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Progesterone receptor-Src kinase signaling pathway mediates neuroprogesterone induction of the luteinizing hormone surge in female rats

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Abstract

Neural circuits in female rats are exposed to sequential estradiol and progesterone to regulate the release of luteinizing hormone (LH) and ultimately ovulation. Estradiol induces progesterone receptors (PGRs) in anteroventral periventricular nucleus (AVPV) kisspeptin neurons, and as estradiol reaches peak concentrations, neuroprogesterone (neuroP) synthesis is induced in hypothalamic astrocytes. This local neuroP signals to PGRs expressed in kisspeptin neurons to trigger the LH surge. We tested the hypothesis that neuroP-PGR signaling through Src family kinase (Src) underlies the LH surge. As observed in vitro, PGR and Src are co-expressed in AVPV neurons. Estradiol treatment increased the number of PGR immunopositive cells and PGR and Src colocalization. Furthermore, estradiol treatment increased the number of AVPV cells that had extranuclear PGR and Src in close proximity (< 40 nm). Infusion of the Src inhibitor (PP2) into the AVPV region of ovariectomized/adrenalectomized (ovx/adx) rats attenuated the LH surge in trunk blood collected 53 h post-estradiol (50 μg) injection that induced neuroP synthesis. Although PP2 reduced the LH surge in estradiol benzoate treated ovx/adx rats, activation of either AVPV PGR or Src in 2 μg estradiol-primed animals significantly elevated LH concentrations compared to dimethyl sulfoxide infused rats. Finally, antagonism of either AVPV PGR or Src blocked the ability of PGR or Src activation to induce an LH surge in estradiol-primed ovx/adx rats. These

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AUTHOR CONTRIBUTIONS

Timbora Chuon: Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing. **Micah Feri:** Data curation; Formal analysis; Investigation; Writing – review & editing. **Claire Carlson:** Data curation; Formal analysis; Investigation; Methodology; Writing – review & editing. **Sharity Ondrejik:** Data curation; Formal analysis; Methodology; Writing – review & editing. **Paul E Micevych:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Visualization; Writing – review & editing. **Kevin Sinchak:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Visualization; Writing – review & editing.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

results indicate that neuroP, which triggers the LH surge, signals through an extranuclear PGR-Src signaling pathway.

Keywords

anteroventral periventricular nucleus; AVPV; estradiol; kisspeptin; LH surge; neuroprogesteron; rostral periventricular region of the third ventricle; RP3V; Src kinase

1 | INTRODUCTION

Progesterone synthesis and signaling in the hypothalamus are critical events for the luteinizing hormone (LH) surge that induces ovulation and are associated with “estrogen positive feedback” in the rodent.[1–11] Ovulation is coordinated with reproductive behavior and development of the uterine lining to maximize the likelihood that copulation results in fertilization and pregnancy. In mammals, these events are orchestrated by hormones of the hypothalamic-pituitary-ovarian axis.

Circulating concentrations of ovarian estradiol slowly rise during diestrus I and II. This initial rise of estradiol exerts negative feedback on the hypothalamus and pituitary but ultimately initiates the activation of a complex interplay of astrocyte and neuronal circuitry, including the induction of classical progesterone receptor (PGR).[1] As the rat estrus cycle progresses, circulating estradiol concentrations rise rapidly to a peak on the afternoon of proestrus signaling positive feedback, comprising a cascade of events culminating in the preovulatory LH surge, which stimulates follicular ovulation and luteinization of the remaining follicular cells.[12] A critical aspect of positive feedback is the estradiol induction of synthesis and secretion of neuroprogesterone (neuroP) by hypothalamic astrocytes.[10,13] This local neuroP acts on the estradiol-induced PGR in neurons of the rostral periventricular region of the third ventricle (RP3V), which contains the anteroventral periventricular nucleus (AVPV) and preoptic periventricular nucleus that release kisspeptin onto gonadotropin-releasing hormone (GnRH) neurons triggering the LH surge.[14–16] Blocking neuroP synthesis in hypothalamic astrocytes in intact rats on the morning of proestrus or during estradiol treatment in ovariectomized/adrenalectomized (ovx/adx) rats eliminates the LH surge.[8] Moreover, estradiol-induced PGRs are essential to initiate and reach the full magnitude and duration of the LH surge.[1,6] In global PGR-knockout mice[2,17] and in mice with PGR knocked out in kisspeptin neurons only (kissPRKOs), there is no LH surge.[16,18] This latter point is critical because kisspeptin neurons are assumed to transduce steroid information to GnRH neurons, which lack the appropriate steroid receptors, and in this way regulate the surge release of GnRH.[19–22]

Although PGR has long been classified as a transcription factor, it is one of a number of nuclear steroid receptors that can be trafficked to the plasma membrane and interact with other signaling proteins to initiate rapid signaling at the level of the plasma membrane.[23–26] Although it is well-established that PGRs are an integral part of the positive feedback mechanism, whether membrane localized PGRs are involved remains an open question. PGR complexes with Src tyrosine kinase (Src).[23,24] Src is a member of the family of non-receptor tyrosine kinases,[27] which interacts directly with PGR via its SH3 domain.[23]

We have demonstrated a PGR-Src signaling complex elsewhere in the arcuate nucleus of the hypothalamus (ARH).[28] The PGR-B isoform is complexed with Src in the plasma membrane. Infusions of a Src inhibitor, PP2, blocks progesterone facilitation of lordosis in estradiol-primed ovx rats, and PGR antagonism blocks the Src activator facilitation of lordosis behavior.[28] Furthermore, in immortalized adult female RP3V kisspeptin neurons, mHypoA51 cells, PGR-Src signaling was demonstrated. mHypoA51 cells express Src and estradiol-induced PGR.[14,15] Activation of Src in mHypoA51 cells induced kisspeptin release, whereas inhibition of Src activation blocked progesterone activation of mitogen-activated protein kinase (MAPK) signaling and inhibited kisspeptin release.[15] Progesterone also rapidly induced intracellular calcium levels in mHypoA51 cells, which may also be mediated via Src.[14] These results suggest that neuroP acts through a membrane-initiated PGR-Src signaling pathway to activate RP3V kisspeptin neurons. We hypothesized that estradiol-induced neuroP acts on estradiol-induced PGRs that signal through Src in kisspeptin neurons in the AVPV to induce the LH surge.

2 | MATERIALS AND METHODS

2.1 | Animals

For Experiments I and II, ovx Long-Evans rats were purchased from Charles River at 200–225 g in weight or 55–65 days old. Animals were housed in a climate- and light-controlled (12:12 h light/dark photocycle, lights on 6:00 a.m.) room with food and water provided ad libitum. Wound clips were removed within 1 week of the arrival date.

For Experiments III and IV, ovx/adx Long-Evans rats were purchased from Charles River at 200–225 g in weight or 55–65 days old. Drinking water was provided for the entirety of the experiments, consisting of NaCl (0.9%) and corticosterone (10 μL^{-1}; Sigma-Aldrich).[10] Animals were housed in a climate- and light-controlled (12:12 h light/dark photocycle, lights on 6:00 a.m.) room with food and water provided ad libitum. Rats were double-housed prior to cannulation. After cannulation surgery, they were single-housed to prevent head-cap damage. Wound clips were removed within 1 week of the arrival date. All procedures involving animals were approved by the California State University, Long Beach IACUC.

2.1.1 | Steroid and drug treatments—17β-Estradiol benzoate (EB) (2 and 50 μg) doses were dissolved in safflower oil (oil) so that the volume of each injection was 0.1 mL. An EB dose of 2 μg produces physiological circulating levels of estradiol, which primes feedback and reproductive neurocircuits without inducing the LH surge or sexual behavior.[29–33] Additional treatment with more estradiol or progesterone can elicit positive feedback. On the other hand, an EB dose of 50 μg reliably induces an LH surge in ovx/adx Long-Evans rats.[10,34] We have used this paradigm to investigate the synthesis of neuroP and its role in triggering of the LH surge in the female ovx/adx rats.[10,34,35] We understand that this is a supraphysiological dose of estradiol and therefore demonstrated the necessity of neuroP synthesis for ovulation in intact cycling rats.[8] In the present study, rats received s.c. injections into the nape of the neck. Bilateral, site-specific infusions introduced drugs into the AVPV. Src activator was dissolved in sterile saline (50 nmol). PP2, R5020 (PGR agonist) and progesterone were dissolved in sterile dimethyl sulfoxide (DMSO) (50 nmol).

Because progesterone will activate all progesterone receptors (the family of membrane progesterone receptors [mPRs] and the PGR), R5020 was used to selectively activate PGR.[36] R5020 is also a low afinity agonist for glucocorticoid receptors. The total volume infused per side was 0.5 μl. All infusions (vehicles and drugs) were performed with a sterile microinjector (Plastics One) connected to a 25 μl Hamilton syringe thin plastic Tygon™ tubing. Infusions were driven by a microliter syringe pump (Stoelting Co.) at a rate of 1 μl min^{-1}. Microinjectors protruded 2 mm beyond the guide cannulae. Infusion cannulae were allowed to remain in place for 1 min to allow diffusion. Following infusion, stylets were reinserted into the guide cannulae and animals were returned to their home cage until blood collection.

2.2 | Experiment I: PGR-Src double-label immunohistochemistry

To verify the colocalization of PGR and Src within AVPV neurons, ovx rats were treated first with either EB (2 μg; $n = 5$) or oil ($n = 4$) s.c. and 44 h later received a second injection of oil. Animals received these treatments once every 4 days for three cycles. Fortyeight hours after the third EB or oil treatment (the third cycle), rats were deeply anesthetized with isoflurane and transcardially perfused with chilled 0.9% saline followed by PARA (4% paraformaldehyde in 0.1 m Sorenson's phosphate buffer).[31,37] Brains were extracted, post-fixed in PARA for 24 h and cryoprotected. Brains were then coronally sectioned in a cryostat at 20 μm through the region of the AVPV and collected into phosphate-buffered saline (PBS, pH 7.5). Double-label immunohistochemistry was conducted using a polyclonal PGR primary antibody raised in rabbit (Table 1) that recognizes PGR-A and PGR-B, and a monoclonal primary antibody raised in mouse that recognizes Src 416-nonphosphorylated (Table 1). Free-floating brain sections were washed in PBS, followed by incubation in PBS containing 10% MeOH and 3% H_2O_2 for 10 min, then incubated in a non-specific blocking solution containing PBS, 0.2% Triton X-100 (TX), 1% normal goat serum (NGS) and 1% bovine serum albumin for 1 h. Sections were incubated for an additional 48 h at 4°C in the non-specific blocking solution with a cocktail of PGR antibody (dilution 1:2000) and Src (dilution 1:800,000). Negative controls consisted of omission of primary antibodies (see Supporting information, Figure S1). Sections were washed in PBS and then Tris-buffered saline (TBS, pH 7.5) and incubated for 2 h in TBS containing 1% NGS, 0.2% TX and a biotinylated goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC) for PGR (dilution 1:200; Jackson ImmunoResearch Laboratories Inc.) and a rhodamineconjugated goat anti-mouse secondary antibody (TRITC) for Src (dilution 1:200; Jackson ImmunoResearch Laboratories Inc.). Brain sections were rinsed in two final TBS washes, transferred to Tris buffer and mounted onto Superfrost Plus slides (Fisher Scientific). Mounted sections were dried on a slide warmer and cover-slipped using Aqua-Poly/Mount (Polysciences Inc.). Immunohistochemical localizations were visualized with a fluorescence microscope (DM6000; Leica Microsystems) and a Fluoview 1000 confocal laser scanning system (Olympus America Inc.).

2.2.1 | Immunohistochemistry analysis—

To confirm colocalization of immunoreactivity for PGR and Src, sections were analyzed by obtaining confocal Z series projections stacks through the depth of the tissue within the AVPV. Z series projections consisted of approximately five to seven images that spanned the depth of

the immunopositive staining with an interval of 2 μm. Three-dimensional reconstructions were used to determine colocalization of PGR and Src immunoreactivity.[38,39] FITC was visualized with an emission filter in the range 465–495 nm and a bandpass filter in the range 515–555 nm. TRITC was visualized with an emission filter in the range 515–550 nm and a bandpass filter in the range 600–640 nm.

Analyses of single- and double-labeled AVPV neurons were performed on photomicrographs of PGR and Src immunofluorescence staining in the AVPV using ImageJ cell counter, version 1.32j, (National Institutes of Health). Images were adjusted for brightness and contrast using Photoshop, version 7.0 (Adobe Systems Inc.) in one sitting prior to analysis in ImageJ.[40,41] Within ImageJ, a consistent oval overlaid on the AVPV provided a standard area to quantify Src and PGR positive stained cells across all sections. Counts were obtained from three AVPV regions per animal. Quantitative measurements of Src and PGR colocalization were performed by counting the number of AVPV neurons that expressed extranuclear staining for Src, nuclear staining for PGR, or both Src and PGR. A mean number of immunopositive neurons per section was calculated per animal. To determine the proportion of Src or PGR immunoreactive neurons that co-express Src and PGR, a percentage was obtained by dividing the number of neurons co-localizing Src- and PGR-immunopositivity by the total number of Src-only or PGR-only immunoreactive neurons, and multiplying by 100.

2.3 | Experiment II: Duolink proximity ligation assay

The results of Experiment I indicated that PGR and Src colocalization increased with estradiol treatment. To determine the potential for PGR and Src interactions in the AVPV and whether estradiol increases interactions between PGR and Src, a Duolink Proximity Ligation Assay (PLA; Sigma-Aldrich) analysis was performed on free-floating AVPV tissue sections from EB and oil treated ovx rats as described in Experiment I (n = 4 per treatment group). For the Duolink PLA, tissues were incubated with primary antibodies against PGR and Src. Tissues were then incubated with secondary antibodies conjugated to oligonucleotides that form a circular template if the distance between the oligonucleotides is < 40 nm.[42] Complementary-labeled, fluorescencetagged, oligonucleotide probes were used to detect and amplify the PGR-Src proteins in close apposition for visualization by confocal microscopy. Free-floating sections were washed in PBS prior to incubation in a Duolink PLA blocking solution, and then incubated for 48 h in PBS solution containing a mouse monoclonal Src antibody (Table 1). We used an antibody that detects nonphosphorylated Src at tyrosine-416 (dilution 1:800,000) and a rabbit polyclonal PGR antibody (dilution 1:2000) (Table 1). Tissue sections were then processed in accordance with the manufacturer's instructions, transferred into Tris buffer and then mounted onto Superfrost Plus slides (Fisher Scientific). Mounted sections were dried on a 37° C slide warmer and incubated in Hoechst (Sigma-Aldrich), dried again, and cover-slipped using Aqua-Poly/Mount (Polysciences, Inc.). Duolink PLA was visualized with a fluorescence microscope (DM6000; Leica) and a Fluoview 1000 confocal laser scanning system (Olympus America Inc.).

2.3.1 | Duolink PLA analysis—With Duolink PLA, extranuclear red fluorescence punctate staining indicates a positive interaction – PGR and Src in close proximity. All images were adjusted with brightness and contrast using Photoshop, version 7.0, in one sitting prior to analysis.[40,41] Within ImageJ, a consistent oval that covered most of the AVPV was used to provide a standard area to quantify number of positive PLA Src-PGR stained cells and Hoechst stained nuclei across all sections. Counts were obtained from three AVPV regions per animal using ImageJ cell counter, version 1.32j. The ratio of positive PLA stained cells in the AVPV was calculated by dividing the number of positive PLA stained cells by the number of Hoechst stained nuclei in the AVPV. A mean ratio of positive PLA staining/number of nuclei per section was calculated for each animal.

2.4 | Experiment III: Inhibition of Src and the LH surge

To test whether Src inhibition prevents the EB-induced LH surge, ovx/ adx rats ($n = 4$ per treatment group) were implanted with bilateral cannulae (PlasticsOne) aimed at the AVPV 13 days after ovx/adx surgery. On day 21 post ovx/adx surgery,[10] all animals received bilateral AVPV infusions of Src inhibitor (PP2, 50 nmol) (Table 2) or DMSO at 11:00 a.m. (Figure 1A). On the same day, all animals then received EB (50 μg s.c.[10,34]) at 12:00 p.m. On days 22 and 23 post ovx/adx surgery, all animals received bilateral AVPV infusions of either PP2 or DMSO at 11:00 a.m. On day 23, animals were deeply anesthetized with isoflurane at 1730 h (just prior to lights out), and quickly decapitated. Trunk blood was collected to measure serum LH levels, and brains were collected to confirm cannula placement. LH was measured in serum with an enzyme-linked immunosorbent assay (ELISA).

2.5 | Experiment IV: Activation of Src and the LH surge

We tested whether Src activation, similar to neuroP, triggers the LH surge in rats primed with a subthreshold EB dose of 2 μg. Although this dose of EB did not induce neuroP synthesis or an LH surge, it upregulated PGR (Figure 2 and Table). One week before EB treatment, adult ovx/adx Long-Evans rats ($n = 4$ per treatment group) were implanted with a bilateral cannula aimed at the AVPV (Figure 1B). At 21 days post ovx/adx surgery, all animals were treated with EB (2 μg; s.c.) at 12:00 p.m. All animals received two sequential infusions into the AVPV starting 47 h post EB (11:00 a.m. colony time) for the first infusion and 15:30 p.m. for the second infusion. Group 1 received two infusions of DMSO: one at 11:00 a.m and the following at 15:30 p.m. Group 2 received DMSO at 11:00 a.m. followed by progesterone at 15:30 p.m. Group 3 received saline at 11:00 a.m., followed by Src activator at 15:30 p.m. Group 4 received an initial infusion of progesterone receptor antagonist (RU486) at 1100 hours followed by Src activator at 15:30 p.m. Group 5 received Src inhibitor (PP2) at 11:00 a.m. followed by Src activator at 15:30 p.m. All animals were deeply anesthetized at 17:30 p.m. just prior to lights out in the colony room, and trunk blood was collected. Serum LH levels were measured by ELISA.

2.6 Experiment V: Src mediation of PGR signaling

To test whether Src mediates the PGR induction of the LH surge in subthreshold (2 μg of EB) treated rats, bilateral cannulae targeted at the AVPV were implanted to site specifically

infuse sequentially paired drug treatments (n = 4 per treatment group). The first AVPV infusion was either DMSO, Src inhibitor (PP2) or PGR antagonist (RU486) at 11:00 a.m. on day 2 of the experiment. At 15:30 p.m., all animals received a subsequent infusion of a PGR agonist (R5020) and trunk blood was collected at 17:30 p.m. R5020 instead of progesterone was infused into the AVPV to demonstrate that PGRs were activated and not members of the family of mPRs.[36] Although R5020 does bind glucocorticoid receptors, it has a much lower binding affinity for mPRs, which are present on Kiss neurons.[15,43–45] Although R5020 can bind to the glucocorticoid receptor,[46] activation of glucocorticoid receptors inhibits the LH surge.[47] Thus, is R5020 has a significant interaction with glucocorticoid receptors in the AVPV, we expect to see a lack of induction of the LH surge in R5020 infused animals. Serum LH levels were measured using ELISA.

2.6.1 | Stereotaxic surgery for implantation of guide cannulae—Animals were deeply anesthetized with isoflurane 13 days after ovx/adx surgery (2%–3% in equal parts oxygen; Western Medical Supply Inc.), injected with an analgesic (Rimadyl 1 mg mL^{-1}; Western Medical Supply Inc.) and secured into a stereotaxic frame (Stoelting Co.). Self-tapping bone screws were inserted into the skull to anchor the dental cement and cannula to the cranium. Bilateral cannulae (22 gauge, 8 mm below pedestal) were then implanted and aimed at the AVPV (coordinates from Bregma: 0° angle; anterior +0.1 mm, lateral 0.7 mm, ventral −6.5 mm, −3.3 mm tooth bar). After dental cement secured the cannula to bone screws, a stylet was placed in the guide cannula, protruding no more than 0.5 mm beyond the opening of the guide cannula to keep the cannula patent and covered with a head cap. After surgery, animals were single-housed and received oral antibiotics through the drinking water for 5 days (0.5 mg mL^{-1} trimethoprim and sulfamethoxazole; Western Medical Supply Inc.). Animals were allowed to recover for 7 days prior to steroid treatment and drug infusion.

2.6.2 | Serum collection and cannula tract confirmation—For Experiments III, IV and V, animals were deeply anesthetized with isoflurane and decapitated at 17:30 p.m. on day 23, 53.5 h following EB treatment (Figure 1). Immediately following decapitation, trunk blood and brains were collected. Blood clotted at room temperature for 90 min and then centrifuged at 2000 g for 15 min at room temperature. Serum was collected and stored at −80°C until analyzed for LH by ELISA. Extracted brains were flash frozen on dry ice and stored at −80°C until sectioned for cannula placement. To determine cannula placement, brains were mounted onto a chuck using HistoPrep medium, sectioned in the coronal plane at 20 μm through the AVPV, and directly mounted onto Superfrost™ Plus slides. Slides were allowed to dry on a slide warmer and stored at −80°C until thionin stained to visualize cannula placement with bright-field microscopy. Two cannula placements outside of the AVPV for Experiment 3 (PP2 infusion) did not reduce LH serum levels from the control group. One set of infusion needles went through the ventral border of the brain at the level of the AVPV, and the other was anterior to the AVPV. For Experiment 4 (DMSO-Progesterone, Saline Src Activ) and Experiment 5 (DMSO-R5020), five cannula placements were outside of the AVPV and did not induce expected increases in LH serum levels from controls. Two sets of infusion needles went through the ventral border of the brain at the level of

the AVPV. Two sets were posterior to the AVPV at the level of the of the suprachiasmatic nucleus.

2.6.3 | Rat LH sandwich ELISA—Serum LH concentrations were measured by rat LH sandwich ELISA as specified by the vendor (Shibayagi via BioVendor).[9] Standards and samples were run in duplicate, and results of the ELISA were measured by a colorimetric microplate reader at 450nm (VarioSkan 2.2; Thermo Scientific Inc.). The mean LH level was calculated for the duplicate samples. The intra-assay coefficients of variation were estimated in two replicated assays of serum samples for all experiments. For Experiments III, IV and V, intra-assay intervals were 8.9%, 8.0% and 6.8% respectively, and the inter-assay coefficient of variation was 7.5%.

2.7 | Statistical analysis

All statistics were performed using SigmaPlot, version 11.0 (Systat Software, Inc.). Data presented as the mean ± SEM and effects were considered significant at the $p < .05$ level for all tests. In Experiment I, a two-way ANOVA was used to analyze the effects of estradiol on number of immunopositive stained cells for either PGR or Src followed by a post-hoc Holm–Sidak (HS) for ANOVA results at the $p < .05$ level. The effect of estradiol on the number of neurons that colocalized immunopositive staining for PGR and Src was analyzed by a *t* test. Percentage data were square root transformed prior to analysis by a *t* test. In Experiment II, the mean ratio of nuclei with PGR-Src positive interactions per section was analyzed by a *t* test. For Experiment III, *t* tests were used to compare serum LH concentrations between two groups. In Experiments IV and V, one-way ANOVA was used to compare serum LH concentrations among the different groups and followed by all pairwise post-hoc analysis by the HS test for ANOVA results at the $p < .05$ level.

3 | RESULTS

3.1 | Experiment I: Colocalization of PGR and Src

PGR stained cells were used for estimating the number of PGR expressing neurons. Although we expected some extranuclear PGR immunostaining, PGR-immunoreactivity was predominately associated with the nucleus (Figure 2B–D,F,J). Src positive staining was localized to the cytoplasm and plasma membrane (Figure 2A,C–E,G–J). Colocalization was visualized as red, nuclear PGR staining associated/ surrounded by green extranuclear Src staining (Figure 2C,D,G–J). Significant main effects were observed in the mean number of PGR and Src immunopositive cells (two-way ANOVA, $F = 144.077$, $df = 1, 17$, $p < .001$), as well as for hormone treatment (two-way ANOVA, $F = 40.251$, $df = 1, 17$, $p < .001$). Furthermore, there was a significant interaction between the main effects (two-way ANOVA, $F = 31.061$, $df = 1, 17$, $p < .001$). The number of Src immunoreactive AVPV neurons was greater than the number of PGR immunopositive neurons, independent of steroid treatment (HS, EB: $t = 4.830$, $p < .001$; oil: $t = 11.798$, $p < .001$) (Figure 2). EB increased significantly the number of AVPV PGR stained cells compared to oil treatment (Holm-Sidak, $t = 8.427$, $p < .001$) (Figure 2). By contrast, EB treatment did not alter the number of Src immunopositive cells compared to oil treatment (HS, $t = 0.545$, $p = .594$) (Figure 2). The number of cells that were immunopositive for both PGR and Src was

increased by EB treatment compared to oil (*t*-test, $p < .001$, $df = 7$, $t = 8.995$) (Figure 2). EB treatment also increased the percentage of Src immunopositive cells that expressed PGR staining (*t*-test, $p < .001$, $df = 7$, $t = 7.989$) (Figure 2), but not the percentage of PGR immunopositive cells that expressed Src (*t*-test, $p = .976$, $df = 7$, $t = -0.0309$) (Figure 2). This population of PGR/Src neurons is consistent with the hypothesis that neuroP may activate an AVPV PGR-Src signaling pathway to induce the LH surge.

3.2 | Experiment II: PGR-Src interactions

To verify the PGR-Src interactions in the AVPV, Duolink PLA showed PGR and Src in close apposition (< 40 nm) as extranuclear punctate red fluorescence staining that was associated with a Hoechst stained nucleus (blue), but not within nuclei (Figure 3). The ratio of positive PGR-Src PLA staining to Hoechst-stained nuclei was significantly greater in 2 μg EB-treated rats compared to oil treated rats, indicating an estradiol-induced increase in number of neurons with PGR-Src in close proximity (*t*-test, $p < .001$, $df = 6$, $t = -7.774$) (Figure 3I).

3.3 | Experiment III: Src activation and the LH surge

The results from Experiments I and II suggest that PGR and Src were co-expressed in AVPV neurons and were within a distance to allow for interaction. To test whether Src activation would induce an LH surge, rats were treated with 50 μg of EB and infused with DMSO or Src inhibitor (PP2) into the AVPV (Figure 4A,B). The DMSO infused rats had surge levels of LH in their serum[9,10,34] (Figure 4C). Infusion of PP2 significantly decreased LH concentrations compared to DMSO-infused rats (*t*-test, $df = 6$; $t = 3.511$, $p = .015$) (Figure 4C). The attenuation of the LH surge following Src inhibition is consistent with our hypothesis that Src mediates neuroP signaling that triggers the LH surge.

3.4 | Experiment IV: Src signaling stimulates the LH surge

To determine whether Src signaling induces the LH surge via interaction with PGR, we compared ovx/adx rats treated with subthreshold EB-priming and infused with progesterone to rats in which Src was activated or inhibited in the AVPV. As expected, progesterone treatment of EB+P primed ovx/adx rats induced significantly higher levels of LH compared to DMSO treated (one-way ANOVA, $df = 4, 15$, $F = 20.221$, $p < .001$; HS, $t = 6.595$, $p < .001$, Figure 5). Src activator treatment in EB treated rats also increased LH levels compared to DMSO treated animals (HS, $t = 3.455$, $p < .01$) (Figure 5). However, LH levels induced by progesterone treatment were greater than those achieved with Src activator (HS, $t = 3.140$, $p < .01$) (Figure 5). Pretreatment with either PGR antagonist (RU486) or Src inhibitor (PP2) significantly reduced levels of LH induced by Src activator (HS, RU486: $t = 3.310$, $p = .01$; PP2: $t = 4.610$, $p = .01$) (Figure 5). LH levels among the DMSO + DMSO, RU486 + Src activator, and PP2 + Src activator were not significantly different from one another (DMSO + DMSO vs. RU486 + Src activator, $t = 0.145$, $p = .887$; DMSO + DMSO vs PP2 + Src activator, $t = 0.156$, $p = .226$ and RU486 + Src activator vs. PP2 + Src activator, $t = 0.130$, $p = .213$) (Figure 5). Together, these results implicated a PGR-Src interactive signaling mechanism underlying the LH surge.

3.5 | Experiment V: PGR signals through Src to induce the LH surge

We tested whether Src signaling mediates the PGR induced LH surge. Treatment with the PGR agonist, R5020, produced significantly elevated levels of LH compared to DMSO (one-way ANOVA, $df = 3, 12$; $F = 10.867$, $p < .001$; DMSO + R5020 vs. DMSO + DMSO, HS, $p < .05$) (Figure 6). Furthermore, pretreating with a Src inhibitor (PP2) or PGR antagonist (RU486) significantly reduced LH levels compared to the DMSO + R5020 group (HS, PP2 + R5020, $t = 4.71$; $p < .001$; RU486 + R5020, $t = 4.47$; $p < .001$). Groups infused with PP2 and RU486 prior to R5020 were not significantly different from the DMSO infused rats (HS PP2 + R5020, $t = 0.07$; RU486 + R5020, $t = 0.31$; $p > .05$) (Figure 6). These results were consistent with the idea that Src activation mediates the PGR induction of the LH surge.

For Experiments III to V, the cannula placements that missed the AVPV were as follows. Two cannula placements outside of the AVPV for Experiment III (PP2 infusion) did not reduce LH serum levels from the control group. One set of infusion needles went through the ventral border of the brain at the level of the AVPV, and the other was anterior to the AVPV. For Experiments IV (DMSO-Progesterone, Saline Src Activ) and Experiment V (DMSO-R5020), five cannula placements were outside of the AVPV and did not induce expected increases in LH serum levels from controls (data not shown). Two sets of infusion needles went through the ventral border of the brain at the level of the AVPV. Two other placements were posterior to the AVPV at the level of the of the suprachiasmatic nucleus. These were not included in the analysis.

4 | DISCUSSION

The major findings of these experiments were: (1) a subset of AVPV neurons co-expressed PGR and Src immunopositive staining (Figure 2); (2) PGR and Src were in close proximity (Figure 3); (3) estradiol priming increased the number of AVPV neurons that co-expressed PGR and Src; and (4) neuroP activated an AVPV PGR-Src signaling pathway that triggered the LH surge. Our studies demonstrated a pathway through which extranuclear PGR-Src signaling mediates the neuroP induction of an LH surge. AVPV infusions of the PGR, and not mPR, agonist, R5020, induced an LH surge in EB primed rats, indicating that the classical PGR mediated the neuroP induction of the LH surge (Figure 6). Moreover, this surge LH was attenuated by antagonism of PGRs with RU486 (Figure 6). Importantly, the estradiol-induced, neuroP-mediate LH surge was attenuated by inhibiting Src activation in the AVPV (Figure 4). Similarly, inhibition of Src blocked the R5020 induction of the LH surge (Figure 6), indicating that PGR-mediated induction of the LH surge requires Src signaling. Furthermore, when Src was activated in the AVPV of subthreshold EB treated rats, surge levels of LH were observed (Figure 5). Finally, our results were congruent with the idea that PGR and Src signaling mediating the surge are not arranged “in series” or “in parallel,” but act as an interdependent signaling complex relying on both proteins. PGR antagonism with RU486 blocked the activation of Src (Figure 5), and Src inhibition blocked R5020 activation of PGR that was necessary for the LH surge (Figure 6). Previous studies have indicated that the PGR-B isoform is upregulated and primarily involved in positive feedback.[14] These results are congruent with data that PGR-B, but not PGR-A, interacts

with the SH3 domain of Src24 implying that neuroP signaling is through the PGR-B isoform. Taken together, these data are consistent with the hypothesis that neuroP activates an AVPV PGR-Src signaling pathway to trigger the LH surge.

NeuroP induction of the LH surge is mediated by the release of kisspeptin from AVPV neurons[9] that requires estradiol priming and neuroP.[14,15] Our present findings obtained in vivo indicate that neuroP activated a PGR-Src pathway in the AVPV region to induce the LH surge, presumably at the level of kisspeptin neurons. In the rat, RP3V kisspeptin neurons in vivo express PGR,[48] and we demonstrated that PGR and Src were colocalized in AVPV neurons. As expected for a mechanism underlying positive feedback, estradiol increased the number of neurons expressing both PGR and Src. The present results provide in vivo support of previous findings in cultured kisspeptin neurons, mHypoA51 cells, derived from adult female mice that are a model for RP3V kisspeptin neurons.[14,15] The mHypoA51 cells express both PGR and Src, and estradiol-priming increases PGR expression as well as increasing PGR-B levels at the plasma membrane.[14] In co-culture experiments, estradiol induced neuroP in hypothalamic astrocytes, which in turn augmented kisspeptin release from mHypoA51 cells.[15] Src activation produced a similar release of kisspeptin from mHypoA51 cells, supporting the idea that neuroP is rapidly signaling through a PGR-Src complex located in the plasma membrane. Downstream, PGR-Src signaling activated a MAPK pathway and the release of intracellular stores of calcium.[14,15] The present study expanded the previous observations by demonstrating that estradiol increased the co-expression and proximity of PGR and Src in AVPV neurons providing evidence of potential interaction.

Our double-label immunohistochemistry revealed a population of AVPV neurons that co-express PGR and Src. As expected, estradiol priming increased the number of AVPV neurons that were PGR immunopositive. Although we counted neurons with positive nuclear PGR staining, it is likely that a subpopulation of PGR are trafficked to the membrane as seen with PGR in vitro[14,15] and nuclear estrogen receptors (e.g., ERα, ERβ[49,50]). Based on the in vitro results obtained in mHypoA51 cells, estradiol treatment increased levels of PGR determined by membrane biotinylation.[15] As expected, as a result of the many signaling functions mediated by Src, the number of Src immunopositive (416-NP) AVPV neurons was greater than the number of PGR immunopositive neurons and was not increased by estradiol priming. Thus, the increase in Src neurons that co-localize with PGR after estradiol treatment was a result of the induction of PGR. Analyses of AVPV PGR and Src immunostaining likely underrepresented total populations of PGR and Src because optimal antigen binding by antibodies is reduced by double-labeling immunohistochemistry techniques. However, our results clearly indicate the potential for PGR-Src interactions due to physical proximity and demonstrated a role for this interaction in positive feedback of the LH surge.

Although we have not formally demonstrated that the PGR-Src interactions exist within a specific AVPV neuronal population, it is very likely that an important population is the kisspeptin neurons. In vivo 40%–60% of RP3V kisspeptin neurons express PGR.[48,51] Mice with PGRs knocked out specifically in kisspeptin neurons (kissPRKOs) lack an LH surge, demonstrating that PGR expression in kisspeptin neurons is essential for induction of the LH surge.[16,18] Blocking progesterone synthesis inhibits estradiol induction of the LH surge.

However, subsequent infusion of progesterone or kisspeptin into the diagonal band of Broca rescues the LH surge, suggesting that RP3V kisspeptin signaling is downstream of neuroP signaling.[9] In vitro mHypoA51 neurons, a model of AVPV kisspeptin neurons, express Src and membrane-associated PGRs.[15] Furthermore, stimulation with either progesterone or PGR-specific agonist (R5020) induced rapid Src phosphorylation, Src induced MAPK pathway activation and Src mediated the release of kisspeptin.[15] Our results also indicated that estradiol increased the levels of PGR and Src in close proximity, suggesting that PGR-Src signaling capability is increased. Therefore, PGR-Src signaling is likely to be acting directly on AVPV kisspeptin neurons that mediate the neuroP triggering of the LH surge.

In previous studies, as well as in the present study, a subthreshold priming dose of estradiol (2 μg of EB) upregulated PGRs but did not stimulate neuroP synthesis or the LH surge.[9] However, an additional infusion of progesterone or kisspeptin into the hypothalamus induced the surge.[9] In the present study, we demonstrated that infusion of Src activator into the AVPV was sufficient to induce the LH surge in subthreshold estradiol-primed rats. The increase in LH concentration induced by Src activation in the AVPV was blocked by pretreating with Src inhibitor (PP2) or PGR antagonist (RU486). Similarly, the increased LH concentration after PGR activation (R5020) in AVPV neurons was blocked by pretreating with either PP2 or RU486. These results suggested that neuroP signaled through Src in kisspeptin neurons inducing kisspeptin release to trigger the LH surge. The ability of RU486 to block Src induction of the LH surge, as well as the ability of PP2 to block PGR induction of the LH surge, suggests that PGR and Src signaling is neither “in series” nor “in parallel”. If PGR and Src signaling pathways were serial, then the activation of the downstream signaling pathway would trigger an LH surge in the presence of an antagonist for PGR but not Src. If the PGR and Src signaling pathways were organized “in parallel”, we should be able to stimulate an LH surge with either a progesterone or Src when the other pathway is antagonized. Thus, neuroP signaling is not dependent on simultaneous activation of both PGR and Src, but both signaling pathways were needed to induce the LH surge. If either PGR or Src were bound to a respective antagonist, an LH surge could not be triggered by activating PGR or Src. These results suggest that PGR and Src are forming an interdependent signaling complex.23 Although we interpret the actions of the infused drugs to be acting on the AVPV neurons, infusions may extend outside the AVPV; thus, the drugs may be acting on PGR/Src expressing neurons that are lateral to the AVPV region (Figures 2–4).

Although Src activation significantly elevated LH concentrations, these concentrations were significantly lower than those induced by progesterone infusions into the AVPV. These results indicate that Src signaling is necessary for PGR induction of the LH surge. It is possible that infusing a higher dose of Src activator may have produced similar LH levels as progesterone. However, Src may not be the only mechanism through which PGR signals. Several explanations are possible: (1) PGR is simultaneously activating another signaling pathway. Results obtained in vitro have demonstrated that estradiol-induced membrane PGR mobilizes intracellular calcium stores.14 Currently, it is not clear whether Src is involved in that pathway. (2) NeuroP may activate other progesterone receptors. In vitro, and in vivo kisspeptin cells express mPR that are not related to classical PGRs.[15,52] Although these novel mPRs are not regulated by estradiol, they may provide an additional signaling pathway

that along with PGR to trigger the LH surge. Our use of a PGR agonist, R5020, to activate PGR and not mPR (Figure 6) indicated that PGR-Src signaling is sufficient to induce the LH surge, but did not eliminate or elucidate a role for mPR in triggering the neuroP induction of the LH surge. To decide which or both of these possibilities are extant will require further experiments.

The present results are congruent with previous results reported from one of our laboratories. PGR-Src signaling in the ARH rapidly facilitates sexual receptivity.[28] Sexual receptivity (lordosis behavior) induced by estradiol and progesterone was abrogated when Src was inhibited.[28] In the ARH, co-immunoprecipitation demonstrated the potential for PGR-Src complex formation.[28] This is similar to PGR-Src localization and signaling that induced the LH surge in the present set of experiments.

In summary, in our model for neuroP induction of the LH surge, initial estradiol priming induces an increase PGR expression in kisspeptin neurons, whereas higher doses of estradiol upregulate neuroP synthesis secreted from hypothalamic astrocytes (Figure 7). Estradiol further upregulates PGR-Src colocalization and their close proximity within the AVPV. During positive feedback, peaking levels of estradiol on proestrus induce the synthesis neuroP, which then signals through a PGR-Src pathway. The PGR-Src complexes are likely localized to AVPV kisspeptin neurons. When activated by neuroP, the PGR-Src complex induces kisspeptin release, activating GnRH neurons that triggers the LH surge. Together, these experiments are consistent with the hypothesis that a PGR-Src signaling complex in the AVPV is necessary to stimulate kisspeptin neurotransmission that triggers the LH surge, critical for ovulation and luteinization of follicular cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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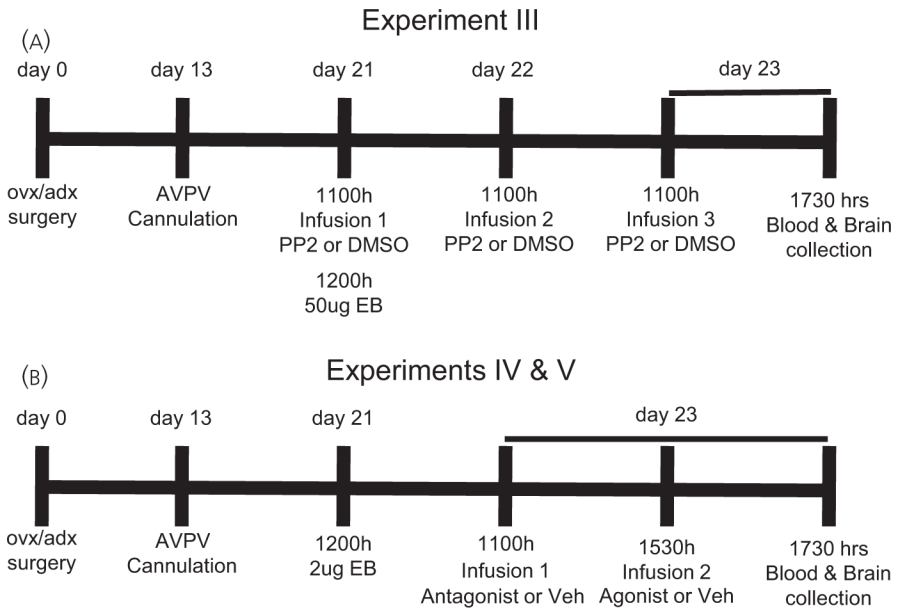
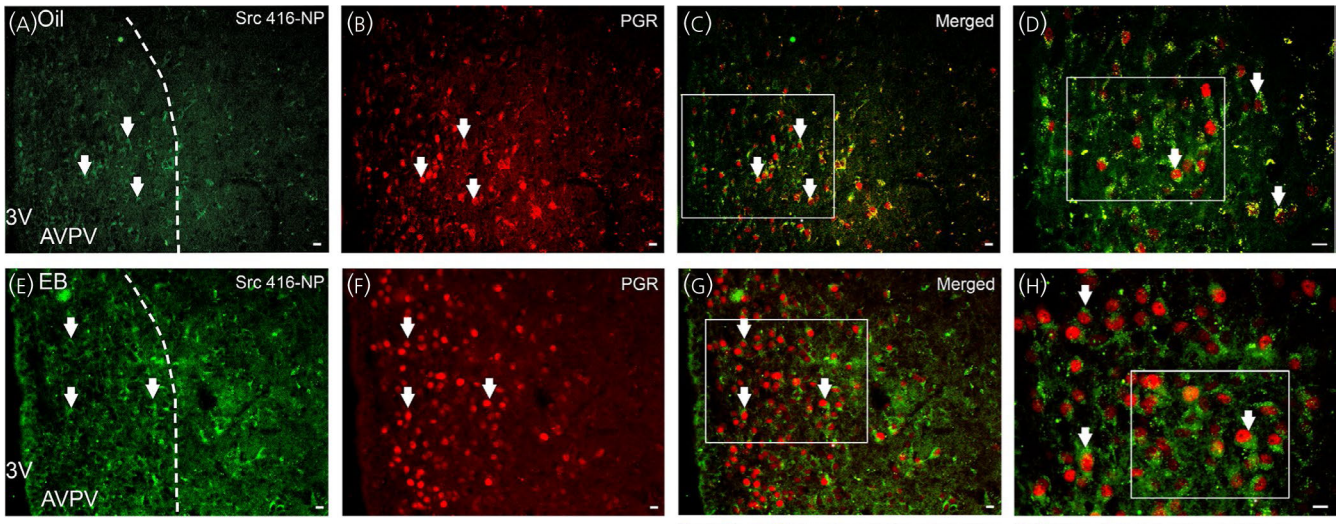


FIGURE 1.

Experimental methods timelines for experiments III to IV. Treatment days are above the line. Day 0 is the day of ovariectomy and adrenalectomy (ovx/adx) surgery. The timing of anteroventral periventricular nucleus (AVPV) cannulation surgery, s.c. 17β-estradiol benzoate (EB) treatment, AVPV drug infusions (infusion) and blood and brain collection are indicated below the line. Timelines (24-h clock) for Experiment III (A); Experiment IV and V (B) are colony time. DMSO, dimethyl sulfoxide; Veh, infusion vehicle



Steroid treatment	PGR	Src	Colocalized
Oil	11.5 ± 4.5	127.8 ± 9.7#	5.2 ± 2.3
2 μg EB	90.3 ± 23.0*	134.9 ± 14.9#	39.9 ± 8.7*

	%Src with PGR	%PGR with Src
Oil	4.1 ± 1.8	44.6 ± 11.1
2 μg EB	29.6 ± 6.1*	44.7 ± 4.6

FIGURE 2.

Estradiol increased the number immunopositive progesterone receptor (PGR) neurons and PGR-Src co-expressing neurons. Photomicrographs of immunohistochemical staining of classical PGR and Src 416-nonphosphorylated (NP) and their co-expression in the anteroventral periventricular nucleus (AVPV) of oil- and 17β-estradiol benzoate (EB)-treated animals. Src 416-NP (green: A and E) and PGR (red: B and F) immunopositive neurons are co-localized (examples indicated by arrows) at the level of the AVPV (insets in C and G are enlarged in D, H and insets in D and H are enlarged in I, and J, respectively). Dotted outline (A, D) indicates the AVPV region where cells were counted. TRITC labeled PGR positive immunoreactivity (red) was mainly localized in the nucleus of neurons. Fluorescein isothiocyanate (FITC) labeled Src positive immunoreactivity (green) was extranuclear and localized to the perikarya and extended to some neuronal processes. Arrowheads in (I) and (J) indicate Src 416-NP immunopositive neurons that do not express PGR. The number of immunopositive AVPV PGR neurons was significantly increased by EB treatment compared to oil, but the number of Src stained cells was not affected. The number of neurons that were immunopositive for both PGR and Src was increased significantly by EB treatment compared to oil treatment (Table). Furthermore, the percentage of PGR immunopositive cells that expressed Src remain unchanged, but EB treatment significantly increased the percentage of Src immunopositive cells that stained positive for PGR (Table). *Significantly greater than oil treatment within group ($p < .001$); #Significantly greater than PGR within treatments ($p < .001$). Scale bars = 10 μm. 3V, third ventricle

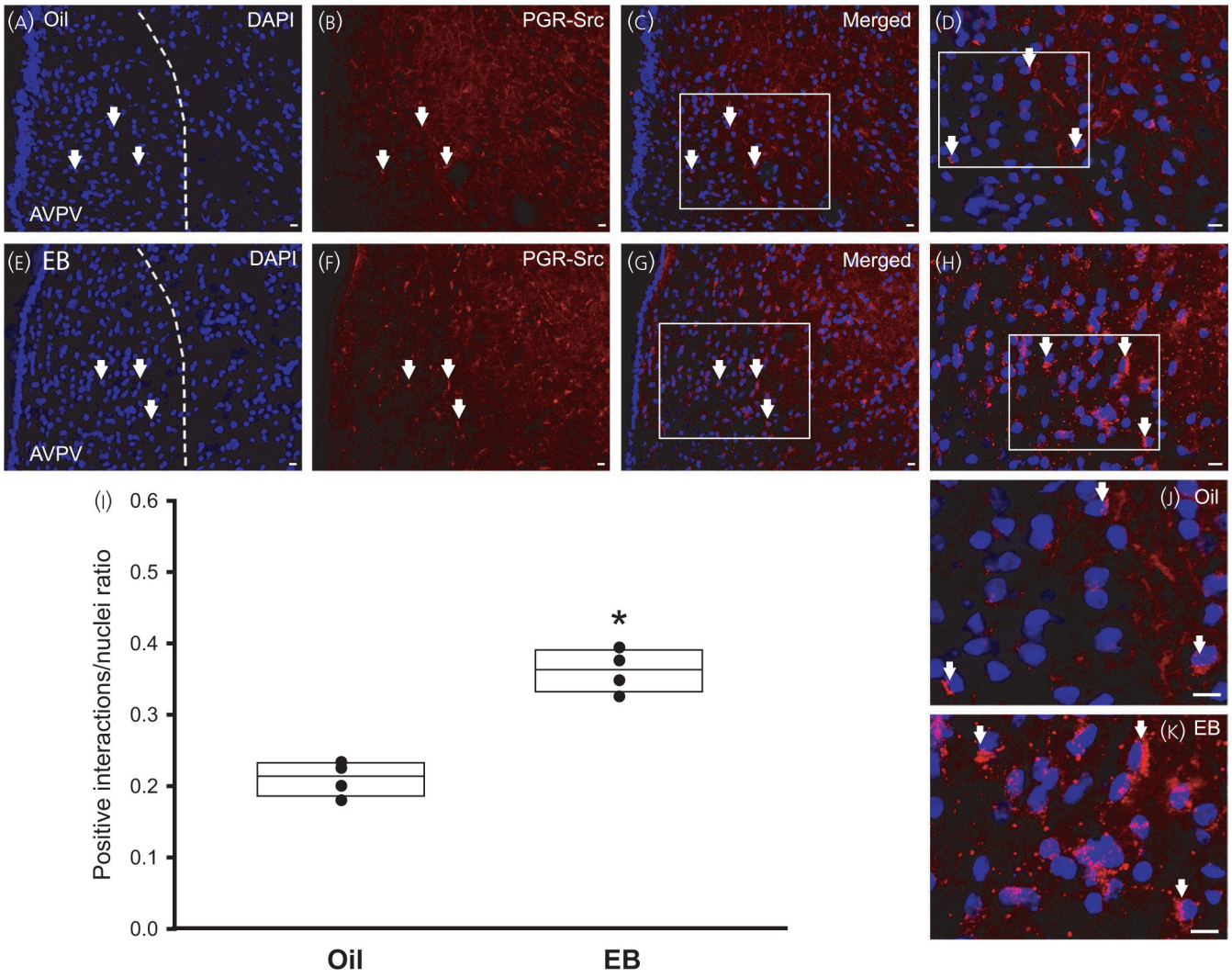


FIGURE 3.

Estradiol increased the number of anteroventral periventricular nucleus (AVPV) neurons with extranuclear progesterone receptor (PGR) and Src in close proximity. Photomicrographs of Duolink proximity ligation assay (PLA) of classical progesterone receptor (PGR) and Src Tyr417-NP (Src) interactions in the AVPV of oil (A–D and J) and 17β-estradiol benzoate (EB)-treated animals (E–H and K). Positive Duolink PLA interactions (red punctate staining) indicates PGR and Src within < 40 nm of each other at the level of the AVPV. Extranuclear PGR-Src PLA staining was associated with Hoechst-stained nuclei (blue; arrows). EB treatment (E–H and K) significantly increased the mean ratio of positive PLA staining for PGR-Src to Hoechst-stained nuclei (I) compared to oil-treated animals (A–D, J). *Significantly greater compared to the oil treatment group ($p <$ .05). Scale bars = 10 μm. DAPI, 4',6-diamidino-2-phenylindole

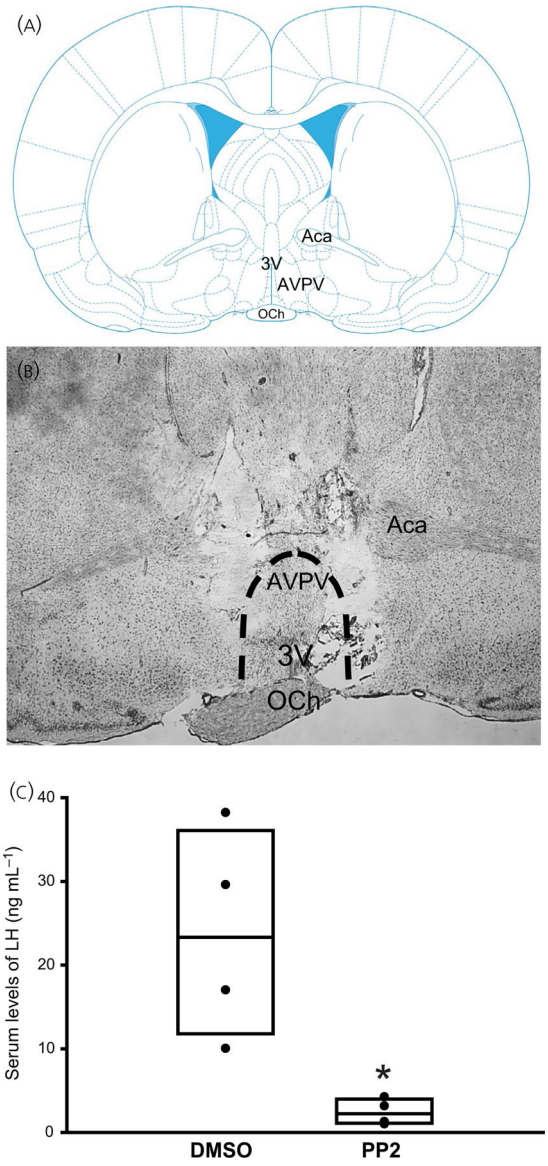


FIGURE 4.

Site-specific infusions inhibiting Src activation at the level of the anteroventral periventricular nucleus (AVPV) blocked the estradiol-induced lutenising hormone (LH) surge in ovariectomized/adrenalectomized (ovx/adx) rats. Adult ovx/adx Long-Evans rats were implanted with a bilateral guide cannula aimed at the AVPV region (A). Brains were coronally sectioned at 20 μm and thionin stained to visualize cannula tract placement and infusion site (B). All animals received an infusion into the AVPV of either dimethyl sulfoxide (DMSO) or Src antagonist (PP2) at 11:00 a.m., and then were treated with 50 μg of estradiol benzoate (EB) at 12:00 p.m. On the next 2 days, animals were infused daily with DMSO or PP2 (11:00 a.m.) and trunk blood was collected just prior to lights out in the colony on day 2 (Figure 1A, timeline). PP2 treatment significantly reduced serum LH concentrations compared to DMSO infused EB-treated ovx rats (C). *Significantly less than DMSO treatment ($p < .05$). 3V, third ventricle; Aca, anterior commissure; Och, optic chiasm

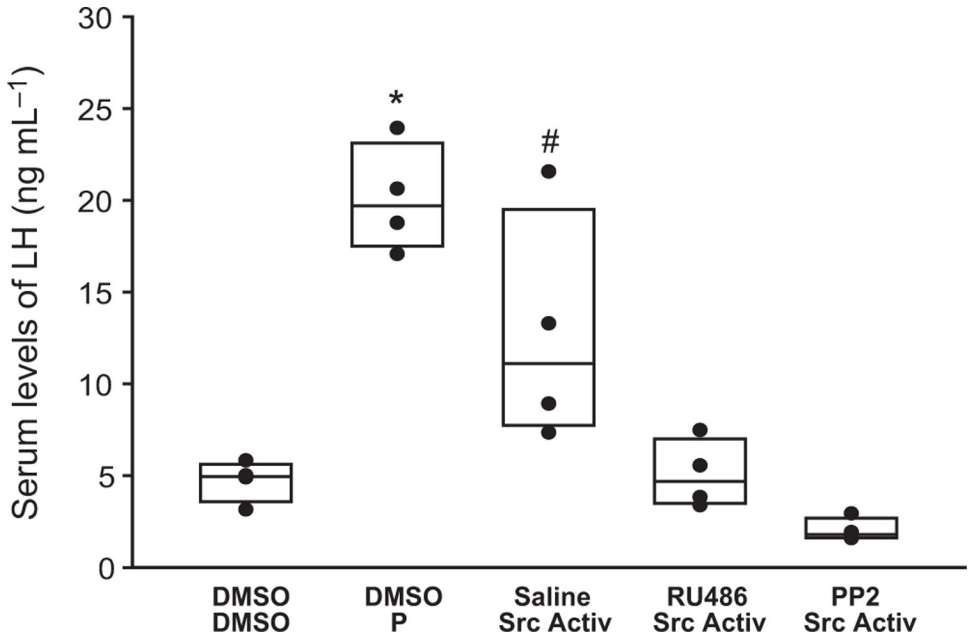


FIGURE 5.

Interdependence of Src signaling with progesterone receptor (PGR) to induce the lutenising hormone (LH) surge. All animals were treated with 2 μg of 17β-estradiol benzoate (EB) and received two sequential infusions. The first set of anteroventral periventricular nucleus (AVPV) infusions consisted of dimethyl sulfoxide (DMSO), saline, RU486 (PGR antagonist) or PP2 (Src inhibitor) administered 6.5 h prior to blood collection. The second infusion administered was either DMSO, progesterone (P) or Src activator (Src Activ) administered 2 h prior to blood collection. DMSO + P and Saline + Src Activ treated animals had significantly higher serum LH concentrations compared to DMSO + DMSO, PP2 + Src Activ and RU486 + Src Activ treated animals, and DMSO + P was significantly greater than Saline + Src Activ. *Significantly greater than all other treatment groups ($p <$ .001). #Significantly different from all other treatment groups ($p <$ .001)

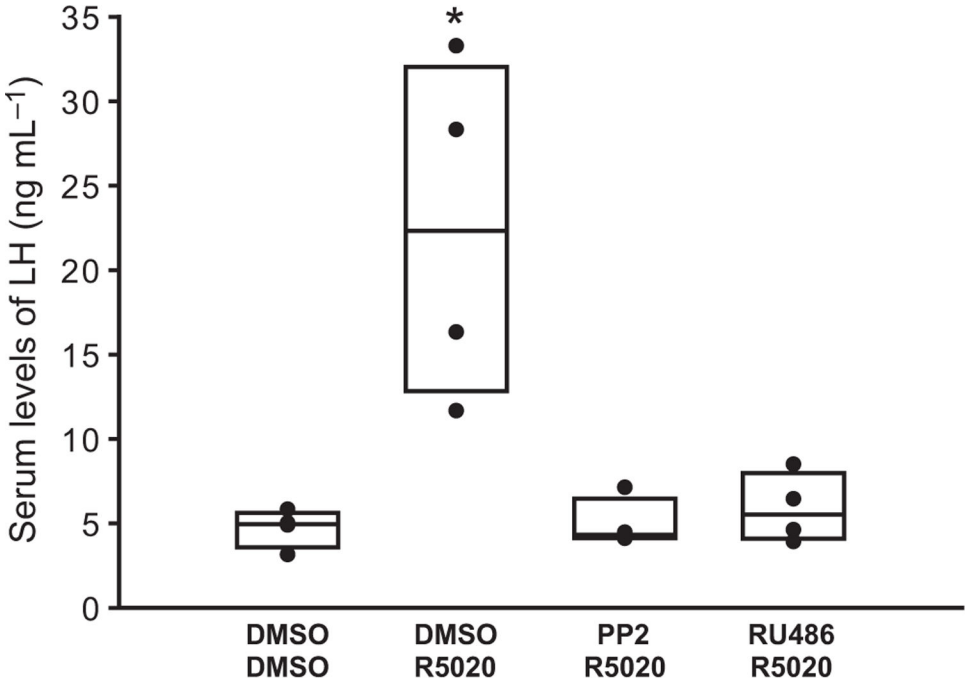


FIGURE 6.

Anteroventral periventricular nucleus (AVPV) progesterone receptor (PGR) mediate progesterone induction of the lutenising hormone (LH) surge via Src signaling. AVPV infusion of PGR specific agonist, R5020, induces the LH surge in 17β-estradiol benzoate (EB)-primed ovariectomized/ adrenalectomized (ovx/adx) rats. All animals were treated with 2 μg of EB and received two sequential AVPV infusions. The first set of AVPV infusions was either dimethyl sulfoxide (DMSO), PP2 (Src antagonist) or RU486 (PGR antagonist) 6.5 h prior to blood collection. The second infusion was either DMSO or R5020 administered 2 h prior to blood collection. EB-primed rats that received AVPV infusions of DMSO + R5020 had a significantly higher serum LH concentration compared to animals that received PP2 or RU486 treatment prior to R5020 and the DMSO + DMSO control animals. *Significantly greater than all other groups ($p < .05$)

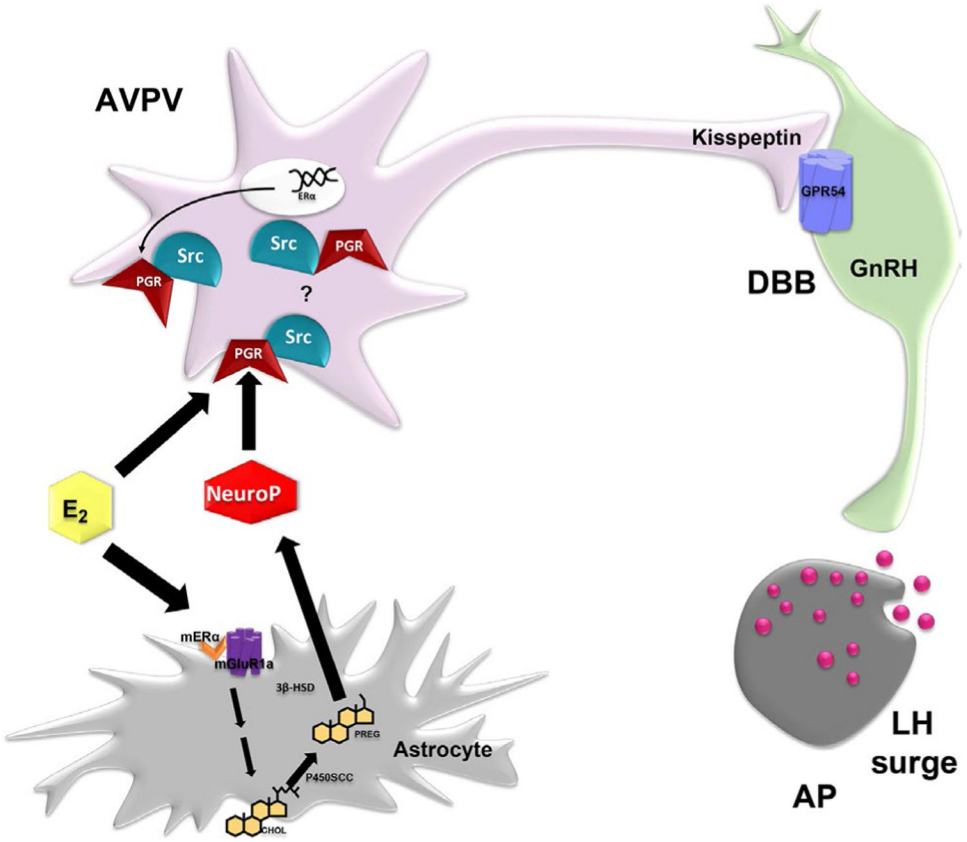


FIGURE 7.

Proposed circuit of estradiol-induced neuroP in anteroventral periventricular nucleus (AVPV) cells. Proestrous levels of estradiol (E_2) induce progesterone receptors (PGR) in neurons and stimulate membrane estrogen receptor-α (mERα) inducing neuroprogesterone (neuroP) synthesis in astrocytes. This neuroP binds to membrane PGR, which activates Src family kinase (Src) signaling in kisspeptin neurons to release kisspeptin. This kisspeptin stimulates gonadotropin releasing hormone (GnRH) neurons to trigger the luteinizing hormone (LH) surge release from the anterior pituitary (AP). The present results support the idea that PGR and Src signaling mediating the LH surge are an interdependent signaling complex. Antagonism of PGR or Src prevents either PGR- or Src-mediated induction of the LH surge. 3β-HSD, 3β hydroxysteroid dehydrogenase; CHOL, cholesterol; DBB, diagonal band of Broca; GPR54, kisspeptin receptor; mGluR1a, metabotropic glutamate receptor-1a type; P450scc, P450 side chain cleavage; PREG, pregnenolone. Modified from Micevych et al.[43]

TABLE 1

Primary Antibodies for immunohistochemical and Duolink Proximity Ligation Assay experiments

Antigen target	Species raised in	Dilution	Company	Catalog no.
PGR	Rabbit	1:2000	Cell Signaling	D8Q2J
Src 416-NP	Mouse	1:800,000	Cell Signaling	7G9

TABLE 2

Drugs administered

Steroid/drug	Action	Vehicle	Concentration	Company	Catalog no.
Src Activator	Src family agonist	Saline	50 nmol	Santa Cruz Biotechnology	SC-3052
Progesterone	Progesterone receptor agonist	DMSO	50 nmol	Sigma-Aldrich	P0130
R5020	PGR-specific agonist	DMSO	50 nmol	PerkinElmer	NLP004005MG
RU486	Progesterone receptor inhibitor	DMSO	50 nmol	Sigma-Aldrich	M8046
PP2	Src family inhibitor	DMSO	50 nmol	Tocris	1407