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Estradiol potentiates inhibitory synaptic transmission in the oval bed nucleus of the striaterminalis of male and female rats

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ABSTRACT

17ß-Estradiol (E2) is a potent neuromodulator capable of producing changes in inhibitory synaptic transmission by either changing pre-synaptic GABA release or post-synaptic $GABA_A$ receptor function. Physiologically, E2 is important for energy homeostasis, influencing food consumption, body weight, adipose tissue metabolism and energy expenditure. E2 may influence energy homeostasis through estrogen receptor-rich regions such as the oval bed nucleus of the stria-terminalis (ovBNST). However, the neurophysiological effects of estradiol within the ovBNST remain largely unknown. Understanding how E2 affects inhibitory transmission may elucidate the ovBNST's contribution to energy homeostasis. Here, using brain slice electrophysiology, we saw that E2 produced a long-term potentiation (LTP) of $GABA_A$ synaptic transmission (LTP^{GABA}) in the ovBNST in male rats. E2 acted on estrogen receptors α and G-protein coupled estrogen receptors (GPER), involved protein kinase activation and required an intact endocannabinoid system. The effects of E2 in males were sensitive to 24 h of food deprivation. In females, E2 was 100-fold more potent at producing LTP^{GABA} ovBNST compared to male rats and involved all three known subtypes of estrogen receptors (ERα, ERß, and GPER). These results demonstrate that E2 is a potent neuromodulator of inhibitory synaptic transmission within the ovBNST of both sexes to potentially regulate energy homeostasis.

1. Introduction

The estrogen 17ß-Estradiol (E2) is a potent neuromodulator capable of controlling the excitability of neurons and synaptic transmission in various brain areas (Fetehi and Fetehi-Hassanabad, 2008; Grassi et al., 2009; Smejkalova and Woolley, 2010; Galvin and Ninan, 2014). Depending on brain regions, E2 either facilitates or suppresses GABA-mediated inhibitory synaptic transmission through a combination of post-synaptic and pre-synaptic effects (Schultz et al., 2009; Huang and Woolley, 2012; Tian et al., 2013; Tabatadze et al., 2014; Mukherjee et al., 2017). For instance, in the hippocampus of female rats, E2 suppresses inhibitory synaptic transmission by promoting post-synaptic endocannabinoid synthesis to inhibit pre-synaptic GABA release (Huang and Woolley, 2012; Tabatadze et al., 2014). In contrast, E2 potentiates inhibitory synaptic transmission in the basolateral amygdala of female rats through G-protein coupled estrogen receptors (GPER; Tian et al., 2013). E2 also has the capacity to alter $GABA_A$ subunit mRNA expression throughout the brain and modulate $GABA_A$ receptor kinetics (Herbison and Fenelon, 1995; Pierson et al., 2005; Shen et al., 2005; Tian et al., 2013). Most studies investigating E2's impact on inhibitory synaptic transmission have used female rodents (often ovariectomized). It remains unclear whether modulation of inhibitory synaptic transmission by E2 is sexually dimorphic.

An important function of estrogens is to participate in energy homeostasis by influencing food consumption, body weight, adipose tissue and energy expenditure. Specifically, E2 is an anorectic, reducing food consumption and weight gain, while increasing energy expenditure (Geary et al., 2001; Musatov et al., 2007; Sharma et al., 2013; Davis et al., 2014). The oval bed nucleus of the stria-terminalis (ovBNST), a subcortical brain region involved in energy homeostasis, contains both an abundance of estrogen receptors and the enzyme aromatase which is necessary to endogenously synthesize E2 (Laflamme et al., 1998; Dong et al., 2001; Zhao et al., 2007). The ovBNST is a convergence node incorporating descending and ascending exteroceptive and interoceptive information important in maintaining energy balance (Dong et al., 2001). Specifically, the rat ovBNST receives gustatory and olfactory sensory information from the cortex, as well as, information on visceral stomach distention and glucose levels

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from the nucleus of the solitary tract (McDonald et al., 1999; Rinaman and Schwartz, 2004; Rinaman, 2010). Activation of ovBNST inputs to the lateral hypothalamus directly promotes feeding behaviour in mice (Jennings et al., 2013). The ovBNST is also implicated in stress-induced weight loss caused by a reduction in food consumption (Roman et al., 2012, 2014). However, E2′s function in the ovBNST remains completely unknown. Further understanding of how E2 affects inhibitory transmission may elucidate the role of the ovBNST in energy homeostasis regulation.

The goal of this study was to determine if E2 can modulate GABA inhibitory synaptic transmission in the ovBNST of male and female rats. Based on evidence from other brain regions, we hypothesize that E2 would alter inhibitory synaptic transmission in female rodents. From detailed findings in the hippocampus, we further hypothesize that E2 will alter inhibitory synaptic transmission through the endocannabinoid system. Due to both the ovBNST's and E2′s importance in the maintenance of homeostasis, we hypothesize that the effects of E2 would be sensitive to a metabolic challenge.

2. Methods

2.1. Animals

Long Evans rats (Charles River, St-Constant, QC) weighing 125–175 g and aged 38–46 days old (68 males; 46 females) were pair-housed in clear Plexiglas cages. C57BL/6 N mice weighing 20–25 g and aged 36–66 days old (7 males) were pair housed in Plexiglas cages. The floors were lined with bedding (Beta Chip, NEPCO, Warrenburg, NY) and the cages were located in a climate-controlled colony room (21 ± 1 °C; humidity 40–70%) on a reversed 12-hr light/dark cycle (9:00 A.M. lights off – 9:00 P.M. lights on) and electrophysiological preparations occurred at the beginning of the dark cycle. The rats were allowed to acclimatize for a minimum of 7 days upon arrival to the facility. Rat chow (LabDiet rodent feed #5001, PMI Nutrition International, Brentwood, MO) and water were provided ad libitum in the home cages. All the experiments were conducted in accordance with the Canadian Council on Animal Care guidelines for use of animals in experiments and approved by the Queen's University Animal Care Committee.

2.2. Food deprivation

Acute food deprivation was conducted in the home-cage in the colony room. Four singly housed male rats underwent a 24-hour acute food deprivation at the beginning of the dark cycle. The following morning, the acutely food deprived rats were prepared for electrophysiology.

2.3. Estrous cycle monitoring

Estrous cycle in female rats was monitored using cotton tipped swabs (Q-Tips, Toronto, ON) dipped in ambient temperature double distilled H_2O. The swab was inserted into the rat vagina and turned gently against the vaginal wall and cells were transferred onto cleaned glass slides. Slides were examined under transmitted light microscopy at 10-20x magnification. The phases of the estrous cycle were determined using previously detailed biological markers (Goldman et al., 2007). Female rats had to successfully undergo their estrous cycle at least twice and be either in the estrus (n = 20) or diestrus (n = 20) phase of the estrous cycle before being prepared for electrophysiology.

2.4. CB_1R transgenic mouse ($cnr1^{-/-}$)

Seven male (4 knockout [KO, homozygous CB_1-deficient mice, $cnr_1-/-$] and 3 wild type littermates [WT, $cnr_1+/+$]) transgenic mice were generated by Dr. Jean-François Bouchard at the Université de Montréal (Montréal, Québec). This targeted mutant was created and characterized by the research team of Pr. Beat Lutz, University of Mainz, Germany[2]. *Strain development:* a construct containing the entire open reading frame of *cnr1* gene flanked by two loxP sites, two homology arms, and a FRT-flanked PGK-neo cassette, was generated and electroporated into E14 mouse embryonic stem cells to obtain the floxed-neo allele. Germ-line transmission was reached by standard procedures. Mice bearing the floxed-neo allele were then crossed with transgenic mice expressing Cre recombinase ubiquitously. Cre-mediated recombination was proved by Southern blot hybridization. Mice carrying a germ-line transmissible deletion of cnr_1 were backcrossed for five generations into C57BL/6 N (Charles Rivers Laboratories). *Expression of CB_1R:* lack of CB_1R expression in the visual system and the central nervous system of KO mice was shown by in situ hybridization and by immunohistochemistry. *Known phenotype:* Consistent with previously reported CB_1-deficient mouse lines, KO mice did not show any gross morphological defects and had normal neurological reflexes (righting, postural, eye blink, ear twitch and whisker orienting reflexes).

2.5. Surgical

Five male rats received bilateral-gonadectomy (GDX) with a single midline ventral skin incision on the scrotum. The GDX surgeries were performed by Charles River Laboratories (Charles River, St-Constant, QC). Six female rats that received bilateral ovariectomy (OVX) via a lumbar incision were anesthetized with an isoflurane-oxygen mixture (induction 5%; maintenance 1.5%). Rats undergoing either GDX or OVX were given at minimum two weeks to recover before being prepared for electrophysiology. A two-week recovery time was chosen to reduce the impact of the surgery on electrophysiological results and to provide sufficient wash-out time for remaining gonad or ovary produced sex steroid hormones.

2.6. Slices preparation and electrophysiology

The rats and mice were euthanized (approximately 10:00 am) under deep isoflurane anesthesia (5% at 5 L/min). The brains were rapidly removed and kept in an iced-cold physiological solution containing (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 6 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 12.5 D-glucose, equilibrated with 95%O_2/5%CO_2. The brains were cut in coronal slices (250 µm) with a vibrating-blade microtome (Leica VT-1000, Leica Canada, Concord, ON) in the physiological solution (2 °C). Brain slices containing the BNST were incubated at 34 °C for 60 min before being transferred to a recording chamber constantly perfused (3 ml/min) with the physiological solution (34 °C). $GABA_A$-inhibitory post-synaptic currents (IPSC) were recorded in the whole-cell voltage-clamp configuration using glass micropipettes (3.5 MOhm) filled with a solution containing (in mM): 70 $Cs^+MeSO_3^-$, 58 KCl, 0.5 EGTA, 7.5 HEPES, 1.2 $MgCl_2$, 12 NaCl, 1 Mg-ATP, 0.3 GTP, and 1 phosphocreatine. $GABA_A$-IPSCs were evoked by local fiber stimulations with tungsten electrodes (FHC, Bowdoin, ME) using a bipolar stimulus isolator (World Precision Instruments, Sarasoto, FL) in the presence of the AMPA antagonist DNQX (50 µM). Recording electrodes were placed in the ovBNST for all but one experiment (Fig. 1A) whereby it was placed in the principal bed nucleus of the stria terminalis (prBNST) (Fig. 1D). Regardless of the region examined, the stimulating electrodes were placed 100–500 µm dorsal from the recorded neurons, and $GABA_A$-IPSCs we evoked with a paired pulse (10–100 µA, 0.1 ms duration, and 50 ms apart) at 0.1 Hz. E_{Cl} in these conditions was approximately −32 mV such that evoked $GABA_A$ postsynaptic currents were inward when neurons were voltage-clamped at −70 mV (Dumont and Williams, 2004). Detailed methodology for recordings of ovBNST $GABA_A$-IPSC was previously published (Krawczyk et al., 2011). Following a 5-min steady baseline recording, neurons were subjected to perfusions of various drugs, followed by a 20 min (minimum) post-

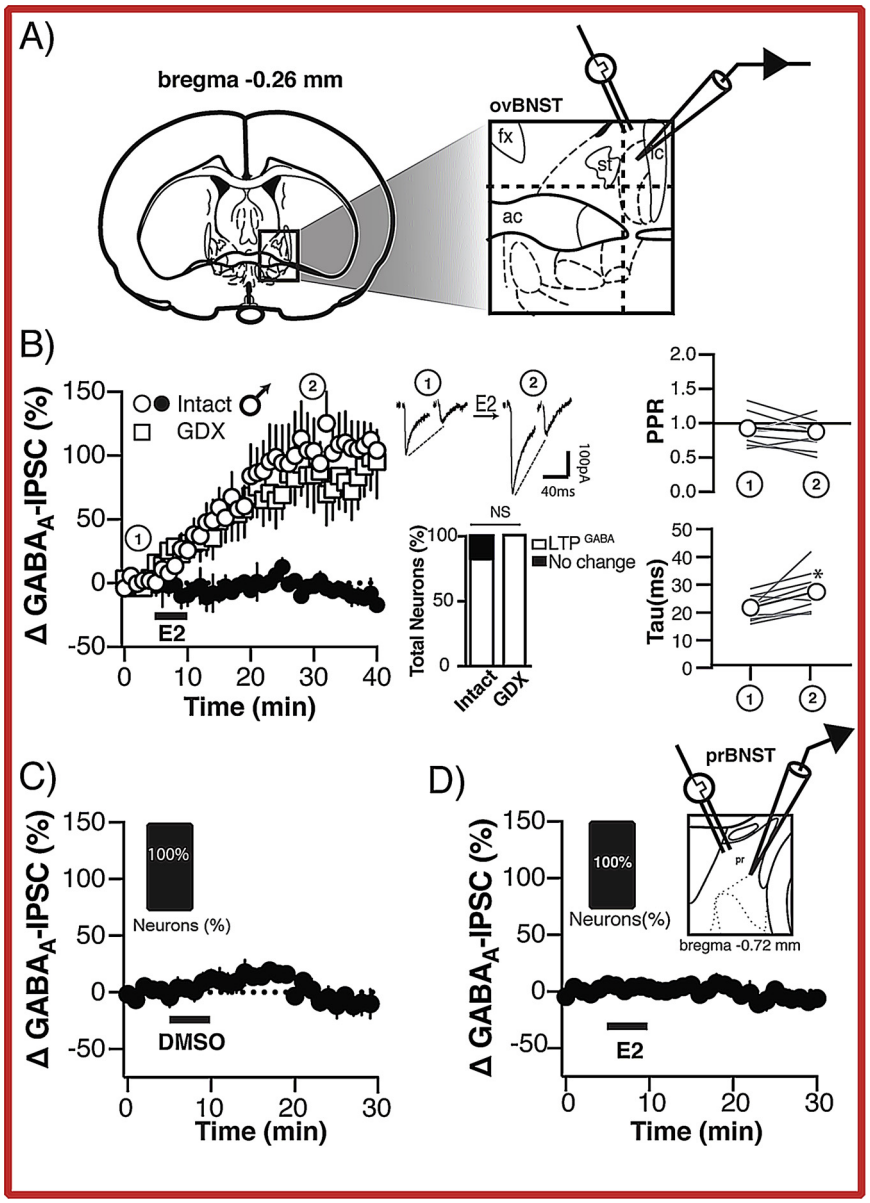

Fig. 1. (A) Schematic illustrating stimulating and recording electrodes placements within the ovBNST (adapted from Paxinos and Watson, 2005). Dotted lines indicate the medial and ventral limits of the recordings that were accordingly restrained to the oval (ov) BNST. E2 produces LTP^{GABA} in intact and GDX male rats. **(B–D)**, binned (1 min, 6 events) electrically-evoked ovBNST GABA$_A$-IPSCs as a function of time recorded in brain slices prepared from **(B)** Intact (n_{cells} = 11; n_{rats} = 8), **(C)** GDX (n_{cells} = 6; n_{rats} = 4), **(B)** DMSO (n_{cells} = 6; n_{rats} = 3), (D) prBNST (n_{cells} = 9; n_{rats} = 6). In B, C and D, white circles and squares represent LTP^{GABA} and black circles represent no change. In B, C and D, the circles represent rats with their gonads intact and the squares represent rats with their gonads removed. In **B**, representative GABA$_A$-IPSCs before and 30 min after 1 nM E2 application. The circled 1 and 2 represent the time course for representative trace, PPR and TAU. The black traces represent LTP^{GABA} Scale bars: 100 pA, 40 ms. * = $p < 0.05$.

induction recording. Total charge transfer was quantified with 2 possible outcomes following pharmacological bath application: 1- Drug-induced long-term potentiation (LTP^{GABA}; > 20% deviation from baseline), and 2-no change, either 20 or 30 min post-drug application, using previously established criteria that used paired-T tests (Naughton, 2016; Normandeau et al., 2018a; Normandeau et al., 2018b, Hawken et al., 2019). LTP^{GABA} required the IPSC of the neuron to be consistent the duration of the recording. Paired-pulse ratios (PPR) were calculated by dividing the second (S2) by the first (S1) event peak amplitude. Peak amplitudes for S1 and S2 were calculated from a baseline value measured immediately before the stimulus artefacts; as such in cases where S1 did not fully decay, the baseline for S2 differed from that of S1. GABA$_A$ time constant (Tau) were calculated and compared between baseline and 20–30 minutes post-drug application for each cell that produced LTP^{GABA}.

2.7. Drugs

Stock solutions of 17β-estradiol (E_2, 1 μM and 10 nM), AM251 (10 mM), PPT (10 μM), DPN, (10 μM), G-1 (10 μM), Staurosporin (SSP;100 μM), CID 16,020,046 (10 mM) and DNQX (100 mM) were prepared in DMSO (100%). All drugs were further dissolved in the physiological solutions at the desired concentration and the final DMSO concentration never exceeded 0.1%. Drugs were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada) or R&D Systems (Minneapolis, MN, USA).

2.8. Statistical analyses

One-way ANOVAs were used to compare variance between groups and tests of simple effects if ANOVAs yielded statistical significance. In order to investigate changes in GABA$_A$-IPSCs total charge transfer following drug perfusion, the baseline area under the curve (AUC) was compared to the post-bath application AU [(post AUC – baseline AUC)/baseline AUC) * 100 − 100]. Measurements of the AUC was done with Axograph X. In graphs denoting electrophysiology time-course, each data point represents the average of 1-min bins (6 evoked GABA$_A$-IPSCs) across recorded neurons. Neuronal responses rates were compared using the Fisher's exact probability test across all groups. The magnitude of LTP^{GABA} was compared using unpaired t-Tests. PPRs and Tau were compared before and after drug application using paired t-Tests. *P values* ≤ 0.05 were considered statistically significant. Data are

reported as mean ± SEM. All statistical analyses were done with GraphPad Prism (GraphPad Software).

3. Results

3.1. E2 mediated long-term potentiation of $GABA_A$ synaptic transmission (LTP^{GABA}) in the male rat oval Bed Nucleus of the Stria Terminalis (ovBNST)

Acute (5 min) bath application of 1 nM solution of E2 resulted in a robust (± 100%) LTP^{GABA} in 81% of recorded male ovBNST neurons (n_{cells} = 11; n_{rats} = 8). E2-mediated LTP^{GABA} was gonad independent in males, as both the potency and response rate were unaffected by gonad removal (t_{13} = 0.521, $p \leq 0.611$, $p \leq 0.514$, respectively; n_{cells} = 6; n_{rats} = 4; Fig. 1B). Paired-pulse ratio analyses suggested that E2-mediated LTP^{GABA} (1 nM) did not result from a change in the probability of GABA release but rather from postsynaptic mechanisms (Baseline:0.90 +/-0.076, E2: 0.833 +/-0.07, t_8 = 1.003, $p \leq 0.345$; Fig. 1B). Accordingly, E2 (1 nM) significantly increased the decay time constant of $GABA_A$-inhibitory postsynaptic currents (IPSCs) (Baseline: 21.651 +/-1.51 ms, E2: 27.055 +/-2.535 ms, t_8 = 2.639, $p \leq 0.029$; Fig. 1 B). The effect of E2 on inhibitory synaptic transmission was not ubiquitous across all BNST regions because E2 had no effect on $GABA_A$-IPSCs in the principal nucleus of the BNST (n_{cells} = 9; n_{rats} = 6; Fig. 1D). We found that application of DMSO, the vehicle used in each experiment, had no effect on inhibitory transmission in every neuron examined (n_{cells} = 6; n_{rats} = 3; Fig. 1C)

The percentage of neurons displaying E2-mediated LTP^{GABA} was significantly reduced by the non-selective protein kinase inhibitor SSP (28%; $p \leq 0.023$; n_{cells} = 7; n_{rats} = 3; Fig. 2A), indicating that E2 increased inhibitory GABA transmission through a protein kinase and most likely in a non-genomic way. The CB1 receptor inverse agonist/antagonist AM-251 also significantly reduced the number of neurons that responded to E2 (28%; $p \leq 0.049$; n_{cells} = 7; n_{rats} = 3; Fig. 2B) suggesting that, similar to previous findings in the female brain hippocampus (Huang and Woolley, 2012; Tabatadze et al., 2014), E2-increased GABA transmission involves endocannabinoids. To confirm if the effects of E2 were CB1 dependent, we used transgenic $cnr_1^{-/-}$ male mice. In male WT mice, 1 nM of E2 produced LTP^{GABA} in 50% of the neurons in the ovBNST (n_{cells} = 12; n_{rats} = 3; Fig. 2C, F). Compared to intact male rats, the potency of E2 was halved in WT mice, suggesting either the effect or potency of E2 may not be ubiquitous across rodents (t_{11} = 2.883, $p \leq 0.014$; Fig. 2C). In $cnr_1^{-/-}$ mice, however, the effect of 1 nM E2 was completely abolished ($p \leq 0.005$ compared to WT; n_{cells} = 13; n_{rats} = 4; Fig. 2C, F).

While, we found strong evidence for the effects of E2 being mediated through the CB1 receptor, the CB1 inverse agonist AM251 did not completely abolish the effect of E2. CB1 receptors are known to mediate pre-synaptic changes and E2 caused post-synaptic kinetic changes. We, therefore, aimed to find other possible downstream targets of E2. The 'orphan receptor' GPR55 had recently been proposed to be a putative endocannabinoid receptor (Ryberg et al., 2007). The GPR55 antagonist CID halved the magnitude of E2-induced LTP^{GABA} in intact male rats ($t(12)$ = 3.4635, $p \leq 0.004$; n_{cells} = 12; n_{rats} = 6; Fig. 2D), but did not significantly reduce the number of neurons producing LTP^{GABA} in response to E2 (41%; $p \leq 0.089$; Fig. 2F). In the ovBNST, AM251 does not appear to have any agonistic properties to GPR55 (Hawken et al., 2019). This finding implied that E2 may act through both CB_1R and GPR55 receptors to produce maximal LTP^{GABA} in male rats.

Finally, the effects of E2 were sensitive to metabolic challenges as the potency of 1 nM E2 was significantly reduced by a 24-hour caloric restriction when compared to intact male rats (t_{10} = 2.633, $p \leq 0.025$; n_{cells} = 14; n_{rats} = 4; Fig. 2 E). However, 24 h-caloric restriction did not significantly reduce the percentage of neurons responding to 1 nM E2 (42%, $p \leq 0.099$; Fig. 2F).

3.2. Sexually dimorphic E2-induced LTP^{GABA} in the ovBNST

E2 (1 nM) produced LTP^{GABA} with comparable potency and response rate in the ovBNST of female rats euthanized at estrus (n_{cells} = 15; n_{rats} = 6), diestrus (n_{cells} = 13; n_{rats} = 7), or after ovariectomy (n_{cells} = 9; n_{rats} = 6) suggesting the effect of E2 was estrous phase- and gonad-independent ($f_{2,19}$ = 0.919, $p \leq 0.416$; $p \leq 0.277$, respectively; Fig. 3A). Since E2's effects were estrous phase independent, we collapsed the data of estrus and diestrus for the remainder of the experiments. In contrast to males, E2 (1 nM) significantly increased the probability of release of GABA as measured by the PPR in intact female rats (Baseline: 0.924 +/-0.047, E2: 0.82+/-0.035; t_{17} = 2.568, $p \leq 0.02$; Fig. 3A). E2 significantly increased $GABA_A$ decay time-constant in intact female rats, an effect similar to male brains (Baseline: 27.128+/-2.667 ms, E2: 30.983+/-3.201 ms; t_{17} = 3.309, $p \leq 0.004$; Fig. 3A). These results suggest that unlike males, where E2 acted exclusively post-synaptic, E2 increased inhibitory transmission through both pre- and post-synaptic mechanisms in females.

We further examined the ovBNST's response to different doses of E2. In males we examined 0.01 nM (n_{cells} = 13; n_{rats} = 5), 0.1 nM (n_{cells} = 11; n_{rats} = 6), and 100 nM (n_{cells} = 9; n_{rats} = 4). In females we examined 0.1 pM (n_{cells} = 9; n_{rats} = 3),1 pM (n_{cells} = 8; n_{rats} = 4), 0.01 nM (n_{cells} = 12; n_{rats} = 4) and, 0.1 nM (n_{cells} = 10; n_{rats} = 4). First, we observed an inverted u-shaped dose response curve for both males and females, indicating that for both sexes there appears to be a negative feedback mechanism when exposed to higher concentrations of E2 (Fig. 3B). Second, the potency of E2 was greater in females (logEC50: -12.98) than males (logEC50: -10.76), as the peak effect in females occurred at 0.01 nM E2, while the peak effect in males required 1 nM E2 ($f_{1,37}$ = 8.84, $p \leq 0.005$; Fig. 3B). Third, in further support of a greater potency for E2 in females, it only required a concentration of 0.1 nM E2 to produce a reduced LTP^{GABA}, while in males this form of negative feedback only occurred at a concentration greater than 1 nM (Fig. 3B). Finally, there was no statistically significant differences between males and females in the number of neurons that responded to 1 nM, 0.1 nM, and 0.01 nM E2 concentrations ($p \leq 0.269$, $p \leq 1$, $p \leq 1$, respectively; Fig. 3B).

3.3. Sexually dimorphic involvement of estrogen receptors subtypes in E2-induced LTP^{GABA}

The best-known physiological effects of E2 are mediated by estrogen-receptor alpha (ERα), estrogen-receptor beta (ERß), and GPER. In males, bath application of the ERα agonist PPT (1 nM; n_{cells} = 9 ;n_{rats} = 7) and the GPER agonist G-1 (1 nM; n_{cells} = 9;n_{rats} = 5) produced LTP^{GABA} in most recorded neurons (77% and 66%, respectively; Fig. 4A, C, D). PPT produced LTP^{GABA} rapidly suggesting extranuclear ERα's in the ovBNST acting through non-genomic mechanisms. In contrast to E2, 1 nM PPT increased the probability of GABA release suggesting that ERα may act pre-synaptically in male rats (t_6 = 2.359, p = 0.056; Fig. 4 A). In contrast, G-1 had no effect on the probability of GABA release which indicates that the contribution of GPER in E2-mediated LTP^{GABA} was, in males, postsynaptic (t_5 = 1.973, p = 0.105; Fig. 4C). Both PPT and G-1 significantly increase $GABA_A$ decay time-constant suggesting post-synaptic alteration in $GABA_A$ receptors function (t_6 = 2.691, p = 0.036, t_5 = 2.743, p = 0.04, respectively; Fig. 4A, C). ERß may not be involved in estrogenic modulation of ovBNST GABA transmission in male rats since the agonist ERß DPN had a measurable effect in one neuron only (n_{cells} = 9 ;n_{rats} = 6; Fig. 4B, D).

In females, however, PPT (n_{cells} = 10 ;n_{rats} = 4), DPN (n_{cells} = 11;n_{rats} = 5) and GPER (n_{cells} = 8;n_{rats} = 4) all induced LTP^{GABA} in a significant number of neurons, suggesting a contribution of all 3 subtypes of estrogen receptors (60%, 45%, 75%, respectively; Fig. 4A, B, C, D). PPT and DPN produced LTP^{GABA} rapidly suggesting extranuclear ERα and ERß's in the ovBNST acting through non-genomic

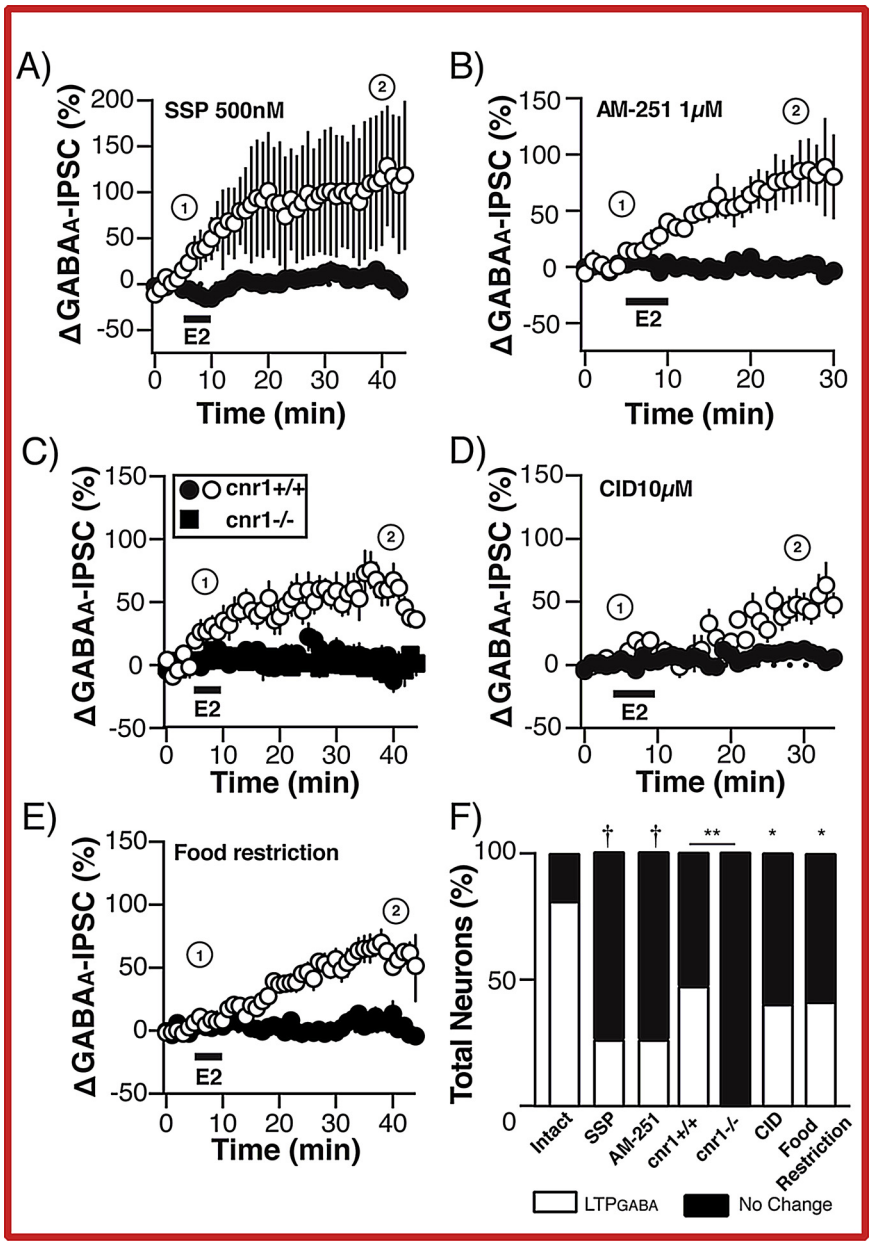

Fig. 2. E2 produces LTP^{GABA} through a protein kinase and CB_1R dependent mechanism in male rodents. **(A–F)**, binned (1 min, 6 events) electrically-evoked ovBNST $GABA_A$-IPSCs as a function of time recorded in brain slices perfused with 1 nM E2 and prepared from **(A)** 500 nM SSP (n_{cells} = 7; n_{rats} = 3), **(B)** 1μM AM251 (n_{cells} = 7; n_{rats} = 3), **(C)** WT and *cnr1*$^{-/-}$ mice (n_{cells} = 12; n_{rats} = 3; n_{cells} = 13; n_{rats} = 4, respectively), **(D)** 10 μM CID (n_{cells} = 12; n_{rats} = 6), and **(E)** 24 h acute FDR (n_{cells} = 14; n_{rats} = 4). In **A**, **B**, **C**, **D**, and **E**, white circles represent LTP^{GABA} and black circles and squares represent no change responses. In **C**, circles represent wild-type mice with their CB1R receptors intact and squares represent knock-out mice without CB1R's. * = $p < 0.05$ represents a significant reduction in E2 induced LTP^{GABA} **(F)** Percentage of cells that responded as either LTP^{GABA} or No Change across experimental conditions. The circled 1 and 2 represent the time course for representative trace. Scale bars: 200 pA, 10 ms. * = $p < 0.05$. † = $p < 0.05$ compared to Intact male rats.

mechanisms. PPR analyses revealed that PPT and DPN acted pre-synaptically whereas G-1 did not affect the probability of GABA release, most likely acting post-synaptically (t_5 = 3.287, p = 0.021, t_4 = 3.911, p = 0.017, t_5 = 0.012, p = 0.99, respectively; Fig. 4A, B, C). Interestingly, PPT, DPN and G-1 significantly increased $GABA_A$ decay time-constant, suggesting postsynaptic effects as well (t_5 = 4.576, p = 0.006, t_4 = 5.598, p = 0.005, t_5 = 5.302, p = 0.003, respectively; Fig. 4 A, B, C).

4. Discussion

Bath-application of E2 resulted in a long-lasting potentiation of $GABA_A$ synaptic transmission in ovBNST neurons of brain slices prepared from adult male rats. In most responsive neurons, E2-mediated LTP^{GABA} was postsynaptic, required estrogen receptors (ER) ERα and GPERs, involved downstream regulation of either/or CB1 and GPR55 receptors, and phosphorylation processes in addition to being sensitive to an acute metabolic homeostatic challenge. In the female ovBNST, E2 acted on all three known ERs, ERα, ER β and GPERs and was 100 times more potent at potentiating $GABA_A$ synaptic transmission.

E2 influences synaptic transmission at GABA synapses in most studied regions of the brain in rodents (Parducz et al., 1993; Saleh and Saleh, 2001; Mukherjee et al., 2017). In the hippocampus, E2 pre-synaptically rapidly reduces inhibitory transmission in adult female rats, but, has no effect in males (Huang and Woolley, 2012; Tabatadze et al., 2014). We observed a different situation in the ovBNST whereby E2 rapidly increased inhibitory synaptic transmission in both sexes. The effect of E2 in the ovBNST was similar to other regions of the extended amygdala, notably the basolateral amygdala where E2 increases GABA-mediated synaptic transmission in brain slices prepared from OVX female mice (Tian et al., 2013).

Depending on the concentration, E2 changed the probability of GABA release in both sexes. Moreover, activation of ERα receptors by PPT, but not the activation of GPER by G-1, changed the probability of release of GABA in both sexes. These findings indicate that E2 binding to ERα in both sexes facilitated pre-synaptic GABA release. A 1 nM concentration of E2 and ERα and GPER activation increased the $GABA_A$ decay time constant in both sexes, indicating a change in the kinetics of

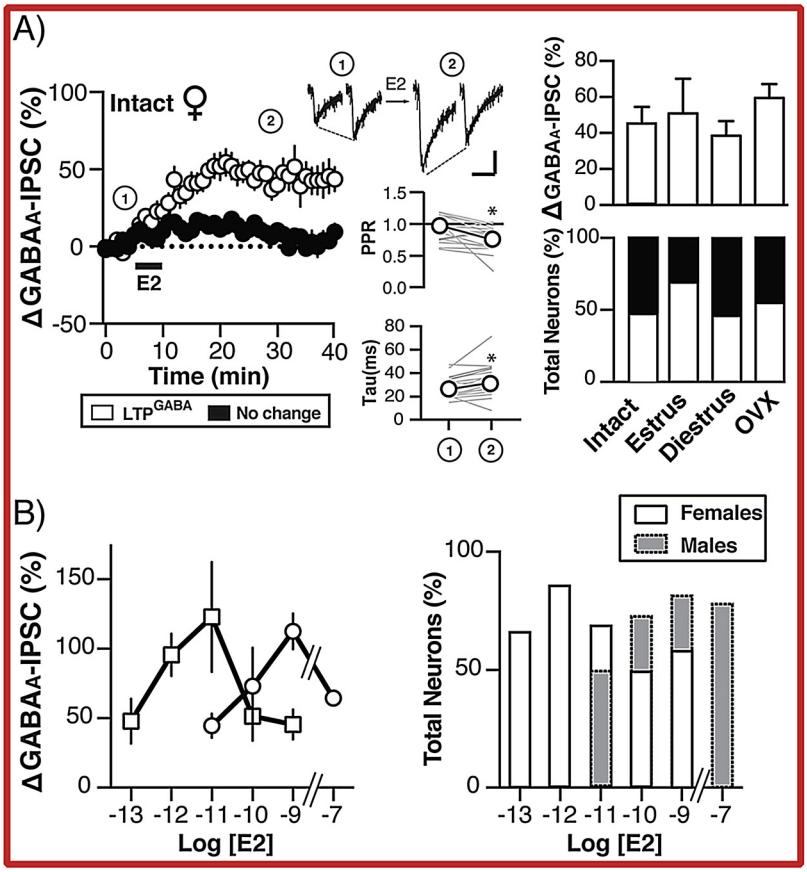

Fig. 3. Dose-response of E2 in both male and female rats **(A)**, Binned (1 min, 6 events) electrically-evoked ovBNST $GABA_A$-IPSCs as a function of time recorded in brain slices prepared from **(A)** intact female rats (n_{cells} = 28; n_{rats} = 13). White circles represent LTP^{GABA} and black circles represent no change responses. Representative $GABA_A$-IPSCs trace before and 25 min after 1 nM E2 application. The circled 1 and 2 represent the time course for representative trace, PPR and TAU. The black traces represent LTP^{GABA} Scale bars: 100 pA, 40 ms. Average E2 induced LTP^{GABA} across female hormonal conditions: Estrus (n_{cells} = 15; n_{rats} = 6), Diestrus (n_{cells} = 13; n_{rats} = 7) and OVX (n_{cells} = 9; n_{rats} = 6). Percentage of cells that responded as either LTP^{GABA} or no Change across experimental conditions. E2 produces an inverted-U dose response in the ovBNST. **(B)** Male 100 nM (n_{cells} = 9; n_{rats} = 4), Male 0.1 nM (n_{cells} = 11; n_{rats} = 6), Male 0.001 nM (n_{cells} = 13; n_{rats} = 5), Female 0.1 nM (n_{cells} = 10; n_{rats} = 4), Female 0.01 nM (n_{cells} = 12; n_{rats} = 4), Female 1 pM (n_{cells} = 8; n_{rats} = 4), and Female 0.1 pM (n_{cells} = 9; n_{rats} = 3). Males are represented by the white circle and females are represented by the white square. Percentage of cells that responded as LTP^{GABA} in males and female rats across experimental conditions. *=$p < 0.05$.

post-synaptic $GABA_A$ receptors. Together, these results corroborate that E2 produced LTP^{GABA} in the ovBNST through both pre- and post-synaptic mechanisms.

A change in $GABA_A$ decay time constant may indicate a change in subunit composition. E2 increases the mRNA expression of the $GABA_A$ α3 subunit (Pierson et al., 2005). Inserting α3 into a $GABA_A$ receptor composed of α1β2γ2S, replacing α1, significantly increases the $GABA_A$ decay time constant (Gingrich et al., 1995). One explanation is that E2 in the ovBNST of rats likely produced LTP^{GABA} through increasing $GABA_A$ α3 expression. Alternatively, E2 increases α2 subunit mRNA in various regions of the brain (Pierson et al., 2005; Calza et al., 2010 Tian et al., 2013). As α2 subunits provides the $GABA_A$ receptor a faster activation time and a slower deactivation time than the α1 subunit, it is possible that E2 in the ovBNST may be increasing α2 expression (Lavoie et al., 1997). Future studies should examine which if any $GABA_A$ subunits E2 affects within the ovBNST.

In the hippocampus, E2 mediates a protein kinase dependent LTD^{GABA} by promoting pre-synaptic CB_1R suppression of GABA release (Huang and Woolley, 2012; Tabatadze et al., 2014). Similarly, CB_1Rs are located on the pre-synaptic bouton of synapses and reduce inhibitory synaptic transmission in the ovBNST (Puente et al., 2010). The effect of E2 in the ovBNST male rodents was also protein kinase- and CB_1R-dependent although in the opposite direction than in the hippocampus. PPR measures indicated greater GABA release following E2 application in the ovBNST. Therefore, unlike in the hippocampus, E2 may interfere with the activation of pre-synaptic CB_1Rs in the ovBNST. It is likely that E2 binding to ERα may activate a protein kinase that interferes with production of the CB_1R endocannabinoid ligands, removing the tonic inhibition of pre-synaptic GABA release to produce LTP^{GABA}. If E2 suppresses the production of endocannabinoid ligands, the GPR55 receptor antagonist CID can additionally occlude the effects of E2. In the ovBNST both CB1R and GPR55 alter IPSC's by producing pre-synaptic changes (Hawken et al., 2019). Thus, E2 may increase inhibitory synaptic transmission pre-synaptically by interfering with the endocannabinoid system within the ovBNST.

ERs and CB_1Rs influence appetite and the maintenance of energy homeostasis (Di Marzo et al., 2001; Geary et al., 2001; Cardinal et al., 2012; Davis et al., 2014). E2 reduces food consumption and controls energy balance/metabolism through a combination of ERα, ERß and GPER (Geary et al., 2001; Liang et al., 2002; Musatov et al., 2007; Yepuru et al., 2010; Sharma et al., 2013; Davis et al., 2014). Furthermore, the nucleus of the solitary track innervates the ovBNST upon changes to either blood glucose levels or stomach distention (Imeryuz et al., 1997; Rinaman, 1999; Holst, 2007; Nakade et al., 2007; Sandoval et al., 2008; Kreisler et al., 2014). In mice, the ovBNST helps maintain energy homeostasis by promoting food consumption through a circuitry involving the lateral hypothalamus (Jennings et al., 2013). Acutely food restricted male rats had reduced E2 neurophysiological potency although without significantly changing the percentage of responding neurons. This contrasts with the effect of acute food restriction on GPR55- and CB1R -mediated modulation of ovBNST GABA transmission (Hawken et al., 2019) and suggests that E2 is acting upstream of those mechanisms. We did not examine the effects of 24-hour FDR in females due to its known effects on the estrous cycle and circulating hormone levels, however, if the female ovBNST shares the same neurocircuitry as the male's the effects may be similar (Bronson and Marsteller, 1985; Dong et al., 2001). The metabolic challenge may have caused a reduction in ER expression. Alternatively, the metabolic challenge may have affected endocannabinoid production or CB_1R expression, which could occlude the effects of E2. In the ovBNST, low-frequency stimulation (LFS) produces activity-dependent LTP^{GABA} by promoting the activation of GPR55 in sated rats. However, after 24-hour FDR LFS produces a long-term depression of GABA by promoting CB1R activation (Hawken et al., 2019). It is possible that E2 and ER's are involved in mediating the switch in this system following a metabolic challenge. Future studies will need to examine how E2 may interact with CB_1R and

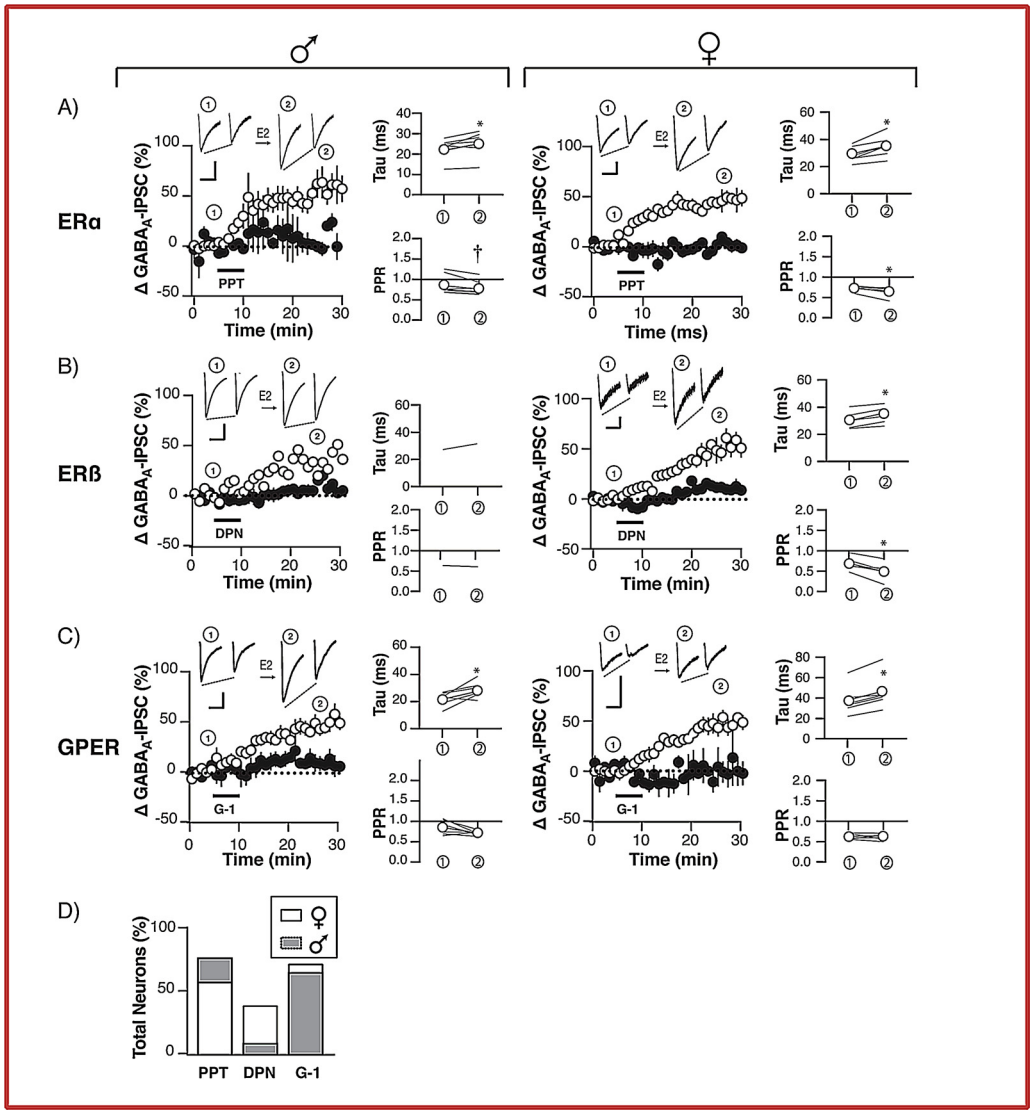

Fig. 4. The effects of ERα, ERβ, and GPER on LTP^{GABA} in both sexes. **(A–C),** binned (1 min, 6 events) electrically-evoked ovBNST GABA$_A$-IPSCs as a function of time recorded in brain slices prepared from **(A)** 1 nM PPT(Male: $n_{cells} = 9$;$n_{rats} = 7$; Female: $n_{cells} = 10$;$n_{rats} = 4$), **(B)** 1 nM DPN (Male: $n_{cells} = 9$;$n_{rats} = 6$; Female: $n_{cells} = 11$;$n_{rats} = 5$) and **(C)** 1 nM G-1 (Male: $n_{cells} = 9$;$n_{rats} = 5$; Female: $n_{cells} = 8$;$n_{rats} = 4$). In **A**, **B** and **C**, white circles represent LTP^{GABA} and black circles represent no change responses. The circled 1 and 2 represent the time course for the PPR, TAU and representative traces. Black traces are LTP^{GABA}. Scale bars: 200 pA, 10 ms.**(D)** Percentage of cells that responded as LTP^{GABA} across experimental conditions. * = $p < 0.05$, † = $p < 0.06$.

GPR55 in the ovBNST to maintain energy homeostasis.

E2 was 100-fold more potent in the female ovBNST. In both sexes, dose-response curves had inverted-U shapes, indicating that in the ovBNST the increased production of E2 either by local aromatase or the gonads may reduce the effect of E2-mediated LTP^{GABA}. Aromatase in the ovBNST is located at presynaptic terminals (Zhao et al., 2007). Thus, aromatase's location would allow inputs to influence inhibitory synaptic transmission within the ovBNST through the production of E2. However, it remains unknown what the circulating concentrations of E2 are within the ovBNST of intact male and female rats. Within the hippocampus, male, estrus and diestrus rats have a circulating concentration of ˜8.4 nM, ˜1 nM and ˜0.7 nM of E2, respectively (Hojo et al., 2009). It remains possible that the natural state of ovBNST neurons is within the reduced potency range of both sexes. Both the aromatatization and the metabolism of E2 may therefore influence LTP^{GABA} in the intact ovBNST.

The metabolism of E2 is a possible explanation for the reduction of E2′s potency on inhibitory synaptic transmission at greater concentrations in males and females. E2 is metabolized into the estrogen's estriol or estrone, and greater concentrations of E2 may increase their production. Estriol is 80-fold and estrone is 12-fold less potent than E2 (Hall, 2015). If high concentrations of E2 were rapidly metabolized into either estriol or estrone, it would explain the reduction in LTP^{GABA}. Intriguingly, estriol in the presence of E2 has anti-estrogenic properties (Kuiper et al., 1997; Melamed et al., 1997). Specifically, estriol can act as an antagonist to the GPER (Lappano et al., 2010). Interestingly, E2 induced LTP^{GABA} is produced, in part, by GPER in both sexes. Therefore, it is possible that the negative feedback we found at higher concentrations of E2 was mediated by estriol's anti-estrogenic properties.

There were no observable differences in the effects of 1 nM E2 between females either in the estrus or diestrus phase of the estrous cycle. Furthermore, the response to bath application of 1 nM E2 was unaffected in male and female rats without gonads for at least 2 weeks. These results suggest that the expression of GABA-modulating ERs was gonad independent and locally regulated in the ovBNST of both sexes. Alternatively, it remains possible that we did not see an effect of estrous cycle or OVX at 1 nM as it was not the most potent dose of E2. More potent doses (1 pM or 0.01 nM) of E2 may be influenced by the ovaries. Regardless, we can conclude that the reduced effect of higher concentrations of E2 on IPSC's appears to be gonad independent. Although the expression of ERs in the ovBNST appears to be gonad independent, estrogens produced by either the testes or the ovaries may influence inhibitory synaptic transmission in this region. In female rats, the estrous cycle determines the amount of E2 that is produced by the ovaries (Shaikh, 1971; Nequin et al., 1979). The fluctuations in production of sex steroid hormones during the various phases of the estrous cycle directly alter food consumption and metabolism (Brobeck et al., 1947). We examined female rats in estrus and diestrus phases of the estrous

cycle as food consumption, metabolism and compulsive behaviours differ between the two (Tarttelin and Gorski, 1971; Wurtman and Baum, 1980; Roberts et al., 1989; Parker et al., 2001). Therefore, it remains possible that ovarian produced E2 can influence the ovBNST to meet the different biological demands of the estrous cycle. To confirm this hypothesis, future studies should use *in-vivo* electrophysiological recordings in either anesthetized or freely moving female rats at different phases of the estrous cycle to measure other relevant electrophysiological properties such as firing rate and the action potential width (Blume et al., 2017).

In males, pharmacologically activating ERα and GPER produced LTP^{GABA}. However, in females, pharmacologically activating all three ERs produced LTP^{GABA}. The rapid induction of LTP^{GABA} suggested non-genomic extranuclear ERα in both sexes and ERβ in females. Although unknown in rats, the female mouse ovBNST does not contain any ERß protein (Mitra et al., 2003). One possibility is that the female rat ovBNST contains either pre or post-synaptic located ERβ protein. Alternatively, the agonist DPN has a 70-fold higher binding affinity and a 170-fold higher potency for ERß than ERα, such that DPN-induced LTP^{GABA} could be ERα-dependent (Meyers et al., 2001). In conjunction with E2 being 100-fold more potent in females, this data indicates that there may either be functional differences or greater amounts of ERα in females than males. There are eleven types of neurons and three different activity patterns in the ovBNST of male rats (Larriva-Sahd, 2006; Hammack et al., 2007). The effect of E2 was rather homogeneous in both sexes affecting the majority of ovBNST neurons, suggesting that E2 modulates multiple subtypes of neurons (Larriva-Sahd, 2006). The eleven anatomically-described subtypes of ovBNST neurons may not all express ERs to the same extent, explaining perhaps the variability observed in the response rates to varying doses of E2 in both sexes. Future studies may want to examine the expression patterns of ER's and link those to the neurophysiological properties of E2 in this brain region.

5. Conclusion

Our study reveals that E2 robustly potentiates inhibitory synaptic transmission in the ovBNST in both male and female rats. This is to our knowledge the first study to examine the neurophysiology of sex steroid hormones in the ovBNST. This study along with recently published work (Hawken et al., 2019) further support the potential role of the ovBNST in energy metabolism, pinpointing to a potential location for E2-mediated modulation of energy homeostasis that could be sex-dependent. This could have important implication for sex-based differences in compulsive behaviours including alcohol, sugar, and other substance use disorders (Pleil et al., 2016; Pleil and Skelly, 2018, Maracle et al, 2019). We further characterized the effects of ERα, ERβ and GPER on inhibitory synaptic transmission in both sexes. However, while we began to characterize the molecular mechanisms behind these effects, there is work that remains to be conducted to determine the full mechanisms of each ER affects inhibitory synaptic transmission. Understanding the function of estrogens in maintaining energy homeostasis may provide valuable insight into how a disruption in estrogenic function promotes the development and maintenance of psychiatric disorders across sexes.

Conflict of interest

The authors declare no competing financial interests.

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