The membrane estrogen receptor ligand STX rapidly enhances GABAergic signaling in NPY/AgRP neurons: role in mediating the anorexigenic effects of 17β-estradiol

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The membrane estrogen receptor ligand STX rapidly enhances GABAergic signaling in NPY/AgRP neurons: role in mediating the anorexigenic effects of 17β-estradiol

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The membrane estrogen receptor ligand STX rapidly enhances GABAergic signaling in NPY/AgRP neurons: role in mediating the anorexigenic effects of 17β-estradiol. Am J Physiol Endocrinol Metab 305: E632–E640, 2013. First published July 9, 2013; doi:10.1152/ajpendo.00281.2013.—Besides its quintessential role in reproduction, 17β-estradiol (E2) is a potent anorexigenic hormone. E2 and the selective Gq-coupled membrane estrogen receptor (Gq-mER) ligand STX rapidly increase membrane excitability in proopiomelanocortin (POMC) neurons by desensitizing the coupling of GABAB receptors to G protein-coupled inwardly rectifying K+ channels (GIRKs), which upon activation elicit a hyperpolarizing outward current. However, it is unknown whether E2 and STX can modulate GABAergic signaling in neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons. We used single-cell RT-PCR and whole cell patch clamping with selective pharmacological reagents to show that NPY/AgRP cells of mice express the GABAB-R1 and -R2 receptors and are hyperpolarized by the GABAB agonist baclofen in an E2-dependent manner. In males, E2 rapidly attenuated the coupling of GABAB receptors to GIRKs, which was blocked by the general PI3K inhibitors wortmannin and LY-294002 or the selective p110α inhibitors, but not bafilomycin A1 orwortmannin with E2 or simply applying STX always enhanced the GABAB response. Thus, in NPY/AgRP neurons, activation of the Gq-mER by E2 or STX enhances the E2-stimulated inwardly-rectifying K+ channels (GIRKs), which upon activation elicit a robust, hyperpolarizing outward current. Conversely, E2 attenuates the orexigenic actions of NPY and suppresses its expression (9, 30, 40). In addition, E2 reduces secretion of the peptide from immortalized hypothalamic neurons (11). We have shown that E2 decreases the membrane excitability of NPY/AgRP neurons, at least partially, through increasing the expression of KCNQ5 channels, which augments the M-current (36). However, whether this anorectic steroid also modulates synaptic GABAB currents, as in POMC cells, remains unknown. We hypothesized that, if baclofen, a GABA receptor agonist, inhibits NPY/AgRP cells, then E2, potentially via the Gq-mER, would enhance the coupling of the GABAB receptor to GIRKs, thereby reducing membrane excitability and curbing food consumption. Here, we used single-cell RT-PCR and visualized whole cell patch clamping with selective pharmacological reagents to investigate these possibilities.

MATERIALS AND METHODS

Animal care. All animal treatments complied with guidelines from the National Institutes of Health and received approval from the Institutional Animal Care and Use Committee at Oregon Health & Science University or Western University of Health Sciences. NPY-GFP transgenic mice (from Dr. Brad Lowell, Harvard University) (47) of either sex lived under controlled temperature (25°C) and photoperiod (12:12 h-light-dark) at Oregon Health & Science University and received food and water ad libitum. Following gonadectomy under 2% isoflurane, mice received a dose of 4 mg/kg carprofen (Rimadyl; Pfizer Animal Health, New York, NY) and then recovered for 1 wk before experimentation.

Two cell populations residing within the arcuate nucleus (ARC) of the hypothalamus compose a critical circuit for regulating energy homeostasis: neuropeptide Y (NPY)/agouti-related peptide (AgRP) and proopiomelanocortin (POMC) neurons (12). Selective stimulation of NPY/AgRP neurons via optogenetics evokes intense feeding (3), and ablation of these neurons in adults causes starvation, evidently due to loss of inhibitory signaling to the brain stem (24, 52, 53). Similar and other approaches have revealed that POMC cells control the exact opposite actions in energy balance (3, 13, 32, 39, 50, 54).

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We have shown previously (32) that the membrane-initiated signaling of E2 attenuated the GABA<sub>B</sub>-GIRK channel coupling in POMC neurons similarly in intact male and ovarioctomized female mice. Therefore, we used the same models to study the pharmacology of the rapid E2-induced actions in NPY/AgRP neurons. Due to the heterogeneous effects of E2 on the GABA<sub>B</sub> response in gonadectomized animals (see RESULTS), we decided to use ovarioctomized females to study the expression of the P<sub>13K</sub>p110β isoform in individual NPY/AgRP neurons and also used these cells to ascertain GABA<sub>B</sub>-R1 and -R2 expression.

In previous studies, the Gq-mER ligand STX showed a clear anorectic effect in female guinea pigs (37, 50). Since our in vitro experiments suggested that STX would also be anorectic in males, we wanted to compare food intake in both sexes. Topkea guinea pigs (Elm Hill Breeding Labs, Chelmsford, MA) of either sex (n = 10 males, n = 12 females) lived under controlled temperature (21–25°C) and photoperiod (12:12-h light-dark) at Western University of Health Sciences and received food and water ad libitum. Following gonadectomy under ketamine-xylazine (33 and 6 mg/kg sc, respectively) supplemented with 2% isoflurane, guinea pigs recovered for 4–8 days before initiation of the feeding studies (see below).

Drugs. E<sub>2</sub> (Steraloids, Wilton, NH), the ER antagonist ICI 182,780 (Fulvestrant; Tocris, Minneapolis, MN), and the selective ER<sub>α</sub> agonist PPT (propyl pyrazole triol, Tocris) were dissolved in 100% ethanol (1, 10, and 1 mM stock, respectively). Tetrodotoxin (TTX; channel blocker; Alomone Labs, Jerusalem, Israel) and baclofen (GABA<sub>B</sub> agonist; Sigma, St. Louis, MO) were dissolved in water (1 and 40 mM stocks, respectively). Wortmannin (PI3K inhibitor, Sigma), LY-294002 (PI3K inhibitor; EMD Millipore, Billerica, MA), and 9-(1-(phenylamino)ethyl)-4H-pyrido[1,2-a]pyrimidin-4-one; synthesized by Dr. Kevan Shokat, UC San Francisco) were dissolved in DMSO (1, 40, and 20 mM stocks, respectively).

STX [2-(4-hydroxyphenyl)-3-phenylpent-2-enoic acid [4-(2-dimethylaminoethoxy)phenyl]amide, E-nenatiomer], a Gq-mER selective ligand, was produced by AAPharmaSyn (Ann Arbor, MI) under contract according to the published synthetic protocol (Tobias et al., 2006). STX was dissolved in 100% ethanol (1 mM stock) or polypropylene glycol (PPG, 20 mg/ml) for in vitro and in vivo experiments, respectively.

Feeding experiments. We chose to use guinea pigs for the feeding experiments on the basis of our previous publications that the diphermylacyamide compound STX decreases food intake and weight gain following ovarioctomy in females (32, 37, 50) and the fact that STX is bioavailable (F = 22%) and centrally active in the female guinea pig (35). For the feeding studies, a Comprehensive Lab Animal Monitoring Systems (CLAMS; Columbus Instruments, Columbus, OH) recorded daily food intake in adult gonadectomized male (n = 10) and female guinea pigs (n = 12) as previously described (35). Animals were given 3 days to acclimate to their CLAMS chambers and daily handling/weighing procedures. After acclimation, analysis of food intake under ad libitum conditions took place for 7 days. Each morning (0800), animals were weighed, injected with STX (3 mg sc) or vehicle (150 µl sc), and then returned to their CLAMS chambers.

Preparation of hypothalamic slices. Following rapid decapitation of 2- to 3-mo-old mice, brains were extricated and placed in ice-cold, carbogenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) cutting solution containing (in mM) 2 KCl, 2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 10 HEPES, 208 sucrose, 10 dextrose, and 1 CaCl<sub>2</sub>. Hypothalamic blocks were dissected and secured within the vibratome well. Five 250 µM coronal slices from the block were cut and then placed in an auxiliary chamber with carbogenated artificial cerebral spinal fluid (aCSF) containing (in mM) 124 NaCl, 5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, 5 HEPES, 10 dextrose, 26 NaHCO<sub>3</sub>, 2 MgSO<sub>4</sub> (or 2 MgCl<sub>2</sub> if BaCl<sub>2</sub> was applied in experiment), and 2 CaCl<sub>2</sub>. 

Electrophysiology. After at least 1 h of recovery following collagenation, the slices were transferred to a recording chamber on an Olympus BX51WI with infrared differential interference contrast imaging and fluorescence. Slices received continuous perfusion of warmed (35°C), carbogenated aCSF at a rate of 2 ml/min by peristaltic pump. Drugs were diluted from stock solutions with aCSF in open-top 60-ml syringes and perfused throughout the recording chamber by the same pump. All cells were perfused with 0.5 µM TTX 5 min before and then during recording.

Whole cell voltage (−50 mV) and current-clamp recording were acquired using 3- to 4-mΩ borosilicate pipettes (1.5 mm OD; World Precision Instruments, Sarasota, FL) filled with internal solution containing (in mM) 128 potassium gluconate, 10 NaCl, 2 MgCl<sub>2</sub>, 10 HEPES, 11 EGTA, 3 ATP, and 0.25 GTP (pH 7.26, 296 mOsm). pCLAMP 9 software was used to acquire and analyze data from an Axopatch 200A amplifier linked to Digidata 1322A and Minidigi 1A digitizers (Molecular Devices, Sunnyvale, CA). Only stable cells showing <20% change in access resistance throughout the experiment were analyzed. The −10-mV liquid junction potential was corrected offline. Current-voltage relationships were established for each cell by stepping the cell from −50 to −110 mV in 10-mV increments. The slope conductance was calculated between −60 and −80 mV. Under basal conditions, the NPY/AgRP cells in this study showed a mean RMP of −51.1 ± 0.6 mV (n = 113) and average input resistance of 2.3 ± 0.2 GΩ (n = 116).

The protocol depicted in Fig. 1 was used to determine the effect of various pharmacological manipulations on coupling of GABA<sub>B</sub> receptors to GIRK channels: after obtaining a stable whole cell configuration, cells were perfused with 0.5 µM TTX for 5 min. Baclofen was then perfused at concentration of 10 µM to reach a steady-state outward current for the first response (R1). After washout of the drug, the current decreased to its resting level. Perfusion of vehicle or the selected treatment took place for 15 min, and then a second response to baclofen (10 µM) was elicited (R2). The magnitude (outward current) of each response was measured, and the effects were expressed as a percentage of R2/R1.

NPV/AGRP cell harvesting and reverse transcription. Single-cell harvesting took place as previously described (7, 55). Briefly, following microdissection from the slice, the ARC was incubated in aCSF containing protease (1 mg/ml) for 15–17 min at 37°C and then washed four times in low Ca<sup>2+</sup> (1 mM) aCSF and two times in regular aCSF. Trituration of the tissue with flamed Pasteur pipettes of progressively smaller tip diameter allowed single cells to disperse onto a glass-bottomed 60-mm dish. Healthy cells adhered to glass after 12 min, and then unhealthy cells and debris were discarded with the continuous flow of carbogenated aCSF via peristaltic pump (2 ml/min).

Dispersed cells were patched with a Xenoswitch microinjector system (Sutter Instruments, Navato, CA) using standard glass pipettes (1.5 mm OD/0.84 ID; World Precision Instruments, Sarasota, FL). Gentle negative pressure applied to the pipette allowed harvesting of cells with minimal collection of aCSF. The contents of the pipette were collected in 0.5-ml tubes containing 1 × Invitrogen Superscript III Buffer, 15 U of RNasin (Promega), and 10 mM dithiothreitol.
(DTT) in 5 μl of diethylpyrocarbonate (DEPC)-treated water. Following collection, the cells were frozen on dry ice and later reverse-transcribed by adding dNTPs (0.5 mM, Promega), random primers (100 ng/tube, Promega), and anchored oligo(dT)20 primer (400 ng/ tube, Invitrogen) to the tube. The cocktail was then heated to 65°C for 5 min and then cooled on ice for 5 min before addition of Superscript III reverse transcriptase (100 U/tube, Invitrogen), RNAsin (15 U), 5 min and then cooled on ice for 5 min before addition of Superscript III reverse transcriptase (100 U/tube, Invitrogen), RNAsin (15 U), and DEPC-treated water to a final volume of 20 μl. Reverse transcription (RT) followed accordingly: 25°C for 5 min, 50°C for 60 min, 70°C for 15 min, and 4°C for 5 min. aCSF collected near dispersed cells underwent RT as a negative control. Cells and tissue used for negative controls received the same processing as above, but without reverse transcriptase (–RT).

**Primer design.** The NPY, GABA<sub>B</sub>-R1, and GABA<sub>B</sub>-R2 primer sequences were as previously published (36, 58). Mouse PI3K p110<sub>α</sub> primers (218 bp product; acc. no. NM_029094; forward primer, 812–833 nt; reverse primer 1010–1029 nt) were designed using the Clone Manager software (Sci Ed Software, Cary, NC). To avoid genomic DNA amplification, primer pairs crossed intron-exon boundaries.

**Single-cell PCR.** Single cell PCR (SC-PCR) required 2–3 μl of cDNA template in a 30-μl PCR mix. Following established protocols (33, 56), 40 to 50 cycles of amplification took place with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). Ethidium bromide applied to a 2% agarose gel allowed visualization of PCR products, which were confirmed with sequencing.

**Statistical analysis.** One-way ANOVA with Newman-Kuels post hoc test was used to compare groups for the electrophysiology experiments. A repeated-measures, multifactorial ANOVA followed by the least significant difference (LSD) test was used to compare groups for the electrophysiology experiments. A repeated-measures, multifactorial ANOVA followed by the least significant difference (LSD) test was used to compare groups for the electrophysiology experiments.

**RESULTS**

**NPY/AgRP cells respond to the GABA<sub>B</sub> agonist baclofen and express GABA<sub>B</sub>-R1 and -R2 mRNA.** Since the effects of GABA<sub>B</sub> agonists such as baclofen have not been established in NPY/AgRP neurons, we measured the outward current (voltage clamp, −50 mV) in response to baclofen in the presence of TTX (0.5 μM; to block action potential-derived synaptic input). Virtually all NPY/AgRP neurons (n = 128) responded to baclofen. A cumulative dose response to the drug applied at 1, 3, 10, and 30 μM concentrations correspondingly generated a mean steady-state outward current of 4.0 ± 1.1 pA (n = 9), 8.4 ± 1.4 pA (n = 23), 14.6 ± 2.1 pA (n = 22), and 19.9 ± 3.5 pA (n = 11) in males and 5.8 ± 2.5 pA (n = 2), 7.7 ± 2.2 pA (n = 3), 21.6 ± 4.8 pA (n = 5), and 28.7 ± 5.9 pA (n = 7) in ovariectomized females (Fig. 2A). A few cells (n = 3) additionally received 65 μM baclofen to confirm that 30 μM elicited a maximum response. Normalizing these data to percent maximal outward current revealed that males and females shared a statistically equivalent EC<sub>50</sub> of 4.3 and 4.7 μM, respectively (Fig. 2B). This is similar to the EC<sub>50</sub> for baclofen-mediated inhibition of POMC neurons (31). In current clamp, 10 μM baclofen hyperpolarized NPY/AgRP cells (n = 11) by 16 ± 2 mV and inhibited firing (Fig. 2C). I-V relationships generated before and after application of the drug showed a reversal potential near E<sub>K</sub> (−83 mV) and a 54 ± 0.1% (n = 29) increase in slope conductance (Fig. 2D). BaCl<sub>2</sub> (500 μM) abrogated the baclofen response (data not shown), which is consistent with the GABA<sub>B</sub> receptors activating GIRK channels (20, 28, 41). Single-cell RT-PCR on 40 neurons from four animals confirmed that 87.5% of NPY/AgRP neurons expressed GABA<sub>B</sub>-R1 and -R2, respectively (Fig. 3, A and B).

**E<sub>2</sub> attenuates but STX enhances GABA<sub>B</sub> signaling in male NPY/AgRP cells.** We used the whole cell voltage clamp protocol depicted in Fig. 1 to measure the rapid effects of E<sub>2</sub> on GABA<sub>B</sub> coupling to GIRK channels in NPY/AgRP neurons, as previously described in POMC cells (31). We used a concentration of 10 μM baclofen to ensure a robust response. Under control conditions (no intervening drug) in intact males, 10 μM baclofen elicited a large response that subsided during a 20-min washout period, and a second, equal-amplitude response thereafter (n = 7; Fig. 4, A and G), suggesting that no rundown or desensitization occurred. Application of E<sub>2</sub> (100 nM) during the washout period, which showed no effect on holding current, significantly attenuated the response (outward current) by 42.6 ± 3.5% (n = 8; P < 0.01; Fig. 4B). E<sub>2</sub> did not alter reversal potential (E<sub>K</sub>) for the GABA<sub>B</sub>-mediated effects.
We have previously shown that the EC50 for E2 in other ARC neurons is 7.5 nM, and the Ki for estrogen receptor antagonist ICI 182,780 is 0.3 nM (21). We opted to use a 100 nM concentration of E2 to promote more rapid pharmacokinetics in the slice (19, 27, 31). However, 10 nM E2 similarly attenuated the baclofen response by 52.0 ± 6.5% (n = 3).

We recently demonstrated that E2 may use a PI3K signaling pathway to attenuate the GABAB response in POMC neurons (26) and hypothesized that E2 may utilize the same pathway in NPY/AgRP neurons. Coperfusion of the general PI3K blockers wortmannin (100 nM; n = 5) or LY-294002 (10 μM; n = 6) with E2 attenuated the suppression of the response (Fig. 4G). Furthermore, on the basis of the recent work highlighting the specific roles of PI3K isoforms in energy balance (2), we found that TGX-221 (11 nM), a selective inhibitor of PI3K p110β, potently blocked the suppression of the response by E2 (n = 4; Fig. 4C). ERα has been shown to complex with PI3K in hypothalamic and cortical neurons (11, 43), so we decided to test specifically if the attenuation of the GABAB response could be mimicked by the ERα-selective agonist PPT (15). Indeed, PPT (100 nM) replicated the effects of E2 to attenuate the GABAB response in NPY/AgRP neurons by 46.7 ± 13.8% (n = 4, P < 0.001; Fig. 4D).

![Fig. 3. Most ARC NPY/AgRP neurons express GABAB-R1 and -R2, whereas only about one-third express PI3K p110β. A: gel representing mRNA expression of PI3K p110β, GABAB-R1, and GABAB-R2 in individual NPY/AgRP neurons from an ovariectomized female mouse. B: bar graph summarizing average transcript expression level across 4 ovariectomized females. Ten cells per animal were assayed for the GABAB receptors, while 12-29 (average of 19) cells per animal were examined for PI3K p110β.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00281.2013)

![Fig. 4. The estrogen receptor (ERα) ligand propyl pyrazole triol (PPT) and the Gq-coupled membrane estrogen receptor (Gq-mER) ligand STX differentially modulate the GABAB response in NPY/AgRP cells from intact male mice. A–F: representative traces of GABAB responses before and after application of E2, PPT, or STX, with or without pharmacological manipulations (see below). Experiments were conducted as shown in Fig. 1. Dotted line represents baseline current. Vhold = −50 mV. Vertical scale bars represent 20 pA; all horizontal ones represent 5 min. For illustrative purposes, most of the 15-min vehicle or treatment period between GABAB responses (R1 and R2) is removed. Other small breaks in the recording signify removal of slightly prolonged return to baseline current following baclofen application. G: bar graphs summarizing effects of E2, STX, or PPT (all 100 nM) on the GABAB response (baclofen, 10 μM) in NPY/AgRP neurons from intact males. Baclofen elicited 2 equal-amplitude responses during perfusion of vehicle (n = 7), but E2 suppressed the response (n = 8). Coperfusing general PI3K inhibitors [wortmannin (WRT), 100 nM, n = 5; LY-294002 (LY), 10 μM, n = 4] or the p110β inhibitor TGX-221 (TGX, 11 nM, n = 6) with E2 reversed this effect. PPT mimicked the effects of E2 (n = 4). STX augmented the response (n = 5) but was rendered ineffective by coperfusing an ER antagonist [ICI 182,780 (ICI), 1 μM, n = 4]. **P < 0.01, ***P < 0.001, vs. vehicle control group.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00281.2013)
In contrast to the ERα agonist PPT, the Gq-mER ligand STX (100 nM), which shows an ~1 million-fold reduced binding affinity for the nuclear ERs (α/β) (31, 32), enhanced the GABA\textsubscript{B} response by 66.7 ± 13.5\% (n = 5, P < 0.001; Fig. 4E). Coperfusion of the ER antagonist ICI 182,780 (1 μM) abrogated the STX-mediated augmentation of the GABA\textsubscript{B} response (n = 4; Fig. 4F). These data suggest that STX acts specifically on the membrane ER (Gq-mER) that we previously showed modulates POMC neuronal excitability and energy homeostasis (21, 31, 32).

E\textsubscript{2} and STX enhance GABA\textsubscript{B} signaling in NPY/AgRP cells from gonadectomized animals. When we utilized our protocol (Fig. 1) in ovariectomized females, 10 μM baclofen elicited a robust response, which was consistently replicated following washout with vehicle (n = 4; Fig. 5, A and G). Application of E\textsubscript{2} (100 nM) during the washout period, however, revealed three significantly different sets of responses compared with the control group. In 29\% (7 of 24) of the neurons, E\textsubscript{2} significantly enhanced the response by 40.4 ± 3.7\% (P < 0.05; Fig. 5B), whereas in 46\% (11 of 24) of the neurons, E\textsubscript{2} significantly enhanced the response by 53.5 ± 6.6\% (P < 0.01; Fig. 5D). In the remaining 25\% of the neurons (6 of 24), there was no net effect of E\textsubscript{2} on the GABA\textsubscript{B} response (Fig. 5C). We believe the apparent lack of an effect in some NPY/AgRP neurons represents an equal balance between the ERα-mediated attenuation and the Gq-mER-mediated enhancement of the GABA\textsubscript{B} response (see Discussion).

The different responses to E\textsubscript{2} (enhancement, attenuation, no effect) showed no apparent anatomic segregation (Fig. 6), but coperfusing the PI3K blocker wortmannin (100 nM) with E\textsubscript{2}, which blocked the attenuation of the baclofen response by E\textsubscript{2} in the male, isolated the enhancement of the GABA\textsubscript{B} response in female NPY/AgRP neurons (53.9 ± 18.7\% increase; n = 4, P < 0.05; Fig. 5E). In support of the role of PI3K in mediating the attenuation of the GABA\textsubscript{B}-GIRK channel coupling, single-cell RT-PCR analysis revealed that 33.3 ± 7.6\% of NPY/AgRP neurons (4 mice, 76 cells total) from ovariectomized females expressed PI3K p110β mRNA (Fig. 3, A and B).

STX (100 nM), which selectively targets the Gq-mER, strictly enhanced the GABA\textsubscript{B} response in ovariectomized females by 59.7 ± 22.0\% (n = 4, P < 0.05; Fig. 5F), similar to the males. A lower concentration of STX (10 nM) similarly enhanced the GABA\textsubscript{B} response (50.2 ± 4.7\%, n = 3).

Interestingly, NPY/AgRP neurons from orchidectomized males responded to E\textsubscript{2} in a similar manner to those from ovariectomized females (P = 0.76; Fisher exact probability

![Fig. 5](http://ajpendo.physiology.org/)
test): in 42% (3 of 7) of the neurons, E2 attenuated the GABA_B response by 35.5 ± 9.8%, whereas it enhanced the response by 84.0 ± 23.0% in 29% (2 of 7) of the neurons (data not shown). E2 showed no apparent effect in the remaining 29% (2 of 7) of cells. Coperfusing wortmannin with E2 isolated the enhancement of the baclofen response (49.7 ± 20.7% increase; n = 4, P < 0.05; Fig. 5G) in NPY/AgRP cells from orchidectomized males, similar to ovariectomized females.

**STX application in vivo reduces food consumption.** We have previously reported that STX reduces food consumption in female guinea pigs (35, 50). Here, we confirmed these findings and extended them to gonadectomized males. Daily subcutaneous injections of STX (at 0800) for 1 wk significantly reduced daily food consumption by greater than 20% in males and females (n = 5–7/group, P < 0.0001; F-value = 22.6; df = 1; Fig. 7). Although STX did not affect body weight over this short period of administration, our previous work showed that longer-term treatment significantly reduces post-ovariectomy weight gain in females (32, 37). These findings are consistent with the in vitro effects of STX on NPY/AgRP and POMC neurons (21, 31, 32).

**DISCUSSION**

We have shown for the first time that NPY/AgRP cells respond to the GABA_B agonist baclofen and that E2 modulates this robust inhibitory response. Low concentrations of E2 (10 nM) suppressed GABA_B signaling in all NPY/AgRP cells from intact male mice, whereas E2 suppressed the signaling in only about one-third of NPY/AgRP neurons from ovariectomized females and orchidectomized males. In contrast, the Gq-mER-selective ligand STX (10 nM) augmented GABA_B signaling in both sexes by greater than 50% and curbed food consumption in guinea pigs. Based on our current findings in NPY/AgRP neurons and previous work in POMC neurons (21, 31, 32, 50), the Gq-mER regulates NPY/AgRP and POMC neurons in a reciprocal manner by enhancing or attenuating GABA_B receptor coupling to GIRK channels, respectively.

GABAergic neurotransmission accounts for the vast majority of inhibitory synaptic activity in the hypothalamus (17, 46), and it is essential for some of the functions of NPY/AgRP neurons (45, 52). Deletion of GABA from NPY/AgRP neurons renders the mice lean and resistant to obesity (45), and chronic infusion of a GABA_A partial agonist into the parabrachial nucleus prevents starvation following NPY/AgRP cell ablation (52). Furthermore, important metabolic signals such as ghrelin and leptin require GABAergic transmission within the ARC for full efficacy (45, 48).

Although GABAergic synaptic activity and its role in the control of energy homeostasis have been extensively studied in POMC neurons (18, 21, 31, 32, 48), very little is known about this signaling in NPY/AgRP neurons. Here, we show for the first time that the GABA_B agonist baclofen robustly hyperpolarizes NPY/AgRP cells, which corroborates the finding that most of these neurons express GABA-R1 and -R2 (Fig. 2). The reversal potential near E_K, curvilinear I/V plot, and sensitivity to barium blockade suggest that the GABA_B receptor is coupled to GIRK channels, as we have previously demonstrated for POMC and other hypothalamic neurons (22, 23, 49, 57).

Besides its critical role in reproduction, it has long been recognized that E2 is an anorexigenic hormone, based on the findings that E2 replacement attenuates post-ovariectomy weight gain in rodents through decreasing food intake and increasing energy expenditure (4, 10, 12, 29, 35, 54). These actions of E2 are thought to be mediated in large part by the transcriptional activity of ERα, since a loss-of-function mutation in the receptor is associated with hyperinsulinemia and obesity in humans (42), and globally deleting it causes obesity in mice (14, 16). Furthermore, conditional deletion of ERα in POMC neurons of female mice results in hyperphagia and weight gain (54), which is not surprising given that E2 regulates the expression of a plethora of genes associated with the control of energy homeostasis (25, 37).
We have identified a novel Gq-mER signaling pathway that is also important for the anorexigenic actions of E2 on POMC neurons (21, 31, 32). We have designed a Gq-mER-selective ligand, STX, that potently increases the membrane excitability in POMC cells through attenuating GABA<sub>B</sub>-GIRK coupling via a PLC-PKC-PKA signaling pathway (31). In addition, STX attenuates the presynaptic, CB1 receptor-mediated decrease in glutamate release, further enhancing the excitability of these neurons (50). STX predictably mimics the anorexigenic effects of E2 to reduce food intake and weight gain following ovariectomy (32, 37).

In contrast to its excitatory actions in POMC neurons, the “nonselective” E2 both enhanced and attenuated the GABA<sub>B</sub> receptor-GIRK channel coupling in NPY/AgRP cells of gonadectomized mice, whereas the selective Gq-mER ligand STX always enhanced the coupling. Moreover, in intact males, E2 and the selective ER<sub>α</sub> agonist PPT attenuated GABA<sub>B</sub> receptor-GIRK channel coupling, whereas STX enhanced the coupling in an ICI 182,780-sensitive manner. These data collectively suggest that E2 suppresses or augments GABA<sub>B</sub>-mediated currents in these orexigenic neurons through binding ER<sub>α</sub> or a putative Gq-mER, respectively. We previously have shown that NPY/AgRP neurons express ER<sub>α</sub> transcript and protein (36), and the cloning of the Gq-mER is a work in progress. Since the NPY/AgRP neurons that differentially responded to E2 showed no physiological (i.e., resting membrane potential or input resistance) or anatomic (Fig. 6) differences, we believe the direction of the response depends on the expression level of ER<sub>α</sub> vs. Gq-mER. Indeed, the Gq-mER ligand STX always enhanced the response to baclofen in both males and females. The present in vitro findings support our previous in vivo findings that STX, but not E2, treatment significantly decreases NPY mRNA expression in the ARC of ovariectomized female guinea pigs (37). Overall, E2 is clearly anorexigenic in females because of its pronounced excitatory effects on POMC neurons (13, 31, 32, 50).

The pathway by which E2-ER<sub>α</sub> suppresses GABA<sub>B</sub> signaling in NPY/AgRP neurons appears to require PI3K, specifically the catalytic p110<sub>β</sub> subunit. We previously reported that E2 also suppresses GABA<sub>B</sub> signaling in POMC neurons, in part via a PI3K signaling pathway (26). Interestingly, deleting p110<sub>β</sub>, but not p110<sub>α</sub>, in POMC cells results in diet-induced obesity, whereas mice with the same deletion in NPY/AgRP neurons resist obesity (2). These findings would support the argument that the p110<sub>β</sub> subunit plays a vital role in regulating energy homeostasis. Single-cell PCR verified that NPY/AgRP neurons from ovariectomized females expressed p110<sub>β</sub> mRNA (Fig. 3), and blockade of PI3K or selective blockade of the p110<sub>β</sub> catalytic subunit abrogated the inhibitory effects of E2 on GABA<sub>B</sub> receptor-GIRK channel coupling. Since the selective ER<sub>α</sub> agonist PPT mimicked the inhibitory effects of E2 on the coupling, presumably increasing membrane excitability, the PI3K signaling pathway may underlie the stimulatory effects of NPY on GnRH and LH secretion in females (1, 5).

Indeed, NPY mRNA expression increases in the ARC at the time of the preovulatory LH surge in female rats (6). The Gq-mER signaling pathway in NPY/AgRP and POMC neurons may, therefore, be specific for the control of energy homeostasis, whereas the ER<sub>α</sub>-PI3K pathway in NPY/AgRP neurons may be exclusive for the reproductive pathway.

The anorexigenic actions of E2 are critical throughout the lifespan of women, who show increased risk for insulin resistance, central adiposity, and cardiovascular disease during menopause (8). Although E2 replacement (i.e., hormone replacement therapy, HRT) can help reverse these effects, HRT also increases the risk for cancer and stroke (38, 51). Selective activation of Gq-mER, on the other hand, elicits robust anorexigenic effects without the systemic risks associated with activating transcription factors ER<sub>α</sub> and ER<sub>β</sub> (35).

Here, we have shown that E2 via a putative Gq-mER rapidly enhances the coupling of GABA<sub>B</sub> receptors to GIRQ channels in NPY/AgRP neurons, thereby increasing the inhibitory tone in these orexigenic cells. Our previous work has shown that STX exerts the exact opposite effect on POMC neurons (31, 32), which serve an opposing role in controlling energy homeostasis. Thus, STX has the potential to significantly decrease the risk for insulin resistance and obesity in menopausal women without the peripheral side effects associated with traditional HRT.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

Rapid estrogen signaling in NPY/AGRP neurons


