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Estrogenic regulation of reproduction and energy homeostasis by a triumvirate of hypothalamic arcuate neurons

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Abstract

Pregnancy is energetically demanding and, therefore, by necessity reproduction and energy balance are inextricably linked. With insufficient or excessive energy stores a female is liable to suffer complications during pregnancy or produce unhealthy offspring. Gonadotropin releasing hormone neurons are responsible for initiating both the pulsatile and subsequent surge release of luteinizing hormone to control ovulation. Meticulous work has identified two hypothalamic populations of kisspeptin (Kiss1) neurons that are critical for this pattern of release. The involvement of the hypothalamus is unsurprising as its quintessential function is to couple the endocrine and nervous systems, coordinating energy balance and reproduction. Estrogens, more specifically 17β-estradiol (E2), orchestrate the activity of a triumvirate of hypothalamic neurons within the arcuate nucleus (ARH) that govern the physiological underpinnings of these behavioral dynamics. Arising from a common progenitor pool, these cells differentiate into ARH kisspeptin, proopiomelanocortin (POMC), and agouti related peptide/neuropeptide Y (AgRP) neurons. Although the excitability of all these subpopulations is subject to genomic and rapid estrogenic regulation, Kiss1 neurons are the most sensitive, reflecting their integral function in female fertility. Based on the premise that E2 coordinates autonomic functions around reproduction, we will review recent findings on how Kiss1 neurons interact with GnRH, AgRP, and POMC neurons as well as how the rapid membrane-initiated and intracellular signaling cascades activated by E2 in these neurons are critical for control of homeostatic functions supporting reproduction. In particular, we will highlight how Kiss1 and POMC neurons conspire to inhibit AgRP neurons and diminish food motivation in service of reproductive success.

Keywords

Hypothalamus; Kisspeptin neurons; Neuropeptide Y; agouti-related peptide; proopiomelanocortin

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1. Estrogenic regulation of reproduction through kisspeptin neurons

Successful reproduction is the core measure of evolutionary fitness and the quintessential function of the hypothalamus is to link the nervous and endocrine systems to support this physiological process. Within the brain, gonadotropin-releasing hormone (GnRH) neurons drive pubertal development and regulate the reproductive cycle. Postnatal GnRH neurons predominantly reside in the medial septum, diagonal band, and preoptic area (POA) of rodents (1–3), but are also found in the basal hypothalamus of sheep (2), guinea pigs (4), and primates including humans (5, 6). From these regions projections are sent to the median eminence to secrete GnRH in a pulsatile fashion, stimulating anterior pituitary gonadotrophs to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). In females, LH and FSH in turn cause the synthesis and release of estrogen and progesterone from the ovaries. Following puberty, these ovarian hormones provide the requisite negative and positive feedback to maintain a normal cycle (7–10). This process is not linear, but rather relies on precisely timed GnRH pulses preceding a final surge to elicit LH release which causes ovulation and stimulates ovarian steroidogenesis. Significant effort has been expended elucidating the neural circuits involved and how estrogens regulate their activity to orchestrate the female reproductive cycle, which will be summarized in this section.

Classically, estrogenic signaling is viewed through estradiol (E2) activation of primarily ERα (11, 12), but also ERβ (13) receptors. Found in the cytosol, ERα or ERβ bind with estrogens and dimerize prior to translocation to the nucleus. Through interactions with estrogen response elements (EREs) present in certain gene promotors, estrogens can regulate transcription (14–16). In addition to cytosolic ERα and ERβ, E2 can activate membranebound estrogen receptors (mERs) to mediate rapid non-genomic actions (17, 18), though certain second messenger cascades such as phosphorylation of cAMP response element-binding protein can ultimately lead to alterations in gene expression as well (19–23). While ERα and ERβ can act as mERs, there are also the G-protein coupled estrogen receptor (GPER/GPR30/GPER1) (24, 25) and an as-yet unidentified Gq-coupled receptor (Gq-mER) (26, 27). These estrogen signaling pathways frequently co-exist and can produce divergent outcomes between cell types. For a more detailed overview of estrogen signaling cascades in hypothalamic neurons, please refer to Stincic et al., 2018 (28).

Despite the clear synchronization of GnRH neuronal activity with circulating estrogen levels (29), GnRH neurons are devoid of ERα (30–32). This revelation prompted a search for either estrogen signaling via alternative pathways or extrinsic synaptic inputs. Though ERβ is found in GnRH neurons (33–38), deletion of ERβ in either the whole animal or conditionally in GnRH neurons typically only impairs rather than eliminates fertility (39–41) by reducing the amplitude without affecting the timing of LH release (42, 43). The role of rapid estrogenic signaling is less clear but may assist in maintaining the GnRH surge for the requisite duration (44). So, while non-ERα signaling pathways contribute to GnRH release, mounting evidence has supported the existence of an extrinsic pulse generator.

Kisspeptin (Kiss1), transcribed from the *Kiss1* gene, is a neuropeptide primarily produced by two populations of hypothalamic neurons found in the arcuate nucleus (ARH) and anteroventral periventricular/periventricular nuclei (AVPV/PeN) (45–49). Kiss1, also

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referred to as Kisspeptin-54, is the endogenous ligand of G protein-coupled receptor 54 (GPR54, *aka* Kiss1R) (50). Kiss1 neurons and the Kiss1Rs are indispensable for normal pubertal development (51–54) and fertility (55–57). Centrally administered kisspeptin robustly stimulates GnRH and gonadotropin secretion in both pre-pubertal and adult animals (45 , 58). Mutations in GPR54 cause autosomal recessive idiopathic hypogonadism in humans and deletion of GPR54 or Kiss1 in mice results in defective sexual development and reproductive failure (51, 53). GnRH neurons robustly express *Gpr54* mRNA (59), and conditional deletion of the gene produces offspring that do not progress through puberty, have reduced gonadal size and are infertile (60). Underscoring the importance of Kiss1 signaling on GnRH function, the infertility phenotype in global GPR54 knockouts can be rescued by reintroducing the receptor solely in GnRH neurons (60). Kiss1 neurons express high levels of ERα and ERβ in both the AVPV/PeN (31, 55, 61) and the ARH (62). Therefore, estrogenic regulation of Kiss1 signaling could communicate the reproductive state of an animal to GnRH neurons.

GnRH neurons are strongly depolarized by kisspeptins. *In vitro* brief kisspeptin bath application produces a long-lasting depolarization in most adult GnRH neurons, regardless of sex (63–67). However, the responsiveness of GnRH neurons is much smaller in juvenile and prepubertal male mice despite a near constant level of *Kiss1R* mRNA expression across development (63). Based on cell signaling studies, kisspeptin excites GnRH neurons primarily through activation of canonical transient receptor potential 4 (TRPC4) channels (66, 68–70) and to a lesser extent through inhibition of inwardly rectifying K^+ channels (66–68, 71, 72). TRPC channels can be activated by G-protein coupled receptors (73, 74) or participate in store-operated calcium entry (75). *Trpc4, Hcn1, Cav1.3, Cav 2.2, and Cav 2.3* mRNA expression are all increased (59), which would presumably enhance responsiveness to kisspeptin. These findings demonstrate that E2 and kisspeptin signaling can drive GnRH/LH release but do not reveal how the pulses or surge are generated.

One of the more striking features of estrogenic regulation of Kiss1 neurons is the divergent effects on the ARH and AVPV/PeN populations. $Kiss1^{ARH}$ neurons co-express and release neurokinin B (NKB) and dynorphin (Dyn) (76–78), leading to their nickname of “KNDy neurons” (79). When circulating levels of E2 are high, $Kiss1^{ARH}$ neurons display enhanced expression of *Vglut2* while *Kiss1* mRNA expression is attenuated, biasing neurotransmission from peptidergic to glutamatergic signaling (80). $Kiss1^{ARH}$ neurons form a reciprocal network with other $Kiss1^{ARH}$ neurons (80) as well as sending projections to $Kiss1^{AVPV/PeN}$ neurons and GnRH “dendrons” and terminals in the median eminence (3, 81–84). Because $Kiss1^{AVPV/PeN}$ neurons do not express TACR3s (NKB receptor) or Kiss1Rs (85, 86), $Kiss1^{ARH}$ inputs to the AVPV/PeN population are essentially silent when E2 levels are low (80). However, as E2 levels peak in the lead up to proestrus and the LH surge, synchronization of $Kiss1^{ARH}$ neurons will exert an excitatory glutamatergic input to both $Kiss1^{AVPV/PeN}$ neurons and distal processes of GnRH neurons (69, 80, 87, 88). In this manner, the $Kiss1^{ARH}$ neurons can act as the pulse generator while driving up $Kiss1^{AVPV/PeN}$ activity that later initiates the LH surge.

The AVPV/PeN expresses high levels of ERα and ERβ, and the actions of the gonadal steroid hormones on Kiss1 neurons are mediated, at least in part, via nuclear-initiated

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signaling (transcriptional) mechanisms (31, 55, 61). For example, following E2 treatment *Kiss1* mRNA expression is upregulated in the AVPV/PeN (47). Furthermore, E2 treatment enhances the expression of *Vgat* and tyrosine hydroxylase to, respectively, support GABAergic and dopaminergic signaling in $Kiss1^{AVPV/PeN}$ neurons (89, 90). Proestrus levels of E2 also elevate $Kiss1^{AVPV/PeN}$ excitability through positive regulation of currents such as the h-, T-type calcium and a persistent sodium current (91–93) (95). Together with previous observations that in rodents lesions of the AVPV/PeN or ER antagonist implants in the region abrogate the positive feedback effects of E2 (96–99) many have hypothesized that E2 acts on $Kiss1^{AVPV/PeN}$ neurons to induce the positive feedback on GnRH and LH secretion. More recently, experiments have shown that high frequency optogenetic stimulation of $Kiss1^{AVPV/PeN}$ neurons evokes kisspeptin release that activates TRPC4 channels in order to depolarize and excite GnRH neurons (87). However, despite the necessity of $Kiss1^{AVPV/PeN}$ neurons for the GnRH/LH surge (55, 87, 100, 101), these neurons are not involved in the pulsatile release of LH. Rather multi-unit recordings point to a different origin of pulse generator activity (83, 102–105).

Though the ARH was long speculated to be the origin of patterned activity, only relatively recently were $Kiss1^{ARH}$ neurons identified as the responsible neuronal subpopulation (81, 87, 106, 107). *In vivo* optogenetic stimulation of $Kiss1^{ARH}$ neurons proved capable of eliciting pulsatile GnRH and, subsequently, LH release in the mouse (106, 108). *In vitro* recordings, however, were necessary to reveal the neurocircuitry required to synchronize ARH Kiss1 activity (Figure 1) (87). To summarize, E2 levels are low prior to onset of LH pulses at which time $Kiss1^{ARH}$ neurons produce and co-release the neuropeptides NKB and dynorphin. $Kiss1^{ARH}$ neurons form a network of excitatory reciprocal connections through NKB release. Dynorphin then presynaptically inhibits further release, reducing the activity of all networked $Kiss1^{ARH}$ neurons (87). This sequence repeats, giving rise to oscillations in neural activity. Next, as E2 levels rise in advance of ovulation, expression of NKB, dynorphin, and kisspeptin genes falls (47, 79, 81) as *Vglut2* mRNA expression and glutamate release probability dramatically rise (80). This causes a progression from peptidergic to amino acid transmission and affects not only inputs from $Kiss1^{ARH}$ to $Kiss1^{AVPV/PeN}$ neurons, but also to other ARH neurons (see below). The $Kiss1^{ARH}$ glutamatergic excitation of $Kiss1^{AVPV/PeN}$ neurons also contributes to kisspeptin-mediated excitation of GnRH neurons in the preovulatory state (87, 109). Furthermore, the precise timing of these events is synchronized to circadian rhythms through vasopressin (AVP) projections from the suprachiasmiatic nucleus to the $Kiss1^{AVPV/PeN}$ neurons such that ovulation occurs immediately prior to activity onset (110). Kiss1 AVPV/PeN and ARH neurons make reciprocal connections (3), which would explain why silencing of $Kiss1^{ARH}$ neurons causes a diurnal shift in eating patterns and obesity (111). Finally, although $Kiss1^{AVPV/PeN}$ neurons are responsible for the LH surge, $Kiss1^{ARH}$ neurons can amplify the surge when E2 is low or paired with progesterone (109).

2. Hypothalamic neurons link reproduction and energy homeostasis

$Kiss1^{ARH}$, proopiomelanocortin (POMC), and neuropeptide Y/agouti-related peptide (AgRP) neurons arise from a common precursor pool (112, 113) and together these neurons govern both reproduction and energy homeostasis. The close juxtaposition of these neuronal

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subpopulations to the median eminence, a circumventricular organ, enables circulating indicators of energy state (*e.g.* blood glucose, leptin, and insulin) to reach these neuronal populations which then convey the information downstream targets (114, 115). ARH neurons are also able to sense E2 levels by virtue of robust estrogen receptor expression (116–118), establishing a point of crosstalk between endocrine and homeostatic signaling in the brain. Historically ARH regulation of energy balance was viewed as a "tug-of-war" between two ARH subpopulations, pro-opiomelanocortin (POMC) and neuropeptide Y/agouti-related peptide (AgRP) neurons. However, Kiss1 neurons and kisspeptin/GPR54 signaling has been revealed to also influence metabolism (119), which has led to the idea that these three populations form a "triumvirate" of neurons to regulate energy homeostasis.

AgRP neurons are considered orexigenic and, regardless of energy state, can drive rapid food consumption (120–123). However, recent findings have challenged this "classical" homeostatic model of ARH function. A long-held assumption was that AgRP activity persisted during food consumption until post-ingestional effects emerged (*e.g.,* elevated blood glucose and insulin); however *in vivo* photometry revealed that detection of food, depending on energy state and palatability, is sufficient to rapidly inhibit AgRP while exciting POMC neurons (124). Therefore, anticipation and extrinsic factors also influence the connectivity between and output of ARH neurons. Regardless, AgRP neurons do not send direct projections to GnRH neurons, but instead act indirectly through GABAergic inhibition of Kiss1 neurons (125). Therefore, low energy stores would lead to increased AgRP excitability and signaling to decrease Kiss1 and, consequently, GnRH activity. Indeed, chemogenetic activation of AgRP neurons prolongs the estrous cycle, whereas targeted ablation of AgRP neurons attenuates the inhibitory tone on Kiss1 neurons (125) and restores fertility in obese or leptin deficient mice (126). Fasting activates AgRP neurons (127), which reduces fertility and expression of *Kiss1* in the ARH (128). Therefore, persistent AgRP activity acts as an indicator of undernutrition and inhibits reproduction.

Conversely, POMC neurons are active when energy stores are replete and decrease food intake following stimulation (120, 129, 130). In addition to the amino acid neurotransmitters GABA (131, 132) and glutamate (131), POMC neurons release a diverse complement of neuropeptides. The POMC precursor peptide is processed into several neuropeptides including, but not limited to, α-melanocyte stimulating hormone (α-MSH, excitatory) and β-endorphin (inhibitory) (133). In contrast to AgRP neurons, POMC neurons send direct projections to GnRH neurons, and the selective μ-opioid agonist DAMGO ([D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin), which mimicks β-endorphin's action (134), hyperpolarizes (inhibits) GnRH neurons through activation of a K^+ conductance (135–138). Naloxone, an opioid antagonist, stimulates GnRH release (139–143) and increases LH production (144, 145), suggesting POMC β-endorphin signaling inhibits GnRH activity and reproductive function (146), whereas selective activation of the α-MSH pathway is stimulatory (146).

More straightforward is Kiss1 to POMC neuronal signaling. Kisspeptin administered *icv* reduces food intake (147), optogenetic stimulation of Kiss1^{ARH} neurons elicits glutamatergic (80, 148) and kisspeptin mediated excitation of POMC neurons (149). Reciprocal connections are made between POMC and Kiss1 neurons (150). POMC neuronal signaling may also use Kiss1 neurons as an intermediary with GnRH neurons since

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a subpopulation of Kiss1$^{AVPV/PeN}$ neurons expresses melanocortin 4 receptor (MC4R) (49), and in sheep the majority of Kiss1^{ARH} neurons express the mRNA for MC3R (151). Blockade of melanocortin signaling in peripubertal females decreases *Kiss1* mRNA expression in the Kiss1^{ARH} neurons (146), and MC4R knockout impairs fecundity (152). Moreover, overexpression of AgRP, an inverse agonist for MCRs, causes infertility (126, 146, 153). While the specifics of POMC inputs to Kiss1 neurons remains unclear, one would assume these projections act to limit food intake in favor of reproductive behaviors.

Due to the metabolically demanding nature of pregnancy, it is no surprise that neurons involved in metabolism also affect fertility. Women suffering with anorexia often exhibit amenorrhea (154–157) and miscarriage risk is heightened in underweight females (158). Unsurprisingly, when energy reserves are low, AgRP neurons limit reproductive functions (125). Metabolism and kisspeptin neurons also affect one another. For example, hypogonadism due to undernutrition is associated with decreased *Kiss1* mRNA expression across a range of ages and species (128, 159–162), Conversely, a high-fat diet is associated with elevated levels of NKB expression and precocious puberty in female rats (163), demonstrating that disruptions of energy homeostasis in either direction can affect kisspeptin signaling. In service of their antagonistic roles to maintain energy balance, AgRP and POMC neurons are inversely regulated by glucose and metabolic hormones including leptin and insulin (164–166). Leptin, a hormone produced by white adipocytes (167), signals the total body energy stores. Mice deficient in leptin or lacking the requisite receptor present an infertile, obese phenotype (168, 169). Since GnRH neurons lack leptin receptors (170, 171), leptin regulation of GnRH neurons is indirect. ARH neurons are one potential line of communication and ablation of AgRP neurons is sufficient to reverse the obese phenotype (126), but as previously stated AgRP neurons do not directly contact GnRH neurons. However, Kiss1^{ARH} neurons express leptin receptors (172, 173), and similar to POMC neurons, leptin depolarizes and increase their firing (173). In contrast, leptin hyperpolarizes AgRP neurons via opening K^+-ATP (174) and Kv2.1 channels (175). Without the inhibitory effects of leptin AgRP neurons become highly active (176) and inhibit Kiss1^{ARH} and Kiss1$^{AVPV/PeN}$ neurons (Figure 2) (125) as well as POMC neurons. Therefore, AgRP activity will be relayed to GnRH neurons, at least in part, through Kiss1 and POMC neurons. Injection or overexpression of leptin into lean mice accelerates the onset of puberty (177, 178), and obesity is associated with precocious puberty in females (179, 180). Therefore, undernutrition and adiposity will affect leptin levels, which in turn influence not just fertility but pubertal timing.

Short-term indices of energy balance also affect ARH function through both genomic and rapid signaling mechanisms (Figure 2). Furthermore, the ARH is a critical control center for peripheral insulin sensitivity and glucose metabolism (181–185). In lean animals, rising blood glucose levels after meal consumption cause insulin release. Circulating insulin easily reaches the ARH neurons adjacent to the median eminence, and *in vitro* perfusion of insulin into the ARH rapidly depolarize POMC neuron by activating inositol triphosphate 3-kinase signaling pathways (186) and ultimately TRPC5 channels (185). TRPC channels can function as both receptor- or store-operated channels opened, respectively, by membrane delimited receptors or depletion of Ca^{2+} stores (75, 187). At the same time AgRP neurons are hyperpolarized by insulin through activation of ATP-sensitive potassium channels (185).

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However, neurons and peripheral tissues can become resistant to insulin and develop glucose intolerance. Acute activation of AgRP neurons rapidly but transiently impairs glucose and insulin tolerance (188) through NPY signaling that limits glucose uptake in brown adipose tissue (BAT) (189). This phenomenon could be a temporary physiological response to simultaneously limit energy expenditure while promoting foraging (121, 190). However, loss of insulin and glucose sensitivity can decouple ARH neurons from homeostatic feedback, promoting or perpetuating obesity (191–193). High-fat diet induced obesity also leads to leptin and ghrelin resistance as well as increased NPY/AgRP neuronal excitability (175, 194). Interestingly, while knockout of leptin receptors in AgRP neurons delays puberty (195), childhood obesity is highly associated with precocious puberty (196–198). In an obese state, TRPC channels associate with the endoplasmic reticulum protein stromal-interaction molecule (STIM1) to function as store-operated and, hence, are no longer opened by insulin (75). E2 protects females from developing insulin resistance in the brain by downregulating STIM1 in POMC neurons, increasing their excitability, and preventing conversion of TRPC to store-operated channels (199). Knockdown of *Stim1* mRNA in $Kiss1^{ARH}$ cells enhances TRPC5 currents in response to senktide and protects against HFD-induced obesity (200). More importantly, E2 also protects against insulin resistance by preventing high-fat diet related upregulation of SOCS-3 (suppressor of cytokine signaling 3), thereby preserving insulin signaling in female rodents (199). In addition, insulin significantly increases *Pomc* mRNA expression within 72 hours following *icv* administration (201). Therefore, circulating estrogens are vital for maintaining insulin sensitivity throughout the female reproductive cycle and are neuroprotective against insulin resistance in obese states.

The ventromedial nucleus of the hypothalamus (VMH) neurons has long been recognized as an integral site for energy balance (202) that also provides positive estrogen feedback (203). Dorsomedial VMH neurons express leptin and insulin receptors (204–208), but ERα-labeled neurons appear restricted to the ventrolateral region (209). The majority of VMH neurons also play a role in glucose sensing (210, 211), displaying excitation with increasing glucose concentrations (212). Only sparse projections are sent outside the VMH; however, a few axons reach the ARH, dorsomedial hypothalamus (DMH), and paraventricular nucleus of the hypothalamus (PVH) (213, 214). On a gross functional scale, the VMH acts anorexigenically with electrical stimulation decreasing food intake (215), and lesions or injections of ERα interference RNA causing hyperphagic and obese phenotypes (216–219). Steroidogenic factor-1 (SF-1) is expressed exclusively within a subpopulation of VMH neurons (220) that innervate and excite POMC neurons (221–226). SF-1 neurons also send excitatory projections to GnRH neurons (227, 228). ERα signaling appears to stimulate energy expenditure through SF-1 neurons (114), and SF1 ablation diminishes LH secretion (229). NPY inhibits VMH neurons (230) through Y1 receptors (231), attenuating VMH-mediated excitation of POMC neurons (232). Therefore, in addition to direct reciprocal connections NPY and POMC neurons utilize VMH SF-1 neurons as an intermediary regulator. Brain-derived neurotrophic factor (BDNF) is an important protein in the differentiation and survival of neurons through development (233) that also plays a role in adult energy balance, particularly in a number of VMH neurons (234, 235). BDNF is co-expressed by most, but not all (~60%) SF-1 neurons (236) and appears to participate in

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energy balance (237), specifically by decreasing meal size (238–241). BDNF and ERα are co-localized in the VMH (242), and the anorexigenic efficacy of BDNF is largely dependent on E2 levels (243). So while there are clear links between the VMH and ARH in regards to metabolism and reproduction, whether or not Kiss1 neurons participate is unknown and deserves further study. If synapses are made in either direction with Kiss1 neurons, one would assume these to be excitatory and anorexigenic.

Kiss1 neurons also regulate energy balance through their projections to nuclei outside the mediobasal hypothalamus (*i.e.,* ARH and VMH). The PVH is important for neuroendocrine and autonomic regulation of numerous functions such as food intake (244). $Kiss1^{ARH}$ and $Kiss1^{AVPV/PeN}$ neurons make close contact with PVH AVP and oxytocin neurons, and optogenetic stimulation of each Kiss1 population can, respectively, elicit postsynaptic glutamatergic or GABAergic responses, demonstrating a direct functional input to the PVH (3). As the PVH is heterogeneous in cell type and is composed of subregions with distinct projection patterns (245), further work remains to be done elucidating the functional significance of these Kiss1 inputs. Concerning the other aspect of the control of energy homeostasis, the DMH is important in thermoregulation through energy expenditure(246) (247–249). Leptin, in addition to inhibiting ARH AgRP neurons (176), also activates neurons in the DMH to drive BAT thermogenesis (250–253). Global knockout of Kiss1R/GPR54 (brain and peripheral tissues) selectively induces an obese phenotype in female mice (254–256). However, Kiss1R knockout constrained to BAT produces a lean phenotype with increased metabolism and body temperature (257), suggesting both direct and indirect signaling pathways. $Kiss1^{ARH}$ fibers do make close contact with leptin receptor expressing neurons in the DMH, and optogenetic stimulation elicits direct glutamatergic postsynaptic responses (3). Activation of leptin receptor-expressing DMH neurons robustly induces energy expenditure (258). Conversely, silencing these neurons decreases expenditure and increases food intake (259) with the latter effect likely the consequence of a lost inhibitory input to AgRP neurons (260). Estrogenic signaling may also play a role in adapting to changes in ambient temperature as E2 increases cFos immunocytochemical labeling in the DMH when animals are exposed to cold (261). However, the DMH exhibits low expression of ERα and ERβ (262), which would suggest either involvement of GPER, Gq-mER or an E2-sensitive input. Indeed, projections from leptin-sensitive neurons in the ARH (263), which are estrogen-sensitive, and the DMH (264) are crucial for communicating hormonal signals of energy balance to PVH neurons, which lack leptin receptors (204) and sparsely express ERα mRNA (265). AVP and OT neurons in the PVH as well as corticotrophin releasing hormone neurons do express ERβ (266) (267). Therefore, the steroid (E2)-sensitive $Kiss1^{ARH}$ neurons are likely not only vital for controlling pulsatile release of GnRH/LH but also coordinating energy homeostasis with reproductive activities through projections to regions such as the PVH and DMH.

3. E2 regulation of ARH circuitry and energy balance

More subtle changes are present in signaling and behavior during healthy reproductive cycles as E2 levels naturally rise and fall. Food intake, specifically sweet foods, decreases during the follicular phase (high E2) of the menstrual cycle (268–270). Female rodents also eat up to 25% less food during the evening following the LH surge (271, 272), which

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could represent the delay between E2-initiated transcription changes and protein synthesis. Decreases in food consumption are due to smaller meal sizes that are not fully compensated for by a slight increase in meal frequency (270, 273–275) , which may speak to how satiety is affected. Intact female rodents exhibit a “scalloping” pattern of activity, in which running activity increases on the night of proestrus (276, 277). Ovariectomy eliminates this effect and leads to an overall decrease in motor activity (278–280) as well as increased food intake (27, 275, 281–285); however, E2 replacement alone is sufficient to restore normal energy balance (23, 27, 275, 278–280). With respect to estrogenic signaling, ERα knockout mice develop an obese phenotype reflective of that observed following ovariectomy (286). Moreover, metabolic deficits are reversed by restoration of ERα, despite lacking the ERE targeting domain, which emphasizes the importance of non-classical signaling (287). Different models of ERβ knockout mice present a range of phenotypes from an impaired ovulatory cycle (288) to complete infertility (289). However, selective deletion of the ERE domain produces only a mild phenotype, once again underscoring the importance of non-classical ERβ signaling (290). GPER knockout mice display sexual dimorphism; although both sexes exhibit reduced energy expenditure and increased body weight, this phenotype emerges six weeks later in female rodents (291). While each subpopulation of ARH neurons exhibits different effects of estrogenic regulation, in general the signaling of anorexigenic neurons is enhanced while orexigenic signaling is attenuated. These are just a few examples of how estrogens are pleiotropic regulators, influencing communication between POMC, AgRP, and Kiss1 neurons in the ARH to coordinate reproduction and energy balance (Figure 2).

POMC neurons are one means by which the anorexigenic effects of E2 are communicated. Disruption of POMC signaling typically results in a positive energy balance. For example, E2 promotes physical activity by upregulating *Mc4r* gene expression in neurons in the VMH, sensitizing them to POMC inputs (292). POMC-specific deletion of ERα is sufficient to induce hyperphagia and increased heat production (114). The contradictory thermogenesis phenotype could be due to higher circulating levels of E2, which is suggested by blunted negative feedback of E2 on LH release that produces abnormal estrous cycles. Regardless, estrogenic signaling is of tantamount importance to the anorexigenic function of POMC neurons in females. There is an upregulation of *Pomc* mRNA expression with high circulating levels of E2 (i.e., proestrus/surge levels) (293, 294) when compared to ovariectomized females rodents (295, 296). Processing of the POMC precursor peptide into β-endorphin is also enhanced by E2 (297, 298). In addition, glutamatergic signaling is supported by E2 through increased expression of *Vglut2* mRNA which manifests functionally as a higher release probability (150, 299). Furthermore, acutely applied E2 or Gq-coupled mER ligand STX also increases the probability of glutamate release (150), possibly by decoupling $GABA_B$ receptors from G protein-coupled inwardly rectifying K^+ (GIRK) channels in POMC nerve terminals (27, 300). E2 also enhances the overall excitability of POMC neurons through upregulation of calcium channel subunits and receptor-operated TRPC channels (199, 301). Therefore, POMC neuronal excitability and neurotransmission is potentiated during proestrus to reduce food intake.

Since E2 is an anorexigenic hormone, it is logical that estrogenic signaling inhibits AgRP neuronal activity (302). Initially it was thought that AgRP neurons unilaterally sent

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inhibitory GABAergic projections to POMC neurons (123, 164); however, channelrhodopsin assisted circuit mapping has shown POMC neurons send reciprocal projections to AgRP neurons, primarily releasing β-endorphin and glutamate (Figure 2) (150). Initial reports noted optogenetic stimulation of POMC neurons most commonly results postsynaptic GABA-mediated currents in unidentified ARH neurons (131). However, other studies focused on responses in AgRP neurons found that optogenetic activation of POMC neurons rarely elicits a fast GABAergic or a slow excitatory (*e.g.* α-MSH) current (120, 123, 150). Few AgRP neurons express *Mc4r* but surprisingly nearly half express *Mc3r* (303). Therefore, the infrequency of GABA and melanocortin mediated responses suggests segregated neurotransmission from POMC neurons (304–307). The predominance of POMC glutamatergic and the infrequency of GABAergic inputs seems contradictory since one would not expect a satiety neuron to excite a hunger neuron. However, enhanced glutamate release from POMC neurons would exert an overall inhibitory tone since E2 upregulates mGluR7 (Group II/III mGluR) mRNA expression in NPY/AgRP neurons (Figure 2) (80). Together these changes in transcription will enhance POMC inhibition of AgRP neurons when circulating E2 is high.

Perhaps, Kiss1 and POMC neurons set the tone of homeostatic circuits based on the reproductive state of the female. As E2 levels peak preceding ovulation, the neuronal activity of AgRP neurons will be suppressed, unless energy reserves are critically low, to reduce food motivation. For example, fasting enhances AgRP activity and signaling by rapidly rewiring circuits (308). In those circumstances AgRP neurons will strongly inhibit Kiss1 neurons (125), disrupting the estrous cycle. Despite initial reports (309), AgRP neurons do express ERα (302); however, under normal physiological conditions rapid E2 signaling bidirectionally adjusts the activity AgRP neurons. For example, E2 alters the ability of $GABA_B$ receptors to activate G protein inwardly rectifying K^+ (GIRK) channels, either strengthening or weakening the coupling based on the relative expression of ERα versus Gq-mER at the time (302, 310). That is not to say that some genomic estrogenic regulation is also present. For example, E2 also decreases AgRP excitability through increased transcription of *Kcnq5* mRNA, enhancing the inhibitory M-current (311). These findings suggest that estrogenic signaling uses both genomic and rapid mechanisms to regulate POMC and Kiss1 function but relies primarily on membrane-initiated signaling for adjusting the activity of AgRP neurons.

4. Conclusions and Future Directions

In summary, estrogenic signaling governs a tripartite collaboration between ARH neurons that regulates GnRH neurons to synchronize energy balance and fertility to maximize reproductive success. When energy reserves are low, POMC activity will be minimal accompanied by a nominal amount of glutamate release onto AgRP neurons (Figure 2). With sufficient energy stores AgRP neurons become less active, leading to disinhibition of POMC neurons (312). Next, POMC activity firing rates rise to the higher frequencies (~20 Hz) (313) necessary to elicit synaptic release of β-endorphin that inhibits AgRP neurons via activation of μ-opioid receptors (150). When circulating E2 levels are high, such as during proestrus, POMC and $Kiss1^{ARH}$ neurons have enhanced glutamate release probability onto AgRP neurons (80). However, simultaneously increased expression of the

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Group III metabotropic glutamate receptor 7 postsynaptically in AgRP neurons causes this to be a net inhibitory input (80, 150). With the same enhanced glutamate release, $Kiss1^{ARH}$ neurons will excite POMC neurons through Group I mGluRs (80). As genomic changes lag while genes are expressed and transcribed rapid, estrogenic signaling likely eases transitions between states (low vs high E2). The positive and negative feedback between ARH neuronal subpopulations may serve to prevent small and/or transient fluctuations in the energy state of the animal from triggering dramatic shifts in the balance of ARH function (312) and enable E2 to bias, but not dictate, interactions between ARH neurons. The functional output of these neural circuit dynamics would manifest behaviorally as changes in reward salience and motivation. Briefly, when a female has sufficient energy stores to support a pregnancy, ovulation is induced, and priorities switch from ingestive behavior to mating. Indeed, ovariectomized female rodents find sucrose more rewarding than E2-treated females (314). In addition, when *Vglut2* mRNA is deleted from $Kiss1^{ARH}$ neurons, this E2 protective effect is lost (80), suggesting that glutamatergic inhibition of AgRP neurons combined with excitation of POMC neurons may be an underlying mechanism.

Although there is little doubt that estrogenic signaling is necessary for Kiss1 neurons to control GnRH and LH release, estrogens clearly orchestrate communication between Kiss1, AgRP, and POMC neurons to regulate energy balance in the service of optimizing reproductive success. Still much work remains to be done to elucidate POMC inputs to ARH and AVPV/PeN Kiss1 neurons (146). Finally, GnRH neurons have variable responses to NPY, α-MSH, and β-endorphin (315) with at least the potency of the μ-opioid response regulated by E2 (316). Therefore, metabolic cues may be communicated directly by POMC and AgRP neurons or through Kiss1 neurons to GnRH neurons. Future studies should be aimed at further unraveling the complexity of the neuronal signaling by this triumvirate of arcuate neurons (Figure 2).

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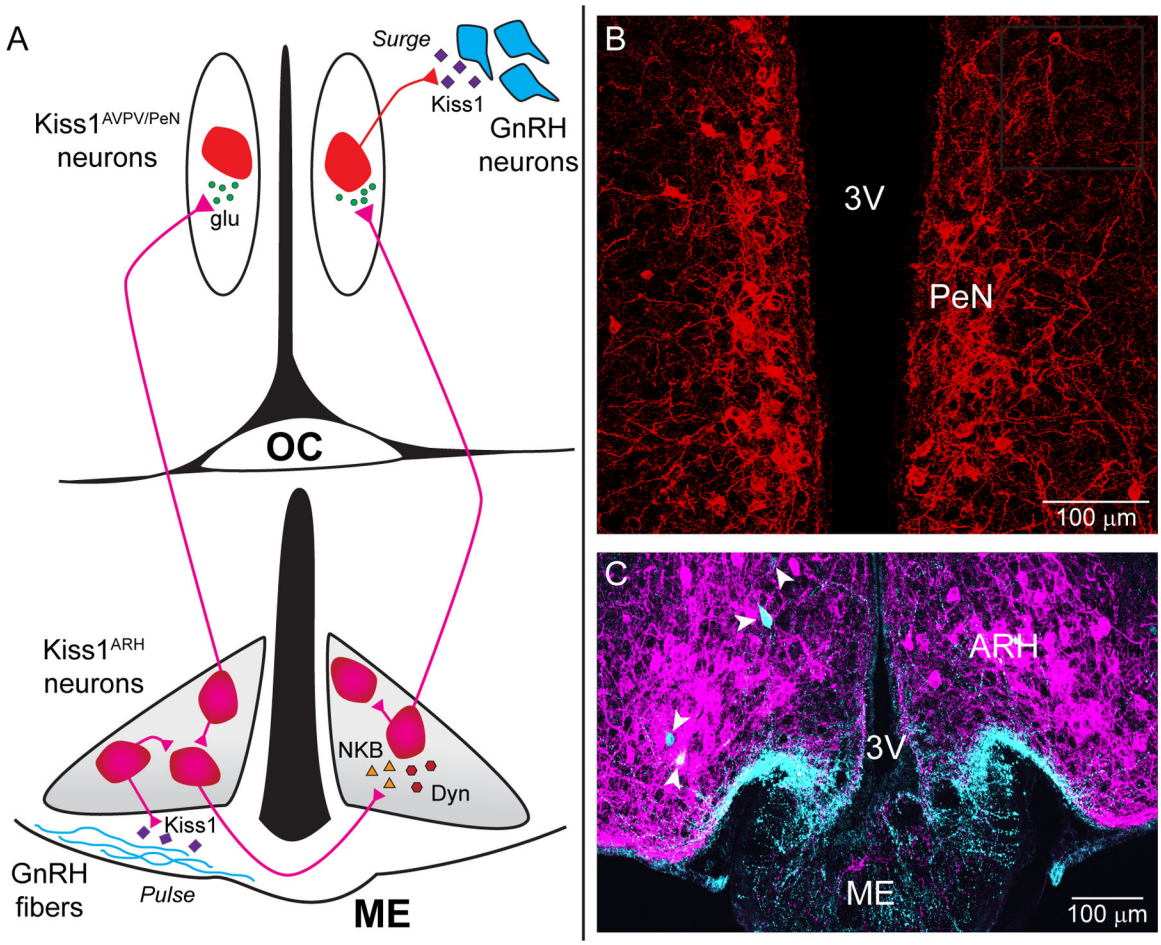

Figure 1. Estradiol governs signaling between Kiss1 and GnRH neurons to drive LH release.

A) Prior to proestrus, estradiol (E2) levels are low and kisspeptin (Kiss1) neurons (bottom, magenta) in the arcuate nucleus of the hypothalamus (ARH, grey region) release neurokinin B (NKB, triangles) that depolarizes and recruits other Kiss1^{ARH} neurons. Dynorphin (Dyn, hexagons) is co-released and acts presynaptically to modulate (inhibit) the release of NKB. Together the two peptides govern the synchronous activity of Kiss1^{ARH} neurons and promote kisspeptin release (diamonds) that stimulates pulsatile gonadotrophin-releasing hormone (GnRH) release from fibers (cyan) in the median eminence (ME). As estradiol levels rise Kiss1^{ARH} neurons transition from peptidergic to primarily fast glutamatergic (circles) neurotransmission to communicate with the Kiss1$^{AVPV/PeN}$ neurons, which stimulates burst-firing of Kiss1$^{AVPV/PeN}$ neurons. E2 also enhances the excitability and kisspeptin release of these rostral Kiss1 neurons (top, red) to robustly excite GnRH neurons via activation of the GPR54 signaling cascade, thereby stimulating the release of GnRH at the time of the preovulatory surge. Kisspeptin, GPR54, NKB, Tacr3 and GnRH are all required for normal fertility. **B)** Confocal micrograph of the PeN containing Kiss1 neurons found along the third ventricle. **C)** Confocal micrograph showing labeled Kiss1 cell bodies (magenta) and a few GnRH cell bodies (cyan with white arrowheads) in the ARH as noted in Herde et al. (J. Neuroscience 2013; 33:12689-97). GnRH fibers from the preoptic run along the ventral ARH into the ME. (3V: third ventricle; AVPV: anteroventral periventricular;

ARH: arcuate nucleus of the hypothalamus; Kiss1: kisspeptin; ME: median eminence; MnPO: median preoptic nucleus; OC: Optic Chiasm; PeN: periventricular nucleus)

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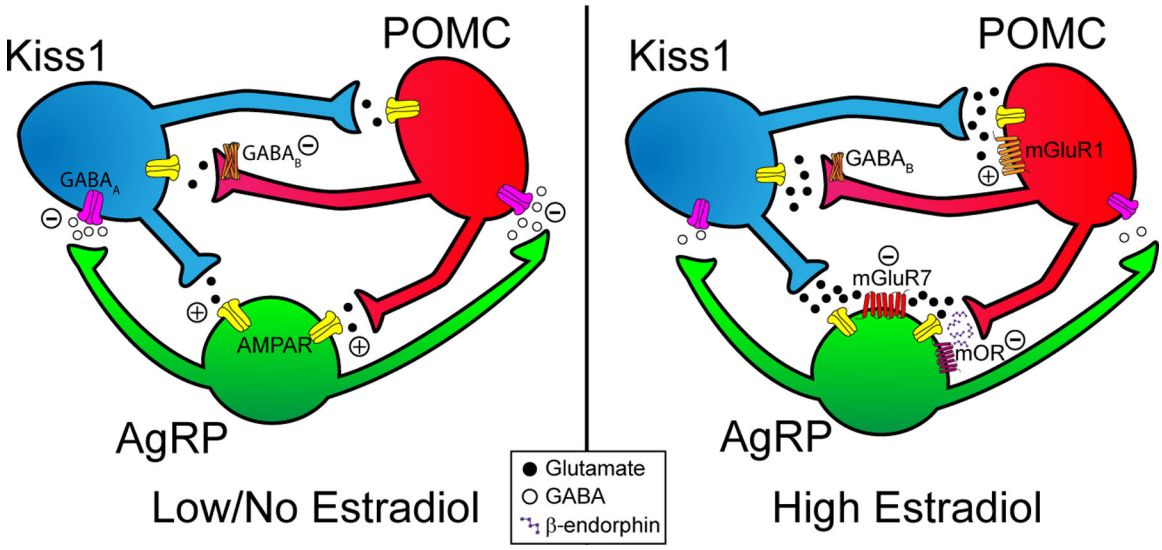

Figure 2. Tri-synaptic circuit in the arcuate nucleus of the hypothalamus.

(**Left panel**) When circulating estradiol levels are low, AgRP neurons will release the inhibitory neurotransmitter GABA onto Kiss1 and POMC neurons. POMC and Kiss1 neurons will release a trickle of glutamate onto AgRP neurons, exciting them through AMPA receptors. (**Right panel**) When circulating levels of E2 are high in proestrus or with E2-replacement in ovariectomized females, AgRP will display reduced neuronal excitability and GABA release. In POMC neurons the coupling of metabotropic $GABA_B$ receptors to GIRK channels is attenuated, further diminishing GABAergic inhibition. Simultaneously in $Kiss1^{ARH}$ neurons *Vglut2*, *CaV3.1*, and *Hcn1,2* mRNA expression is upregulated to enhance glutamate release as well as the excitatory T-type calcium and h-currents (80). Therefore, POMC and Kiss1 neurons will be disinhibited/excited at the same time their glutamate release probability is enhanced through increased *Vglut2* mRNA transcription. However, E2 will also increase expression of the inhibitory mGluR7 receptor in AgRP neurons such that the greater glutamate release will recruit these extrasynaptic receptors, causing an overall inhibitory input. Additionally, POMC neurons will produce more β-endorphin to further inhibit AgRP neurons. Taken together, reciprocal connections between POMC and Kiss1 neurons will act synergistically to increase their activity and excitability while both inhibiting AgRP neurons to decrease food motivation in favor of reproductive behavior. (AgRP: neuropeptide Y/agouti-related peptide; Kiss1: kisspeptin; mOR: μ-opioid receptor; mGluR1: Group I metabotropic glutamate receptor 1; mGluR7: Group III metabotropic glutamate receptor 7; POMC: proopiomelanocortin).