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Central aspects of systemic estradiol negative and positive feedback on the reproductive neuroendocrine system

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Abbreviations: ERα, estrogen receptor alpha; GFP, green-fluorescent protein; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; OVX, ovariectomized; OVX+E, ovariectomized with estradiol implant; OVX+E+E, ovariectomized with estradiol implant plus estradiol injection; GABA, gamma-aminobutyric acid; PSC, postsynaptic currents; EPSC, excitatory postsynaptic currents; KERKO, kisspeptin-specific ERα □□□□□□□□□; AVPV, anteroventral periventricular nucleus; CRISPR, clustered regularly interspaced short palindromic repeats.

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Abstract

The central nervous system regulates fertility through the release of gonadotropin-releasing hormone (GnRH). This control revolves around the hypothalamo-pituitary-gonadal axis, which operates under traditional homeostatic feedback by sex steroids from the gonads in males and most of the time in females. An exception is the late follicular phase in females, when homeostatic feedback is suspended and a positive feedback response to estradiol initiates the preovulatory surges of GnRH and luteinizing hormone (LH). Here we briefly review the history of how mechanisms underlying central control of ovulation by circulating steroids have been studied, discuss the relative merit of different model systems, and integrate some of the more recent findings in this area into an overall picture of how this phenomenon occurs.

Introduction

GnRH neurons form the final common central output pathway controlling fertility in vertebrates. Their output is regulated primarily by homeostatic sex steroid feedback. During the preovulatory period of the mammalian female reproductive cycle in spontaneously ovulating species, however, the feedback action of estradiol switches from negative to positive feedback. This initiates a surge of GnRH, and subsequently LH, release and ultimately triggers ovulation. A central signal is required for ovulation in most mammals. In some species, including rabbits, ovulation is induced by copulation; this association made it possible to study the neural link to reproduction as early as the 18th century. In 1797, Jon Haighton recounted to the Royal Society his observation that, in rabbits, sex made “by sympathy the ovarian vesicles enlarge, project, and burst” (1). Haighton rejected the hypothesis that semen directly stimulated the ovary to release an egg because he had severed the Fallopian tubes. He conjectured sympathy, or crosstalk, between the vagina and ovaries through the nervous system occurred to induce ovulation. The study of the brain’s role in ovulation accelerated in the early 20th century. In 1936, Marshall and Verney induced ovulation when they passed electrical current through a rabbit’s brain (2). A year later, Harris refined their work when he induced ovulation by electrically stimulating a specific region of the brain, the hypothalamus (3).

A neural signal was also postulated to be necessary for ovulation in animals that do not require copulation to ovulate, i.e., spontaneous ovulators. Humans, non-human primates, sheep, rodents, and many other mammals ovulate spontaneously at the end of the follicular phase of the reproductive cycle (proestrus in rodents). Studying spontaneous ovulation became possible as techniques, such as the vaginal smear, were developed to follow cycle stage in live animals.

In 1950, Everett and Sawyer delayed spontaneous ovulation by anesthetizing rats with phenobarbital on the afternoon of proestrus. In their control animals, ovulation occurred between 1 and 2 am on the morning of estrus (lights off at 7 pm), but anesthesia delayed ovulation by 24 hours if administered during a critical period (3 – 5 pm before lights off) the previous day (4). They hypothesized that a neural signal initiated spontaneous ovulation during this period. Eight years later, Critchlow stimulated the hypothalamus directly to trigger "spontaneous" ovulation (5). In the 1950s, hypothalamic pathologies were first associated with both hypogonadism and precocious puberty in humans (6), further supporting a central role in the regulation of fertility.

The study of the brain's role in reproduction did not occur in isolation, as a role was also emerging for the pituitary. In 1921 and 1922, Evans and Long noted that injecting pituitary extract into a rat's peritoneal cavity enlarged its ovaries and disrupted its estrous cycles (7-9). Similarly, surgical removal of the pituitary caused ovarian atrophy, and pituitary transplants beneath the hypothalamus (site of the *sella turcica,* home of the pituitary) restored estrous cycles and spontaneous ovulation (10,11). When the pituitary was transplanted to sites outside of the *sella turcica,* however, reproduction was not restored (12). These studies supported two early hypotheses: first, the pituitary may be important for reproduction in spontaneously ovulating species, and second, communication with the hypothalamus is necessary for pituitary control of reproduction.

Support for the hypothalamo-pituitary control of ovulation and reproduction continued to expand through the 20th century. A releasing factor in the hypothalamus had long been postulated to initiate pituitary hormone release to control reproduction. By 1971, Schally had isolated and sequenced 11.4 mg of GnRH from the hypothalami of 240,000 pigs (13). This GnRH is made and released by a small population (800 – 2500 neurons in mammals) that is scattered through the preoptic area and anterior hypothalamus (14). Many of these neurons project to and secrete GnRH into the median eminence, from where it is carried down long portal vessels into the capillary beds of the anterior pituitary. There, GnRH binds to receptors on pituitary gonadotropes to trigger the release of two hormones, follicle stimulating hormone (FSH) and LH. The release of these hormones stimulates follicular maturation and the production of sex steroids in the ovaries. Ovarian steroids provide feedback on the pituitary and hypothalamus to regulate hormone release. Collectively, hypothalamus, pituitary, and ovaries control complex hormonal interactions to precisely coordinate the reproductive cycle. The focus of this review is

on systemic feedback; for recent reviews of a potentially interesting role for neural steroids in this process the reader is referred to a recent review on this by Terasawa (15).

Modes of estradiol feedback regulation of the hypothalamus and pituitary

In mammals, ovarian estradiol was soon linked with ovulation induction (16), and studies showed that estradiol differentially regulates pulsatile vs surge modes of GnRH release via negative and positive feedback, respectively. For the majority of the reproductive cycle, GnRH is released in pulsatile manner and drives the pulsatile release of gonadotropins (17-20). Estradiol is traditionally referred to as having negative feedback actions on pulsatile hormone release. A closer examination of the actions of estrogens suggests this nomenclature is somewhat misleading. The term negative feedback arises from the observation that mean LH levels are lower in estrogen-treated than in ovariectomized (open feedback loop) animals (21-23). This is attributable primarily to a reduction in pulse amplitude as frequency of GnRH and LH release are often increased, or at least not suppressed, in higher estrogen states produced by either steroid replacement in the physiologic range or natural progression towards the late follicular phase (22,24-28). For historical consistency, we will refer to this action of estradiol as negative feedback, but wish to clarify the term to mean the action of estradiol to modulate the pulsatile pattern of GnRH/LH that characterizes much of the female cycle.

In most mammals, there is a switch from pulsatile GnRH to a continuous surge of GnRH release at the end of the follicular phase that is induced by estradiol positive feedback. There is little evidence of episodic secretion during the surge suggesting it is a different mode of secretion or a continuous mode superimposed upon the episodic mode (29-32). There remains some controversy over whether or not a GnRH surge exists in humans. It is certainly clear that in old-world primates a consistent GnRH pulse frequency can generate reproductive cyclicity at least over a few months (33,34). This led to the postulate that GnRH is permissive for LH surge generation in these species, rather than deterministic. Other indirect measures of GnRH release have suggested there is actually a decrease in GnRH during the LH surge in monkeys and women (35-37). Estradiol positive feedback at the pituitary appears to be stronger in these species, evidenced by the ability of estradiol to induce an LH surge in males and the ability of transplanted ovaries to produce cyclic hormonal changes reminiscent of the menstrual cycle in males (38,39). This question is difficult to resolve without direct measurement of GnRH release itself. This is not currently possible in humans but in rhesus monkeys preovulatory, estradiol-

induced and progesterone-induced increases in GnRH release during the LH surge have been observed (30,40,41), suggesting this phenomenon may also exist in humans.

Models to study estradiol feedback

Because of the availability of a vast array of genetic and other technical tools, much of the work to understand the neurobiology underlying these different modes of GnRH release has been done in rodent species, specifically laboratory mice. Three primary hormone replacement models have been used to induce negative and positive feedback in mice and were recently compared directly (42). Early work in mice utilized paradigms consisting of ovariectomy (OVX) with low estradiol replacement for approximately a week, followed by an estradiol rise on its own (E rise model) or in combination with a subsequent progesterone rise (43). Another paradigm is to ovariectomize mice and replace with a constant high physiologic level of estradiol (OVX+E) (44). This model takes advantage of a diurnal change in the feedback action of estradiol in these species. Specifically, in rodents ovulation is tightly coupled to time-of-day, and the GnRH/LH surges begin 1-2 hours before lights out in nocturnal species (4,32) and a similar time before lights on in diurnal species (45). In mice, rats and hamsters, the OVX+E paradigm induces daily LH surges in the late afternoon, hence has been referred to as the daily surge model (44,46,47). In OVX+E mice, LH release is suppressed in the morning (AM) and increased in the afternoon (PM) relative to ovariectomized mice that do not receive estradiol (OVX). This pattern persists in brain slices with GnRH firing rates and release suppressed in the AM relative to the PM in OVX+E mice (44,48).

Of note all of these models deviate from the natural estrous cycle, and all have advantages and disadvantages. On the negative side, constant estradiol, even at physiologic levels, is not characteristic of the estrous cycle. Further, all of these OVX+E models operate on a different duration than the typical cycle, with the E rise model being longer and the daily surge being shorter. On the plus side, all of these paradigms permit the study of estradiol feedback in genetic models that are not capable of generating an estradiol rise on their own. The differences in these models also can make it possible to probe different aspects of positive feedback. In the E rise model, the switch between negative and positive feedback relies on both an increase in estradiol and on time of day. In the daily surge model, the switch between negative and positive feedback relies on time of day. An interesting biological question that remains to be answered is

whether or not the underlying neurobiological mechanisms are the same in both of these models and how they compare to the natural cycle.

Daily surge vs the cycle

The daily surge model has been used to characterize changes in multiple intrinsic and fast-synaptic properties during the switch from negative to positive feedback (49-54). As this dataset has grown, it became increasingly important to compare at least some of the changes induced by this model to those that occur during the cycle. This was particularly important as the amplitude of the proestous surge was observed to be larger than the estradiol-induced LH surge (55,56). To do this, we examined three parts of the estrous cycle. Diestrous PM is a time of relatively low estradiol that is characterized by pulsatile LH release. Proestrous AM is a time when exposure to high estradiol needed for surge induction has occurred, but the LH surge has not yet been triggered. Proestrous PM is the time of estradiol positive feedback and the LH surge. GnRH neuron firing rate (diestrous and proestrous PM only), GABAergic fast synaptic transmission, GnRH neuron excitability, and action potential properties were examined (Figure 1). Firing rate of GnRH neurons determined by extracellular recordings of GFP-identified GnRH neurons in brain slices prepared on the afternoon of diestrous vs proestrous were strikingly similar to those observed in the daily surge model from OVX+E AM vs OVX+E PM neurons, respectively (56). Further, the larger amplitude of the proestrous LH surge was shown to be attributable at least in part to increased pituitary responsiveness to GnRH (56). These observations suggest that the final output of the reproductive neuroendocrine system (GnRH release) is likely to be similar in the daily surge model and during the natural proestrous surge.

Whole-cell recordings were used to examine synaptic and intrinsic properties of GnRH neurons during the cycle. The number of action potentials fired in response to fixed current injection is one way to characterize the integrated sum of the intrinsic properties of a neuron; this is often termed excitability. GnRH neuron excitability on diestrous PM was strikingly similar to that in OVX AM, OVX PM and OVX+E AM in the daily surge model (54,57). Similarly the positive feedback states (OVX+E PM and proestrous PM) were comparable in excitability and greater than that observed during the negative feedback/open loop conditions. We were initially surprised that OVX+E AM cells were not less excitable than cells from OVX mice as other properties, including potassium and calcium currents, are altered by estradiol in the daily surge models in manners that would typically reduce excitability. Computational modeling suggested an inverse relationship between the conductance and voltage-dependence of inactivation of a

transient potassium current in GnRH neurons accounted for the similarity between OVX and negative feedback states (OVX+E AM) (54).

Of interest in this regard, the excitability of GnRH neurons recorded on proestrous AM was reduced compared to diestrous PM. The same shifts in response to cycle stage were observed for GABAergic transmission to GnRH neurons, with transmission during the low estradiol negative feedback state of diestrous PM being lower than during positive feedback on proestrous PM, but GABA input during the high estradiol negative feedback of proestrous AM being the lowest frequency. These results were again initially surprising. The ability of a high physiologic and even pharmacologic level of estrogen to induce positive feedback is consistent (43,58,59), but *in vivo* the negative feedback actions of constant estradiol on GnRH release appeared to be stronger than those of the estradiol rise during the cycle (28,58). These observations had led us to postulate that a likely limitation of the daily surge model was that negative feedback was stronger than would be typical during the cycle. Together these newer data suggest that a possible limitation of the daily surge model is rather that negative feedback in this model effectively recapitulates that of lower estradiol states of diestrus, but may fall short of the stronger negative feedback that emerges on the morning of proestrus.

The existence of a daily central signal for ovulation such as observed in the daily surge model was identified in the middle of the last century in studies that demonstrated that barbiturate anesthesia during a critical period on proestrus blocked ovulation for 24 hours in rats (4). Ovulation can occur on a daily basis during the breeding season in many fish and bird species (60,61). Daily ovulation *per se* has not been observed in placental mammals but the LH surge and ovulation occurs at a particular time of day in some mammals. This is especially observed, as mentioned above, in rodents. Interestingly, LH surges in women occur more often during late sleep/early wake hours (62,63), and shiftwork, which can disrupt the circadian clock, is linked to menstrual cycle irregularities and increased time to pregnancy (64-66).

If a daily neural signal for ovulation can exist, why don't mammals ovulate daily? This may be attributed in part to the time needed for a follicle to mature to the point that it can produce sufficient estradiol to trigger positive feedback. Of interest in this regard, *tau* mutant hamsters, in which the free-running period is ~20 hours vs. just under 24 hours in the wild type, exhibit estrous cycles lasting five circadian days, or about 100 hours. This is similar in duration to the typical four-day (96 hour) estrous cycle in wild type golden hamsters (67). Daily LH surges are induced during subjective afternoon in OVX+E *tau* hamsters, and the period of consecutive LH

surges was shorter than in wild type hamsters (68). These observations are consistent with the postulate that follicle maturation and subsequent estradiol production are limiting and that the reproductive cycle does not result from a mere counting of circadian days. The provision of a constant high physiologic estradiol level, such as in the OVX+E daily surge model, would circumvent this limitation, allowing a central signal to occur on a daily basis as observed.

Are synaptic and/or intrinsic changes needed to produce increased GnRH neuron output during positive feedback?

The daily surge model has produced data indicating that both synaptic and intrinsic properties of GnRH neurons are altered by estradiol feedback mode (50-54,69,70). Performing these studies typically required optimizing recording conditions to isolate a single variable. Further, most experiments were done in voltage-clamp mode, which fixes membrane potential to observe and quantify currents, but at the same time precludes the membrane potential from responding to changes in intrinsic properties. To begin to address the question of whether intrinsic changes and/or synaptic changes are needed to generate increased GnRH neuron firing during positive feedback we utilized dynamic clamp (71). GABA is the primary fast synaptic input to GnRH neurons in adults and can be excitatory even in adulthood (72,73). We mined our previous recordings of GABA transmission to GnRH neurons in the daily surge model (44), and selected traces that were representative of OVX (open loop), OVX+E AM (negative feedback) and OVX+E PM (positive feedback) conditions. Conductance trains mimicking these patterns were then applied in random order to GnRH neurons from these same animal models, effectively mixing or matching intrinsic properties of the recorded cell with the type of synaptic input (Figure 2). This approach revealed that both the synaptic inputs and intrinsic properties were important for the increased firing rate observed during positive feedback (72,73). Specifically, the GABA conductance train from positive feedback induced more firing in all animal models, suggesting increased input frequency was important, and this positive feedback train was most effective in cells recorded during positive feedback, indicating the intrinsic properties during positive feedback poise the cell to be more responsive to excitatory synaptic input.

It is important to point out that additional factors not examined in this study may contribute to surge generation. For example, estradiol can alter excitatory fast glutamatergic transmission to GnRH neurons, and spines where glutamate afferents may synapse onto activated GnRH neurons are increased on proestrus (53,74,75). It is also important to point out that in other

animal models, no change in GABA PSC frequency has been reported during positive feedback (76). Arguing against a lack of a role for GABA in surge generation, specific knockout of estrogen receptor alpha (ERα) from GABA neurons blocks positive feedback (77), although this could be attributable to reduced release of cotransmitters such as kisspeptin that would be activated by estradiol action (78) as many kisspeptin neurons utilize GABA as a co-transmitter (79,80).

Where does estradiol act for negative and positive feedback?

A persistent question about estradiol feedback has been where it occurs. This is because this feedback requires classical signaling via ERα (81), which GnRH neurons typically do not express in detectable levels (82,83). Estradiol feedback is thus likely transmitted to GnRH neurons by ERα-expressing afferents (84). Kisspeptin is a neuromodulator that stimulates GnRH neurons (85,86). These neurons project to GnRH neurons and are directly but differentially responsive to estradiol (87-89). Specifically, the mRNA for kisspeptin is increased by estradiol in the kisspeptin neurons of the anteroventral periventricular (AVPV), postulated to underlie positive feedback, but decreased in kisspeptin neurons of the arcuate nucleus, postulated to underlie negative feedback. To begin to determine the role of ERα in these cells, whole-body knockout of ERα from kisspeptin cells was done using Cre-lox technology. These KERKO mice have disrupted cycles and do not exhibit estradiol-induced LH surges (90-92). This suggests ERα in kisspeptin cells may be critical for both estradiol negative and positive feedback. Relatively little was known about the properties of these kisspeptin neurons and how they respond to estradiol. We thus began to characterize these properties in control and KERKO mice.

AVPV kisspeptin neurons were found to be more excitable during estradiol positive feedback on proestrus PM than during negative feedback on diestrus PM (93) (Figure 3). This increased firing was attributable to estradiol; adding progesterone did not produce a further elevation in firing rate. Burst firing by these neurons followed the same pattern, being increased during positive feedback whether occurring during the cycle or induced by estradiol. Both electrophysiological recordings measuring ionic currents and mRNA expression of these ion channel genes in pooled cells suggest several ionic conductances that can underlie burst firing are expressed by AVPV kisspeptin neurons, including hyperpolarization-activated cation channels, T-type calcium channels, and persistent sodium channels, and are regulated by estradiol (93-96). Further support of a role for estradiol comes from studies in KERKO mice.

AVPV kisspeptin neurons were less excitable, fired fewer bursts and no longer changed firing rate in response to estradiol (97).

Estradiol feedback also modulates synaptic transmission to AVPV kisspeptin neurons, increasing glutamate transmission and suppressing hyperpolarizing GABAergic transmission to these cells, indicating that estradiol tilts the balance toward excitatory inputs during positive feedback (98,99). Coupled with estradiol upregulation of intrinsic conductances underlying bursting firing, AVPV kisspeptin neurons are poised to increase output during positive feedback to drive the GnRH/LH surge.

KERKO mice are a useful tool but lack both temporal and spatial regulation of ERα. Because Cre-lox will delete ERα as soon as *Kiss1* is expressed there can be developmental changes in these cells or their networks (100,101). Further, the deletion of ERα from all kisspeptin cells makes it impossible to assess independently the role of AVPV and arcuate kisspeptin neurons. We thus used CRISPR/Cas9 to target *Esr1* in the AVPV of adult mice (97). This approach successfully reduced ERα expression in AVPV kisspeptin neurons from ~75% in controls to about 25% in knockdown mice. These mice exhibited typical cycles but had markedly blunted proestrous and estradiol-induced LH surges. Further, their electrophysiologic properties resembled those in KERKO mice. These studies suggest ERα in AVPV kisspeptin neurons is required for estradiol action on their intrinsic membrane excitability and that these effects are activational, rather than organizational.

Kisspeptin neurons in the hypothalamic arcuate nucleus (also called KNDy neurons for their coexpression of kisspeptin, neurokinin B and dynorphin) are postulated to mediate estradiol negative feedback regulation of pulsatile GnRH/LH pulse as well as to generate LH pulses (87,102). Short-term extracellular recordings of these cells in OVX vs. OVX+E mice during negative feedback did to reveal any differences in firing pattern (98), although an effect of steroids on a longer-term firing pattern of these cells, similar to that observed in males, cannot be excluded (103). In, KERKO mice, however, firing rate of arcuate kisspeptin neurons in brain slices was markedly increased, as was LH pulse frequency *in vivo* (98). Estradiol also altered synaptic transmission to these cells, suppressing spontaneous glutamatergic transmission. Of note, the direction of regulation of glutamate transmission to these two kisspeptin populations is opposite.

Targeting the same CRISPR approach to the arcuate kisspeptin neurons produced a similar reduction in percent of neurons expressing ERα. In striking contrast to the mice in which the

AVPV was targeted, mice with reduced ERα expression in the arcuate kisspeptin neurons had disrupted estrous cycles, with an increasing tendency to remain in estrus. This is similar to mice in which ERα was deleted from *Tac2*-expressing neurons via Cre-lox technology (92); the overlap of ERα and *Tac2* expression in the brain is largely represented by the arcuate kisspeptin neurons. In the targeted CRISPR knock down, arcuate kisspeptin neurons also exhibited increased firing rate and increased levels of glutamatergic transmission. Together with the above, these findings suggest arcuate kisspeptin neurons mediate at least some aspects of negative feedback via ERα. These observations are further consistent with a key role for these cells in generating pulsatile secretion, as normal LH pulse frequency modulation is critical for producing cyclic changes in steroids.

Conclusions and future directions

Application of newer methodologies to the old question of how the action of estradiol switches from negative to positive feedback has brought increased understanding and generated new questions. At the GnRH neuron, both fast-synaptic and intrinsic changes appear to contribute to initiating a robust GnRH surge, but the nature of these signals can be further refined. The postulated roles of AVPV kisspeptin neurons in positive feedback and arcuate neurons in negative feedback have been supported, but how these signals are generated in these cells and then conveyed to GnRH neurons remains largely a mystery. Mechanistic studies of population synchrony and the neurobiology of the interactions between kisspeptin neurons and GnRH neurons need to be pursued. Further investigation of the nature of the estradiol-sensitive inputs to kisspeptin neurons may reveal additional interactions among these cells and/or new populations to study in the question of estradiol feedback.

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Figures and legends

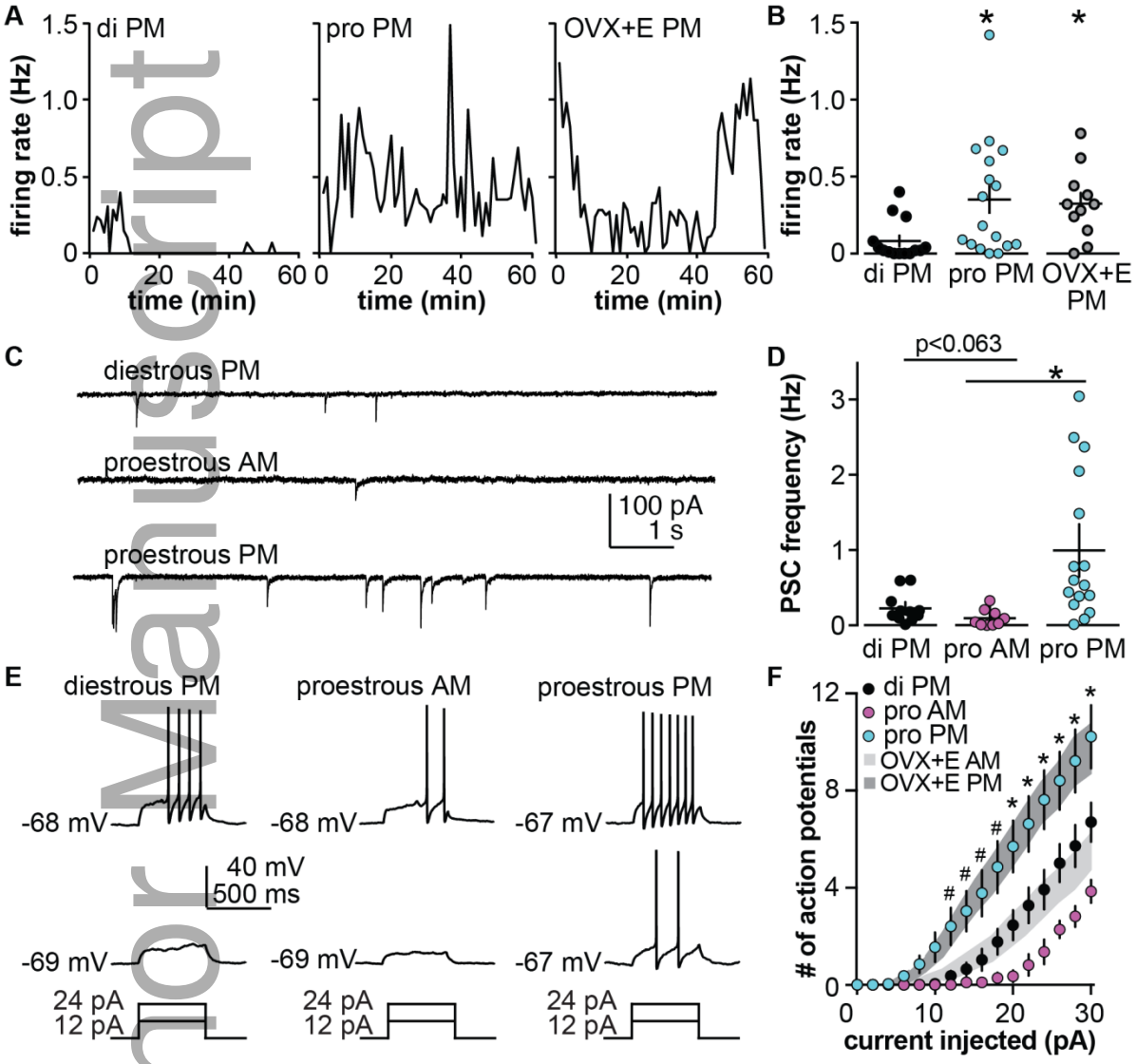

Figure 1. Comparison of daily surge model with estrous cycle. A, B. Representative firing patterns (A) and individual values and mean ±SEM firing rate (B) of GnRH neurons from diestrous, proestrous or OVX+E mice recorded in the PM. C, D. Representative recordings (C) and individual values and mean ±SEM frequency (D) of spontaneous GABAergic postsynaptic current (PSCs) in GnRH neurons from diestrous PM, proestrous AM and proestrous PM mice. E. Representative current-clamp recordings from diestrous PM, proestrous AM and proestrous PM mice. F. Mean±SEM number of action potentials in these groups; grey shaded areas show

range of SEM for the same experiment in GnRH neurons from OVX+E AM and OVX+E PM mice. * p<0.05. A and B adapted from (56), C-F adapted from (54,57) with permission.

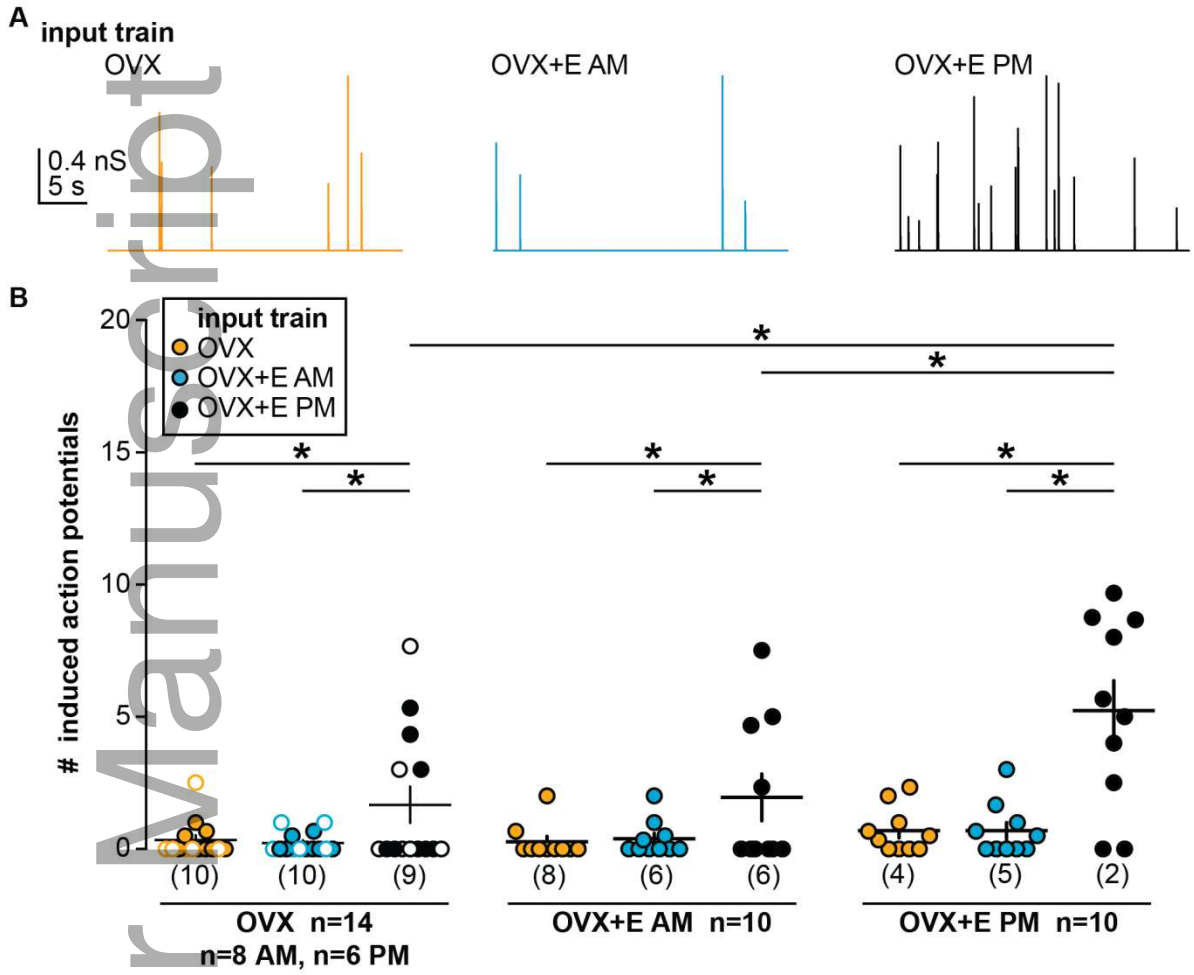

Figure 2. Both synaptic input and intrinsic properties contribute to increased GnRH neuron firing during positive feedback. A. Representative conductance trains from OVX (orange), OVX+E AM (blue), and OVX+E PM (black) conditions. B. Individual values and mean ± SEM spikes induced during individual postsynaptic conductances in input each train in cells from all three animal models. In the OVX group, open circles denote cells recorded in the PM and closed circles denote cells recorded in the AM. Numbers in parentheses along x-axis indicate number of cells not firing any spikes. *p<0.05 two-way repeated-measures ANOVA/Fisher's LSD test. From (71) with permission.

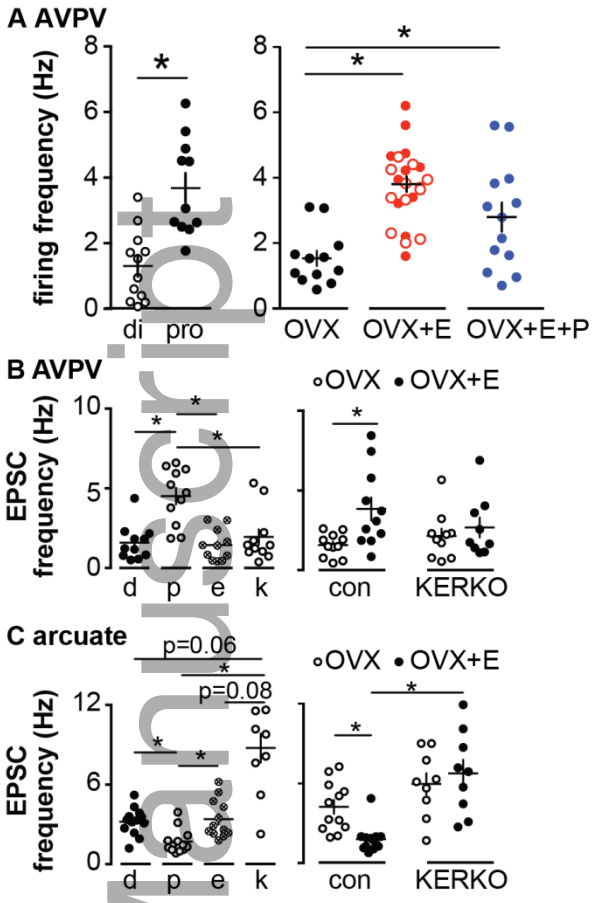

Figure 3. Estradiol regulation of firing rate and EPSC frequency in kisspeptin neurons of the hypothalamus. A. AVPV kisspeptin neuron firing rate is elevated during proestrus (left) and by estradiol (right). Open symbols in OVX+E were injected with vehicle at the time of progestin injection, closed symbols were uninjected controls. B, C. Spontaneous glutamatergic EPSC frequency is regulated by cycle stage and estradiol in both AVPV (B) and arcuate (C) kisspeptin neurons. Estradiol regulation is lost in KERKO mice. From (93,98) with permission.

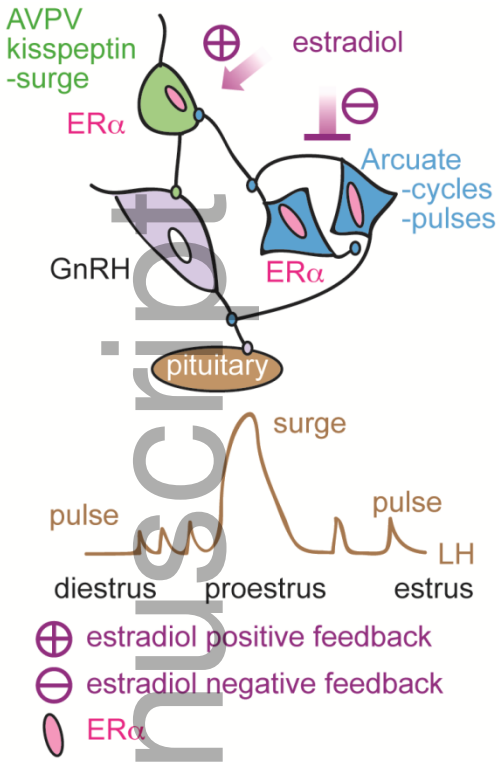

Figure 4. Schematic diagram of proposed feedback actions of estradiol via AVPV and arcuate kisspeptin neurons. From (97).