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Changes in GABAergic transmission to and intrinsic excitability of gonadotropin-releasing hormone (GnRH) neuron during the estrous cycle in mice

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Changes in GABAergic transmission to and intrinsic excitability of gonadotropin-releasing hormone (GnRH) neuron during the estrous cycle in mice.

Abbreviated title: Estrous-cycle dependent changes in GnRH neurons

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Abbreviations: AHP, afterhyperpolarization potential; ERα, estrogen receptor alpha; GFP, green-fluorescent protein; GnRH, gonadotropin-releasing hormone; KW, Kruskal-Wallis; LH, luteinizing hormone; OVX, ovariectomized; OVX+E, ovariectomized and estradiol implant; OVX+E+E, ovariectomized and estradiol implant plus estradiol injection; FWHM, full width at half maximum.

Abstract

Gonadotropin-releasing hormone (GnRH) neurons form the final common central output pathway controlling fertility and are regulated by steroid feedback. In females, estradiol feedback action varies between negative and positive; negative feedback typically regulates episodic GnRH release whereas positive feedback initiates a surge of GnRH, and subsequently LH, release ultimately triggering ovulation. During the estrous cycle, changes between estradiol negative and positive feedback occur with cycle stage and time of day, with positive feedback in the late afternoon of proestrus in nocturnal species. To test the hypotheses that synaptic and intrinsic properties of GnRH neurons are regulated by cycle stage and time of day, we performed whole-cell patch-clamp studies of GnRH neurons in brain slices from mice at two times considered negative feedback (diestrous PM and proestrous AM) and during positive feedback (proestrous PM). GABAergic transmission can excite GnRH neurons and was higher in cells from proestrous PM mice than cells from proestrous AM mice and approached traditional significance levels relative to cells from diestrous PM mice. Action potential response to current injection was also greater in cells from proestrous PM mice than the other two groups. Interestingly, the hormonal milieu of proestrous AM provided stronger negative feedback on both GnRH neuron excitability and GABAergic postsynaptic current amplitude than diestrous PM. These observations demonstrate elements of both synaptic and intrinsic properties of GnRH neurons are regulated in a cycle-dependent manner and provide insight into the neurobiological mechanisms underlying cyclic changes in neuroendocrine function among states of estradiol negative and positive feedback.

Significance statement

Infertility affects 15-20% of couples; failure to ovulate is a common cause. Understanding how the brain controls ovulation is critical for new developments in both infertility treatment and contraception. GnRH neurons are the final output pathway for central control of fertility and produce a signal that ultimately initiates ovulation in response to estradiol positive feedback. We studied how the reproductive cycle regulates both synaptic transmission to GnRH neurons and excitability of these cells. Both GABAergic transmission to GnRH neurons and GnRH neuron excitability are decreased during stages the estrous cycle characterized by negative feedback by gonadal steroids, compared to the late afternoon of proestrus, when positive feedback and ovulation occur.

63 **Introduction**

64 GnRH neurons form the final hormonal output pathway through which the central nervous
65 system controls reproduction. GnRH triggers the release of the anterior pituitary hormones, LH
66 and follicle-stimulating hormone (Schally et al., 1971), which in turn promote sex steroid
67 production and gametogenesis. In both sexes, gonadal steroid feedback controls GnRH release
68 and pituitary response to GnRH (Karsch et al., 1987; Levine and Ramirez, 1982; Moenter et al.,
69 1991). For most of the female reproductive cycle, the steroid estradiol suppresses total
70 GnRH/LH release (negative feedback). However, at the end of the follicular phase (proestrus in
71 rodents), sustained rising levels of estradiol switch from suppressing GnRH/LH release to
72 inducing a surge of GnRH/LH release (positive feedback) (Moenter et al., 1991; Czieselsky et
73 al., 2016). The LH surge triggers ovulation.

74 In rodents, ovulation is tightly coupled to time-of-day (Everett and Sawyer, 1950; Sarkar et al.,
75 1976). GnRH/LH surges typically begin ~1-2 hours before lights out in nocturnal species with
76 this positive feedback mode of hormone release being confined to the proestrous phase of the
77 estrous cycle. Several paradigms for inducing positive feedback “surge” hormone release have
78 been developed to study this phenomenon, with most involving ovariectomy and estrogen
79 replacement (Norman et al., 1973; Legan and Karsch, 1975; Bronson and Vom Saal, 1979a;
80 Bronson, 1981; Christian et al., 2005). Most studies of the biophysical properties of GnRH
81 neurons during estradiol negative and positive feedback have made use of estrogen
82 replacement surge-induction models.

83 To lay a basis for understanding how synaptic and intrinsic properties of GnRH neurons change
84 between conditions of estradiol negative and positive feedback within the normal estrous cycle,
85 we examined the rates of GABAergic fast synaptic transmission, the primary fast synaptic input
86 to GnRH neurons, as well as GnRH neuron excitability, measured as action potential firing rate
87 in response to current injection, and action potential properties. We compared proestrous PM,

the time of positive feedback, with a low estradiol negative feedback stage (diestrous PM) and a high estradiol negative feedback stage (proestrous AM). We hypothesized that the transition among cycle stages induces changes in the intrinsic properties of GnRH neurons and GABAergic fast synaptic transmission to these cells.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich, unless noted.

Animals. Transgenic mice expressing green fluorescent protein (GFP) under the control of the GnRH promoter (Tg(Gnrh1-EGFP)51Sumo MGI:6158457) (GnRH-GFP mice) were used (Suter et al., 2000). Mice were housed on a 14-h light:10-h dark cycle with lights off at 6 P.M. (eastern standard time). Teklad 2916 chow (Envigo) and water were available ad libitum. Estrous cycles of adult females aged 60-128 days were monitored by vaginal cytology to determine estrous cycle stage; mice were studied on diestrus or proestrus. Uterine mass was measured at the time of brain slice preparation to confirm cycle stage as it is directly proportional to circulating estradiol levels (Shim et al., 2000). Uterine mass was within the published range for diestrus (n=10, 47.3 ± 2.7 mg) and proestrus (AM, n=8, 131.3 ±10.6 mg; PM n=11, 121.5 ± 2.7 mg)(Silveira et al., 2016). Uterine mass was lower on diestrus (one-way ANOVA/Tukey's $F_{(2, 26)}$, 68.3, $p<0.0001$) than either time of day on proestrus and was not different between proestrous AM and PM ($p>0.4$).

Brain Slice Preparation. All solutions were bubbled with 95% O_2/5% CO_2 throughout the experiments and for at least 15 min before exposure to tissue. Brain slices for AM recording were prepared 8.5 to 9.5 h before lights out; slices for PM recordings were prepared 1.5-2.5 h before lights out. The time of PM slice preparation corresponds to 30 min before the onset "surge peak" window through the end of that window as defined in previous work (Christian and Moenter, 2007). The brain was rapidly removed and placed in ice-cold sucrose saline solution containing the following (in mM): 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25

113 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$, at pH 7.6 and 345 mOsm. Coronal (300 µm) slices were
114 cut with a VT1200S Microtome (Leica Biosystems). Slices were incubated in a 1:1 mixture of
115 sucrose saline and artificial CSF (ACSF) containing (in mM) 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10
116 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 2.5 $CaCl_2$, at pH 7.4 and 305 mOsm, for 30 min at
117 room temperature (~21 to 23°C). Slices were then transferred to 100% ACSF at room
118 temperature for 0.5-5 h before recording.

119 *Data Acquisition.* During recording, slices containing the preoptic area and anterior
120 hypothalamus, which contain the majority of GnRH neuron somata, were placed into a chamber
121 continuously perfused with ACSF at a rate of 2 ml/min with oxygenated ACSF heated to 29.5-
122 31.5°C with an inline-heating unit (Warner Instruments). GFP-positive cells were visualized with
123 a combination of infrared differential interference contrast and fluorescence microscopy on an
124 Olympus BX50WI or BX51WI microscope. Borosilicate glass capillaries (1.65-mm OD x 1.12-
125 mm ID; World Precision Instruments, Inc.) were pulled by using a Flaming/Brown P-97 unit
126 (Sutter Instrument Company) to make recording pipettes. Pipettes measured 2-4.5 MΩ when
127 filled with (in mM): 125 K gluconate, 20 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl2, 4 MgATP, and 0.4
128 NaGTP, 300 mOsm, pH 7.2 with NaOH for current-clamp recordings or when filled with (in mM):
129 140 KCl, 10 HEPES, 5 EGTA, 0.1 CaCl2, 4 MgATP, and 0.4 NaGTP, 300 mOsm, pH 7.2 with
130 NaOH for recording GABAergic PSCs. Pipettes were wrapped with Parafilm (Bemis) to reduce
131 capacitive transients; remaining transients were electronically cancelled. Pipettes were placed in
132 contact with a GFP-positive neuron using an MP-285 micromanipulator (Sutter Instrument
133 Company). All potentials reported were corrected online for liquid junction potential of −14.2 mV
134 (Barry, 1994). Recordings were made with an EPC-10 dual patch-clamp amplifier (HEKA
135 Elektronik) and Patchmaster software (HEKA Elektronik). Experiments were analyzed offline
136 using custom software (DeFazio and Moenter, 2002; DeFazio et al., 2014) written in IgorPro
137 (Wavemetrics).

Experimental Design. Comparisons of the properties of GABAergic transmission to GnRH neurons and the intrinsic firing properties of GnRH neurons in response to current injection were made among cells in brain slices from diestrous PM, proestrous AM and proestrous PM mice.

Whole-cell patch-clamp. After achieving a >1 GΩ seal and the whole-cell configuration, membrane potential was held at -60 mV between protocols. Series resistance (Rs), input resistance (Rin), and holding current (Ihold) were measured every 2-3 min using a 5 mV hyperpolarizing step from −60 mV (mean of 20 repeats, 20 ms duration, sampled at 100 kHz and filtered at 10 kHz). Only recordings with a Rin of >500 MΩ, Ihold of −50 to 20 pA, stable Rs of <20 MΩ, and a stable Cm between 8.5 and 23 pF were used for analysis.

Spontaneous GABAergic postsynaptic currents (sPSCs) were measured in voltage-clamp at a holding potential of -70 mV. Current was sampled at 10 kHz and filtered at 10 kHz. ACSF contained 20 μM D-APV, and 20 μM CNQX to block ionotropic glutamate receptors. At least two 120-second recordings were made for each cell for determining sPSC frequency. Mean±SEM recording time was 591±107 s/cell for diestrous PM (n=11, range 240-1200 s), 457±46 s/cell for proestrous AM (n=9, range 240-600 s) and 536±56 s/cell during proestrus (n=16, range 244-1010 s). A total of 1351, 446 and 7929 sPSC events were recorded during diestrous PM, proestrous AM and proestrous PM, respectively.

To measure activity-independent miniature PSCs (mPSCs), two to three 120-second recordings were made before and during bath application of 1 μM tetrodotoxin (TTX) in a separate set of cells from the diestrous PM and proestrous PM groups.

GnRH neuron excitability was assessed in current-clamp recordings. Direct current was adjusted to keep cells within 2 mV of -69 mV. Membrane potential was sampled at 20 kHz and filtered at 7.3 kHz. Bridge balance (95%) was used for most cells; for a few cells in diestrous PM and proestrous PM groups, bridge balance was not used but results were similar. ACSF

contained 100 μM picrotoxin, 20 μM D-APV, and 20 μM CNQX to block ionotropic GABA and glutamate receptors. Cells were injected with current from 0-30 pA (500 ms, 2 pA steps). This protocol was repeated two to three times per cell and the number of action potentials at each step was averaged. The first spike fired was used to determine the following action potential characteristics: latency from start of the current injection to first spike, firing threshold (membrane potential when the first derivative of the voltage trace exceeds 1 V/s), peak amplitude relative to threshold, full width at half-maximum (FWHM), rate-of-rise, and time and amplitude of after-hyperpolarization potential (AHP, both relative to threshold).

Statistical Analyses Data were analyzed using Prism 7 (GraphPad) or SPSS (IBM) and are reported as the mean ± SEM. The number of cells per group is indicated by n. No more than two cells were used per animal with at least four animals tested per group. One cell from the diestrous PM GABA transmission group was identified as an outlier by ROUT (robust regression and outlier removal) with a strict Q coefficient of 0.01 and was excluded from all data sets. Data distribution was determined using a Shapiro-Wilk test for normality. Amplitudes of sPSC were binned at 5pA intervals and histograms constructed of the mean on a per cell basis. Interevent intervals were binned at 0.1s and plotted as a cumulative probability; events in cells from the proestrous AM group were sufficiently infrequent that the histogram of these data was not informative. Recordings with zero events were excluded from interevent interval analysis; values reported are thus an underestimate of interevent interval as the maximum that could be considered was two minutes. ANOVA analyses did not assume equal subgroup sizes. Tests are specified in the results and legends. A p value <0.05 was accepted as significant.

Results

GABAergic transmission to GnRH neurons is increased during proestrus.

In the daily surge model, GABAergic transmission is decreased during negative feedback and increased during positive feedback relative to OVX controls (Christian and Moenter, 2007). To

examine if GABA transmission to GnRH neurons is modulated between phases of the estrous cycle during which physiologic negative vs positive feedback are observed, spontaneous GABAergic postsynaptic currents (sPSCs) were recorded from GnRH neurons in brain slices obtained from diestrous PM, proestrous AM (both negative feedback) or proestrous PM (positive feedback) mice. Representative recordings are shown in Figure 1A, and recording parameters in Table 1. Frequency of spontaneous GABAergic PSCs (sPSCs) was increased during proestrous PM relative to proestrous AM and approached traditional significance values vs diestrous PM (Figure 1B, diestrous PM n = 11, proestrous AM, n=9, proestrous PM n = 16, Kruskal-Wallis/Dunn's, $p=0.063$ proestrous PM vs diestrous PM, $p<0.001$ proestrous AM vs proestrous PM). Interestingly, although mean frequency of GABA transmission from cells recorded on diestrous PM was not different from proestrous AM, the cumulative probability distribution of sPSC interevent interval averaged by cell differed significantly among all groups. Specifically, the distribution was shifted towards shorter intervals on proestrous PM and longer intervals for proestrous AM, both being different from the intermediate distribution for diestrous PM events and from one another (Figure 1C Kruskal-Wallis/Dunn's, proestrous AM vs both proestrous PM and diestrous PM, $p<0.0001$; diestrous PM vs proestrous PM, $p<0.0001$). Cumulative distributions can be skewed by one or two high frequency cells; in these data sets, the median and interquartile range followed the same pattern as the mean (diestrous PM 0.18 Hz [IQR 0.10-0.32], proestrous AM 0.02 Hz [0.001-0.16], proestrous PM 0.57 Hz [0.30-1.91]).

Amplitude of sPSCs was also markedly suppressed in cells from proestrous AM mice (Figure 1D, E, one-way ANOVA/Tukey, proestrous AM $p<0.05$ vs diestrous PM, proestrous AM $p<0.005$ vs proestrous PM). Consistent with this observation, the peak of the amplitude histogram was significantly left-shifted for proestrous AM cells vs diestrous PM and proestrous PM cells (Figure 1F, Kruskal-Wallis/Dunn's, $p<0.001$). No difference was observed in decay time between 90% and 10% of the maximum current amplitude (Figure 1G, ANOVA, $p>0.2$).

GABAergic transmission is primarily activity independent and does not change between diestrus and proestrus

Increased GABAergic PSC frequency during proestrus may be due to an increase in presynaptic activity and/or synaptic release sites on GnRH neurons. To differentiate between these mechanisms, PSC frequency and amplitude were recorded before and during treatment with the voltage-gated sodium channel blocker tetrodotoxin (TTX, Figure 2A). TTX treatment isolates activity-independent neurotransmission, which is proportionate to the number of functional synaptic connections as well as to release probability at individual release sites (Auger and Marty, 2000; Kaeser and Regehr, 2014). Because the frequency of overall GABAergic transmission was very low in cells recorded on proestrous AM, they were excluded from this analysis. Neither PSC frequency nor amplitude (Figure 2B-D, two-way repeated-measures ANOVA/Bonferroni) were altered during TTX treatment (n=6 cells diestrous PM, n=5 cells proestrous PM). An increase in PSC decay time during TTX was detected by ANOVA, but *post hoc* tests did not detect differences within cycle stage (Figure 2E, two-way repeated-measures ANOVA, TTX: $F_{(1,9)}$=6.4, Bonferroni: p=0.22 for both groups). Collectively these data indicate that most synaptic transmission observed in the slice is activity independent and that this does not change between the cycle stages examined.

GnRH neuron excitability is increased during positive feedback.

To investigate if GnRH neuron excitability is also modulated during the estrous cycle, we measured GnRH neuron response to depolarizing steady-state current injections (0-30 pA, 2 pA steps, 500ms). Figure 3A shows representative responses to +12 and +24 pA injections. The rheobase current (the minimum current required to initiate spikes) was lowest on proestous PM during positive feedback and highest on proestrous AM (Figure 3C, diestrous PM n = 9, proestrous AM n = 7, proestrous PM n = 9, one-way ANOVA/Tukey, $p<0.05$ diestrous PM vs both proestrous AM and proestrous PM, $p<0.0001$ proestrous AM vs proestrous PM). Once

237 firing was initiated, GnRH neurons from proestrous PM mice fired more spikes. Specifically, at
238 current steps from 12-30 pA, cells recorded on proestrous PM fired more spikes than cells from
239 either diestrous PM mice or proestrous AM mice (Figure 3B, two-way repeated-measures
240 ANOVA/Fisher's LSD, $p<0.05$). Differences were also observed between the two negative
241 feedback stages examined; at current steps ≥20 pA, cells from proestrous AM mice fired fewer
242 spikes than cells from diestrous PM mice. Input resistance was lower in cells recorded on
243 proestrous AM; this could contribute to fewer spikes being fired in this group (Table 1).

244 A number of action potential properties were also altered among the cycle stages examined,
245 including action potential threshold being hyperpolarized on proestrous AM vs diestrous PM
246 (Figure 3E, ANOVA/Tukey, $p<0.01$) and rate-of-rise being lower on proestrous AM than
247 proestrous PM (Figure 3H, Kruskal-Wallis/Dunn's, $p<0.05$). Full-width at half-maximum was
248 greater on proestrous AM than both other groups (Figure 3G, ANOVA/Tukey, $p<0.0001$).
249 Afterhyperpolarization time was reduced on proestrous PM compared to diestrous PM (Figure
250 3J, Kruskal-Wallis/Dunn's, $p<0.05$). No cycle-dependent changes were observed in time to first
251 spike (spike latency, Figure 3D, ANOVA $p=0.0792$), AP spike amplitude (Figure 3F, ANOVA,
252 $p>0.5$), or AHP amplitude (Figure 3I, ANOVA $p>0.5$).

253 **Discussion**

254 The female reproductive cycle is characterized by one of the rare examples of positive feedback
255 in physiology, specifically the induction of a surge mode of GnRH and LH release at the end of
256 the follicular phase (proestrous PM in nocturnal rodents). This is largely attributed to exposure
257 to high sustained levels of estradiol from the mature Graafian follicle(s) (Docke and Dorner,
258 1965). Here we show that GABAergic transmission to GnRH neurons and GnRH neuron
259 excitability are both increased during positive feedback (proestrous PM) relative to two different
260 representations of negative feedback, diestrous PM and proestrous AM, which, interestingly,
261 also diverged from one another in some aspects.

The present observations support and extend work in an OVX+E mouse model in which the switch from estradiol negative to positive feedback occurs solely on a time-of-day basis. Estradiol must be elevated near peak follicular phase levels for several hours to initiate the switch to positive feedback (Evans et al., 1997). The levels of estradiol achieved in the daily surge model are in the physiologic range, but are persistently, rather than cyclically, elevated, and the result is daily changes from negative to positive feedback. Because estradiol level is similar during negative and positive feedback, it is not an estradiol rise that triggers the change between states in this model. This differs from the estrous cycle in which an estradiol rise is viewed as the trigger for the transition. The question is thus raised of whether or not underlying mechanistic differences observed between feedback states in the daily surge model are the same as those during the cycle. The similar increase in GnRH neuron excitability and GABA transmission observed during positive feedback in the present work in cycling mice to published observations in the OVX+E daily surge model indicates at least some of the neurobiological mechanisms underlying the feedback switch are similar between these models. Consistent with these observations, GnRH neuron firing rate has also been shown to be similar during positive feedback whether induced by OVX+E or occurring spontaneously on the afternoon of proestrus (Silveira et al., 2016).

Of interest, the two negative feedback stages studied also diverged from one another with regard to some of the parameters examined. Specifically, cells studied on the morning of proestrus were less excitable and had smaller amplitude GABAergic PSCs than cells on the afternoon of diestrus. Both estradiol and progesterone change with cycle stage and either or both may underlie these observations. Progesterone typically provides negative feedback on GnRH release and firing rate (Moenter et al., 1991; Barrell et al., 1992; Pielecka et al., 2006). In the present studies we have no measure of progesterone, but it is likely that the influence of this steroid would be greater on diestrous PM than proestrous AM. Based on uterine mass, we can

surmise that estradiol levels were higher on the morning of proestrus than on the afternoon of diestrus. The reduced excitability and transmission observed on proestrous AM relative to diestrous PM may thus be a consequence of the increase in estradiol inducing a biphasic feedback response, with negative feedback preceding positive feedback. In this regard, administration of a surge-inducing dose of estradiol to ovariectomized females initially suppresses and then increases GnRH release (Moenter et al., 1990). Similar observations have been made during reproductive cycles of sheep and women, in which the amplitude of LH pulses is reduced as the follicular phase proceeds, coincident with rising estradiol levels. In the present study, some action potential parameters appeared to change sooner upon exposure to the cyclical rise in estradiol (e.g., the hyperpolarization of threshold) than others (e.g., increased rate of rise). Still others exhibited biphasic changes upon exposure to the estradiol rise (e.g., rheobase and FWHM). Together these observations suggest both that estradiol action during the mouse cycle is biphasic and that the negative feedback signal provided by high estradiol before transition to positive feedback on proestrus is stronger than that produced by the hormonal milieu on diestrus. This indicates the strong negative feedback observed in the AM of the OVX+E daily surge model may more closely resemble proestrous AM than diestrous PM.

The shift from negative to positive feedback from proestrous AM to proestrous PM is consistent with the biphasic effects of estradiol, but may be augmented by other steroid changes. In addition to its role during negative feedback, progesterone can also amplify the LH surge in rats and mice (Bronson and Vom Saal, 1979b). Studies have also identified central changes induced by progesterone and ligand-independent actions of the progesterone receptor as important for positive feedback (Chappell et al., 1999; Chappell and Levine, 2000; Micevych and Sinchak, 2011). The amplitude of the proestrous LH surge in mice is greater than the estradiol-induced surge, attributable at least in part to augmented pituitary response to GnRH on proestrus (Silveira et al., 2016). In women, progesterone administration during the late follicular phase

augments LH pulse amplitude, which could be attributable to increased amplitude GnRH release and/or increased responsiveness to endogenous GnRH, but does not alter pulse frequency, which would require central action (Hutchens et al., 2016). Of interest to the site of progesterone action, the excitability parameters observed in the present study on the afternoon of proestrus, when both progesterone and estradiol from the ovary were present before brain slice preparation, are remarkably similar to those during positive feedback in OVX+E daily surge mice (Adams et al., 2018), which have been exposed to only circulating estradiol for at least two days. These observations may indicate boosting effects of progestins on LH surge amplitude occur independent of GnRH neurons at the level of the anterior pituitary; such action could be directly upon the pituitary and/or indirectly via alterations of other neuroendocrine factors that affect LH release such as gonadotropin-inhibitory hormone (Son et al., 2012).

In addition to the parameters examined in the present study, it is likely that other synaptic and intrinsic properties of GnRH neurons change with cycle stage. With regard to the former, estradiol suppresses glutamatergic excitatory fast synaptic transmission during negative feedback in the daily surge model (Christian et al., 2009), and increased glutamatergic transmission on proestrus in rats (Tada et al., 2013). In another estrogen-induced surge model, the density of spines, often considered a termination point for glutamatergic inputs, was increased in GnRH neurons expressing cFos as a marker of elevated neuronal activity during the surge (Chan et al., 2011). With regard to intrinsic properties, a decrease in both transient A-type and sustained delayed rectifier potassium currents (DeFazio and Moenter, 2002; Pielecka-Fortuna et al., 2011) and an increase in both low and high-voltage activated calcium currents have been reported during positive feedback using different estradiol regimens (Zhang et al., 2009; Sun et al., 2010). Similar changes in specific voltage-gated ion channels may underlie the changes in excitability observed among cycle stages in the present study. Of note, the lower excitability of cells recorded on the morning of proestrus and lower input resistance compared to

337 either the afternoon of diestrus or negative feedback (OVX+E AM) in the daily surge model may
338 suggest greater changes in these and perhaps other conductances occur during the morning of
339 proestrus (Adams et al., 2018).

340 The concept that estradiol regulates synaptic properties of GnRH neurons to bring about the
341 switch from negative to positive feedback were not supported in recent work using another LH-
342 surge induction model in which OVX mice are treated with basal estradiol replacement then an
343 additional estrogen injection to mimic the proestrous estradiol rise (Bronson and Vom Saal,
344 1979b; Bronson, 1981). No differences were observed in sPSC or mPSC frequency between
345 negative feedback (OVX+basal E, slices made 4.5-5 hours before lights out, recordings made 1-
346 3.5 hours before lights off) and positive feedback (OVX+basal E+E injection, slices made 1.5-2
347 hours before lights out, recordings 1 hour before to 1.5 hours after lights out) (Liu et al., 2017).
348 Despite this difference, both models reliably produce an LH surge. This could indicate that
349 changes in GABAergic PSC frequency may not be necessary for initiating positive feedback but
350 may mark cotransmission of other substances such as kisspeptin (Lee et al., 2010; Piet et al.,
351 2018). In this regard, knockout of estradiol receptor alpha from GABAergic neurons eliminates
352 estradiol positive feedback (Cheong et al., 2015). Of note, this would remove ERα from a large
353 percentage of kisspeptin neurons in the AVPV that utilize GABA as a co-transmitter; the lack of
354 a surge may reflect reduced activation of these neurons (Cravo et al., 2011; Frazao et al.,
355 2013). Another possibility is that the overlap of recording time relative to lights out in the former
356 study precluded detection of a difference between negative and positive feedback. If time of day
357 interacts with estradiol to generate the changes observed in synaptic transmission to GnRH
358 neurons, as suggested by the present data comparing proestrous AM and PM and previous
359 work in the daily surge model, it is possible that the switch to positive feedback levels of
360 transmission had already occurred based on basal estradiol alone. Of note, the frequency of

synaptic transmission in that study is higher in all groups that we have observed either in daily surge or cycling mice.

The LH surge is critical for ovulation, reproduction and the continuation of species. The present studies add to a literature that indicates multiple factors can influence the switch from negative to positive feedback, and further indicates that the mechanisms producing negative feedback are also changing throughout the cycle. Feedback stage-dependent shifts in both GnRH neuron intrinsic excitability and fast-synaptic inputs likely contribute to the increase in firing rate and GnRH release during positive feedback.

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491 **Table 1: Whole-cell recording properties for Figures 1-3**

Mean±SEM of GnRH whole-cell passive properties from Figure 1			
	diestrous PM	*proestrus AM*	*proestrus PM*
Input resistance (MΩ)	929±42	1336±160*	1034±63
Capacitance (pF)	14.7±0.7	13.0±0.8	14.5±0.7
Series resistance(MΩ)	13.2±0.6	13.5±1.0	14.6±0.7
Holding current (pA)	-13.6±4.2	-0.15±4.7#	-17.4±2.8
*p<0.05 vs diestrous PM; #p<0.01 vs proestrous PM, Tukey's			
ANOVA parameters for comparison of GnRH passive properties (Figure 1)			
Input resistance (MΩ)	$F_{(2,33)}$=4.84		
Capacitance (pF)	$F_{(2,33)}$=1.32		
Series resistance (MΩ)	$F_{(2,33)}$= 0.951		
Holding current (pA)	F $F_{(2,33)}$=5.38		
Mean±SEM of GnRH whole-cell passive properties from Figure 2			
	diestrous PM	*proestrus PM*	
Input resistance (MΩ) before TTX during TTX	 1053±88 846±83	 1113±145 775±91	
Capacitance (pF) before TTX during TTX	 15.8±0.8 15.0±0.8	 13.2±0.9 13.8±1.0	
Series resistance(MΩ) before TTX during TTX	 12.1±0.9 13.3±1.5	 12.3±0.6 14.7±0.8	
Holding current (pA) before TTX during TTX	 -16.4±3.5 -25.1±6.3	 -19.4±2.6 -28.5±3.8	
Two-way repeated measures ANOVA for comparison of GnRH passive properties among groups (Figure 2)			
	group	*TTX*	*group x TTX*
Input resistance (MΩ)	$F_{(1,9)}$=0.001	$F_{(1,9)}$=36.0***	$F_{(1,9)}$=2.1
Capacitance (pF)	$F_{(1,9)}$=2.5	$F_{(1,9)}$=0.1	$F_{(1,9)}$=6.9*
Series resistance (MΩ)	$F_{(1,9)}$=0.4	$F_{(1,9)}$=6.0*	$F_{(1,9)}$=0.7
Holding current (pA)	$F_{(1,9)}$=0.3	$F_{(1,9)}$=14.1**	$F_{(1,9)}$=0.01
Mean±SEM of GnRH whole-cell passive properties from Figure 3			
	diestrous PM	*proestrous AM*	*proestrous PM*
Input resistance (MΩ)	1125±150	667±43*	1361±144
Capacitance (pF)	13.7±0.7	13.8±0.7	12.5±0.8
Series resistance(MΩ)	13.5±0.9	11.9±0.9	13.5±1.4
Holding current (pA)	-0.7±5.2	-2.8±6.3	-10.1±4.4
*p<0.05 vs diestrous PM, Tukey's			
ANOVA parameters for comparison of GnRH passive properties (Figure 3)			
Input resistance (MΩ)	$F_{(2,22)}$=6.65		
Capacitance (pF)	$F_{(2,22)}$=1.02		
Series resistance (MΩ)	$F_{(2,22)}$=0.62		
Holding current (pA)	KW=3.36		

Figure Legends

Figure 1. GABAergic sPSC frequency is highest on proestrous PM. **A.** Representative sPSC recording from a neuron in each group. **B.** Individual values and mean ± SEM of spontaneous GABAergic PSC frequency (Kruskal-Wallis, KW=14.4, *p<0.05 Dunn's). **C.** Mean by cell cumulative probability distribution of interevent interval (IEI) for each group (Kruskal-Wallis, KW=191, *p<0.0001, Dunn's). **D.** By-cell average of all sPSC from all cells in each group. **E.** Individual values and mean ± SEM of sPSC amplitude (ANOVA $F_{(2,33)}$=6.69, *p<0.05, **p<0.005 Tukey). **F.** Histogram of mean by cell sPSC amplitude distribution (Kruskal-Wallis, KW=23.9, proestrous AM vs both diestrous PM and proestrous PM, *p<0.001, Dunn's). **G.** Individual values and mean ± SEM of sPSC time decay time between 90% and 10% of the maximum event amplitude (ANOVA $F_{(2,33)}$=1.34).

Figure 2. Blocking action potentials does not affect GABAergic PSC frequency or amplitude in diestrous or proestrous mice. **A.** Representative recordings from a representative neuron in each group before (control or con, top) and during (bottom) TTX treatment (from n=6 cells diestrous PM, n=5 cells proestrous PM). **B.** Individual values and mean ± SEM of GABAergic PSC frequency . **C.** Average of all PSC traces for control or ttx periods from all cells in each group. **D-E** Individual values and mean ± SEM for: **D,** PSC amplitude, **E,** decay time between 90% and 10% of the maximum current amplitude. No statistical differences were detected using two-way repeated-measures ANOVA/Bonferroni test (B, cycle stage: $F_{(1,9)}$=1.3; TTX: $F_{(1,9)}$=1.6; cycle stage x TTX: $F_{(1,9)}$=0.0; D, cycle stage: $F_{(1,9)}$=0.3; TTX: $F_{(1,9)}$=0.6; cycle stage x TTX: $F_{(1,9)}$=0.5; E, cycle stage: $F_{(1,9)}$=0.5; TTX: $F_{(1,9)}$=6.4 (p=0.01); cycle stage x TTX: $F_{(1,9)}$=0.9)

Figure 3. GnRH neuron excitability is increased on proestrus vs diestrus. **A.** Representative traces from a neuron in each group during 500 ms current injections of 12 and 24 pA (current injection protocol below). **B.** Mean ± SEM spikes elicited for each current injection step (two-way repeated-measures ANOVA cycle stage: $F_{(2,22)}$=10.2, current: $F_{(15,330)}$=93.03, interaction: $F_{(30,330)}$=9.503, #p<0.05 diestrous PM vs proestrous PM and p<0.05 proestrous AM vs proestrous PM; *p<0.05 among all three groups, Fisher's LSD). **C-H** Individual values and mean ± SEM for: **C,** rheobase current (ANOVA $F_{(2,22)}$=12.8, *p<0.05, **p<0.0001), **D,** latency to first spike (ANOVA $F_{(2,22)}$=2.85, p=0.0792), **E,** action potential threshold (ANOVA $F_{(2,22)}$=6.18, *, p<0.01 Tukey), **F,** action potential amplitude (ANOVA, $F_{(2,22)}$=0.676), **G**, full-width at half-maximum (ANOVA $F_{(2,22)}$=26.2, **p<0.0001 Tukey), **H**, action potential rate of rise (Kruskal-Wallis, KW=6.69, *p<0.05 Dunn's), **I**, afterhyperpolarization potential (AHP) amplitude (ANOVA $F_{(2,22)}$=0.252), and **J**, AHP time (Kruskal-Wallis, KW=7.03, p<0.05 Dunn's).