HHS Public Access

Author manuscript

Neuroendocrinology. Author manuscript; available in PMC 2021 January 01.

Published in final edited form as:

Neuroendocrinology. 2020 ; 110(3-4): 172–184. doi:10.1159/000503006.

Differential roles of hypothalamic AVPV and arcuate kisspeptin neurons in estradiol feedback regulation of female reproduction

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Abstract

Mammalian reproductive function includes puberty onset and completion, reproductive cyclicity, steroidogenesis, gametogenesis, fertilization, pregnancy and lactation; all are indispensable to perpetuate species. Reproductive cycles are critical for providing the hormonal milieu needed for follicular development and maturation of eggs but cycles, in and of themselves, do not guarantee ovulation will occur. Here we review the roles in female reproductive neuroendocrine function of two hypothalamic populations that produce the neuropeptide kisspeptin, demonstrating distinct roles in maintaining cycles and ovulation.

Keywords

ovulation; CRISPR; estradiol; reproduction; kisspeptin; AVPV; arcuate

The reproductive axis and estradiol feedback

Gonadotropin-releasing hormone (GnRH) neurons integrate central, peripheral and external cues to generate the central output that regulates fertility [1,2]. GnRH neurons reside primarily in the preoptic area (POA) and anterior hypothalamus. These neurons project to the median eminence and release GnRH near the primary capillaries of the hypophyseal portal vasculature, which carry this decapeptide to the pituitary where it activates the synthesis and secretion of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [1,3]. GnRH is released in an episodic, or pulsatile, manner that is critical for pituitary function [4–6]. High GnRH pulse frequency favors LH synthesis and release, whereas low GnRH pulse frequency preferentially promotes FSH [7–9]. FSH and LH regulate gametogenesis and steroidogenesis [10]. The sex steroids, including estradiol, progesterone and testosterone, feed back to the brain to regulate GnRH release, and on the

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Reprints requests should be addressed to corresponding author

Disclosure statement: The authors have nothing to disclose.

pituitary to regulate the responsiveness of gonadotropes to GnRH [11–16]. In males and during most of the female reproductive cycle, sex steroids suppress GnRH neuron activity and release via negative feedback [17–22]. During the preovulatory stage of the female cycle (late follicular phase or proestrus in rodents), a sustained elevation in estradiol causes a switch of estradiol action from negative to positive feedback, thus inducing elevated GnRH neuronal activity and causing a preovulatory surge of GnRH, and subsequent LH, release [6,23–25]. The LH surge triggers ovulation. There is some debate in higher primates about the necessity of the GnRH surge for ovulation, as an LH surge can occur without a change in episodic release in women [26], and an unchanging frequency of GnRH pulse administration can induce cycles in monkeys in which endogenous GnRH was ablated [4]. In this regard, it is worth noting that in monkeys GnRH surges have been observed along with the preovulatory or estradiol-induced LH surge [27,28]. In rodents and sheep, a surge release of GnRH is required for the LH surge [25,29,30]. The investigation of estradiol feedback regulation of the hypothalamus has focused mainly on ovarian estradiol as it is the predominant signal to generate the switch between negative and positive feedback. For a recent review on the possible role of local neurosteroids in these processes, the reader is directed to Terasawa, et al. [31].

With the help of the advanced genetic and other technical tools available, much of the work to understand central control of fertility has been done in rodents, specifically laboratory mice. To study systemic estradiol regulation, both stages of the estrous cycle characterized by negative (diestrus) and positive (proestrus) feedback, and hormone manipulation have been used [32,33]. With regard to the latter, one paradigm utilizes ovariectomy (OVX) with low estradiol replacement (OVX+E, negative feedback), followed by a subsequent estrogen injection several days later to induce positive feedback (OVX+E+E); this is referred as estradiol rise model [34]. Another paradigm utilizes OVX and with or without a constant high physiological level of estradiol [17]. This daily surge model exhibits a diurnal switch in estradiol feedback, with LH levels lower in estradiol-treated than OVX mice due to estradiol negative feedback in the morning (OVX+E AM), and higher in estradiol-treated mice in the afternoon due to positive feedback (OVX+E PM). As with LH *in vivo*, GFP-identified GnRH neurons in brain slices prepared from this model exhibit low firing rates and release frequency in OVX+E AM mice and high firing and release frequency in OVX+E PM mice [17,18]. Fast-synaptic transmission to and intrinsic membrane properties and ionic conductances of GnRH neurons are both altered by estradiol in this model [35–40]. Similar changes occur between positive feedback during the cycle [33,41]. Recent studies further demonstrated that GnRH neurons integrate fast-synaptic and intrinsic changes to increase firing rates during positive feedback [42].

Estrogen receptors involved in systemic estradiol feedback

The physiological responses to systemic estradiol in regulating reproductive functions are primarily mediated by two known subtypes of nuclear receptor, estrogen receptor α (ERα) and estrogen receptor β (ERβ) [43–45], as well as membrane-associated receptors (GPR30 and mER) [46–48]. In rodents and humans, the two nuclear receptors are encoded by *Esr1* (human ESR1) and *Esr2* (human ESR2), respectively. Both ERα and ERβ typically act as ligand-activated transcription factors [49], by either binding directly to estrogen response

elements (EREs) or interacting with other proteins to alter gene expression [50,51]. ERα and ERβ can also modulate non-genomic membrane-associated signaling cascades [47,52–55]. Genomic and nongenomic actions of ERs are evident for many reproductive processes [50,56]. For example, signaling via the estrogen response element (ERE) is needed for estradiol-induced changes in GnRH neuron firing rate [57], whereas non-classical signaling also plays a role in this response at the pituitary [58]. Mice with global knockout of ERα or ERβ show distinct reproductive deficits. ERαKO mice are infertile and have disrupted reproductive tracts including hypoplastic uteri, and large hemorrhagic cysts and absence of corpora lutea in the ovaries [59]. In contrast, ERβKO mice are fertile but have fewer and smaller litters [59,60]. Further, ERαKO, but not βERKO, female mice exhibit atypically elevated serum LH in ovary-intact mice compared to their littermate controls. Likely related to this, estradiol replacement in OVX ERαKO females does not reduce LH [61]. A neuron-specific ERα KO mouse model shares similarly impaired negative feedback as the ERαKO mice, as well as disrupted positive feedback marked by an absence of estradiol-induced LH surge release [62]. Together these observations suggest that estradiol negative and positive feedback rely on estrogen signaling via ERα. Although tightly regulated by estradiol, GnRH neurons do not express detectable level of ERα; their response is thus at least in part attribute to estradiol action through upstream ERα-expressing neurons [63,64]. The upstream neurons that have by far been the subject of the most investigation for its role in estradiol feedback over the past fifteen years are kisspeptin neurons.

Kisspeptin signaling

The discovery of the link between KISS1 (produce kisspeptin) and KISS1R (produce kisspeptin receptors) genes and puberty and fertility comes from human studies. Patients carrying mutations in either of these genes exhibit idiopathic hypothalamic hypogonadism, impaired pubertal maturation, and low-amplitude LH pulses [65,66]. Transgenic mice that lack *Kiss1* or *Kiss1r* exhibit a similar hypothalamic hypogonadism phenotype [66,67]. Kisspeptin is expressed in several organs including gonads, pancreas, colon, pituitary, and brain [68,69]. In mouse hypothalamus, kisspeptin expression is restricted to two regions: the arcuate nucleus and the anteroventral periventricular nucleus (AVPV) [70], Both populations express ERα, ~99% in the arcuate and ~70% in the AVPV [71]. Arcuate kisspeptin expression is similar in both sexes, whereas AVPV kisspeptin expression is more extensive in females [72]. When estradiol is elevated, kisspeptin mRNA expression is increased in the AVPV and decreased in the arcuate nucleus [73,74].

Projections from kisspeptin neurons to GnRH neurons vary with species [75–77]. In the mouse, kisspeptin fibers from the AVPV form direct appositions to GnRH cell bodies, whereas fibers from arcuate kisspeptin neurons are primarily apposed to GnRH processes that are running through the arcuate nucleus to the median eminence [78–80]. Both of these configurations support a direct kisspeptin-GnRH connection. *In situ* hybridization for *Kiss1r* and *Kiss1r* promoter-driven *lacZ* demonstrate GnRH neurons express kisspeptin receptors [81,82]. Bath application of kisspeptin to brain slices robustly increases GnRH firing activity and release [18,83,84]; kisspeptin injection *in vivo* increases GnRH release and subsequent LH release [85]. From a loss-of-function standpoint, blockade of kisspeptin action by injecting an antibody or a specific antagonist decreases GnRH activity, LH release and

estrous cyclicity [72,86]. Deletion of *Kiss1r* from GnRH neurons recapitulates the *Kiss1r* KO phenotypes; re-introducing *Kiss1r* expression to GnRH neurons in *Kiss1r* KO mice rescue the deficits [87]. These observations indicate kisspeptin-GnRH circuitries are critical for normal reproduction. The differential regulation of kisspeptin by estradiol in arcuate and AVPV kisspeptin neurons set up the working hypothesis that these regions have distinct roles in mediating estradiol negative and positive feedback, respectively [88].

Arcuate kisspeptin neurons and estradiol negative feedback

Evidence that suggested a link between arcuate nucleus neurons and LH pulses came from early lesion studies, well before the discovery of kisspeptin: ablation of the arcuate nucleus abolished LH pulses in rats and monkeys [89,90]. Further, correlation of peaks in neuronal multi-unit activity (MUA) in the mediobasal hypothalamus (MBH), which contains the arcuate, with pulsatile LH release was demonstrated in several species including monkeys, goats, sheep and rodents [91–94]. In rats, sheep and goats, only a small percent of GnRH neurons are in the MBH, suggesting other neurons in MBH are involved in generating MUA peaks and perhaps pulse generation.

Identification of a role of kisspeptin in fertility refocused attention on the arcuate region, as arcuate kisspeptin neurons exhibit two characteristics needed for steroid regulated GnRH/LH pulse frequency. First, their activity is associated with pulsatile LH release [95]. Second, they can directly sense steroid feedback [77]. This brings up the intriguing possibility that generation and steroid regulation of GnRH pulses may be combined into one system. Arcuate kisspeptin neurons coexpress two additional peptides involved in regulating reproduction: neurokinin B (NKB) and dynorphin A, and are often referred to as KNDy neurons [77,96,97]. Intracerebroventricular (ICV) injection of NKB and dynorphin alters LH pulse frequency and associated MUA activity peaks in goats, and LH pulse frequency in ewes: NKB increased the frequency of MUA peaks in the arcuate and LH pulse frequency. In contrast, dynorphin inhibited MUA peaks and LH secretion, whereas blocking its action increased frequency of both central and pituitary output [98,99]. Bath application of these two peptides to mouse brain slides has a corresponding effect on KNDy neurons; NKB is excitatory, dynorphin inhibitory [100–102]. These changes may be attributable to direct action of the peptides on KNDy neurons, as these neurons form interconnect circuits [102] and most KNDy neurons express the NKB receptor, NK3R, with a smaller percentage of these cells expressing the dynorphin-specific kappa-opioid receptor (KOR) [97,103]. Long-term monitoring of KNDy neuron activity revealed that they exhibit spontaneous peaks and nadirs in firing rate [104]. This pattern can be altered when NK3R is activated; blocking KOR, however, did not affect the patterns [104]. This difference may be explained by the observation that fewer KNDy neurons express KOR than NK3R [105], although the higher efficacy of *in vivo* KOR blockade vs in brain slices may indicate dynorphin acts via cell populations not present in the brain slice to inhibit LH pulses *in vivo*. Together these observations point to a hypothesis that KNDy neurons form an interconnected network that is modulated by its peptide products to determine their rhythmic output to GnRH neurons, thus affecting LH pulses.

To test the sufficiency of KNDy neurons to trigger a pulse of LH release, studies were conducted using Cre-dependent mice and adeno-associated viral (AAV) vectors. Activation of KNDy neurons using optogenetic channelrhodopsin2 (ChR2) *in vivo* triggers a pulse of LH release, confirming their capability to elicit LH release either directly or indirectly [106]. The calcium indicator GCaMP6 and fiber photometry were utilized to estimate bulk arcuate kisspeptin neuron activity based on fluctuations in calcium-sensitive fluorescence. Increases in fluorescence of KNDy neurons correlates with LH pulse release [95,107]. From a loss-of-function standpoint, KNDy neuron ablation was achieved by delivering diphtheria toxin A to mice expressing the toxin receptor in kisspeptin cells. When done on postnatal day 20, when the diphtheria toxin receptor is primarily expressed in arcuate but not AVPV kisspeptin neurons [108], these mice exhibit persistent diestrus as adults, suggesting KNDy neurons are required for establishing/maintaining cyclicity [109]; of note, the integrity of the AVPV kisspeptin population was not assessed in adult mice in these studies. Similarly, blocking the release of neuropeptides and neurotransmitters from KNDy neurons using a Cre-dependent AAV expressing the light chain of tetanus toxin halted the reproductive cycle, with mice again remaining in diestrus; these mice also had decreased LH levels [110]. These *in vivo* studies further suggest KNDy neurons are at least a component of the pulse-generating or conveying system.

Besides displaying rhythmic activity, KNDy neurons are also capable of receiving and being regulated by steroids, including estradiol. This modulation happens at multiple levels. First, estradiol decreases kisspeptin, NKB and NK3R expression in these cells [73,74,97]. Second, estradiol reduces the excitatory effect of NKB and enhances the inhibitory effect of dynorphin on KNDy neuron firing rate [100,111]. The effects of steroids on KNDy neuron firing rate is complicated. In early short-term extracellular recordings of these cells, results were inconsistent but typically revealed no effect of castration in females or males [100,101]. More recent work examining OVX vs OVX+E females demonstrated a trend for estradiol to reduce firing rate; no statistical difference was revealed in two-way ANOVA analysis (Figure 1A) [112], but a direct comparison of these groups with greater power in a recent preliminary report revealed a suppression in OVX+E females [113]. There is only one study of long-term firing pattern, which was done in male mice. This work suggest sex steroids, including estradiol, modulate firing patterns of KNDy neurons but not the overall mean firing rate over a couple hours [104]. Fast synaptic inputs to KNDy neurons are also regulated by estradiol, as estradiol decreases glutamatergic input frequency (Figure 1C) and GABAergic input amplitude to KNDy neurons [112,114]. These suggest that estradiol may alter KNDy neuron activity both directly and via modulating afferent systems. With regard to the latter, the interconnected nature of KNDy neurons and their use of glutamate as a cotransmitter [71] may indeed be a direct KNDy neuron network effect, whereas GABA changes are more likely via distinct cells.

The importance of estradiol-sensing in KNDy neurons is demonstrated by two Cre-lox genetic mouse models: a kisspeptin-specific ERα knockout (KERKO) and an NKB (*Tac2* gene produce)-specific ERα knockout (TERKO) [115,116]. In KERKO mice, deletion of ERα from kisspeptin cells leads to advanced vaginal opening during development but disrupted cyclicity (persistent estrus) in adults [116]. In this model, however, ERα is removed from both AVPV and arcuate kisspeptin neurons as well as other kisspeptin cells

located centrally and peripherally, making the interpretation of a specific role of arcuate kisspeptin neurons difficult. In the TERKO model, ERα is removed largely from the arcuate kisspeptin neurons in the brain [115]. TERKO mice also exhibited advanced vaginal opening and prolonged estrus, similar to the KERKO mice, suggesting the phenotypes may largely attribute to KNDy neurons, and that ERα in kisspeptin cells, particularly KNDy neurons, is critical for reproductive function including puberty and cyclicity.

KERKO mice have also been used to study the mechanisms of how estradiol modulates KNDy neuron activity and LH pulse generation. To test if KERKO mice are able to respond to negative feedback regulation, plasma LH levels were measured in OVX and OVX+E control and KERKO mice. The post-OVX rise in KERKO mice was reduced compared to controls, but estradiol was able to reduce LH levels in both groups [117]. These findings led to the postulate that arcuate kisspeptin neurons may not be necessary to mediate negative feedback. Examination of LH pluses with frequent sampling revealed elevated pulse frequency in ovary-intact KERKO mice compared to estrous controls, suggesting frequency modulating effects of estradiol are likely at least in part mediated by ERα in kisspeptin cells [112]. Interestingly, KERKO mice are also less responsive to kisspeptin and GnRH challenge in terms of LH release [112]. Biophysical studies of KNDy neurons in KERKO mice further reveal several critical roles ERα plays. KNDy neurons in KERKO mice exhibit elevated firing rate (Figure 1 A), and received elevated spontaneous and action potential-independent glutamatergic transmission compared to controls [112] (Figure 1 C). Further, when OVX vs OVX+E mice were compared, estradiol suppressed glutamatergic transmission and firing rate in controls but not KERKO mice (Figure 1 A and C). This suggests the lack of ERα in kisspeptin cells leads to a lack of response to estradiol in these cells [112]. The loss of ERα signaling and subsequent elevated LH pulse frequency may contribute to the disrupted cyclicity [112], as modulation of GnRH/LH frequency is critical for maintaining normal cyclicity.

Although informative to understand estradiol negative feedback on GnRH-KNDy network, this Cre-lox based KERKO model has its own caveats. Specifically, it is impossible to distinguish activational and organizational roles of ERα, as ERα is deleted as soon as the kisspeptin gene turns on, before birth in KNDy neurons and before puberty in AVPV kisspeptin neurons [108,118]. Further, ERα is deleted from all kisspeptin cells. Spatial and temporal precision is needed to dissect the role of KNDy neurons in reproduction. To overcome these caveats, a CRISPR-Cas9 approach was employed to reduce ERα in the arcuate kisspeptin neurons in adult mice [119]. This model utilized the Cre-lox system to express the Cas9 protein in kisspeptin cells. Then, in adult mice, an AAV vector that expresses an sgRNA that targets *Esr1* (ERα gene) was injected into the arcuate region (Arc-AAV-*Esr1*). As a control, an AAV vector targeting the *lacZ* gene was introduced in the same manner (Arc-AAV-*lacZ*); these mice were not different from control females for the parameters measured [119]. To reduce the caveats of CRISPR off-target effects, two independent sgRNAs were used and independently tested; no detectable differences were found between these. This approach achieved partial (~65%) knockdown of ERα in kisspeptin cells specifically in the arcuate region. Despite the partial nature of this knockdown, these Arc-AAV-*Esr1* mice exhibited disrupted reproductive cyclicity, spending prolonged time in estrus, and reduced response to kisspeptin and GnRH administration

compared to Arc-AAV-*lacZ*; both of these responses are similar to the KERKO mode (Figure 2 A and C) [112,115,116,119]. In contrast to KERKO mice, which exhibit increased LH-pulse frequency, no changes of pulse frequency were observed in Arc-AAV-*Esr1* mice (Figure 2A). This may be attributable to these mice being singly housed, whereas the KERKO mice examined for LH pulses were group housed; single housing can increase stress, which makes pulses harder to detect [120].

In brain slices, KNDy neurons in Arc-AAV-*Esr1* mice also shared several biophysical similarities with cells from KERKO mice. Specifically, these neurons tend to be more active (Figure 1 B, $p<0.05$ two-tailed t-test of log-normalized data, $p<0.14$ two-tailed Mann-Whitney U test of original data) and receive more glutamatergic inputs compared to Arc-AAV-*lacZ* (Figure 1 D) [112,119]. Taken together, ERα in KNDy neurons is required to maintain the typical function of the KNDy neuron network and female cycles, independent of developmental roles.

AVPV kisspeptin neurons and estradiol positive feedback

As with the arcuate region and pulses, the AVPV region was associated with surge generation long before the discovery of kisspeptin. AVPV neurons are sexually dimorphic, with more neurons in females, and many express ERα [121,122]. They exhibit increased cFos expression, an immediate early gene with expression often correlated with increased neuronal activity, during the LH surge when GnRH release is elevated [123,124]. Lesions of the AVPV block the preovulatory as well as the estradiol-induced LH surge [125–127]. AVPV kisspeptin neurons, as a subset of AVPV neurons, share these characteristics: they are sexually dimorphic, ERα positive (more than 70%), and express cFos during the LH surge [78,88,128–130]. At least 1/3 of AVPV kisspeptin neurons communicate with GnRH cell bodies; these neurons almost all express ERα [131]. Elevated activity often correlates with neurotransmitter and neuropeptide release, thus cFos expression may indicate increased activity and release from AVPV kisspeptin neurons to their efferent projections including GnRH neurons. Besides expressing the potent GnRH stimulator kisspeptin, AVPV kisspeptin neurons coexpress tyrosine hydroxylase (~70%), and utilize GABA (~75%) and glutamate (~20%) [71,132], both of which excite GnRH neurons, as cotransmitters [133,134]. The role of dopamine, a product of TH-expressing neurons, on GnRH neuron function is not very well defined. When TH is knocked out of kisspeptin cells, mice exhibit normal reproduction [135], suggesting kisspeptin and GABA might be the main resource for GnRH excitation [136]. Together these observations help to build a model that AVPV kisspeptin neurons are regulated by estradiol to increase activity during the GnRH/LH surge.

To investigate this model at a more mechanistic level, studies were conducted to test if AVPV kisspeptin neurons are more excitable during estradiol positive feedback (proestrus) compared to negative feedback (diestrus). Extracellular recordings of GFP-identified neurons in brain slices were made of GFP-identified AVPV kisspeptin cells. These neurons firing more action potentials and exhibit a greater degree of rapid action potential bursts on proestrus compared to diestrus and estrus (Figure 1 E, left) [137,138]. Hormonal manipulations (OVX+ E, OVX+ E+ progesterone) suggest that it is primarily estradiol that mediates these changes as OVX+E mice recapitulate the firing characteristics observed in

cells from proestrous mice, whereas OVX+E+P mice are not different from those receiving only estradiol replacement (Figure 1 E, right). Several ionic conductances have been identified in AVPV kisspeptin neurons, including hyperpolarization-activated cation channels, T-type calcium channels, and persistent sodium channels [137,139,140]. All three of these channels have been demonstrated to promote burst firing and pace-making in other neurons [141–143]. Both electrophysiological recordings measuring ionic currents and mRNA expression of these ion channel genes in pooled AVPV kisspeptin cells suggest these conductances are upregulated by estradiol [137,139,140]. These increases in burst-related ionic conductances in AVPV kisspeptin neurons may contribute to the increased firing activity of these neurons during the time of the GnRH/LH surge provide a mechanism for how estradiol modulates AVPV kisspeptin neuronal firing to facilitate positive feedback. Besides exhibiting cycle/estradiol-dependent ionic conductances, AVPV kisspeptin neurons also received increased excitatory glutamatergic inputs (Figure 1 G) and decreased inhibitory GABAergic transmission during positive feedback [112,114], tilting the balance toward excitation during positive feedback. These observations suggest that estradiol-sensing positive feedback circuitry may extend beyond AVPV kisspeptin neurons, as their upstream inputs are also modulated by estradiol.

To test if stimulation of AVPV kisspeptin neurons is sufficient to generate LH secretion *in vivo*, ChR2 was targeted to these cells. Photostimulation of AVPV kisspeptin neurons for ~15 minutes induced LH release secretion of similar amplitude to the endogenous LH surge, but the time course was more similar to a pulse than a prolonged surge release [136]. These results demonstrate activation of AVPV kisspeptin cells likely increases GnRH and thereby LH release, but also suggest induction of surge release may need prolonged activation of AVPV kisspeptin neurons.

The KERKO model has also been used to study role of ERα in kisspeptin cells, including AVPV kisspeptin neurons, in positive feedback. KERKO mice remain in estrus and exhibit high circulating estradiol, thus an estradiol rise surge model was used to study estradiol positive feedback. KERKO mice failed to generate estradiol-induced LH surges, suggesting ERα in kisspeptin cells is required for estradiol positive feedback [117]. From a biophysical aspect, AVPV kisspeptin neurons from KERKO mice are less excitable (Figure 1 F) and receive fewer glutamatergic inputs compared to littermate controls (Figure 1 G). Further, these typically estradiol-sensitive parameters also no longer regulated by estradiol in OVX vs OVX+E mice (Figure 1 F and G) [112,119]. This indicates that ERα plays a necessary role in modulating the excitability of AVPV kisspeptin neurons to trigger LH surge [119]. Although informative, this model also has the caveats mentioned above regarding a lack of spatial and temporal precision. These caveats are potentially more serious in the AVPV population as kisspeptin cell number in the AVPV drops when estradiol is removed.

The CRISPR approach again provides space and time-specific regulation of ERα in AVPV kisspeptin neurons. Reduction of *Esr1* in the AVPV region was achieved as above using kisspeptin-Cre targeting of Cas9 and delivery of sgRNAs targeting the *Esr1* gene (AVPV-AAV-*Esr1*) in adulthood [119]. In AAV-AVPV- *Esr1* mice, ~35% of kisspeptin neurons express ERα vs 70% in control mice that received AAV-AVPV-*lacZ*. To test if ERα deletion in AVPV kisspeptin neurons alters neuronal firing properties in OVX+E mice, whole-cell

recordings were paired with *post hoc* testing (immunofluorescence of biocytin labeled recorded cells or single-cell qPCR) to determine expression of ERα in each recorded cell. In OVX+E mice with AAV-AVPV-*Esr1*, only ERα negative AVPV kisspeptin neurons exhibited decreased firing rate and bursts compared to AVPV-AAV-*lacZ* infected cells and AAV-AVPV-*Esr1* uninfected cells [119]. These responses are similar to changes that occur in these cells in KERKO mice, suggesting the primary effect of estradiol on the intrinsic electrophysiological properties of these cells is activational.

From a systemic aspect, these mice maintained normal cyclicity for at least two months post surgery; in contrast, disruptions of cyclicity when the arcuate kisspeptin population was targeted began to emerge within three weeks (Figure 2 D) [119]. It is possible that the remaining ERα expressing AVPV kisspeptin neurons are sufficient to maintain cyclicity. Alternatively, cyclicity may be maintained by other neurons, such as the arcuate KNDy neurons. In a recent study, genetic deletion of ERα broadly in the preoptic area and AVPV region, but not arcuate of adult female mice produced persistent estrus and decreased the amplitude of the estradiol-induced LH surge using the estradiol rise surge-induction model [144]. In the area covered by this knockdown many cells besides AVPV kisspeptin neurons express ERα [145] making it difficult to ascribe these results to a specific cell type.

Despite having normal cycles, AVPV-AAV-*Esr1* mice had, at best, blunted LH surges, both proestrus and estradiol induced (Figure 2 B). Although a reduced proestrous surge could be attributable to reduced estradiol levels, this caveat is minimized by the demonstration that estradiol-induced surges are also reduced. Further, the estradiol levels produced in the AVPV-AAV-*Esr1* mice are sufficient to induce vaginal cornification. Consistent with blunted LH surges, ovarian histology showed reduced or absent corpra lutea in two-thirds of knockdown mice compared to AVPV-AAV-*lacZ* control mice [119]. These results support and extend much research in the field by demonstrating that ERα in AVPV kisspeptin neurons is important for positive feedback and LH surge generation.

Conclusion and future directions

Application of modern genetic approaches to the long-existing questions of where estradiol acts to bring about negative and positive feedback has brought more insights into the regulation of GnRH/LH pulses and surges. There is now strong evidence that arcuate and AVPV kisspeptin neurons play distinct roles in mediating the response to systemic changes in estradiol, regulating cyclicity and the LH surge, respectively. Because CRISPR-mediated changes were induced in adults, we can conclude that the observations are not attributable to a loss of ERα action during development (Figure 3).

Several major areas of investigation remain regarding the kisspeptin-GnRH circuitry. First, AVPV and arcuate kisspeptin neurons receive and process estradiol signals, but we do not know if estradiol action in their estrogen-sensitive afferents is critical. It is possible that reducing ERα in our target kisspeptin populations merely blocked processing of incoming signals from the true first-order responding cells. Are these signals from upstream cells required and, if so, what and where are these neurons. Further, do AVPV kisspeptin neurons require the input from arcuate kisspeptin neurons to mediate the switch from negative to

positive feedback? Multi-region spectrum-specific fiber photometry approaches may be applied to monitor simultaneously the activity of AVPV and arcuate neuronal activity in each distinct reproductive stage [146,147]. This approach could also be utilized to test if there is synchrony between arcuate kisspeptin neurons and GnRH neurons.

Second, how AVPV and arcuate kisspeptin neurons convey their feedback modulation to GnRH neurons is not completely understood. Does increased AVPV and/or arcuate kisspeptin neuronal activity lead to increased neurosecretion? Assuming from work on other systems that the answer is yes, are the cotransmitters and other peptides in these cells important or is kisspeptin the primary player? Further, is all of the kisspeptin communication received directly by GnRH neurons? Studies in global Kiss1r knockout mice have suggested that replacing these receptors only in GnRH neurons restore fertility [87]. Further work on these mice, however, has demonstrated that aspects of steroid feedback, gonadal structure, and gonadotropin release are not fully restored [148]. Consistent with this latter finding, kisspeptin treatment increases fast synaptic transmission to GnRH neurons, indicating there are indirect pathways involving at least GABA and glutamate through which this neuromodulator may influence GnRH output [149]. One population of interest in this regard are neurons that utilize nitric oxide (NO) as a neurotransmitter; NO has been proposed as a synchrony signal to GnRH neurons [150,151]. Further, nitric oxide synthase (nNOs) expressing neurons in the median preoptic nucleus (MnPO) region also express *Kiss1r* [152], making them another possible intermediate between kisspeptin and GnRH neurons. Comprehensive projection mapping and direct stimulation and/or inhibition of nNOs neurons *in vivo* are needed to test this postulate.

Third, it is still not clear how estradiol regulates gene expression profiles to change intrinsic neuronal activity of AVPV and arcuate kisspeptin neurons. Of particular interest in this regard are the mechanisms underlying the many-hour delay from achieving a surge-inducing level of estradiol and the onset of the GnRH/LH surge. The length of this delay and the ability to remove estradiol before the surge is initiated without affecting it imply genomic mechanisms [12], but detailed temporal profiling of gene expressing spanning this gap is lacking. Advanced single-cell and/or single-nucleus sequencing approaches may shed light on the steps involved in estradiol feedback regulation on these cells. Recently, two droplet-based single-cell RNA-sequencing studies of the arcuate and POA region provide intriguing data for identify different populations of neurons and their transcriptomes, including the two kisspeptin populations; this detailed information allows potential reimagining of how different neuronal populations are related to one another [153,154]. Future studies should include studying these neurons at precise times under different hormone treatments and/or distinct cycle stages to reveal the time course of estradiol-dependent gene expression profiles, and generate hypotheses for future physiological investigations.

Support

Supported by National Institute of Health/*Eunice Kennedy Shriver* National Institute of Child Health and Human Development R01 HD41469.

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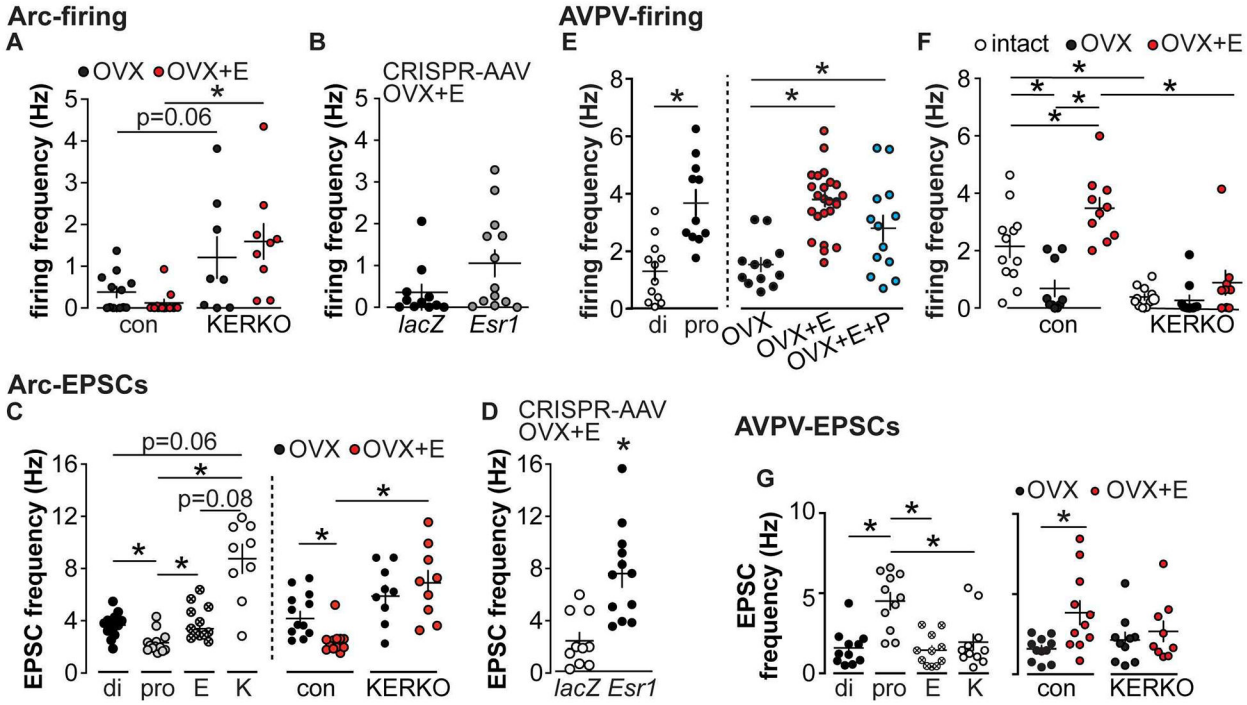

Figure 1.

Estradiol regulation of firing rate and EPSC frequency in arcuate and AVPV kisspeptin neurons of the hypothalamus. A, Firing rate of arcuate kisspeptin neurons is elevated in cells from KERKO compared to control mice. B, Firing rate is not different between cells from Arc-AAV-*Esr1* and Arc-AAV-*lacZ* OVX+E mice. C, Spontaneous glutamatergic EPSC frequency is regulated by cycle stage (left) and estradiol (right) in arcuate kisspeptin neurons. E, estrus, K, KERKO. D, Knockdown of ERα targeted to the arcuate increases glutamatergic inputs to arcuate KNDy neurons. E, Firing rate of AVPV kisspeptin neurons is elevated during proestrus (left) and by estradiol (right). F, The firing rate decreases in cells from KERKO compared to control and is no longer estradiol-sensitive. G, Spontaneous glutamatergic EPSC frequency is regulated by cycle stage (left) and estradiol (right) in AVPV kisspeptin neurons. *, $p<0.05$; adapted from [112,119,137] with permission.

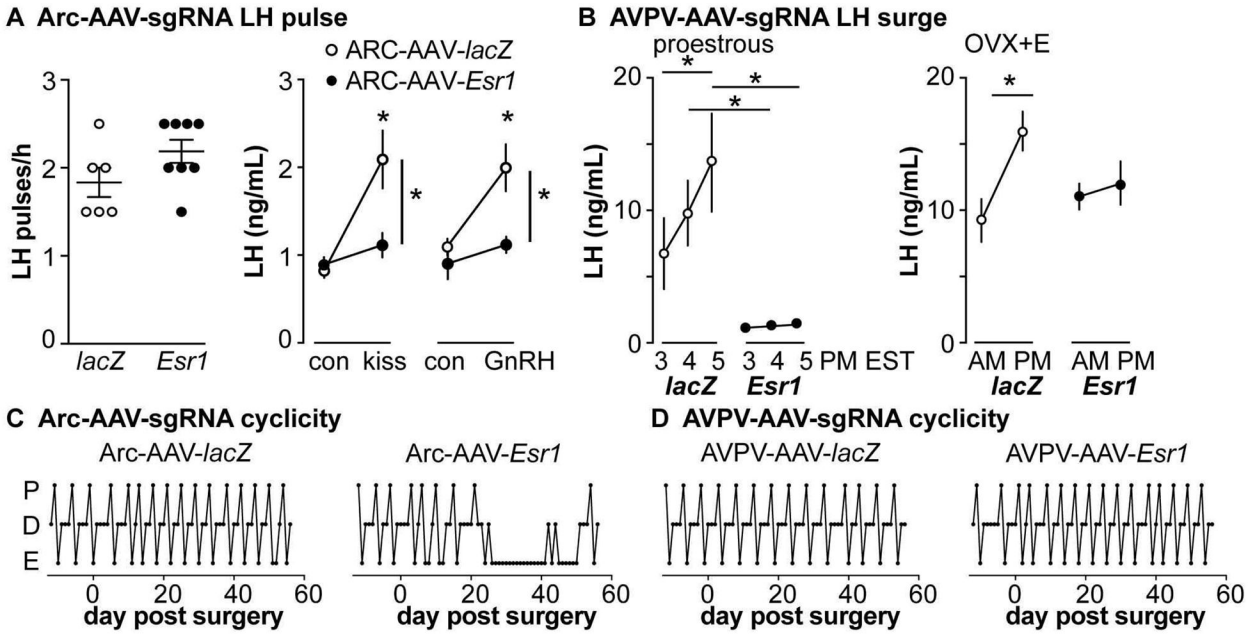

Figure 2.

Distinct roles of arcuate and AVPV kisspeptin neurons in regulating estradiol feedback and reproductive function. A, Knockdown of ERα in arcuate kisspeptin neurons did not alter LH-pulse frequency on estrus (left) but desensitized the LH response to kisspeptin and GnRH challenge. B, Knockdown of ERα in AVPV kisspeptin neurons blunted the proestrous (left) and estradiol-induced (right) surges. C and D, Knockdown of ERα in arcuate (C) but not AVPV (D) kisspeptin neurons alter reproductive cyclicity. Adapted from [119].

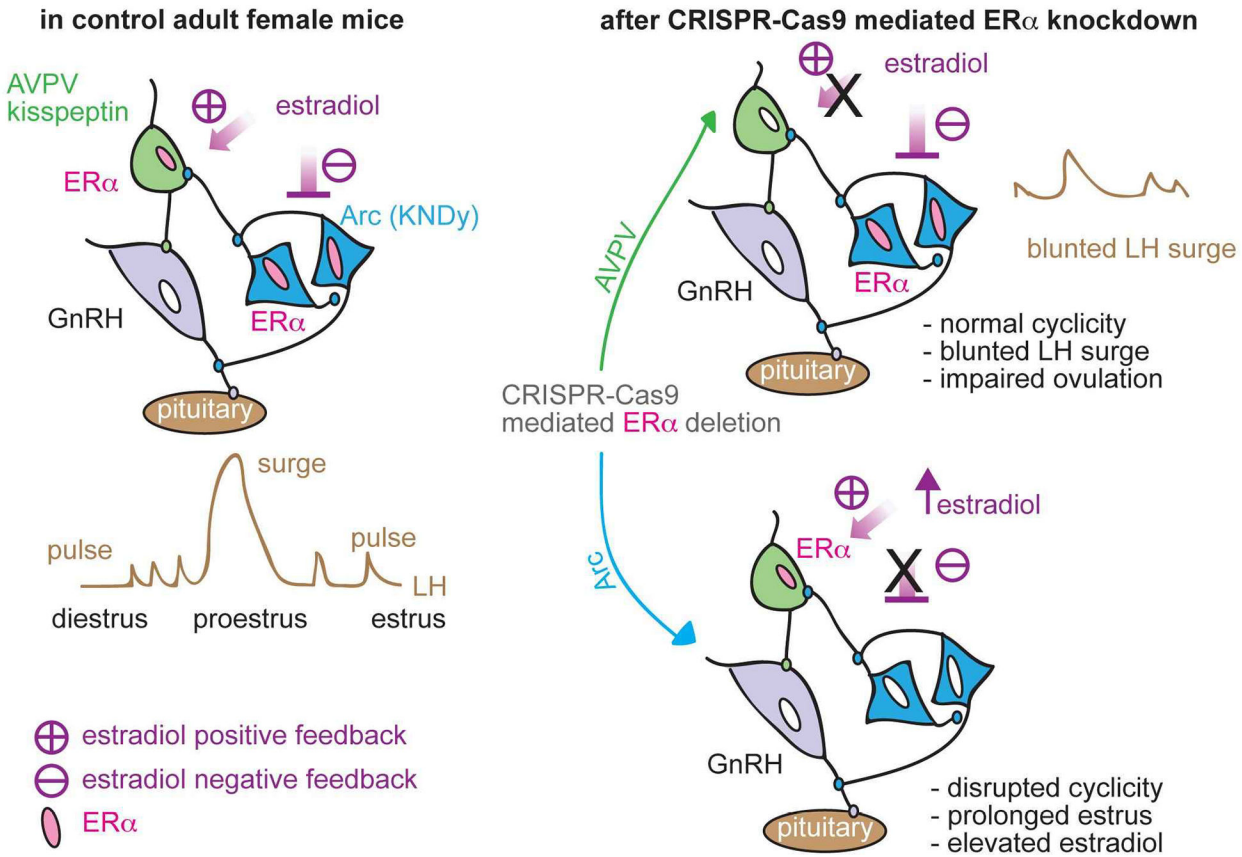

Figure 3.

Schematic diagram of estradiol feedback regulation on ERα in AVPV and arcuate kisspeptin neurons in adulthood. Knockdown of ERα in AVPV kisspeptin neurons blunted LH surge but did not alter reproductive cyclicity whereas knockdown of ERα in arcuate kisspeptin neurons disrupted the cyclicity. From [119].