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MODULATORY INFLUENCES OF ESTRADIOL AND OTHER ANOREXIGENIC HORMONES ON METABOTROPIC, $G_{i/o}$ -COUPLED RECEPTOR FUNCTION IN THE HYPOTHALAMIC CONTROL OF ENERGY HOMEOSTASIS

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Abstract

The appetite suppressant actions of estradiol are due to its ability to attenuate orexigenic signals and potentiate anorexigenic signals. The work from my laboratory has shown that male guinea pigs are more sensitive to the hyperphagic and hypothermic effects of cannabinoids than their female counterparts. Cannabinoid sensitivity is further dampened by the activational effects of estradiol. This occurs via the hypothalamic feeding circuitry, where estradiol rapidly attenuates the cannabinoid CB1 receptor-mediated presynaptic inhibition of glutamatergic input onto anorexigenic proopiomelanocortin (POMC) neurons in the arcuate nucleus. This disruption is blocked by the estrogen receptor antagonist ICI 182,780, and associated with increased expression of phosphatidylinositol-3-kinase (PI3K). Moreover, the ability of estradiol to reduce both the cannabinoid-induced hyperphagia and glutamate release onto POMC neurons is abrogated by the PI3K inhibitor PI 828.

The peptide orphanin FQ/nociceptin (OFQ/N) activates opioid receptor-like (ORL)1 receptors to hyperpolarize and inhibit POMC neurons via the activation of postsynaptic G protein-gated, inwardly-rectifying (GIRK) channels. We have demonstrated that the fasting-induced hyperphagia observed in ORL1-null mice is blunted compared to wild type controls. In addition, the ORL1 receptor-mediated activation of GIRK channels in POMC neurons from ovariectomized female rats is markedly impaired by estradiol. The estrogenic attenuation of presynaptic CB1 and postsynaptic ORL1 receptor function may be part of a more generalized mechanism through which anorexigenic hormones suppress orexigenic signaling. Indeed, we have found that leptin robustly suppresses the OFQ/N-induced activation of GIRK channels in POMC neurons. Furthermore, its ability to augment excitatory input onto POMC neurons is blocked by PI 828. Thus, estradiol and other hormones like leptin reduce energy intake at least partly by activating

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Author Disclosures

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PI3K to disrupt the pleiotropic functions of $G_{i/o}$ -coupled receptors that inhibit anorexigenic POMC neurons.

Keywords

cachexia; cannabinoid; sex difference; estradiol; nociceptin; leptin; phosphatidylinositol-3-kinase

1. Introduction

It has long been known that hypothalamic lesions produce obesity [1], and over the past several decades the scientific community has witnessed an explosion in the number of investigations into the hormonal and synaptic determinants underlying the hypothalamic control of energy homeostasis. The hypothalamic feeding circuitry comprises both orexigenic and anorexigenic components. The orexigenic components can be found in the lateral hypothalamic area (LHA) housing the orexin- and melanin-concentrating hormone (MCH)-containing somata [2], as well as the in the arcuate nucleus (ARC) where the neuropeptide Y (NPY)/agouti-related peptide (AgRP)- and ghrelin-containing somata reside [3–5]. Conversely, anorexigenic components can be found in the ARC as well as the hypothalamic paraventricular nucleus (PVN) that contain the cell bodies of the proopiomelanocortin (POMC) and corticotropin-releasing hormone (CRH) neurons, respectively [2,6]. The hypothalamic ventromedial nucleus (VMN) is also involved in satiety, as lesions of this region cause profound hyperphagia and obesity [7,8]. There is extensive synaptic reciprocity and interconnectivity between the nuclei comprising the hypothalamic feeding circuitry, which is also sensitive to circulating levels of nutrients and peripheral hormones such as glucose, insulin, ghrelin and leptin [9,10]. Interestingly, all of the afferent elements of the hypothalamic feeding circuitry project to the PVN, where they make synaptic contact with the CRH neurons residing there [9,10]. Thus, the CRH neurons serve as a point of convergence and synaptic integration that is important for generating an efferent response to changes in the prevailing energetic environment.

The neurons within the hypothalamic feeding circuitry express signaling molecules such as phosphatidylinositol-3-kinase (PI3K) and AMP-activated protein kinase (AMPK) that mediate the glucose sensing and changes in neuronal activity that occur upon leptin and insulin receptor activation [11–14]. These and other hormones can also influence the levels of orexigenic endocannabinoids such as 2-arachidonoylglycerol (2-AG) within the nuclei comprising the hypothalamic feeding circuitry. For example, the orexigenic effect of ghrelin, as well as its ability to presynaptically inhibit glutamatergic input onto parvocellular neurons in the PVN, is due to augmented biosynthesis of 2-AG in the hypothalamus that then increases hypothalamic AMPK activity [15] via CB1 receptor activation. Conversely, anorexigenic leptin decreases hypothalamic endocannabinoid levels, which are elevated in obese Zucker (*fa/fa*) rats [16,17]. In addition, leptin diminishes the glucocorticoid-stimulated, endocannabinoid-mediated decrease in glutamatergic input onto parvocellular PVN neurons, an effect that is absent in *fa/fa* rats [17]. Moreover, CB1 receptors are expressed in the LHA [18], PVN [2] and the VMN [19], and administration of CB1 receptor agonists like tetrahydrocannabinol (THC) and anandamide into these hypothalamic nuclei

elicits a hyperphagic response [20,21]. Furthermore, in the LHA endocannabinoids retrogradely inhibit GABAergic input onto MCH neurons [22], and the elevated endocannabinoid biosynthetic capacity seen with diet-induced obesity and in *ob/ob* mice enhances the retrograde inhibition of GABAergic input onto orexin neurons [18]. While it is clear that cannabinoids can impact the homeostatic control of energy balance, they can also influence hedonic aspects of consumption; particularly in relation to highly palatable foods. For example, cannabinoids influence synaptic transmission in the nucleus accumbens, a structure containing the terminal fields of the mesolimbic dopamine neurons mediating natural and drug-induced reward [23,24]. In addition, diet-induced obesity is associated with a down-regulation of CB1 receptors in extrahypothalamic regions like the nucleus accumbens; suggesting that endogenous cannabinoids provide the initial impetus for the ingestion of palatable food [25]. Indeed, administration of the endogenous cannabinoid agonists 2-AG and anandamide into the nucleus accumbens stimulate feeding and augment the “liking” of intraoral sucrose [26,27]. By contrast, blockade of CB1 receptors with Rimonabant diminishes dopamine release in the nucleus accumbens caused by ingestion of highly palatable food [28]. Interestingly, the cell bodies of mesolimbic dopamine neurons from peripubertal female rats are more sensitive to endocannabinoid-mediated depolarization-induced suppression of GABA_A receptor-mediated inhibition (DSI) than those from their male counterparts [29]. However, given the burgeoning wealth of information on the neuroendocrine regulation of the hypothalamic feeding circuitry, we focus here on the modulation of orexigenic, metabotropic G_{i/o}-coupled receptors such the CB1 and opioid receptor-like (ORL)1 receptors by the anorexigenic hormones estradiol and leptin.

2.1 Sex Differences in the Cannabinoid Regulation of Energy Homeostasis

The literature is replete with examples of sex differences in cannabinoid-regulated biology. For example, female rodents are more sensitive to the antinociceptive effect of cannabinoids than their male counterparts [30,31]. They are more sensitive to the locomotor actions of cannabinoids as well [30–32]. In humans, women are more responsive to the cannabinoid-induced orthostatic hypotension that occurs when transitioning from the supine to the standing position [33]. They are more susceptible to the visuospatial memory impairment caused by cannabinoids as well [34]. Self-administration studies have revealed that female rats more rapidly acquire stable cannabinoid intake than males, and maintain a higher rate of responding [35]. Likewise, developmental studies have uncovered that females rats are more vulnerable to the effects of adolescent cannabinoid exposure on subsequent behavioral and biochemical changes (e.g., anhedonia, CB1 receptor downregulation) occurring in adulthood [36]. Similar studies have shown sexually disparate, behavioral interactions between subchronic nicotine exposure during adolescence and acute cannabinoid administration in adulthood [37].

While the considerable majority of the evidence presented above indicates that females are more responsive to cannabinoids, the converse is true with regard to energy homeostasis. For example, CB1 receptor agonists administered into the fourth ventricle of male rats are ~10X more potent in stimulating the consumption of sweetened condensed milk than they are in females [38]. In addition, we have demonstrated that CB1 receptor agonists produce a

greater increase in cumulative energy intake in orchidectomized male guinea pigs than they do in their ovariectomized female counterparts [39]. They also produce a reduction in core body temperature that is ~0.5 °C greater in orchidectomized males than that observed in ovariectomized females [39].

2.2 Estrogenic Modulation of Cannabinoid-Induced Changes in Energy Homeostasis

The sex differences that we observed in the cannabinoid regulation of energy balance occurred in castrated animals. This begged the question: what are the activational effects of estradiol in modulating cannabinoid-induced changes in energy balance in the female? Estradiol is a well-documented anorexigenic hormone that, in humans, diminishes energy intake during the periovulatory phase of the menstrual cycle [40] and, in rodents, decreases consumption during the estrus phase of the estrous cycle [41]. It does so, in general, by attenuating orexigenic signaling and potentiating anorexigenic signaling within the hypothalamic feeding circuitry [42]. Using an established treatment paradigm schematically represented by the timeline shown in Figure 1, we found that estradiol *per se* increased the expression of the PI3K p85 α regulatory subunit in the ARC (Figure 2). Western blotting of ARC tissue microdissected from two to three 1mm-thick coronal slices spanning its rostral-caudal extent as described previously [43–45] revealed clear phosphorylated Akt (pAkt) and total Akt bands measuring 60 KDa in weight (Figure 3). Estradiol administered two hours prior to tissue harvesting increased the amount of pAkt in the ARC compared to vehicle-treated controls (Figure 3). As we have previously reported [43–46], estradiol also decreased cumulative energy intake, as well as several measures of energy expenditure such as O₂ consumption, CO₂ production and metabolic heat production (Figure 4). The estradiol-induced decreases in energy intake, but not energy expenditure, were blocked by the PI3K inhibitor PI 828 (Figure 4). Most importantly, we have found that estradiol rapidly attenuates cannabinoid-induced hyperphagia and hypothermia [46], the former of which is restored when PI 828 is co-administered along with the cannabinoid receptor agonist WIN 55,212-2 into the third ventricle of ovariectomized, estradiol-treated female guinea pigs (Figure 5). These findings are in keeping with the fact that estradiol decreases the hypothalamic expression of the CB1 receptor [47], and that levels of anandamide and 2-AG in the rodent hypothalamus are reduced during proestrus relative to diestrus [48].

We then endeavored to resolve this estrogenic diminution of the cannabinoid-induced increase in energy intake down to the level of the hypothalamic feeding circuitry. Our electrophysiological studies revealed that the cannabinoid agonist WIN 55,212-2, upon activation of a metabotropic, G_{i/o}-coupled receptor like the CB1 receptor, decreased the number of excitatory synaptic events impinging on anorexigenic POMC neurons per unit time in vehicle-treated hypothalamic slices (Figure 6). This was evident upon examination of either the representative traces of membrane current, or the cumulative distribution plots of the interval between contiguous miniature excitatory postsynaptic currents (mEPSCs), under baseline control conditions and in the presence of WIN 55,212-2. By contrast, bath application of estradiol for a mere 10 minutes markedly blunted the ability of WIN 55,212-2 to decrease the number of excitatory synaptic events per unit time (Figure 6). However, co-perfusion of the estrogen receptor antagonist ICI 182,780 along with estradiol enabled WIN 55,212-2 to significantly decrease the number of excitatory synaptic events per unit time to a

level observed in recordings from vehicle-treated slices (Figures 6 and 7). Likewise, PI 828 abrogated the estrogenic attenuation of the ability of WIN 55,212-2 to decrease in mEPSC frequency (Figure 7). Collectively, this demonstrates that estradiol activates PI3K to diminish the cannabinoid-induced increase in energy intake that occurs, at least in part, via a reduction in the cannabinoid-induced presynaptic inhibition of excitatory input onto POMC neurons. In view of this rapid estrogenic attenuation of the cannabinoid-induced decrease in glutamate release onto POMC neurons, it therefore should not be surprising that estradiol *per se* increases basal mEPSC frequency in POMC neurons as well as POMC expression in the ARC [43,49,50]. We and others have shown that these rapid effects of estradiol on POMC neurons are due to activation of estrogen receptor (ER) α and a G_q -coupled membrane ER (G_q -mER)[42,44,51]. These ERs can be trafficked and tethered to the plasma membrane via caveolins and palmitoylation, as has been shown in the ARC, hippocampus and vascular endothelial cells [52–54].

2.3 Anorexigenic Hormone Modulation of opioid receptor-like (ORL)1 receptor-mediated signaling in POMC Neurons

The question arises: can estradiol negatively modulate the coupling of other orexigenic, $G_{i/o}$ -coupled receptors to their effector systems in POMC neurons? One such likely candidate is the ORL1 receptor, which is found in abundance in the mediobasal hypothalamus [55–58]. The ORL1 receptor is the cognate receptor of the endogenous peptide orphanin FQ (aka nociceptin; OFQ/N) [57,59]. When activated by OFQ/N, the ORL1 receptor hyperpolarizes and thereby inhibits POMC neurons via activation of a G protein-gated, inwardly-rectifying K^+ (GIRK) channel [60]. This observation is consistent with the fact that: 1) OFQ/N stimulates energy intake in rats [61,62], 2) OFQ/N prolongs deprivation-induced consumption and decreases c-fos expression in POMC neurons at the time of meal termination [63], and 3) ORL1 knockout blunts fasting-induced hyperphagia [64]. In electrophysiological recordings of POMC neurons from vehicle-treated, ovariectomized female rats, we found that OFQ/N produced a robust outward current that returned to baseline levels of holding current upon its clearance from the slice (Figure 8A). This outward current was associated with an increase in conductance, and reversed polarity around the Nernst equilibrium potential for K^+ (Figure 8B and 8C). These are characteristic features associated with activation of GIRK channels. However, in recordings of POMC neurons from estradiol-treated animals, this OFQ/N-induced activation of GIRK channels was markedly diminished (Figure 8).

Do other anorexigenic hormones negatively modulate the function of orexigenic, $G_{i/o}$ -coupled receptors? One likely candidate is the adipokine leptin that is the product of the *ob* gene [65]. Once released by adipocytes, it activates its cognate receptor that is expressed in several hypothalamic appetite-regulating centers to suppress energy intake via negative feedback [22,65–70]. Rodents and humans lacking either the leptin receptor or leptin itself exhibit several features that are characteristic of perturbed energy homeostasis; including hyperphagia, increased adiposity, hyperglycemia, hyperinsulinemia and dyslipidemia [65,71–73]. In addition, obesity seen under a variety of pathophysiological conditions is most often associated with hyperleptinemia and leptin resistance [72,74–76].

Leptin receptor activation elicits a complex signal transduction cascade, one that shares several features in common with estrogen receptor signaling [77]. The receptor has a cytoplasmic domain that, following activation and subsequent dimerization, allows for the association of Janus kinases (JAKs). This interaction leads to the activation of JAKs that, in turn, facilitates the recruitment and tyrosine phosphorylation of other molecules like signal transducer and activator of transcription (STAT)-3, insulin receptor substrate, forkhead box protein, mammalian target of rapamycin, extracellular signal-regulated protein kinase (ERK), phospholipase C (PLC), and PI3K/Akt [12,13,78–81].

In POMC neurons, and in steroidogenic factor (SF)1-containing neurons in the VMN, this signaling pathway leads to a membrane depolarization that increases the probability of generating action potentials [13,68,82,83]. This increase in firing rate ultimately is due to an inward current caused by the leptin-induced activation of postsynaptic transient receptor potential C (TRPC) channels [81]. On the other hand, leptin hyperpolarizes NPY/AgRP neurons [84] and attenuates the fasting-induced increase in neuronal firing [85], which may be attributed to the activation of postsynaptic K_{ATP} channels in these cells [86]. Leptin also disrupts endocannabinoid-mediated DSI in MCH [22] and orexin [18] neurons in the LHA. Conversely, it has been reported to decrease inhibitory GABAergic input onto POMC neurons [87]. Leptin receptors are also expressed in glutamatergic neurons in the VMN that play an important role in the leptin-induced changes in energy balance [19,88,89]. Similar to estradiol, we found that a brief exposure to leptin, in the presence of 2-APB to block the depolarizing effect of TRPC channel activation [81], markedly attenuated the reversible, OFQ/N-induced outward current in POMC neurons from ovariectomized female rats, as well as the associated increase in conductance (Figure 9). We also found that leptin increased the number of excitatory synaptic events per unit time impinging on POMC neurons from ovariectomized female rats, which can be readily ascertained upon examination of both the representative traces of membrane current and the quantile plot depicting the significant leptin-induced shift in the cumulative probability distribution for the interval between contiguous mEPSCs (Figure 10A and 10C). Importantly, this leptin-induced increase in mEPSC frequency is completely reversed by PI 828 (Figure 10B and 10C). Thus, both estradiol and leptin can negatively modulate the coupling of orexigenic, $G_{i/o}$ -coupled receptors to their effector systems. The resultant increase in POMC neuronal activity occurs through facilitation of excitatory neurotransmission, antagonism of GIRK channel currents, and activation of TRPC channels in these cells via stimulation of PI3K.

3. SIGNIFICANCE

The results gathered by our laboratory over the past few years have uncovered sex differences in, and modulatory effects of anorexigenic hormones on, the neuroendocrine regulation of energy homeostasis. Male guinea pigs are more responsive than females to cannabinoid-induced increases in energy intake and decreases in core body temperature, and estradiol further dampens these effects in females. These findings are consistent with those observed in the rat [38], and have clear clinical relevance. In the phase II trial and randomized, placebo-controlled studies touting the efficacy of THC and dronabinol to ameliorate cachexic symptoms and promote appetite in patients with cancer [90] and HIV/AIDS [91,92], the considerable majority of study participants were men. However, in

another clinical trial in which the gender ratio of the cancer patients was more equitably distributed, the results were not as compelling [93]. This reinforces the idea that gender and endocrine status needs to be taken into account in developing rational therapies for the treatment of HIV/AIDS- or cancer-related cachexia. Thus, it is predicted that CB1 receptor agonists would be less effective in normally cycling women, especially around the time of ovulation, and more effective under hypoestrogenic conditions such as anorexia nervosa, in which dronabinol promotes weight gain [94], and menopause. Regarding the latter, ~80% of the participants in the RIO-North America obesity trial were women with an average age of ~45 years that showed significant improvement in body weight, waist circumference and blood lipid profile following long-term treatment with the CB1 receptor antagonist Rimonabant [95]. Were it not for an increase in the incidence of dysphoric side effects (e.g., nausea, anxiety and, to a lesser extent, depression) relative to that experienced in placebo-treated controls, this drug undoubtedly would still be on the market to this day. While baseline female reproductive status was not specifically evaluated in this study, it is quite reasonable to assume that a substantial fraction of the study participants were women who were either poised to enter into their climacteric or fully menopausal. With this in mind, the Rimonabant-induced amelioration of the perturbed energy balance observed in the RIO-North America obesity trial might be explained, at least in part, by its ability to block the enhanced endocannabinoid tone resulting from the hypoestrogenic state experienced by a large percentage of the study participants.

The sex difference in, and estrogenic influence on, the cannabinoid regulation of energy homeostasis observed at the behavioral level is accompanied by parallel underlying changes in the cellular physiology at POMC synapses. In females, CB1 receptors couple to and thereby activate Kv4.2 channels associated with a transient A-type K^+ current in POMC neurons by reducing the amount of hyperpolarization required to transition the channels from the inactivated to the closed state [96], whereas in males they couple to GIRK channels that elicit a sizable outward current and membrane hyperpolarization [97]. Estradiol antagonizes the cannabinoid-induced activation of the Kv4.2 channels in POMC neurons, and attenuates the cannabinoid-induced presynaptic inhibition of glutamatergic input impinging upon these cells [46,98]. It does so by activating $ER\alpha$ and the G_q -mER [44]. This ER-mediated disruption of CB1 receptor/effector coupling in and around POMC neurons can be attributed to activation of PI3K, which is an upstream signaling molecule responsible for a variety of estradiol-mediated effects ranging from synaptogenesis [99] to endothelial nitric oxide production [100]. The pathway through which estradiol impairs CB1 receptor-mediated signaling also includes protein kinase C (PKC) and neuronal nitric oxide synthase (nNOS), as inhibitors of these enzymes rescue both the cannabinoid-induced hyperphagia and presynaptic inhibition of glutamate release onto POMC neurons in the presence of the steroid [44,45]. These synaptic dynamics are illustrated in Figure 11. Finally, the estradiol-induced activation of PI3K is involved along with PLC, PKC and protein kinase A in uncoupling other metabotropic, $G_{i/o}$ -coupled receptors like the $GABA_B$ and μ -opioid receptors from their GIRK channels in POMC neurons [51,101].

In addition, estradiol diminishes the coupling of ORL1 receptors from their postsynaptic GIRK channels in POMC neurons, and reduces the OFQ/N-induced presynaptic inhibition of glutamatergic input onto these cells [102]. Activation of ARC ORL1 receptors by OFQ/N

elicits orexigenesis [62], due in large part to reduced excitability of POMC neurons involved in meal termination [63]. This reduced POMC neuronal activity is the result of the ORL1 receptor-mediated hyperpolarization caused by activation of postsynaptic GIRK channels as well as the decreased glutamate release onto these cells [60,64,102]. Moreover, fasting-induced hyperphagia, as well as the pleiotropic actions of OFQ/N on POMC neurons, are appreciably blunted in ORL1 receptor null mice [64]. Thus, the disruption of the function of the orexigenic, $G_{i/o}$ -coupled ORL1 receptor expressed in POMC neurons and its excitatory synaptic inputs represents another physiologically relevant example of how estradiol exerts its anorexigenic actions. Furthermore, it is consistent with the estrogenic attenuation of the ORL1 receptor-mediated antinociception that occurs via an ERK-dependent mechanism [103].

Most interestingly, leptin can also impair ORL1 receptor/GIRK channel coupling in POMC neurons. This observation cannot be attributed simply to a matter of synaptic integration because TRPC channels, whose activation by leptin would exert opposing influences on membrane current (or potential) [81], were blocked by 2-APB. Therefore, the most parsimonious explanation is that leptin hampers the ability of OFQ/N to activate GIRK channels in POMC neurons. The signaling pathways of estradiol and leptin share many components in common including, but not limited to, STAT-3 and PI3K [77]. Both hormones also increase excitatory synaptic input onto POMC neurons [43,77]. This excitatory input arises from SF1-containing, leptin receptor- and CB1 receptor-bearing neurons in the VMN that are sensitive to fasting and reduced in *ob/ob* mice [10,19,88,89,104]. We have shown previously that this excitatory input is obliterated following application of ionotropic glutamate receptor antagonists [64,98]. In addition, we found that the leptin-induced increase in glutamatergic neurotransmission received by POMC neurons is due to the activation of a PI3K pathway. This same signaling molecule is also critical for the leptin-induced activation of TRPC channels and the subsequent increase in the excitability of these cells [13,81]. Moreover, OFQ/N hyperpolarizes and thereby inhibits these same leptin receptor-expressing VMN neurons via activation of GIRK channels [105]. Collectively, these findings illustrate the anatomical convergence and dynamic regulatory interplay between the SF1-containing, leptin receptor- and CB1 receptor-bearing VMN neurons and the anorexigenic POMC neurons, and how their activity can be profoundly influenced by estradiol, leptin, cannabinoids and OFQ/N. In conclusion, our findings lend considerable insight into the pleiotropic mechanisms through which these anorexigenic hormones modulate the function of orexigenic, $G_{i/o}$ -coupled receptors. Given that they also help coordinate the timing of sexual behavior with ovulation, fertilization and implantation [42,106–110], they help to establish the inexorable link between energy homeostasis and reproduction. Thus, during times of heightened female sexual receptivity, as seen during behavioral estrus around the time of ovulation, the primary focus is on finding an appropriate male counterpart with which to mate. Other appetitive behaviors related to energy intake are secondary. If successful fertilization has been achieved, then the converse would be true in the luteal phase, during which time an increased energy demand is created by the blastocyst being implanted into estradiol-primed, progesterone-stimulated uterine endometrium. On the other hand, primary amenorrheic individuals like those suffering from anorexia nervosa are both hypoestrogenemic and hypoleptinemic [111,112]. Refeeding in

these individuals increases body weight, body mass index, leptin and luteinizing hormone, which is associated with increased estradiol levels and a resumption of menstruation [112–114].

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Abbreviations

2-AG	2-arachidonoylglycerol
AgRP	agouti-related peptide
AMPK	AMP-activated protein kinase
ARC	hypothalamic arcuate nucleus
CRH	corticotropin-releasing hormone
DSI	depolarization-induced suppression of inhibition
ERK	extracellular signal-regulated protein kinase
GIRK	G protein-gated, inwardly-rectifying K ⁺
JAK	Janus kinase
LHA	lateral hypothalamic area
MCH	melanin-concentrating hormone
mEPSC	miniature excitatory postsynaptic current
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
OFQ/N	orphanin FQ/nociceptin
ORL	opioid receptor-like
PI3K	phosphatidylinositol-3-kinase
PLC	phospholipase C
PKC	protein kinase C
POMC	proopiomelanocortin
PVN	hypothalamic paraventricular nucleus
SF	steroidogenic factor
STAT	signal transducer and activator of transcription
THC	tetrahydrocannabinol
TRPC	transient receptor potential C

VMN hypothalamic ventromedial nucleus

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Highlights

1. CB1 and ORL1 receptors are orexigenic, $G_{i/o}$ -coupled receptors.
2. Estradiol and leptin physiologically antagonize the function of these receptors.
3. This may be a fundamental mechanism by which anorexigenic hormones suppress appetite.

Figure 1.

Timeline for behavioral testing in the female guinea pig. Stereotaxic guide cannula implantations and ovariectomies were performed 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 given seven days to recover after the first surgery and six days of recovery after the second; at which time the *in vivo* experiments were initiated. The analyses for energy balance were performed in a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments), as described in the timeline. After three days of acclimation to the CLAMS chamber, energy intake and expenditure for each animal were monitored around the clock for five days. Each morning at 8:00 a.m., treatments of either CB1 receptor agonist WIN 55,212-2 (3 µg; i.3.v.) and/or PI 828 (30 µg; i.3.v.) or their cremephor/ethanol/saline (1/1/18; v/v/v) vehicle were given. Every other day animals were pretreated with EB (10µg; s.c.) or its sesame oil vehicle (0.1mL) 15 minutes prior to the central administration of the aforementioned drugs. At the end of the five-day monitoring period, the animals were anesthetized with 32% isoflurane, rapidly decapitated, and the brain removed. 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 hours. The ARH was then microdissected from the slices for subsequent determination of PI3K p85α regulatory subunit mRNA expression and Akt phosphorylation via quantitative polymerase chain reaction and Western blotting, respectively.

Figure 2. Estradiol benzoate (EB) increases PI3K expression in the ARC. Bars represent means and vertical lines 1 SEM of the PI3K, p85α regulatory subunit mRNA expression determined in ARC tissue. *, Values measured in EB- and AM251-treated animals that are significantly different (Mann-Whitney U-test; $p < .05$; $n = 5 - 8$) than those measured in vehicle-treated controls. Adapted from [43] (MDPI AG Basel).

Figure 3.

EB activates Akt in the ARC. **A**, Representative Western blots illustrating the levels of Akt, pAkt, and the loading control GAPDH in the ARC microdissected from EB- and vehicle-treated animals. **B**, Composite bar graph illustrating the pAkt/Akt ratio determined in ARC microdissections from animals treated 2 hours prior with either EB or its sesame oil vehicle. Bars represent means and vertical lines 1 S.E.M., respectively. *, Values from EB-treated animals that are significantly different (Mann-Whitney U-test; $P < 0.05$; $n = 6$) from vehicle-treated controls.

Figure 4.

EB decreases energy intake and expenditure, the former of which is dependent on the activation of PI3K. The composite bar graphs illustrate whether the PI3K inhibitor PI 828 (30 µg; i.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/PI 828-treated animals that are significantly different (P<0.05; one-way ANOVA/LSD; n = 4 – 10) than those from vehicle-treated controls.

Figure 5.

PI3K mediates the estrogenic attenuation of the cannabinoid-induced hyperphagia. This composite bar graph illustrates the ability of PI 828 to rescue the increase in energy intake caused by WIN 55,212-2 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; 4 – 10) from vehicle-treated controls.

Figure 6.

Estradiol (E_2) rapidly attenuates the cannabinoid receptor agonist-induced presynaptic inhibition of glutamate release onto POMC neurons via an ER receptor-mediated mechanism. **A**, Spontaneous mEPSCs recorded in a vehicle-treated ARC neuron at baseline (left) and following exposure to 1 μ M WIN 55,212-2 (right). To the right of the membrane current traces is the cumulative probability plot illustrating the increase in interval (inverse of frequency) between contiguous mEPSCs in the cell on the left. To the right of the cumulative probability plot is the double-labeling of this same neuron that is immunopositive for β -endorphin (**a**, biocytin-streptavidin-AF488 labeling; **b**, β -endorphin immunofluorescence visualized by AF546; **c**, composite overlay). **B**, Spontaneous mEPSCs in a cell perfused with 100 nM E_2 at baseline (left) and following exposure to 1 μ M WIN 55,212-2 (right). To the right of the membrane current traces is the cumulative probability plot illustrating the interval between contiguous mEPSCs that substantiates the lack of cannabinoid effect in the E_2 -treated slice containing the cell on the left. To the right of the cumulative probability plot is the double-labeling of this same neuron that is immunopositive for cocaine-and amphetamine-regulated transcript (CART; **a**, biocytin-streptavidin-AF488 labeling; **b**, CART immunofluorescence visualized by AF546; **c**, composite overlay). **C**, Spontaneous mEPSCs recorded in a neuron co-treated with 100 nM E_2 and 1 μ M ICI 182,780 at baseline (left) and following exposure to 1 μ M WIN 55,212-2 (right). To the right of the membrane current traces is the cumulative probability plot illustrating the ability of ICI 182,780 to restore the cannabinoid receptor agonist-induced decrease in mEPSC frequency. To the right of the cumulative probability plot is the double-labeling of this same neuron that is immunopositive for α -melanocyte-stimulating hormone

(α -MSH; **a**, biocytin-streptavidin-AF488 labeling; **b**, α -MSH immunofluorescence visualized by AF546; **c**, composite overlay). *, Distribution of the mEPSC interval in the presence of WIN 55,212-2 that is significantly different (Kolmogorov-Smirnov test, $p < .05$) than that observed under basal conditions. Printed with permission from [43] (MDPI AG Basel).

Figure 7.

The estrogenic attenuation of the cannabinoid-induced decrease in glutamate release is blocked by PI 828. Spontaneous mEPSCs in a cell perfused with E₂ and 10 μ M PI 828 at baseline (left) and following exposure to 1 μ M WIN 55,212-2 (right). To the right of the membrane current traces is the cumulative probability plot illustrating the interval between contiguous mEPSCs that substantiates the ability of PI 828 to restore the inhibitory effect of WIN 55,212-2 on mEPSC frequency. To the right of the cumulative probability plot is the composite bar graph illustrating the efficacy of ICI 182,780 and PI 828 to block the E₂-induced attenuation of the decrease in mEPSC frequency caused by WIN 55,212-2. Bars represent means and vertical lines 1 SEM of the cannabinoid receptor agonist-induced decrease in mEPSC frequency. *, Distribution of the mEPSC interval in the presence of WIN 55,212-2 that is significantly different (Kolmogorov-Smirnov test, $p < .05$) than that observed under basal conditions. #, Values measured in E₂-treated slices that are significantly different (Kruskal-Wallis/median-notched Box-and-Whisker analysis; $p < .05$; $n = 5 - 8$) than those measured in the other treatment conditions. Printed with permission from [43] (MDPI AG Basel).

Figure 8.

Estradiol attenuates OFQ/N-induced postsynaptic currents. A) Membrane current traces from identified POMC neurons recorded in the presence of 1 µM TTX showing that 2 µg EB given 30 hours earlier to ovariectomized female rats had reduced OFQ/N-induced outward currents compared to the recorded neurons from vehicle-treated control animals. The solid arrows represent the commencement of the OFQ/N & TTX application, whereas the broken arrows denote the cessation of the application. B) This I/V plot illustrates the reduced OFQ/N induced currents in neurons from EB treated animals compared to oil vehicle controls. C) Composite bar graph illustrating the marked reduction in slope conductance (g) caused by EB compared to oil vehicle control animals. * = $P < 0.05$, multi-factorial ANOVA/LSD, $n = 16 - 19$. Printed with permission from [102](S. Karger AG, Basel).

Figure 9.

Leptin attenuates the OFQ/N-induced activation of postsynaptic K^+ currents in ARC neurons from ovariectomized female rats. **A**, Membrane current traces from a POMC neuron, generated during a whole-cell voltage clamp recording at a holding potential of -60 mV in the presence of 1μ M TTX, that depict the robust and reversible outward current caused by 1μ M OFQ/N. **B**, Membrane current traces generated as in **A** that show how leptin pretreatment (in the presence of 2-APB) reduces the OFQ/N-induced activation of GIRK channels. The arrows denote the termination of drug application. **C**, This I/V plot, generated from a series of depolarizing and hyperpolarizing voltage pulses (10 mV increments; 150 msec in duration) ranging from -50 to -130 mV, illustrates the OFQ/N-induced currents measured at different voltages that were diminished by leptin pretreatment. **D**, Composite bar graph illustrating how leptin dampens the OFQ/N-induced increase in g that was calculated between -60 and -80 mV, as well as between -100 and -130 mV, via linear regression. * = $P < 0.05$, multi-factorial ANOVA/LSD; $n = 13$.

Figure 10.

The leptin-induced increase in glutamatergic input is prevented following inhibition of PI3K. The traces of membrane current, generated during whole-cell voltage clamp recordings at a holding potential of -75 mV in the presence of 500 nM TTX and 10 μ M SR 95531 to block $GABA_A$ receptor-mediated input, show the mEPSCs in recordings of POMC neurons from ovariectomized female rats under basal conditions and in the presence of 100 nM leptin, alone (**A**) and following pretreatment with PI 828 (**B**; 10 μ M). The cumulative probability plots on the **right** illustrate the interval distribution between >100 contiguous mEPSCs, and substantiate the ability of PI 828 to eliminate the leptin-induced increase in mEPSC frequency. * = $P < 0.05$, Kolmogorov-Smirnov. **C**, Composite bar graph that summarizes the leptin-induced enhancement of glutamatergic input onto POMC neurons, which occurs via a PI3K pathway. Bars represent means and vertical lines 1 S.E.M. of the mEPSC frequency that was normalized to values observed under baseline conditions within their designated treatment groups. * = $P < 0.05$, Kruskal-Wallis/median-notched Box-and-Whisker analysis, $n = 4 - 6$.

Figure 11.

Schematic for how estradiol disrupt CB1 receptor-mediated signaling at glutamatergic inputs onto POMC neurons. Estrogens rapidly uncouple cannabinoid CB1 receptors from their effector systems in the glutamatergic nerve terminal by activating nNOS. This is achieved through an increase in PI3K, which enhances phosphatidylinositol triphosphate (PIP₃), in turn up-regulating Akt leading to augmented nNOS [45]. The increased enzyme activity may reduce endocannabinoid tone either by inhibiting synthesis, enhancing breakdown, or promoting reuptake and removal from the synaptic cleft. Alternatively, the nitric oxide produced may act transynaptically in a retrograde fashion to uncouple the CB1 receptor from its effector system(s) in the glutamatergic nerve terminal.