Effects of Estrous Cycle, Estrogen and Progesterone Administration on the Antidromic and Orthodromic Reaction Threshold of Medial Preoptic Neurons in the Rat Hypothalamus

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ABSTRACT. In the present study, attempts were made to evaluate the effects of the estrous cycle and the administration of estradiol (Ovx-E) or progesterone (Ovx-P) after ovariectomy on the threshold of antidromic (AD) and orthodromic (OD) responses of neurons in the medial preoptic area (POA) induced by electrical stimulation of the median eminence-arcuate nucleus (ARC) of the hypothalamus. Single units were evaluated with extracellular recording. The threshold of AD and OD response in POA neurons declined during estrus and proestrus of the estrous cycle and in the Ovx-E group, but increased in the Ovx-P group and during diestrus. The number of neurons with the inhibitory OD response increased during proestrus and in the Ovx-E group, but decreased during diestrus. The neurons with the excitatory OD response increased in the Ovx-P group and during diestrus, and decreased during proestrus. The threshold of the inhibitory and excitatory OD response decreased during proestrus, estrus and in the Ovx-E group, but increased in the Ovx-P group. It is apparent from these studies that estrogen appears to activate the neurons of the POA receiving inhibitory synaptic input via the axonal collateral branches of the ARC neurons, and that progesterone seems to activate the neurons of the POA receiving excitatory synaptic input.—KEY WORDS: antidromic response, medial preoptic neuron, orthodromic response, ovarian hormone, threshold.

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Synaptic connections between the neurons of the medial preoptic area (POA) and those of the median eminence-arcuate nucleus (ARC) in the rat hypothalamus have already been clarified electrophysiologically [4–6, 9, 12, 28, 34–36]. The parvocellular neurosecretory cells in the outer layer of the ARC have been shown to control the anterior pituitary by releasing neurohormones such as luteinizing hormone-releasing hormone (LHRH) into the hypophyseal portal vein [32, 39]. LHRH neurons are present in the POA [2, 3, 8, 14, 18], which is also thought to contain the neural mechanism controlling LHRH serection, or the LHRH pulse generator [7, 20, 23]. It remains an unsettled question whether the cells constituting the LHRH pulse generator are LHRH neurons themselves or another cell group. The LHRH pulse generator is the mechanism by which LHRH is released into the hypophyseal portal vein through intermittent, burst-like firing of the neurons of the anterior hypothalamus, especially the POA. This mechanism is thought to control the reproductive mechanism directly or via sex steroids [23]. Progesterone administration after injection of estradiol benzoate in ovariectomized rats causes a lordosis response in almost all animals. This would suggest the presence of nerve cells selectively taking up and concentrating estradiol in the hypothalamus, and in particular the POA, in order to control sexual behavior [33].

There remain, however, many questions concerning basic aspects of the inhibitory and excitatory synaptic transmission system in POA neurons. In order to clarify these, we examined the influence of the estrous cycle and the administration of estrogen and progesterone after ovariectomy on the threshold of antidromic (AD) and orthodromic (OD) responses in POA neurons induced by ARC stimulation.

MATERIALS AND METHODS

Animals and anesthesia: Adult female rats of a Sprague-Dawley strain weighing 200–300 g were used for the experiments and maintained at a room temperature of 23±1°C, humidity of 40–60%, 14 hour illumination, and food (CE2, Nihon Clea, Tokyo) and water administered *ad libitum*. Vaginal smears were taken at 8:30 a.m. and examined daily microscopically at least for 3 successive cycles. All experimental rats had regular 4-day estrous cycles. Animals were classified into the following 7 experimental groups. Rats, showing proestrous, estrous, metestrous and diestrous stages of the estrous cycle; and rats, administered subcutaneously

with 20 μg estradiol benzoate (Ovx-E) and 10 mg progesterone propionate (Ovx-P) after ovariectomy were used. Ovariectomized rats were used 10–20 days after a surgical operation. The Ovx-E group was injected two times with 10 μg estradiol dissolved in 0.1 m*l* sesame oil in the morning of the experimental day and at 24 hr prior to the examination. The Ovx-P group was treated with 5 mg progesterone dissolved in 0.1 m*l* sesame oil at 24 hr before the experiment. Ovariectomized rats as the control (Ovx-Oil) were injected subcutaneously with only oil of the same volume as Ovx-E and Ovx-P. Animals were anesthetized at 9:00 a.m. with urethane (1.0–1.2 g/kg), given as a single intraperitoneal injection and placed in the headholder of a stereotaxic apparatus (SR-6, Narishige, Tokyo).

Recording and stimulating electrodes: A stainless-steel microelectrode made of an insect pin about 0.4 mm in diameter was used. The tip of the electrode was ground so as to be less than 1 μm in diameter in an electrolytic grinding solution. The electrode was insulated using Insulex E (Clark Electromedical Instruments, Ltd., England). The resistance of these electrodes was 0.5–4.0 megohms in saline. The recording electrode was connected to the input of a preamplifier (DAM-5A-641, W-P Instruments Inc., U.S.A.) through a high impedance probe. A bipolar stimulating electrode made of a nichrome wire about 0.125 mm in diameter was used. Insulation and fixation were carried out with epoxylite resin. It was connected with a digital stimulator (ME-6012, ME Commercial Co., Tokyo) having a variable intensity. The electrical stimulus to ARC was a single 1-msec biphasic rectangular wave, and principally, stimulation was applied at a frequency of once a second for 30 sec. The response of the POA neuron to ARC stimulation was classified as AD, OD or no response.

Recording and stimulating sites: The recording and stimulating electrodes were inserted in the POA and ARC according to the coordinates of Albe-Fessard *et al.* [1]. These were placed at anterior (A) 8.0 and lateral (L) 0.5 mm for a recording electrode in the POA; at A 5.8, L 0.3 and ventral (V) 1.1 mm for a stimulating electrode into ARC. The recording area was confined to a region within V 2.8–4.3 mm in depth between the suprachiasmic nucleus and commissura anterior of the hypothalamus. For unit recording, the microelectrode was mounted in the micromanipulator of the stereotaxic instrument and action potentials were led to an amplifier.

Observation and recordings of POA units: Action potentials were monitored visually and acoustically, displayed on a digital memory or monitoring oscilloscope (VC-9, Nihon Kohden, Tokyo). The amplified outputs were also led to an audio-amplifier and loudspeaker, oscilloscope, spike counter and to the external trigger of a square-wave pulse generator (ME-2100, MEAC system, ME Commercial Co., Tokyo). Triggered 1 msec biphasic square waves were supplied from the pulse generator to the driver amplifier of one channel of a polygraph (Nihon Kohden, Tokyo) and to a tape recorder (DFR-3515, Sony data recorder, Sony Magnescale Inc., Tokyo). The data were stored on a magnetic tape for later analysis, as well as a digital memory oscilloscope (DM-1562 Toshiba, Tokyo Electronic Ind. Co., Ltd., Tokyo) from which spikes were photographed directly with a polaroid camera (M75D, King CRT, Asanuma & Co., Ltd., Tokyo) using an instant black and white film (FP-3000B, Fiji Film Co., Tokyo). A single unit was initially recorded from active POA neurons extracellularly. ARC was then stimulated repeatedly to measure the minimum current required to induce AD and OD responses to obtain the threshold value (mA). The intensity of the current for stimulation was twice the threshold value in the evaluation of AD and OD responses to ensure a stimulus response. The criteria of AD response included a constant latency of the evoked spike, collision of antidromic potentials with spontaneous orthodromic ones and the ability of the neuron to follow stimuli delivered at above 60 Hz. OD responses of the POA neurons were classified into inhibitory OD responses with decreased spontaneous firing activity, excitatory OD responses with facilitation of firing activity, and lack of response to electrical stimulation of the ARC. Principally, the inhibiting and exciting orthodromic discharges of the POA neurons during and after ARC stimulation were monitored acoustically by a sound of the loudspeaker, displayed visually on a digital memory and monitoring oscilloscopes, and photographed directly with an instant black and white film. Finally, either inhibitory or excitatory OD responses were determined on a photograph. The effect of estrous cycle and the administration of estrogen and progesterone after ovariectomy on the threshold of the AD response, inhibitory OD response, excitatory OD response and lack of response in POA neurons was studied. The surgical operation and recording procedures were described in the previous publica-

tion [36].

Confirmation of the position of inserted electrodes in the brain: At the end of each experiment, the recording and stimulating sites were stimulated with 30 μA cathodal current through the electrode for 30 sec to deposit a spot of iron which indicated the site where the electrode reached. Brains were fixed by cardiac perfusion with 50 m*l* of saline containing 3% potassium ferrocyanide and ferricyanide and 50 m*l* of 10% formalin. The marking sites were confirmed by microscopic observation of 50 μm serial sections stained with neutral red.

RESULTS

In POA neurons exhibiting an AD response to electrical stimulation of the ARC, the threshold in 20 cells was studied for each test section. In POA neurons exhibiting an OD response, the threshold was measured in 30 cells during proestrus, estrus, metestrus and diestrus of the estrous cycle, and in 50 cells in Ovx-E, Ovx-P and Ovx-Oil groups.

The threshold for an AD response in a POA neuron was 0.40±0.12 mA in Ovx-E ($P<0.01$) and 0.43±0.11 mA during estrus ($P<0.05$), a significant decrease from the control value (Ovx-Oil) of 0.50±0.13 mA. A significant increase was seen in Ovx-P, 0.65±0.18 mA ($P<0.005$), and during diestrus, 0.60±0.11 mA ($p<0.01$). The threshold for an OD response in POA neurons was 0.46±0.15 mA in Ovx-E ($P<0.005$), 0.47±0.13 mA during estrus ($P<0.005$) and 0.50±0.16 mA during proestrus ($P<0.01$), all significantly decreased from 0.58±0.12 mA in Ovx-Oil, and conversely, a significant increase was seen in Ovx-P, 0.65±0.14 mA ($P<0.01$), compared with the control group (Fig. 1). Friedman Two-way Analysis of Variance yielded a statistically significant P-value (AD response: $Xr^2=23.19$, $P<0.003$ and OD response: $Xr^2=10.66$, $P<0.013$) in the distributions of threshold values to induce AD and OD responses measured on the different days of the estrous cycle.

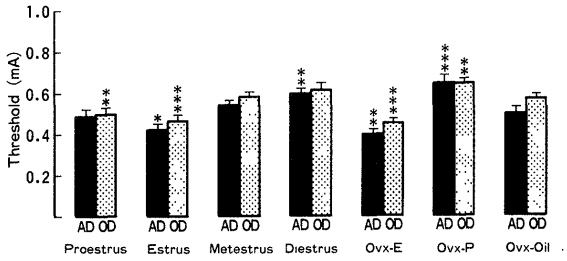

Fig. 1. Threshold values for antidromic and orthodromic responses in preoptic neurons to electrical stimulation of the median eminence-arcuate nucleus in the 7 experimental groups of rats. Bars indicate the mean±SEM. Abbreviations represent AD (■): antidromic response and OD (⊡): orthodromic response. Asterisks represent *: $P<0.05$, **: $P<0.01$, ***: $P<0.005$ as the levels of the statistically significant difference between threshold values for AD and OD responses in each test group and control values in Ovx-Oil by Student's *t (Welch)* test. There were significant regressions in the distributions of threshold values to induce AD and OD responses in each stage of the estrous cycle by Friedman Two-way Analysis of Variance, AD: $Xr^2=23.19$, $P<0.003$ and OD: $Xr^2=10.66$, $P<0.013$.

The OD responses in POA neurons were classified into 3 groups, an inhibitory OD response with a decrease in spontaneous firing activity, an excitatory OD response with an increase in spontaneous activity and an OD response with no change in the spontaneous activity. The number of neurons exhibiting an inhibitory response (inhibitory neurons) was increased during proestrus (33%) and in Ovx-E (32%), but decreased during diestrus of the estrous cycle. The percentage of neurons with the inhibitory OD response was the lowest in the control Oxv-Oil (14%). The number of neurons with the excitatory OD response was increased in Ovx-P (50%) and during diestrus (43%). The number of neurons with the excitatory OD response, as with the inhibitory OD response described above, was the fewest in Ovx-Oil (10%). Chi-square analysis between these three types of neurons in each test section and the corresponding three types in the control group revealed a significant difference in each test section

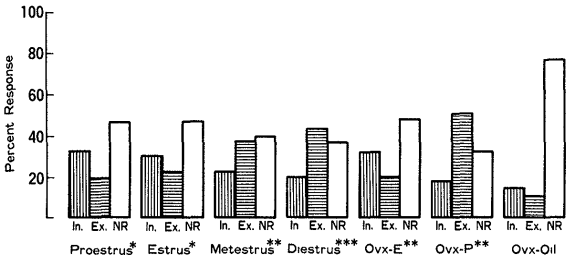

Fig. 2. The percentage of neurons with inhibitory orthodromic response, excitatory orthodromic response and no response in the preoptic area to electrical stimulation of the median eminence-arcuate nucleus in the 7 experimental groups of rats. Abbreviations represent In. (▥): neurons with inhibitory orthodromic response, Ex. (▤): neurons with excitatory orthodromic response and NR (□): neurons with no response. Asterisks represent *: $P<0.05$, **: $P<0.01$, ***: $P<0.005$ as the level of the statistically significant difference between X^2 values in each test group and control values in Ovx-Oil by Chi-square test.

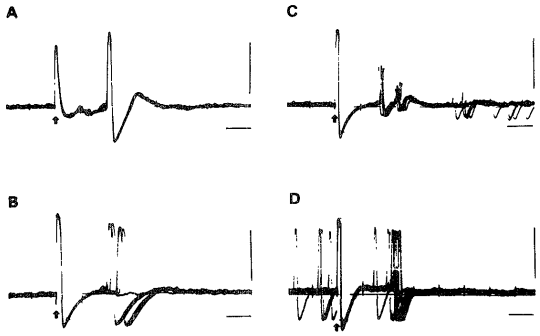

Fig. 3. Representative patterns of the antidromic response, inhibitory orthodromic response, excitatory orthodromic response and no response in preoptic neurons to electrical stimulation of the median eminence-arcuate nucleus. A, B, C and D show five superimposed reactions obtained with barely threshold stimuli. Arrows show artifact of the electrical stimulation. A: antidromic response. B, C and D: orthodromic response to repeated stimulation of the median eminence-arcuate nucleus. B: no response in terms of spontaneous firing activity. C: excitatory orthodromic response with facilitation of spontaneous firing activity. D: inhibitory orthodromic response with a decrease in spontaneous firing activity. Calibrations mark 5 msec and 1 mV.

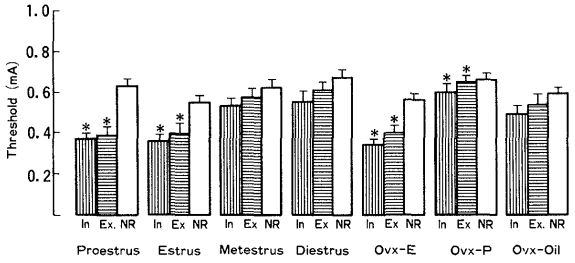

Fig. 4. Threshold of the inhibitory orthodromic response, excitatory orthodromic response and no response in preoptic neurons to electrical stimulation of the median eminence-arcuate nucleus in the 7 experimental groups of rats. Bars indicate the mean±SEM. Abbreviations represent In. (▥): inhibitory orthodromic response, Ex. (▤) : excitatory orthodromic response and NR (□): no response. Asterisks represent *: $P<0.05$ as the level of the statistically significant differnece between threshold values in each test group and control values in Ovx-Oil by Student's *t (Welch)* test.

(Fig. 2). Examples of the AD response, inhibitory OD response, excitatory OD response and no response are shown in Fig. 3.

The threshold of the inhibitory OD response was significantly lowered in Ovx-E, during estrus and proestrus at 0.34±0.10 mA, 0.36±0.07 mA and 0.37±0.08 mA, respectively, compared to the control level of 0.49±0.14 mA in Ovx-Oil ($P<0.05$), but increased in Ovx-P with a value of 0.60±0.12 mA ($P<0.05$). The threshold for the excitatory OD response was significantly lowered during proestrus, estrus and in Ovx-E at 0.38±0.11 mA, 0.40±0.12 mA and 0.41±0.11 mA, respectively, compared to the control level in Ovx-Oil of 0.54±0.13 mA ($P<0.05$). The threshold for the group exhibiting no response was statistically unchanged compared to the control value of 0.61±0.11 mA in Ovx-Oil, in each test section (Fig. 4). Throughout the estrous cycle no significant (In.: $P>0.09$, Ex.: $P>0.06$ and NR: $P>0.55$) regressions were observed in the distributions of threshold values.

DISCUSSION

The outer layer of the ARC contains the axonal endings of the infundibular neurons which regulate the anterior pituitary, and a few other neuronal elements. Yagi and Sawaki [40] localized the cell bodies of the infundibular neurons for the first time by recording the retrograde action potentials to determine the anatomical characteristics. This method of retrograde fixation has been used to localize the cell bodies of infundibular neurons in the ventromedial nucleus, dorsomammillary nucleus, anterior periventricular nucleus, suprachiasmic nucleus, medial preoptic nucleus and arcuate nucleus [11, 21, 22, 26, 27, 30, 31]. The projection of neurons on these nuclei was demonstrated histologically [10, 24, 25]. The cell bodies of the neurons of these nuclei are thought to received inhibitory or excitatory synaptic impulses via the cylindrodendrites of the infundibular neurons.

Yagi [37, 38] suggested the presence of neurons in the POA with a shortened latency period and increased firing frequency in response to the estrogen administration in ovariectomized rats, suggesting that these are estrogen sensitive neurons. Autoradiography further revealed the presence of cells in the medial preoptic nucleus, amygdaloid nucleus, septal nucleus and ventromedial nucleus which selectively take up and concentrate estradiol [33]. Receptor proteins specifically binding to the administered estradiol were noted in the cytoplasm of these cells. After being bound to these proteins, estradiol is transferred to the nerve cell nucleus to activate specific genes involved in the synthesis of related enzymes [29].

Many questions remain, however, as to the synaptic connection between the axon terminal of the POA neurons and ARC neurons as well as the

influence of estrogen and progesterone on the input of the inhibitory and excitatory neurons to POA neurons via the axonal anastomotic branches of the infundibular neurons. The effect of estrous cycle, estrogen and progesterone administration were therefore studied in rats to determine the stimulation threshold for the AD response and inhibitory and excitatory responses of POA neurons. POA neurons exhibiting an AD response to repeated ARC simulation showed neither a decrease nor augmentation of spontaneous firing activity during and after stimulation. These results are in agreement with the reports of Dyer *et al.* [6]. The AD response is a reaction which occurs when electrical activity is induced and recorded from the cell body of a single neuron following the stimulation of its axon terminal. Consequently, this electrical phenomenon is not influenced by excitatory or inhibitory neurotransmitters, since it is not mediated by a synapse, and therefore, does not result in either inhibition or excitation of the spontaneous firing activity.

OD responses of the POA neurons, on the other hand, were classified into an inhibitory OD response with a decrease in spontaneous firing activity by electrical stimulation of the ARC, an excitatory OD response with an increase in spontaneous firing activity and no response with neither an increase nor decrease in firing activity. The number of neurons exhibiting the inhibitory neuron characteristics was increased during proestrus and in Ovx-E compared with the control group, but decreased during diestrus of the estrous cycle. The number of neurons with excitatory OD response, conversely, was increased in Ovx-P and during diestrus. Kelly *et al.* [15] reported that in thirty-six medial preoptic-septal neurons (mPOA-S) from normal cycling female rats tested by microelectrophoretically administered with 17β-estradiol (17βES) and 17α-estradiol (17αES) hemisuccinate, twelve of mPOA-S units responded with inhibition to 17βES, and that none responded to 17αES. Furthermore, Kelly *et al.* [16] noted that 17βES brought direct and rapid changes in the firing activity of neurons, and that the non-antidromically identified neuron was a site for these effects, suggesting that the responses differed significantly throughout the estrous cycle. It is apparent from their studies that estrogen has significant effects on the spontaneous firing and on the antidromic and orthodromic activations in POA neurons.

The threshold of the AD response in POA neurons in response to electrical stimulation of the ARC was depressed in Ovx-E and the group administered with estrogen compared with the untreated ovariectomized control group (Ovx-Oil), and was elevated in Ovx-P and during diestrus of the estrous cycle. The threshold of the inhibitory and excitatory OD responses was decreased in Ovx-E, during proestrus and estrus, but increased in Ovx-P. A significant difference from the control group was noted in each of the above study sections. Kawakami and Sawyer [13] suggested for the first time that estrogen might lower the threshold of electrical stimulation of the anterior hypothalamus-preoptic area necessary for the induction of paradoxical sleep in the rabbit. On the other hand, Koizumi and Yamashita [17] asserted that an antidromic volley set up by stimulation of the posterior pituitary has been shown to produce a hyperpolarization of the somatic membrane of considerable magnitude and duration following a brief excitation of the cell. Furthermore, Lincoln [19] reported that the threshold was lowered by a combination of estrogen and progesterone treatment in the persistent estrous rat. It is interesting to note that the spayed estrogen-treated rats did not show the same degree of depression in spontaneous firing activity, which might indicate that both estrogen and progesterone are necessary to produce the depression in activity observed in the group with persistent estrus. The depression of the threshold may reflect some depolarizing neural input to the axons of neurons in the POA.

These findings are compatible with the notion that estrogen changes the membrane potential in the direction of depolarization, and that progesterone hyperpolarizes the membrane potential and raises the threshold. Threshold changes may be also influenced by excitatory and inhibitory neurotransmitters. These changes in the membrane potential are probably caused by structural changes of the ionic channels in the electric field.

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