The role of neuronal nitric oxide synthase (nNOS) in the estrogenic attenuation of cannabinoid-induced changes in energy homeostasis

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ABSTRACT

Since estradiol attenuates cannabinoid-induced increases in energy intake, energy expenditure and transmission at proopiomelanocortin (POMC) synapses in the hypothalamic arcuate nucleus (ARC), we tested the hypothesis that neuronal nitric oxide synthase (nNOS) plays an integral role. To this end, whole animal experiments were carried out in gonadectomized female guinea pigs. Estradiol benzoate (EB; 10 µg; s.c.) decreased incremental food intake as well as O₂ consumption, CO₂ production and metabolic heat production as early as 2 hours post administration. This was associated with increased phosphorylation of nNOS, as evidenced by an elevated (pnNOS)/nNOS ratio in the ARC. Administration of the cannabinoid receptor agonist WIN 55,212-2 (3µg; i.c.v.) into the third ventricle evoked hyperphagia as early as 1 hour post administration, which was blocked by EB and restored by the non-selective NOS inhibitor L-NAME (100µg; i.c.v.) when the latter was combined with the steroid. Whole-cell patch clamp recordings showed that 17β-estradiol (E₂; 100nM) rapidly diminished cannabinoid-induced decreases in mEPSC frequency, which was mimicked by pre-treatment with the NOS substrate L-arginine (30µM) and abrogated by L-NAME (300µM). Furthermore, E₂ antagonized endocannabinoid-mediated depolarization-induced suppression of excitation, which was nullified by the nNOS-selective inhibitor NPLA (10µM). These effects occurred in a sizable number of identified POMC neurons. Taken together, the estradiol-induced decrease in energy intake is mediated by a decrease in cannabinoid sensitivity within the ARC feeding circuitry through the activation of nNOS. These findings provide compelling evidence for the need to develop rational, gender-specific therapies to help treat metabolic disorders like cachexia and obesity.

Key words: estradiol, nNOS, POMC, cannabinoid, energy balance
Introduction

Naturally occurring and synthetic cannabinoids aid in the regulation and central control of energy homeostasis and peripheral metabolic processes through managing bodily functions such as, food intake, gastrointestinal motility and secretion, fat and carbohydrate disposition, mitochondrial respiration and core body temperature (for review see (Borgquist and Wagner 2013). Both exo- and endocannabinoids stimulate hyperphagia (Cota et al. 2003; Fride et al. 2005) and hypothermia (Fitton and Pertwee 1982; Hillard et al. 1999). This control occurs through intricate interactions between the gut, liver, pancreas, brainstem, hypothalamus and limbic forebrain via endogenous ligands such as anandamide, 2-arachidonyl glycerol (2-AG), and exogenous agonists like WIN 55,212 and the CB1 receptor (Borgquist and Wagner 2013). CB1 receptor agonists increase the amount of time spent eating and decrease time spent resting, whereas CB1 receptor antagonists decrease the amount of time spent eating and increase the time spent grooming (Escartín-Pérrez et al. 2009). We have previously shown that cannabinoids exert their actions on feeding behavior and metabolism in a sexually differentiated manner, with males being more sensitive to cannabinoid CB1 receptor activation than females (Farhang et al. 2009). This could be attributed in part to lower hypothalamic cannabinoid receptor binding site density in females versus male rats (Riebe et al. 2010) as well as the pleiotropic actions of cannabinoids at anorexigenic proopiomelanocortin (POMC) synapses in the hypothalamic arcuate nucleus (ARC) (Farhang et al. 2009).

It is well known that estradiol decreases energy intake, core body temperature and body weight in various species (Butera and Czaja 1984; Palmer and Gray 1986; Johnson et al. 1994; Stephenson and Kolka 1999). Considerable evidence suggests that this may be due, at least in part, to rapid membrane-initiated estrogen signaling mechanisms. Membrane estrogen receptor (mER)α found within the hippocampus uses calveolin-1 protein to functionally couple with the metabotropic glutamate receptor mGluR1a (Boulware et al. 2007). 17β-estradiol (E₂) activates a protein kinase C (PKC) pathway through activation of mGluR1a to increase intracellular Ca²⁺ and progesterone synthesis (Dewing et al. 2008; Kuo et al. 2010). Similarly, studies in rodents have demonstrated that the estrogenic changes in energy homeostasis involve the activation of Gq-coupled mERs (Roepke et al. 2010; Washburn et al. 2013; Santollo et al. 2013). This can lead to a decrease in the ability of orexigenic cannabinoids to evoke hyperphagia (Kellert et al. 2009; Washburn et al. 2013). At the cellular level, E₂ rapidly reduces the ability of the cannabinoid receptor agonist WIN 55,212-2 to decrease the frequency of miniature excitatory postsynaptic currents (mEPSCs) impinging on POMC neurons (Kellert et al. 2009; Washburn et al. 2013). These effects are reversed when E₂ is co-perfused with the competitive estrogen receptor (ER) antagonist ICI 182,780 (Jeffery et al. 2011). While estradiol has been shown to downregulate hypothalamic CB1 receptors (Riebe et al. 2010), the effectiveness of WIN 55,212-2 to decrease mEPSC frequency was restored when phosphatidylinositol-3-kinase (PI3K) or PKC inhibitors were co-perfused with E₂ (Jeffery et al. 2011; Washburn et al. 2013). In the hypothalamic paraventricular nucleus (PVN), the Src/kinase pathway mediates an ERβ-induced increase in phosphorylation levels of neuronal nitric oxide synthase (nNOS) by activating an intervening PI3K/Akt pathway (Gingerich and Krukoff 2008). This is similar to the estrogenic regulation of NOS in endothelial cells, where calveolin-1-dependent translocation of ERα to the plasma membrane is critical for its ability to interact with Src and ultimately PI3K. This interaction, in turn, enables Akt-dependent phosphorylation/activation of eNOS (Haynes et
In addition, both nNOS expression and phosphorylation/activation in the hypothalamus vary over the course of the estrous cycle, with the highest levels observed during proestrus (Sica et al. 2009; Parkash et al. 2010). Moreover, fasting is reported to decrease nNOS levels in the ARC and VMN; an effect that is reversed upon refeeding (Otukonyong et al. 2000). Therefore, it appears that an estradiol-induced increase in activated nNOS should lead to nitric oxide production, which would act to antagonize cannabinoid-mediated presynaptic inhibition of excitatory input onto POMC neurons.

The purpose of the present study was to test the hypothesis that the estrogenic attenuation of cannabinoid-induced changes in energy homeostasis is due, at least in part, to the activation of nNOS in the ARC. To this end, we conducted whole-animal experiments in ovariectomized female guinea pigs to determine estrogen-induced changes in energy balance and nNOS activation in the ARC and VMN microdissected from hypothalamic slices. We also examined whether NOS inhibition could restore cannabinoid hyperphagia in estrogen-treated animals. In vitro electrophysiologic recordings were performed to explore whether nNOS inhibition could similarly rescue the cannabinoid-induced presynaptic inhibition of excitatory input onto ARC POMC neurons in E2-treated slices, and whether nNOS substrate loading could act in lieu of E2 to diminish this presynaptic inhibition of transmitter release in vehicle-treated slices.

Materials and Methods

Animals. All animal procedures described in this study are in accordance with institutional guidelines based on National Institutes of Health standards and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences. Female Topeka guinea pigs (450 - 700 g; 50 – 75 days old) were acquired from Elm Hill Breeding Labs (Chelmsford, MA, USA) or bred in our animal care facility, maintained under controlled temperature (69-73°F) and a coordinated light cycle of (12 hrs. on: 12 hrs. off), and provided with food and water ad libitum.

Surgical Procedures. To better resolve how estradiol influences cannabinoid sensitivity within the hypothalamic feeding circuitry, two survival surgeries were conducted. Stereotaxic guide cannula implantations, as well as ovariectomies, were performed as previously described after inducing anesthesia with ketamine/xylazine (33mg/kg & 6 mg/kg, respectively; s.c.), while maintaining the anesthetic plane with 1.5-2% isoflurane. Animals were subject to stereotaxic surgery 13 days prior to experimentation, during which we inserted a 22-gauge guide cannula 1mm above the third ventricle. Animals were secured in a stereotaxic frame by fitting the incisors over a tooth bar and inserting blunt ear bars into the ear canals. The surgery was performed using aseptic techniques. First, the scalp was opened by making a 2-2.5 cm incision down the midline of the skull beginning at the front of the orbits towards the occipital lobe with a #10 scalpel blade. A single hole was drilled and the dura layer cut so that a guide cannula could be slowly lowered at an angle 4° from the vertical plane (to avoid puncturing the mid-sagittal sinus) to its desired location using the following coordinates (in mm, measured from bregma and the top of the cerebral cortex) - M/L: -0.7mm, A/P: -2.1, D/V: -9.8, tooth bar: -5.5 (Luparello et al. 1964; Tindal 1965). Three 3.2 mm bone anchor screws were then inserted into pre-drilled holes to secure the guide cannula placement. Dental cement/acrylic was used to affix the stainless steel screws.
and cannula to the skull. Finally, a stylette was inserted into the guide cannula to prevent cerebrospinal fluid from entering the shaft of the cannula. Animals were allotted seven days of recovery prior to the ovariectomies, and an additional six days of recovery prior to the start of the in vivo experiments.

*Drugs.* Unless otherwise indicated all drugs were purchased through Tocris Cookson, Inc. (Bioscience Minneapolis, MN, USA). For the behavioral experiments, estradiol benzoate (EB; Steraloids, Newport, RI, USA) was initially prepared as a 1 mg/mL stock solution in punctilious ethanol. A known quantity of this stock solution was added to a volume of sesame oil sufficient to produce a final concentration of 100 μg/mL, following evaporation of the ethanol. The cannabinoid receptor agonist \((R)-(+)\)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55,212-2) was dissolved in cremephor/ethanol/0.9% saline (CES; 1/1/18; v/v/v) at a concentration of 1.5 μg/μL, and delivered in a total volume of 2 μL. The NOS inhibitor N-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was dissolved in CES at a concentration of 50 μg/μL, and given in a total volume of 2 μL.

For the electrophysiological experiments, the voltage-gated Na\(^+\) channel blocker tetrodotoxin (TTX) with citrate (Alomone Labs, Jerusalem, Israel), was dissolved in Ultrapure H\(_2\)O to a stock concentration of 1 mM and diluted further with artificial cerebrospinal fluid (aCSF) to a working concentration of 500 nM. The GABA\(_A\) receptor antagonist 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide (SR 95531) was dissolved in Ultrapure H\(_2\)O to a stock concentration of 10 mM and the stock concentrations were diluted further with aCSF to the working concentration of 10 μM. E\(_2\) (Steraloids) was dissolved in punctilious ethanol to a stock concentration of 1 mM and diluted further with aCSF to a working concentration of 100 nM. WIN 55,212-2 was dissolved in dimethylsulfoxide (DMSO) to 1 mM stock concentrations and the stock concentrations were diluted further with aCSF to the working concentration of 1 μM. L—NAME was dissolved in Ultrapure H\(_2\)O to a stock concentration of 30 mM and the stock concentrations were diluted further with aCSF to the working concentration of 300 μM. The selective nNOS inhibitor N\(^5\)-[Imino(propylamino)methyl]-L-ornithine hydrochloride (NPLA) was dissolved in Ultrapure H\(_2\)O to a stock concentration of 10 mM and the stock concentrations were diluted further with aCSF to the working concentration of 10 μM. The NOS substrate L-arginine was dissolved in Ultrapure H\(_2\)O to a stock concentration of 30 mM and the stock concentrations were diluted further with aCSF to the working concentration of 30 μM. Aliquots of the stock solutions were stored at -20°C until needed.

*Feeding and Metabolic Studies.* The analyses for energy balance were performed in a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments; Columbus, OH, USA) as previously described and validated (Farhang et al. 2010; Washburn et al. 2013; Qiu et al. 2014). Food intake was monitored around the clock for five days in ovariectomized female guinea pigs; each with a guide cannula implanted into the third ventricle of the brain 13 days prior to acclimation in the CLAMS chambers, and 16 days prior to the start of the monitoring period. A meal was defined as an event in which an animal consumed ≥ 10 mg of food. Once the animal had eaten at least this threshold amount, the computer logged this event as a meal in the experimental data file the instant the animal withdrew its head from the food dish. We calculated meal frequency as the number of meals consumed per unit
time, and meal size as the amount of food eaten in a given hour divided by the number of meals in the same hour. We also measured O$_2$ consumption, CO$_2$ production and the metabolic heat production as indices of energy expenditure. Each morning at 8:00 a.m., the animals were given either the CB1 receptor agonist WIN (3μg; I.3.V), alone and in combination with the NOS inhibitor L-NAME (100μg; I.3.V), or their CES vehicle. Every other day animals were injected with EB (10μg; s.c.) or its sesame oil vehicle. All doses of EB, estrogen, WIN 55,212-2 and L-NAME used in these in vivo studies were determined after careful consultation of the literature, or derived from our previously published work (Landi et al. 2002;Merroun et al. 2014;Kellert et al. 2009;Reis et al. 2010;Cope et al. 2010;Hama and Sagen 2011;García-Juárez et al. 2012).

Western Blot Analysis. Unless otherwise stated, all primary antibody solutions were prepared in TBS containing 0.1% of Tween 20 and Odyssey blocking buffer at a 1:1,000 dilution. Primary antibodies directed against the following antigens were used: glyceride-3-phosphate dehydrogenase (GAPDH; 1:10,000; Millipore, Billerica, MA, USA), total nNOS (Santa Cruz, Dallas, TX, USA), NOS phosphorylated at the serine residue located in the 1412$^{th}$ position of the amino acid sequence (pnNOS; ABCAM, Cambridge, MA, USA). At the end of the five-day experimental period, animals were anesthetized with 32% isofluorane and rapidly decapitated. Following brain removal, two to three coronal slices (1 mm in thickness) spanning the rostral-caudal extent of the ARC were prepared using a guinea pig brain matrix (Ted Pella, Inc.; Redding, CA, USA), and stored in RNAlater (Ambion, Inc.; Austin, TX, USA) for 2-3 hr. The ARH and, for comparision, the ventromedial nucleus (VMN) were then microdissected from the slices. ARH and VMN microdissections were homogenized in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5 M EDTA, 0.5 M EGTA) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA). Protein levels were quantified using a Bradford assay (BioRad Laboratories, Hercules, CA, USA) to establish equal loading into the gel. Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfatepolyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour with odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, 68504) and incubated overnight with primary antibodies at 4°C. They were then washed four times with tris-buffered saline with tween (TBST) for 10 min, followed by incubation with Odyssey infrared-conjugated secondary antibodies diluted 1:10,000 in Odyssey blocking buffer for 2 h at room temperature. After 4x10-min washes with TBST followed by 4x10-min washes with tris-buffered saline (TBS), membranes were scanned using an Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE, 68504). All membranes were probed with GAPDH as a loading control. Levels of pnNOS and nNOS expression was determined by calculating the ratio of phosphoprotein density to total protein density for each experimental group, and then normalizing the ratio to the values observed in vehicle-treated animals.

Electrophysiology. Electrophysiological recordings from arcuate neurons with biocytin-filled electrodes were performed using an in vitro hypothalamic slice preparation as previously described (Jeffery et al. 2011). Briefly, electrode resistances varied from 3 - 8 MΩ. Membrane currents were recorded in voltage clamp with access resistances ranging from 8-20 MΩ, and underwent analog-digital conversion via a Digidata 1322A interface coupled to pClamp 8.2 software (Axon Instruments). The access resistance, as well as the resting membrane potential (RMP) and the input resistance (R$_{in}$), were
monitored throughout the course of the recording. If the access resistance deviated greater than 20% of its original value, the recording was ended.

To evaluate whether E2 modulates cannabinoid-induced presynaptic inhibition of excitatory input onto ARC neurons via a nNOS pathway, we first monitored mEPSC frequency and amplitude from a holding potential of -75 mV using an internal solution in which Cs⁺ was substituted for K⁺. We perfused slices with aCSF containing TTX (500 nM) and SR 95531 (10μM) to block GABA_A receptor-mediated synaptic input, alone or in combination with either E₂ (100nM) or its EtOH (0.01%; v:v) vehicle. These agents were bath applied for 3-4 minutes prior to the attainment of 3-4 minutes worth of baseline mEPSC frequency and amplitude. E₂ or EtOH were then perfused along with WIN 55,212-2 (1μM) for an additional 3-4 minutes, and 3-4 more minutes of data were collected. In some experiments, slices were pretreated with the nNOS inhibitor L-NAME (300μM) 4 minutes prior to, and then perfused along with E₂ to assess the role of NOS in mediating estrogenic modulation of cannabinoid-induced changes in mEPSC frequency and/or amplitude. Alternatively, we pretreated slices with the NOS substrate L-arginine (30μM) to ascertain whether nitric oxide modulated the effect of estradiol on cannabinoid-induced changes in mEPSC frequency and/or amplitude. The threshold for event detection was set at least three pA below the baseline holding current as assessed from the headstage output, and continuously monitored throughout each 3-4 min recording period. This was done to ensure that the smaller amplitude events were not inadvertently omitted from the analysis. Synaptic events were detected and analyzed using Clampfit 8.2 (Axon Instruments) in combination with the SigmaPlot (IBM/SPSS, New York, NY, USA) and StatGraphics (StatPoint, Inc., Warrenton, VA, USA) programs. When we analyzed the data to determine mEPSC frequency and amplitude for the ≥100 contiguous synaptic events per condition, we poured over each 250-msec sweep in the entire range to ensure that each event that we included in the analysis bore the classic kinetic profile of a fast EPSC. We used this information to evaluate cannabinoid-induced alterations in mEPSC frequency and amplitude as assessed from cumulative probability plots.

Next, we set out to determine whether endocannabinoids retrogradely inhibit excitatory input via the depolarization-induced suppression of excitation (DSE; Kreitzer and Regehr 2001; Ohno-Shosaku et al. 2002). Prior to executing the DSE protocol, we monitored spontaneous EPSCs (sEPSCs) from a holding potential of -75 mV for 3-4 min to establish baseline frequency and amplitude in the presence of SR 95531 to block GABA_A receptor-mediated synaptic input, alone or in combination with either: 1) E₂ or its EtOH vehicle to ascertain the ability of the steroid to modulate the extent of the DSE, 2) the CB1 receptor antagonist AM-251 (1 μM) to assess the role of the CB1 receptor and 3) E₂ combined with the nNOS-selective inhibitor NPLA (10 μM) to assess the role of nNOS signaling in the estrogenic modulation. The doses of the drugs used in these electrophysiological experiments were chosen based on our prior work as well as the published work of others (Palmer et al. 1988; Radomski et al. 1990; Moore and Handy 1997; Zhang et al. 1997; Yu et al. 1997; Ho et al. 2007; Washburn et al. 2013). To elicit DSE, cells were given a 60-mV depolarization (0.75 – 3 seconds in duration). These pulses were delivered every 60 seconds for up to 15 consecutive trials. Data were analyzed by looking at the average post-stimulation amplitude and frequency acquired from at least 3 separate trials over 5-second bins up to 20-seconds normalized to that observed under basal conditions.
Immunohistochemistry. Following electrophysiological recording, slices were fixed with 4% paraformaldehyde in Sorensen's phosphate buffer (pH 7.4) for 90-180 min (Kellert et al. 2009). They then were immersed overnight in 20% sucrose dissolved in Sorensen's buffer, and frozen in Tissue-Tek embedding medium (Miles, Inc., Elkhart, IN, USA) the next day. Coronal sections (20 μm) were cut on a cryostat, and mounted on slides. These sections were washed with 0.1 M sodium phosphate buffer (pH 7.4), and then processed with streptavidin-AF488 (Molecular Probes, Inc., Eugene, OR, USA) at a 1:300 dilution. After localizing the biocytin-filled neuron via fluorescence microscopy, the slides containing the appropriate sections were processed with a polyclonal antibodies directed against either β-endorphin (Immunostar, Inc., Hudson, WI, USA; 1:400 dilution), α-melanocyte-stimulating hormone (α-MSH, Immunostar; 1:200 dilution) or cocaine-amphetamine-regulated transcript (CART; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA; 1:2000 dilution) using fluorescence immunohistochemistry (Kellert et al. 2009).

Statistical Analyses
Comparisons between two groups were made with the Mann-Whitney U test. Comparisons between more than two groups were performed using either the one-way, multifactorial or rank-transformed multifactorial analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test, or alternatively via the Kruskal-Wallis test followed by analysis of the median-notched, box-and-whisker plot. Comparisons of the mEPSC interval distributions were evaluated via the Kolmogorov-Smirnov test. Differences were considered statistically significant if the probability of error was <5%.

Results
Experiment #1: The Effects of EB and the cannabinoid receptor agonist WIN 55,212-2 on nNOS Activation in the ARC and VMN
Since we know that estradiol disrupts cannabinoid signaling at POMC synapses via a pathway that involves PI3K and PKC (Jeffery et al. 2011; Washburn et al. 2013), and that it also activates nNOS in the PVN via a Src/PI3K/Akt pathway (Gingerich and Krukoff 2008), we proposed that nNOS activation represents a final common pathway through which the steroid dampens cannabinoid-induced changes in energy homeostasis. As shown in Figure 1A, Western blotting yielded clear pnNOS and total nNOS bands registering at 160 kDa in both the ARC and VMN. EB (10 μg; s.c.) administered 2 hours prior increased the intensity of the pnNOS band; resulting in a nearly four-fold elevation in the pnNOS/NOS ratio in samples from the ARC (Figure 1B). By contrast, the cannabinoid receptor agonist WIN 55,212-2 (3 μg; i.3.v.) markedly decreased the intensity of the pnNOS band (Figure 1A), and diminished the pnNOS/NOS ratio in both the ARC and VMN (Figure 1B; rank-transformed multifactorial ANOVA/LSD, F\text{drug} = 60.58, P < 0.0001, df = 2, F\text{region} = 1.54, P < 0.22, df = 1; F\text{interaction} = 1.69, P < 0.20, df = 2; n = 5)

Experiment #2: The Role of nNOS in mediating EB-Induced Changes in the Cannabinoid Regulation of Energy Intake
We then used in vivo behavioral studies in ovariectomized female guinea pigs to examine the effects of estradiol on energy intake and expenditure. As shown in Figure 2A, the EB-induced activation of nNOS
in the ARC was associated with reduced cumulative food intake (one-way ANOVA/LSD, F = 3.21, P < 0.05; n = 5), O₂ consumption, (F = 5.10, P < 0.009; n = 5), CO₂ production (F = 5.79, P < 0.006; n = 5) and metabolic heat production (F = 4.40, P < 0.02; n = 5). The decrease in energy intake, but not the various measures of energy expenditure, was blocked by the NOS inhibitor L-NAME (100 μg; i.3.v.). After seeing the effects of EB *per se* on energy homeostasis, as well as its ability to activate nNOS in the ARC, we investigated whether prior delivery of L-NAME could restore the hyperphagic effect of the cannabinoid receptor agonist WIN 55,212-2 administered into the third ventricle in EB-treated animals. For this experiment, we used a one-hour time point because we wanted to see if EB could negatively modulate the hyperphagia caused by WIN 55,212-2 prior to the onset of its own hypophagic response (see Figure 2A). As shown in Figure 2B, the hyperphagic effect of centrally administered WIN 55,212-2 is rapidly and completely blocked by EB within one hour after delivery, and sustained at all subsequent time points observed up to 24 hours post administration (not shown). L-NAME alone had no effect on energy intake in vehicle- or EB-treated animals, and did not alter the increase in energy intake caused by WIN 55,212-2 in vehicle-treated animals. It did, however, prevent the EB-induced blockade of the hyperphagic effect of the agonist, thereby enabling an increase in consumption similar to that seen in vehicle-treated animals (multi-factorial ANOVA/LSD, F<sub>steroid</sub> = 4.29, df = 1, p < 0.04, F<sub>WIN 55,212-2</sub> = 11.48, df = 1, p < 0.001, F<sub>interaction</sub> = 4.09, df = 1, p < 0.05; n = 5). This suggests that nNOS is involved in the estrogenic uncoupling of cannabinoid-induced hyperphagia in our guinea pig model.

**Experiment #3: The Role of nNOS in Mediating E₂-induced Changes in the Cannabinoid Regulation of Excitatory Synaptic Input onto Anorexigenic POMC Neurons**

We made recordings from a total of 294 ARC neurons. These cells had a RMP of -52.5 ± 0.9 mV and a R<sub>i</sub> of 727.2 ± 47.8 MΩ. Eighty-two of these neurons exhibited conductances like the hyperpolarization-activated cation current and A-type K<sup>+</sup> current that are characteristic of POMC neurons (Ibrahim et al. 2003; Kelly et al. 2009). Of these, 67 were immunopositive for POMC neurons like the one shown in Figure 3. To further our understanding of how these changes in energy homeostasis are occurring on a cellular level, we then examined how estradiol could impact cannabinoid-induced changes in excitatory synaptic input onto POMC neurons. The representative membrane current traces, corresponding quantile plots and the composite bar graph demonstrate that WIN 55,212-2 (1 μM) reduced the number of excitatory synaptic events per unit time in recordings from vehicle-treated slices (Kolmogorov-Smirnov, K-S statistic = 2.54558, P < 0.0001; Mann-Whitney U-test, W = 12.5, P < 0.03; n = 8), and that E₂ (100 nM) appreciably attenuated this effect (Figure 4; K-S statistic = 1.06912, P < 0.21; n = 8). Since E₂ inhibits the ability of WIN 55,212-2 to decrease mEPSC frequency, coupled with the fact that L-NAME restored the hyperphagia caused by WIN 55,212-2 in the presence of EB, we wanted to explore the role of NO in the estrogenic attenuation of the cannabinoid-induced decrease in excitatory synaptic input. As shown in Figure 5A & 5C, WIN 55,212-2 clearly reduced mEPSC frequency in recordings from slices pre-treated for 16-20 minutes with E₂ and L-NAME (300 μM; Kolmogorov-Smirnov, K-S statistic = 2.06316, P < 0.0005; Mann-Whitney U-test, W = 30.0, P-value < 0.009; n = 5). By contrast, this effect was markedly diminished in the presence of the NO substrate L-arginine (30 μM; K-S statistic = 0.725691, P < 0.69; Figure 5B & 5C; n = 6).
To test whether estradiol could also modulate retrograde endocannabinoid signaling we then attempted to elicit DSE during recordings from hypothalamic slices. We found that a 60-mV depolarizing stimulus (0.75 – 3 seconds in duration) delivered to the postsynaptic neuron in vehicle-treated slices decreased the frequency and amplitude of sEPSCs impinging upon it (Figure 6A & 7; n = 11). The onset of this effect was observed within five seconds (Kruskal-Wallis/ median-notched Box-and-Whisker analysis; frequency: test statistic = 19.3284, P < 0.0003; amplitude: test statistic = 24.2295, P < 0.0001), sustained for at least 15 seconds (frequency: test statistic = 17.1405  P < 0.0007; amplitude: test statistic = 21.8684, P < 0.0001), and appeared to return to baseline levels by 20 seconds after the stimulus (frequency: test statistic = 7.57239, P < 0.06; amplitude: test statistic = 9.97297, P < 0.02). This stimulus-induced reduction in sEPSC frequency and amplitude was negated by both E$_2$ (Figure 6B & 7; n = 10) and AM251 (Figure 6C & 7; n = 7). Furthermore, when E$_2$ was bath applied to slices pre-treated with the nNOS-selective inhibitor NPLA (10 μM), post-stimulus decrements in sEPSC frequency and amplitude were subsequently restored to levels similar to those observed in recordings from vehicle-treated slices (Figure 6D & 7; n = 7). Collectively, this indicates that E$_2$ blocks endocannabinoid-mediated DSE by activating a nNOS pathway in POMC neurons.

Discussion

Our results demonstrate that estrogen-induced decreases in energy intake are mediated by a reduction in cannabinoid sensitivity via a mechanism involving the activation of nNOS in the ARC. We base these conclusions on the following findings: 1) EB decreases energy intake and expenditure while activating nNOS in the ARC but not the VMN; 2) the hyperphagic effect of the cannabinoid receptor agonist WIN 55,212-2 is blocked by EB, and rescued by the NOS inhibitor L-NAME in EB-treated animals; 3) the estrogenic attenuation of the cannabinoid-induced decrease in mEPSC frequency in POMC neurons is nullified by L-NAME and mimicked by the NOS substrate L-arginine; and 5) the estrogen-induced decrease in retrograde endocannabinoid-mediated DSE is largely negated by the nNOS-selective inhibitor NPLA.

The estradiol-induced reduction in food consumption currently observed is consistent with the literature reported for other rodent models (Butera and Czaja 1984;Dubuc 1985;Palmer and Gray 1986). This also agrees with findings in human females, in whom energy intake is lowest at the late follicular phase of the ovulatory cycle, when estrogen levels peak and are unopposed by progesterone (Johnson et al. 1994). The estrogen-induced anorexigenesis can be attributed, in part, to the negative modulation of metabotropic receptors expressed in POMC neurons. For example, estrogens rapidly impede postsynaptic G$_{i/o}$-coupled receptors like the GABA$_B$ receptor from G-protein-gated, inwardly rectifying K$^+$ (GIRK) channels by activating a PLC/PKC/PKA pathway (Qiu et al. 2003;Qiu et al. 2006;Qiu et al. 2008). Estrogen also potentiates the appetite suppression caused by fenfluramine-induced serotonin release (Rivera and Eckel 2005). This results in higher activation of SHT$_{2c}$ receptors, which are G$_o$-coupled and in turn activate PLC to hydrolyze phosphatidylinositol bisphosphate. In POMC neurons, this leads to an enhanced firing rate via activation of canonical transient receptor potential channels (Sohn et al. 2011) and the uncoupling of GIRK channels from metabotropic G$_{i/o}$-coupled receptors (Qiu et al. 2007).
Presently, we found that estradiol rapidly blocks cannabinoid-induced hyperphagia and the decrease in mEPSC frequency. This is consistent with what we have shown previously (Kellert et al. 2009; Washburn et al. 2013). The steroid is able to elicit its negative modulatory effect within minutes after bath application to hypothalamic slices, and lasts for at least 24 hours when systemically administered to ovariectomized female guinea pigs (Nguyen and Wagner 2006; Kellert et al. 2009). Estradiol also rapidly occludes the AM251-induced decrease in energy intake, and by 24 hours it negates the hypophagia and increase in excitatory synaptic input onto POMC neurons caused by CB1 receptor antagonism (Nguyen and Wagner 2006; Kellert et al. 2009). In addition, we have demonstrated that the rapid estrogenic attenuation of the cannabinoid-induced increase in energy intake, and decrease in glutamatergic input onto POMC neurons, are mimicked by the diphenylacrylamide compound STX, which activates a G\textsubscript{q}-coupled membrane ER that is neither ER\textalpha nor ER\textbeta (Qiu et al. 2006). Moreover, the ER\textalpha agonist PPT attenuates the cannabinoid-induced hyperphagia within 4 hours, and negates the cannabinoid-induced decrease in glutamate release onto POMC neurons in animals treated 24 hours prior to experimentation (Washburn et al. 2013). This latter finding is in accordance with the fact that nearly 75% of guinea pig POMC neurons express ER\textalpha (Roepke et al. 2007), as opposed to only 25-30% in the mouse (de Souza et al. 2011). Thus, the estrogenic disruption of metabotropic CB1 receptor signaling is due most likely to a combination of ER\textalpha and G\textsubscript{q}-coupled mER activation in guinea pig POMC neurons.

Arguably the most seminal finding of the present study is that estradiol can rapidly antagonize endocannabinoid-mediated DSE. This contrasts with studies in transgenic mice suggesting that endocannabinoid regulation of energy homeostasis is independent from the melanocortin system (Hentges et al. 2005; Sinnayah et al. 2008). In our guinea pig model, we clearly observe that endocannabinoids can inhibit POMC neurons via activation of presynaptic CB1 receptors on glutamatergic nerve terminals, which plays an integral role in the hyperphagia induced in this species. Further support for the use of this guinea pig animal model comes from reports demonstrating that guinea pigs were more sensitive than rats or mice during the development of appetite-suppressing drugs like fenfluramine (Mennini et al. 1991) and fluoxetine (Anelli et al. 1992). Moreover, guinea pigs, like humans, do not have the capability of synthesizing their own vitamin C (Horton et al. 1975; Odumosu 1981). Coupled with the fact that guinea pig POMC neurons also express ER\textalpha to a greater extent than is observed in rats or mice (Roepke et al. 2007; de Souza et al. 2011), this would suggest that these cells may serve as a comparatively more important locus in this species for the regulatory interplay between estradiol and endocannabinoids that helps control energy balance in the female. The DSE that we encountered in POMC neurons is clearly observed within five seconds, and lasts at least 15 seconds after the depolarizing stimulus. This is congruent with that found in the cerebellum, where a brief depolarization of cerebellar Purkinje cells at physiological temperature was found to induce a transient suppression of excitatory synaptic transmission at both parallel fiber synapses and climbing fiber synapses and lasts for 10-20 seconds (Kreitzer and Regehr 2001). Furthermore, in these cells the DSE is prevented by the CB1 receptor antagonist AM251 (Kreitzer and Regehr 2001). Similarly, a depolarization of CA1 pyramidal neurons in hippocampal slices for 7-10 seconds produces a DSE that follows a similar time course (Ohno-Shosaku et al. 2002). The results found in the cerebellum and hippocampus support our findings that DSE is prevalent in short-term neuronal signaling in the
hypothalamus and are mediated by endocannabinoids that are released from depolarized postsynaptic neurons to suppress the excitatory transmission through activation of the CB1 receptor. 

Lastly, our results demonstrate that estrogen suppresses energy intake by rapidly disrupting cannabinoid signaling at POMC synapses via a nNOS pathway. This is in agreement with the fact that nitric oxide mediates the estrogenic potentiation of gonadotropin-releasing hormone secretion in hypothalamic explants caused by the activation of 2-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors (Matagne et al. 2005). There is also considerable evidence to suggest that estradiol-stimulated nitric oxide production represents the final step in this signal transduction pathway. For example, estrogen-induced activation of NOS is dependent upon upstream PI3K/Akt activity, as has been shown in the vascular endothelium (Haynes et al. 2003) and centrally in the hypothalamic PVN (Gingerich and Krukoff 2008). The Src/PI3K/Akt signaling pathway leading to increased nNOS activation in the PVN is initiated by ERβ (Gingerich and Krukoff 2008). The nitric oxide thus formed may very well serve as a retrograde messenger that increases the strength of excitatory synapses during long-term potentiation (Schuman and Madison 1991;Volgushev et al. 2000;Hardingham and Fox 2006;Di et al. 2009;Fenselau et al. 2011). In addition, the rapid estrogenic attenuation of the CB1 receptor-mediated decrease in glutamate release onto POMC neurons involves the activation of PI3K and PKC pathways (Jeffery et al. 2011;Washburn et al. 2013). Estrogens have also been found to activate PI3K to functionally uncouple GABA\textsubscript{B} receptors from GIRK channels in POMC neurons (Malyala et al. 2008), and the PI3K inhibitors wortmannin and LY294002 significantly reduced estrogen-mediated GABA\textsubscript{B} receptor desensitization in these cells (Malyala et al. 2008). This estrogen activation can be very rapid as shown by the finding that in NG108-15 neurons estrogens stimulate the phosphorylation of Akt within 30 minutes in a PI3K-sensitive manner (Akama and McEwen 2003). These observations serve to reinforce the importance of PI3K as an upstream messenger of estrogenic signaling and subsequent activation of nNOS. While nitric oxide may very well be synthesized in the POMC neurons themselves, it may also be the case that nNOS itself is suppressing the expression of enzymes involved in endocannabinoid biosynthesis like sn-1-selective diacylglycerol lipase and/or N-acyl-phosphatidyl-ethanolamine-selective phospholipase D (Borgquist and Wagner 2013). Alternatively, it could be that nNOS is localized to the upstream bouton, where it enhances the expression of endocannabinoid degrading enzymes like monoacylglycerol lipase, or even the rate of endocannabinoid removal from the synaptic cleft (Bisogno et al. 2001;HashimotoDani et al. 2007;Straiker et al. 2009). This latter scenario is given additional credence due to the finding that in transgenic mice POMC neurons per se do not express nNOS (Leshan et al. 2012). These possibilities will ultimately serve as the basis for future experiments along these lines. 

In conclusion, our results indicate that the estradiol up-regulates nNOS activity in POMC neurons to disrupt the activation of presynaptic CB1 receptors by endocannabinoids. Our data helps to elucidate how rapid estrogenic signaling alters cellular responsiveness to cannabinoids to help bring about estrogen-induced changes in energy balance. Given that the cannabinoid regulation of energy homeostasis is sexually differentiated (Farhang et al. 2009), this supports the need for rational, gender-based therapies for the treatment HIV/AIDS- and cancer-related cachexia as well as obesity.

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Figure Legends

Figure 1. EB and the cannabinoid receptor agonist WIN 55,212-2 (WIN) modulate nNOS activation in the mediobasal hypothalamus. A) Representative Western blots illustrating the levels of nNOS, pnNOS, and
the loading control GAPDH in the ARC and VMN microdissected from vehicle-, EB- and WIN 55,212-2-treated animals. The vertical lines represent demarcations between bands run on different gels. **B** Composite bar graph illustrating the pnNOS/nNOS ratio determined in ARC and VMN microdissections from vehicle-, EB- and WIN 55,212-2-treated animals. Bars and vertical lines represent means and 1 S.E.M., respectively. *, Values from drug-treated animals that are significantly different (P<0.05; rank-transformed multifactorial ANOVA/LSD; n = 5) than those from vehicle-treated controls.

Figure 2. **A**) EB decreases energy intake and expenditure, the former of which is dependent on the activation of nNOS. The composite bar graphs illustrate whether the nNOS inhibitor L-NAME (100 µg; i.3.v.) can block the changes in food intake, O2 consumption, CO2 production and metabolic heat production caused by EB (10 µg; s.c.). Bars and vertical lines represent means and 1 S.E.M., respectively. *, Values from EB-treated and EB/L-NAME-treated animals that are significantly different (P<0.05; one-way ANOVA/LSD; n = 5) than those from vehicle-treated controls. **B**) The hyperphagic effect of WIN 55,212-2 (3 µg; i.3.v.) is blocked by EB, and rescued by L-NAME in EB-treated animals. Bars represent means and vertical lines 1 S.E.M. of the amount of food eaten in the one hour following the various treatment conditions. *, Values of food intake in animals treated with WIN 55,212-2 that are significantly different (P< 0.05; multi-factorial ANOVA/LSD; n = 5) from vehicle-treated controls. #, Values from L-NAME-treated animals that are significantly different (P< 0.05; multi-factorial ANOVA/LSD; n = 5) than those from vehicle-treated animals.

Figure 3. A color photomicrograph of an ARC POMC neuron from which an electrophysiological recording was taken. **A**) Biocytin labeling of the neuron filled throughout the recording and visualized with AF488. **B**) Immunoreactivity for β-endorphin located in the soma and varicosities of the cell in A as visualized by AF488. **C**) Merged photomicrographs from **A-B** illustrating that the ARC neuron (denoted by the arrows) that was double-labeled with biocytin and β-endorphin immunoreactivity.

Figure 4. E2 attenuates the cannabinoid-induced decrease in mEPSC frequency in POMC neurons. Membrane current traces and cumulative probability distributions showing **A**) the decrease in mEPSC frequency caused by the CB1 receptor agonist WIN 55,212-2 in a recording from a vehicle-treated slice, and **B**) the diminution of the cannabinoid-induced decrease in mEPSC frequency in a recording from an E2-treated slice. **C**) A composite bar graph that illustrates the estrogenic attenuation of the cannabinoid-induced decrease in mEPSC frequency. Bars represent means and vertical lines 1 S.E.M. of the mEPSC frequency that was normalized to values observed under baseline conditions.*, P<0.05, Kolmogorov-Smirnov (**A**); Mann-Whitney U-test (**C**; n = 8).

Figure 5. The estrogenic attenuation of the cannabinoid-induced decrease in mEPSC frequency in POMC neurons is nullified by the NOS inhibitor L-NAME and mimicked by the NOS substrate L-arginine. Membrane current traces and cumulative probability distributions showing **A**) the decrease in mEPSC frequency caused by WIN 55,212-2 in a recording from a slice that was co-treated with E2 and L-NAME, and **B**) the dampening of the cannabinoid-induced decrease in mEPSC frequency in a recording from a slice that was treated with L-arginine. **C**) A composite bar graph that illustrates how the cannabinoid-
induced decrease in mEPSC frequency is rescued by L-NAME in E₂-treated slices, and obstructed in slices treated with L-arginine. Bars represent means and vertical lines 1 S.E.M. of the mEPSC frequency that was normalized to values observed under baseline conditions.*, P<0.05, Kolmogorov-Smirnov (A); Mann-Whitney W test (C; n = 5-6).

Figure 6. E₂ antagonizes endocannabinoid-mediated depolarization-induced suppression of excitation (DSE) in POMC neurons by activating a nNOS pathway. To the left are the representative membrane current traces that illustrate the changes in sEPSC frequency and amplitude elicited by DSE during recordings in slices treated with either vehicle (A), E₂ (B), the CB1 receptor antagonist AM251 (C) or E₂ and the nNOS-selective inhibitor NPLA (D). The rectangular wave under the arrow labeled DSE represents the truncated change in membrane current caused by the three-second, 60-mV depolarizing voltage command. To the right are graphical depictions of the DSE-induced changes in raw frequency and amplitude (relative to the last five seconds of the baseline control period) for each of the representative traces in the four different treatment conditions.

Figure 7. The composite bar graphs in A and B illustrate the DSE-induced changes in sEPSC frequency and amplitude, respectively, observed during treatment with vehicle, E₂, AM251, or E₂ and NPLA. Bars and vertical lines represent means and 1 S.E.M., respectively. *, Values of post-stimulus sEPSC frequency and amplitude that are significantly different (P<0.05; Kruskal-Wallis/median-notched Box-and-Whisker analysis; n = 7-11) than those observed under basal conditions.
A. 

**E₂ & 300 μM L-NAME**

Baseline

1 μM WIN 55,212-2

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B. 

**30 μM L-arginine**

Baseline

1 μM WIN 55,212-2

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C. 

![Graph showing mEPSC frequency](image-url)