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Sexually Dimorphic Regulation of NK-1 Receptor-Mediated Electrophysiological Responses in Vagal Primary Afferent Neurons

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Received 13 December 1999; accepted in final form 16 March 2000

INTRODUCTION

Activation of estrogen receptors, which are widely distributed throughout the central and peripheral nervous systems (Bettini et al. 1992; Papka et al. 1997), is critical for the development of sexual dimorphism. Estrogen can also produce numerous neuromodulatory effects. In the CNS, estrogen modulates the firing patterns and synaptic connectivity of hypothalamic neurons involved in reproductive behavior in female rats (Wong and Moss 1992). In the peripheral nervous system, the neuronal activity and receptive field size of afferent fibers innervating the uterus show variation following the estrous cycle (Robbins et al. 1992). The effects of estrogen on the nervous system are not only limited to areas obviously related to reproductive behavior. For example, following estrogen administration, the size of facial mechanoreceptive fields of trigeminal neurons is increased (Bereiter and Barker 1975). Additionally, there are numerous reports of gender differences in pain susceptibility and the response to analgesic agents, such as morphine (Baamonde et al. 1989; Cicero et al. 1996). These latter observations support a role for estrogen as a modulator of sensory processing.

Estrogen also increases gene expression of numerous peptides (Watters and Dorsa 1998) and receptors (Gazzaley et al. 1998), including neuromedin [substance P (SP)] receptors (Vilablana and Hanley 1997). SP is released from peripheral nerve endings of small diameter afferents eliciting peripheral reactions characterized as neurogenic inflammation (Lembeck and Holzer 1979). SP is also released centrally from primary afferent nerve endings in the spinal dorsal horn, where it contributes to the transmission of nociceptive information (De Koninck and Henry 1991). It has been recently demonstrated that SP is released from the cell bodies of primary afferent neurons (Huang and Neher 1996). Primary afferent neurons not only release SP, but some (dorsal root ganglion neurons) are also excited by SP (Dray and Pinnock 1982; Inoue et al. 1995; Li and Zhao 1998; Spiegelman and Pui 1990) while others [nodose ganglion neurons (NGNs) of the ferret] are inhibited by this neuropeptide (Jafri and Weinreich 1996, 1998). These actions presumably reflect the existence of SP autoreceptors in membranes of primary afferent neurons.

We have reported that most adult primary vagal afferent somata from male guinea pig are electrophysiologically unresponsive to exogenously applied SP. However, following algesin-induced inflammation in vitro (Weinreich et al. 1997) or in vivo (Moore et al. 1999b), ~80% of NGNs exhibit an NK-2 receptor-mediated depolarizing response to bath-applied SP. In a preliminary series of experiments with NGNs from female guinea pigs, we noted that, in contrast to male NGNs, significant numbers of control NGNs were depolarized by SP. Therefore, expression of tachykinin receptors in guinea pig NGNs may be sexually dimorphic.

To determine whether estrogen regulates SP responsiveness of guinea pig NGNs, we examined four groups of animals with various estrogen levels: (1) ovariectomized females (OVX), (2) OVX females with 17β-estradiol treatment (OVX + E2), (3) near-term pregnant females, and (4) control males. Our results suggest that estrogen may modulate SP responsiveness in these neurons.
reveal a gender difference in the expression of functional NK-1 tachykinin receptors in guinea pig NGNs.

**METHODS**

**Animals**

Reproductively mature female Hartley guinea pigs (4–6 mo old, bred at the University of Maryland) were anesthetized with ketamine (80 mg/kg ip)/xylazine (1 mg/kg im) and surgically ovarioectomized through bilateral flank incision. After at least 100 days, some ovarioectomized animals were anesthetized, and 21-day continuous-release pellets containing 0.25 mg 17β-estradiol (Innovative Research of America, Sarasota, FL) were inserted in the neck subcutaneously. Twenty days after the insertion of pellets, animals were anesthetized with ketamine/xylazine, and both nodose ganglia were removed. Nodose ganglia from ovarioectomized animals not treated with estrogen were used as the control group for 17β-estradiol–treated female animals. Near-term pregnant guinea pigs (~60 days gestation; term, 65 days) were used as the pregnant animal group. A subset of male guinea pigs (2–5 mo old) was subject to castration to examine the contribution of testosterone to the regulation of SP responsiveness. Guinea pigs were anesthetized as described above, to allow the removal of both testicles through bilateral scrotal incisions. These animals were allowed to recover at least 7 days prior to removal of nodose ganglia. Gангlia from noncastrated male guinea pigs served as controls. The University of Maryland Institutional Animal Care and Use Committee approved all methodology used in these experiments.

**Tissue preparation and cell culture**

Dissected ganglia were cleaned of adhering connective tissue and debris in ice-cold 4°C Lockes solution (in mM): 136 NaCl, 5.6 KCl, 1.2 MgCl₂, 2.2 CaCl₂, 14.3 NaHCO₃, 1.2 NaH₂PO₄, and 10 dextrose, equilibrated with 95% O₂–5% CO₂, pH 7.2–7.4. Isolated NGNs were prepared following the procedure outlined by Moore et al. (1999a), except ganglia were kept at 4°C for 6–8 h with collagenase (10 mg/ml) and dispase (10 mg/ml) in Hank’s balanced salt solution then incubated in the same solution at 37°C for 15 min. NGNs were subsequently dissociated by trituration with fire-polished Pasteur pipettes of diminishing diameters. Isolated NGNs were resuspended in KS). Cell suspensions (0.15 ml) were transferred onto circular poly-d-lysine (Sigma Chemical, St. Louis, MO)–coated glass coverslips (Bellco, Vineland, NJ) in a 24-well culture plate and maintained at 37°C. NGNs were held in culture for 2–48 h prior to electrophysiological recording. Coverslips were transferred to the recording chamber and superfused with Lockes solution via a gravity flow system (2–3 ml/min). The bath level was lowered to <50 μm above the neurons using an adjustable aspirator to minimize electrode stray capacitance. A concentration of 100 nM SP was used throughout this study because it evoked robust responses (>10 mV depolarization) but produced minimal desensitization with repeated exposure. SP was bath applied for 30 s, and antagonists were bath applied at least 5 min prior to the re-application of SP. When SP was administered multiple times, at least 3 min was allowed between applications.

**Electrophysiology and drug applications**

Electrical properties and responses to SP were recorded intracellularly using conventional “sharp” glass micropipettes having DC resistances of 30–100 MΩ when filled with 3 M KCl. Current- and voltage-clamp recordings were made with an Axoclamp-2A amplifier (Axon Instrument, Foster City, CA) in either bridge (bandwidth 10 kHz) or discontinuous mode (sample rate 5 kHz, bandwidth 0.3–3 kHz). In discontinuous mode, the headstage voltage was continually monitored to ensure that the sampled voltage reached steady state. Membrane input resistance (Rᵢ) was monitored by measuring changes in the amplitude of electrotonic voltage transients produced by 100-pA hyperpolarizing current pulses, 200 ms in duration. Neurons were included in this study if they had a stable resting membrane potential (less than −50 mV), action potential overshoot >20 mV, and a Rᵢ > 20 MΩ. Data acquisition and analysis were performed with pClamp 6.2 software and a Digidata 1200 interface (Axon Instruments).

The recording chamber was mounted on the stage of a compound microscope equipped with Hoffman optics (x400) to visualize neurons for intracellular impalement. Coverslips in the recording chamber were superfused with Lockes solution via a gravity flow system (2–3 ml/min). The bath level was lowered to <50 μm above the neurons using an adjustable aspirator to minimize electrode stray capacitance. A concentration of 100 nM SP was used throughout this study because it evoked robust responses (>10 mV depolarization) but produced minimal desensitization with repeated exposure. SP was bath applied for 30 s, and antagonists were bath applied at least 5 min prior to the re-application of SP. When SP was administered multiple times, at least 3 min was allowed between applications.

**Statistics**

Data are expressed as means ± SE. Z-test was used to compare the percentage of neurons responding to SP, and one-way ANOVA was used to compare response characteristics among the groups. P < 0.05 was considered significant. Statistical analyses were performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

**RESULTS**

**SP responsiveness and serum 17β-estradiol level**

Most NGNs from adult male guinea pig nodose ganglia do not show electrophysiologically detectible responses to bath applied 100 nM SP (Moore et al. 1999a,b, Weinreich et al. 1997). By contrast, 41% of acutely isolated NGNs from ovarioectomized and estrogen treated female guinea pig (OVX + E₂, 6 animals) were depolarized an average of 17 mV by 100 nM SP (Table 1 and Fig. 1). Some of the neurons fired action potentials during the rising phase of the depolarization (Figs. 1C and 2). The traces in Fig. 1A illustrate a typical SP response recorded in the same neuron in either current clamp or voltage clamp. Both responses were accompanied by an increase in membrane conductance. During the peak of the SP response, Rᵢ fell to about one-third of the value recorded before SP application (see Table 1). The serum 17β-estradiol values in OVX + E₂ females averaged 23.9 ± 3.3 pg/ml (mean ± SE, n = 8). SP responses were also observed in NGNs prepared from pregnant females (9 animals; Fig. 1B). There were no significant differences in the magnitude of the membrane depolarization, change in Rᵢ, duration of response (P > 0.1) as...
were reversibly blocked by 100 nM CP99,994 applied prior to and during SP application. The sample records in Fig. 2, A and C, show the reversible block by CP99,994 recorded in a NGN from OVX + E2 female and a control male NGN. SR48,968 and SB223412-A did not block SP responses (Fig. 2B) from any group. Taken together, these data suggest that NK-1 receptors mediate SP responses in NGNs from control males and females, with high and low serum estrogen concentrations.

**Effect of testosterone on SP responsiveness**

The percentage of SP responsive NGNs from control males and females with low serum estrogen levels were comparable (Table 1). To rule out a contribution of testosterone on SP response rate in males, we tested SP responsiveness in orchiectomized male animals (3 animals, 57 neurons). NGNs dissociated 1 wk after orchiectomy did not show any difference in response rate (18%, 10/57), receptor type (SP responses were blocked by CP99,994), response amplitude (17 ± 3.1 mV), response duration (69 ± 15.1 s), or membrane conductance change (38 ± 9.3%). This suggests that lower levels of testosterone in females, compared with male guinea pigs, do not contribute to their increased responsiveness to SP.

**DISCUSSION**

Our primary observation is that elevated serum 17β-estradiol, achieved either by pregnancy or by chronic administration of estrogen, directly or indirectly up-regulates NK-1 receptor-mediated SP responses in female primary vagal afferent neurons. These results suggest that estrogen can modulate sensory processing through regulation of functional receptor expression.

**TABLE 1. Depolarizing substance P responses recorded in isolated nodose ganglion neurons from four groups**

<table>
<thead>
<tr>
<th>SP response</th>
<th>OVX + E2</th>
<th>Pregnant</th>
<th>OVX</th>
<th>Control Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>%NGNs responding</td>
<td>41 (15/37)</td>
<td>19 (21/109)</td>
<td>13 (11/83)</td>
<td></td>
</tr>
<tr>
<td>Duration, s</td>
<td>64 ± 5.3</td>
<td>71 ± 6.0</td>
<td>69 ± 6.6</td>
<td>57 ± 7.4</td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>17 ± 2.1</td>
<td>16 ± 1.7</td>
<td>15 ± 1.9</td>
<td>13 ± 2.0</td>
</tr>
<tr>
<td>Base $R_{in}$, MΩ</td>
<td>65 ± 5.1</td>
<td>68 ± 6.7</td>
<td>71 ± 11.2</td>
<td>73 ± 12.8</td>
</tr>
<tr>
<td>Peak $R_{in}$, MΩ</td>
<td>37 ± 6.2</td>
<td>39 ± 2.9</td>
<td>38 ± 4.0</td>
<td>47 ± 7.9</td>
</tr>
<tr>
<td>%Decreased $R_{in}$</td>
<td>44 ± 5.0</td>
<td>37 ± 3.7</td>
<td>30 ± 5.6</td>
<td>34 ± 7.0</td>
</tr>
</tbody>
</table>

Serum estradiol, pg/ml

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>
| Values are means ± SE. Numbers in parentheses depict ratio of number of responding cells to total number of cells. Base $R_{in}$, input resistance before SP response; Peak $R_{in}$, input resistance at the peak of SP response; %Decreased $R_{in}$, (base $R_{in}$ − peak $R_{in}$) × 100/base $R_{in}$ SP, substance P, ovariectomized; E2, 17β-estradiol; NGNs, nodose ganglion neurons; ND, not determined. * $P = 0.018$ for comparison between OVX + E2 and OVX; $P = 0.002$ between OVX + E2 and control male with $t$-test. † $P = 0.002$ for comparison between pregnant and OVX; $P < 0.001$ between pregnant control and pregnant male with $t$-test. In addition to the $t$-test, we have calculated the proportion of responding NGNs in each animal and determined the mean and SE of proportions for each experimental group. These values were for $\text{OVA} + \text{E2}$ (n = 6; 44.8 ± 6.3%), Pregnant (n = 9; 44.8 ± 9.4%), OVX (n = 9; 21.8 ± 4.8%), and Male (n = 9; 12.0 ± 4.0%). One-way ANOVA analysis reveals that there is no difference ($P > 0.1$) in duration, amplitude, base $R_{in}$, peak $R_{in}$, and %decrease $R_{in}$ among all groups.

well as percentage of SP responsive neurons ($P = 0.842$) between pregnant and the OVX + E2 females (Table 1). The serum 17β-estradiol values in pregnant females averaged 16.0 ± 2.4 pg/ml (n = 4). For comparison, we examined SP responses in NGNs from OVX females (9 animals) and from control males (11 animals). The serum level of 17β-estradiol was 3.9 ± 0.3 pg/ml (n = 8) in OVX females. The percentage of SP responsive neurons in these two groups was significantly ($P < 0.005$) lower than in the groups with elevated serum estrogen (OVX + E2 and pregnant females). Only 19 and 13% of OVX animals and control males, respectively, were depolarized by SP (Table 1). However, the magnitude of the responses, their duration, and changes in $R_{in}$ produced by SP were similar to values observed in NGNs from animals with elevated serum estradiol (Table 1). There was no significant difference in the percentage of SP responsive NGNs (P = 0.361) between OVX females and control males. Furthermore, several passive and active membrane properties of NGNs were not significantly different ($P > 0.186–0.736$) among four groups (Table 2).

**Pharmacological characterization of NK receptor type**

The time course and magnitude of the SP responses, recorded in either current or voltage clamp in NGNs from female guinea pigs (and the responses in male guinea pigs, Fig. 1C), appeared similar to “unmasked” NK-2 receptor-mediated SP responses recorded in male NGNs following allergic inflammation (Weinrech et al. 1997) or exogenous application of serotonin (5-HT) (Moore et al. 1999a). To determine the nature of the tachykinin receptor subtype underlying SP responses in female NGNs, we treated NGNs from all groups with selective NK-1, NK-2, or NK-3 receptor antagonists (100 nM); CP99,994, SR48,968, or SB223412-A, respectively. SP responses from all animal groups, including control male NGNs,
Estrogen can have both acute and chronic effects on nervous tissues. Acute effects occur within seconds to a few minutes after estrogen exposure and disappear within seconds of estrogen removal. For example, bath applied 17β-estradiol reversibly potentiates kainate-induced currents in acutely dissociated hippocampal CA1 neurons within 3 min (Gu and Moss 1996). This potentiation was mimicked by 8-bromo-cAMP and enhanced by a phosphodiesterase inhibitor, suggesting that this action of 17β-estradiol is mediated by a second messenger, in particular, cAMP. Membrane estrogen receptors or cytosolic components have been suggested as possible mechanisms for these acute effects (Moss et al. 1997; Woolley 1999). Classically, estrogen affects neurons through genomic mechanisms. Estrogen can diffuse across the plasma membrane of neurons and bind to specific intracellular receptors. These hormone-receptor complexes enhance the transcription of many genes and induce the synthesis of specific proteins including membrane ligand-activated receptors. Genomic mechanisms usually requires hours to days. In the present study, we treated guinea pigs chronically with 17β-estradiol for 21 days, a time period sufficient for estrogen to exert genomic effects on neurons and at a dose that produces serum levels similar to that measured during pregnancy.

There are at least three possible mechanisms through which estrogen might regulate the SP responsiveness of NGNs. First, some NGNs innervating the uterus may respond to SP follow-

![Figure 2](http://jn.physiology.org/)

**FIG. 2.** Effects of selective tachykinin receptor antagonists on SP-induced membrane depolarization recorded in current-clamp mode. A: pretreatment with a selective NK-1 receptor antagonist CP99,994 (100 nM) completely blocked the membrane depolarizing response to a 30-s application of 100 nM SP recorded in NGN isolated from an OVX + E2 female. This cell had a resting membrane potential of −61 mV and a resting $R_\text{in}$ of 70 MΩ. SP response returned to near control values following 10 min of superfusion with control Locke solution. B: SP responses recorded from another NGN isolated from an OVX + E2 female were unaffected by pretreatment with a selective NK-2 receptor antagonist, SR48,968 (100 nM), or the selective NK-3 receptor antagonist, SB223412-A (100 nM). Resting membrane potential was −59 mV, and a resting $R_\text{in}$ was 80 MΩ. C: CP99,994 (100 nM) completely blocked SP-induced membrane depolarization of a NGN from a control male. The SP response returned to near control values after superfusion with normal Locke solution. Resting membrane potential was −65 mV, and a resting $R_\text{in}$ was 80 MΩ. Downward deflections in all traces are electrotonic voltage transients evoked by 100-pA (200-ms) constant-current pulses to monitor changes in $R_\text{in}$. Horizontal bars indicate times that SP and tachykinin antagonists were applied. Unlabeled bars are times of SP application. Calibration bar applies to A–C.

**TABLE 2.** Passive and active membrane properties of acutely isolated nodose ganglion neurons from four groups

<table>
<thead>
<tr>
<th></th>
<th>OVX + E2</th>
<th>Pregnant</th>
<th>OVX</th>
<th>Control Male</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter, μm</td>
<td>39 ± 0.9</td>
<td>39 ± 0.8</td>
<td>40 ± 0.5</td>
<td>39 ± 0.5</td>
<td>0.530</td>
</tr>
<tr>
<td>RMP, mV</td>
<td>−62 ± 1.3</td>
<td>−63 ± 0.7</td>
<td>−62 ± 0.7</td>
<td>−61 ± 0.7</td>
<td>0.332</td>
</tr>
<tr>
<td>$R_\text{in}$ MΩ</td>
<td>53 ± 4.0</td>
<td>53 ± 3.1</td>
<td>54 ± 3.3</td>
<td>48 ± 2.6</td>
<td>0.524</td>
</tr>
<tr>
<td>Rheobase, nA</td>
<td>0.90 ± 0.11</td>
<td>0.90 ± 0.06</td>
<td>0.94 ± 0.07</td>
<td>0.84 ± 0.05</td>
<td>0.736</td>
</tr>
<tr>
<td>AP amplitude, mV</td>
<td>92 ± 1.8</td>
<td>93 ± 1.2</td>
<td>90 ± 1.1</td>
<td>89 ± 1.5</td>
<td>0.186</td>
</tr>
<tr>
<td>Overshoot, mV</td>
<td>32 ± 1.9</td>
<td>31 ± 1.2</td>
<td>29 ± 1.2</td>
<td>28 ± 1.4</td>
<td>0.208</td>
</tr>
<tr>
<td>AP half-duration, ms</td>
<td>0.95 ± 0.05</td>
<td>1.00 ± 0.09</td>
<td>1.02 ± 0.05</td>
<td>1.15 ± 0.06</td>
<td>0.297</td>
</tr>
</tbody>
</table>

Values are means ± SE. Number of neurons for OVX + E2 was 37; for Pregnant, 79; for OVX, 109; and for Control Male, 83. RMP, resting membrane potential; AP, action potential. Rheobase, the minimum amount of depolarizing current needed to evoke single AP in 40–60% of the trials. Data recorded at 33–37°C. * One-way ANOVA analysis.
ing estrogen-induced changes in the uterus. A subpopulation of NGNs innervates the uterus (Ortega-Villalobos et al. 1990), and their peripheral nerve terminals show diverse changes following the estrous cycle in the rat (Robbins et al. 1992). Signal molecules could thus travel in vagal afferent axons from the uterus to the NGNs and trigger expression of NK responses. Although this may be a plausible explanation for the expression of SP responses in some NGNs, the number of SP responsive neurons in O VX + E2 and pregnant females (41%) are likely to exceed the number of NGNs innervating the uterus.

Second, estrogen may up-regulate SP responsiveness by altering the animal’s susceptibility to inflammation. It is well known that immune responses can be influenced by sex hormones (Grossman 1984). In general, females have stronger humoral and cell-mediated immunity. A more vigorous immune response not only makes females more resistant to infections, but it also exaggerates responses to autoantigens and can induce autoimmune disease (Ansar Ahmed et al. 1985). In addition, estrogen contributes to hypersensitivity reactions such as allergic rhinitis and asthma by augmenting mast cell degranulation (Vliagothi et al. 1992), and by up-regulation of histamine receptors (Hamano et al. 1998). Thus tachykinin receptors might also be up-regulated secondary to activation of immune cells. Although tractable, this explanation is in conflict with results from our previous studies. Following immunological activation of mast cells in vitro (Weinreich et al. 1997) or in vivo (Moore et al. 1999b), functional NK-2 receptors, not NK-1 receptors, are expressed in male guinea pig NGNs.

Last, and most plausibly, estrogen may directly act on estrogen receptors in NGNs, leading to increased SP responsiveness. Estrogen might simply increase the number of SP receptors by enhancing transcriptional processes or trigger the translocation of SP receptors from cytosol to the cell surface. It is also possible that estrogen activates or augments a regulator component of the SP receptors without changing the number of receptors. This interpretation is supported by the finding that estrogen receptor protein and mRNA are localized in the rat NGNs (Papka et al. 1997) and that physiological serum levels of 17β-estradiol correlate with the percentage of neurons expressing functional NK-1 receptor-mediated responses. Finally, preliminary data reveals that 17β-estradiol can increase SP responsiveness in isolated NGNs. Eleven of 36 NGNs incubated with 50 nM 17β-estradiol for >30 min showed SP response (14 ± 4.2 mV), while only 3/30 NGNs treated with vehicle responded.

In summary, we have shown that gender differences in the SP responses in guinea pig vagal afferent neurons are due to a sexually dimorphic expression of functional NK-1 receptors. Differences in estrogen levels are most likely responsible for the higher percentage of SP-responsive NGNs in females. These results illustrate that estrogen can modulate sensory processing via control of functional tachykinin receptor expression in primary afferent neurons.

The authors thank E. Lancaster and Drs. K. A. Moore and M. S. Gold for helpful comments on an earlier version of this manuscript. We also thank G. Pinkas for technical assistance.

This work was supported by National Institute of Health Grants NS-22069 to D. Weinreich and HL-49999 to L. P. Thompson.

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