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Modulation of the arcuate nucleus-medial preoptic nucleus lordosis regulating circuit: a role for GABA_B receptors

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Abstract

Estradiol rapidly activates a microcircuit in the arcuate nucleus of the hypothalamus (ARH) that is needed for maximal female sexual receptivity. Membrane estrogen receptor- α complexes with and signals through the metabotropic glutamate receptor-1 α stimulating NPY release within the ARH activating proopiomelanocortin (POMC) neurons. These POMC neurons project to the medial preoptic nucleus (MPN) and release β -endorphin. Estradiol treatment induces activation/internalization of MPN μ -opioid receptors (MOR) to inhibit lordosis. Estradiol membrane action modulates ARH gamma-aminobutyric acid receptor-B (GABA_B) activity. We tested the hypothesis that ARH GABA_B receptors mediate estradiol-induced MOR activation and facilitation of sexual receptivity. Double label immunohistochemistry revealed expression of GABA_B receptors in NPY, ER α and POMC expressing ARH neurons. Approximately 70% of POMC neurons expressed GABA_B receptors. Because estradiol initially activates an inhibitory circuit and maintains activation of this circuit, the effects of blocking GABA_B receptors were evaluated before estradiol benzoate (EB) treatment and after at the time of lordosis testing. Bilateral infusions of the GABA_B receptor antagonist, CGP52432, into the ARH prior to EB treatment of ovariectomized rats prevented estradiol-induced activation/internalization of MPN MOR, and the rats remained unreceptive. However, in EB treated rats, bilateral CGP52432 infusions 30 minutes before behavior testing attenuated MOR internalization and facilitated lordosis. These results indicated that GABA_B receptors were located within the lordosis-regulating ARH microcircuit and are necessary for activation and maintenance of the estradiol inhibition of lordosis behavior. Although GABA_B receptors positively influence estradiol signaling, they negatively regulate lordosis behavior since GABA_B activity maintains the estradiol-induced inhibition.

Keywords

lordosis behavior; μ -opioid receptor

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Introduction

Estradiol acts through multiple estrogen receptor- α (ER α) signaling pathways to facilitate sexual receptive behavior (lordosis). In the arcuate nucleus of the hypothalamus (ARH), estradiol initially binds to membrane ER α (mER α) that complexes with and signals through metabotropic glutamate receptor-1a (mGluR1a; reviewed in Micevych and Mermelstein, 2008; Sinchak and Micevych, 2003). This rapidly causes the release of neuropeptide Y (NPY) that induces β -endorphin (β -END) release from ARH neurons that project to the medial preoptic nucleus (MPN). β -END in the MPN activates/internalizes μ -opioid receptors (MOR) and inhibits lordosis. The subsequent deactivation of MPN MOR by progesterone facilitates lordosis in estradiol primed rats (Sinchak and Micevych, 2001). The importance of the activation of this inhibitory circuit is underlined by experiments in which MOR was inhibited by naloxone at the time of estradiol treatment, which blocked the facilitation of lordosis (Torii et al., 1999; see Sinchak and Micevych, 2003) for review). Thus, these initial actions of estradiol are important for the ultimate coordination of excitatory and inhibitory neurocircuits that facilitate lordosis. As part of our longstanding goal of characterizing neurotransmission that mediates the actions of estradiol to turn on the ARH-MPN MOR lordosis inhibitory circuit and then to deactivate the inhibition, we tested the role of gamma-aminobutyric acid-B (GABA_B) receptors in mediating estradiol activation of this circuit.

Previous studies have implicated GABA in the regulation of lordosis. GABA-ergic regulation of lordosis behavior has been studied in the ventromedial nucleus of the hypothalamus (VMH) and the medial preoptic area. Depending on the timing of the experimental manipulation, GABA has been reported to either facilitate or inhibit lordosis behavior (Agmo et al., 1989; Masco et al., 1986; reviewed in Paredes and Agmo, 1992; Salzberg et al., 2002). However, knockdown of the synthetic enzymes for GABA, GAD 65 & 67, in the VMH blocked the ability of estradiol and progesterone priming to induce sexual receptivity, suggesting that GABA transmission mediates steroid priming important for the facilitation of lordosis behavior (McCarthy et al., 1994). The need for GABA neurotransmission for the facilitation of lordosis is similar to the requirement for opioid activation during the initial exposure to estradiol for the ultimate facilitation of lordosis behavior (Torii and Kubo, 1994; Torii et al., 1995, 1996, 1999), and may be related to activation of specific GABA receptors. Although GABA actions have been attributed to the VMH, GABA_B receptor activity in the ARH POMC neurons is modified by steroids (Kelly and Wagner, 1999; Kelly et al., 1992; for review see Micevych and Kelly, 2012). Additionally, GABA_B receptors mGluR1 may interact to augment mGluR1 signaling (Hirono et al., 2001), or modulate PKC to enhance L-type voltage-gated Ca²⁺ channels (VGCC; Bray and Mynlieff, 2011; Park et al., 2010).

Thus, we tested the hypothesis that GABA_B receptors mediate estradiol activation of the ARH-MPN lordosis circuit. In Experiment 1, we used double-label immunohistochemistry to determine whether GABA_B receptors are expressed within subpopulations of NPY, POMC and ER α neurons of the ARH lordosis circuit. In Experiment 2 we tested the hypothesis that GABA_B receptors mediate the initial estradiol signaling that rapidly activates MPN MOR to inhibit lordosis. In Experiment 3, we tested whether GABA_B receptors mediated the estradiol-induced inhibition of lordosis and activation of MPN MOR 30 hours after estradiol treatment. According to our hypotheses, we expected that GABA_B receptor antagonism before estradiol priming would block estradiol-induced MPN MOR activation and facilitation of lordosis. We also expected that blocking GABA_B receptors 30 hours after estradiol would deactivate MPN MOR, facilitating lordosis behavior.

Methods

Animals

Adult male and ovariectomized (ovx) female Long-Evans rats (200–225 g) were purchased from Charles River Laboratory Inc. (Wilmington, MA). The supplier performed the bilateral ovariectomy surgeries. Animals were housed two per cage in a climate and light controlled room (12/12 L/D cycle, lights on 0600 h) with food and water available *ad libitum*. The California State University, Long Beach and UCLA IACUC Committees approved all procedures.

Experiment 1: Colocalization of GABA_B receptors with ER α , NPY or POMC in ARH—To determine which neurons of the ARH microcircuit expressed GABA_B receptors, double-labeling experiments were done on ARH tissue sections with antibodies raised against GABA_{B1} receptors, POMC, ER α and NPY. OvX rats were anesthetized with isoflurane (2–3% in equal parts oxygen and nitrous oxide) and fixed in a stereotaxic frame. Each rat was implanted with a guide cannula (28 gauge; Plastics One, Roanoke, VA) directed at the lateral ventricle (coordinates from bregma: anterior, -1.0mm; lateral, -1.4mm; ventral, -3.5mm from dura; tooth bar, -3.3mm; modified from Paxinos and Watson, 1986) using standard stereotaxic procedures. The cannulae were secured to the skull with dental acrylic and stainless steel bone screws. Stylets were placed in the guide cannulas that protruded < 0.5 mm beyond the opening of the guide cannulas. Animals were singly housed after surgery, received oral antibiotics (trimethoprim and sulfamethoxazole; 0.4mg/ml; Hi-Tech Pharmacal, Amityville, NY) dissolved in the drinking water, and were allowed to recover for seven days before infusion. Neuropeptides are rapidly transported out of the neuronal perikarya, reducing the ability to demonstrate their presence within a given cell body. To increase the accumulation of NPY and POMC in the cell soma, colchicine was used to block axonal transport of neuropeptides. Animals were treated with colchicine (100 μ g) dissolved in artificial cerebrospinal fluid and delivered into the lateral ventricle by infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.5 μ l/minute. The infusion needle (28 gauge) protruded 2 mm beyond the opening of the cannula and was allowed to remain in place for one minute after infusion to allow for diffusion of drug treatment. After infusion, obturators were reinserted into the guide cannulas, and animals were returned to their home cage. Animals were anesthetized 48 hours later and transcardially perfused as described below.

Experiments 2 & 3: GABA_B receptor regulation of estradiol-induced MPN MOR activation and sexual receptivity—Experiments 2 & 3 were done to ascertain whether GABA_B receptor activation influenced estradiol actions in the ARH on MOR activation and lordosis. Approximately one week after arrival, all ovx rats were implanted with indwelling bilateral cannulae with screw-on head caps and dummy stylet inserts (Plastics One Inc., Roanoke, VA) using standard aseptic stereotaxic surgical techniques under isoflurane anesthesia. Guide cannulae were aimed at the ARH (tooth bar = -3.3; coordinates (mm) from bregma: AP = -2.8; L = 0.6; V = -7.1; modified from Paxinos and Watson, 1986). Rats were allowed to recover for one week before drug infusions and behavioral testing.

On the day of cannula implantation, all rats received a subcutaneous injection of 2 μ g estradiol benzoate (EB) dissolved in safflower oil (injection volume: 0.1 ml). In these experiments, rats were treated once every four days with EB for four treatments. This is a priming dose of estradiol that produces peak circulating levels of estradiol that are equivalent to proestrous levels, but does not facilitate lordosis without either subsequent progesterone or other pharmacological manipulations (Asarian and Geary, 2002; Dewing et al., 2007; Jones et al., 2013; Priest et al., 1995; Sanathara et al., 2011; Sinchak and

Micevych, 2001). We use an estradiol treatment paradigm in which EB is injected once every four days to mimic the rise and fall of estradiol during the estrous cycle (Asarian and Geary, 2002; Priest et al., 1995). This paradigm has been useful in examining neuropeptide expression, receptor activation and induction of sexual receptivity. In Experiment 2, a GABA_B receptor antagonist, [3-[[[3,4-dichlorophenyl)methyl]amino]propyl](diethoxymethyl) phosphinic acid (CGP52432, 8 nmol in 0.5 μ l saline) or 0.5 μ l saline was infused bilaterally into the ARH 30 minutes prior to the third EB treatment. Females were tested for sexual receptivity 30 hours after the third EB treatment. To determine whether GABA_B receptors mediate the initial estradiol-induced MPN MOR activation all ovx rats received the same CGP52432 or saline infusion received for behavioral testing 15 minutes before the fourth EB treatment. All ovx rats were anesthetized with isoflurane and perfused 30 minutes after the fourth EB treatment. In Experiment 3, CGP52432 or saline was infused into the ARH 29.5 hours after the third EB treatment and ovx rats were tested for sexual receptivity 30 minutes later. To determine whether GABA_B receptors mediate the sustained estradiol-induced MPN MOR activation, ovx animals received an ARH infusion of either CGP52432 or saline 29.5 hours after the fourth EB. An infusion pump (0.5 μ l/min) was used to deliver drugs or vehicle into the ARH bilaterally. Injectors were left in for one minute after completion of the infusion to allow for dispersal of the infused solution. In Experiment 3, all rats were anesthetized with isoflurane and perfused 30 minutes after drug or vehicle treatment, which was 30 hours after the fourth EB treatment.

Behavioral Testing

In Experiments 2 and 3, ovx animals were tested for sexual receptivity 30 hours after EB treatment. The female rat was placed in a behavioral chamber (Plexiglas 75 cm $\times$ 75 cm $\times$ 75 cm box) with cage bedding covering the floor, with a sexually experienced, adult stimulus male rat (the male rat was introduced to the chamber at least 10 minutes prior to the placement of the female rat). The male rats were allowed to sexually mount the female rat 10 times. The number of times that she showed lordosis to the vigorous mounting was recorded, and the lordosis quotient (LQ) was calculated by dividing the number of lordosis responses by the total number of mounts multiplied by 100. After 10 mounts by the male the female was returned to her home cage (Dewing et al., 2007; Dewing et al., 2008; Sanathara et al., 2011; Sinchak and Micevych, 2001). A square root transformation was performed on LQ data and then comparisons by t-test were performed (Sigma Stat V3.5). Results were compared using Cohen's d for pairwise comparisons.

Perfusion

In all experiments, animals were anesthetized and transcardially perfused with chilled 0.9% saline, followed by 4% paraformaldehyde dissolved in 0.2 M Sorensen's phosphate buffer, (pH 7.4). Brains were removed and placed in fixative overnight at 4°C. The fixative was replaced with 20% sucrose in phosphate buffer to cryoprotect the tissue. Brains were blocked, sectioned (20 μ m) on a cryostat and collected into chambers filled with PBS for immunohistochemistry and to confirm cannula placement (Dewing et al., 2007; Dewing et al., 2008; Sanathara et al., 2011; Sinchak and Micevych, 2001).

Immunohistochemistry

Experiment 1: GABA_B receptor colocalization: To determine whether the GABA_B receptor was expressed in NPY cells, sections containing the ARH were first incubated with a mouse primary antibody directed against the GABA_{B1} receptor (1:1500; Abcam, Cambridge, MA) overnight at 4°C. Sections were washed with Tris-buffered saline (TBS), sequentially incubated in TNB blocking buffer and horse anti-mouse secondary antisera (Vector Laboratories, Burlingame, CA) for one hour. Sections were then washed in TNT

before incubation with streptavidin-horseradish peroxidase (1:100; Perkin Elmer, Inc., Waltham, MA) for 30 minutes, washed, and then incubated for five minutes in fluorescein-conjugated tyramide (Tyramide Signal Amplification kit; 1:50; Perkin Elmer). Tissue was then washed with TBS and then incubated with blocking solution for one hour before incubation with rabbit primary antibody directed against NPY (1:2000; Immunostar, Hudson, WI) for two nights. Sections were then washed with TBS before incubation in Texas Red conjugated goat anti-rabbit secondary antibody (1:100; Vector) for three hours. Sections were washed again with TBS and a final wash with Tris buffer (TB) and mounted onto SuperFrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and coverslipped using Aqua-Poly/Mount anti-fade media (Polysciences, Inc., Warrington, PA).

To determine whether the GABA_{B1} receptor was expressed in POMC neurons, sections containing ARH were first incubated with mouse primary antibody directed against the GABA_{B1} receptor and then processed for fluorescence as described above, using fluorescein-conjugated tetramethylrhodamine (Tyramide Signal Amplification kit; 1:50; Perkin Elmer). Tissue was then washed with TBS and incubated with blocking solution for one hour before incubation with chicken primary antibody directed against POMC (1:500; Abcam) overnight. Sections were then washed with TBS before incubation with donkey anti-chicken fluorescein isothiocyanate secondary antibody (1:100, Jackson ImmunoResearch, West Grove, PA) for three hours. Tissue was then washed again in TBS followed by Tris buffer before mounting and coverslipping as described above.

To determine whether the GABA_{B1} receptor was expressed in cells also expressing ER α , sections containing ARH were first incubated with mouse primary antibody directed against GABA_{B1} and then processed for fluorescence as described above using fluorescein-conjugated tyramide. Tissue was then washed in 0.2% Triton-X 100 in TBS, followed by a 15 minute incubation with 0.1 M glycine. Sections were then incubated with blocking solution for 30 minutes before incubation with rabbit primary antibody directed against ER α (1:10000; Millipore, Billerica, MA) for three nights. Sections were then incubated with Texas Red conjugated goat anti-rabbit secondary antibody and mounted and coverslipped as described above.

Colocalization Image Analysis

All immunohistochemically labeled sections were examined using a Zeiss Axioskop 2 equipped with epifluorescent illumination, Axiovision digital camera, and Axiovision digital image analysis system (Zeiss) to determine colocalization. Fluorescein (FITC) and rhodamine were imaged with a 488-nm and 550-nm emission filter and a 515-540 nm bandpass filter, respectively. Immunoreactive staining was considered colocalized when two immunofluorescent markers were present in the same cell. The overlapping fluorochromes (green and red) produce a yellow signal. Immunolabeled cells were counted in 22 sections of each ARH at a magnification of 250X. To prevent double counting, the formula of Abercrombie was applied (Abercrombie, 1946).

Experiments 2 & 3: MOR immunoreactive intensities—MOR immunoreactive intensities (a measure of MOR activation/internalization) was visualized with immunohistochemistry on free-floating sections collected through the MPN, as described previously (Dewing et al., 2007; Eckersell et al., 1998; Mills et al., 2004; Sanathara et al., 2011; Sinchak and Micevych, 2001). Fluorescein detection of MOR antibody (1:5000; Neuromics Antibodies Edina, MN) was done using a Tyramide Signal Amplification kit as per the manufacturer's protocol (Perkin Elmer). Sections were then placed in 0.1 M Tris buffer (pH 7.5) and mounted on Superfrost Plus slides. Slide mounted sections were dried

on a 37°C slide warmer for 15 minutes and immediately coverslipped using Aqua-Poly/Mount mounting medium.

MOR Image Analysis

The relative activation of the MPN MOR was estimated in the dorsal region of the MPN by measuring the area of positive MOR immunofluorescence staining in epifluorescent photomicrographs (Dewing et al., 2007; Dewing et al., 2008; Sanathara et al., 2011). An increase in immunostaining intensity is correlated with increased MOR internalization, a measure of receptor activation (Dewing et al., 2007; Dewing et al., 2008; Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001; reviewed in Sinchak and Micevych, 2003). Photographs were captured in grayscale with a Leica DFC360 monochrome fluorescence camera mounted on a 6000B Leica Microscope with Leica software. FITC labeling was detected using BP 485/20 excitation filter and BP 537/45 suppression filter. Brightness and contrast were adjusted for in Adobe Photoshop (version 7.0; Adobe Systems Inc., San Jose, CA) and then imported into ImageJ software (version 1.32j; National Institutes of Health, Bethesda, MD). In ImageJ, the “pixel inverter” function was selected and calibrated. To measure the MOR immunofluorescence staining intensity within each photomicrograph, a 60 µm diameter circle was positioned on the dorsal MPN and positive MOR immunofluorescence staining measured. Within the same section, an area of background staining was measured. The intensity level of MOR immunofluorescence was calculated in arbitrary units (AU) by subtracting the background staining from the positive MPN MOR immunostaining. MOR activation/internalization appeared as distinct cytoplasmic immunoreactive puncta (Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001).

Results

Experiment 1 – Distribution of GABA_B in ARH neurons

GABA_B receptor immunoreactive neurons were scattered throughout the ARH (Figure 1). As previously reported, ERα neurons were observed throughout the ARH (Dewing et al., 2007). POMC immunoreactive neurons were observed along the rostrocaudal extent of the ARH, especially in the lateral part of the nucleus. These POMC immunoreactive cell bodies were larger than those stained for ERα or NPY (Figure 1). Ventrally, POMC immunoreactive neurons extend laterally outside the cytoarchitectonic borders of the nucleus (Jirikowski et al., 1986; Khachaturian et al., 1985). These neurons outside the borders of the ARH were not included in the colocalization analysis. A large percentage of the POMC immunoreactive neurons were also stained with antisera raised against GABA_B (69.9 ± 4.2%; Table 1, Figure 1). Approximately 32% of the ERα and 28% of the NPY neurons were colocalized with GABA_B immunoreactivity. While significant GABA_B colocalization was observed within NPY and ERα immunoreactive populations the colocalization with POMC immunoreactivity was much greater (Table 1, Figure 1).

Experiment 2 – Blocking GABA_B receptors prior to estradiol activation of the lordosis-regulating circuit

Behavior—Control ovx animals that received ARH saline infusions 30 minutes prior to the 2 µg EB priming exhibited, as expected, a low mean level of sexual receptivity (Figure 2). ARH infusion of the GABA_B receptor antagonist, CGP52432, 30 minutes prior to 2 µg EB priming had no effect on sexual receptivity (LQ) compared to saline treated rats (t-test: df = 13, P < 0.84, t = 0.209; Cohen's d: d = 1.46, r = 0.59; Figure 2).

MOR immunohistochemistry—Infusion of CGP52432 into the ARH 30 minutes prior to EB treatment reduced MPN MOR activation/internalization as measured by MOR

immunoreactive intensity compared to saline treated, EB primed rats (t-test: $df = 13$, $P = 0.02$, $t = 2.64$; Cohen's $d: d = 0.11$, $r = 0.06$; Figure 2), indicating that antagonism of ARH GABA_B receptors blocked estradiol-induced MPN MOR activation.

Experiment 3 – Blocking GABA_B receptors at the time of testing sexual receptivity reversed estradiol-induced MOR activation and facilitated lordosis

Behavior—In EB primed, ovx, nonreceptive rats, blocking GABA_B receptor activation with CGP52432 in the ARH 29.5 hours after EB treatment facilitated sexual receptivity 30 minutes later as measured by LQ compared to saline treated rats (t-test: $df = 11$, $P < 0.001$, $t = 19.4$; Cohen's $d: d = 9.43$, $r = 0.98$; Figure 3). These data indicate that inhibition of GABA_B receptors facilitates lordosis in EB primed rats.

MOR immunohistochemistry—ARH infusion of CGP52432 29.5 hours after the final EB treatment significantly decreased MPN MOR immunostaining intensity compared to saline infused rats (t-test: $df = 11$, $P = 0.046$, $t = -2.25$; Cohen's $d: d = 1.35$, $r = 0.56$; Figure 3) indicating that GABA_B receptor signaling induces β -END release in the MPN that activates MOR.

Discussion

The major finding of this study is that GABA_B receptor activity in the ARH modulates estradiol-induced activation of the ARH microcircuit that regulates sexual receptivity. Inhibition of GABA_B receptors in the ARH prior to EB treatment prevented estradiol-induced activation/internalization of MOR in the MPN. Similarly, blocking GABA_B receptors immediately before testing lordosis behavior reversed the persisting estradiol-induced MOR activation/internalization and facilitated lordosis. Both effects demonstrate the importance of GABA_B receptors for estradiol activation of the lordosis circuit.

The MOR internalization studies indicate that GABA signaling through GABA_B receptors mediates both initial and sustained estradiol-induced activation of β -END release into the MPN. We have previously demonstrated that the activation of MOR is a consequence of estradiol membrane signaling requiring mER α transactivation of mGluR1a in the ARH (Dewing et al., 2007; Dewing et al., 2008). Within the ARH, estradiol activates NPY-Y1 receptors leading to a release β -END into the MPN to activate MOR inhibiting lordosis behavior (Mills et al., 2004). GABA_B receptor blockade prior to EB treatment blocked estradiol-induced MPN MOR internalization/activation. To facilitate lordosis behavior, the estradiol-induced MOR-mediated inhibition must be reversed (Sinchak & Micevych 2001). When either opioid receptor antagonists or progesterone was given to estradiol treated rats 30 hours post-EB, lordosis was facilitated. Similarly, when GABA_B receptors were blocked at this time, the estradiol-induced MPN MOR internalization/activation was reversed and lordosis behavior facilitated. These results are consistent with the hypothesis that GABA signaling through GABA_B receptors is important for estradiol membrane-initiated activation of the ARH lordosis microcircuit that induces β -END neurotransmission.

Inhibition of GABA_B receptors in the ARH prior to estradiol treatment blocked the estradiol-induced MPN MOR activation and did not enhance sexual receptivity. This transient MOR-mediated inhibition is necessary for lordosis behavior, as initially demonstrated by Torii and colleagues (Torii and Kubo, 1994; Torii et al., 1995, 1996, 1999) who gave naloxone (a classical opioid receptor antagonist) prior to estradiol priming. We confirmed the importance of MOR by examining lordosis in a MOR knockout mouse in which full sexual receptivity could not be achieved compared to the wildtype controls (Sinchak et al., 2005). Moreover, the present results are consistent with the seemingly paradoxical finding of McCarthy et al., (1994) who suggested that GABA transmission in

the mediobasal hypothalamus was essential for the facilitation of lordosis behavior. Knockdown of the enzymes, GAD₆₅ and GAD₆₇, prevented GABA synthesis and facilitation of lordosis. Our present study revealed that activation of GABA_B receptors is a component of the estradiol-induced activation of the inhibitory circuit necessary for full sexual receptivity. Thus, by preventing GABA synthesis, GABA_B receptors could not be activated through estradiol stimulation and lordosis behavior was attenuated.

We speculate that the functions of this circuit are: 1) to match dose and duration of ovarian steroid exposure with appropriate sexual behavior and 2) to synchronize sexual behavior with ovulation and uterine development so that copulation results in successful pregnancy. In our hands, 2 µg is a behaviorally subthreshold dose of EB. This dose rapidly brings on-line the ARH-MPN lordosis inhibitory circuit and maintains its activity for up to 48 hours while simultaneously up-regulating facilitative circuits through transcriptional and nontranscriptional mechanisms, which require 20-30 hours. When the estradiol-induced inhibition is relieved approximately 20-30 hours after estradiol lordosis is facilitated (Dewing et al., 2007; Mills et al., 2004; Sanathara et al., 2011; Sinchak and Micevych, 2001). In the intact rat, the estradiol-induced inhibition is relieved by progesterone on the evening of proestrus facilitating sexual receptivity. MPN MOR are activated on diestrous day one and two of the cycle and then deactivated on the evening of proestrus (Sinchak and Micevych, 2003). In the steroid deprived ovx rat MPN MOR are deactivated, and she is not receptive since the facilitative circuits are neither up-regulated nor activated. Thus, the estradiol-induced inhibition produced by the ARH-MPN circuit is not required. In the present experiments, we observed the necessity for GABA_B receptor activation in this circuit. When GABA_B receptor was antagonized 30 hours after EB treatment, the estradiol-induced activation of MPN MOR was reduced and lordosis facilitated. These results indicate that estradiol-induced MOR activation had been rapidly induced and maintained in part by GABA_B signaling. Interestingly, the effect of antagonizing GABA_B receptors mimics the action of progesterone in this circuit (Sinchak and Micevych, 2001). Whether progesterone acts through silencing GABA_B receptors will require further study that is beyond the scope of this report.

GABA_B receptors are co-expressed in ARH ERα, NPY and POMC neurons, suggesting that GABA_B receptor signaling could affect various neurons along the ARH microcircuit mediating lordosis behavior. However, the degree of colocalization of GABA_B receptors strongly implicates the POMC neuron as the primary site of action. The colocalization experiments were done in tissue from ovx rats because estradiol has been shown to down-regulate POMC and ERα expression and its action on NPY expression is controversial (Holland et al., 1998; Lauber et al., 1990; Mills et al., 2004; Simerly and Young, 1991; Tong et al., 1990; Treiser and Wardlaw, 1992; Wilcox and Roberts, 1985).

In order to build up the neuropeptides in the cell bodies in our study, animals were treated with a microtubule inhibitor: colchicine. While colchicine may affect the expression of neuropeptides, it is one of the only methods that can effectively demonstrate native neuropeptides in the cell body. The effects of colchicine on NPY mRNA levels are brain region dependent; in some areas an increase was noted but in others there was no effect (Cortes et al., 1990). In the worst case, we may have overestimated the colocalization of NPY with GABA_B receptor.

In the present study, based on a percentage of colocalization (70%), the primary site of GABA action through GABA_B receptors appears to be in POMC neurons. Inhibition of GABA_B receptors reduced β-END release indicating that GABA_B activation induces β-END release. GABA activation of GABA_B receptors decreases neuronal excitation through opening GIRK channels (Luscher et al., 1997; Sodickson and Bean, 1996). In the guinea pig

ARH, estradiol acts directly on POMC neurons to uncouple the GABA_B receptor from the inhibitory GIRK thereby increasing the excitation of POMC neurons (reviewed in Micevych and Kelly, 2012). Therefore, if estradiol is reducing the coupling of the GABA_B receptor to the GIRK channel, then inhibition of GABA_B receptor signaling should increase β -END release resulting in greater MOR activation/internalization in the MPN. However, our results suggest that GABA_B receptor activation mediates estradiol-induced activation of the β -END release and MPN MOR activation. Thus, in the rat the interaction of estradiol and GABA_B receptor interaction appears to be quite different from the guinea pig. One possibility is that estradiol may switch upstream GABA_B signaling (GABA_B receptors were also expressed in ER α and NPY neurons) from inhibition to facilitation of the POMC neuron. Such a steroid-induced switch has been described for adrenergic signaling in the VMH (Etgen et al., 2001). In the presence of estradiol, GABA_B receptor signaling may change NPY-Y1 receptor coupling, which is nominally inhibitory (Klenke et al., 2010; Melnick, 2012). Alternatively, estradiol may directly mediate VGCC (Bray and Mynlieff, 2011; Park et al., 2010; Rhim and Miller, 1994), or induce G-protein activation of PKC (Dewing et al., 2008) and PKA that enhance L-type VGCC currents (Bray and Mynlieff, 2011; Park et al., 2010). Thus, estradiol may enhance POMC excitation through reduction of GABA_B receptor coupling to the GIRK and switching the GABA_B receptor signaling mechanism. It is also possible that GABA_B receptors modulate synaptic input upstream of POMC neurons. This is suggested by the colocalization of GABA_B receptors in ER α and NPY neurons. In this scenario, GABA_B receptors may enhance the activity of mER α -mGluR1a signaling that activates POMC neurons projecting to the MPN as observed in the cerebellum (Hirono et al., 2001).

In summary, estradiol-induced activation of POMC neurons that project to the MPN and regulate lordosis behavior involves GABA transmission in the ARH. We determined that activity at the GABA_B receptor, which is widely distributed in the ARH, modulates estradiol membrane-initiated signaling that activates POMC neurons to regulate sexual receptivity in the rat.

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Highlights

- ❑ GABA_B receptors are co-expressed in arcuate nucleus ER α , NPY and POMC neurons
- ❑ Nearly 70% of arcuate nucleus POMC neurons express GABA_B receptors
- ❑ Estradiol activates POMC neurons that inhibits lordosis
- ❑ GABA_B receptor antagonism blocked estradiol activation of POMC neurons and facilitated lordosis
- ❑ GABA_B receptors positively modulate estradiol signaling regulating POMC neurons and lordosis

Figure 1.

GABA_B receptors are expressed in ERα, POMC & NPY neurons in the ARH.

Photomicrographs of immunohistochemical colocalization of GABA_{B1} receptors with ERα, POMC and NPY. GABA_{B1} immunoreactivity was widely distributed throughout the rostro-caudal, and medio-lateral extent of the nucleus. GABA_B receptor colocalization with ERα tended to be in the lateral parts of the ARH. NPY immunoreactivity, like that of GABA_{B1}, was more uniformly distributed, but lower levels of GABA_B receptors colocalization was observed compared to POMC. Approximate area of ARH is outlined in the first panel.

Figure 2.

Inhibition of GABA_B receptors prior to EB treatment blocks rapid estradiol activation of medial preoptic nucleus (MPN) μ -opioid receptors (MOR). A-C) Photomicrographs illustrating MPN MOR immunoreactive fiber densities used for MOR intensity measures 30 minutes after EB treatment. MPN MOR positive immunoreactive staining (MORi) before (A) and after contrast and brightness manipulation (B-C) for rats bilaterally infused with either saline (B) or GABA_B receptor antagonist, CGP52432 (CGP; C) into the ARH 30 minutes prior to 2 μ g EB treatment and brains collected 30 minutes after EB. D) Bilateral ARH infusion of CGP 30 minutes prior to EB treatment reduced MPN MORi intensity compared to saline treated rats 30 minutes after EB ($P = 0.020$). E) Similar ARH CGP infusions did not increase sexual receptivity (Lordosis Quotient) compared to saline treated, EB primed rats ($P < 0.838$). A-C) * indicates MPNc. D & E) * = significantly different from Saline group.

Figure 3.

Inhibition of GABA_B receptors 29.5 hours after EB priming blocks the maintained estradiol activation of MPN MOR and facilitates sexual receptivity. A-C) Photomicrographs illustrating MPN MOR immunoreactive fiber densities used for MORi intensity measures 30 hours after EB treatment. MPN MORi positive staining before (A) and after contrast and brightness manipulation (B-C) for rats bilaterally infused with either saline (B) or CGP (C) into the ARH 29.5 hours after 2 μ g EB treatment and brains collected 30 hours after EB treatment. D) CGP was bilaterally infused into the ARH 29.5 hr after EB and 30 minutes later MPN MOR intensities were decreased in EB treated rats compared to saline ARH infused controls ($P = 0.046$). E) Similar ARH CGP infusions increased sexual receptivity in EB primed rats compared to saline infused rats ($P < 0.001$). A-C) * indicates MPNc. D & E) * = significantly different from other treatment group.

Table 1

Proportion of immunohistochemically double-labeled ARH neurons

	% GABA _B	% ER α	% NPY	% POMC
ER α	32.3 $\pm$ 2.7	--		
NPY	27.6 $\pm$ 1.3		--	
POMC	69.9 $\pm$ 4.2			--
GABA _B	--	30.0 $\pm$ 2.4	4.4 $\pm$ 0.2	17.4 $\pm$ 3.6