

**Serotonin 5-HT_{2C} Receptor Signaling in Hypothalamic POMC Neurons:
Role in Energy Homeostasis in Females**

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Number of Text pages: 37

Number of figures and tables: 7 figures and 1 table

Number of references: 43

Number of words in Abstract: 251

Introduction: 496

Discussion: 1495

Abbreviations: POMC, proopiomelanocortin; GABA, γ -amino butyric acid; E₂, 17 β -estradiol; GABA_B, γ -amino butyric acid B receptor; GIRK, G protein-coupled inwardly rectifying potassium (K⁺); mER, membrane estrogen receptor; PLC, phospholipase C; PKC δ , protein kinase C delta; PKA, protein kinase A; 5-HT, 5-hydroxytryptamine, serotonin; scRT-PCR, single-cell reverse transcription polymerase chain reaction; qPCR, quantitative polymerase chain reaction; DOI, (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; RS102221 hydrochloride, 8-[5-(2,4-Dimethoxy-5-(4-trifluoromethylphenylsulphonamido)phenyl-5-oxopentyl)] 1,3,8-triazaspiro[4.5] decane-2,4-dione hydrochloride; ORG 3363, 1,2,3,4,10,14b-Hexahydro-2-methyldibenzo [c,f]pyrazino[1,2-a]azepine hydrochloride, R-enantiomer; PIP₂, phosphatidyl-inositol 4,5 bisphosphate; icv, intracerebroventricular; PVN, paraventricular nucleus; GDX, gonadectomized; TTX, tetrodotoxin; BIS, bisindolymaleimide I hydrochloride; DHT, dihydrotestosterone; U-73122, 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; U-73343, 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; wortmannin, (1S,6br,9aS,11R,11bR) 11-(Acetyloxy)-1,6b,7,8,9a,10,11,11b-octahydro-1-(methoxymethyl)-9a,11b-dimethyl-3H-furo [4,3,2-de] indeno[4,5,-h]-2-h]-2-benzopyran-3,6,9-trione; spiperone, 8-[4-(4-Fluorophenyl)-4-oxobutyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one hydrochloride; m-CPP, 1-(3-Chlorophenyl)piperazine hydrochloride; MK212, 6-Chloro-2-(1-piperazinyl) pyrazine hydrochloride; aCSF, artificial cerebral spinal fluid; DTT, dithiothreitol; DEPC, diethylpyrocarbonate; RT, murine leukemia virus reverse transcriptase; dNTPs, deoxynucleotide triphosphates; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; T, testosterone; IP₃, inositol 1,4,5 triphosphate; DAG, diacylglycerol; ACVII, adenylyl cyclase VII.

ABSTRACT

Hypothalamic POMC neurons play a critical role in the regulation of energy balance, and there is a convergence of critical synaptic input including GABA and serotonin on POMC neurons to regulate their output. Previously, we found that E_2 reduced the potency of the $GABA_B$ receptor agonist baclofen to activate GIRK channels in hypothalamic POMC neurons through a mER via a $G_{\alpha q}$ -PLC-PKC δ -PKA pathway. We hypothesized that the mER and neurotransmitter receptor signaling pathways converge to control energy homeostasis. Since $5-HT_{2C}$ receptors mediate many of the effects of serotonin in POMC neurons, we elucidated the common signaling pathways of E_2 and 5-HT in guinea pigs using single-cell RT-PCR, real time RT-PCR and whole-cell patch recording. Both $5-HT_{2C}$ and $5-HT_{2A}$ receptors were co-expressed in POMC neurons. The $5-HT_{2A/C}$ agonist DOI desensitized the $GABA_B$ response in a dose-dependent manner, which was antagonized by the selective $5-HT_{2C}$ receptor antagonists, RS 102221 and ORG 3363. The $5-HT_{2C}$ receptor was $G_{\alpha q}$ -coupled to PLC activation and hydrolysis of plasma membrane PIP_2 to directly inhibit GIRK channel activity. Co-application of the two agonists at their EC_{50} concentrations (DOI, 20 μ M and E_2 , 50 nM) produced additive effects. Although there was a significant sex difference in the effects of E_2 on baclofen responses, there was no sex difference in $5-HT_{2C}$ receptor-mediated effects. Finally, both DOI and estrogen (icv) inhibited feeding in ovariectomized female mice. Therefore, the $G_{\alpha q}$ signaling pathways of the mER and $5-HT_{2C}$ receptors may converge to enhance synaptic efficacy in brain circuits that are critical for maintaining homeostatic functions.

Introduction

The hypothalamus is a key CNS center for controlling many homeostatic processes, and hypothalamic POMC neurons are critical neurons in these hypothalamic circuits (Elmqvist et al., 1999; Cone, 2005; Gropp et al., 2005; Luquet et al., 2005). POMC neurons modulate the excitability of hypothalamic neurons that control reproduction, stress responses, fluid balance, temperature and appetite through direct synaptic contacts. In addition, POMC neurons project to other brain areas (e.g., midbrain) to control motivated behaviors such as sexual and maternal behavior. There is a heavy projection of serotonin fibers from the dorsal and median raphe nuclei to the arcuate and PVN, and there is a dense expression of 5-HT_{2A} receptors in the PVN and 5-HT_{2C} receptors in the arcuate (Gundlah et al., 1999). Serotonin analogs and drugs that increase the activity of central serotonergic pathways have been developed and widely used as appetite suppressants (Breisch et al., 1976; Bickerdike, 2003). Indeed, the 5-HT reuptake inhibitor/5-HT releaser d-fenfluramine and selective 5-HT_{2C} receptor agonists activate α -MSH (POMC)-containing neurons (Heisler et al., 2002; Heisler et al., 2006), but the cellular mechanism (s) of the 5-HT_{2C} receptor agonist action have not been elucidated.

Interestingly, serotonin neurons are targets of ovarian and testicular steroids (Bethea, 1993). Traditionally these steroid effects are attributed to activation of the nuclear receptors (McEwen, 2001). In non-human primates, estrogens up-regulate tryptophan hydroxylase in midbrain 5-HT neurons (Bethea et al., 1998), decrease serotonin transporter mRNA expression in midbrain raphe (Bethea et al., 1998) and decrease expression of the 5-HT_{2C} receptor in a number of hypothalamic nuclei (Gundlah et al., 1999). Specifically, E₂ modulates many of the homeostatic functions through the

transcription factors ER α and ER β (Couse and Korach, 1999). However in contrast to the relatively slow genomic effects of E₂, we have identified a putative mER that is G α_q -coupled to a PLC-PKC-PKA pathway (Qiu et al., 2003). E₂ reduces the potency of the GABA_B receptor agonist baclofen to activate G-protein-coupled, inwardly rectifying K⁺ channels in hypothalamic neurons, and this membrane-delimited signaling pathway also plays a critical role in the control of energy homeostasis (Qiu et al., 2006).

It is noteworthy that serotonergic drugs (i.e., selective serotonin re-uptake inhibitors) and E₂ are effective in alleviating postmenopausal symptoms in women (Stearns et al., 2002). It maybe that E₂ and serotonin, via mER and 5-HT_{2C} receptors, respectively, synergize to regulate energy metabolism in postpubertal females. Therefore, understanding the actions of E₂ and serotonin on POMC neurons may provide insight into fundamental differences between females and males in the hypothalamic control of feeding and energy homeostasis. In the present study, we sought to elucidate the cellular cascades activated by 5-HT in comparison to E₂ in hypothalamic POMC neurons using whole-cell recording, scRT-PCR and real time RT-PCR techniques and their functional consequences at the whole animal level. Our findings delineate the 5-HT_{2C} signaling pathway in males and females and determine its convergence with the mER signaling pathway in arcuate POMC neurons to control energy homeostasis.

Materials and Methods

Animals and Treatments

All animal procedures described in this study are in accordance with institutional guidelines based on National Institutes of Health standards. Male and female Topeka

guinea pigs (400-600 gm), bred in our institutional breeding facility, and female multicolor guinea pigs (400-500 gm; Elm Hill Breeding Labs, Chelmsford, MA) were used in these experiments. The guinea pigs were maintained under constant temperature (26°C) and light (on between 6:30 A.M. and 8:30 P.M.). Animals were housed individually, with food and water provided *ad libitum*. They were gonadectomized under ketamine-xylazine anesthesia (33 and 6 mg/kg, respectively, s.c.) 5-7 d before experimentation and were given sesame oil vehicle (0.1 ml, s.c.) 24 h before experimentation. Serum estrogen concentrations were measured in the GDX females by radioimmunoassay (ONPRC Radioimmunoassay Core) from trunk blood collected on the day of experimentation and were <10 pg/ml. An additional group of female 11-12-week-old C57Bl/6J mice (Jackson Labs) used for feeding study were housed upon arrival on a 12-h light/12-h dark cycle (lights off at 18:00 h) with free access to standard pellet diet and water.

Drugs

All drugs were purchased from Calbiochem (La Jolla, CA) unless otherwise specified. TTX (Alomone Labs, Jerusalem, Israel) was dissolved in Milli-Q H₂O and further diluted with 0.1% acetic acid (final concentration, 1 mM), pH 4-5. E₂ was purchased from Steraloids (Wilton, NH), recrystallized to ensure purity, and dissolved in 100% ethanol to a stock concentration of 1 mM. T and DHT (Steraloids) was also dissolved in 100% ethanol. The PKC inhibitors BIS (100 μM), and rottlerin (10 mM), the PLC inhibitor, U73122 (20 mM), the less active analog U73343 (20 mM), the PI-4-kinase inhibitor wortmannin (10 mM), RS 102221 hydrochloride (10 mM) and Spiperone hydrochloride (20 mM, Tocris, Ellisville, Missouri), were dissolved in DMSO. PIP₂ was dissolved in the pipette solution at

concentration of 5 μ M. The solution was sonicated intermittently on ice for 30 min. Sonification was repeated each time before filling a new pipette. DOI (20 mM), m-CPP (10 mM), MK212 (10 mM) and ORG 3363 (from NV Organon, 20 mM) were dissolved in H₂O. The G_{αq}-binding protein designed to mimic the C terminus of the G_{αq}-subunit and the G_{αs}-binding protein designed to mimic the C terminus of the G_{αs}-subunit were synthesized by PeptidoGenic Research (Livermore, CA). The peptide sequence for G_{αq} peptide was Ac-LGLNLKEYNLV-OH, and the peptide sequence for G_{αs} peptide was CRMHLRQYELL. The peptides were also dissolved in H₂O. Aliquots of the stock solutions were stored at -20 °C until needed.

Electrophysiology

Adult Topeka guinea pigs were gonadectomized 6-10 days before each experiment. Each animal was quickly killed by decapitation, the brain rapidly removed from the skull and a block containing the hypothalamus immediately dissected. The hypothalamic block was submerged in cold (4 °C) oxygenated (95% O₂, 5% CO₂) aCSF containing the following constituents, in mM: NaCl, 124; KCl, 5; NaHCO₃, 26; NaH₂PO₄, 2.6; dextrose, 10; HEPES, 10; MgSO₄, 2; CaCl₂, 2 at 4 °C. Coronal slices (300-350 μ m) through the caudal-rostral extent of the arcuate nucleus were cut with the aid of a vibratome. The slices were transferred to a multiwell auxiliary chamber containing oxygenated aCSF and kept there until electrophysiological recording after ~2 h. During recording, slices were maintained in a chamber perfused via a peristaltic pump with warmed (35 °C) oxygenated aCSF at a rate of 1.5 ml/min. Microelectrodes (resistances 3-6 M Ω) were fabricated from borosilicate glass pipettes (1.5 mm O.D) and filled with an internal solution (pH 7.30)

containing the following constituents, in mM: K-Gluconate, 128; NaCl, 10; MgCl₂, 2; EGTA, 11; HEPES, 10; ATP, 1; GTP, 0.25, and 0.25% biocytin. Standard whole-cell voltage clamp procedures were followed using an Axopatch 200A amplifier (Axon Instruments). Signals were digitized with a Digidata 1200 and analyzed using pClamp 7.0 software (Axon Instruments). The liquid junctional potential of -10 mV was corrected in the data analysis. Current and voltage traces were also recorded on an analog chart recorder (Gould Instruments). Following the formation of a >1GΩ seal, intracellular access was achieved by suction, and only those cells that showed less than 10% change in access resistance throughout the recording were included in this study. All the responses to baclofen were measured in voltage clamp as outward currents ($V_{\text{hold}} = -60$ mV). For the electrophysiology analysis, only cells with gigaohm or better seals were included in this study.

The protocol for drug administration in the whole-cell patch voltage-clamp experiments (V_{hold} , -60 mV) was followed as described in a previous publication (Qiu et al., 2003). After seals were formed and the whole-cell configuration was obtained, slices were perfused with TTX (1 μM) for 5 min. The first GABA_B receptor-mediated response was generated by perfusing baclofen (at EC₅₀ concentration of 5 μM) until a steady-state outward current was obtained (R_1). After drug washout, the current returned to its predrug resting level. The cells were then treated with serotonin receptor agonist drugs DOI and/or other drugs for 15 min, baclofen (5 μM) was perfused again, and R_2 was measured. The effects of serotonin receptor agonist drugs or other drugs on the baclofen response are expressed as a percentage of R_2 over R_1 .

Composite dose–response curves were generated from the following logistic equation fitted by computer (SigmaPlot 8.0, San Jose, CA) to the data: $\Delta I_{\max} = 100 \times ([\text{agonist}]^n / ([\text{agonist}]^n + EC_{50}^n))$, where ΔI_{\max} is the maximum outward current for a given agonist, EC_{50} represents the agonist potency, and n is the Hill slope.

Immunocytochemistry

Following electrical recording, the slices were prepared for fluorescence immunocytochemistry as described previously (Qiu et al., 2003). Briefly, the slices were fixed with 4% paraformaldehyde in Sorensen's phosphate buffer, pH 7.4, for 120 min, immersed overnight in 20% sucrose dissolved in Sorensen's buffer, and frozen in OCT embedding medium and prepared for immunocytochemistry as described previously (Kelly and Rønnekleiv, 1994). Briefly, coronal sections (20 μm) were cut on a cryostat (model 1720 Digital Cryostat; Leitz, Wetzlar, Germany) and mounted on Fisher SuperFrost Plus slides. Sections were washed for 5 min with 0.1 M sodium phosphate buffer, pH 7.4, and then streptavidin-Cy2 (1:7,500-1:10,000; Jackson ImmunoResearch, West Grove, PA) was applied for 2 h. The reaction was terminated by washing with buffer. The slices were scanned for the injected neuron with a Nikon (Melville, NY) Eclipse 800 fluorescence microscope. After localization of the biocytin-filled neurons, the slides containing the appropriate sections were processed for the presence of β -endorphin using fluorescence immunohistochemistry as described previously (Kelly and Rønnekleiv, 1994). Briefly, the sections with the biocytin-identified neurons were incubated overnight with a polyclonal β -endorphin antibody (kindly provided by Dr. Robert Eskay, NIH) at 1:5000 and washed in 0.1 M phosphate buffer, followed by incubation with a donkey anti-rabbit IgG-Cy3 at 1:500

(Jackson ImmunoResearch). The sections were washed with sodium phosphate buffer, and coverslips were applied using a glycerolglycine buffer (2:1; pH 8.6) containing 5% N-propylgallate (Sigma-Aldrich) to reduce photobleaching. Immunostained cells were analyzed and photographed using a Nikon E800 microscope.

Dispersed Single-cell RT-PCR

Arcuate single cell harvest from guinea pig hypothalamic slices was performed as described (Qiu et al., 2003). Briefly, coronal hypothalamic slices (350 μ m) were cut on a vibratome and placed in an auxiliary chamber containing oxygenated aCSF. The slices were allowed to recover for 1-2 h in the chamber before dispersion. The arcuate nucleus of the hypothalamus was microdissected and incubated in 2-3 ml of aCSF containing 1 mg/ml protease XIV (Sigma-Aldrich) for ~15 min at 37 °C. The tissue was then washed three times in 1 volume of low-calcium aCSF and two times in normal aCSF. The cells were isolated by trituration with flame-polished Pasteur pipettes, dispersed on a dish, and perfused continuously with aCSF at a rate of 1.5 ml/min. Cells were visualized using a Nikon inverted microscope, and individual neurons were patched and harvested into the patch pipette by applying negative pressure. The content of the pipette was expelled into a siliconized microcentrifuge tube containing 5 μ l of the following solution: 0.5 μ l of 10X buffer (100 mM Tris-HCl, 500 mM KCl, 1% Triton X-100; Promega, Madison, WI), 15 U of RNasin (Promega), 0.5 μ l of 100 mM DTT, and DEPC-treated water (Ambion, Austin, TX). In addition, hypothalamic tissue was homogenized, and total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) according to the protocol of the manufacturer. The harvested cell solution and 25 ng of hypothalamic total RNA in 1 μ l were denatured for 5

min at 65 °C and cooled on ice for 5 min, and then single-stranded cDNA was synthesized from cellular RNA by adding 50 U of RT (Applied Biosystems, Foster City, CA), 1.5 µl of 10X buffer, 2 mM MgCl₂, 0.2 µl of dNTPs, 15 U of RNasin, 10 mM DTT, 100 ng of random hexamers (Promega), and DEPC-treated water to a final volume of 20 µl. Cells and tissue RNA used as negative controls were processed as described above but without RT. The reaction mixtures were incubated at 42 °C for 60 min, denatured at 99 °C for 5 min, and cooled on ice for 5 min.

Primers listed in Table 1 were designed using the Clone Manager Software (Scientific and Educational Software, Cary, NC) and synthesized by Invitrogen (Carlsbad, CA). PCR was performed using 3 µl of cDNA template (2 µl for GAPDH) from each RT reaction in a 30 µl of PCR reaction volume containing the following: 3 µl of 10X buffer, 2.4 µl of 25 mM MgCl₂ (2 mM final concentration), 0.2 mM dNTPs, 0.2 µM forward and reverse primers, 2 U of Taq DNA polymerase (Promega), and 0.22 µg of TaqStart antibody (Clontech, Palo Alto, CA). Taq DNA polymerase and TaqStart antibody were combined and incubated at room temperature for 5 min, and the remainder of the reaction contents were added to the tube and incubated at 94 °C for 2 min. PCR reactions for 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} went through 42-47 cycles of amplification according to the following protocols: 20 sec denaturation (94 °C), 30 sec annealing (59-62 °C), 30 sec elongation (72 °C), with a final 72 °C extension for 5 min. POMC and GAPDH PCR went through 45 and 37 cycles of amplification, respectively, in two steps: 30 sec denaturation (94 °C), 45 sec annealing (65-67 °C) with a final 72 °C extension for 5 min. Ten microliters of the PCR products were visualized with ethidium bromide on a 1.5% agarose gel.

Real- Time RT-PCR

Total RNA was extracted from the microdissected arcuate nucleus of GDx female guinea pigs (n=6) using the RNAqueous-Micro kit (Ambion, Austin TX). The RNA was treated with DNase I using the DNA-free kit (Ambion) according to manufacturer's instructions and quantified using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Reverse transcription was carried out with 200 ng total RNA using 50 U RT (Applied Biosystems, Foster City, CA), 1.5 μ l 10X buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 15 U RNasin, 10 mM DTT, 100 ng random hexamers and DEPC-treated water to a final volume of 20 μ l. As a negative control, RNA was processed as described above but without RT. The reaction mixtures were incubated at 42 °C for 60 min, denatured at 99 °C for 5 min and cooled on ice for 5 min.

qPCR was performed using an equivalent of 1.5 ng total RNA (3 μ l of a 1:20 dilution of cDNA template), 0.5 μ M forward and reverse primers and the Power SybrGreen PCR mastermix (Applied Biosystems) in a 20 μ l reaction volume. The qPCR reaction for 5-HT_{2C}, 5-HT_{2A}, 5-HT_{1A}, and GAPDH contained 10 μ l 2X mastermix, 0.5 μ M forward and reverse primers, 3 μ l cDNA and nuclease-free water to a 20 μ l final volume. qPCR was performed on samples in the ABI Prism 7500 Fast machine in triplicate under the following conditions: 95 °C, 10 min; 40 cycles of amplification at 95 °C, 15 sec and 60 °C, 1 min followed by a dissociation step for melting point analysis with 35 cycles of 95 °C for 15 sec, 60 °C to 95 °C in increments of 1 °C for 1 min and 95 °C for 15 sec. Standard curves using diluted cDNA from guinea pig hypothalamus (1:5, 1:10, 1:50, 1:100, 1:500) (Fig. 6e) were prepared to determine the efficiency of the primers. The slopes of the standard

curves for 5-HT_{2C}, 5-HT_{2A}, 5-HT_{1A}, and GAPDH were -3.1, -3.2, -3.2, and -3.3, respectively (Fig. 6e). The efficiency was calculated for each primer pair using the following formula: $E=10^{(-1/m)}-1$, m =slope (Pfaffl, 2001; Livak and Schmittgen, 2001). The efficiencies were 100% for all transcripts. The similar efficiencies between the primer pairs allowed us to make quantitative estimates between 5-HT_{2C}, 5-HT_{2A}, and 5-HT_{1A} mRNA expression. The amplification data were analyzed by the ABI 7500 System v 1.3.0 software and calculated using the $\Delta\Delta$ CT method (Livak and Schmittgen, 2001).

For quantification of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor mRNA, expression differences were analyzed using qPCR. The $\Delta\Delta$ CT method was used to calculate relative mRNA expression (Livak and Schmittgen, 2001) (Fig. 6f). 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor mRNA values were normalized to the endogenous control gene GAPDH. 5-HT_{2C} was used as the calibrator and as a reference to which 5-HT_{2A}, 5-HT_{1A} were compared. The relative target gene expression was calculated using $2^{(-\Delta\Delta CT)}$ where ΔCT = target CT – control CT, $\Delta\Delta CT$ = ΔCT target - ΔCT calibrator. Mean and S.E.M. were calculated using Prism 4 software (Graphpad Software Inc., San Diego, CA) (Fig. 6f).

Feeding Study

Eleven-to-twelve week-old female mice anesthetized by intraperitoneal injection of 0.15 ml mouse cocktail (ketamine-xylazine-saline, 1:1:8) were overiectomized and then kept anesthetized with isoflurane during the icv cannulation procedure. The mice were placed into a stereotaxic instrument (Cartesian Instruments, Bend OR), the cranial surface cleaned and a cannula placed into the third ventricle as described previously (Cepoi et al., 2004). Briefly, a small hole was drilled and a sterile stainless steel guide cannula (25

gauge, 1.1 cm long, with an obturator stylet placed within) was implanted at midline, 0.825 mm posterior to bregma and 4.8 mm below bregma based on *the Atlas of the Mouse Brain* (Franklin and Paxinos, 1997). Mice were housed individually and allowed to recover for 10 days. Thereafter, the animals were adapted repeatedly for at least 3 weeks to the experimental procedure which included a brief restraint in a procedure bag during which time the icv injection was performed. To test the effects of the compounds on feeding following an overnight fast, mice were placed in clean cages with bedding material and free access to water, but without food for 16 hours (17:00 h-09:00h). At the end of the fast, each mouse was lightly restrained, the obturator stylet removed from the guide cannulae and saline (0.9% NaCl), E₂ (0.012 nmol) or DOI (110 nmol) in 2 µl total volume was infused over a 1 min period. Another 1 min period was allowed for diffusion of the drugs before removing the injection needle. The mice were put back into their cages with a pre-weighed food pellet (Purina Mouse Chow, 5144). Body weight and pellet weights were determined at 1, 2, 6 and 24 hours post injection. The correct cannulae placement was confirmed by injecting methylene blue dye at the end of the study and visualizing the location of the dye in brain slices.

Statistical Analysis

Comparisons between groups were performed using a one-way or two-way ANOVA and Bonferroni post-test for the tissue analysis and whole-animal experiments and a one-way ANOVA for the electrophysiological experiments with post hoc Newman-Keuls paired analysis. Differences were considered statistically significant if the probability of error was <5%.

Results

Sex differences in the estrogen-mediated desensitization of the GABA_B response in hypothalamic neurons. Whole-cell recordings were made in arcuate neurons (n = 153) from GDX male and female guinea pigs. A subgroup of these neurons (n = 56) was identified using dual-labeling immunocytochemistry (Fig. 1), and 40% of the neurons were β -endorphin positive (i.e., POMC neurons). For the electrophysiology analysis, only cells with gigaohm or better seals were included in this study. There was no difference in the mean resting membrane potential (males: -55.5 ± 1.3 mV (n=21) versus females: -55.0 ± 0.5 mV (n=102), $I_{\text{hold}} = 0$ pA) or the mean input resistance (males: 0.96 ± 0.3 G Ω versus females: 0.92 ± 0.1 G Ω) between the two groups. Similar to our previous findings in mice (Qiu et al., 2006), there were no sex difference in the mean outward current induced by 5 μ M baclofen (males: 37.7 ± 3.8 pA, n=43; females: 34.7 ± 2.4 pA, n=88). However, there was a significant sex difference in the effects of E₂ on the baclofen response in GDX guinea pigs. POMC neurons from GDX male guinea pigs were less sensitive to the effects of estrogen by about 15% (Fig. 1). T at a higher concentration (1 μ M) could attenuate the baclofen response (Fig.1a,b) in GDX male guinea pigs but still was not as efficacious as E₂. However, DHT (1 μ M), which cannot be aromatized to E₂, had no effect on the baclofen response (Fig. 1a,b), suggesting an estrogenic response.

Activation of the 5-HT_{2C} receptor desensitizes the GABA_B response in male and female POMC neurons. The anorectic effects of serotonergic drugs are thought to act

via increasing the activity of POMC cells. 5-HT_{2C} agonists (m-CPP and MK212) increase the firing frequency of male POMC neurons (Heisler et al., 2002), but the cellular signaling pathways mediating serotonin's effects are not known. However, the attenuation of the prominent GABA and opioid inhibitory input through the uncoupling of G_{i/o} receptors from GIRK channels might account for the increase in POMC neuronal firing following exposure to serotonin specific 5-HT_{2A/C} agonists (Heisler et al., 2002). In this experiment, we used the whole-cell recording method to measure the effects of DOI on the activation of the GIRK conductance by the GABA_B receptor agonist baclofen. For assessing DOI modulation of the GABA_B response, we used an EC₅₀ concentration (5 μM) of baclofen and the protocol described previously (Qiu et al., 2003). A robust outward current was measured in response to baclofen that subsided after washout of the GABA_B agonist (Fig. 2a). The application of baclofen 20 min later elicited the same robust response, suggesting that desensitization and rundown were not occurring in response to successive applications of 5 μM baclofen. However, if DOI (20 μM) was applied during the interim period (i.e., after the washout of the first application of baclofen), there was a significant (p<0.005) decrease of 30% in the response to a second application of baclofen (Fig. 2a). DOI alone had no effect on the holding potential, and current-voltage relationships generated before and during the application of DOI (20 μM) showed that this drug did not change the reversal potential for the baclofen-mediated response: control E_{baclofen}, -92.7 ± 2.3 mV, n=11; versus after DOI, E_{baclofen}, -95.1 ± 3.7 mV, n=11. In addition, both 5-HT_{2C} receptor agonist MK212 and 5-HT_{2C/1B}-receptor agonist m-CPP also could significantly inhibit the baclofen response and even gave a more robust inhibition (40%) of the baclofen response. Furthermore, the effects of DOI were blocked by the 5-HT_{2C} receptor

antagonists ORG 3363 (10 μ M) or RS 102221 (20 μ M), but not the 5-HT_{2A} receptor antagonist spiperone (20 μ M) when co-perfused with DOI (Fig. 2a,b). Treatment with ORG 3363, RS 102221 or spiperone alone had no effect on the baclofen response (data not shown). In contrast to the sex differences in the E₂ desensitization of the GABA_B response, there was no difference between males and females in the DOI-mediated effects (71.5 ± 2.3 , R₂/R₁, n=13 for females versus 78.7 ± 1.7 , R₂/R₁, n=4 for males). Therefore, we characterized the signaling pathway of the 5-HT_{2C} receptor in female arcuate (POMC) neurons.

5-HT_{2C} receptor mediated attenuation of the GABA_B response depends on activation of G_{αq}. Early studies showed that 5-HT₂ receptor subtypes couple to G_{αq} protein and activate PLC (de Chaffoy de Courcelles et al., 1985; Conn et al., 1986). Therefore, we next examined the involvement of specific signaling proteins in the DOI-mediated modulation. Since 5-HT_{2A/C} receptors are G_{αq} protein coupled and PKC is the downstream pathway, we first examined whether or not activation of PKC is critical for DOI modulation of the GABA_B response using a PKC inhibitor. If activation of the PKC pathway is involved, then the effect of DOI on GABA_B responses should be blocked by inhibiting PKC. To test this, we applied a PKC inhibitor BIS, which is a selective inhibitor of PKC that does not distinguish between the conventional, novel, and atypical isoforms of PKC, and a selective PKC δ inhibitor rottlerin. We have established that BIS (100 nM) and rottlerin (5 μ M) blocked the inhibition of baclofen responses by E₂ (Qiu J et al., 2003). But as shown in Figure 3, after ~15 min of dialysis with BIS (100 nM), the DOI-induced reduction of the GABA_B response was not attenuated. Furthermore, both BIS (100 nM) and rottlerin (5

μM) did not block the inhibition of baclofen response by m-CPP, a selective 5-HT_{2C} agonist ($p > 0.05$, m-CPP, 71.17 ± 2.24 , $n=3$ versus m-CPP + BIS, 73.16 ± 3.06 , $n=7$; m-CPP versus m-CPP + rottlerin 68.92 ± 4.01 , $n=3$). These results would indicate that the suppression of the GABA_B response by DOI requires PLC activation but not the activation of PKC. To examine whether the DOI-mediated inhibition of the GABA_B response depended on the activation of G_{αq}, arcuate neurons were dialyzed with a peptide (11 amino acids) that mimics the C-terminal binding site of G_{αq}. A similar strategy has been used by Carr and coworkers (Carr et al., 2002) to abrogate 5-HT₂ receptor signaling in prefrontal cortical neurons. Indeed in cells dialyzed with this peptide (200 μM), the DOI-mediated reduction of the GABA_B response was blocked significantly (Fig. 3a,b) compared with cells dialyzed with a control peptide (11 amino acids) that mimics the C-terminal domain of G_{αs} (Fig. 3a,b).

Desensitization of GABA_B response is dependent on 5-HT_{2C} activation of PLC and hydrolysis of plasma membrane PIP₂. In light of the above results for a primary role for G_{αq} in DOI-mediated inhibition, we tested whether the activation of PLC might also play a role. Since the specific PKC inhibitor BIS did not block the effects of DOI, this indicated that the PLC, which is downstream from the activation of G_{αq} protein and a well known G_{αq} effector, may be responsible for the DOI effect. Therefore, we focused on pathways downstream of PLC to further elucidate the DOI-mediated signaling pathway. To determine whether the activation of PLC is required for the DOI-induced inhibition of the GABA_B response, neurons were treated with the broad-spectrum PLC inhibitor U73122 (10 μM), which was perfused in the extracellular bathing media. Under these conditions

there was no difference between the first baclofen response and second one in the presence of U73122 (U73122 treated versus control group, 95.57 ± 2.44 , $n=3$; 96.48 ± 5.9 , $n=5$), but the DOI-mediated reduction of GABA_B response was blocked (Fig. 3c,d), whereas the less active PLC inhibitor U73343 at the same concentration had no effect (Fig. 3c,d). Furthermore, whole-cell dialysis with PIP₂ (5 μ M) also attenuated the DOI-mediated inhibition, and there were no significant differences between the first baclofen responses with and without PIP₂ dialysis. Moreover, the addition of PI-4-kinase inhibitor wortmannin at 10 μ M potentiated the inhibition of DOI on the baclofen response (Fig. 3d). Therefore, the 5-HT_{2C} receptor signals through PLC and PIP₂, which is different from the mER signaling pathway (Qiu et.al., 2003). Since DOI and E₂ act through divergent G_{oq} signaling pathways, we investigated the convergence of the 5-HT_{2A/C} receptor agonist DOI and E₂ on the GABA_B response. Concentration–response curves (Fig. 4b) showed that DOI rapidly attenuated the GABA_B response in a concentration-dependent manner with 50% inhibition at 16.5 μ M for DOI. Therefore based on the previous published EC₅₀ (46.0 nM) for the E₂- mediated desensitization of the GABA_B response (Qiu et al., 2006), we co-applied both agonists at their EC₅₀ concentrations and found that the effects were additive (Fig.4a,c).

5-HT_{2A/C} receptor agonist DOI and estrogen reduce food intake and body weight in GDX female mice. As proof of principle that the mER and serotonin 5-HT_{2C} signaling pathways are physiologically important, we investigated the acute effect of DOI and E₂ in attenuating the weight gain in the female (Fig. 5). Female mice were ovariectomized, allowed to recover for 10 days and, thereafter, were adapted repeatedly to the icv injection

procedure as described in the Methods. Changes in food intake and body weight gain were measured after overnight food-deprivation. Based on a dose response to DOI (10, 20, 110 nM), no effect was observed with 10 nM. Following 20 nM DOI, inhibition of food intake ($p < 0.05$) but not body weight gain was observed at 6 h (data not shown). Following 110 nM DOI, food intake as well as body weight gain was strongly inhibited starting already at 1 h post DOI injection as compared with saline, vehicle controls (Fig. 5 a,b; $p < 0.05-0.005$). The greatest effects on body weight and food intake after DOI treatment were observed at 6 h. At that time point, similar to DOI, E_2 albeit at a significantly lower dose, attenuated the weight gain and food intake in GDX female mice (Fig.5 c,d).

Expression of 5-HT₂ receptor mRNA transcripts in arcuate (POMC) neurons from GDX guinea pigs. Based on our electrophysiology results, we examined which serotonin receptors are expressed in arcuate (POMC) neurons in the guinea pig. Previous findings have identified 5-HT_{2C} receptors in male mouse POMC neurons (Heisler et al., 2002). Using scRT-PCR, we measured 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor transcripts in 54 male and 57 female arcuate neurons including POMC neurons from 4 animals each (Fig.6a). Overall, the distribution of these receptors in arcuate neurons was similar in males ($69 \pm 13\%$, $47 \pm 6\%$, $51 \pm 8\%$; $n=4$) and females ($81 \pm 12\%$, $62 \pm 8\%$, $37 \pm 6\%$; $n=4$) for 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptors, respectively. Based on the frequency distribution of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor mRNAs specifically in POMC positive neurons in GDX females ($n=38$ cells), 84% of these neurons expressed 5-HT_{2C} receptor mRNA, 76% expressed 5-HT_{2A} receptor mRNA and 42% expressed 5-HT_{1A} receptor mRNA (Fig. 6b). The degree of co-expression of these receptors in POMC neurons was

also determined as illustrated in figure 6b, and found to be 71% for 5-HT_{2A} and 5-HT_{2C} and 26% for all three receptors (Fig. 6b). Using RT-PCR and real time PCR to quantify the level of expression of serotonin receptor mRNAs in the microdissected arcuate nucleus, we found that 5-HT_{2A} receptors were most highly expressed followed by 5-HT_{2C} receptors (Fig 6 c-f; $p < 0.05$). The expression of 5-HT_{1A} was significantly lower than that of 5-HT_{2A} and 5-HT_{2C} receptor transcripts ($p < 0.001$; Fig. 6c-f). Therefore, the scRT-PCR and qPCR data support the electrophysiological data that 5-HT_{2C} and 5-HT_{2A} receptors are highly expressed in the arcuate and co-expressed in the majority of POMC neurons.

Discussion

In the present study we have characterized the signaling pathway of the serotonin 5-HT_{2C} receptor in hypothalamic (POMC) arcuate neurons. Activation of this G_{αq}-coupled receptor desensitized the GABA_B response in neurons. Acute activation of the mER with E₂ also desensitized the GABA_B receptor response, but unlike the mER signaling pathway, the 5-HT_{2C} receptor pathway led to direct hydrolysis of PIP₂ to affect GIRK channel function. Although there was a clear sex difference in the efficacy of E₂ to activate mER, there was no difference in the 5-HT_{2C} mediated response between males and females. Finally, E₂ and the 5-HT_{2C} agonist DOI, albeit at a higher dose, were effective in reducing food intake and body weight gain in fasted, GDX females, which highlights the physiological importance of the mER and 5-HT_{2C} signaling pathways in mediating POMC neuronal excitability.

Serotonin affects arcuate (POMC) neuronal activity through multiple receptor-mediated mechanisms. Both 5-HT_{2A} and 5-HT_{2C} receptors are localized to the medial basal hypothalamus, and activation of these G_{αq}-coupled receptors excites these neurons (Heisler et al., 2002; Wright et al., 1995). In particular POMC neurons are excited in response to d-fenfluramine, 5-HT, or 5-HT_{2C} receptor agonists like m-CPP and MK 212 (Heisler et al., 2002). However, the underlying mechanism (s) for the excitation of hypothalamic neurons has not been elucidated. Our data show that the G_{αq}/PLC –coupled pathway of 5-HT_{2A/C} receptors causes an inhibition of the baclofen response, and provide the first evidence that depletion of PIP₂, rather than the phosphorylation of the channel, is a key step in this pathway based on the following: First, PKC inhibitors did not block the inhibition of the baclofen response by the 5-HT_{2A/C} receptor agonist DOI or 5-HT_{2C} receptor agonist m-CPP. Second, the DOI-mediated reduction of the GABA_B response was significantly attenuated by the PLC inhibitor U73122 compared with cells perfused with the less active inhibitor U73343. Third, whole-cell dialysis with PIP₂ attenuated the DOI inhibition of the GABA_B response, and the addition of wortmannin greatly potentiated the inhibition of DOI on baclofen responses. Lastly, intracellular dialysis with a peptide fragment of G_{αq} abrogated the 5-HT_{2C} receptor interaction with G-protein, which indicates that 5-HT_{2A/C} receptors are specifically coupled to G_{αq} protein. Thus, we conclude that 5-HT_{2A/C} receptor mediated inhibition of GIRK channels involves PLC activation by G_α subunits of the G_{αq} family and that the receptor-mediated hydrolysis of plasma membrane PIP₂ is the critical mediator. It is worth noting that although both 5-HT_{2A} and 5-HT_{2C} receptors are G_{αq}-coupled, the inhibition of the baclofen response in POMC neurons is mainly through the 5-HT_{2C} receptor since the 5-HT_{2C} selective agonists m-CPP and

MK212 attenuated baclofen responses in POMC neurons. Moreover, the selective 5-HT_{2C} antagonists Org 3363 and RS 102221, but not the 5-HT_{2A} antagonist spiperone, potently blocked the actions of DOI in guinea pig arcuate (POMC) neurons indicating that the inhibition of the GABA_B response is through the 5-HT_{2C} receptor. This is compatible with previous findings that the 5-HT_{2C} receptor agonists show greater efficacy for activating the PLC pathway, whereas 5-HT_{2A} receptor agonists have relatively greater efficacy for activating the phospholipase A₂ pathway (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003). The PLC hydrolysis of PIP₂ and inhibition of GIRK channels is not unique to 5-HT_{2C} receptors since other G_{αq}-coupled receptors have different propensities for activating this pathway depending on the subcellular localizations of the GPCR's relative to the proximity of PLC and GIRK channels (Cho et al., 2005).

Interestingly, GABA_B, μ-opioid and 5-HT_{1A} receptors are all expressed in POMC neurons (Kelly et al., 1992; Lagrange et al., 1994; Qiu et al., 2003; present findings). All of these receptors are G_{αi/o}-coupled to activation of GIRK channels, which uniformly inhibit POMC neuronal activity. Similar to our findings with the 5-HT_{2C} receptor mediated desensitization of the GABA_B response in POMC neurons, activation of 5-HT_{2A} receptors can desensitize 5-HT_{1A} receptors and increase the excitability of CRH neurons as measured by ACTH release (Zhang et al., 2001). The heterologous desensitization in these paraventricular nucleus neurons has not been characterized but may be via PKC-mediated phosphorylation of GIRK channels (Brown et al., 2005) or by PLC-mediated PIP₂ depletion (Brown et al., 2005; Cho et al., 2005). Therefore, the PLC mediated PIP₂ depletion maybe a common signaling pathway for 5-HT_{2A/C} receptors in hypothalamic neurons.

Evidence from several species indicates that food intake and body weight are influenced both by changes in endogenous estrogens and by exogenous estrogenic treatments (Czaja and Goy, 1975). Butera and Czaja (1984) have shown that the anorexigenic effects of E_2 are attributable to the direct actions of the steroid in the arcuate-ventromedial hypothalamic nuclei, and recently we have shown that E_2 and STX, a selective ligand for the mER, attenuated the weight gain in female guinea pigs following ovariectomy (Qiu et al., 2006). The present findings with icv administration of E_2 in GDX female mice corroborate the findings in the guinea pig for a central action of steroid to regulate energy homeostasis. Therefore, this membrane-delimited signaling pathway may play a vital role in the control of energy homeostasis. It is interesting that eating disorders are much more prevalent (95%) in young women as compared to men, but the reasons for this difference are not clear (Sodersten et al., 2006). In animal models there are clear sex differences in food intake and energy homeostasis with hormone treatment. Estrogen reduces food intake and body weight in females and males, although E_2 is more effective in females and T in males (Czaja and Goy, 1975; Czaja, 1984). We found that T at a higher concentration could mimic, but the non-hydrolyzable androgen DHT could not mimic the effects of E_2 . Moreover, E_2 was more efficacious in female than in male arcuate (POMC) neurons, but there was no sex difference in the actions of 5-HT_{2A/C} agonists. Taken together, the sex differences in the control of feeding and energy homeostasis may be due, in part, to a greater efficacy of E_2 in females versus males to dis-inhibit POMC neurons via the mER signaling pathway.

Serotonin 5-HT_{2C} receptors have also been strongly implicated in inhibiting feeding. For example, the selective 5-HT_{2C} antagonist, RS-102221, increases food intake and body

weight when injected intraperitoneally (Bonhaus et al., 2006), and 5-HT_{2C} receptor-deficient mice are hyperphagic, obese, and refractory to threshold anorexic doses of d-fenfluramine (Tecott et al., 1995; Vickers et al., 1999), a drug that blocks the reuptake of 5-HT and stimulates its release (Heal et al., 1998). In contrast, 5-HT_{2A} receptor knockout mice do not exhibit an obesity phenotype, so one may assume that 5-HT_{2A} receptors are not critically involved in energy homeostasis (Zhou et al., 2005). We found that 5-HT_{2C} mRNA was highly expressed and co-localized with 5-HT_{2A} receptor in POMC neurons. This would disagree with in situ hybridization results in rhesus (female) monkeys showing that 5-HT_{2C} receptor mRNA is highly expressed in the arcuate region, whereas the 5-HT_{2A} receptor mRNA is more localized to the PVN (Gundlach et al., 1999). In addition, (Heisler et al., 2002) found that up to 80% of POMC neurons express 5-HT_{2C} receptor mRNA. However, the role of the 5-HT_{2C} and 5-HT_{2A} receptors in different physiological processes may lie in their coupling to distinctive signaling pathways.

Based on our cellular electrophysiological data we have proposed a model for the convergence of the mER and 5-HT_{2C} signaling pathways in arcuate (POMC) neurons (Figure 7). It is noteworthy that the downstream signaling pathway of baclofen inhibition by 5-HT_{2C} receptor agonists in arcuate (POMC) neurons is different from mER in which E₂ desensitizes the GABA_B response via a G_{αq}-PLC-PKCδ-PKA pathway (Qiu et al., 2003). These differences may be due to the different compartmentalization of the receptors. Recently, it was reported that the signaling components for G-protein activation in neurons are compartmentalized or pre-assembled (Lober et al., 2006). GPCR's, G-proteins and other effector molecules can pre-assemble into stable signaling complexes (Rebois and Hebert, 2003). Therefore, the 5-HT_{2C} receptor and mER may be pre-assembled into

complexes with different downstream effectors that converge on GIRK channels. For example, Brown and colleagues (Brown et al., 2005) have shown that although protein kinases (i.e., PKC δ) mediate the inhibition of GIRK_{1/2} channels by the muscarinic M₃ receptor, re-synthesis of PIP₂ is required for complete recovery from inhibition. Therefore, PIP₂ turnover is critical for GIRK channel function. Our data would support this idea since 5-HT_{2C} receptors can inhibit the GIRK channels by an independent pathway from mER, but the two pathways converge on the same population of GIRK channels as shown by the additive effects of the two agonists given together. As proof of principle, we have found that both E₂ and DOI are effective to inhibit feeding in GDX female mice, and as predicted from the cellular findings, E₂ was more potent than DOI to inhibit food intake and weight gain. Therefore, the G α_q signaling pathways of mER and 5-HT_{2C} receptors may converge to enhance synaptic efficacy in brain circuits that are critical for maintaining homeostatic functions.

Acknowledgments: The authors would like to recognize the skilled technical assistance of Ms. Rebecka Amodei and Mr. Scott Kuhn. The authors would like to recognize Dr. Jan Kelder (NV Organon) for his generous gift of Org 3363.

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Footnotes

This study was supported by United States Public Health Service grants NS 43330, NS38809, DK 68098.

Legends for Figures

Figure 1. Sex differences in the effects of E₂ on baclofen responses in GDX guinea pigs. a. Representative traces of the baclofen response in the presence of T (1 μM) or DHT (1 μM) in male arcuate (POMC) neurons. b. Bar graphs summarizing the effects of the E₂ (100 nM), T (1 μM) and DHT (1 μM) in arcuate (POMC) neurons. E₂ was more efficacious in female than in male arcuate (POMC) neurons. T at a higher concentration (1 μM) could mimic, but DHT could not mimic the effects of E₂. Bars represent the mean ± S.E.M. of 3-11 cells tested per group. *, p<0.05, E₂ in females vs. males; and DHT versus testosterone in males; #, p<0.05, T versus control. Since there was no difference in female (96.6 ± 6%, n = 8) and male (96.0 ± 5%, n = 3) control cells, the data were combined for the control bar. c. a representative POMC neuron that responded to DOI. Arcuate neurons were filled with biocytin during the whole-cell recording. Left panel, Biocytin-streptavidin-Cy₂ labeling of a small fusiform arcuate neuron. Scale bar, 20 μm. Middle panel, Immunocytochemical staining of β-endorphin in the same neuron (arrow). Right panel, Overlay of left and middle panels.

Figure 2. Activation of 5-HT_{2C} receptors desensitizes the GABA_B response in POMC neurons. a. Representative traces of the effects of DOI and ORG 3363 on the baclofen responses. b. Bar graphs summarizing the effects of 5-HT_{2C} receptor agonists, MK-212 (10 μM) and m-CPP (10 μM), 5-HT_{2A/C} receptor agonist DOI (20 μM), 5-HT_{2A} receptor antagonist spiperone (20 μM), 5-HT_{2C} receptor antagonists ORG 3363 (10 μM) and RS

102221 (20 μ M). Bars represent the mean \pm S.E.M. of 4-14 cells. *, $p < 0.05$ and **, $p < 0.01$, versus vehicle-control.

Figure 3. 5-HT_{2C} receptor-mediated attenuation of the GABA_B response depends on activation of G_{αq}, but not PKC. Cells were dialyzed for 15 min before baclofen application with BIS (100 nM), G_{αq} (200 μ M) or G_{αs} peptide (200 μ M), which were included in the patch pipette solution. a. Representative traces of the baclofen responses in the presence of PKC inhibitor, BIS or G_{αq} peptide. b. Bar graphs summarizing the effects of the broad spectrum PKC inhibitor BIS and G_α inhibitors. Bars represent the mean \pm S.E.M. of 4-14 cells. * $p < 0.05$, G_{αq} peptide plus DOI versus G_{αs} peptide plus DOI; and ** $p < 0.01$ versus vehicle control. c,d. 5-HT_{2C} receptor-mediated attenuation of the GABA_B response depends on activation of PLC. c. Representative traces of the baclofen responses in the presence of PIP₂ or the PLC inhibitor U73122. Cells were dialyzed for 15 min before baclofen application with phosphatidylinositol bisphosphate (PIP₂, 5 μ M), which was included in the patch pipette solution. d. Bar graphs summarizing the effects of PIP₂, PLC inhibitor U73122 (10 μ M) and the inactive analog of U73122, U73343 (10 μ M) and PI-4-kinase inhibitor wortmannin (wort, 10 μ M). Bars represent the mean \pm S.E.M. of 3-14 cells. *, $p < 0.05$, U73122 plus DOI versus U73343 plus DOI; ** $p < 0.01$, versus vehicle control; ***, $p < 0.005$, versus vehicle control; and ###, $p < 0.005$, Wort versus DOI plus Wort.

Figure 4. Effects of DOI and E₂ on GABA_B response were additive. a. Representative traces of the baclofen response in the absence or presence of DOI (20 μ M) alone or DOI plus E₂ (50 nM) in arcuate (POMC) neurons. b. Cells were perfused with different

concentrations of DOI (0, 10, 50, 100, and 300 μM). Data are presented as mean \pm S.E.M. ($n = 4\text{--}14$ cells/data point). Based on a logistics equation fit to the data points (see Materials and Methods), the EC_{50} value of the inhibition of baclofen response by DOI was 16.5 μM . The Hill slope for DOI was 1.8. c. Bar graphs summarizing the effects of DOI (20 μM) and E_2 (50 nM). E_2 or DOI at their EC_{50} concentrations could significantly inhibit the baclofen response, and the effects were additive. Bars represent the mean \pm S.E.M. of 5-14 cells. **, $p < 0.01$; and ***, $p < 0.005$, versus vehicle-control; #, $p < 0.05$, E_2 or DOI versus DOI plus E_2 .

Figure 5. 5-HT_{2A/C} receptor agonist and estrogen inhibit feeding in GDX female mice.

a. 5-HT_{2A/C} receptor agonist DOI reduces food intake in GDX female mice. DOI (110 nmol) given i.c.v. to overnight food-deprived, ovariectomized mice produced a potent inhibition of cumulative food intake. Data are shown as means \pm S.E.M.; $n=6$ for each group. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$, compared with saline vehicle-treated mice at all time points measured (two-way ANOVA and Bonferroni post-test). b. 5-HT_{2A/C} receptor agonist DOI reduced body weight gain in GDX female mice. DOI (110 nmol) given i.c.v. to overnight food-deprived GDX female mice produced a potent inhibition of body weight gain. Data are shown as means \pm S.E.M.; $n = 6$ for each group. *, $p < 0.05$, ***, $p < 0.005$, compared to saline vehicle-treated mice at all time points measured (two-way ANOVA and Bonferroni post-test). c,d. E_2 , similar to DOI reduced feeding at the 6 h time-point. Bar graphs summarizing the effects of DOI and E_2 (0.012 nmol) on food intake (c) and body weight gain (d). Bars represent the mean \pm S.E.M. $n = 5\text{--}7$ mice per group, ***, $p < 0.005$, **, $p < 0.01$, DOI or E_2 group versus their respective control groups.

Figure 6. 5-HT_{2C} receptor mRNA is highly expressed in POMC neurons. a. A representative gel illustrating the mRNA expression of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptors in arcuate neurons. All neurons expressed GAPDH (data not shown). One cell contained no RT as a negative control (-RT). Other controls included aCSF from the vicinity of the harvested cells and a water blank (data not shown). b. Diagram showing the distribution and co-expression of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor transcripts in arcuate POMC neurons from GDX females. 5-HT_{2C} and 5-HT_{2A} were detected in the majority of POMC neurons, 84% and 76% respectively, whereas 5-HT_{1A} receptor mRNA was detected in only 42% of POMC neurons. c. qPCR analysis of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor mRNA expression in the microdissected arcuate nucleus, using the SYBR green method. Cycle number is plotted against the normalized fluorescence intensity (Mean Delta Rn) to visualize the PCR amplification. The cycle threshold (Ct) value (dotted line) is the point in the amplification at which the sample values were calculated. d. The superimposed melting curves for GAPDH, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} depict a single product. e. The standard curve regression line produced slope values of -3.1, -3.2 and -3.2 which translate into similar efficiencies of 100%. f. Quantitative analysis of 5-HT_{2C}, 5-HT_{2A} and 5-HT_{1A} receptor mRNA expression in the arcuate nucleus from oil-treated GDX female guinea pigs (mean ± S.E.M.; n=6). 5-HT_{2A} and 5-HT_{1A} are compared to 5-HT_{2C} (One-way ANOVA, a, p<0.05; b, p<0.001; c, p<0.001).

Figure 7. A cellular model of the rapid signaling of E₂ and 5-HT in hypothalamic neurons. Schematic overview showing the mER and 5-HT_{2A/C} receptors-mediated

modulation of neurotransmitter regulated, G protein-coupled receptors in arcuate (POMC) neurons. Black is used to summarize the results of the current experiments. The cellular cascade activated by E_2 (see Qiu et al., 2003) is shown in gray. E_2 activates a mER that is $G_{\alpha q/11}$ -coupled to activation of PLC that catalyzes the hydrolysis of membrane-bound PIP_2 to IP_3 and DAG. Calcium is released from intracellular stores (endoplasmic reticulum) by IP_3 , and DAG activates $PKC\delta$. Through phosphorylation, ACVII activity is upregulated by $PKC\delta$. The generation of cAMP activates PKA, which can rapidly uncouple $GABA_B$ and μ -opioid (μ) receptors from their effector system through phosphorylation of a downstream effector molecule (e.g. the inwardly rectifying K^+ channel, or GIRK). 5-HT_{2C} receptor mediated inhibition of GIRK channels involves PLC activation by G_α subunits of the $G_{q/11}$ family and receptor-mediated hydrolysis of plasma membrane PIP_2 (PIP_2 depletion) is the critical mediator. Therefore, 5-HT_{2C} receptor and mER intracellular signaling pathways converge to dis-inhibit arcuate (POMC) neurons.

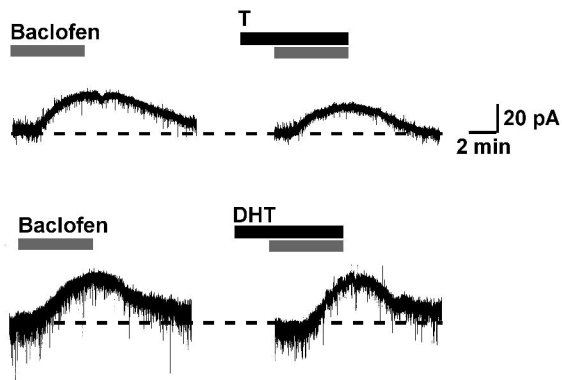
Table 1. Primer sequences used for single-cell RT-PCR and qPCR.

Name	Product Length	Primer sequence	Base pair #	Accession #
POMC ¹	206 bp	TTG CTG GCC CTC CTG CTT CA CTC TCG GCT CTT CTT ATC TG	37-57 251-231	S78260
5-HT _{2C} ¹	252 bp	GCA CCA TGC CAG GCT ATC AAC GTT GGA GAA AGC CCT GCG GTA	1-21 252-232	EF212439
5-HT _{2A} ¹	348 bp	ACC GCT ATG TTG CCA TCC AGA A AAT GGT TGT CAG GGT GAT CAG	80-101 427-406	M85162
5-HT _{1A} ¹	302 bp	TCC GAC GTG ACC GTC AGC TA AAT GGT TGT CAG GGT GAT CAG	85-104 386-367	AF488978
GAPDH ²	212 bp	CAT CCA CTG GTG CTG CCA AG GTC CTC GGT GTA GCC CAA GA	123-142 334-315	CPU51572
5-HT _{2C} ³	146 bp	TAT CGC TGG ATC GGT ATG ATC CCT CAG TCC AAT CAC	151-168 296-279	EF620035
5-HT _{2A} ³	100 bp	CCA TAG CCG CTT CAA CTC CAG CCC AAA GAC TGG AAC	111-129 210-193	M85162
5-HT _{1A} ³	92 bp	TCG GTT CCT TGG CGG TTA C GCC CAG AGT CCA CTT GTT GAG	107-125 198-178	EF620034

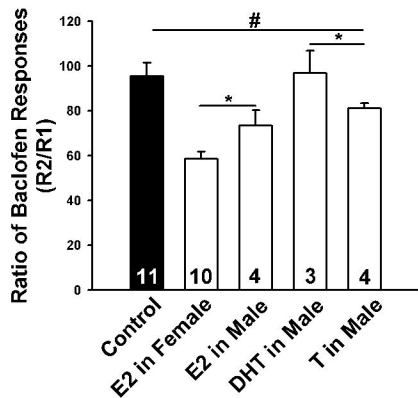
The sense primer is listed first with the antisense primer below. ¹Primers used for single-cell RT-PCR; ²primers used for both single-cell and qPCR; ³primers used for qPCR.

Figure 1

a



b



c

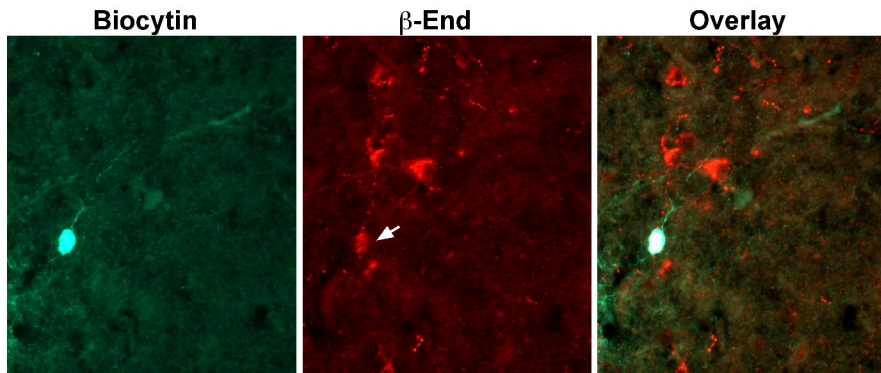


Figure 2

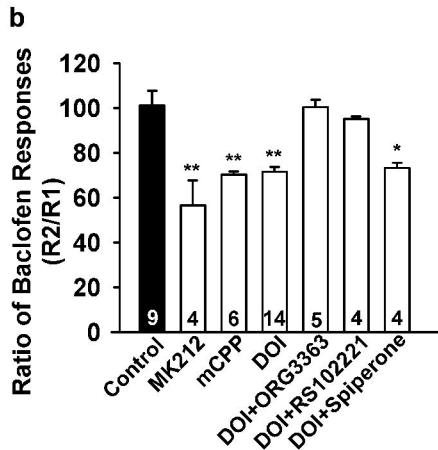
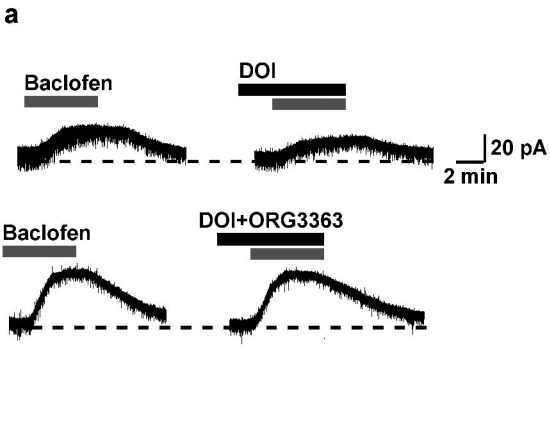


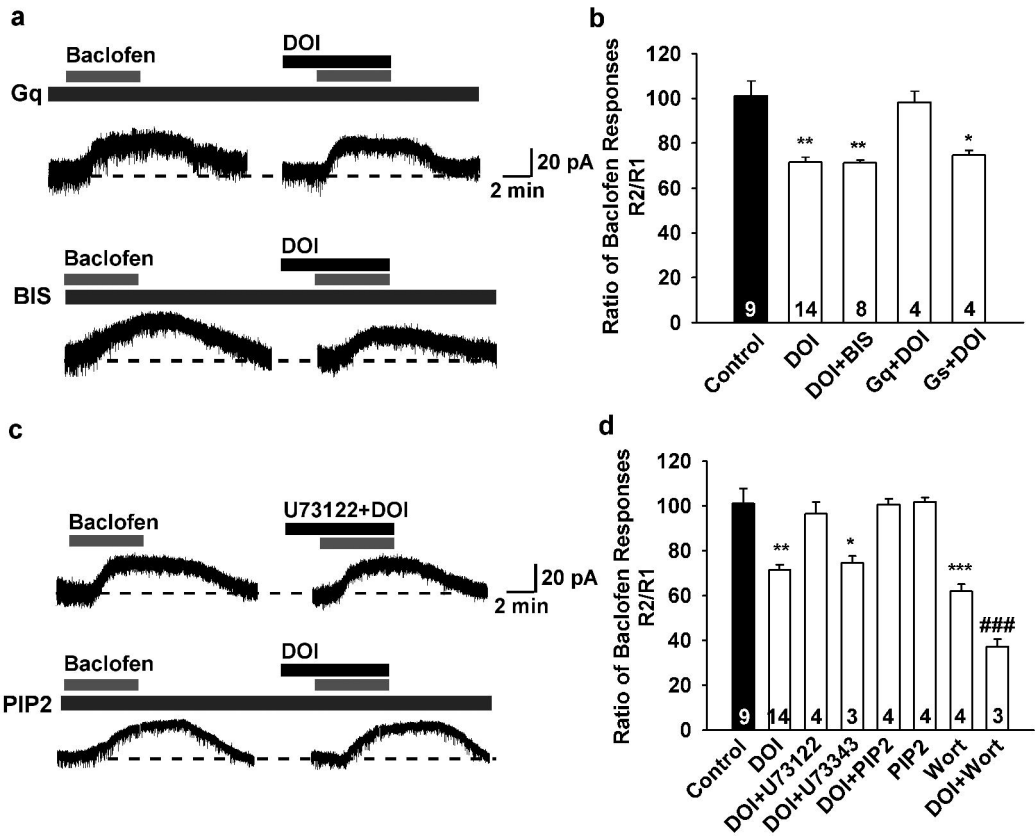
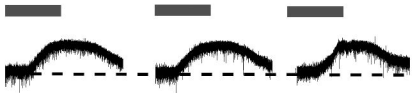
Figure 3

Figure 4

a

Baclofen



Baclofen

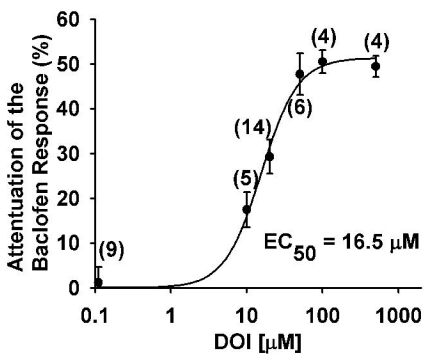
DOI

E2+DOI



20 pA
2 min

b



c

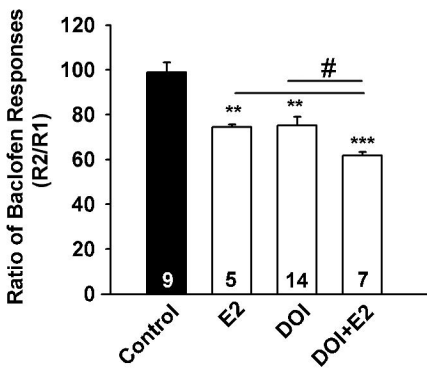


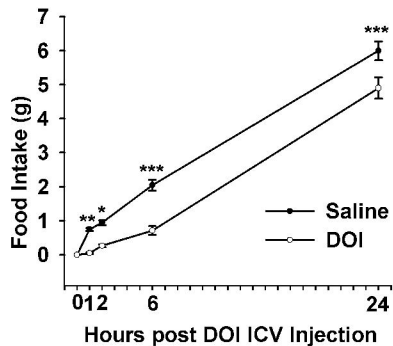
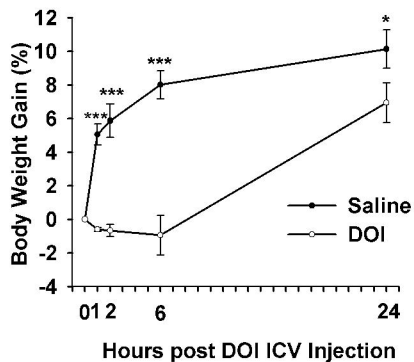
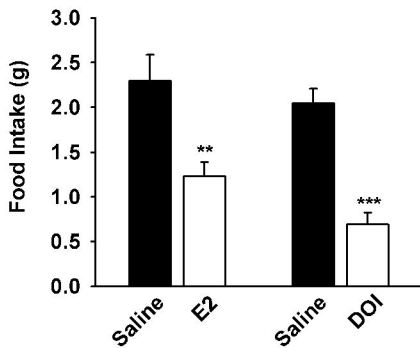
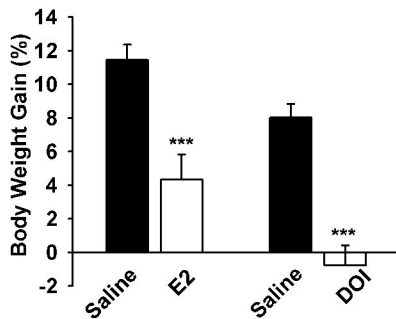
Figure 5**a****b****c****d**

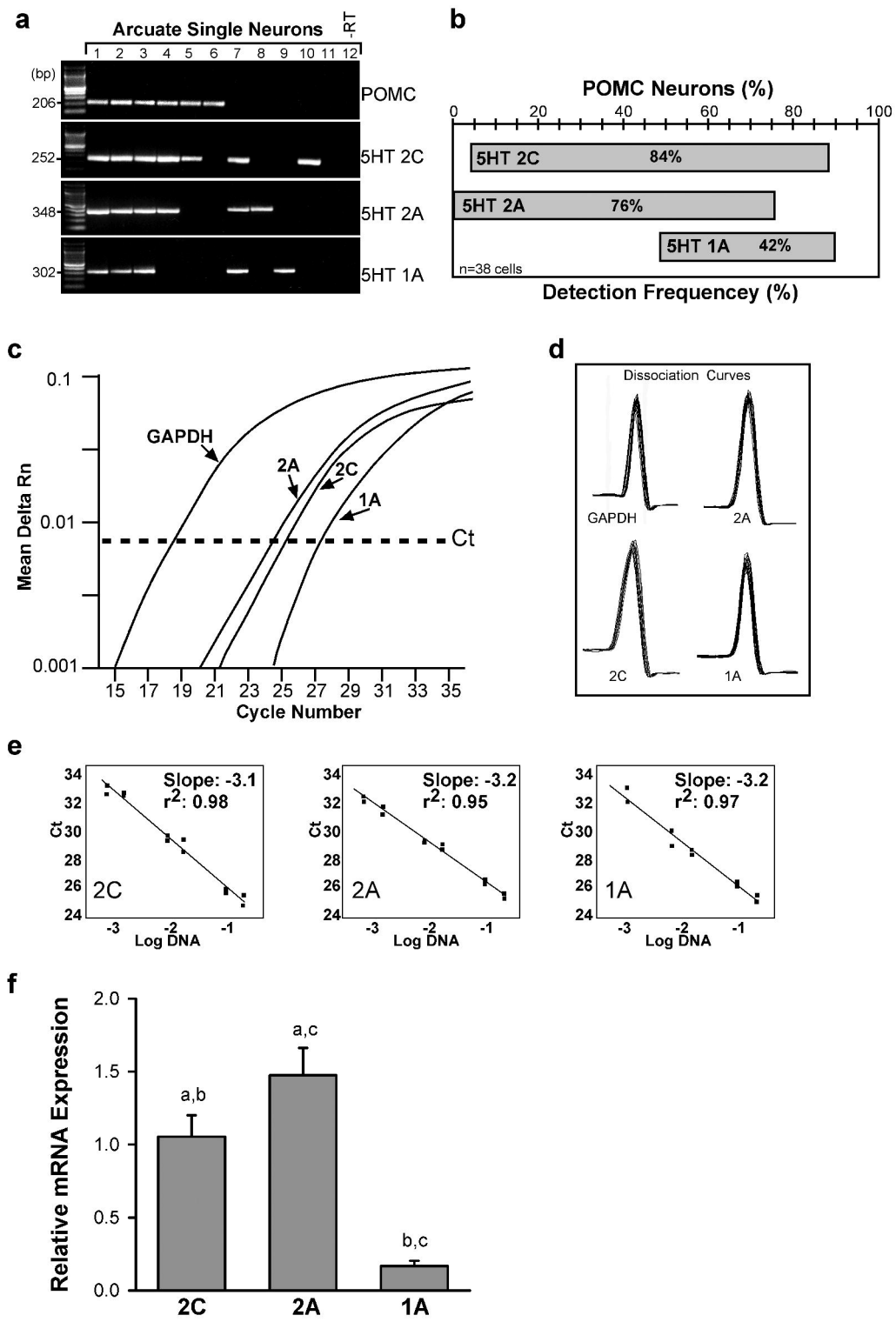
Figure 6

Figure 7

