Oxytocin Cells in the Supraoptic Nucleus Receive Excitatory Synaptic Inputs from the Contralateral Supraoptic and Paraventricular Nuclei in the Lactating Rat

Kazumasa Honda1, Ayako Sudo1 and Kentaro Ikeda1

1Faculty of Nursing and Welfare Sciences, Fukui Prefectural University, Fukui 910-1195, Japan

Abstract. The present experiments were undertaken to examine whether oxytocin cells in the supraoptic nucleus receive synaptic inputs from the contralateral supraoptic nucleur or paraventricular nucleus. Using urethane-anesthetized lactating rats, extracellular action potentials were recorded from single oxytocin or vasopressin cells in the supraoptic nucleus. Electrical stimulation was applied to the contralateral supraoptic nucleus or paraventricular nucleus, and responses of oxytocin or vasopressin cells were analyzed by peri-stimulus time histogram or by change in firing rate of oxytocin or vasopressin cells. Electrical stimulation of the contralateral supraoptic nucleus or paraventricular nucleus did not cause antidromic excitation in oxytocin or vasopressin cells but caused orthodromic responses. Although analysis by peri-stimulus time histogram showed that electrical stimulation of the contralateral supraoptic nucleus or paraventricular nucleus caused orthodromic excitation in both oxytocin and vasopressin cells, the proportion of excited oxytocin cells was greater than that of vasopressin cells. Train stimulation applied to the contralateral supraoptic nucleus or paraventricular nucleus at 10 Hz increased firing rates of oxytocin cells and decreased those of vasopressin cells. The results of the present experiments suggest that oxytocin cells in the supraoptic nucleus receive mainly excitatory synaptic inputs from the contralateral supraoptic nucleus and paraventricular nucleus. Receipt these synaptic inputs to oxytocin cells may contribute to the synchronized activation of oxytocin cells during the milk ejection reflex.

Key words: Milk ejection burst, Neural connection, Oxytocin cell, Paraventricular nucleus, Supraoptic nucleus

Materials and Methods

Animals and surgery

Animals were handled in accordance with the Guidelines for the Care and Use of Laboratory Animals of Fukui Prefectural University. Adult female Wistar rats (250–320 g B.W.) were used on days 8–12 of lactation under urethane anesthesia (1.1 g/kg, i.p.). An inguinal mammary gland was cannulated with a polyethylene tube to measure intramammary pressure, which was used to detect milk ejection (see the Electrophysiology section). A silicone cannula was inserted into the right atrium through the right jugular vein for injection of oxytocin, which was used to check whether measurement of intramammary pressure worked well. The rat was then fixed prone in a stereotaxic frame. All surgical procedures for the brain were performed by dorsal approach. Three small holes were drilled in the skull for insertion of recording and stimulating electrodes.

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Correspondence: K. Honda (e-mail: kazhond@fpu.ac.jp)
electrodes. The stereotaxic coordinates of Paxinos and Watson [15] were used for insertion of electrodes. A side-by-side stimulating electrode comprised of stainless steel wire (200 µm diameter), which was slanted laterally at an angle of 6 degrees to the vertical line, was inserted into the neurohypophysis (4.0 mm anterior to the interaural line, midline, 0–0.3 mm dorsal to the interaural line) in order to antidromically identify neurons in the SON projecting to the neurohypophysis. After securing the stimulating electrode inserted into the neurohypophysis with acrylic resin and self-tapping screws in the skull, the same type of stimulating electrodes were inserted into the right SON (7.8 mm anterior to the interaural line, 1.7 mm lateral to the midline, 0.3 mm dorsal to the interaural line) or PVN (7.2 mm anterior to the interaural line, 0.5 mm lateral to the midline, 2.0 mm dorsal to the interaural line). These stimulating electrodes were also secured in place with acrylic resin and self-tapping screws in the skull.

**Electrophysiology**

A glass micropipette (tip diameter, 1 µm; impedance, 20–30 MΩ) filled with 0.5 M sodium acetate containing 2% Pontamine sky blue 6B (Tokyo Chemical Industry, Tokyo, Japan) was introduced into the left SON. Pontamine sky blue 6B was used to mark the recorded site when it was necessary. Extracellular recordings were then made from single neurons. Recorded neurons were further identified as projecting to the neurohypophysis by their antidromic responses to electrical stimulation of the neurohypophysis (Fig. 1). Identified SON neurons were further divided into two groups according to their response to suckling. Eight to 11 pups were applied to a mother’s nipples, and the milk ejection reflex was induced. Neurons that showed a brief high frequency burst of action potentials approximately 10–20 sec before milk ejection that was detected by a sharp increase in intramammary pressure were designated as putative OT cells [1, 5, 16–18] (Fig. 2), and neurons that did not show bursts before milk ejection were classified as putative vasopressin (VAP) cells [18] (Fig. 2). Then neurons were tested for their response to electrical stimulation of the contralateral SON or PVN. To collect data for peri-stimulus time histograms (PSTH), a hundred sets of electrical stimulus pulses (2 monophasic pulses with a 5-msec interval; current intensity, 1 mA; pulse duration, 0.5 msec) were applied to the contralateral SON or PVN at 2-sec intervals. When the number of spikes for 25 msec after stimulation increased by more than 100% compared with the number before stimulation, the response was regarded as orthodromic excitation (OD+). When the silent period continued for more than 25 msec after electrical stimulation, the response was regarded as orthodromic inhibition (OD–). When the two above-mentioned responses were observed consecutively, the response was regarded as orthodromic inhibition followed by excitation (OD–+). In some of the cells analyzed by PSTH, the effects of train electrical stimulation of the contralateral SON or PVN on firing rate were also analyzed (Table 1). Electrical stimulus pulses (monophasic pulse; current intensity, 1 mA; pulse duration; 0.5 msec) were applied to the contralateral SON or PVN at 10 Hz, 5 Hz and 2 Hz for 100 sec. Mean firing rates for 100 sec before stimulation and those during stimulation were compared.

**Fig. 1.** Examples of the oscilloscope records obtained during antidromic identification of a SON cell. All traces were obtained from an average of eight sweeps. a: A trace shows averaged sweeps demonstrating constant latency. The neurohypophysis was stimulated at an inverse triangle. b: A trace shows the collision test, whereby a cell is antidromically activated immediately after the occurrence of a spontaneous (orthodromic) action potential so that the antidromic and orthodromic potentials collide and cancel each other in the middle of an axon (\(\frac{1}{2}\Delta\)). c: A trace shows the frequency following two shocks delivered at more than 50 Hz.

**Fig. 2.** Criteria for classification of oxytocin and vasopressin cells. Neurons that showed a brief high-frequency burst of action potentials approximately 10–20 sec before each milk ejection, detected by a sharp increase in intramammary pressure, were designated as OT cells (left traces). Neurons that did not show a burst before each milk ejection were classified as VAP cells (right traces). OT, oxytocin; VAP, vasopressin.

**Table 1.** Numerical summary of neurosecretory cells recorded from the SON

| Cell type | OT | VAP | Unclassified |
|-----------|----|-----|--------------|
| AD cell   | 39 | 36  | 31           |

**Stimulated site**

| Stimulated site | SON | PVN | SON | PVN | - |
|-----------------|-----|-----|-----|-----|---|
|                 | 19  | 20  | 15  | 21  | - |

**Type of analysis**

| Type of analysis | PSTH | FR | - |
|-----------------|------|----|---|
|                 | 19   | 6  | - |
|                 | 20   | 11 | - |
|                 | 15   | 3  | - |
|                 | 21   | 13 | - |

AD, antidromically identified as projecting to the posterior pituitary; OT, oxytocin; VAP, vasopressin; SON, supraoptic nucleus; PVN, paraventricular nucleus; PSTH, response was analyzed by peristimulus time histogram; FR, some of cells analyzed by PSTH also analyzed for their response to train electrical stimulation by change of firing rate.
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Histology

After the end of each experiment, a direct current of 1 mA was passed through each stimulating electrode for 5 sec to mark stimulated sites. After perfusion fixation with 10% formaldehyde solution (37% formaldehyde solution diluted to 10%) containing 10% sucrose, the brain was cut coronally into 50-µm sections using a freezing microtome and stained with Neutral Red. Then the stimulated sites were examined histologically and illustrated on the atlas of the rat brain of Paxinos and Watson [15] (Fig. 7 and raw data not shown). Recordings were only included in the analysis if the tip of the stimulating electrode was confirmed to have been located in the SON or PVN.

Results

A total of 106 single units that showed antidromic response to electrical stimulation of the posterior pituitary were recorded from the SON of 22 lactating rats (Table 1). Of these, thirty-nine cells showed a brief high-frequency burst of action potentials approximately 10–20 sec before reflex milk ejection and were designated as putative OT cells [1, 5, 16–18] (Fig. 2). Thirty-six cells did not show bursts before reflex milk ejection and were classified as putative VAP cells [18] (Fig. 2). The remaining 31 cells could not be classified because the mother rats did not show the milk ejection reflex during recording of these cells. Nineteen and 20 putative OT cells were tested for their response to electrical stimulation of the contralateral SON and PVN, respectively. Fifteen and 21 putative VAP cells were tested for their response to electrical stimulation of the contralateral SON and PVN, respectively. None of cells tested showed antidromic response to electrical stimulation of the contralateral SON or PVN. If cells showed antidromic response to electrical stimulation of the contralateral SON or PVN, spikes of the same number as that of stimulation (one hundred) should be detected at constant time after stimulation in PSTH analysis. But such spikes were not detected in any PSTH analysis (see Figs. 3 and 4).

Analysis by PSTH

Thirteen out of 19 (68%) putative OT cells were orthodromically excited following electrical stimulation of the contralateral SON, and the remaining 6 were unresponsive (Fig. 3 and Table 2). The latency from electrical stimulation to excitation was 36.0 ± 4.1 msec (N=13, mean ± standard error). On the other hand, only 4 out of 15 (27%) putative VAP cells showed orthodromic excitation following electrical stimulation of the contralateral SON, and 2 cells showed orthodromic inhibition. The latency of excitatory responses was 27.5± 2.0 msec (N=4). The remaining 9 putative VAP cells were unresponsive. There was a significant difference between the proportion of putative OT cells that showed an OD+ response to electrical stimulation of the contralateral SON and that of putative VAP cells that showed an OD+ response (χ² test, P<0.05).

Twelve out of 20 (60%) putative OT cells were orthodromically excited following electrical stimulation of the contralateral PVN,
and one was inhibited. The remaining 7 were unresponsive (Fig. 4 and Table 2). The latency from electrical stimulation to excitation was $33.9 \pm 4.7$ msec ($N=12$). On the other hand, 6 out of 21 (29%) putative VAP cells showed orthodromic excitation following electrical stimulation of the contralateral PVN, and 4 cells showed orthodromic inhibition followed by excitation. The latency of excitatory responses was $53.3 \pm 11.1$ msec ($N=6$). The remaining 11 putative VAP cells were unresponsive. Although there was no significant difference between responses of putative OT and VAP cells to electrical stimulation of the contralateral PVN, it was noticed that a higher proportion of putative OT cells tended to show orthodromic excitation than putative VAP cells.

**Table 2.** Numerical summary of response of OT and VAP cells in the SON to electrical stimulation of the contralateral SON and PVN, analyzed by PSTH

| Stimulated site | SON | PVN |
|-----------------|-----|-----|
| Type of response | OD+ | OD− | UN | OD+ | OD− | OD− | UN |
| OT cell         | 13 (68)* | 0 (0) | 6 (32) | 12 (60) | 0 (0) | 1 (5) | 7 (35) |
| VAP cell        | 4 (27) | 2 (13) | 9 (60) | 6 (29) | 4 (19) | 0 (0) | 11 (52) |

Numbers in each column indicate numbers of cells, and those in parentheses indicate percentage. OT, oxytocin; VAP, vasopressin; SON, supraoptic nucleus; PVN, paraventricular nucleus; OD+ orthodromic excitation; OD−, orthodromic inhibition; UN, unresponsive. * Responses of OT and VAP cells to electrical stimulation of the SON were significantly different ($\chi^2$ test, $P<0.05$).

**Table 3.** Mean firing rate of OT and VAP cells in the SON before and during electrical stimulation of the contralateral SON and PVN

| Stimulated site | SON | PVN |
|-----------------|-----|-----|
| Cell type       | OT (6) | VAP (3) | OT (11) | VAP (13) |
| MFR before stimulation | $1.10 \pm 0.46$ ** | 2.93 | $1.02 \pm 0.23$ | $2.50 \pm 0.69$ |
| MFR during stimulation | $6.38 \pm 1.39$ ** | 1.08 | $1.71 \pm 0.49$ | $1.89 \pm 0.40$ |

Electrical stimulus pulses were applied at 10 Hz for 100 sec. MFR, mean firing rate (Hz); OT, oxytocin; VAP, vasopressin; SON, supraoptic nucleus; PVN, paraventricular nucleus. Numbers in each parenthesis indicate number of cells analyzed. ** $P<0.01$, paired $t$-test.

**Analysis by rate meter recording**

Electrical stimulus pulses with a current intensity of 1 mA applied to the contralateral SON at 10 Hz increased the mean firing rate of putative OT cells (Fig. 5 and Table 3). The increase was statistically significant (paired $t$-test, $P<0.01$). It was noticed that putative VAP cells tended to show a reduced firing rate during train electrical stimulation.

Although electrical stimulus pulses with a current intensity of 1 mA applied to the contralateral PVN at 10 Hz also increased the mean firing rate of putative OT cells (Fig. 6 and Table 3), the increase was not statistically significant (paired $t$-test, $P>0.05$). It was also noticed that putative VAP cells tended to show a reduced firing rate...
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Discussion

The synchronization of the bursting activity of OT cells during the milk ejection reflex may include intranuclear and internuclear synchronization. The former was suggested to occur by local glutamatergic drive onto OT cells [14]. The latter mechanisms are not fully known at present. Our previous studies on the DMH showed that the cells in the DMH on one side projected to the SON in both sides of the hypothalamus. However, there were not many cells in the DMH projecting to the SON in both sides of the hypothalamus [11–13]. So it seems unlikely that such a small number of DMH cells fully support synchronization of 9000 OT cells in the hypothalamus. The present experiments were therefore undertaken to examine the existence of excitatory drives between OT cells in the bilateral hypothalamus. If such excitatory drives between OT cells of different magnocellular nuclei exist, they might contribute to synchronization of burst discharge of OT cells during the milk ejection reflex.

None of neurosecretory cells in the SON showed antidromic excitation in response to electrical stimulation of the contralateral SON or PVN. These results were similar to those of Takano et al., who examined connections between the bilateral SON [19], but were different from those of Saphier and Feldman, who examined connections between the ipsilateral PVN and SON [20, 21]. The latter authors reported that some of neurosecretory neurons in the PVN or SON were antidromically activated by electrical stimulation of the ipsilateral SON or PVN, respectively. Morphological studies have not demonstrated monosynaptic neural connection between magnocellular cells in the different nuclei, i.e., between the SON or PVN and contralateral SON [22, 23]. Therefore, in the present experiment, it was suggested that the orthodromic response of putative OT or VAP cells in the SON induced by electrical stimulation of the contralateral SON or PVN was mediated by a polysynaptic pathway.

We do not know the extent of stimulus current spread. So, it is difficult to exclude the possibility that we stimulated outside of the PVN or SON. In two rats, the stimulating electrodes aimed to stimulate the PVN were displaced from the magnocellular part of the PVN, and none of the cells recorded from these animals responded to the electrical stimulation applied through these electrodes. These results suggest that the extent of stimulus current spread in the present experiment was highly restricted.

A high ratio of putative OT cells in the SON showed orthodromic excitation following electrical stimulation of the contralateral SON or PVN. Although some putative VAP cells also showed orthodromic excitation following similar stimulation, there were fewer of these types of cells than putative OT cells. These result were also similar to those of Takano et al. [19] but were different from those of Saphier and Feldman [20, 21]. In addition to the difference between analyzed connections, i.e., contralateral vs. ipsilateral, the difference in animals used might constitute another reason for the difference in the results obtained. Lactating rats were used in the present experiments. On the other hand, Saphier and Feldman used male rats [20, 21]. It is known that OT cells of the hypothalamo-neurohypophysial system undergo reversible morphological changes whenever they are strongly stimulated. These changes include an increase in oxytocinergic synaptic contact with OT cell and of double synapses in contact with OT cells in lactating rats [24, 25]. Therefore, these changes may contribute to communication not only among oxytocin cells in the same nucleus but also among those in separate nuclei. So, it would be interesting to compare the results of the present experiments with those of non-lactating female rats. A small number of cells showed orthodromic inhibition or orthodromic inhibition followed by excitation in response to electrical stimulation of the contralateral
SON or PVN. These results suggest inhibitory neural connections also exist between bilateral OT or VAP cells, although the physiological significance of these inhibitory connections is not known at present.

Train stimulation of the SON or PVN increased firing rates of putative OT cells in the contralateral SON but not those of putative VAP cells. These results also showed that OT cells receive excitatory synaptic inputs from the contralateral SON and PVN in lactating rats. Putative VAP cells were rather inhibited during train stimulation of the contralateral SON or PVN, which may reflect a rise in blood pressure by vasopressin released into blood circulation following train electrical stimulation of the SON or PVN.

Although the results of the present experiments do not directly demonstrate that OT cells receive synaptic input from OT cells in the contralateral hypothalamus, it is suggested that there are excitatory synaptic connections between OT cells in the bilateral hypothalamus. In addition to our previous results indicating that some DMH cells projected to the SON in both sides of the hypothalamus [11–13], the results of the present experiment suggesting the existence of excitatory connections between OT cells in the bilateral hypothalamus would be a good base supporting a synchronization of burst discharges of OT cells in separate magnocellular nuclei.

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