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17 β -Estradiol Attenuates Excitatory Neurotransmission and Enhances the Excitability of Rat Parabrachial Neurons In Vitro

Mohammad Fatehi,^{1,2} Jeffrey A. Zidichouski,^{1,2,3} Samuel B. Kombian,⁴ and Tarek M. Saleh^{1,2*}

¹Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Canada

²Prince Edward Island Health Research Institute, Charlottetown, Canada

³Institute for Nutrisciences and Health, National Research Council of Canada, Charlottetown, Canada

⁴Department of Applied Therapeutics, Faculty of Pharmacy, Kuwait University, Safat, Kuwait

The steroid hormone 17 β -estradiol and its respective receptors have been found in several cardiovascular nuclei in the central nervous system including the parabrachial nucleus. In a previous study, we provided evidence that 17 β -estradiol attenuated an outward potassium conductance in parabrachial neurons of male rats, using an in vitro slice preparation. In this study we sought to enhance the comprehensive information provided previously on estradiol's postsynaptic effects in the parabrachial nucleus by directly examining whether 17 β -estradiol application will modulate excitatory synaptic neurotransmission. Using a pontine slice preparation and whole-cell patch-clamp recording, bath application of either 17 β -estradiol (20–100 μ M) or BSA-17 β -estradiol (50 μ M) decreased the amplitude of evoked excitatory postsynaptic currents (from 30–60% of control) recorded from neurons in the parabrachial nucleus. The paired pulse ratio was not significantly affected and suggests a post-synaptic site of action. The inhibitory effect on the synaptic current was relatively long-lasting (non-reversible) and was blocked by the selective estrogen receptor antagonist, ICI 182,780. Furthermore, 17 β -estradiol reduced the maximum current elicited by a ramp protocol, increased the input resistance measured between resting membrane potential and action potential threshold and caused an increase in the firing frequency of the cells under current-clamp. In summary, 17 β -estradiol caused 3 effects: first, a depolarization; second, a reduction in evoked excitatory postsynaptic potentials; and third, an enhancement of action potential firing frequency in neurons of the parabrachial nucleus. These observations are consistent with our previous findings and support a role for estrogen in modulating neurotransmission in this nucleus.

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Key words: autonomic; patch-clamp electrophysiology; excitatory postsynaptic currents; estrogen

The parabrachial nucleus (PBN) located in the brainstem, receives afferents from and projects to several forebrain regions implicated in autonomic regulation (Bernard et al., 1993; Bester et al., 1997; Huang et al., 2003; Kang et al., 2004). The presence of estrogen receptors (ER α and ER β) on cell bodies, axons, and terminals of autonomic regulatory nuclei throughout the neuraxis including the parabrachial nucleus has been documented (Turcotte and Blaustein, 1993). Previous work in our laboratory has shown that bath application of 17 β -estradiol attenuated an outwardly rectifying potassium conductance through a tyrosine-kinase linked process when recoding from PBN neurons in vitro (Fatehi et al., 2005). This estrogen-induced postsynaptic effect was not mimicked by the inactive stereoisomer 17 α -estradiol, but could be reproduced by bath application of the membrane impermeable-form, BSA-conjugated 17 β -estradiol indicating a non-nuclear site of action of estradiol. The observation that estradiol attenuated an outward potassium conductance in PBN neurons in vitro possibly resulting in increased PBN neuronal excitability, may be in contrast to what has been reported as an action of this hormone in vivo. Microinjection of estrogen into the PBN of male rats, while recording extracellular neuronal activity in the thalamus in vivo, rapidly attenuated visceral afferent neurotransmission to the thalamus (Saleh and Saleh, 2001). Other

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*Correspondence to: Tarek M. Saleh, Chief Research Officer, PEIHRI, Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, P.E.I. C1A 4P3, Canada. E-mail: tsaleh@upe.ca

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in vivo studies have shown that estrogen acts to enhance GABAergic neurotransmission through the PBN, thereby decreasing the excitability of neurons and neurotransmission through this nucleus (Saleh and Saleh, 2001). Occlusion of the middle cerebral artery (MCAO) has also been shown to increase PBN neuronal activity and release estradiol into the extracellular fluid surrounding the PBN (Saleh et al., 2003). Pre-treatment with estradiol prevented this ischemia-induced increase in PBN neuronal activity recorded in vivo (Saleh et al., 2003). Electrical stimulation of PBN neurons in vivo results in an increase in renal sympathetic nerve activity and bilateral microinjection of 17β -estradiol into the PBN decreased sympathetic nerve activity (Saleh et al., 2000, 2003; Saleh and Connell, 2003a). This further suggests that estrogen may exert an inhibitory influence on PBN neurons or neurotransmission in vivo.

The inhibitory effect of estrogen observed in the studies mentioned above in vivo seems to be contrary to what is observed in vitro. For example, evidence in the hippocampal slice preparation indicates that estradiol caused prolongation of the excitatory postsynaptic potentials (EPSPs) and increased incidence of repetitive firing in response to synaptic stimulation in the striatum radiatum (Wong and Moss, 1992). In this same nucleus, estradiol has also been shown to enhance active ionic conductances, such as voltage-dependent high- and low-threshold Ca^{2+} currents, in CA1 pyramidal cells (Wooley et al., 1997). Furthermore, rapid, non-genomic effects of estrogen have been reported whereby estradiol pretreatment enhanced the response to synaptic stimulation or glutamate application (Wooley et al., 1997) and potentiated kainate-induced currents (Gu and Moss, 1998).

Due to this apparent discrepancy in the responses observed in vivo versus in vitro, as well as the nucleus specificity of estradiol's actions on neurotransmission, we sought out to directly determine if estrogen had a modulatory role on glutamate-mediated excitatory neurotransmission in the PBN in vitro using whole-cell patch-clamping. This information will prove useful for clinicians and researchers interested in the cardiovascular implications of this hormone in males.

MATERIALS AND METHODS

Slice Preparation

All efforts were made to minimize the number of animals used in this study. The handling and maintenance of animals met the guidelines of the Canadian Council on Animal Care and were approved by the University of Prince Edward Island Animal Care Committee (protocol #04-032). Male Sprague-Dawley rats (Charles River, Montreal, PQ, Canada) weighing 150–200 g were deeply anaesthetized with isoflurane (Abbott Laboratories, Saint-Laurent, PQ, Canada) vapour in a closed environment and then decapitated. After quick removal, the brain was immersed in ice-cold ($2\text{--}3^{\circ}\text{C}$) artificial cerebrospinal fluid (aCSF) with the following composition (in mM): 125 NaCl, 2.5 KCl, 11 D-glucose, 18 NaHCO_3 , 1.2 NaH_2PO_4 , 1.2 MgCl_2 , 2.5 CaCl_2 (pH 7.4) that was continuously bubbled with 95% O_2 /5% CO_2 . Coronal slices (300 μm thick) containing the PBN were prepared from a tissue

block of the brain maintained in ice cold carbogenated aCSF, using a vibratome (Model 1000 plus, Ted Pella Inc., Redding, CA). Three to four slices containing the PBN were usually obtained from each brain. Slices were incubated in a temperature-controlled chamber ($27\text{--}29^{\circ}\text{C}$) containing aCSF bubbled with a mixture of 95% O_2 and 5% CO_2 for at least 45 min before the initiation of recording. Because the PBN is bilaterally located in pontine coronal sections, in most cases, each slice was hemisected and one half section was then transferred to a 500 μl recording chamber and submerged in continuously flowing extracellular solution (3–4 ml/min) gassed with 95% O_2 /5% CO_2 .

In Vitro Electrophysiological Recordings

The area from which neurons were recorded was restricted to a crescent-shaped region in the dorsolateral surface of the pons that is bordered dorsally by the ventral spino-cerebellar tract and ventrally by the *brachium conjunctivum*. These boundaries delineate the location of neurons believed to be responsive to baroreceptor activation (Jhamandas et al., 1991). “Blind” whole-cell patch-clamp recordings (Blanton et al., 1989) were carried out on neurons from the central, dorsal and external lateral subnuclei of the parabrachial nucleus (PBN) using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA). All experiments were carried out at temperatures of $30 \pm 1^{\circ}\text{C}$. A tight gigohm seal ($\geq 2\text{ G}\Omega$) on each cell was obtained using micropipettes (with 5–8 $\text{M}\Omega$ resistance when filled with the internal solution) pulled from thin-walled (outer diameter, 1.5 mm) glass capillary tubes (KG-33; Garner Glass Co., Claremont, CA) by a Flaming-Brown micropipette puller (Model P-87; Sutter Instruments Co., Novato, CA). The composition of the internal solution was (in mM): 130 K-gluconate, 6 NaCl, 10 HEPES, 2.5 Na-ATP, 0.1 Na-GTP; pH and osmolarity adjusted to 7.3 (with KOH) and 275–285 mOsmol/l, respectively. The external solution had the same composition as that used for slice preparation. The pH and osmolarity of the external solution were adjusted to 7.4 (with HCl) and 285–295 mOsmol/l, respectively. The fast electrode capacitance was first compensated before breaking into the cell and access resistance routinely ranged between 15–35 $\text{M}\Omega$. After whole-cell configuration was achieved, capacitive transients were cancelled by using an automatic compensation function of the MultiClamp 700B (about 70–80%) and were monitored periodically. Access (series) and input resistances of all cells were also monitored and recorded periodically throughout the experiment by applying a 20 mV hyperpolarizing pulse for 20–40 ms. Only those cells that showed $<15\%$ change in access resistance over the period of experiments were included in the analysis of the data. Synaptic transmission was evoked by positioning a bipolar tungsten stimulating electrode within the external lateral or dorsomedial subnuclei of the PBN. For synaptic current recordings, all cells were voltage clamped at a holding potential (V_h) of -65 mV , which was near their resting potential. In some experiments, voltage ramps were applied ranging from $-130\text{--}+20\text{ mV}$ (4 sec duration). The current produced in response to this protocol was recorded to produce an I–V curve. Data acquisition and analysis were carried out using Clampex and Clampfit 9.2 software respectively (Axon Instruments, Union City, CA). All data were acquired at a sampling rate of 6.6 kHz and filtered at 3 kHz. Each stored trace was an average of two successive responses to presynaptic stimulation or to a ramp pro-

Fig. 1. 17 β -Estradiol inhibits evoked excitatory postsynaptic currents (EPSCs) recorded in PBN cells in a concentration-dependent manner. **A:** Representative current traces recorded from 3 cells ($V_h = -65$ mV) in the presence of picrotoxin (50 μ M), showing the extent of inhibition of the synaptic response induced by various concentrations of 17 β -estradiol. **B:** A concentration-response curve for 17 β -

estradiol on the evoked EPSC amplitude. The calculated EC_{50} was 19.8 μ M. **C:** An averaged normalized time-effect plot generated from 10 cells that were exposed to 50 μ M 17 β -estradiol for the duration indicated by the length of the line. Note that after 10 min, each point on the graph is plotted every minute.

tolcol depolarization. Drugs were applied to the cells by bath perfusion of the slices with aCSF containing the final concentration of the drug.

Statistics

EPSC amplitudes were measured from baseline to peak and considered as the excitatory synaptic strength at the chosen stimulus intensity. Responses were normalized by taking the mean of the last four responses before drug application and dividing the rest of the values by this mean. Therefore, data are expressed as mean percentage change from control values $\pm$ standard error of the mean (SEM). Each individual cell served as its own internal control. For comparison of various groups, means were analysed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test. Results expressed as percentages of control were considered to be nonparametric data and analysed by employing the Mann-Whitney U -test. Statistical significance was determined at $P \leq 0.05$. Graphing was carried out using Excel (Microsoft, Mississauga, ON, Canada), GraphPad Prism (Prism Software, San Diego, CA), and CorelDraw (Corel Software, Ottawa, ON, Canada) software.

Drugs

Isoflo (isoflurane) was purchased from Abbott Laboratories (Saint-Laurent, QC, Canada). ICI 182,780 was obtained from

Tocris (Ellisville, MO), respectively. 17 β -Estradiol, BSA-17 β -estradiol (17 β -estradiol 6-(*O*-carboxymethyl) oxime:BSA fluorescein isothiocyanate conjugate; 5–10 mol steroid per mol BSA), tetrodotoxin (TTX), and all of the salts used in the aCSF were purchased from Sigma (St. Louis, MO). Appropriate stock solutions were made and diluted with aCSF just before application.

RESULTS

Data presented were obtained from either voltage- or current-clamp recordings from neurons located in central sub-nuclei of the lateral parabrachial nucleus (PBN). Electrophysiological criteria set for accepting cells were: resting membrane potential (RMP) more negative than -55 mV, evoked excitatory postsynaptic current (EPSC) or evoked excitatory postsynaptic potential (EPSP) latency <5 msec, and action potential amplitude >80 mV measured from the resting membrane potential. For voltage-clamp experiments, holding potential was -65 mV. To show that the negative current signal after the stimulus artefact recorded under voltage-clamp conditions was a synaptic response, in a few experiments either TTX (1 μ M) or cadmium (100 μ M) were bath applied and completely abolished the signal (data not shown). Thus, taking the latter observations and the average slope of activation (2.7 ± 0.8 msec) and decay (9.6 ± 1.7 msec) of

the responses together, we are confident that the recorded responses were glutamate-mediated EPSCs. Furthermore, these evoked EPSCs were completely blocked by the non-NMDA receptor antagonist, CNQX (10 μ M; data not shown).

Effect of 17 β -Estradiol on Evoked Excitatory Postsynaptic Currents

All synaptic events recorded under voltage clamp at a holding potential of -65 mV were inward currents. In all experiments, 50 μ M picrotoxin, a GABA_A receptor-chloride channel blocker, was added to the aCSF thereby eliminating any IPSC contamination of the excitatory responses. At this holding potential and inclusion of picrotoxin, the evoked synaptic events recorded in this study were predominately non-NMDA receptor-mediated excitatory responses. Bath application of 17 β -estradiol (20–100 μ M) for 3–5 min in the presence of picrotoxin (50 μ M) consistently reduced the amplitude of evoked EPSCs in a concentration-dependent manner (Fig. 1A,B). The onset of action was between 1–2 min with a peak effect observed after 3–5 min of application (Fig. 1C). A maximum synaptic depression of $56.0 \pm 4\%$ of control was observed at a concentration of 50 μ M 17 β -estradiol ($n = 10$; $P < 0.01$; Fig. 1C), with a calculated EC₅₀ value of 19.8 μ M. Representative recordings shown in Figure 1A, illustrate the inhibitory effect of 17 β -estradiol on EPSCs. In some experiments (data not shown), we observed spontaneous miniature inward currents under voltage-clamp conditions ($V_h = -65$ mV), which were similarly attenuated after exposure to 17 β -estradiol. No significant recovery of either the EPSC (Fig. 1A,C) or the miniature inward currents was observed 20–30 min after washout of the drug.

Involvement of Estradiol Receptors in the Inhibition of Evoked Excitatory Postsynaptic Currents

To further verify whether this effect of the hormone was receptor-mediated, we treated slices with two different concentrations of 17 β -estradiol after pre-treatment of cells with aCSF containing the estradiol receptor antagonist, ICI 182,780 (200 μ M, dissolved in 0.05% v/v DMSO). Similar to our previous finding (Fatehi et al., 2005), exposure of the brain slices to DMSO alone (up to 0.1% v/v), or ICI 182,780 (200 μ M dissolved in 0.05% v/v DMSO) had no significant effect on resting membrane current or on synaptic currents recorded in these cells (data not shown). The inhibitory effects of 20 and 50 μ M 17 β -estradiol on the evoked excitatory postsynaptic currents were significantly attenuated in the presence of ICI 182,780 (Fig. 2). For example, under control conditions, 20 μ M 17 β -estradiol significantly depressed the evoked EPSC by $26.7 \pm 5.6\%$ ($n = 6$; $P < 0.05$). In the presence of 200 μ M ICI 182,780, however, the same concentration of 17 β -estradiol only slightly reduced the evoked EPSC by only $5.6 \pm 4.5\%$ ($n = 5$; $P > 0.05$).

Fig. 2. The inhibitory effect of 17 β -estradiol is partially mediated by classical estrogen receptors. **A**: Representative current traces demonstrating the inhibitory effect of 17 β -estradiol is attenuated in the presence of ICI 182,780 (200 μ M). **B**: Summary of the antagonistic action of ICI 182,780 (200 μ M) on the inhibitory effect of estradiol on EPSC amplitude at two different concentrations (* $P < 0.05$, ** $P < 0.01$, # $P < 0.05$ vs. control, one-way analysis of variance followed by a Tukey-Kramer multiple comparison test, $n = 4$ –5 cells).

Estradiol has been reported previously to produce its neuronal effects by activating receptors located on either the surface of the membrane or intracellular receptors (Gu and Moss, 1998; Fatehi et al., 2005). To determine if the inhibitory effect of 17 β -estradiol was a result of activation of cell surface receptors, we carried out further experiments using a membrane impermeable form of 17 β -estradiol, bovine serum albumin-conjugated 17 β -estradiol (BSA-17 β -estradiol). Similar to the action of 17 β -estradiol, bath application of BSA-17 β -estradiol (50 μ M) also reduced the excitatory postsynaptic currents by $44.0 \pm 4.0\%$ ($n = 5$; $P < 0.05$) (Fig. 3). This inhibition was also markedly attenuated ($-19.0 \pm 7.0\%$; $n = 3$; $P < 0.05$ compared to control effect) (Fig. 3) when BSA-17 β -estradiol was applied to cells that had been pre-treated with ICI 182,780 (200 μ M).

Fig. 3. 17β -Estradiol-induced inhibition of the EPSC is primarily mediated by an estradiol receptor on the cell membrane. **A**: Superimposed current traces of the evoked EPSC illustrating the inhibitory effect of the membrane impermeable analog, BSA- 17β -estradiol (50 μ M). **B**: Representative traces showing that ICI 182,780 (50 μ M) partially antagonizes the inhibitory effect of BSA- 17β -estradiol (50 μ M) on the EPSC. **C**: Bar graph summarizing the inhibitory effect of BSA- 17β -estradiol (BSA-E2; 50 μ M) on the EPSC, which is attenuated by ICI 182,780 (50 μ M) pretreatment ($*P < 0.05$, $**P < 0.01$, $^{\#}P < 0.05$ vs. control, one-way analysis of variance followed by a Tukey-Kramer multiple comparison test, $n = 4$ –10 cells).

Effects on Paired-Pulsed Synaptic Responses

To determine if the inhibitory effect of 17β -estradiol on the excitatory synaptic transmission was due to a presynaptic or postsynaptic mechanism, another set of experiments was carried out on responses using a paired-pulse protocol (100 msec interval between the first and the second pulse). The paired-pulse ratio is a common index used to determine change in neurotransmitter release probability from the presynaptic terminal (Creager et al., 1980; Clark et al., 1994). When a pair of stimuli was applied, two excitatory postsynaptic currents were recorded. 17β -Estradiol (20 μ M) had an equal inhibitory effect on the first and the second response

Fig. 4. 17β -Estradiol does not alter paired-pulse ratio. **A**: Superimposed representative traces taken at the beginning and after 20 min of recording showing no change in paired-pulse depression over time. **B**: Superimposed representative traces showing equal 17β -estradiol (20 μ M)-induced inhibition of the first (P1) and second (P2) responses to a paired pulse stimulation protocol. **C**: Bar graph summarizing the lack of change in paired-pulse ratio following exposure to 17β -estradiol (20 μ M; $n = 5$).

resulting in no change in the paired pulse ratio (0.35 ± 0.7 vs. 0.38 ± 0.5 in control, $P > 0.05$; $n = 5$) (Fig. 4).

Effects on Responses to Slow Ramp Depolarization

To determine if 17β -estradiol modulated postsynaptic conductances to produce the observed decrease in EPSC amplitude, we measured the effect of estradiol application on steady-state currents activated by slow voltage ramps. 17β -Estradiol at relatively low concentrations (≤ 20 μ M) did not produce any current in these cells but the steady state I-V curve generated by the slow voltage ramps in the presence of 20 μ M 17β -estradiol intersected the curve produced under control conditions at a relatively negative potential (-80 to -85 mV) (Fig. 5A). An estimate of the chord conductance between -65 mV (resting membrane potential) and -40 mV, showed that 17β -estradiol also caused an increase in the

Fig. 5. 17 β -Estradiol decreases the outwardly rectifying conductance. **A:** Superimposed representative current traces illustrating the inhibitory effect of 17 β -estradiol (20 μ M) on responses to a slow voltage ramp applied from -130 to $+20$ mV. Note the presence of action potentials (sharp spikes) following exposure to 17 β -estradiol (20 μ M). **B:** Bar graph summarizing the effect of 17 β -estradiol (20 μ M) on ramp responses recorded from PBN cells (* $P < 0.05$, $n = 4$ cells).

resistance of the cells (200.5 ± 20 M Ω compared to 110 ± 10 M Ω in control, $n = 5$, $P < 0.05$). Furthermore, the amplitude of maximum current at 10 mV was reduced by $30 \pm 6\%$ of control ($n = 5$; $P < 0.05$) (Fig. 5). In addition to this direct postsynaptic effect, 20 μ M 17 β -estradiol also seemed to increase the excitability of these neurons, as it resulted in action potential generation in most cells around -50 mV after the ramp depolarization (Fig. 5A; note spikes in the response recorded after exposure to 20 μ M 17 β -estradiol).

Effects on Resting Membrane Potential and Action Potentials

To determine if the 17 β -estradiol-induced increase in input resistance of PBN neurons was physiologically-relevant, we tested if it affected neuronal excitability of these cells by examining its effects on action potentials. As observed, bath application of low concentrations of

Fig. 6. 17 β -Estradiol causes a moderate depolarization, and increases action potential firing frequency in PBN neurons. **A:** Representative traces illustrating the effects of 17 β -estradiol (50 μ M) on resting membrane potential, evoked postsynaptic potential amplitude and action potential firing frequency. Upper panel shows the current pulse (20 pA) used to trigger action potentials before (middle panel) and following (lower panel) exposure to 17 β -estradiol (50 μ M). Note that the actions potentials have been truncated for clarity. **B:** A bar graph summarizing the effect of 17 β -estradiol (20 μ M) on the resting membrane potential, evoked postsynaptic potentials amplitude and action potential firing frequency recorded from PBN cells (* $P < 0.05$, $n = 3$ –5 cells).

17 β -estradiol in current clamp mode did not induce any measurable change in the resting membrane potential. This suggests that 17 β -estradiol does not alter a resting or leak conductance. However, 17 β -estradiol at higher concentrations (≥ 50 μ M) caused a significant membrane depolarization (11 ± 3 mV; $P < 0.05$; $n = 3$) (Fig. 6). In addition, current clamp experiments showed that 17 β -estradiol (50 μ M) decreased the amplitude of evoked excitatory postsynaptic potentials by $24.0 \pm 5.0\%$ of control ($P < 0.05$; $n = 3$) (Fig. 6) that is consistent with our voltage clamp findings. This effect was not due to a change in the driving force, as EPSPs from cells in which the change in RMP was compensated for by direct current injection were also inhibited by a similar percent change from control ($P < 0.05$; $n = 3$; data not shown).

When action potentials were triggered by a 20 pA, 200 msec step of depolarizing current, 17 β -estradiol (50 μ M) increased the frequency of action potential firing by $188.0 \pm 19.0\%$ ($P < 0.05$; $n = 3$) (Fig. 6).

DISCUSSION

The present investigation delineates the pharmacologic effects of 17 β -estradiol on excitatory neurotransmission in the PBN. It also evaluates the effects of this biologically-active form of estradiol on resting membrane potential and on electrically evoked potentials. In summary, the important findings of this study are: 1) 17 β -estradiol attenuates evoked excitatory postsynaptic currents and potentials in the PBN; and 2) 17 β -estradiol causes a postsynaptic membrane depolarization and increases action potential firing frequency, while significantly reducing the maximum current observed at the more depolarized voltage potentials used in this study. Considering that these effects taken together seem contradictory based on the existing data, our interpretation is that it is possible that individual neurons are excited, but the synaptic network is inhibited, which overall results in reduced neuronal excitability. The functional significance of this suggestion is discussed below.

Mechanism of Action on Excitatory Neurotransmission

It is well established that evoked excitatory postsynaptic currents recorded from neurons in the PBN are mediated mainly by non-NMDA-receptor activation (Zidichouski et al., 1996; Saleh et al., 1997; Chen et al., 2000). Because it was our objective to investigate for any possible effect of 17 β -estradiol on only excitatory responses, all experiments were carried out in the presence of 50 μ M picrotoxin. This concentration of picrotoxin has been shown to be sufficient for eliminating GABAergic inhibitory transmission after afferent fibre stimulation (Zidichouski et al., 1996; Saleh et al., 1997; Chen et al., 2000). The results presented here support the conclusion that 17 β -estradiol at 20–100 μ M concentration, attenuates excitatory synaptic neurotransmission within the PBN. This estradiol-induced inhibitory effect seemed to be receptor mediated because it was significantly attenuated by the estradiol receptor antagonist, ICI 182,780. The possibility of additional receptor-independent effects of 17 β -estradiol could not be excluded with certainty, however, due to the high concentration of ICI 182,780 used did not fully antagonize the inhibitory effect of this hormone. This modulation of excitatory transmission by 17 β -estradiol may be dependent on the activation of cell membrane estradiol receptors as it was mimicked by BSA-17 β -estradiol, a membrane impermeable analogue. This effect is consistent with the observation that 17 β -estradiol blocked synaptic activity in other autonomic nuclei such as the amygdala and nucleus of the solitary tract (Womble et al., 2002; Xue and Hay, 2003).

The observation that the EPSCs did not recover after washout of estradiol for 20–30 min in this study, is consistent with observations made with other steroids

and peptides after bath application (Wooley et al., 1997; Gu and Moss, 1998; Wooley, 1999). It is believed that the “sticky” nature of these drugs does not allow for recovery and that these drugs could be activating more long term temporal or even genomic changes in the cell. It is documented that estradiol can activate a variety of intracellular signalling pathways (Lee and McEwen, 2001; Simoncini and Genazzani, 2003). These may result in long-lasting alterations in glutamate receptor sensitivity. In general, 17 β -estradiol acts via at least two broad-spectrum mechanisms, genomic and non-genomic, to regulate morphology, metabolism, and electrical properties of various cells. The genomic effects of 17 β -estradiol are not immediate and, in fact may take several hours or days to be observed. In contrast, the non-genomic effects of 17 β -estradiol are comparatively much more immediate and normally occur within a few minutes of exposure. Although we intended to focus on non-genomic effects of 17 β -estradiol and specifically on excitatory synaptic neurotransmission in cells located in the central PBN, it might not be reasonable to exclude that a genomic effect could also be occurring. However, the potential genomic effects of the drug would be limited by the fact that bath application of estradiol was only done once per brain slice, thus limiting the potential of observing effects that may be related to a genomic change.

A recognised approach to distinguish presynaptic from postsynaptic actions on synaptic transmission is the use of a paired-pulse protocol (Creager et al., 1980; Zucker, 1989; Clark et al., 1994; Saleh et al., 1997; Martín and Pozo, 2004). Because we did not observe any significant change in the paired-pulse ratio in the present study, this data suggests that the inhibitory effect of 17 β -estradiol on excitatory neurotransmission in the PBN synapse is likely occurring via a postsynaptic site of action. Interestingly, both increases and decreases in synaptic transmission have been observed with 17 β -estradiol (Wooley, 1999; Kelly et al., 2003). The majority of studies, and now ours, seem to indicate that the effect of estradiol on synaptic transmission within central nervous system nuclei is predominantly inhibitory (Weiland and Orchinick, 1995; Wooley, 1999; Womble et al., 2002; Xue and Hay, 2003). It has also been shown, however, that this steroid can cause significant potentiation of excitatory postsynaptic responses and induce LTP in hypothalamic and hippocampal synapses (Gu and Moss, 1998; Foy et al., 1999; Rudick and Woolley, 2003; Shiroma et al., 2005).

Effect on Cell Excitability

Although the data on synaptic transmission would suggest that 17 β -estradiol may minimize PBN neuronal excitability, the postsynaptic experiments indicate that the overall effect of 17 β -estradiol on these cells is excitatory. Indeed, in current clamp experiments, we could record concurrently both a decrease in EPSP amplitude and an increase in AP firing frequency. A recent study in our laboratory showed that 17 β -estradiol blocked

outward potassium currents in neurons recorded within the PBN (Fatehi, et al., 2005). According to the latter observation, it was suggested that the hormone caused cellular excitation. In the present study, we have provided direct evidence to show that 17β -estradiol indeed increases cell excitability as shown by the increase in the frequency of firing of action potentials generated in response to depolarizing current injection. The depolarization, most likely occurring via blocking of potassium channels (Fatehi et al., 2005) coupled with the increase in input resistance in the presence of 17β -estradiol, may underlie our consistent observation of an increase in PBN neuronal excitability. In this regard, 17β -estradiol is not the only neuromodulator that causes excitatory synaptic inhibition while increasing excitability. Other neuromodulators such as cholecystokinin have been shown to depress excitatory synaptic transmission that is preceded by depolarization of neurons leading to increased action potential firing (Kombian et al., 2003). As mentioned previously, 17β -estradiol may influence postsynaptic membrane responsiveness to various electrical and chemical stimuli by modulating several structures or mechanisms including ion channels, receptors and receptor-mediated intracellular signalling.

In conclusion, our data indicate that estradiol attenuates excitatory neurotransmission in the PBN and increases the excitability of these neurons through multiple mechanisms. This synaptic effect, likely post-synaptically mediated, indicates that 17β -estradiol changes the responsiveness of PBN neurons to excitatory inputs without influencing the availability of glutamate. The pathophysiological consequences of such actions are not currently clear. We speculate that by acting to reduce excitatory neurotransmission in the PBN, estradiol may influence the function of this nucleus as it is a key integration and relay site for autonomic visceral afferent information travelling to higher CNS centres such as the thalamus and cortex. For example, in a stroke model involving permanent occlusion of the middle cerebral artery (MCAO), ischemia involving the insular cortex (a forebrain autonomic nucleus with projections to the PBN) resulted in excessive excitation of PBN neurons (Saleh et al., 2004). Further, *in vivo* microdialysis studies in our laboratory have shown that after MCAO, a three-fold increase in extracellular concentration of estradiol in the PBN was observed (Saleh et al., 2004). The extracellular concentration rose from approximately 35 pg/ml before MCAO, to almost 100 pg/ml (~ 367 pM) within 10 min post-MCAO. When this release of estradiol was prevented by adding letrozole (an aromatase inhibitor that depletes endogenous estrogen) to the dialysate, PBN neuronal activity was significantly greater after MCAO, and the cardiovascular consequences (sympathoexcitation) were also greater (Saleh et al., 2004). Direct injection of estradiol (0.5 μ M in 200 nl) bilaterally into the PBN 30 min before MCAO, however, significantly attenuated the ischemia-induced excitation of PBN neurons (Saleh et al., 2005). This suggests that the endogenous (physiologic) concentration of estradiol is inadequate to attenuate neuronal excitability and prevent the autonomic and cardiovascular consequences of stroke,

but that higher, non-physiological doses (such as those determined to be effective in this study), may be required to prevent excessive excitation of PBN neurons in cerebrovascular disease. Also, given the nature of bath application, it would be reasonable to suspect the concentration would be lower at the tissue or cellular level. The potential uses of estrogen supplementation in the prevention or treatment of ischemia-induced neuronal cell death has been extensively provided by Wise et al. (2005). Understanding such modulatory effects of estradiol may be beneficial with respect to the central regulation of autonomic and cardiovascular homeostasis or perhaps in the understanding of pathological or therapeutic interventions, such as increasing CNS estrogen levels, to treat neurogenic arrhythmias.

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