sakuma and Pfaff, 1979). A direct projection from the vlVMN to the MCG has been reported to send facilitative signals for lordosis. The nature of these axons is sexually dimorphic (Sakuma and Pfaff, 1981). The connection between the VMN and the MCG is the most critical neural tract for induction of estrous states.

In the telencephalon, the lateral septum (LS) exerts a lordosis-inhibiting influence, since destruction of this nucleus (Nance et al., 1974; Kondo et al., 1990) or injection of ibotenic acid into the LS (Tsukahara and Yamanouchi, 2001) facilitates lordosis behavior. The medial and lateral amygdala have facilitative and inhibitory influences, respectively (Masco and Carrer, 1980). The stria terminalis is also involved in lordosis regulation (Takeo et al., 1995). In the diencephalon, in addition to the VMN, many nuclei regulate lordosis behavior. The POA also plays an inhibitory role in regulating lordosis (Powers and Valenstein, 1972; Takeo et al., 1993; Sakuma, 1994). The habenula plays a facilitative role in lordosis regulation (Masco and Carrer, 1980). In the midbrain, in addition to the MCG, serotonergic neurons of the dorsal raphe nucleus (DR) are an important inhibitory regulator of lordosis (Kakeyama and Yamanouchi, 1996). The reticular nucleus in the midbrain is also involved in the control of the MCG (Pfaff, 1980). These regions may influence the function of the VMN directly or indirectly. However, the nuclei that send axons to the vlVMN and efferent pathways from the vlVMN to the nuclei involved in regulation of lordosis are not clear.

In addition, recently, the right and left VMN has been found to influence the ERα expression in each region, because in ovariectomized rats, lesions in the right or left VMN increase ERα expression in the contralateral side (Shimogawa et al., 2014). In the present experiment, to clarify the efferent and afferent neural connections and potential bilateral connections of the VMN that are involved in lordosis regulation, anterograde and retrograde neural tracers were injected into the left side of the VMN, specifically into the ventrolateral region, and histochemical analyses were performed from the telencephalon to the midbrain in ovariectomized rats.

2. Materials and methods

2.1. Animals

Seven-week-old female Wistar rats were purchased (Takasugi Experimental Animal, Saitama, Japan) and maintained on a controlled light-dark cycle (14L:10D, lights off at 19:00) with constant temperature (22–24 °C). Food and water were freely accessible. All experiments were conducted according to the regulations for Animal Experimentation at Waseda University (Approval No. 2011-A003, 2012-A004, 2013-A037).

One week after purchase, all animals were ovariectomized under isoflurane anesthesia to eliminate the influence of sex steroids. A retrograde neural tracer, fluoro-gold (FG: Biotium, Inc. Hayward, CA, USA), or an anterograde neural tracer, phaseolus vulgaris-leucoagglutinin (PHA-L: Vector Laboratories, Inc. Burlingame, CA, USA), was then injected into the left side of the VMN, specifically, the ventrolateral part to clarify neural connections and tracts involved in the regulation of lordosis behavior.

2.2. Injection of retrograde tracer

One week after the ovariectomy, FG (8% solution dissolved in distilled water) was injected iontophoretically into the left VMN through a glass micropipette (tip diameter 40–50 μm). Rats were fixed in a stereotaxic instrument in which the incisor bar was set 3.3 mm below the interaural line under isoflurane anesthesia. The tip of the micropipette was lowered to a point 2.5 mm caudal to, 9.8 mm below, and 0.8 mm left of the bregma. An alternating current of 2 μA was applied for 8–14 min using a Midgard Precision Current Source (Stoelting-Muromachi Kikai, Tokyo, Japan). Six days after FG injection, animals were deeply anesthetized with pentobarbital sodium solution (50 mg/kg body weight, Somnopentyl, Kyoritsu Seiyaku, Tokyo) and then perfused intracardially with ice-cold 50 mM phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde–50 mM phosphate buffer. Brains were removed and post-fixed overnight with the same fixative used for perfusion. Brains were further immersed in 30% sucrose–50 mM PBS for 5 days at 4 °C. Serial coronal brain sections (50-μm thickness) were taken with a cryostat and collected. Sections mounted on slides were dehydrated through a graded series of ethanol, cleared by xylene, and then coverslipped with marinol.

Brain sections were observed under a fluorescence microscope (U-LH100HGAPO, Olympus, Tokyo, Japan). The FG injection site and the distribution of FG-labeled neuronal cells were plotted onto the rat brain map of Paxinos and Watson (2007). Sections were observed under stimulation with a UV excitation filter and white-colored cells were identified as FG cells.

Digital images of FG cells were obtained with a Polaroid Digital Microscope Camera PDMCII/OL (Olympus) and stored in a computer.

2.3. Injection of anterograde tracer

One week after the ovariectomy, PHA-L (2.5% in sodium phosphate buffer, pH 8.0) was injected iontophoretically into the left VMN through a glass micropipette (tip diameter 10–20 μm). Rats were fixed in a stereotaxic instrument in which the incisor bar was set 3.3 mm below the interaural line under isoflurane anesthesia. The tip of the micropipette was lowered to a point 2.5 mm caudal to the bregma, 9.8 mm below the bregma, and 0.8 mm left of the bregma according to a rat brain atlas. An alternating current of 5 μA was applied for 18–20 min using a Midgard Precision Current Source (Stoelting-Muromachi Kikai). Eleven days after PHA-L injection, coronal brain sections (50-μm thickness) were obtained by the process same as above.

For PHA-L immunostaining, free-floating sections were incubated with 0.6% H_2O_2–50 mM PBS for 30 min at room temperature (RT) before and after rinsing thrice with 50 mM PBS for 10 min. Sections were then incubated with 5% normal goat serum (NGS, Chemicon, CA, USA)–0.1% Triton X-100–50 mM PBS at RT for 90 min, and then incubated with rabbit anti-PHA-L (1:300, Vector Laboratories, Inc.)–0.1% Triton X-100–50 mM PBS for 72 h at 4 °C. After washing thrice with 50 mM Tris–HCl buffered saline (TBS) for 10 min, sections were reacted with Envision Plus System-HRP (Dako, Glostrup, Denmark) for 30 min at RT. After rinsing thrice with 50 mM TBS for 10 min, sections were reacted with a Metal Enhanced DAB Substrate Kit (34065, Thermo Fisher Scientific, IL, USA) for visualization of PHA-L immunoreactivity. Immunostained sections mounted on slides were dehydrated through a graded series of ethanol, cleared by xylene, and then coverslipped with marinol. These sections were examined by light microscopy and digital images were stored on a computer. The PHA-L injection site and the distribution of anterograde tracer-labeled fibers and cells were traced onto the rat brain atlas of Paxinos and Watson (2007).

3. Results

3.1. FG-infusion area: retrograde labeling

In all 11 rats, fluorescence was observed in the right side of the VMH between 1.89 and 3.36 mm posterior to the bregma, and the injection site extended around a 1.2-mm area in the