Dynorphin and GABA<sub>A</sub> Receptor Signaling Contribute to Progesterone’s Inhibition of the LH Surge in Female Mice

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Progesterone can block estrogen-induced luteinising hormone (LH) surge secretion and can be used clinically to prevent premature LH surges. The blocking effect of progesterone on the LH surge is mediated through its receptor in the anteroventral periventricular nucleus (AVPV) of the hypothalamus. However, the underlying mechanisms are unclear. The preovulatory LH surge induced by estrogen is preceded by a significant reduction in hypothalamic dynorphin and gamma-aminobutyric acid (GABA) release. To test the detailed roles of dynorphin and GABA in an LH surge blockade by progesterone, ovariectomized and 17β-estradiol capsule-implanted (OVX/E<sub>2</sub>) mice received simultaneous injections of estradiol benzoate (EB) and progesterone (P) or vehicle for 2 consecutive days. The LH level was monitored from 2:30 pm to 8:30 pm at 30-minute intervals. Progesterone coadministration resulted in the LH surge blockade. A continuous microinfusion of the dynorphin receptor antagonist nor-BNI or GABA<sub>A</sub> receptor antagonist bicuculline into the AVPV from 3:00 pm to 7:00 pm reversed the progesterone-mediated blockade of the LH surge in 7 of 9 and 6 of 10 mice, respectively. In addition, these LH surges started much earlier than the surge induced by estrogen alone. However, 5 of 7 progesterone-treated mice did not show LH surge secretion after microinfusion with the GABA<sub>B</sub> receptor antagonist CGP-35348. Additionally, peripheral administration of kisspeptin-54 promotes LH surge-like release in progesterone treated mice. These results demonstrated that the progesterone-mediated suppression of the LH surge is mediated by an increase in dynorphin and GABA<sub>A</sub> receptor signaling acting though kisspeptin neurons in the AVPV of the hypothalamus in female mice.

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Reproductive cyclicity in mammals is regulated by the interplay between estradiol (E<sub>2</sub>), progesterone (P), gonadotropin-releasing hormone (GnRH), and gonadotropin hormones (1). Progesterone is produced by the corpus luteum and inhibits hypothalamic GnRH and consequently gonadotropin secretion (2). Moreover, when administered before or concurrent with E<sub>2</sub>, P inhibits E<sub>2</sub> positive feedback and abolishes the preovulatory GnRH/luteinising hormone (LH) surge (1, 3). This inhibition of the LH surge by P has been identified in many species, including rodents (3), sheep
(4), primates (5), and humans (6–8). Clinically, this P effect has been used as an alternative to a gonadotropin-releasing hormone (GnRH) analogue for suppressing premature LH surges in controlled ovarian stimulation (COS) for in vitro fertilization (IVF) cycles (6–8). Despite its physiological and clinical importance, the mechanism by which P blocks the preovulatory LH surge is not fully understood.

Progestosterone has been shown to block the LH surge by acting centrally to inhibit the surge of GnRH secreted by the hypothalamus (4). In rodents, the hypothalamic nuclei that control positive and negative feedback are different. The anteroventral periventricular nucleus (AVPV) is thought to underlie the LH surge by mediating different. The anteroventral periventricular nucleus (AVPV) is thought to underlie the LH surge by mediating the positive feedback effects of E2 and the arcuate nucleus (ARC), which contains kisspeptin/neurokinin-B/dynorphin (KNDy) neurons and mediates steroid negative feedback actions on pulsatile LH secretion (9, 10). A recent study showed that progesterone's inhibitory effects on the LH surge are mediated by its receptor in the AVPV (11). The lack of colocalization of P receptors (PRs) with GnRH neurons (12) suggests that this action must occur via other neurons expressing steroid receptors. The control of GnRH activity and subsequent LH release involves numerous neurotransmitter systems (13), including the dynorphin (14) and gamma-aminobutyric acid neurons in the paraventricular nucleus and POA (15) pathways, which modulate GnRH neurons within the hypothalamus.

It is known that the majority of dynorphin neurons express PR messenger ribonucleic acid (mRNA) (16). It is generally thought that P inhibits GnRH pulse frequency though dynorphin neurons (17, 18). However, the role that dynorphin plays on LH surge regulation is controversial. Previous studies have suggested that a decrease in dynorphin inhibitory input to the preoptic area (POA) is a prerequisite for LH surge secretion in rodents (19), and by acting through kappa-opioid receptors (KOR), dynorphin could block the LH surge and ovulation (20). However, another study has demonstrated that a substantial k-opioid tone is still present during LH surge initiation (21).

Additionally, GABA plays an important role before the occurrence of the LH surge (22). Gamma-aminobutyric acid neurons in the paraventricular nucleus and POA express PRs in monkeys (23). The infusion of a GABA agonist into the POA in rats has been shown to attenuate the LH surge (24). It is noteworthy that GABA can mediate both excitation and inhibition through the ionotropic receptor GABA_A depending on the intracellular chloride concentration of the GnRH neurons and inhibition through the metabotropic receptor GABA_B (25, 26). However, whether GABA, especially GABA located in the AVPV, is involved in P inhibition of the LH surge is still unclear.

In the present study, we monitored LH surge profiles in OVX/E2 capsule + estradiol benzoate (EB) + P-treated female mice subjected to a bilateral continuous microinfusion of a potent KOR (nor-BNI), GABA_A receptor (bicuculline [BIC]), or GABA_B receptor (CGP-35348) antagonist, respectively, into the AVPV, to investigate the potential rescue of the P-induced inhibition of the LH surge by the decreased activity of the dynorphin and GABA signaling pathways in the AVPV. Additionally, we monitored LH secretion in OVX/E2 capsule + EB + P-treated female mice receiving peripheral administration of kisspeptin-54 to investigate whether the ability of GnRH neurons to respond to kisspeptin remains intact.

**Materials and Methods**

**Animals**

Female, adult (10 weeks old) C57BL/6 mice weighing 20–25 g were obtained from Charles River (Margate, UK), housed individually under controlled temperature (22 ± 2 °C) and light conditions (12:12 hours light:dark, with lights on at 7:00 AM), and given a standard maintenance diet (Special Diet Services, Wagstaf, UK) and water ad libitum. All procedures were conducted in accordance with the British Home Office Animals Scientific Procedures Act 1986, and all experimental protocols were approved by the Animal Welfare and Ethical Review Body at King's College London.

**Surgical procedures**

All surgical procedures were carried out under anaesthesia with ketamine (100 mg/kg intraperitoneal [i.p.]; Pharmacia and Upjohn Ltd, Crawley, UK) and Rompun (10 mg/kg i.p.; Bayer, Leverkusen, Germany). Only mice that had exhibited at least 3 consecutive 4- to 5-day estrous cycles were selected for experimentation. They were subjected to bilateral OVX and implanted with a silastic capsule (Sanitech, Havant, UK) containing 17β-estradiol (1 μg per 20 g of body weight) (27). To assess the effects of dynorphin and GABA antagonists on the hypothalamic AVPV, animals were secured in a motorized Kopf stereotaxic frame and surgical procedures were performed using a robotic stereotaxic system (Neurostar, Tubingen, Germany). A small hole was drilled in the skull at a location above the AVPV. The stereotaxic coordinates used to target the AVPV (0.25 mm lateral, 0.26 mm anterior to the bregma and at a depth of 5.3 mm below the surface of the skull) were obtained from the Paxinos and Franklin mouse brain atlas (28). A bilateral guide cannula (26-gauge; Plastics One, Roanoke, Virginia) was then targeted towards the AVPV at the same time as the OVX surgery. The guide cannula was secured using dental cement (Dental Filling Ltd., Swindon, UK) and fitted with a dummy cannula (Plastics One) to maintain patency. The mice were housed in individually ventilated...
cages after surgery and other parameters were controlled in each cage.

**Blood sampling procedure**

After a 3- to 5-day recovery period postsurgery, the mice were handled daily to acclimatize them to the tail-tip blood sampling procedure (29). The tip of the mouse's tail was excised using a sterile scalpel for subsequent blood sample collection (30). After a 1-hour acclimation period, blood samples (4 µl) were collected at 30 minute intervals using a pipette tip dipped in heparinized saline (50 U/ml heparin sodium/ml normal saline; CP Pharmaceuticals, Wrexham, UK), and the whole blood was immediately diluted with 56 µl of 0.2% BSA in 1 M PBS containing 0.05% Tween 20 (PBS-T), vortexed, and snap frozen on dry ice. Blood samples were frozen at -80 °C for a later assay to determine LH concentrations.

**Effect of an intra-AVPV infusion of nor-BNI, BIC, or CGP-35348 on progesterone-mediated inhibition of the LH surge**

The treatment of OVX/E₂ capsule-implanted mice with EB has been validated to induce an LH surge (30) and facilitates the study of the mechanisms underlying this key reproductive process. Six to 10 days after the OVX/E₂ surgery, mice with a diestrous-like vaginal cytology were administered a subcutaneous (sc) injection of EB (1 µg/20 g of body weight) and an i.p. injection of P (100 µg/20 g of body weight) at 8:30 am, followed by a second i.p. injection of P at 4:30 pm. This is designated Day 1 of treatment. The following day, Day 2, this hormone regime was repeated, and is an established protocol that results in the inhibitory effect of P on the estrogen-induced LH surge (3, 11). The dose of P was chosen to maintain a serum P level higher than the level after ovulation (11, 31). As positive controls for the inhibitory effects of P, a separate group of OVX/E₂ capsule + EB-treated mice were injected with vehicle (50 µl of peanut oil, i.p., Sigma-Aldrich, Gillingham, UK) instead of P. These steroid regimes are illustrated in Fig. 1.

Intra-AVPV injections of a selective antagonist for the dynorphin activated KOR (nor-BNI; Tocris Bioscience, Bristol, UK), GABA₃ receptor antagonist (BIC; Sigma-Aldrich), GABA₆ receptor antagonist (CGP-35348; Sigma-Aldrich), or artificial cerebrospinal fluid (aCSF) were administered to separate groups of OVX/E₂ capsule + EB + P mice to identify the roles of dynorphin and GABA signaling in the P-mediated inhibition of the LH surge. On the afternoon of Day 2 (Day 1 is the day on which the EB and P treatments started), a bilateral internal cannula (Plastics One) with extension tubing preloaded with nor-BNI, BIC, CGP-35348, or aCSF was inserted into the guide cannula and extended 0.5 mm beyond the guide cannula tip to reach the AVPV. The distal ends of the tubing were extended outside of the animal cage and connected to 10-µl Hamilton syringes (Waters Ltd., Elstree, UK) secured in a PHD 2000 programmable syringe pump (Harvard Apparatus, Massachusetts), thereby allowing constant infusion without disturbing the animals during the experiment.

The infusion dose for nor-BNI (0.1 µg administered at a rate of 300 nl/h in aCSF on each side) (32), BIC (6 ng administered at a rate of 300 nl/h in aCSF on each side) (33) or CGP-35348 (0.4 µg administered at a rate of 300 nl/h in aCSF on each side) (34) was based on previous research and our preliminary studies. The infusion was performed from 3:00 pm to 7:00 pm on Day 2. The lights-on phase in our laboratory is from 7:00 am to 7:00 pm, and as 2 hours after the midpoint of the light phase corresponds to the beginning of the critical period for ovulation block by pentobarbital (35), and significant LH surge levels and cfos levels in GnRH neurons and the AVPV is around the light/dark transfer period (36), we chose 3:00 pm to 7:00 pm as the treatment infusion period in our experiments.

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**Figure 1. Timeline of the experiments.** Ovariectomized mice implanted with estradiol (E₂) capsules (OVX/E₂) and showing a diestrous-like vaginal cytology were administered a sc injection of estradiol benzoate (EB: 1 µg/20 g of body weight) and an i.p. injection of progesterone (P: 100 µg/20 g of body weight) at 8:30 am, followed by a second i.p. injection of P at 4:30 pm (Day 1). The following day, designated Day 2, this hormone regime was repeated. As positive controls, the OVX/E₂-treated mice were injected with vehicle (50 µl of peanut oil, i.p.) instead of P. Mice were administered an intra-AVPV infusion of relevant drugs or artificial cerebrospinal fluid as control from 3:00 pm to 7:00 pm on Day 2. Blood samples were collected every 30 minutes between 2:30 pm and 8:30 pm on Day 2 from both experimental groups and assayed for LH.
The OVX/E2+EB + P-treated mice were administered nor-BNI (n = 12), BIC (n = 12), CGP-35348 (n = 10) or aCSF (n = 12) via the intra-AVPV cannulae from 3:00 pm to 7:00 pm and blood samples were collected every 30 minutes between 2:30 pm and 8:30 pm on Day 2, the expected day of the LH surge (Day 1 is the day on which the EB and P treatments began). In addition, a separate group of OVX/E2+EB+oil (positive control; n = 12) were administered an intra-AVPV infusion of aCSF from 3:00 pm to 7:00 pm (300 nl/h of aCSF on each side) on Day 2 as controls. All of the mice in each experiment were independent and used only once. Blood samples were collected every 30 minutes between 2:30 pm and 8:30 pm on Day 2 (Fig. 1).

**Effect of peripheral administration of kisspeptin-54 on progesterone-mediated inhibition of the LH surge**

Separate groups of OVX/E2 capsule + EB + P-treated mice received a single i.p. injection of Kisspeptin-54 (Kp-54, Tocris Bioscience; 1 nmol / 30 g of body weight in 100 μl) (37) (n = 9), or vehicle saline (n = 5) at 3:30 pm on Day 2 of the protocol as shown in Fig. 1. Blood samples were collected every 30 minutes between 2:30 pm and 8:30 pm on Day 2 as described above.

**Brain collection and histological verification of the cannula position**

After experimentation, 0.5 μl of India ink was injected over 5 minutes through the internal cannulae inserted into the guide cannulae for the purpose of site verification. Animals were then euthanized by decapitation. The brain was removed, snap frozen on dry ice, and then stored at -80 °C, followed by sectioning (30 μm) using a cryostat (Bright Instrument Co., Ltd., Luton, UK). Every third section throughout the AVPV region corresponding to the bregma (0.74 to -0.7 mm) was mounted and stained with cresyl violet to evaluate the cannula position. The slides were then viewed under a light microscope and the images were captured using a digital camera (Zeiss, Oberkochen, Germany). Only data from animals with correct cannula placement were analyzed. The remaining mice were excluded from the analysis due to inaccurate probe placement.

**LH measurement**

Blood samples were assessed using an enzyme-linked immunosorbent assay (ELISA), as previously reported (29). A mouse LH standard (mLH; reference preparation, AFP-5306A, NIDDK-NHPP, Bethesda, Maryland), coating antibody (https://antibodyregistry.org/RRID:AB_2665514, monoclonal antibovine LH beta subunit antiserum, 518B7, University of California, California) (38), anti-LH antibody (https://antibodyregistry.org/RRID:AB_2665533; National Hormone & Peptide Program, Torrance, California) (39), and a secondary antibody (http://antibodyregistry.org/RRID:AB_772206, GE Healthcare, Chicago, Illinois) (40) were used to determine the LH concentration. The intra-assay and inter-assay variations were 4.6% and 10.2%, respectively.

**Statistical analysis**

Statistical comparisons of the separate LH values from 2:30 pm to 8:30 pm between groups were performed using one-way ANOVA followed by posthoc Tukey’s multiple comparison test or the Games–Howell test for data with unequal variance. Data are presented as the means ± SEM, and \( P < 0.05 \) was considered statistically significant.

**Results**

**Cannula placement in the AVPV**

The location of each intra-AVPV cannula was confirmed by microscopic histological inspection of cresyl violet-stained brain sections. Only animals with appropriate bilateral cannula placement in the AVPV were included in the analysis (Fig. 2). Of the 58 mice that underwent hypothalamic cannulation, 45 were confirmed to have correct bilateral cannula placement in the AVPV. Data from animals...
with incorrect cannula placement, either on one or both sides were excluded from the analysis. These included: 3 of the 12 OVX/E_2+EB + P-treated administered nor-BNI; 2 of 12 OVX/E_2+EB + P-treated administered BIC; 3 of 10 OVX/E_2+EB + P-treated administered CGP-35348; 3 of 12 OVX/E_2+EB + P-treated administered aCSF; and 2 of 12 OVX/E_2+EB+oil-treated administered aCSF.

Effects of progesterone on the LH surge in OVX/E_2+EB-treated mice

Ten mice with correct intra-AVPV cannula placement underwent the OVX/E_2+EB+oil surge induction protocol and aCSF infusion with tail-tip blood sampling every 30 minutes for 6 hours (from 2:30 pm to 8:30 pm). Of these animals, 8 exhibited an LH surge (Fig. 3) beginning at approximately 5:30 pm before light offs (7:00 pm). Luteinising hormone concentrations prior to surge onset were 1.52 ± 0.05 ng/ml and gradually increased to peak concentrations of 5.81 ± 0.62 ng/ml just before (0.5 hour) lights out, and then was gradually reduced to baseline levels 1.5 hours later. The remaining 2 mice showed no change in LH secretion during the blood sampling period (data not shown). However, P successfully blocked the LH surge, in all 9 OVX/E_2+EB-treated mice with correct intra-AVPV cannula placement that were administered P together with intra-AVPV aCSF infusion (Fig. 3).

Effect of an intra-AVPV infusion of nor-BNI on the LH surge profile in OVX/E_2+EB + P-treated mice

The intra-AVPV infusion of nor-BNI rescued the LH surge in 7 of 9 mice with correct intra-AVPV cannula placement that underwent the OVX/E_2+EB + P protocol (Fig. 4). A significant increase in mean LH concentration was observed between 3:30 pm and 5:30 pm in the EB + P-treated plus nor-BNI infusion group than in the mice subjected to the same protocol but infused with aCSF as control (Fig. 4). The remaining 2 mice showed no change in LH secretion during the blood sampling period (data not shown). In the nor-BNI infusion group, which showed an LH surge, the mean LH peak levels have no significant difference with those of OVX/E_2+EB-treated with aCSF infusion mice (7.2 ± 1.48 vs. 5.81 ± 0.62 ng/ml, respectively, P > 0.05). However, the nor-BNI infusion group exhibited LH surges much earlier than the OVX/E_2+EB-treated group, with the peak of the LH surge in the nor-BNI group occurring approximately 1.5 hours earlier (compare Figs. 3 and 4).

Effect of an intra-AVPV infusion of bicuculline or CGP-35348 on the LH surge in OVX/E_2+EB + P-treated female mice

An intra-AVPV infusion of bicuculline rescued the surge in 6 of 10 OVX/E_2+EB + P-treated mice with correct intra-AVPV cannula placement (Fig. 5A). Moreover, the surge began approximately 2 hours earlier, starting at about 3:00 pm and returning to baseline at 6:00 pm, compared to the surge in the OVX/E_2+EB-oil-treated group administered intra-AVPV aCSF (compare Fig. 5A with Fig. 3). The peak LH surge levels in bicuculline infusion

Figure 3. The inhibitory effects of progesterone (P) on the LH surge in OVX/E_2 capsule + EB mice. Eight of the 10 OVX/E_2+EB+oil-treated mice receiving intra-AVPV infusion of artificial cerebrospinal fluid (aCSF) between 3:00 pm and 7:00 pm on designated Day 2 (see Fig. 1 for steroid hormone protocol) and showed the expected LH surge approximately 1 hour before lights off (7:00 pm). The remaining 2 animals failed to show an LH surge (data not shown). All 9 of the OVX/E_2+EB group treated with P (see Fig. 1) and receiving intra-AVPV infusion of aCSF on Day 2 failed to show an LH surge (same data illustrated in Fig. 3). Mean ± SEM levels of LH are indicated. Significantly different values are indicated by an asterisk (*P < 0.05; **P < 0.01).

Figure 4. Microinfusion of the kappa opioid receptor antagonists, nor-BNI into the AVPV reversed the inhibitory effects of progesterone (P) on the LH surge in OVX/E_2 capsule + EB treated mice. Mean (±SEM) LH levels in 7 of 9 OVX/E_2 capsule + EB + P-treated mice receiving a bilateral intra-AVPV infusion of nor-BNI (0.1 μg administered at a rate of 300 nl/h in artificial cerebrospinal fluid [aCSF] on each side) between 3:00 pm and 7:00 pm on designated Day 2 (see Fig. 1 for experimental timeline) reveal a typical LH surge, but advanced in onset by approximately 2.5 hours. The remaining 2 animals failed to show an LH surge (data not shown). All 9 of the OVX/E_2+EB group treated with P (see Fig. 1) and receiving intra-AVPV infusion of aCSF on Day 2 failed to show an LH surge (same data illustrated in Fig. 3). Significantly different values are indicated by an asterisk (*P < 0.05; **P < 0.01).
groups were similar to the mice in the OVX/E2+EB with aCSF infusion group (5.95 ± 0.66 vs. 5.81 ± 0.62 ng/ml, respectively, \(P > 0.05\)). The remaining 4 mice showed no change in LH secretion during the blood sampling period (data not shown). Of the 7 OVX/E2+EB + P-treated mice with correct intra-AVPV cannula placement that underwent intra-AVPV infusion of CGP-35348, 5 did not show an LH surge during the blood sampling period from 2:30 pm to 8:30 pm (Fig. 5B). Only 2 mice exhibited an LH surge: 1 showed an LH surge starting at approximately 3:30 pm, reaching a peak value of 4.95 ng/ml at 5:00 pm and then gradually returning to baseline at 6:30 pm, while the other one exhibited an LH surge at 5:30 pm, that is, at a similar time to the OVX/E2+EB with aCSF infusion, and reached a peak value of 5.56 ng/ml 1 h later that finally ended at 8:00 pm (Fig. 5B).

Effect of peripheral administration of kisspeptin-54 on progesterone-mediated inhibition of the LH surge

We assessed LH secretion after of a single i.p. injection of KP-54 (1 nmol / 30 g of body weight in 100 μl; \(n = 9\)) or saline (\(n = 5\)) in the OVX/E2+EB + P-treated mice. KP-54 increased plasma LH levels above vehicle control immediately after injection with the highest values occurring 30 minutes postinjection (8.8 ± 0.99 vs. 1.24 ± 0.15 ng/ml, \(P < 0.001\)) and remaining elevated for approximately 2 hours postinjection (4.19 ± 0.32 vs 1.43 ± 0.33 ng/ml, \(P < 0.001\)) before returning to baseline (Fig. 6).

Discussion

The results from the present study provide novel evidence that P-mediated suppression of the LH surge in female mice is mediated by dynorphin-KOR and GABA<sub>A</sub> receptor signaling acting through kisspeptin neurons in the AVPV of the hypothalamus. Intra-AVPV administration of dynorphin-related KOR and GABA<sub>A</sub> receptor antagonists, namely, nor-BNI and bicuculline, respectively, attenuated P-mediated inhibition of the LH surge in female mice. In contrast, GABA<sub>B</sub> receptor activity in the AVPV did not seem to be closely
related to P-mediated suppression of the LH surge, as microinfusion of a GABA_\text{\textsubscript{B}} receptor antagonist, CGP-35348, did not sufficiently rescue the P-induced inhibition of the LH surge. Moreover, peripherally administered KP-54 induced LH surge-like secretion in OVX/E\textsubscript{2}+EB + P-treated mice.

Estrogen provides the signal to the GnRH/LH neurosecretory system to stimulate the LH surge in female mammals, while P can modify this response and sometimes has opposing effects (3, 41, 42). Previous studies have shown that P can block the LH surge if it is administered either coincident with or immediately after the stimulatory estradiol signal (11, 41). In accordance with these data, we confirmed that simultaneously administering a luteal phase level of P and a supraphysiological levels of EB in OVX/E\textsubscript{2} mice blocks the LH surge regardless of the high estrogenic milieu by EB, which normally induces the LH surge. Studies in ewes showed that P could block the positive feedback effects by affecting the synthesis and/or secretion of the neurotransmitter systems that are targeted by E\textsubscript{2}. This is consistent with previous studies showing that dynorphin plays a key role in LH surge timing (19). Few GnRH neurons express KOR receptors, suggesting that dynorphin acts via other intermediates to exert its effects (49).

GABAergic neurons within the GnRH network provide an important regulatory influence on GnRH neurons (50). According to a previous study, GABA release shows a sharp decline during the preovulatory afternoon period, which is closely associated with the onset of the LH surge in female rats (51). Based on our data, GABA_\text{\textsubscript{A}} receptor activity in the AVPV appears to play a critical role in mediating the inhibitory effect of P on the LH surge, as intra-AVPV infusion of bicuculline, a GABA_\text{\textsubscript{A}} receptor antagonist, blocked the P-mediated inhibition of the LH surge in mice. There are a few possible mechanisms to explain the above data: First, P may inhibit LH secretion through allopregnanolone (52), a P metabolite that acts on GABA_\text{\textsubscript{A}} receptors as an allosteric agonist (52, 53). This hypothesis is supported by evidence showing that the inhibition of LH release by allopregnanolone is reversed by bicuculline in steroid-treated rats (54). Second, P may also change GABA_\text{\textsubscript{A}} receptor conformation and affect its synthesis and expression in a specific manner (55). Furthermore, the LH surge started much earlier in the bicuculline infusion group than in the positive control group, consistent with the preliminary conclusion that GABA_\text{\textsubscript{A}} receptors affect the initiation of the LH surge (56). The effect of GABA_\text{\textsubscript{A}} receptor activation on GnRH neurons is controversial, as a recent study showed that directly stimulating GABA neurons in the AVPV evoked a sustained large increase in LH (15). Nevertheless, infusing a GABA agonist into the POA of rats at the estimated time of the surge can effectively suppress the LH surge (24, 33). GABA_\text{\textsubscript{A}} receptor activation has different effects on GnRH neurons in different environments, brain regions, or at different hormone levels (50, 57). Our research supports the notion that GABA_\text{\textsubscript{A}} receptor plays an inhibitory role in GnRH/LH secretion and for the first time provides evidence that P increases GABA levels in the AVPV to extinguish the LH surge. However, CGP-35348, a GABA_\text{\textsubscript{B}} receptor antagonist, did not fully rescue the LH surge, which was consistent with previous studies showing the predominance of GABA_\text{\textsubscript{B}} over GABA_\text{\textsubscript{A}} receptors in mediating LH secretion (58). The explanation for why 2 mice in the CGP-35348 treatment group produced LH surge is unclear. Perhaps this may be due to the dose of GABA_\text{\textsubscript{B}} receptor antagonist used, so further work is required.
Numerous neurotransmitters and neuropeptide systems co-regulate LH surge secretion. Among these, kisspeptin neurons in the AVPV nucleus plays a vital role in the regulation of the preovulatory LH surge (15). Herbison and colleagues have shown c-fos labeling, a marker of neuronal activation, in both AVPV kisspeptin cells and GnRH cells at the time of the presumptive GnRH surge (59). It seems that P prevents the LH surge though interrupting estrogen-kisspeptin-GnRH signaling in the AVPV, as a previous study showed that microinjection of a P receptor antagonist into the AVPV rescued the LH surge blocked by P (11). Kisspeptin signaling is requisite and likely to be downstream of GABA_A receptor and dynorphin signaling, since kisspeptin neurons in AVPV which directly and potently stimulate GnRH neurons express KOR and receive direct functional GABA_A receptor inputs (15, 59–61). GABA generally hyperpolarizes AVPV kisspeptin neurons and there is an estradiol-induced decrease in GABAergic input to these neurons in the afternoon in ovariectomized estradiol-treated mice, allowing for the increase in kisspeptin output critical for eliciting the preovulatory LH surge (62). Leon et al (60) provided conclusive evidence that the GABA_A receptor blockade-induced increase in LH secretion is completely dependent on kisspeptin signaling to GnRH neurons, since LH secretion was abolished in global Grp54 KO mice, but recovered when Gpr54 expression was selectively reintroduced only in GnRH neurons. It has been shown that kisspeptin neurons in the AVPV express KOR and that these cells are robustly hyperpolarized by a KOR agonist (61). In addition, our results suggest that P does not affect the response of GnRH neurons to kisspeptin as peripheral administration of KP-54 successfully promoted LH secretion in all O VX/E2+EB + P-treated mice, consistent with previous research that peripherally administered KP-54 could activate c-FOS in GnRH neurons (37). It therefore appears that the inhibitory effect of P may be mediated by dynorphin and GABA-decreasing kisspeptin release by impairing the activity of the AVPV kisspeptin neurons.

It has been shown that AVPV neurons receive inputs from the suprachiasmatic nucleus (SCN) (63) and that AVPV cells exhibit circadian fluctuations in cAMP levels (64). The time chosen for infusion of drugs in the present study was from 3:00 pm to 7:00 pm on experiment Day 2, which accounts for circadian rhythmicity (35, 36). Our results demonstrate that decreasing GABA_A receptor and dynorphin signaling in the AVPV could advance the timing of the LH surge (19, 56), presumably by increasing kisspeptin level to rescue LH surge. These data are supported by previous finding that kisspeptin expression in the AVPV was temporally increased in females in the late afternoon correspondence with circadian time (62, 65).

There is unequivocal evidence that the POA, encompassing the AVPV kisspeptin neurons, is essential for the positive feedback action of E2 that generates the preovulatory GnRH/LH surge in mice and rats (10, 66–68). In the nonrodent species the arcuate KNDy neurons are considered the major population involved in GnRH/LH surge generation (10, 66–68). Although kisspeptin is found in the POA of nonrodent species, including sheep, monkeys, and humans, and kisspeptin expression is elevated at the time of the spontaneous or E2-induced LH surge, the POA is not essential for LH surge generation, at least not in higher primates (66–68). Therefore, there remains the caveat that the mechanisms involved in progesterone-blocking LH surge might be different between rodent and nonrodent species.

In summary, the results of the present study suggest that the progesterone-induced suppression of the LH surge in female mice is mediated by increased dynorphin and GABA_A receptor signaling in the AVPV acting though local kisspeptin neurons.

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