Differential gene regulation of GHSR signaling pathway in the arcuate nucleus and NPY neurons by fasting, diet-induced obesity, and 17β-estradiol

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Abstract:

Ghrelin's receptor, growth hormone secretagogue receptor (GHSR), is highly expressed in the arcuate nucleus (ARC) and in neuropeptide Y (NPY) neurons. Fasting, diet-induced obesity (DIO), and 17β-estradiol (E2) influence ARC *Ghsr* expression. It is unknown if these effects occur in NPY neurons. Therefore, we examined the expression of *Npy*, *Agrp*, and GHSR signaling pathway genes after fasting, DIO, and E2 replacement in ARC and pools of NPY neurons. In males, fasting increased ARC *Ghsr* and NPY *Foxo1* but decreased NPY *Ucp2*. In males, DIO decreased ARC and NPY *Ghsr* and *Cpt1c*. In fed females, E2 increased *Agrp*, *Ghsr*, *Cpt1c*, and *Foxo1* in ARC. In NPY pools, E2 decreased *Foxo1* in fed females but increased *Foxo1* in fasted females. DIO in females suppressed *Agrp* and augmented *Cpt1c* in NPY neurons. In summary, genes involved in GHSR signaling are differentially regulated between the ARC and NPY neurons in a sex-dependent manner.

Keywords: arcuate nucleus, ghrelin, neuropeptide y, fasting, diet-induced obesity, 17β-estradiol

Glossary: Arcuate Nucleus (ARC), Agouti-related Peptide (AgRP), Carnitine Palmitoyltransferase 1c (CPT-1c), Central Nervous System (CNS), Diet-Induced Obesity (DIO), 17β-estradiol (E2), Estradiol Benzoate (EB), Forkhead Box O1 (FoxO1), Green Fluorescent Protein (GFP), Growth Hormone Secretagogue Receptor (GHSR), Neuropeptide Y (NPY), Proopiomelanocortin (POMC), Uncoupling Protein 2 (UCP2)

1. Introduction

Obesity is a global health concern due, in part, to changes in diet and lifestyle and is a major risk factor for a variety of clinical conditions including cardiovascular disease, type II diabetes, and metabolic syndrome (Mark, 2006). One hallmark of obesity is the dysregulation of the hypothalamic centers that control feeding behavior, energy expenditure, and the peripheral hormones that mediate the communication between the body and the brain (Cai and Liu, 2011). Normally, neuronal populations in the hypothalamus regulate energy homeostasis by responding to circulating nutrients as well as appetite-regulating hormones such as leptin, insulin, and ghrelin (Gao and Horvath, 2007).

The incidence of metabolic disorders associated with obesity exhibit clear sex differences with premenopausal women having fewer metabolic disorders than men. However, metabolic disorders increase dramatically in postmenopausal women (Ford, 2005; Loucks et al., 2007). The loss of the reproductive steroid 17β-estradiol (E2) is, in part, the major cause of these effects especially on body weight gain (Rachoń and Teede, 2010; Stefanska et al., 2015). In ovariectomized rodent models, E2 regulates many aspects of energy homeostasis through both peripheral actions and central mechanisms (reviewed in Mauvais-Jarvis et al., 2013; Roepke, 2009; Shi et al., 2009). E2 suppresses feeding and fat accumulation and augments energy expenditure and activity. To control energy homeostasis, E2 primarily uses the nuclear steroid receptor, ERα, which is highly expressed in the hypothalamus (Roepke, 2009). Total body knockout of ERα or deletion in specific hypothalamic neurons produces an obese phenotype with hyperphagia, higher visceral adiposity, and lower activity and energy expenditure in mice (Geary et al., 2001; Heine et al., 2000; Mamounis et al., 2014; Musatov et al., 2007; Xu et al., 2011).

Among the hypothalamic areas involved in energy homeostasis, the arcuate nucleus (ARC) is of special interest as it is an integration center for homeostatic signals from the periphery and

the central nervous system (CNS). The “first order” ARC neurons central to the control of energy homeostasis are the proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons (Gao and Horvath, 2007). POMC and NPY neurons have opposing actions in the control of energy homeostasis. POMC neurons are anorexigenic primarily through the actions of α-melanocyte-stimulating hormone (α-MSH) via melanocortin receptors (MC3/4) expressed throughout the hypothalamus (Dietrich and Horvath, 2013). NPY neurons are orexigenic primarily through the actions of its neuropeptides, NPY and agouti-related peptide (AgRP), an antagonist for the MC4 receptors. Thus, the actions of POMC and NPY/AgRP neurons are important for the hypothalamic control of feeding and energy expenditure (Gao and Horvath, 2007).

Ghrelin is a brain-gut peptide hormone secreted from the stomach to stimulate food intake by acting on its receptor, growth hormone secretagogue receptor (GHSR). GHSR is expressed throughout the brain and in NPY/AgRP neurons in the ARC (Cowley et al., 2003; Willesen et al., 1999). Ghrelin-expressing neurons are also found in the periventricular region of the hypothalamus dorsal to the ARC (Guan et al., 2003; Mondal et al., 2005). ARC and peripheral ghrelin administration induces NPY/AgRP gene expression (Chen et al., 2004; Goto et al., 2006; Kamegai et al., 2001, 2000) and NPY activation (Wang et al., 2002) and potently depolarizes NPY neurons (Andrews et al., 2008; Cowley et al., 2003). Furthermore, ghrelin activation of GHSR potently excites NPY neurons and controls calcium homeostasis (Andrews et al., 2008; Cowley et al., 2003). These rapid effects of ghrelin potentially involve calcium channels (Kohno et al., 2003) and may involve the inhibition of the M-current as recently reported in striatal neurons (Shi et al., 2013).

Activation of GHSR in NPY neurons initiates a signaling cascade that involves the mitochondrial enzymes uncoupling protein 2 (UCP2) and carnitine palmitoyltransferase 1 (CPT-1) to control *Npy*/*Agrp* gene expression through a forkhead box O1 (FoxO1)-mediated mechanism (Andrews, 2011; Andrews et al., 2008; López et al., 2008a). CPT-1 is involved in malonyl-CoA sensing and fatty acid oxidation, while UCP2 is necessary for reactive oxidation

species (ROS) buffering and mitochondrial biogenesis (Andrews, 2011; López et al., 2008b). Of the three types of CPT-1, hypothalamic neurons express CPT-1c, which does not have acyltransferase activity, while hypothalamic astroyctes express CPT-1a, which does have acyltransferase activity (Wolfgang et al., 2006). FoxO1 is a transcription factor that negatively regulates adipogenesis, mediates insulin-induced gluconeogenesis, and is an effector of *Npy*/*Agrp* gene expression (Cao et al., 2011; Ren et al., 2012).

Fasting and diet-induced obesity (DIO) regulate expression of *Npy*, *Agrp*, *Ghsr*, and *Ucp2* in the ARC (Briggs et al., 2014, 2013, 2010; Coppola et al., 2007; Verhulst et al., 2012), which may be a contributing mechanism underlying the ghrelin resistance seen in DIO (Briggs et al., 2014, 2010; Perreault et al., 2004). Furthermore, a high fat diet (HFD) or DIO inhibits ghrelin's augmentation of hyperphagia (Gardiner et al., 2010; Perez-Tilve et al., 2011). Recently, E2 was shown to increase *Ghsr* expression in the ARC (Frazao et al., 2014). Interestingly, E2 is an anorexigenic steroid and is known to induce *Pomc* and suppress *Npy* gene expression (Roepke, 2009). Because ghrelin is orexigenic, this E2-induced increase in *Ghsr* expression in the ARC may be independent of its effects on feeding and occur in other ARC neurons.

Because GHSR and the components of its signaling pathway are widely expressed in the heterogeneous cell types of the ARC, determinations of gene expression only in the ARC may lead to incorrect assumptions about their modulation in NPY neurons. Therefore, our hypothesis is that there will be significant differences in gene expression between the ARC and NPY neurons due to fasting and DIO. We also hypothesize that there will be distinct sex differences in the response to these dietary influences due, in part, to E2 in females. To address these hypotheses, we characterized the expression of *Ghsr*, *Ucp2*, *Cpt1c, Foxo1*, and *Npy*/*Agrp* in both the ARC and NPY neurons in males after fasting and DIO and in ovariectomized females with or without E2 replacement after fasting and DIO using wild type (WT) C57 mice and GFP-NPY transgenic mice.

2. Materials & Methods

2.1. Animal Care and Experimental Design

Animal experiments described in this project are in accordance with institutional guidelines based on National Institutes of Health standards and have been approved by The Rutgers University Institutional Animal Care and Use Committee. All animals were maintained under controlled temperature and photoperiod (12 h:12 h). All mice, both WT C57BL/6J (Jackson Laboratory) and GFP-NPY ((Dr. Bradford Lowell, Harvard University, (van den Pol et al., 2009), were given free access to food and water except where noted. In experiment #1, males (10-15 weeks of age), 13 WT and 12 GFP-NPY, were either fed *ad libitum* (n=7 WT, n=6 GFP) or fasted (n=6 both) for 24 h prior to tissue collection or cell harvesting. In experiment #2, males (8 weeks of age), 12 WT and 12 GFP-NPY, were fed either a low-fat diet (ND, 10% kCal, n=6) or a high-fat diet (HFD, 45% kCal, n=6) for 12 weeks prior to tissue collection or cell harvesting. In experiment #3, females (10-14 weeks of age), 12 WT and 12 GFP-NPY, were ovariectomized (ovx) under isoflurane anesthesia and allowed to recover for 5 days and fed *ad libitum* on a low-phytoestrogen standard chow (Lab Diets 5V75). Females were then injected 48 h prior to sacrifice with 0.25 μg estradiol benzoate (EB) or oil followed by a second injection of 1.5 μg EB (n=6) or oil (n=6) 24 h prior to sacrifice. The EB injection paradigm yields physiological levels of E2 (Bosch et al., 2013). In experiment #4, females (10-14 weeks old), 12 WT and 12 GFP-NPY, were ovx under isoflurane anesthesia and allowed to recover for 5 days. Females were then injected 48 h prior to sacrifice with 0.25 μg estradiol benzoate (EB) or oil followed by a second injection of 1.5 μg EB (n=6) or oil (n=6) 24 h prior to sacrifice. These females were fasted 24 h prior to euthanasia. In experiment #5, females (8 weeks of age), 32 WT and 24 GFP-NPY, were ovx under isoflurane anesthesia and fed either ND (n=16 WT; n=12 GFP) or HFD (n=16 WT;

n=12 GFP) for 8 weeks prior to tissue collection or cell harvesting. Half of each diet group was administered oil or EB (300 μg/kg) daily perorally via a peanut butter carrier (WT, all groups: n=8; GFP-NPY, all groups: n=6). We chose peroral E2 replacement for the DIO studies to reduce the stress-inducing effects of repeated injection and to maintain a constant systemic level of E2 in the blood (Ingberg et al., 2012). Body weights were measured weekly. All animals were injected with ketamine (100 μl of 100 mg/ml, i.p.) prior to decapitation. All euthanasia occurred at 1000-1100 h. Uteri were weighed in all females.

2.2. Drugs and Diets

Estradiol benzoate (EB) and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) were purchased from Sigma-Aldrich. EB was dissolved in 100% ethanol (Sigma) prior to dissolving in sesame oil (Sigma). Ghrelin and tetrodotoxin (TTX) were purchased from Tocris (Bristol, UK) and dissolved in water. DIO diets were purchased from Research Diets (New Brunswick, NJ): low-fat diet (10% kcal fat; D12450B) and high-fat diet (45% kcal fat; D12451).

2.3. ARC Tissue Dissections and RNA Extractions

At the end of the experiments, males and females were decapitated and the brain was extracted from the skull. The basal hypothalamus (BH) was cut using a brain slicer (Ted Pella,, Redding, CA, USA), into one mm thick coronal rostral and caudal blocks corresponding to plates 42-47 and plates 48-53, respectively, from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2008, 3rd edition) (Paxinos and Franklin, 2008). The BH blocks were transferred to RNAlater (Life Technologies, Grand Island, NE, USA) and stored overnight at 4°C. The entire ARC was dissected from the BH slices using a dissecting microscope for

storage at -80°C. Total RNA was extracted from the combined ARC using Ambion RNAqueous-Micro Kit (Life Technologies) according to the manufacturer's protocol. Total RNA was also DNase I-treated, using the extraction kit, at 37°C for 20 min to minimize any genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Waltham, MA) and an Agilent 2100 Bioanalyzer with RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). Samples with an RNA integrity number greater than 7.0 were used for analysis.

Complementary DNA (cDNA) was synthesized from 200 ng of total RNA using 50 U Superscript III reverse transcriptase (RT) (Life Technologies, Inc.), 4 μl 5x Superscript Buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Mountain View, CA), 100 ng random hexamer primers (Promega, Madison, WI), 40 U/μl RNasin (Promega), and 100 mM DTT in DEPC-treated water (Gene Mate, Kaysville, UT) in a total volume of 20 μl. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. The cDNA was diluted 1:20 with nuclease-free water (Gene Mate, Bioexpress) for a final cDNA concentration of 0.5 ng/μl and stored at -20°C. BH test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

2.3. Cell Harvesting of Dispersed NPY Neurons

All GFP-NPY males and females were killed by decapitation after sedation. The brain was quickly removed from the skull and submerged in cold (4ºC) oxygenated (95% O_2, 5% CO_2) high-sucrose artificial CSF (aCSF, in mmol): 208 sucrose, 2 KCl, 26 $NaHCO_3$, 10 glucose, 1.25 NaH_2PO_4, 2 $MgSO_4$, 1 $MgCl_2$, 10 HEPES (pH 7.3, 300 mOsm). Four coronal slices (250 μm) from the BH were cut on a vibratome and bathed for ~10 min in high-sucrose CSF at 4ºC. The

196 slices were then transferred to an auxiliary chamber where they were kept at room temperature
197 (25ºC) in aCSF consisting of (in mmol): 124 NaCl, 5 KCl, 2.6 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26
198 $NaHCO_3$, 10 HEPES, 10 glucose (pH 7.3, 310 mOsm) for recovery of ~1 h until cell dispersal.
199 The ARC nucleus was microdissected and incubated in 5-10 ml aCSF (pH 7.35, 300 mOsm)
200 containing 1 mg/ml protease for 15 minutes at 37ºC. The tissue was then washed four times in
201 low-calcium CSF (0.1 mM $CaCl_2$) and twice in aCSF. The cells were isolated by trituration with
202 flame-polished Pasteur pipettes, dispersed onto a 60 mm glass-bottomed Petri dish, and
203 perfused continuously with aCSF at a rate of ~2 ml/min. Fluorescent and adjacent, non-
204 fluorescent cells were visualized using a Leica DM-IL inverted fluorescent microscope, patched,
205 and then harvested by applying low negative pressure to the pipette using the Xenoworks
206 manipulator system (Sutter Instruments, Novato, CA). The content of the pipette was expelled
207 into a siliconized microcentrifuge tube containing 1 µl 5X Superscript III Buffer (Life
208 Technologies), 15 U RNasin (Promega), 0.5 µl 100 mM DTT, and DEPC-treated water in a 8 µl
209 volume. GFP-NPY neurons were either harvested as single cells (Fig. 1) or collected into 10-12
210 pools of 5 NPY neurons each from each animal (all other Fig.s).
211 Each harvested cell or pool of cells was reverse transcribed as described previously (Bosch
212 et al., 2013; Roepke et al., 2011). Briefly, the tubes containing the harvested cell solution and
213 two tubes containing 25 ng of hypothalamic total RNA in 1 µl were denatured for 5 min at 65ºC,
214 then cooled on ice for 5 min. Single stranded cDNA was synthesized from cellular RNA by
215 adding 50 U Superscript III RT, 3 µl 5x Superscript Buffer, 5 mM $MgCl_2$, 0.625 mM dNTPs
216 (Clontech), 15 U RNasin, 400 ng anchored oligo(d)T (Life Technologies), 100 ng random
217 hexamers (Promega), 10 mM DTT in DEPC-water in a total volume of 25 µl. One single cell and
218 one tissue RNA tube were used as negative controls and processed as described above but
219 without RT. CSF was also collected every 2-3 pools and analyzed for any contamination.
220 Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at
221 50°C, 15 min at 70°C. In preliminary investigation of *Ghsr* expression in NPY neurons,

harvested single cells (10-15) were collected from male GFP-NPY mice that were either fed (n=6) or fasted (n=6) for 24 hr. *Npy* and *Ghsr* expression were analyzed using standard PCR protocols and gel electrophoresis as previously described (Roepke et al., 2011; Xu et al., 2008). Each reaction was amplified for 50 cycles using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) at an annealing temperature of 60°C. Negative (cell and tissue samples without RT), collected CSF, and positive tissue controls were analyzed with each PCR run.

2.4. Quantitative Real-time PCR

All primers were designed to span exon-exon junctions and synthesized by Life Technologies, using Clone Manager 5 software (Sci Ed Software, Cary, NC). See Table 1 for a listing of all the primer sequences used for single-cell and quantitative real-time PCR (qPCR). For ARC tissue qPCR, 4 μl cDNA template (an equivalent of 2 ng total RNA) was amplified using either PowerSYBR Green master mix (Life Technologies) or SsoAdvanced SYBR Green (BioRad, Hercules, CA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in triplicate to determine the efficiency [$E = 10(^{-1/m})–1$, m = slope] of each primer pair. All efficiencies, expressed as % efficiency, were approximately equal (one doubling per cycle, 90-110%, Table 1).

The relative mRNA expression data were analyzed using the $\Delta\Delta C_q$ method (Livak and Schmittgen, 2001; Pfaffl, 2001; Schmittgen and Livak, 2008). The amplification protocol for all the genes was as follows: initial denaturing at 95°C for 10 min (PowerSYBR) or 3 min (SsoAdvanced) followed by 40-45 cycles of amplification at 94°C for 10 sec (denaturing) and 60°C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec, and 95°C for 5 sec. The reference genes used were *Actb* (β-actin), *Gapdh* (glyceraldehyde-3-phosphate

dehydrogenase), and *Hprt* (Hypoxanthine-guanine phosphoribosyltransferase). Positive (BH) and negative controls (BH no RT) were added to the amplification run as well as one water blank. Quantification values were generated only from samples showing a single product at the expected melting point.

Table 1. Primer sequences for single cell PCR and qPCR.

Gene Name	Product Length	Primer Eff. (%)	Primer sequence	Base pair #	Accession #
Agrp	146	105	F: CTCCACTGAAGGGCATCAGAA R: ATCTAGCACCTCCGCCAAA	287-307 414-432	NM_007427.2
Actb	63	100	F: GCCCTGAGGCTCTTTTCCA R: TAGTTTCATGGATGCCACAGGA	849-867 911-990	NM_007393.3
Cpt1c	191	96	F:GGCTGGCATTGGTCAGAATC R:CGTGCAACCTCAGGAAGTC	719-738 892-910	NM_153679.2
Foxo1	243	96	F:CAATGGCTATGGTAGGATGG R:TTTAAATGTAGCCTGCTCAC	2208-2227 2431-2450	NM_019739
Gapdh	98	93	F: TGACGTGCCGCCTGGAGAAA R: AGTGTAGCCCAAGATGCCCTTCAG	778-797 852-875	NM_008084.2
*Ghsr**	122	111	F: CAGGGACCAGAACCACAAAC R: AGCCAGGCTCGAAAGACT	1003-1022 1107-1124	NM_177330
Hprt	117	107	F:GCTTGCTGGTGAAAAGGACCTC TCGAAG R:CCCTGAAGTACTCATTATAGTC AAGGGCAT	631-658 718-747	NM_013556
*Npy**	182	100	F: ACTGACCCTCGCTCTATCTC R: TCTCAGGGCTGGATCTCTTG	106-125 268-287	NM_023456
Ucp2	194	105	F: CATTGGCCTCTACGACTC R: CGACAGTGCTCTGGTATC	668-685 844-861	NM_011671

Forward primer (F) is listed first with the reverse primer (R) second. AgRP, agouti-related peptide; Cpt-1c, carnitine palmitoyltransferase 1c; Foxo1, forkhead box O1; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ghsr, Growth Hormone Secretagogue Receptor; Hprt, hypoxanthine-guanine phosphoribosyltransferase; NPY, neuropeptide Y; Ucp2, uncoupling protein 2. *also used for single-cell PCR.

Final relative quantitation was calculated using a calibrator of diluted (1:20) cDNA from the BH of an untreated, intact male. The data were reported as relative mRNA expression. To determine the C_q for each transcript, the threshold was consistently set at the lowest point of the exponential curve where the slope of the curve was the steepest for all plates. All gene expression data were calculated using the geometric mean of the C_q values for *Actb*, *Gapdh*,

and *Hprt* to calculate the ΔC_q. The relative linear quantity of target molecules was calculated using the formula $2^{-\Delta\Delta^{CT}}$. The *n*-fold difference was averaged for each treatment.

For quantification of gene expression in NPY pools, two target genes and one reference gene were analyzed in duplicate from each pool of neurons using 4 μl of sample per well with 45 cycles of amplification (Bosch et al., 2013; Roepke et al., 2008, 2011). Relative gene expression was analyzed by first averaging the relative gene expression of 3 pools from each animal. The pool average was then analyzed within each treatment (n = 6 animals, n = 3 pools/animal). Due to the small sample volume (25 μl), only *Gapdh* was used as a reference gene.

2.5. Preparation of Basal Hypothalamic Slices

Slices for electrophysiology were prepared as described previously (Roepke et al., 2012, 2011). Transgenic GFP-NPY mice were killed quickly by decapitation at 1000 h. The brain was rapidly removed from the skull and a block containing the BH was immediately dissected. The BH block was submerged in cold (4°C) oxygenated (95% O_2, 5% CO_2) high-sucrose aCSF (aCSF; in mmol): 208 sucrose; 2 KCl; 26 $NaHCO_3$; 10 glucose; 1.25 NaH_2PO_4; 2 $MgSO_4$; 1 $MgCl_2$; 10 HEPES (pH 7.3; 300 mOsm). Coronal slices (250 μm) from the BH were cut on a vibratome and bathed for ~10 min in high-sucrose CSF at 4ºC. The slices were then transferred to an auxiliary chamber in which they were kept at room temperature (25°C) in aCSF consisting of (in mM): 124 NaCl; 5 KCl; 2.6 NaH_2PO_4; 2 $MgCl_2$; 2 $CaCl_2$; 26 $NaHCO_3$; 10 glucose (pH 7.35; 310 mOsm) until recording (recovery for 1-2 h). A single slice was transferred to the recording chamber which was mounted on an Olympus BX51W1 upright microscope equipped with video-enhanced, infrared-differential interference contrast (DIC) and Exfo X-Cite 120 Series fluorescence light source (Mississauga, Ontario, Canada). The slice was continually perfused

with warm (35°C), oxygenated aCSF at 1.5 ml/min. Targeted neurons were viewed using both IR-DIC and blue excitation light with an Olympus 40X water-immersion lens.

2.6. Visualized Whole-cell Patch Recording

Normal aCSF and pipette solutions were used in electrophysiological recording (Roepke et al., 2012, 2011). Standard whole-cell patch recording procedures and pharmacological testing were as previously described (Roepke et al., 2012, 2011). Whole-cell voltage and current clamp recordings were performed using pipettes made of borosilicate glass and pulled using a P-97 Flaming/Brown Micropipette Puller (Sutter Instrument). Pipettes were filled with normal internal solution consisting of (in mM): 10 NaCl; 128 K-gluconate; 1 $MgCl_2$; 10 HEPES; 1 ATP; 1.1 EGTA; 0.25 GTP (pH 7.3; 300 mOsm) with a 3-5 MΩ resistance. An Axopatch 200A amplifier, Digidata 1322A Data Acquisition System, and pCLAMP software (version 9.2, Molecular Devices) were used for data acquisition and analysis. Input resistance, series resistance, and membrane capacitance were monitored throughout the experiments. Only cells with stable series resistance (< 30 MΩ, < 20% change) and an input resistance > 500 MΩ were used for analysis. The access resistance was 80% compensated, and the calculated liquid junction potential (10 mV) was corrected. To display reversal potential and rectification characteristics of the ligand-activated currents, I-V plots were constructed by voltage steps from -50 to -140 mV at 10 mV increments applied at 1 s intervals from a holding potential of -60 mV. The input resistance was determined from the I-V plot as the ratio of the voltage (-60 to -80 mV) divided by the change in current (pA). In voltage clamp, a deactivation protocol was used to measure the M-current. The deactivation protocol measured the currents elicited during 500 ms voltage steps from -25 to -75 mV after a 300 ms prepulse to -20 mV, which included the membrane potential at which the maximal M-current could be obtained (Roepke et al., 2012, 2011). The

amplitude of M-current relaxation or deactivation was measured as the difference between the initial (< 10 ms) and sustained current (> 475 ms) of the current trace in the control conditions (TTX only, 0.5 μM, 5 min) minus the difference in ghrelin (100 nM, 0.5 μM TTX, 10 min) conditions. The deactivation protocol was repeated twice for each bath solution and averaged for analysis.

2.7. Hormone Serum Analysis

Terminal trunk blood was collected for each WT animal immediately after decapitation. Blood samples were collected using K^+ EDTA tubes. The protease inhibitor AEBSF (1 mg/ml) was quickly added to the sample and mixed. Plasma was isolated after centrifugation and the supernatant was stored at -20°C until analysis. Total ghrelin (EZRGRT-91K; Millipore, Billerica, MA) was measured as per manufacturer's instructions. Total plasma estradiol levels were measured using Mouse/Rat Estradiol ELISA kit (ES180S-100) (Calbiotech, Spring Valley, CA).

2.8. Data Analysis

All data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA) and were expressed as mean ± SEM. In all cases, effects were considered significant at $\alpha \leq 0.05$. All data from quantitative real-time PCR experiments were analyzed using a two-way ANOVA followed by a *post-hoc* Bonferroni multiple comparison test with gene and treatment (fasting, diet, or steroid) as the two factors. Hormone data were analyzed by a two-way ANOVA followed by a *post hoc* Bonferroni multiple comparison test or a Student's *t*-test (unpaired). Comparisons of the I-V plots between control and ghrelin perfusion were performed using a

repeated measures, two-way ANOVA analysis with the *post hoc* Bonferroni multiple comparison test.

3. Results

3.1. Linearity of Ghsr expression in NPY neurons

To initially determine the detectability of *Ghsr* in NPY neurons and its regulation by fasting in males, we harvested individual GFP-NPY neurons from 6 fed males and 6 fasted males and conducted single-cell RT-PCR for *Ghsr* (Fig. 1*A* and *B*). Fasting increased the detection of *Ghsr* from 63.3 ± 4.2% in fed males to 86.7 ± 4.2% in fasted males ($p < 0.01$, Fig. 1B). These data suggest that fasting increases the expression of *Ghsr* specifically in NPY neurons. Furthermore, to determine if the linearity of mRNA expression in pools of NPY neurons compared to that in single neurons, we quantified the expression of *Npy*, *Ghsr*, and *Gapdh* in collection tubes containing 1, 2, 4, or 8, cells/tube from fasted males (n = 6 tubes/cell number from 2 males). Using 4 μl of pool cDNA, NPY primers produced C_q values of 29.8 ± 0.3, 28.8 ± 0.3, 27.8 ± 0.2, and 27.1 ± 0.2, for 1, 2, 4, and 8, cells/tube, respectively. GHSR primers, using 4 μl of pool cDNA, produced C_q values of 36.3 ± 0.2, 35.1 ± 0.1, 34.0 ± 0.2, and 33.0 ± 0.1 for 1, 2, 4, and 8, cells/tube, respectively. GAPDH primers, using 2 μl of pool cDNA, produced C_q values of 32.3 ± 0.2, 31.2 ± 0.4, 30.6 ± 0.3, and 29.4 ± 0.1 for 1, 2, 4, and 8, cells/tube, respectively (Fig. 2*C*). These data suggest that as NPY cell number increases, the quantification of *Npy*, *Ghsr*, and *Gapdh* gene expression increases linearly.

3.2. Effects of Fasting on Gene Expression and Ghrelin

Previously, fasting has been shown to affect NPY neuronal activity (Roepke et al., 2011; Takahashi and Cone, 2005), gene expression (Palou et al., 2009; Roepke et al., 2011), and synaptic inputs (Dietrich and Horvath, 2013). Fasting has also been shown to increase the expression of *Ghsr* expression in the hypothalamus (Kim et al., 2003). To determine if fasting had a specific effect on the expression of the genes involved in the GHSR signaling pathway in the ARC and specifically in NPY neurons, both WT males and GFP-NPY males were either fed or fasted for 24 h prior to tissue collection or cell harvesting. In the ARC, there was a significant effect of fasting ($F(1, 66) = 50.26$, $p < 0.0001$; (Fig. 2*A*)), gene ($F(5, 66) = 8.51$, $p < 0.0001$), and fasting*gene ($F(5, 66) = 8.064$, $p < 0.0001$). Fasting increased the expression of *Npy* ($p < 0.05$), *Agrp* ($p < 0.0001$), *Ghsr* ($p < 0.0001$), and *Ucp2* ($p < 0.05$). In NPY neurons, there was a significant effect of fasting ($F(1, 59) = 38.36$, $p < 0.0001$; (Fig. 2*B*)), gene ($F(5, 59) = 8.866$, $p < 0.0001$), and fasting*gene ($F(5, 59) = 6.934$, $p < 0.0001$). Fasting increased *Npy* ($p < 0.05$), *Agrp* ($p < 0.0001$), *Ghsr* ($p < 0.0001$), and *Foxo1* ($p < 0.001$) expression, but decreased *Ucp2* expression. As expected, fasting increased plasma ghrelin concentrations in males by ~ 73% ($p < 0.001$, Fig. 2*C*).

3.3. Effects of DIO on gene expression and ghrelin

DIO has been shown to affect NPY neuronal activity, gene expression, and ghrelin sensitivity in the ARC (Briggs et al., 2014, 2013, 2010). To determine if DIO had an effect on the GHSR signaling pathway in the ARC and in NPY neurons, WT males and GFP-NPY males were fed a low-fat diet (ND) or a high-fat diet (HFD) for 12 weeks prior to tissue collection or cell harvesting. In the ARC, there was a significant effect of diet ($F(1, 60) = 22.97$, $p < 0.0001$; (Fig. 3*A*)), gene ($F(5, 60) = 2.421$, $p < 0.05$), and diet*gene ($F(5, 60) = 2.644$, $p < 0.05$). DIO suppressed *Npy* ($p < 0.01$), *Agrp* ($p < 0.05$), *Ghsr* ($p < 0.05$), and *Cpt1c* ($p < 0.05$) expression in ARC tissue, In pools of NPY neurons, there was a significant effect of diet ($F(1, 60) = 20.72$, p

387 < 0.0001; (Fig. 3*B*)), gene (F(5, 60) = 3.94, p < 0.01), and fasting*gene (F(5, 60) = 3.337, p =
388 0.01). DIO suppressed *Npy* (p < 0.05), *Agrp* (p < 0.05), *Ghsr* (p < 0.01), and *Cpt1c* (p < 0.05)
389 expression. DIO had no effect on plasma ghrelin concentrations in males (Fig. 3*C*) while
390 doubling cumulative body weight gain over 12 weeks of HFD (ND: 6.5 ± 0.4 g vs. HFD: 13.5 ±
391 1.0 g; p < 0.001; Fig. 3*D*).
392
393 *3.4. Effects of EB and Fasting on Gene Expression and Ghrelin*
394
395 Because E2 is known to modulate ARC gene expression (Roepke et al., 2007; Roepke,
396 2009) including *Ghsr* expression (Frazao et al., 2014), we examined the effects of EB
397 replacement in fed and fasted, ovariectomized (ovx) female mice. To determine if EB had an
398 effect on GHSR signaling pathway in the ARC and in NPY neurons from fed females, WT and
399 GFP-NPY females were ovx and injected with either two daily doses of oil or EB prior to tissue
400 collection or harvesting of cells. In the ARC, there was a significant effect of steroid (F(1, 57) =
401 39.59, p < 0.0001; (Fig. 4*A*)), gene (F(5, 57) = 6.416, p < 0.0001), and steroid*gene (F(5, 57) =
402 5.613, p < 0.001). In fed females, EB replacement increased *Agrp* (p < 0.05), *Ghsr* (p < 0.01),
403 *Cpt1c* (p < 0.001), and *Foxo1* (p < 0.0001) expression in the ARC. In NPY pools, there was a
404 significant effect of steroid (F(1, 58) = 7.766, p < 0.01; (Fig. 4*A*), gene (F(5, 58) = 3.354, p <
405 0.01), and steroid*gene (F(5, 58) = 3.533, p < 0.01). Unlike in the ARC, only *Agrp* expression
406 was higher in EB-treated mice (p < 0.01), while EB replacement suppressed *Foxo1* expression
407 (p < 0.05). These data suggest that the positive effects of EB replacement on *Foxo1* expression
408 in the ARC of fed mice are specific to NPY expressing neurons.
409 To determine if fasting altered the effects of E2 replacement on the GHSR signaling
410 pathway in both the ARC and NPY neurons, WT and GFP-NPY females were ovx and injected
411 with either two daily doses of oil or EB and fasted 24 h prior to tissue collection or harvesting of
412 cells. In the ARC, there was a significant effect of steroid (F(1, 60) = 25.47, p < 0.0001; (Fig.

4*C*)), gene (F(5, 60) = 5.652, p < 0.001), and steroid*gene (F(5, 60) = 6.208, p = 0.0001). EB replacement did not increase *Agrp* expression as it had in the fed females nor did EB replacement affect *Npy* and *Ucp2* expression. However, *Ghsr* expression (p < 0.1) was higher in EB-treated females as was *Cpt1c* (p < 0.05) and *Foxo1* (p < 0.0001). In pools of NPY neurons, In the ARC, there was a significant effect of steroid (F(1, 59) = 7.839, p < 0.01; (Fig. 4*D*)). EB replacement doubled *Foxo1* expression (p < 0.01) but had no effect on any other gene in the pools of NPY neurons.

To determine if fasting had an effect separate from steroid treatment, we analyzed gene expression in ARC tissue within each steroid treatment (oil or EB) using samples from fed and fasted females. As expected, fasting increased *Npy* (p < 0.05) and *Agrp* (p < 0.001) expression in the ARC samples from oil-treated females as well as *Ghsr* (p < 0.05) expression in the ARC (fasting: F(1,58) = 37.36, p < 0.0001; Fig. 4*E*). In EB-treated females, fasting increased *Npy* (p < 0.01) and *Agrp* (p < 0.001) expression but had no effect on any other gene in the GHSR pathway (fasting: F(1,60) = 6.649, p < 0.05; Fig. 4*F*). When diet and steroid were analyzed together for each gene, there were significant effects of diet on *Npy* expression (F(1, 19) = 17.35, p < 0.001) and *Agrp* expression (F(1, 20) = 16.79, p < 0.001). There were also steroid effects on *Ghsr* (F(1, 20) = 4.507, p < 0.05) and a steroid effect (F(1, 20) = 6.894, p < 0.05) and steroid*diet effect (F(1, 20) = 6.894, p < 0.05) on *Cpt1c* expression.

Fasting also increased plasma ghrelin levels in both oil-treated (p < 0.01) and EB-treated (p < 0.001) females while acute EB replacement had no effect on plasma ghrelin levels. There was a significant effect of fasting (F(1,20) = 50.69, p < 0.0001; Fig. 5*A*). There was a significant effect of steroid (F(1, 32) = 89.6, p < 0.0001), fasting (F(1, 32) = 6.281, p < 0.05), and steroid*fasting (F(1, 32) = 4.491, p < 0.05 (Fig. 5*B*). EB replacement 5 days post-ovx increased uterine weights (shown as normalized to body weights) in both fed (p < 0.0001) and fasted (p < 0.0001) females while fasting increased normalized uterine weights of EB-treated females. Interestingly, normalized uterine weights were higher in the fasted females treated with EB than

439 the fed females treated with EB ($p < 0.05$). Plasma E2 levels were higher in EB-treated females
440 ($p < 0.01$; Fig. 5*C*). Fed and fasted samples were reported collectively because no significant
441 differences between fed or fasted females were found (data not shown).
442
443 *3.5. Effects of EB and DIO on gene expression and ghrelin*
444
445 It is well known that E2 replacement suppresses the post-ovariectomy body weight gain in
446 wild type female mice fed a normal chow diet through actions in the CNS including the ARC
447 (Geary et al., 2001; Mamounis et al., 2014; Roepke, 2009). Because DIO and E2 both regulate
448 ARC gene expression (see Figs. 3 and 4), we examined the combined effects of DIO and EB
449 replacement in ovx females by feeding them either a low-fat (10%) or high-fat (45%) diet with
450 oral dosing with oil or EB for 8 weeks. The cumulative body weight gain in both diets was
451 suppressed by oral EB dosing ($p < 0.001$ in ND and $p < 0.0001$ in HFD) and greater in females
452 fed a HFD compared to the ND-fed females ($p < 0.0001$ in oil and $p < 0.01$ in EB, Fig. 6*A*).
453 There was a significant effect of diet ($F(1, 28) = 55.36$, $p < 0.0001$), steroid ($F(1, 28) = 74.44$, p
454 < 0.0001), and steroid x diet ($F(1, 28) = 4.734$, $p < 0.05$). There was no difference in weekly
455 energy intake between oil and EB-treated animals within each diet although, as expected,
456 females fed a HFD had a higher weekly energy intake than the ND-fed females (ANOVA:
457 $F(3,24) = 3.681$, $p < 0.05$; data not shown). Plasma E2 levels, collected 24 h after the last
458 dosing of EB, were higher in both EB-treated females than oil-treated (steroid: $F(1, 28) = 16.70$,
459 $p < 0.001$; Fig. 6*B*). Uterine weights (normalized to body weight) were also higher in EB-treated
460 females ($p < 0.0001$ for both diets) compared to oil-treated (steroid: $F(3,28) = 27.14$, $p < 0.0001$;
461 Fig. 6*C*). Elevated uterine weights are a standard biomarker for physiological levels of E2 (and
462 EB). Plasma ghrelin levels were suppressed by EB replacement in the ND-fed females ($p <$
463 0.05) but not in the HFD-fed females, although ghrelin levels in HFD-EB treated females were
464 significantly lower than the ND-oil treated females ($p < 0.01$). HFD alone decreased plasma

ghrelin levels in oil-treated females ($p < 0.05$) but not in EB-treated females (diet: $F(1,28) = 5.237$, $p < 0.5$; steroid: $F(1,28) = 14.34$, $p < 0.001$; Fig. 6*D*).

In the ARC, neither diet nor steroid had an effect on *Npy* or *Foxo1* expression (Fig. 6*E*). Both diet and EB had an effect on *Agrp* expression (steroid: $F(1, 28) = 11.20$, $p < 0.01$; diet: $F(1, 28) = 11.40$, $p < 0.01$). EB replacement did increase *Agrp* expression by threefold in ND-fed females ($p < 0.01$), which was abrogated in the HFD-fed females. HFD in oil-treated females suppressed *Agrp* expression ($p < 0.05$). EB replacement alone altered *Ghsr* expression in the ARC by two- to threefold in both ND-fed ($p < 0.01$) and HFD-fed ($p < 0.01$) females ($F(1,28) = 20.15$, $p < 0.001$). Regardless of steroid, HFD (oil: $p < 0.05$, EB: $p < 0.05$) suppressed *Ucp2* expression in the ARC to ~ 25% of ND-fed females ($F(1, 28) = 18.30$, $p < 0.001$). There was also an effect of diet on *Cpt1c* expression (diet: $F(1, 28) = 4.726$, $p < 0.05$).

In pools of NPY neurons, steroid and diet had no effect on *Npy*, *Ghsr*, *Ucp2,* or *Foxo1* expression (Fig. 6*F*), but there was interaction of steroid and diet on *Npy* and *Ghsr* expression (*Npy*: $F(1, 20) = 4.472$, $p < 0.05$; *Ghsr*: $F(1, 20) = 4.865$, $p < 0.05$). Both steroid and diet altered *Agrp* expression (steroid: $F(1, 20) = 28.64$, $p < 0.0001$; diet: $F(1, 20) = 21.37$, $p < 0.001$; steroid x diet: $F(1, 20) = 5.163$, $p < 0.05$). *Agrp* expression was augmented in NPY neurons by EB treatment in ND-fed females ($p < 0.001$) and suppressed by HFD in both oil-treated ($p < 0.05$) and EB-treated ($p < 0.001$) females. As in the acute EB-treated females from the Experiment #3 and unlike in the ARC, *Cpt1c* expression in NPY pools was augmented twofold in HFD-fed, oil-treated females ($p < 0.01$) (diet: $F(1, 20) = 12.30$, $p < 0.01$; steroid x diet: $F(1, 20) = 5.778$, $p < 0.05$), indicating that this gene is regulated by HFD (DIO) differentially in NPY neurons compared to the heterogeneous ARC neuronal population.

3.6. Ghrelin Inhibits the M-current in NPY neurons

Previously, we characterized the regulation of the M-current in NPY neurons by fasting in

male mice and E2 replacement in female mice (Roepke et al., 2011). Since ghrelin has recently been shown to inhibit the M-current in striatal neurons (Shi et al., 2013), we hypothesized that ghrelin would also affect the M-current in NPY neurons. Using whole-cell patch clamp electrophysiology in GFP-NPY from fed male mice, ghrelin perfusion depolarized 70.6% (12/17) of NPY neurons by 5.8 ± 0.9 mV (Fig. 7*A*). To determine the effects of ghrelin on M-current activity, we used standard voltage clamp deactivation protocol (Fig. 7B) (Roepke et al., 2012, 2011). Perfusion of ghrelin (100 nM) suppressed the potassium current elicited by the deactivation protocol after 10 min (ghrelin: $F(1,22) = 10.90$, $p < 0.01$; Fig. 7*C*). The peak current at -35 mV was suppressed by ghrelin perfusion by ~50% (12/17, $p < 0.0001$). Furthermore, there was a significant interaction between treatment and responders (ANOVA: $F(1, 30)=18.18$, $p < 0.001$). Interestingly, the peak current in non-responders under control conditions was lower than the responders ($p<0.05$), but after ghrelin perfusion, the peak current was higher in the non-responders ($p<0.05$). Ghrelin perfusion did not affect the M-current in the NPY neurons ($n = 5$) that did not respond to ghrelin perfusion (5/17, Fig. 7*D*).

4. Discussion

Understanding the impact of caloric restriction and high fat diets on the neuroendocrine control of energy homeostasis is key to addressing the obesity epidemic and other metabolic disease. Most studies in this field only examine male rodent models due, in part, to the influence of circulating E2 on energy homeostasis in females during the estrous cycle. Another weakness of this field is the prevalence of studies examining genomic effects of these conditions on the heterogeneous nuclei of the hypothalamus without regard to potential differences in cell-type responses. Therefore, we set out to illustrate the importance of cell-type specific effects and steroid-driven sex differences in the neuroendocrine responses to caloric restriction and DIO using specific neuronal pools coupled with quantitative real-time PCR in intact males and ovx

females with or without E2 replacement. We clearly demonstrate that the regulation of the GHSR signaling pathway by fasting and DIO in both ARC tissue and in pools of NPY neurons occurs in a sex-dependent manner due to the regulation of these genes by E2.

Table 2. A summary of results

	Male		Female						
			Fed vs. Fasted				ND vs. HFD		
Gene Name	Fast	HFD	Oil Fast	EB Fast	Fed EB	Fast EB	Diet	EB	Diet x EB
Npy	↑Arc ↑NPY	↓Arc ↓NPY	↑Arc	↑Arc					* NPY
Agrp	↑Arc ↑NPY	↓Arc ↓NPY	↑Arc	↑Arc	↑NPY		* Arc * NPY	* Arc * NPY	* NPY
Ghsr	↑Arc ↑NPY	↓Arc ↓NPY		↑Arc				* Arc	* NPY
Ucp2	↑Arc ↓NPY						* Arc		
Cpt1c		↓Arc ↓NPY					* Arc * NPY		* NPY
Foxo1	↑NPY				↓NPY	↑NPY			
	Fig. 2	Fig. 3	Fig. 4E	Fig. 4	Fig. 4B	Fig. 4D	Fig. 6E & 6F		

For male fasted and HFD and female fasted experiments: ↑ denotes an increase in expression; ↓ denotes a decrease in expression. For female ND vs. HFD: * denotes significant effect of diet, EB, or interaction. ARC = arcuate nucleus. NPY = pools of NPY neurons. Treatment headings are in comparison to Fed and ND for males. For female fed vs. fasted, Fast is in comparison to fed within steroid treatment in the ARC and EB is in comparison to oil within fed or fasted state in NPY pools.

We showed that fasting and DIO have opposing effects on *Npy*/*Agrp* and *Ghsr* expression in the ARC that is recapitulated in pools of NPY neurons from male mice. However, two genes involved in GHSR signaling, *Ucp2* and *Foxo1*, were differentially regulated in between the ARC and NPY neurons in fasted males. EB replacement in fed, ovx females increased *Agrp*, *Ghsr*, *Cpt1c*, and *Foxo1* in the ARC but only *Agrp* in pools of NPY neurons. In fasted females, EB replacement also increased *Ghsr*, *Cpt1c*, and *Foxo1* expression in the ARC but only *Foxo1* in NPY pools. In ovx, ND-fed females, EB replacement augmented *Agrp*, which was abrogated by HFD in both the ARC and NPY pools; however, regulation of *Ghsr* expression by EB was not affected by HFD in the ARC nor did EB increase *Ghsr* expression in NPY pools. Therefore, our data suggest that fasting, HFD (DIO), and E2 have differential effects on GHSR signaling in

between the heterogeneous ARC and homogenous pools of NPY neurons in a sex-specific manner. A summary of the differential regulation of GHSR signaling molecules is presented in Table 2.

4.1. The Effects of Fasting in Males

Fasting alters ARC gene expression and, in particular, increases expression of orexigenic neuropeptides *(Npy* and *Agrp*) and genes involved in ghrelin signaling (*Ghsr*, *Ucp2*) (Briggs et al., 2013, 2010; Coppola et al., 2007; Palou et al., 2009; Verhulst et al., 2012). In our study, fasting increased *Npy*, *Agrp*, *Ghsr*, and *Ucp2* expression in the ARC. In pools of NPY neurons, fasting increased the neuropeptides and *Ghsr* but decreased *Ucp2* expression. In our study and others (Verhulst et al., 2012), fasting increased plasma ghrelin, a potent orexigenic peptide hormone. Presumably, the elevation of plasma ghrelin and *Ghsr* expression in NPY neurons intensifies neuronal sensitivity to ghrelin to drive the post-fasting hyperphagia (Bagnasco et al., 2003; Becskei et al., 2009; Kinzig et al., 2009; Teubner et al., 2013) and augment *Npy*/*Agrp* expression, which is found in fasted male mice (Verhulst et al., 2012).

Ghrelin binding to GHSR leads to the activation of an AMPK-mediated cascade that stimulates CPT-1, which acylates carnitine with palmitic acid and other long-chain fatty acids. CPT-1 (along with CPT-2) allows the translocation of acyl-carnitine (palmitoylcarnitine) from the cytosol into the mitochondrial matrix for beta-oxidation (Andrews et al., 2008; López et al., 2008a; Wolfgang and Lane, 2011; Wolfgang et al., 2008, 2006). Fatty acid beta-oxidation leads to an increase in reactive oxygen species (ROS), which trigger UCP2 to buffer ROS and promote mitochondrial biogenesis (Andrews et al., 2008; Du et al., 2006; Lam et al., 2005). An increase in UCP2 activity also enhances the efficiency of the transcription of *Npy*/*Agrp* genes, leading to an increase in orexigenic behavior (Andrews et al., 2008). Interestingly, the elevated plasma levels of ghrelin are not correlated with an increase in *Cpt1c* gene expression, which

564 has been previously reported for *Cpt1* (Andrews et al., 2008). Although hypothalamic malonyl-
565 CoA levels decrease during fasting (Hu et al., 2003; Wolfgang et al., 2007), the expression of
566 *Cpt1c* in the ARC and in NPY neurons during fasting is not sensitive to this change.

567 Greater activation of the GHSR signaling cascade due to elevated ghrelin during fasting will
568 augment uncoupling respiration (UCP2 activity) in the mitochondria. Fasting increases *Ucp2*
569 expression in the ARC and in cortical mitochondria (Coppola et al., 2007; Davis et al., 2008), as
570 does ghrelin in the hypothalamus (Andrews et al., 2008). However, *Ucp2* expression is
571 suppressed by fasting in pools of NPY neurons by ~50%. This decrease in *Ucp2* expression
572 contradicts the hypothesis that UCP2 is required for the ghrelin-induced increase in *Npy*/*Agrp*
573 gene expression, neuronal activity, and the drive to feed during negative states of energy
574 balance (fasting) (Andrews et al., 2008), although a reduction in gene expression does not
575 necessarily correlate with a reduction in UCP2 activity, as previously reported with ghrelin
576 administration (Lage et al., 2010).

577 Alternatively, the decrease in *Ucp2* expression, which could interfere with the activation of
578 *Npy*/*Agrp* gene expression, is compensated by the increase in *Foxo1*, the transcription factor
579 that mediates, in part, the regulation of *Npy*/*Agrp* gene expression by ghrelin (Lage et al., 2010).
580 FoxO1 activity in ARC AgRP-expressing neurons regulates locomotor activity, oxygen
581 consumption, food intake, glucose homeostasis, and ghrelin sensitivity (Cao et al., 2011; Kim et
582 al., 2012; Ren et al., 2012). Interestingly, *Foxo1* expression in the ARC was not affected by
583 fasting despite an increase in plasma ghrelin. Previous studies have demonstrated that ghrelin
584 administration augments *Foxo1* expression in the ARC (Lage et al., 2010). However, fasting did
585 increase *Foxo1* expression by two- to threefold in NPY neurons. The difference in expression
586 between the ARC and NPY neurons suggests that the increase in *Foxo1* expression in NPY
587 neurons was offset by a decrease in *Foxo1* expression in other ARC neurons.

588

589 *4.2. The Effects of Diet-induced Obesity in Males*

HFD or DIO induces ghrelin insensitivity in mice (Andrews, 2011; Briggs et al., 2013), possibly through dysregulating the GHSR signaling pathway including CPT-1 and UCP2. For example, HFD or DIO will suppress *Npy*, *Agrp*, and *Ghsr* expression in the ARC in several model species (Briggs et al., 2010; Kurose et al., 2005). Briggs and colleagues (2010) found that DIO suppressed both hypothalamic *Ghsr* expression and ghrelin (total and active) in male mice (Briggs et al., 2010), unlike in our study where DIO had no effect on total ghrelin. However, another study in male rats fed a HFD from weaning to 6 months of age found that hypothalamic *Ghsr* expression was not altered compared to ND-fed males (Priego et al., 2009). We have demonstrated in our study that by 12 weeks of DIO suppressed *Npy*, *Agrp*, and *Ghsr* in both the ARC and specifically in NPY pools. The decrease in NPY neuronal expression of *Ghsr* is a potential mechanism underlying diet-induced ghrelin resistance and NPY insensitivity (Briggs et al., 2013, 2010). The decrease in expression of ghrelin's receptor would certainly lead to attenuation of the fasting-induced hyperphagia (Briggs et al., 2010).

Unlike in fasted males, *Ucp2* expression was unchanged in the ARC of HFD-fed males compared to ND-fed males. Previous studies have demonstrated that whole hypothalamic *Ucp2* expression is elevated by prolonged (33 weeks) HFD in male mice (Kocalis et al., 2012). It is possible that *Ucp2* levels remained unchanged in our study due to differences in expression amongst the various hypothalamic nuclei. Alternatively, unaltered UCP2 levels will serve to maintain mitochondrial ROS buffering capacity in response to elevated fatty acid beta-oxidation due to excess lipids from the HFD. On the other hand, *Cpt1c* expression was suppressed in both ARC and in NPY pools. The reduction in *Cpt1c* expression in HFD-fed males is not involved in fatty acid beta-oxidation during DIO but may be relevant to the malonyl-CoA sensing in the mitochondria and its role in hypothalamic glucose-sensing (Wolfgang et al., 2006). Therefore, the reduction in *Cpt1c* may play a role in the dysregulation of glucose homeostasis by the ARC during DIO.

4.3. Integration of the Effects of 17β-estradiol, Fasting, and DIO in Females

E2 replacement in multiple models and replacement paradigms suppresses *Npy* expression ((Dhillon and Belsham, 2011, reviewed in Brown and Clegg, 2010; Roepke, 2009), NPY orexigenic activity (Santollo and Eckel, 2008), and *Agrp* expression (Cheng et al., 2009; Olofsson et al., 2009). However, in our study, acute EB treatment did not alter *Npy* expression in fed, fasted, or DIO females. Certainly, the differences between our studies and others involve variations in methodology in E2 replacement (injection, capsules, pellets, oral, etc.), rodent models, housing, and strain effects (Nagy et al., 2002; Simon et al., 2013). With regard to *Agrp* expression, it was augmented by EB in fed females but not in fasted females, indicating that fasting is a primary driver of *Agrp* expression and overwhelms the effects of EB. Furthermore, *Npy* and *Agrp* regulation by fasting in females is independent of EB since fasting augmented the expression of both genes in oil-treated and EB-treated females.

Little is known about the interactions of HFD/DIO and E2/EB on *Npy*/*Agrp* expression in the ARC. In our study, *Npy* is not suppressed by HFD in females in both ARC tissue and NPY pools as it is in male mice, which highlights the importance of examining sex differences in neuroendocrine responses to HFD and obesity. Conversely, *Agrp* expression is augmented by EB in ND-fed females and suppressed by HFD in both oil-treated and EB-treated females, as it is in male mice. In NPY neurons, the suppression of *Agrp* expression by HFD in females is not correlated with a suppression of *Ghsr* as it is in male mice, suggesting that DIO-induced ghrelin resistance occurs through different mechanisms in males and females. Interestingly, acute EB treatment affected more genes in the ARC than long-term EB treatment (See Table 2). This divergent response highlights the difference of the physiological relevance of acute vs. long-term E2 replacement paradigms. In most rodent models, circulating E2 rise and fall during the estrous cycle every 4-5 days. Our acute paradigm is similar to this cyclical pattern and closely

642 mimics the proestrous levels of endogenous E2, while the long-term effects may be
643 compensated for by receptor-mediated mechanism such as ERα downregulation due to long-
644 term steroid exposure (Roepke et al., 2008).

645 The consistent effect of EB treatment, whether acute or long-term, to increase *Agrp*
646 expression in our study is not commonly reported. In many studies, E2 effects on *Agrp*
647 expression or secretion are either suppressive or not significant (reviewed in (Brown and Clegg,
648 2010; Roepke, 2009). However, long-term E2 replacement in female mice did produce an
649 increase in *Agrp* expression in the hypothalamus (Cheng et al., 2009). Furthermore, in clonal
650 hypothalamic neurons that express both NPY and AgRP, E2 treatment augments *Agrp*
651 expression depending on the temporal ratio of ERα/ERβ expression wherein greater ERβ
652 activation produces positive *Agrp* expression by E2 (Titolo et al., 2006) to potentially function as
653 a mediator of peripheral hormone signaling on the HPG axis (Sheffer-Babila et al., 2013). Both
654 acute and long-term E2 treatment suppresses ERα expression in the ARC (Roepke et al., 2008;
655 *Yang et al.,* unpublished data), which may be sufficient to alter the ERα/ERβ ratio leading to an
656 increase in *Agrp* expression.

657 Our data suggests that a unique interaction between circulating E2, ghrelin, and *Ghsr*
658 expression in the rodent ARC during fasting and DIO. In previous studies, plasma ghrelin levels
659 were lower in intact female rats than in ovx females (Clegg et al., 2007). E2 replacement also
660 suppresses ghrelin expression in the stomach (Matsubara et al., 2004). In our study, acute E2
661 did not alter plasma ghrelin in fed or fasted females, but long-term EB replacement did suppress
662 ghrelin in ND-fed females. Plasma ghrelin level was also reduced in HFD-fed, oil-treated
663 females, yet EB replacement did not further suppress ghrelin in HFD-fed females. Others have
664 found that both acute and long-term (8 weeks) peripheral ghrelin administration augmented *Npy*
665 and *Agrp* expression in male and female mice (Egecioglu et al., 2008; Goto et al., 2006). The
666 reduction in plasma ghrelin by HFD or EB in females, without the suppression of *Ghsr*

expression in NPY neurons, may counteract the regulation of *Npy* gene expression typically found by HFD-fed animals.

In fed, fasted, or DIO ovx females, EB augmented the expression of *Ghsr* in the ARC but not in NPY neurons, indicating that E2's action on *Ghsr* expression does not directly affect the ARC melanocortin-neuropeptide Y circuitry. While *Ghsr* is expressed in ARC growth hormone releasing hormone (GHRH) neurons, dopaminergic neurons, and KNDy neurons (Frazao et al., 2014; Osterstock et al., 2010; Willesen et al., 1999; Zigman et al., 2006), the effects of E2 on *Ghsr* expression in these neurons are not fully characterized. Frazao and colleagues (2014) reported that E2 administration in ovx females (through capsules inserted during ovx) increased *Ghsr* expression in the ARC and in *Kiss1*-expressing neurons (Frazao et al., 2014). In our study, fasting only increased ARC *Ghsr* expression in oil-treated females. This suggests that the interaction of fasting and E2 differentially regulates *Ghsr* in the ARC. While *Ghsr* expression does not change during the estrous cycle in female rats, ghrelin's effects on food intake are only found during the diestrus cycle, when E2 levels are lower (Sakurazawa et al., 2013). The differential effect on ghrelin sensitivity and *Ghsr* expression may be due, in part, to changes in expression of downstream effectors of GHSR signaling (e.g., *Cpt1c, Foxo1*) as we have found in the ARC and in NPY pools.

EB did not regulate *Ucp2* expression in the ARC or NPY neurons. We did expect to find EB-induced *Ucp2* regulation because previous studies have reported that E2 alters the expression of *Ucp2* in adipocyte and breast cancer cell lines (Nadal-Serrano et al., 2012). Unlike in males, fasting did not have an effect on *Ucp2* expression in either tissue or cell type in females while HFD suppressed *Ucp2* expression in the ARC of females independent of steroid treatment. The differences in *Ucp2* expression between males and females in response to fasting and HFD highlight again the need to examine sex differences in genes controlling energy homeostasis in the hypothalamus.

To our knowledge, this is the first study reporting an effect of E2 (EB) on expression of brain-specific *Cpt1c* gene in the ARC, although a previous study found a small, but significant, reduction in hypothalamic *Cpt1c* expression by E2, when administered by pellet over 2 months (Cheng et al., 2009). In our study, EB replacement 5 days post-ovx increased *Cpt1c* expression in the ARC regardless of fed state but not in NPY neurons. Presumably, *Cpt1c* is ubiquitously expressed in ARC neurons, and elevated expression of *Cpt1c* is correlated with the increase in ARC *Ghsr* expression by EB in other non-NPY neurons (see discussion above). Elevated *Cpt1c* by EB would augment the ability of ARC neurons to respond to changes in malonyl-CoA concentrations, which correlates with feeding behavior (Hu et al., 2003; Wolfgang et al., 2007). Interestingly, EB treatment and HFD in females increased *Cpt1c* in NPY neurons, unlike in male ARC and NPY pools. The elevation of *Cpt1c* may be involved in the resistance of females to DIO-induced impairments of glucose homeostasis since it acts as a sensor for malonyl-CoA and hypothalamic glucose sensing (Wolfgang et al., 2006).

E2, through its nuclear receptors, impacts FoxO1 signaling in the brain, the reproductive tract, breast cancer cells, and other cell lines via phosphorylation through Akt or Pak1 (Kemper et al., 2014; Koh, 2006; Lengyel et al., 2007; Mazumdar et al., 2003; Won et al., 2006). However, there is no data suggesting that E2 directly regulates the *Foxo1* gene in the brain. In our study, *Foxo1* expression was augmented by acute EB treatment in the ARC in fed and fasted females and in NPY neurons in fasted females. Lage and colleagues (2010) found that central ghrelin administration increased FoxO1 and pFoxO1 protein expression in the female hypothalamus, which was correlated with an increase in *Npy*/*Agrp* expression (Lage et al., 2010). However, in our study, E2 suppressed *Foxo1* expression in NPY neurons in fed females suggesting that EB may blunt the effects of ghrelin in NPY neurons by suppressing *Foxo1* expression in the fed state but augment ghrelin's action through an increase in *Foxo1* in fasted females.

718 *4.4. Ghrelin Inhibits the M-current in NPY Neurons*

719

720 In a previous study, we have shown that fasting suppressed the M-current in NPY neurons
721 in males (Roepke et al., 2011). Recently, ghrelin has been shown to inhibit the M-current in
722 striatal neurons through a PLC-PKC-mediated signaling pathway (Shi et al., 2013). Because
723 ghrelin activates Ca^{2+} channels (N-type) in NPY neurons (Kohno et al., 2007, 2003) and in
724 GHRH neurons (Osterstock et al., 2010) and unidentified voltage-gated K^+ channels in GH3
725 cells (Han et al., 2005), we hypothesized that ghrelin would also inhibit the M-current in NPY
726 neurons. In approximately 70% of NPY neurons from fed males, ghrelin perfusion depolarized
727 NPY neurons while suppressing the activity of the M-current. Presumably, in NPY neurons,
728 ghrelin also inhibits the M-current via a similar PLC-PKC-mediated pathway recently
729 characterized in striatal neurons (Shi et al., 2013). Because the M-current is suppressed
730 transcriptionally (reduction in KCNQ2 and KCNQ3 expression) in NPY neurons by fasting
731 (Roepke et al., 2011) while *Ghsr* expression is augmented (the current study), the role of M-
732 current inhibition in the stimulation of NPY neuronal activity during fasting may be reduced,
733 supplanted by another cation channel (Kohno et al., 2007, 2003), or compensated by the
734 increased *Ghsr* expression and activity. This reduction in the M-current may play a role in the
735 attenuation of the ghrelin-induced hyperphagia after food restriction that is found in DIO male
736 mice and hamsters (Briggs et al., 2010; Teubner et al., 2013).

737

738 **5. Conclusion**

739

740 Our experiments have confirmed the effects of fasting, DIO, and E2 on the expression of
741 *Ghsr*, *Npy,* and *Agrp* in the ARC (Briggs et al., 2013, 2010; Brown and Clegg, 2010; Coppola et
742 al., 2007; Palou et al., 2009; Roepke et al., 2008; Verhulst et al., 2012). However, gene
743 expression in pools of NPY neurons does not fully reflect findings in the heterogeneous ARC for

744 other genes involved in GHSR signaling. Clearly, neuronal cell type should be considered when
745 studying the expression of ubiquitously expressed genes and proteins in hypothalamic nuclei.
746 The quantitative analysis of pools of GFP-tagged neurons by real-time quantitative PCR from
747 treated males and females will greatly enhance our understanding of the sex-dependent, cell-
748 type-specific effects of fasting, DIO, and E2 on hypothalamic homeostatic functions.

749 A final concern is the relevance of peripheral vs. central ghrelin (from BH ghrelin neurons)
750 (Guan et al., 2003) in the activation of NPY neurons and other ARC neurons (Frazao et al.,
751 2014). Peripheral administration of ghrelin does activate ARC NPY neurons (Wang et al., 2002),
752 but does not eliminate the role of catecholamine hindbrain neurons in mediating the actions of
753 peripheral ghrelin on feeding behavior and NPY activation (Date et al., 2006, 2002; Emanuel
754 and Ritter, 2010). Therefore, the alterations in plasma ghrelin may not be a significant
755 contributor to the ARC and NPY changes in gene expression.

756 Results from the current study focusing on the genes involved in the GHSR signaling
757 pathway in NPY neurons provide some insight into the interactions of orexigenic ghrelin
758 signaling and anorexigenic E2 signaling in the control of energy homeostasis in females.
759 Indeed, sex differences in ghrelin and GHSR activity are driven, in part, by the interaction of E2
760 with fasting and DIO (Priego et al., 2009). In summary, these studies emphasize the importance
761 of considering cell type and sex while delineating the effects of ghrelin and potentially other
762 peripheral hormones on hypothalamic gene expression and homeostatic functions.

763

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765

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Figure Legends

Fig. 1. Single cell expression and linearity of Ghsr expression in NPY neurons. Fasting increased the percentage of Ghsr-expressing NPY neurons in males shown here in a representative gel (A) and summarized in a bar graph (B). Numbers in bars represent sample size. (C) Linear regression analysis plotting C_q versus the log scale of the number of cells collected in each pool to determine the linearity of mRNA expression in pools of NPY neurons compared to single neurons for *Npy*, *Ghsr*, and *Gapdh* expression collected from fed males (n=6). Data are represented as mean ± SEM. Bar graph was analyzed with a Student's *t*-test ($P < 0.01$).

Fig. 2. Fasting for 24 hr increased expression of GHSR pathway genes in ARC and NPY neurons in males. (A) Fasting increased *Npy*, *Agrp*, *Ghsr*, and *Ucp2* gene expression in the ARC compared to the fed males. (B) Fasting increased *Npy*, *Agrp*, *Ghsr*, and *Foxo1* gene expression in NPY pools while decreasing *Ucp2* in NPY pools. (C) Fasting also increased plasma ghrelin (ng/ml) levels. Data are represented as mean ± SEM normalized to fed males. Data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test (A & B) or Student's *t*-test (C) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Numbers in parentheses or bars represent sample size.

Fig. 3. DIO suppressed expression of GHSR pathway genes in ARC and NPY neurons in males. (A) DIO decreased expression of *Npy*, *Agrp*, *Ghsr*, and *Cpt1c* in ARC tissue. (B) DIO decreased expression of *Npy*, *Agrp*, *Ghsr*, and *Cpt1c* in NPY pools. (C) DIO had no effect on plasma ghrelin levels. (D) HFD increased cumulative body weight gain over 12 weeks in males. Data are represented as mean ± SEM normalized to ND. Data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test (A & B) or Student's *t*-test (C & D) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Numbers in parentheses or bars represent sample size.

Fig. 4. EB differentially regulated expression of GHSR pathway genes between the ARC and NPY neurons in ovx females. In fed females, (A) EB increased *Agrp*, *Ghsr*, *Cpt1c*, and *Foxo1* expression in the ARC and (B) increased *Agrp* expression and decreased *Foxo1* expression in NPY pools. In fasted females, (C) EB increased *Ghsr*, *Cpt1c*, and *Foxo1* expression in the ARC and (D) increased *Foxo1* expression in NPY pools. (E) In oil-treated ARC, fasting increased *Npy*, *Agrp*, and *Ghsr* expression. (F) In EB-treated ARC, fasting increased *Npy* and *Agrp* expression. Data are represented as mean ± SEM normalized to oil (A-D) or fed (E-F). Gene expression data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Numbers in parentheses represent sample size.

Fig. 5. Plasma levels of ghrelin and E2 in oil- and EB-treated females. (A) Fasting increased plasma ghrelin in both oil- and EB-treated, ovx females. (B) EB increased uterine weights in both fed and fasted, ovx females. (C) Plasma E2 levels were higher in EB-treated than oil-treated females. Data are represented as mean ± SEM. Data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test (A & B) or Student's *t*-test (C) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). Numbers in bars represent sample size.

Fig. 6. Interaction of EB replacement and DIO (HFD) in ovx females on expression of GHSR pathway genes in ARC and NPY neurons. (A) EB replacement suppressed post-ovariectomy weight gain in both ND- and HFD-fed females. (B) Plasma E2 was higher in both ND- and HFD-fed females on oral dosing of EB (300 μg/kg/day). (C) Uterine weights were higher in EB-treated females in both ND- and HFD-fed females. (D) HFD and EB suppressed plasma ghrelin.

Asterisks denote comparison to ND-oil. (E & F) Interactions of EB treatment and HFD on gene expression in ARC tissue and NPY pools. Weight and hormone data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). Gene expression data were analyzed by a two-way ANOVA with *post hoc* Bonferroni multiple comparison test within genes (common letters denote similar expression). Data are represented as mean ± SEM normalized to ND-oil. Numbers in bars or parentheses represent sample size.

Fig. 7. Ghrelin suppresses the activity of the M-current in NPY neurons from fed males. (A) 70% (12/17) of NPY neurons were depolarized (+5.8 ± 0.9 mV) by ghrelin (100 nM). (B) Representative traces of the deactivation protocol from NPY neurons in control conditions (TTX, 500 nM) and after ghrelin perfusion (10 min). (C) I-V plot of the deactivation traces illustrates the decrease in current after ghrelin perfusion. (D) Control peak current was 55.8 ± 3.6 pA, and ghrelin peak current was 37.4 ± 3.5 pA in the 12 responders. Letter denotes comparison between control and ghrelin. Data are represented as mean ± SEM. Numbers in bars (A & D) represent sample size. Data (C & D) were analyzed by a two-way ANOVA (C: repeated measures) followed by *post hoc* Bonferroni multiple comparison test (**c** = P < 0.001; **d** = P < 0.0001).

Differential gene regulation of GHSR signaling pathway in the arcuate nucleus and NPY neurons by fasting, diet-induced obesity, and 17β-estradiol

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Highlights

1. Fasting in males had differential effects on expression of *Foxo1* and *Ucp2* expression.
2. In males, DIO suppressed ARC and NPY *Ghsr* and *Cpt1c* expression.
3. In females, E2 augmented ARC *Ghsr*, *Cpt1c*, and *Foxo1* but suppressed NPY *Foxo1*.
4. In females, DIO suppressed ARC *Agrp* and augmented NPY *Cpt1c*.
5. Ghrelin suppressed M-current activity by ~50% in NPY neurons from fed male mice.

Figure 1

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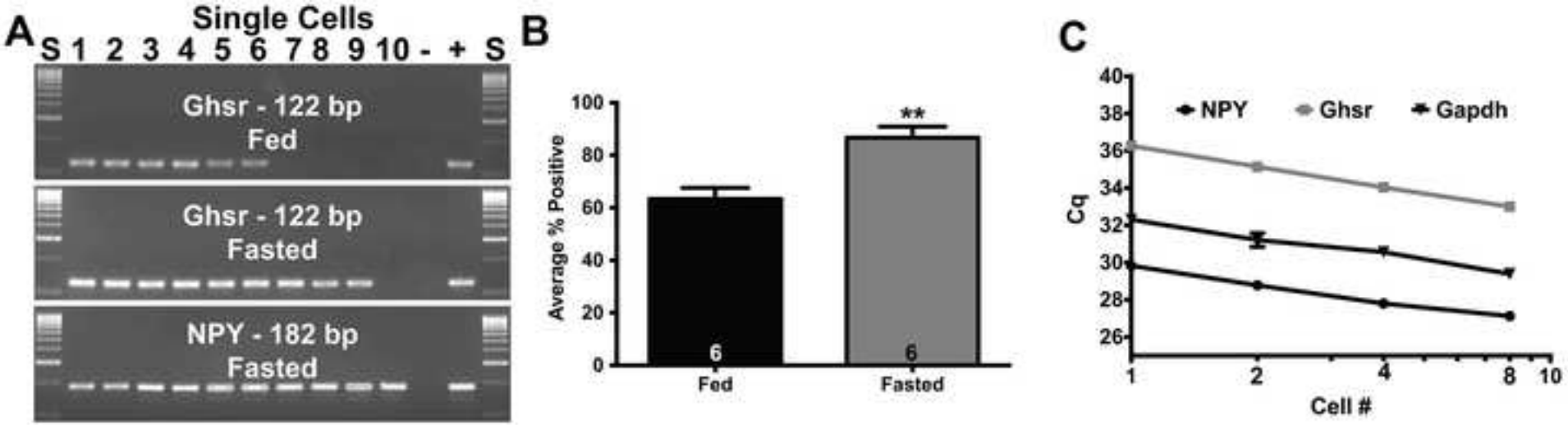

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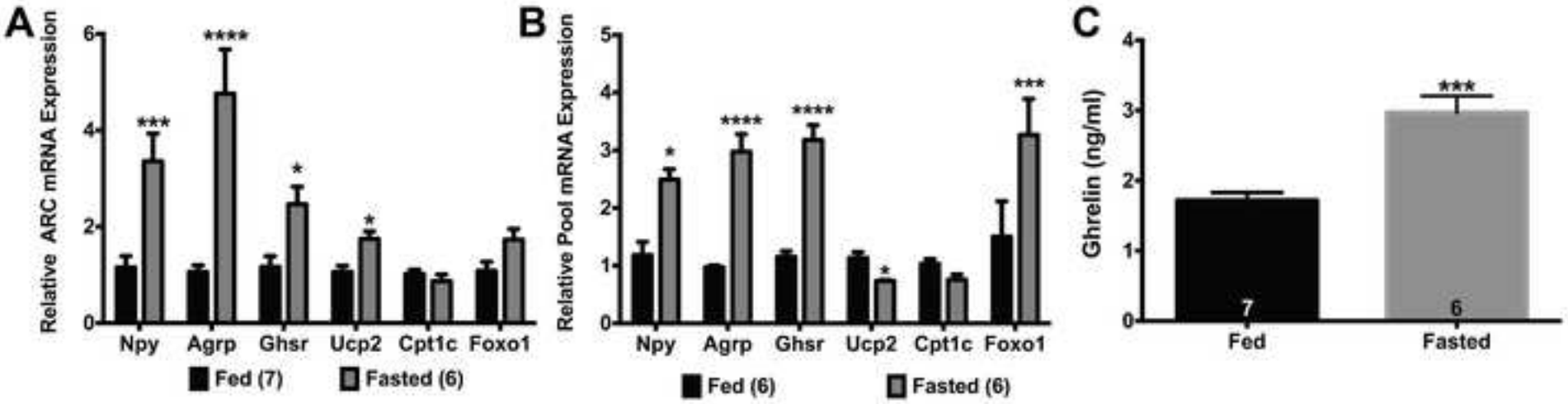

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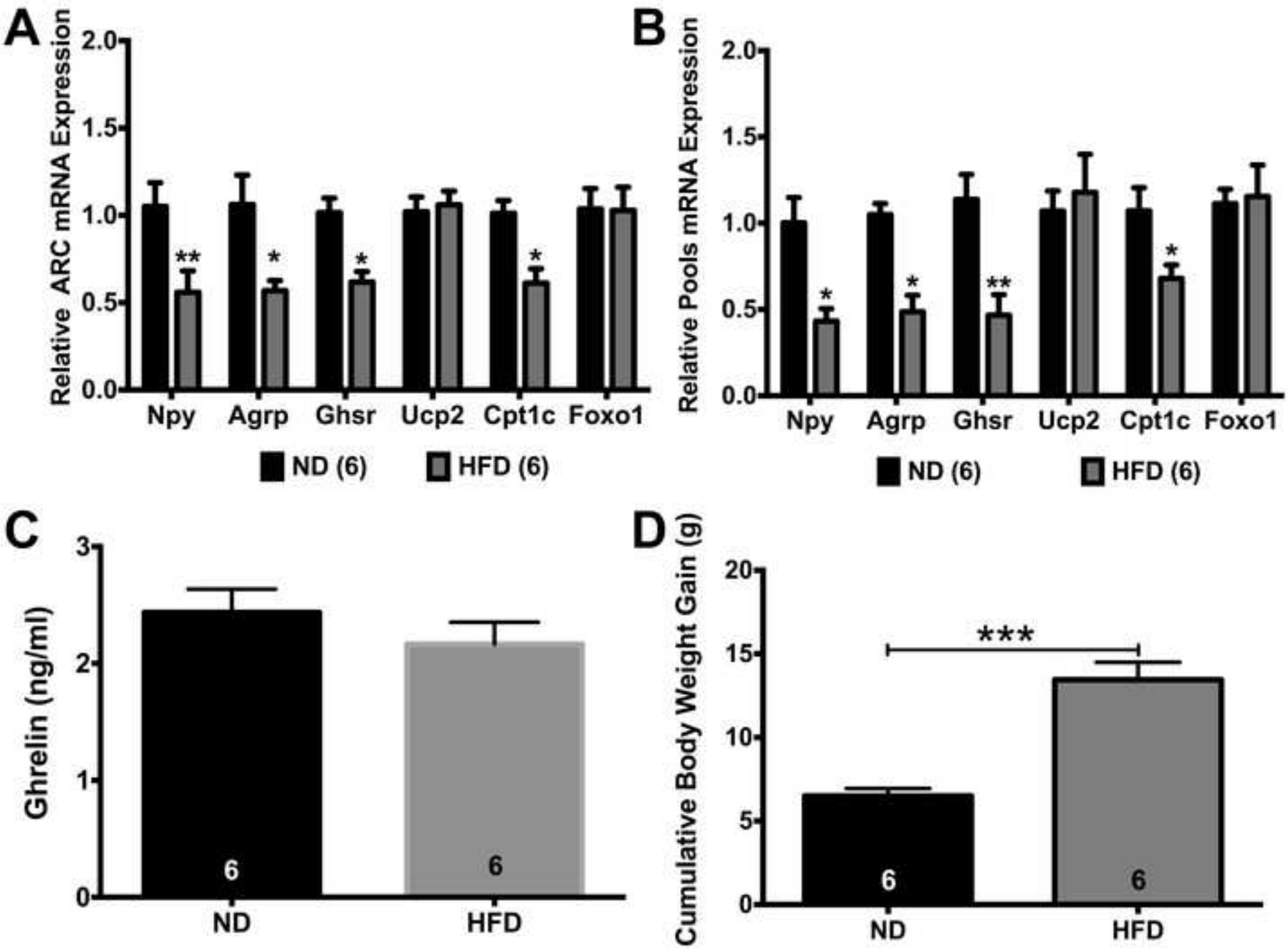

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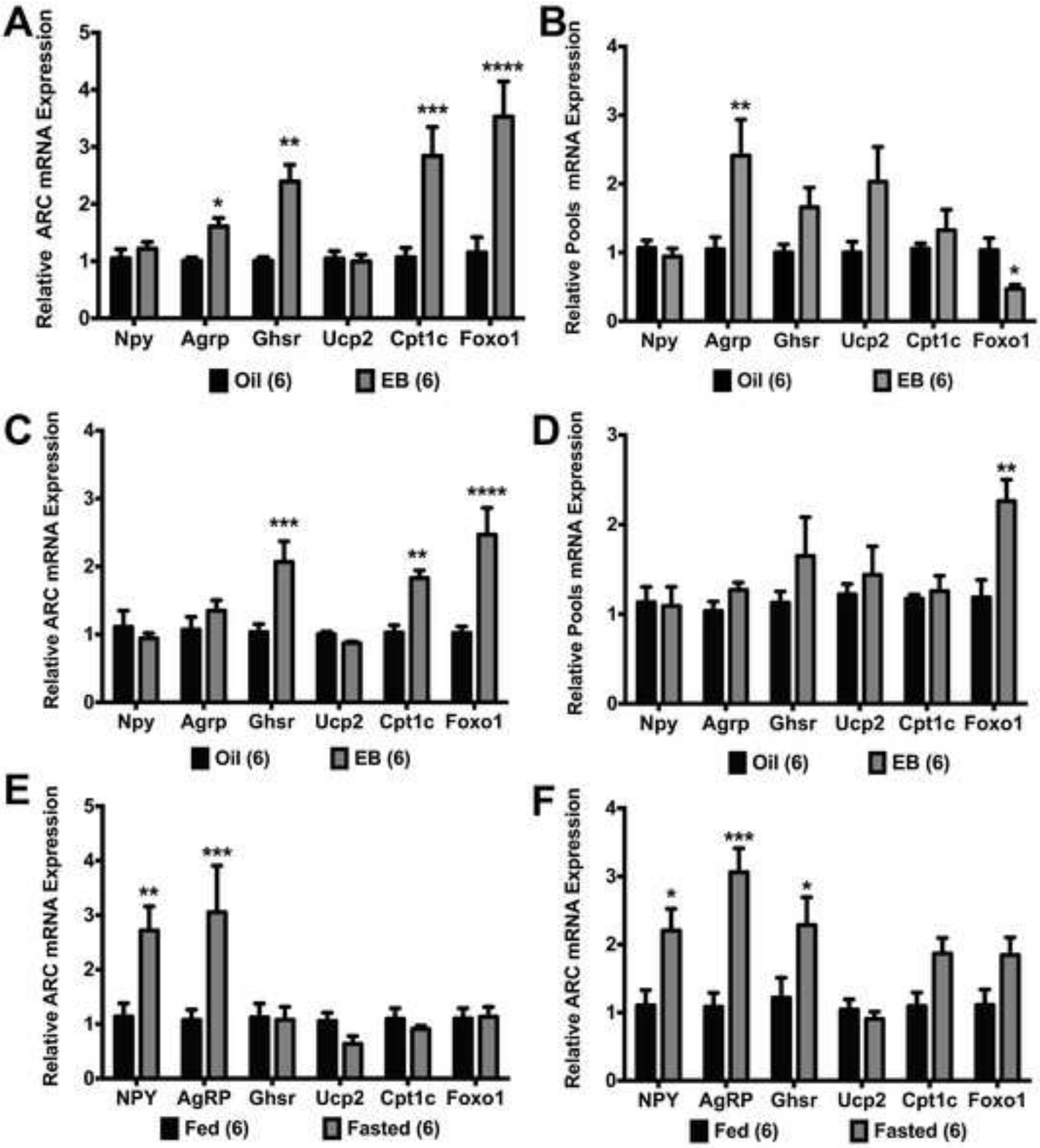

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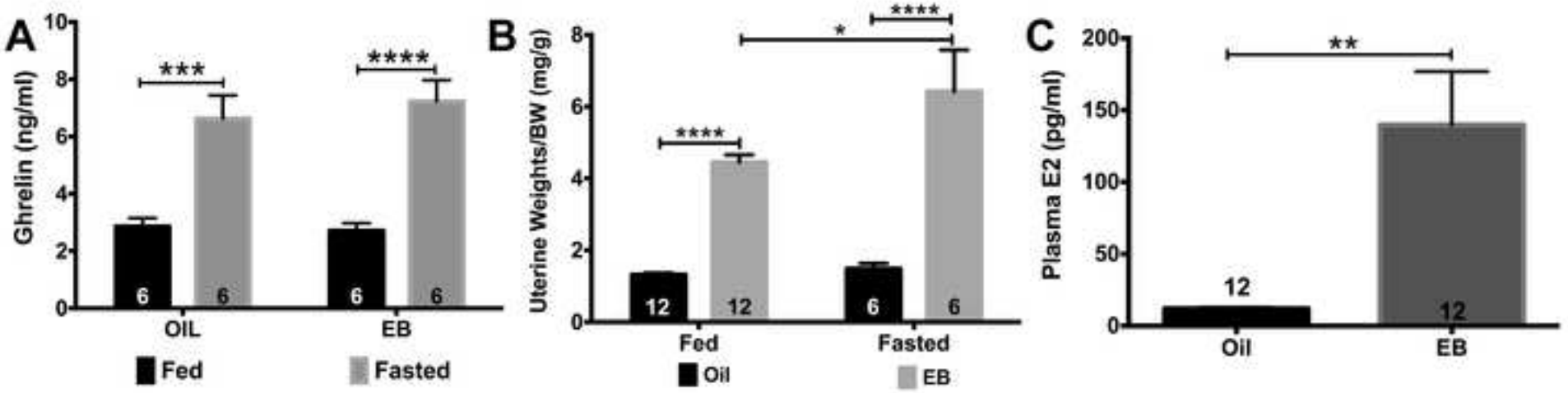

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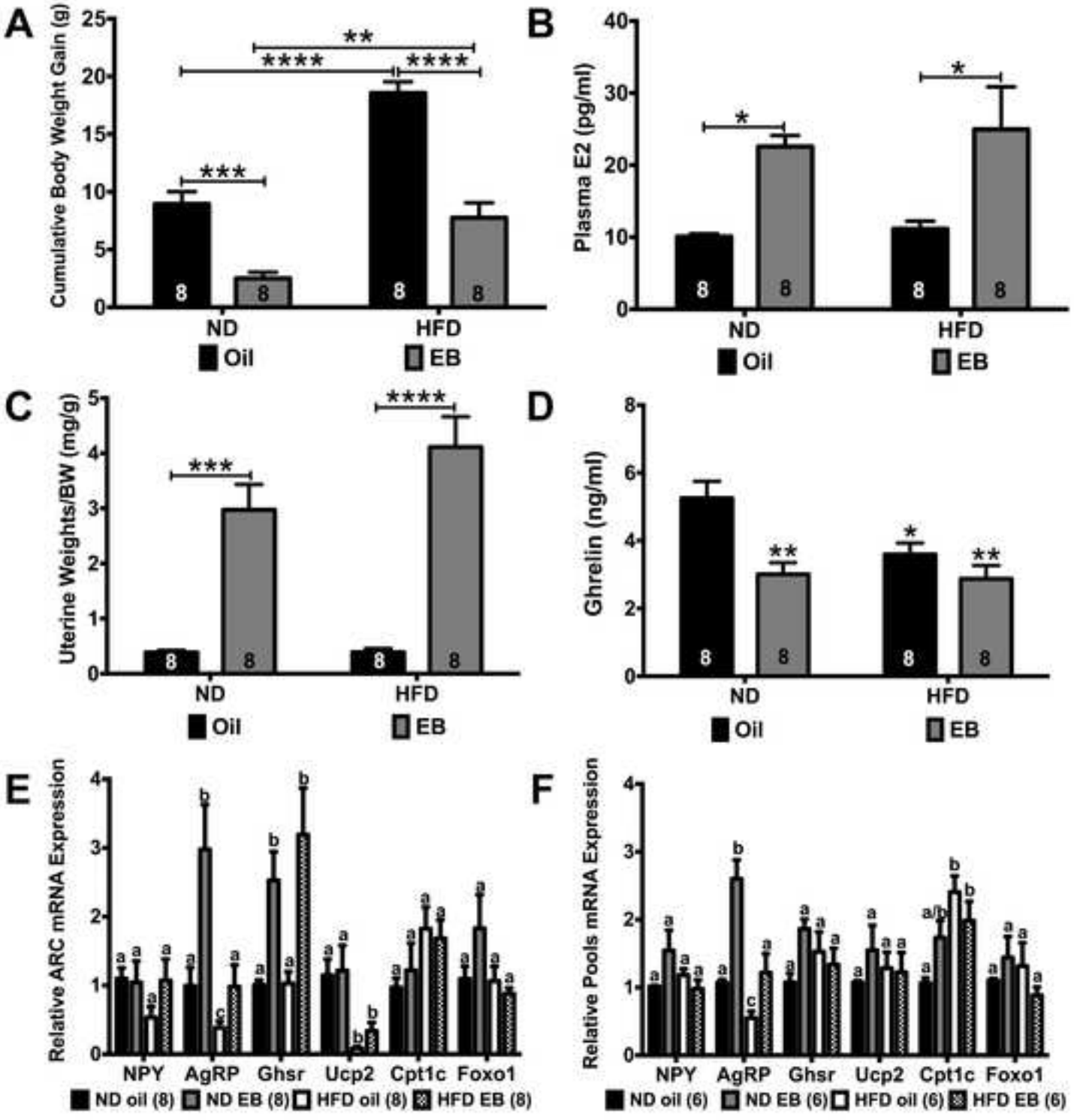

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