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Journal

Neuroendocrinology, 106(2)

ISSN

0028-3835

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Publication Date

2018

DOI

10.1159/000471878

Peer reviewed

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Author manuscript

Neuroendocrinology. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

Neuroendocrinology. 2018 ; 106(2): 101–115. doi:10.1159/000471878.

Estrogen and progesterone integration in an in vitro model of RP3V kisspeptin neurons

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Abstract

Positive feedback on gonadotropin release requires not only estrogen but also progesterone to activate neural circuits. In rodents, ovarian estradiol (E2) stimulates progesterone synthesis in hypothalamic astrocytes (neuroP), needed for the luteinizing hormone (LH) surge. Kisspeptin (kiss) neurons are the principal stimulators of gonadotropin releasing hormone neurons, and disruption of kiss signaling abrogates the LH surge. Similarly, blocking steroid synthesis in the hypothalamus or deleting classical progesterone receptor (PGR) selectively in kiss neurons prevents the LH surge. These results suggest a synergistic action of E2 and progesterone in kiss neurons to affect gonadotropin release. The mHypoA51, immortalized kiss-expressing neuronal cell line derived from adult female mice, is a tractable model for examining integration of steroid signaling underlying estrogen positive feedback. Here, we report that kiss neurons in vitro integrate E2 and progesterone signaling to increase levels of kiss translation and release. mHypoA51 neurons expressed non-classical membrane progesterone receptors (mPRα and mPRβ) and E2-inducible PGR, required for progesterone-augmentation of E2-induced kiss expression. With astrocyte-conditioned media or in mHypoA51-astrocyte co-culture, neuroP augmented stimulatory effects of E2 on kiss protein. Progesterone activation of classical, membrane-localized PGR led to activation of MAPK and Src kinases. Importantly, progesterone or Src activation induced release of kiss from E2-primed mHypoA51 neurons. Consistent with previous studies, the present results provide compelling evidence that the interaction of E2 and progesterone stimulates kiss expression and release. Further, these results demonstrate a mechanism though which peripheral E2 may prime kiss neurons to respond to neuroP, mediating estrogen positive feedback.

Key Terms

ERα. mPR; progesterone receptor; estrogen positive feedback; Src; MAPK

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Disclosure Statement: The authors have nothing to disclose.

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INTRODUCTION

The neuropeptide kisspeptin (kiss) is associated with reproductive functions such as puberty and estrogen feedback on pituitary release of luteinizing hormone (LH). Kiss-expressing cells in the arcuate nucleus (Kiss/Neurokinin B/Dynorphin, a.k.a. "KNDy" neurons) have been implicated in estrogen negative feedback [1,2], while kiss neurons in the rostroperiventricular continuum of the third ventricle (RP3V) mediate estrogenic effects on the LH surge (positive feedback; see [3] for review). Estradiol (E2) stimulates kiss mRNA in the RP3V [4], and kiss stimulates GnRH neurons [e.g., [5]; see [6] for review]. RP3V kiss neurons express ERα, the critical receptor for E2 positive feedback [7–9]. While GnRH neurons express ERβ [10,11], they do not express ERα. Finally, administration of E2 or kiss induces surge release of LH [12–14]. Thus, ERα-expressing RP3V kiss neurons are considered the site of E2 positive feedback onto GnRH neurons. Indeed, mounting electrophysiological evidence supports this role (see [15] for review).

Progesterone (P4) signaling is also a critical component of gonadotropin release. Replacement with E2 in ovariectomized (ovx) rats will induce LH release [16–18], but the LH surge is advanced and augmented by additional replacement with P4 [19–21]. Similarly, the rodent estrous cycle is dependent on P4, in particular, neuroprogesterone (neuroP), which is synthesized locally in hypothalamic astrocytes [22–26]. In gonadally intact rats, the estrous cycle is halted after central infusion of aminoglutethimide (AGT), which inhibits local production of neuroP [27]. AGT also blocks the E2-induced surge in ovx rats, indicating that local production of steroids is "downstream" of E2 signaling [14]. Genetic knockout of classical progesterone receptor (PGR) in kiss neurons attenuates the LH surge in ovx mice [28]. Thus, while there is increasing evidence that neuroP signaling in kiss neurons is important for gonadotropin release, the mechanism of action has not been elucidated. We hypothesize that RP3V kiss neurons are a direct target for neuroP and that integration of E2 and neuroP signaling in kiss neurons is a critical component of the LH surge.

We have characterized a line of kisspeptinergic mHypoA51 neurons, originally isolated and immortalized from adult, female mouse hypothalamus [29]. Cells were purchased commercially (CELLutions Biosystems, Burlingame, ON) and were originally immortalized by retroviral transfer of the SV40 T-Ag [see [30]]. We found that these neurons are an appropriate model to study steroid signaling in RP3V kiss neurons, based on their expression of ERα, PGR, and E2 induction of kiss. Importantly, these cells do not express neurokinin B (NKB), the hallmark of arcuate nucleus kiss neurons that are involved in estrogen negative feedback. Using the mHypoA51 neurons, we report here that astrocyte-derived neuroP augments kiss protein levels and release through both MAPK and Src, activated by classical PGR, which is trafficked to the cell membrane. These results demonstrate a possible mechanism through which ovarian E2 and neuroP signals may be integrated in kiss neurons to mediate estrogen positive feedback onto GnRH neurons. Here we show that astrocyte-derived neuroP augments kiss protein levels in mHypoA51 neurons, suggesting that RP3V kiss neurons may be the critical site of hypothalamic E2/P4 integration underlying the LH surge.

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METHODS

mHypoA51 cultures

mHypoA51 neurons obtained from CELLutions Biosystems (Burlington, ON) are derived from adult, female mouse hypothalamus. Characterization of these cells as a model for AVPV kisspeptin neurons is described in previously [29]. mHypoA51 neurons were thawed and maintained as previously described [29]. Briefly, cells stored in liquid nitrogen were thawed rapidly in a 37°C water bath and maintained in high glucose DMEM media (#11960, GIBCO/Invitrogen; Carlsbad, CA) with 10% qualified fetal bovine serum (FBS; #26140-079; Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (#15140122, Invitrogen). Cells were passaged at 90% confluence and used until the 10th passage.

Immunocytochemistry

mHypoA51 neurons were plated onto poly-D-lysine (PDL)-coated 8-well chambered glass slides (#PEZGS0816; Millipore; Billerica, MA) at a density of approximately 1,500 cells/well. Once cells had attached, slides were fixed in 4% paraformaldehyde (PFA) for 15 minutes, then rinsed in phosphate buffered saline (PBS) and blocked (2% normal goat serum [NGS], 1% bovine serum albumin [BSA] in PBS). Cells were exposed to primary antibody (mPRα or mPRβ) in blocking solution for 48 hours at 4°C. Following PBS rinses, cells were exposed to secondary antibodies (1:200; HRP-linked goat anti-rabbit or donkey anti-goat) in blocking solution for 1 hour. Signal was amplified using a tyramide signal amplification kit (PerkinElmer, Waltham, MA), using the manufacturer's recommended protocol. Slides were rinsed and coverslipped using Hardset medium containing DAPI (Vector Biolabs). Cells were imaged using a microscope (AxioPlan2, Zeiss; Pleasanton, CA) and associated software (AxioVision, Zeiss). Percentages of total cells (defined as containing DAPI signal) expressing either mPRα or mPRβ were quantified.

Astrocytes were plated onto PDL-coated 8-well chambered glass slides after isolation, growth, and purification (see methods for glial culture below). Cells were fixed with 4% PFA for 15 min, rinsed in PBS, and blocked in 3% NGS and 0.4% Triton X-100 in PBS. Cells were then exposed to glial fibrillary associated protein (GFAP) antibody (1:5,000) in blocking solution for 48 hours at 4°C. Following further PBS rinses, cells were exposed to secondary antibody (1:500 Alexa594 Goat anti-rabbit) in blocking solution for 4 hours. Slides were rinsed and nuclei were stained with DAPI (300 nM; #D1306; ThermoFisher; Waltham, MA) for 5 minutes, rinsed in ddH$_2$O, and cover slipped (Fluoromount G; #0100-01, SouthernBiotech; Birmingham, AL). Slides were imaged and quantified for percentage of total cells expressing GFAP immunoreactivity.

Surface Biotinylation

The use of biotinylation to isolate surface proteins has been previously described [31–33]. Briefly, mHypoA51 neurons were grown to near confluence in T75 flasks. Three flasks were combined per experimental treatment. Cells were steroid starved overnight in control media (see above), then treated for 24 hours with 1nM E2 or vehicle (0.1% DMSO). Cells were washed (5 mL/flask) in cold BuPBS and incubated in 0.5 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (ThermoFisher) in PBS for 30 min at 37°C with gentle agitation. Excess biotin

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reagent was quenched with 5 mL cold glycine buffer (50 mM glycine in PBS) and cells were harvested with cell lifters (Corning, New York). Flasks were scraped twice more and rinsed with cold PBS. Cells were centrifuged at 850 × g for 3 min at 4°C. Pellets were resuspended in RIPA lysis buffer containing protease inhibitors (Santa Cruz Biotechnology; Dallas, TX). Cells were homogenized by passage through a 25-gauge needle (10 passes every 10 min for 30 min) and kept on ice between passes. Samples were centrifuged at 14,000 rpm for 5 min at 4°C and supernatants were transferred to new tubes. Proteins (no more than 1500 μg/mL) were incubated with 200 μL of immobilized NeutrAvidin (ThermoFisher) in Pierce™ spin columns for 2 hours at room temperature and spun for 1 minute at 1000 × g. Beads were washed 4 times with RIPA buffer with protease inhibitors. Bound proteins were eluted with SDS-PAGE sample buffer supplemented with 50 μL Laemmli buffer (#1610737; BioRad) with β-mercaptoethanol (# BP176-100; Fisher Scientific) for 1 hour at 37°C. Proteins were electrophoresed as described below.

Primary hypothalamic astrocytes

Primary astrocytes were isolated from 60-day old female C57BL/6 mice (Charles River, Los Angeles, CA) as previously described [24]. Animals were anesthetized and the brains were removed and washed in ice cold HBSS. Whole hypothalami were dissected from brains using a brain matrix (Braintree, Braintree, MA) and microdissection knives (Fine Science Tools, Inc., Foster City, CA). Hypothalami were minced with razor blades and incubated in 9.25 mL PBS + 250 uL 2.5% trypsin without phenol red (Invitrogen) for 30 minutes at 37° C. Cells were gently agitated 3 times during incubation. During the incubation time, 1 mL sterile water was added to a vial of DNase (Sigma, #D4263), which was then kept on ice. Following the 30 min incubation, 500 μL of DNase was added to the conical tube containing the minced hypothalami, and the tissue was left at 37°C for an additional 10 minutes. The supernatant was removed and tissue was triturated in DMEM/F-12 media (Gibco) containing 10% FBS (Life Technologies) and 1% penicillin/streptomycin with L-glutamine. Cells were passed through a 100 μm cell strainer (ThermoFisher) and plated in T75 flasks, with additional FBS (final concentration 20%). Media was changed regularly and FBS was decreased to 10% when astrocytes were healthy and adherent. Prior to experiments, astrocytes were shaken at approximately 200 rpm for 4 hours to cause detachment of oligodendrocytes and non-astrocytic glia (modified from [34]). Immediately afterwards cells were trypsinized and replated into new flasks. Cultures prepared using this method were assessed for astrocyte content via quantification of GFAP immunoreactivity. Counts revealed a population of 98.1 ± 1.0% pure astrocytes (n = 3,343 cells counted; Supplemental Figure 2).

Drug and steroid treatments

All drugs and steroids are listed in Table 2. Concentrations used are based on previous *in vitro* experiments from our laboratory and others (references in Table 2). Src agonist (also published as “YEEI” was used at the minimum concentration that elicited pErk1/2 (1 μM). Cells were steroid starved in phenol red-free medium (# 17-205-CV, Corning) with 5% charcoal-stripped FBS (#100119; Gemini Bio-products; Sacramento, CA), and 1% penicillin/streptomycin with L-glutamine (#10378016; Invitrogen). This served as control

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medium and was used in all steroid treatments. Vehicle (DMSO or ethanol; EtOH) was added as appropriate for controls.

Co-culture

mHypoA51 cells were thawed and maintained as described above. mHypoA51 cells were plated onto 0.4 μm PET mesh inserts (#MCHT06H48; Millipore) suspended in 6-well culture plates (Fig 4A). Meanwhile, astrocytes were plated into separate 6-well plates to later be combined with mHypoA51 neurons. Both cell types were grown in their respective media for 1-2 days, to allow proper attachment of cells. Twenty-four hours prior to co-culture, mHypoA51 cells were treated with either steroid-free media (see above) or media with 1 nM E2. Astrocytes were steroid-starved overnight (approx. 18 hours). Media, with appropriate drugs, was applied to the wells containing astrocytes. Mesh inserts of mHypoA51 neurons were transferred into the astrocyte wells. Additional treatment media was added to the apical surface of the inserts to ensure exposure for both cell types. This co-culture setup allows transfer of media through the insert pores, while the 1 mm distance between the bottom of the inserts and the 6-well plate precludes direct cell-cell contact. Two hours after the combination of cell types, mHypoA51 cells were removed via trypsinization from the inserts, lysed in RIPA lysis buffer, and processed for western blotting (see below).

3βHSD1 knockdown in astrocytes

A combination of four siRNAs (gene accession NM_008293; FlexiTube siRNA #SI01070811, SI01070818, SI01070825, and SI01070832; Qiagen, Valencia, CA) with Lipofectimine RNAiMAX (#13778150; Life Technologies) was used to knock down 3βHSD1, according to the manufacturer's protocol with OptiMEM reduced serum media (#31985070; Life Technologies). Optimal siRNA concentrations were determined in pilot experiments (Fig. 3B, C). For subsequent experiments, siRNA was added (75 pmol or 33 nM per well of 6-well plate, equivalent to 18.75 pmol or 8.3 nM of each of the four siRNAs). Because estradiol can induce 3βHSD1 and steroidogenic activity [35,36] the cells were treated with E2 to maximize any potential 3βHSD1 activity. Briefly, 72 hours after the start of transfection, astrocytes were steroid-starved and treated with 1 nM E2 for 4 hours. Media was then collected and immediately used in conditioned media experiments or kept at −20°C until use.

Groups were analyzed using SigmaPlot for Windows version 12.5 (Systat Software, Inc., San Jose, CA). Where appropriate, Student-Newman-Keuls (SNK) post hoc tests were used (significance set to 0.05).

PCR

PCR was carried out as described previously, using the SYBR Green (Life Technologies) system [35]. First, total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's protocol. RNA quality and quantity was detected on a spectrophotometer (NanoDrop ND 1000; ThermoFisher). Complementary DNA (cDNA) was made with 1.5 μg RNA and the Superscript III First Strand Synthesis System (Life Technologies). PCR reactions were set up in duplicate, in 20 μL volumes, using SYBR GreenER (Life Technologies) and 2 μL cDNA. Primer sequences and concentrations along with annealing

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temperatures are listed in Table 2. Dissociation curves were assessed after each PCR assay and amplicons were electrophoresed on a 2% agarose gel to ensure a single amplified product at the predicted amplicon size. Fold changes in gene products of interest were calculated using the well-established 2^-ddCt method.

Western Blotting

Whole cell homogenates were obtained for all targets, except PGR, using the following protocol. Following treatments, cells were scraped in cold HBSS and centrifuged. Pellets were resuspended in RIPA lysis buffer containing a protease inhibitor cocktail, 1 mM sodium orthovanadate, and 2 mM PMSF (RIPA lysis buffer system, #sc-24948, Santa Cruz Biotechnology). Protein concentrations were assessed using the BCA method (Pierce) and a NanoDrop spectrometer. 25 μg total protein was loaded per lane of a 10-lane 10% tris/glycine gel (NuPage). Samples along with a ladder (#1610374; BioRad) were electrophoresed then transferred onto PVDF membranes using the iBlot2 rapid transfer system (#IB21001; ThermoFisher). Membranes were incubated in blocking buffer (5% non-fat milk in tris-buffered saline with 0.1% Tween-20, TBS-T) for 1 hour and then exposed to primary antibody overnight. Primary antibodies were diluted in 5% milk blocking buffer or 5% BSA in 0.1M TBS-T, per the recommendation of each antibody's manufacturer. Blots were analyzed as described previously [29]. Briefly, band intensities were compared using background subtraction and normalized to a housekeeping gene (GAPDH). This analysis was done with AlphaView Software (Protein Simple; San Jose, CA).

Measurement of Kiss Release

mHypoA51 neurons were plated in 100 mm plates and grown to approximately 90% confluency. For experiments, cells were rinsed in HBSS and treated with 5 mL control media without serum. Media contained 1 nM E2 or vehicle control (0.1% DMSO) and the following protease inhibitors (all from Sigma; dissolved in water unless otherwise indicated): Aprotinin (0.5 μM), Bestatin (60 nM), Leupeptin (10 μM), and Pepstatin A (1.5 μM in EtOH). These protease inhibitors did not affect cell viability as assessed with a Trypan Blue Exclusion Assay and cells appeared healthy upon visual inspection. After a 24-hour treatment with E2 or vehicle, cells were exposed to 1 nM P4, 10 nM R5020 (classical PGR agonist, PerkinElmer), or 1 μM Src activator (denoted as "Src"; #sc-3052; Santa Cruz Biotechnology) for 4 hours. Media was collected and immediately stored at −20°C. Samples were then concentrated using a SpeedVac. A mouse kiss ELISA kit (EKU05505; Biomatik, Wilmington, DE) was used to quantify kiss concentrations. The kit sensitivity was reported as 4.66 pg/mL and the intra-assay and inter-assay variability is <10 and <12, respectively (reported as % of coefficient of variation, see product manual). The ELISA was performed using the manufacturer's instructions. Samples and standards were run in duplicate.

Progesterone Assay

Previous studies have documented the release of neuroP from E2-treated hypothalamic astrocytes [e.g.,[36]]. To measure neuroP, we used an ELISA kit (# ADI-900-011; Enzo Life Sciences). The reported kit sensitivity is 8.57 pg/mL and the intra-assay and inter-assay variability is 7.6 and 6.8, respectively (reported as % of coefficient of variation, see product manual). Astrocytes were steroid-starved overnight in serum-free control medium. Media

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was aspirated and replaced with fresh 5 mL serum-free medium containing 1 nM E2 or 0.1% DMSO (vehicle). Four hours later, the astrocyte-conditioned media was collected, extracted with diethyl ether, desiccated, reconstituted in assay buffer and immediately assayed according to the ELISA kit manufacturer's protocol. A sample of known progesterone concentration was extracted and run in parallel with unknown samples to verify the efficacy of extraction.

Statistical Analyses

All data were analyzed using one- or two-way ANOVAs with SNK post-hoc tests where appropriate, unless otherwise indicated. Significance was set to p 0.05. All statistics were performed with SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA).

RESULTS

mHypoA51 neurons express membrane progesterone receptors (mPRα, mPRβ, and membrane-localized PGR)

Previously, we demonstrated expression of PGR in mHypoA51 neurons, with immunocytochemistry and PCR [29]. While PGR is found predominantly in the nucleus, there is precedent for trafficking of this receptor to the membrane [see [37]]. Here, membrane proteins labeled and purified by surface biotinylation confirmed presence of PGR in cell membrane fractions of mHypoA51 neurons (Supplemental Figure 1). In addition to PGR, a number of membrane progestin receptors (mPRs) have been characterized (e.g., [38]). These receptors, also called PAQRs (progestin and adipoQ receptors) are 7 transmembrane domain proteins associated with rapid intracellular signaling. We now report that mHypoA51 neurons express the membrane progesterone receptors, mPRα and mPRβ, as assayed by PCR and immunohistochemistry (Fig. 1A). mPRα was expressed in 95.17 ± 1.7 % of mHypoA51 neurons while, 86.21 ± 3.9% expressed mPRβ. PCR using primers for mPRα and mPRβ yielded a product which exhibited a single dissociation (melting) peak (at 86.5°C and 85.5°C, respectively) and resulting amplicon bands were at expected sizes (284 bp and 217 bp, respectively) when run on a 2% agarose gel. We previously reported that PGR is upregulated by a 24-hour 1 nM E2 treatment [29]. Here, we tested whether non-classical membrane progesterone receptors mPRα and mPRβ were modulated by the same E2 treatment. No changes were seen in mRNA levels for either mPRα ($t_{10} = -0.250$; $p = 0.8$) or mPRβ ($t_9 = 1.07$; $p = 0.3$) in response to E2 treatment. As a positive control, kiss and PGR mRNA were assayed in the same samples and were found to increase with E2 treatment as previously reported [data not shown; see [29]].

Progesterone signals through the non-receptor tyrosine kinase, Src

Membrane-localized PGR has been reported to act though activation of Src to activate cell signaling [39,40]. In order to determine whether this signaling pathway was active in mHypoA51 cultures, we first assayed mHypoA51 neurons for expression of Src. Src immunocytochemistry revealed that 96.5% of the neurons were Src positive (2,374/2,460 cells; Fig. 2A). Pharmacological stimulation of Src caused phosphorylation of Erk1/2 within 5 min (228.1 ± 53.8% compared to 100.1 ± 4.6% in controls; $t_{13} = -2.205$; $p < 0.05$; one-tailed t-test; Fig. 2B), suggesting that Src is upstream of Erk1/2 in these cells.

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Progesterone induces phosphorylation of Erk1/2 via activation of non-nuclear classical progesterone receptor (PGR)

In mHypoA51 neurons pre-treated with E2, we compared the effects of P4 stimulation with and without 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2), a Src inhibitor. There were significant overall effects of both P4 ($F_{1,12} = 20.487$; $p < 0.001$) and PP2 ($F_{1,12} = 73.755$; $p < 0.001$) as well as an effect of interaction ($F_{1,12} = 18.312$; $p < 0.01$). In E2-primed mHypoA51 neurons, P4 stimulation elicited a marked increase in both p44/42 Erk1/2 phosphorylation to 304.4 ± 45.0% of control ($p < 0.001$; Fig. 2C). Progesterone-induced MAPK phosphorylation was abrogated following PP2 treatment (5.7 ± 1.4% of control for PP2 + P4; $p < 0.001$ compared to P4; Fig. 2C). Because non-classical mPRs have also been associated with Erk1/2 phosphorylation, we used the PGR-specific agonist R5020 to distinguish PGR from mPR signaling. Again, overall effects of R5020 ($F_{1,19} = 6.906$; $p < 0.05$) and PP2 ($F_{1,19} = 15.068$; $p < 0.01$) were observed. In mHypoA51 neurons, a 5 min exposure to R5020 (10 nM) induced Erk1/2 phosphorylation (217.3 ± 29.0% of control; $p = 0.001$; Fig. 2D), which, similar to P4 stimulation, was abrogated by pretreatment with Src inhibitor PP2 (R5020 treatment and PP2 treatment: $p = 0.795$; Fig. 2D). There was no effect of PP2 in non-R5020 treated cells ($p = 0.269$).

NeuroP augments E2-induced kiss mRNA and protein

We have previously characterized the E2-induced upregulation of kiss mRNA in mHypoA51 neurons [29]. In order to test whether P4 could have an additional stimulatory effect, mHypoA51 neurons were treated with P4 following a 24-hour exposure to E2. We compared these treatments to pretreatments with vehicle control or STX, a ligand for a novel membrane estrogen receptor. An overall effect of pretreatment was observed (two-way ANOVA; $F_{2,38} = 12.708$; $p < 0.001$). E2 induced kisspeptin mRNA, as expected (3.07 ± 0.79-fold increase from controls; $p < 0.05$; Fig 3A). Following this 24-hour E2 pre-treatment, P4 (1 nM) caused a marked increase in kisspeptin mRNA (5.06 ± 1.2-fold increase from controls; $p < 0.05$). However, P4 had no effect on kisspeptin mRNA following vehicle or STX pretreatment ($p = 0.991$ and 0.718, respectively).

As we previously demonstrated, E2 stimulated the synthesis and release of progesterone (neuroP) in cultured primary hypothalamic astrocytes isolated from adult, female rat and mouse [24,26,36]. In order to test whether this neuroP would also augment the stimulatory effects of E2 on kiss levels, we knocked down 3βHSD1, the enzyme that converts pregnenalone to progesterone by transfecting astrocytes with siRNA directed toward HSD3B1 mRNA (75 pmol). PCR using specific primers (see Table 3) directed towards the gene product of HSD3B1 revealed an 80% knockdown of mRNA (Fig. 3B). Amplified product exhibited a single peak on dissociation (83°C) and a single band at the expected amplicon size (250 bp). 3βHSD1 protein was knocked down by 62.2 ± 16.1%, as assayed by western blot (Fig. 3C). Media from E2-treated astrocytes in which 3βHSD1 was knocked down had a 67.1% reduction in neuroP compared to media from neg siRNA-treated astrocytes (P4 in neg siRNA-treated astrocyte media: 56.3 ± 7.1 pg/ml vs. 18.5 ± 2.8 pg/ml in media from 3βHSD1 knock-down; $t_7 = -4.944$; $p < 0.01$; Fig 3D). Media from astrocytes transfected with negative control siRNA were comparable to levels previously reported in non-transfected, E2-treated astrocytes [previously reported as 62.6 ± 7 pg/mL; see [36]].

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Astrocyte-derived neuroP augments E2-induced kiss

Next, to determine whether 3βHSD knockdown in astrocytes (i.e., loss of neuroP) affected kiss mRNA, 1 nM E2-treated mHypoA51 cells were exposed to media from 3 groups of astrocytes. The first were transfected with control siRNA and then incubated with vehicle (DMSO) or 1 nM E2 for 4 hours. E2 (1 nM) was added to the vehicle-treated negative siRNA media before exposure to mHypoA51 neurons. This was done so that all mHypoA51 neurons were exposed to E2 and could be compared in the presence or absence of a single variable: neuroP. The third group of astrocytes was transfected with siRNA targeted towards 3βHSD and treated with E2. There was an overall effect of astrocyte media condition on mHypoA51 kiss mRNA levels (one-way ANOVA; $F_{2,30} = 5.332$; $p = 0.01$). Exposure of mHypoA51 neurons to E2-treated astrocyte media caused a significant increase in kiss mRNA (4.18 ± 0.8-fold; $p < 0.01$) versus exposure to vehicle-treated astrocyte media (1.32 ± 0.4-fold; Fig. 3E). Knockdown of 3βHSD1 in astrocytes significantly reduced kiss mRNA to levels that did not differ from the control siRNA + vehicle group (2.3 ± 0.6-fold; $p < 0.05$ compared to neg siRNA + E2 group; $p = 0.276$ compared to control siRNA + vehicle group).

To test whether hypothalamic astrocytes stimulate kiss, mHypoA51 neurons were co-cultured with primary hypothalamic astrocytes (see Fig. 4A for experimental timeline). The baseline condition (set as 100%) was defined as mHypoA51 cells incubated for 24 hours with steroid-free media and then treated with 1 nM E2 during the 2-hour co-culture. In the second condition, mHypoA51 neurons were treated for 24 hours with E2, but not the co-culture (no expected induction of neuroP). Kiss levels were not altered by the 24-hour pre-treatment (110 ± 34.7%; $p = 0.872$; Fig. 4B). However, when mHypoA51 neurons were stimulated with E2 before and during the co-culture with astrocytes, kiss levels increased to 262.2 ± 69.7% of baseline levels in 2 hours ($F_{2,13} = 4.106$; $p < 0.05$, different from E2 pre-treatment/no E2 in co-culture condition [second bar]; Fig. 4B). To determine whether this effect was due to release of neuroP from astrocytes, kiss protein was assayed in the presence of 10 μM aminoglutethimide (AGT; a P450scc inhibitor that blocks pregnenolone synthesis). Astrocytes were steroid starved overnight and treated with AGT for 1 hour prior to co-culture. Significant treatment effects of both E2 ($F_{1,7} = 11.627$; $p < 0.05$) and AGT ($F_{1,7} = 5.768$; $p < 0.05$) were observed. Presence of E2 in co-culture again led to an increase in kiss levels in mHypoA51 neurons (247.9 ± 42.5% compared to controls; $p < 0.01$). AGT abrogated the E2 stimulation of kiss in mHypoA51/astrocyte co-culture, decreasing kiss protein to baseline levels (135.4 ± 25.1% compared to control levels; $p < 0.05$ E2 vs. E2+AGT; Fig. 4C).

Progesterone and Src stimulation cause release of kiss

We had hypothesized that P4 would augment E2-induced kiss protein levels and its release. Media from mHypoA51 neurons was assayed for kiss content to assess release. An overall effect of treatment was observed ($F_{4,37} = 6.558$; $p < 0.01$). A 24-hour treatment with 1 nM E2 (24 hours) did not increase levels of kiss release (in media), however subsequent treatment with 1 nM P4 (4 hours) increased kiss levels to 212.6 ± 34.8% of controls (vs. 103.3 ± 9.8% in E2-only-treated cells; $p < 0.01$; Fig. 5). Similarly, 1 μM Src agonist significantly increased kiss release (195.7 ± 13.6% compared to E2-only treated cells; p <

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0.01). Stimulation with PGR-selective agonist R5020 elicited a non-significant increase in kiss release (151.2 ± 23.1% compared to E2-only-treated cells; p = 0.12).

DISCUSSION

We have previously characterized mHypoA51 neurons, derived from adult female mouse hypothalamus, as a model for anterior hypothalamic (RP3V) kisspeptin neurons [29]. Estradiol has two distinct modes of signaling in mHypoA51 neurons: E2 membrane-initiated signaling, which induces kisspeptin expression, and nuclear E2 signaling, which up-regulates PGR [29]. Here, we investigate the role of astrocyte-derived neuroP signaling in immortalized kiss neurons, within the hypothesis that neuroP augments E2 effects on levels of protein and release of kiss. Understanding steroid signaling in anterior hypothalamic kiss neurons is critical, as these neurons are implicated in the induction of the surge release of GnRH underlying estrogen positive feedback of LH release in female mice.

Using immunocytochemistry, PCR, and surface biotinylation, we report that mHypoA51 neurons express multiple P4 receptors on the cell membrane. These include classical, "nuclear" PGR, translocated to the membrane, as well as the novel mPRs, mPRα and mPRβ from the progestin and adipoQ receptor (PAQR) family of receptors (reviewed in [38,41,42]. The mPRs were initially characterized in fish ovaries but have subsequently been found in the reproductive and nervous systems of a number of mammalian species including humans. While mPRs are not classic G-protein coupled receptors, they contain 7 transmembrane domains and have been shown to signal rapidly through intracellular pathways involving effectors such as MAPK and PKC [see [43] for review]. Unlike PGR, mPRs are expressed only at the cell membrane. The presence of multiple membrane receptors for P4 suggests that rapid, membrane-initiated P4 signaling occurs in kiss neurons. While relatively little is known about PGR signaling at the level of the membrane in the hypothalamus, this signaling pathway appears critical in ovulation and reproduction. PGR has similar palmitoylation sequences to those of ERα [37], which has been demonstrated on the membrane of multiple cell types. Our goal was to determine whether activation of PGR in immortalized kiss neurons was consistent with numerous studies showing that PGR is needed for female reproduction and the LH surge. Both global and cell-specific PGR knockdowns cause complete infertility in rodents. [28,44]. Specifically, loss of PGR expression in kiss neurons disrupts puberty, LH surges, and fertility [28,45]. However, the expression of mPRs in mHypoA51 (kiss) neurons suggests that there may also be a role for these receptors in reproduction. Indeed, these receptors have been implicated in female reproductive behavior [e.g., [46,47]]. However, since mPR expression in immortalized kiss neurons was not modulated by E2, their putative role in reproduction appeared independent of E2. The selective PGR agonist, R5020, did not significantly increase kiss release, suggesting that neuroP may require mPRs for neurotransmitter action of kiss.

To begin examining the cell signaling underlying P4 action in mHypoA51 neurons, we examined Src (a non-receptor tyrosine kinase associated with rapid intracellular signaling cascades) as a mediator of membrane steroid signaling. This kinase has been demonstrated to directly associate with PGR in the cell membrane *in vitro* (human breast cancer cells) [40,48,49] and *in vivo* ([50,51]). Nearly all (96.5%) mHypoA51 neurons express Src. In

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humans, PGR has a proline rich "PXXPXR" motif, which can directly bind to the SH2 domain of Src [40]. Mouse and rat PGR also contain two repeats of this motif, at a.a. 288 and 238. This suggests that a similar PGR-Src interaction could occur in the rodent. Mouse mPRα and mPRβ do not contain these motifs, though this does not exclude the possibility that signaling through these receptors could activate Src indirectly.

Stimulation of this kiss cell line with either P4 or Src activator caused a significant increase in kiss release *in vitro*, suggesting that rapid P4/Src signaling may be functionally important in the RP3V kiss circuit underlying ovulation. Stimulation with P4 or direct pharmacological stimulation of Src in mHypoA51 kiss cells resulted in rapid activation of Erk1/2. P4- and R5020-induced phosphorylation of Src at tyrosine 416 is consistent with the idea of a PGR/Src interaction. Blocking Src activity with PP2 prevented Erk1/2 phosphorylation by P4. These results indicate that Src is necessary and sufficient for activation of Erk1/2, and that for P4 signaling, Src acts upstream of Erk1/2in these kiss cells. To differentiate action via PGR from potential mPR activation, we used R5020, which, at concentrations less than 10 μM, is a PGR-selective agonist [52,53]. R5020 (10 nM) mimicked P4 stimulation by inducing Erk1/2 phosphorylation, which was blocked by the Src inhibitor, PP2. Activation of Src also led to kiss release (Fig. 5), indicating that this effector may also play a physiological role in kiss signaling underlying the LH surge.

While the main source of circulating P4 in rodents is from the luteinized follicles in the ovaries, neuroP is produced locally within the hypothalamus, and this steroidogenesis is critical for the estrous cycle and the LH surge. Gonadally intact, cycling rats given third ventricular infusions of aminoglutethimide (AGT), a CYP11A inhibitor, exhibited arrested estrous cycles, a powerful demonstration that locally synthesized steroids are critical for reproduction [22,27]. It is important to note that in these studies, plasma levels of estradiol were unchanged, indicating that peripheral steroidogenesis was unaffected. Concurrently, hypothalamic levels of neuroP were dramatically reduced. Astrocytes have been demonstrated to be the source of this neuroP [26,54–56], responding to E2 within seconds to mobilize intracellular calcium (Ca^{2+}) and rapidly synthesize and release neuroP.

We demonstrate that P4 augments the stimulatory E2 effect on kiss mRNA. Importantly, this only occurs when cells have been pre-treated with E2 for 24 hours (Fig. 3A). This pretreatment upregulates PGR expression, which appears to be a necessary precursor to P4 action in kiss cells, facilitating the increase of intracellular free Ca^{2+} levels, and stimulation of kiss mRNA [29]. Here, we show that E2 treatment did not modulate mPRα or mPRβ expression levels, suggesting that these non-classical receptors may be constitutively active and do not require E2-induction. Instead, they may cooperate with PGR to induce kiss release. Furthermore, P4 did not affect kiss expression when pre-treated with vehicle or STX, a ligand for a novel membrane receptor [57]. These results are consistent with the idea that ERα activation is necessary for the expression of PGR that mediates neuroP actions on kiss expression and release.

The hypothesis that neuroP plays a stimulatory role in kiss signaling was tested in two ways: i) exposing mHypoA51 neurons to astrocyte-conditioned media, and ii) co-culturing astrocytes and mHypoA51 kiss neurons where the cells shared media without direct contact.

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This second method allowed for two-way cell interactions and enabled us to isolate and analyze each cell type individually. In each of these assays, we reduced neuroP synthesis in astrocytes using siRNA or pharmacological blockade.

Previously, we demonstrated that E2-induced kiss expression required a 24- to 48-hour treatment. In the current experiments, a comparatively short E2 treatment (2-hour) of the mHypoA51/astrocyte co-culture was used. Under these conditions E2-induced neuroP did not facilitate an increase in kiss protein levels in mHypoA51 neurons unless they were pre-treated with E2 (Fig. 4B). The necessity for E2 pre-treatment was underscored by the lack of responsiveness of kiss mRNA levels (Fig. 3A). The inability of P4 to affect kiss message or protein without E2 pre-treatment is likely due to the low basal levels of PGR in mHypoA51 neurons. It also suggests that mPRs themselves are incapable of inducing kiss expression. A 24-hour pre-treatment with E2 induces PGR in mHypoA51 neurons, mirroring *in vivo* data [58–60]. The presence of astrocytes caused a marked increase in kiss levels, suggesting a stimulatory effect of neuroP in mHypoA51 neurons. When AGT was used to block steroidogenesis in co-cultured astrocytes, the stimulatory effect of E2-stimulated astrocytes was diminished, demonstrating that astrocyte synthesis of neuroP is critical for further upregulation of kiss. Moreover, the present results are consistent with preliminary studies *in vivo* where AGT arrests the estrus cycle [27] and blocks the E2-induced LH surge in ovx/adrenalectomized rats [i.e., rats without peripheral sources of P4; [13,14]]. The AGT-blocked LH surge was restored by ventricular infusion of P4, or kiss infusions into site of GnRH neurons, the diagonal band of Broca (DBB). Kiss infusions were effective in eliciting an LH surge, even in the absence of peripheral P4, indicating that E2-induced synthesis of neuroP activates kiss release to induce the LH surge.

Our results support the idea that kiss neurons are the site of E2/neuroP integration underlying the LH surge. Kiss cells express receptors for both E2 and P4 *in vivo* and *in vitro*, and P4 augments the expression and release of kiss. In animal models, additional replacement with P4 augments the E2-elicited LH surge, and blocking hypothalamic synthesis of P4 completely prevents the E2-induced surge. Moreover, knockdown of PGR in kiss cells abrogates reproduction by disrupting the LH surge [28]. Here, we show that P4 facilitated the response of in vitro kiss neurons to E2 effects on kiss expression. Furthermore, co-culture and media sharing assays indicated that astrocyte-derived neuroP is sufficient to augment E2 signaling in immortalized kiss neurons. Thus, results demonstrate E2 induction of PGR at the membrane, and the P4 activation of Src and MAPK signaling that lead to kiss release provide a mechanistic explanation of steroid integration at the level of kiss cells – mediators of estrogen positive feedback.

While these data are from *in vitro* experiments, they (along with our previous publication characterizing mHypoA51 neurons [29]) indicate that these the mHypoA51 cells model *in vivo* RP3V kiss neurons. The rapid timeline of Src and Erk1/2 phosphorylation is more difficult to examine in the whole animal, however it will be interesting to determine whether these cell signaling molecules also mediate P4 effects on the LH surge *in vivo*.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors wish to thank Alexandra K. Scott and Lauren A. Dinh for technical assistance with experiments.

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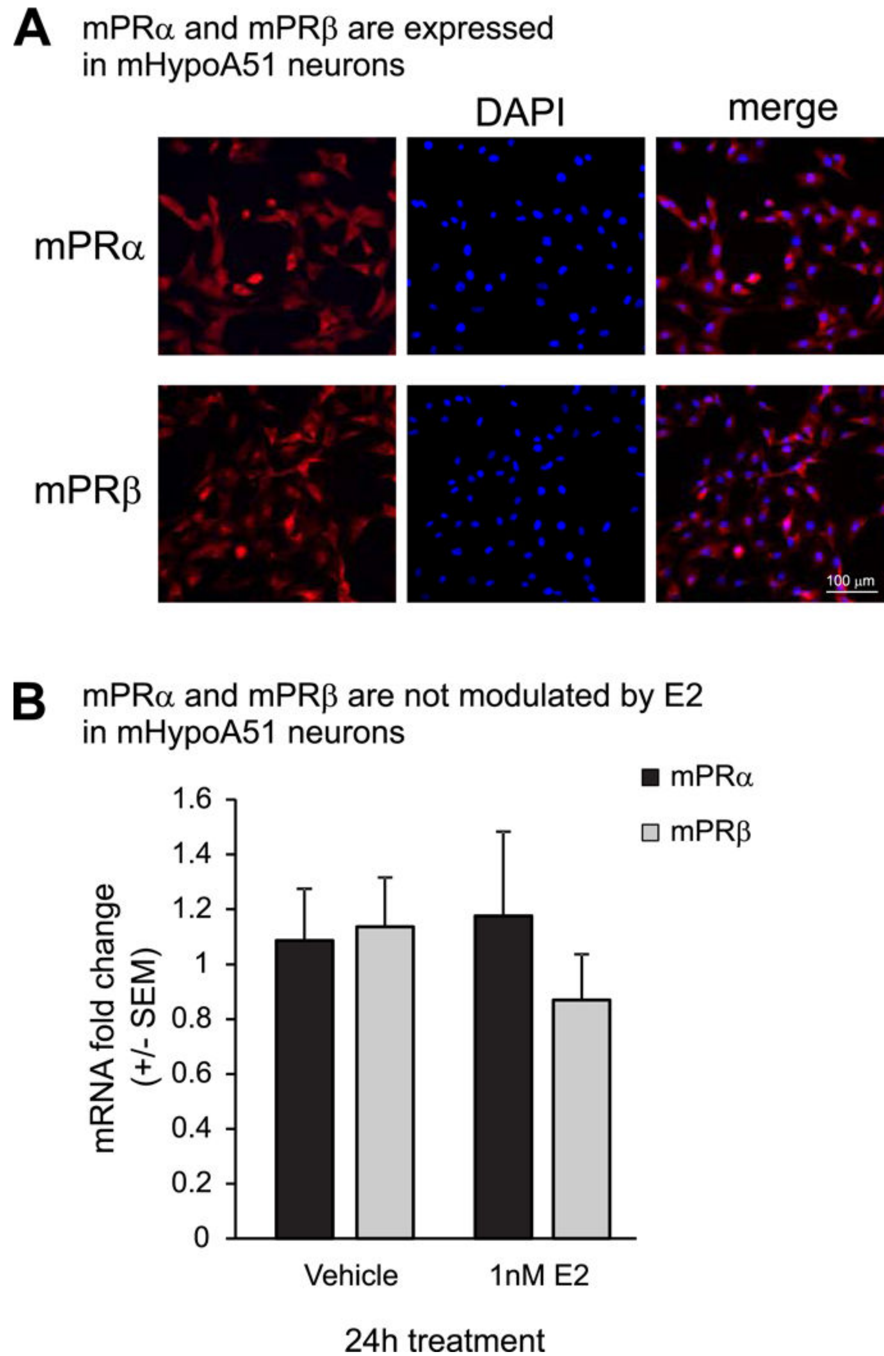

Figure 1.

Non-classical progesterone receptors are expressed on the membrane of mHypoA51 kiss neurons as assayed by immunocytochemistry and PCR (antibody and PCR primary information: see Tables 1 and 3). A. mPRα and mPRβ are expressed in mHypoA51 neurons. Scale bar (100 μm) applies to all images. B. E2 treatment does not affect mPRα and mPRβ mRNA levels in mHypoA51 neurons (one-tailed t-test; $p = 0.8$ for mPRα; $p = 0.3$ for mPRβ).

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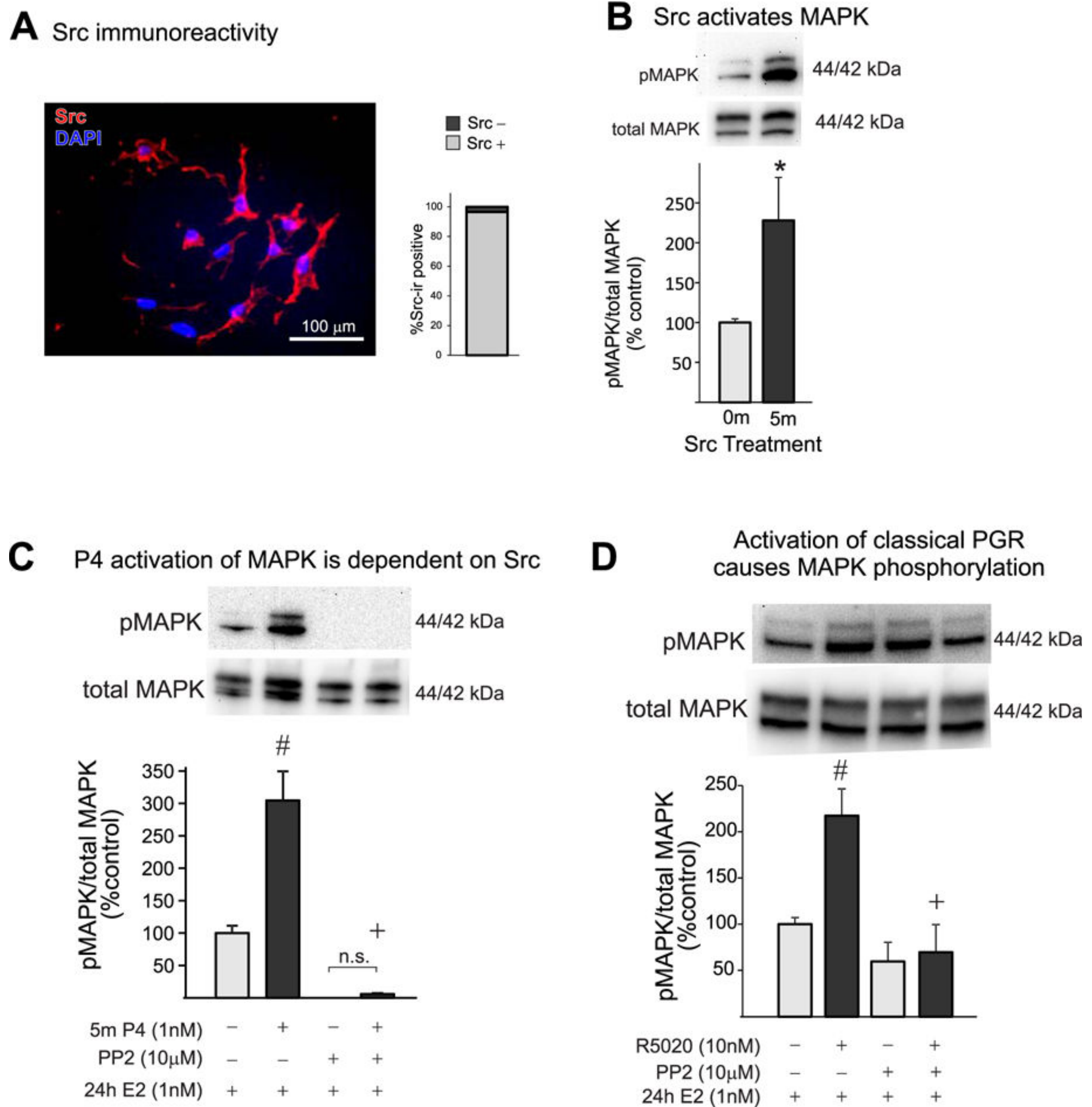

Figure 2.

Rapid progesterone (P4), Src, and Erk1/2 signaling in mHypoA51 kiss neurons. A. Representative image of mHypoA51 kiss neurons showing Src-immunoreactivity, and related quantification of Src-expressing mHypoA51 neurons (96.4%). B. Activation of Src caused an increase in phosphorylated Erk1/2 (pErk1/2) (228.1 ± 53.8% of control levels; $p < 0.05$). C. P4 treatment caused a significant increase in pErk1/2 levels (304.4 ± 45.0% vs. controls, n = 7-8/group). This effect was abrogated following a pre-incubation with PP2, a Src inhibitor. D. A PGR-selective agonist (R5020, 10 nM) caused a significant increase in pErk1/2 ($p = 0.001$). This effect was prevented by a pre-treatment with Src inhibitor PP2 ($p = 0.795$).

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For B-D: * $p < 0.05$, significantly different compared to control; # p 0.001, significantly different from E2 only; + p 0.001, significantly different from E2 + P4 or E2 + R5020 (PP2 effect).

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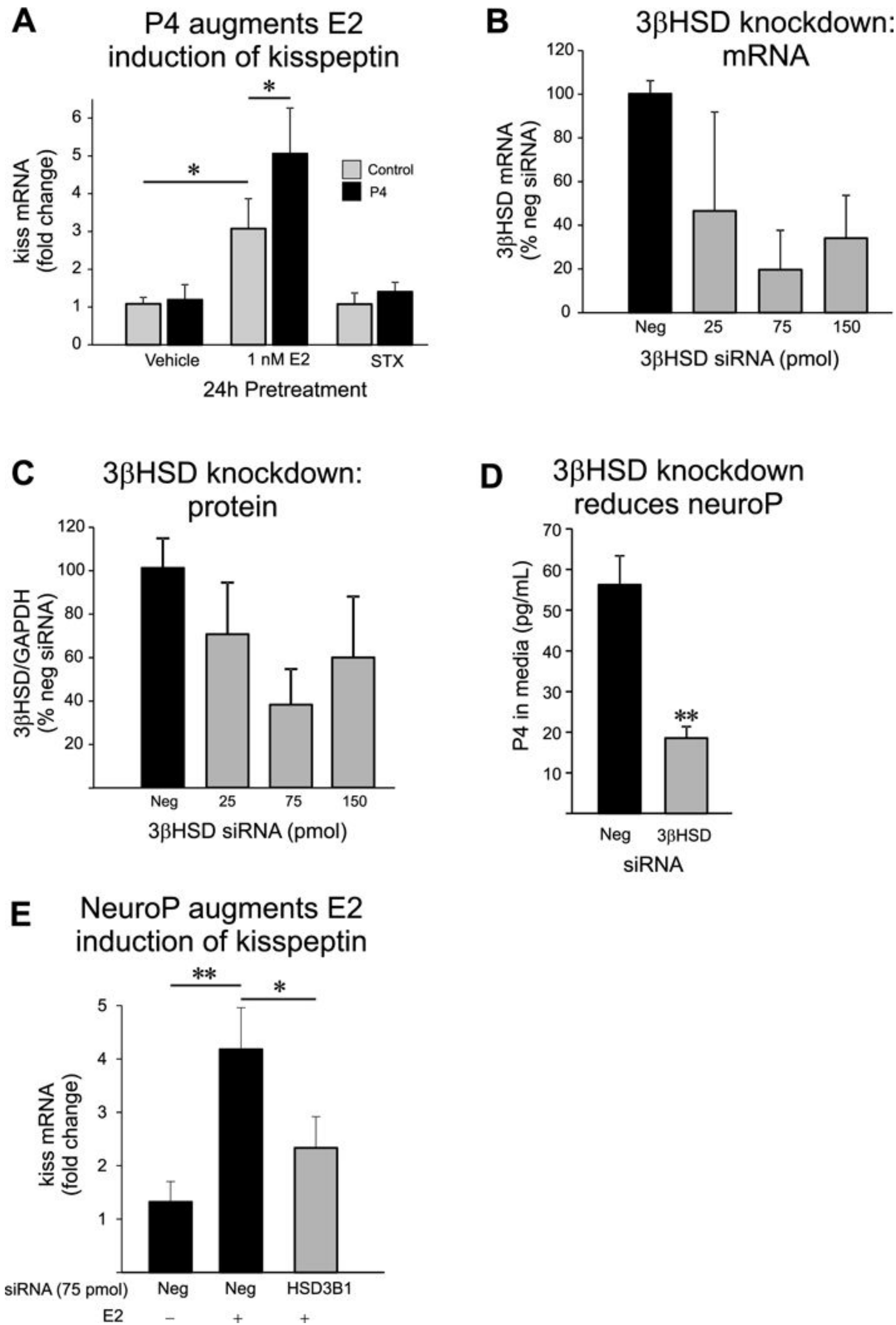

Figure 3.

E2-induced kiss mRNA is augmented by neuroP. A. mHypoA51 neurons were pre-treated for 24 hours with either vehicle (DMSO), 1 nM E2, or 1 nM STX, a ligand for a novel membrane estrogen receptor. Progesterone (P4, 10 nM) or vehicle (H_2O) was then added to cells for an additional 24 hours. P4 augmented the E2-induced increase in kiss mRNA (5.06 ± 1.2-fold increase from controls versus E2 alone: 3.07 ± 0.79-fold increase from controls; $p < 0.05$ P4 treatment within E2 pre-treatment). P4 treatment did not affect kiss mRNA following pre-treatment with either vehicle or STX (n = 4/group). B, C. Dose-response

curves of transfection of primary astrocytes with siRNA directed towards HSD3B1 (encoding 3βHSD1, the enzyme that converts pregnenolone to progesterone). 75 pmol 3βHSD siRNA resulted in an 80% reduction in 3βHSD mRNA and a 60% reduction in 3βHSD protein. All further knockdown experiments were conducted using this dose. (preliminary data; $n = 2$). D. 3βHSD knockdown reduced E2-induced neuroP production from 56.3 ± 7.1 pg/mL to 18.6 ± 2.8 pg/mL, compared to negative siRNA control ($p < 0.01$, one-or two-tailed t-test; $n = 4$/group). E. mHypoA51 cells pre-treated with 1 nM E2 and then exposed to astrocyte-conditioned media. E2, control siRNA treated astrocyte conditioned media increased kiss mRNA (4.18 ± 0.8-fold versus 1.32 ± 0.38-fold in kiss cells exposed to media from non-E2 treated astrocytes). E2 was added to neg siRNA + vehicle media after exposure to astrocytes to control for E2 exposure in mHypoA51 neurons. Knockdown of 3βHSD prevented E2-induced kiss increase (kiss mRNA: 2.33 ± 0.6-fold increase from control siRNA group; $p < 0.05$ compared to control siRNA E2 group). ** $p < 0.01$; * $p < 0.05$

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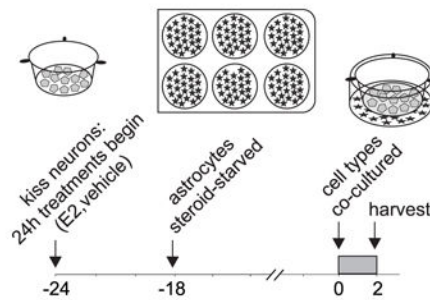

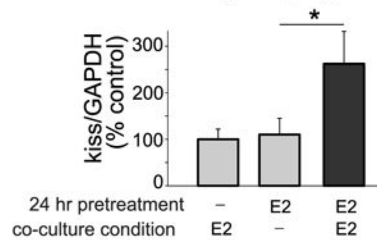

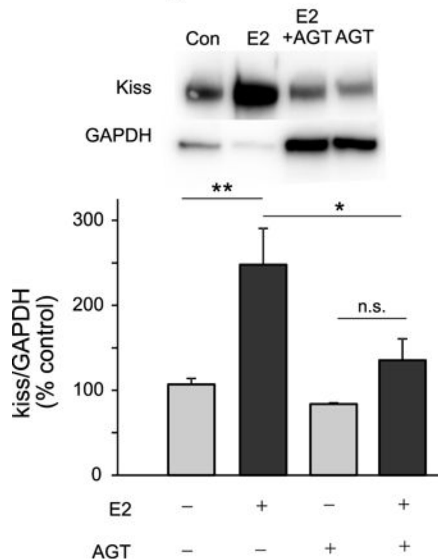

Figure 4.

Co-culture of mHypoA51 kiss neurons and primary hypothalamic astrocytes. A. Schematic illustrating the timeline of co-culture experiments. B. Kiss neurons pre-treated with 1 nM E2 or vehicle (0.1% DMSO) for 24 hours were added to astrocyte cultures. Co-cultures were treated with 1 nM E2 or vehicle. Kiss cells that were pre-treated with E2 and co-cultured with E2 exhibited increased kiss protein levels (262.2 ± 69.7% compared to control levels; $p < 0.05$, significantly different from E2-pretreatment, no E2 in co-culture; one-way ANOVA). Kiss cells that were not pre-treated with E2 or co-cultured without E2 had low levels of kiss

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protein. C. Steroidogenesis in astrocytes is necessary for induction of kiss protein. All kiss cells were pre-treated with 1 nM E2 for 24 hours. Astrocytes were pretreated for 1 hour with aminoglutethimide (AGT; 10 μM) or vehicle (0.1% DMSO) and co-cultures had E2 or vehicle (0.1% DMSO). Presence of E2 in the co-culture increased kiss protein (247.9 ± 42.5% compared to no E2 co-culture condition). AGT pre-treatment of astrocytes decreased kiss levels to 135.4 ± 25.1% of no E2 co-culture condition ($p < 0.05$). E2 had no effect in the presence of AGT ($p = 0.259$). Significance determined by two-way ANOVA (n = 3 experiments). * $p < 0.05$; ** $p < 0.01$

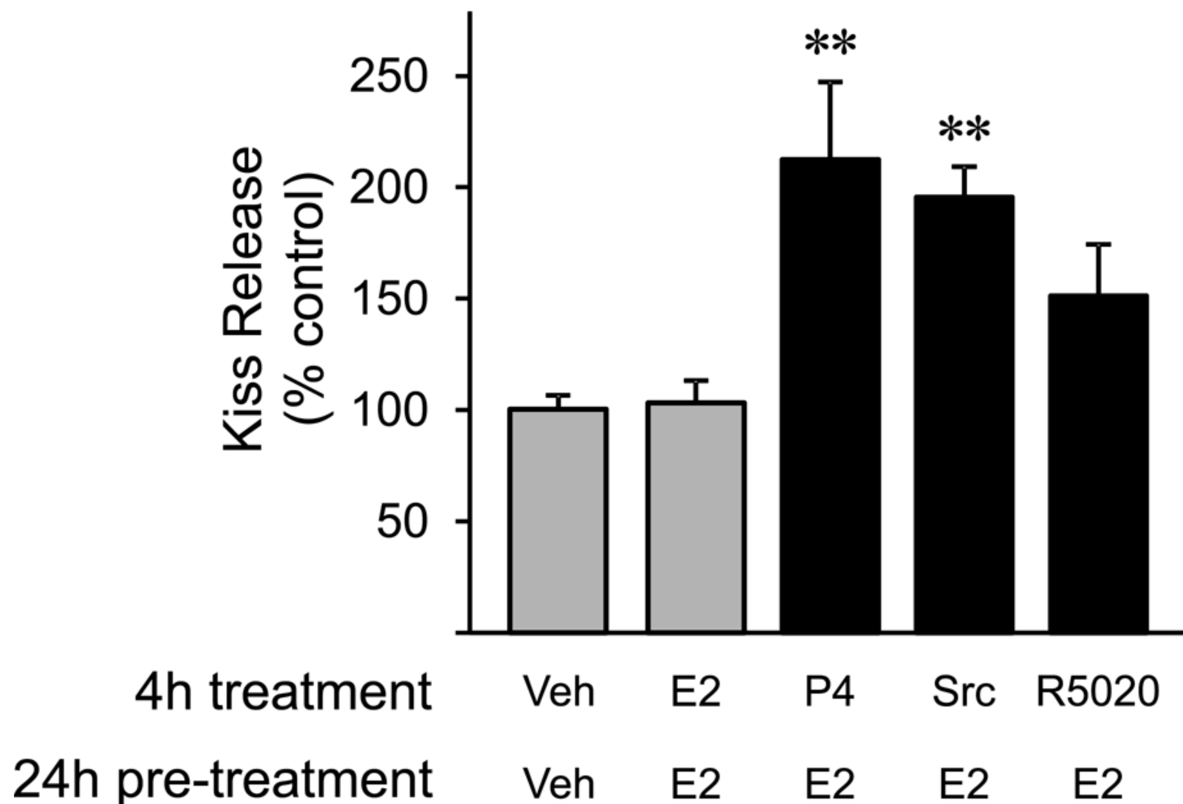

Figure 5.

Kiss release in vitro. A 24-hour E2 treatment did not alter media kiss concentration ($p = 0.912$). P4 (1 nM) following E2 pre-treatment significantly increased media kiss levels ($212.6 \pm 34.8\%$ versus vehicle control; $p < 0.01$ significantly different from E2 alone). Similarly, Src significantly increased kiss release ($195.7 \pm 13.6\%$; $p < 0.01$, with respect to E2 alone; one-way ANOVA with SNK post hoc). There was a non-significant trend towards an increase in kiss release after exposure to the PGR selective agonist, R5020 ($151.2 \pm 23.1\%$ versus vehicle control; $p = 0.12$ with respect to E2 alone). ** $p < 0.01$ significantly different from E2 treatment alone

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Table 1

Antibody Table

Peptide/protein target	Antigen sequence	Name of Antibody	Manufacturer, catalog #	Species raised in; monoclonal or polyclonal	Dilution used
Src (WB)	Recombinant protein, residues 1–110 of human Src	32G6	Cell Signaling, 2123	Rabbit; monoclonal	1:1,000
3βHSD1	Not specified	EPR9686	Abcam, ab150384	Rabbit; monoclonal	1:500
Phospho-Src (WB)	Synthetic phosphopeptide corresponding to residues surrounding Tyr419 of human Src	D49G4	Cell Signaling, 6943	Rabbit; monoclonal	1:1,000
Src (ICC)	Synthetic peptide corresponding to residues at the C terminus of human Src	EG107	Abcam, ab32102	Rabbit; monoclonal	1:1,000
PGR	Synthetic peptide corresponding to residues 533–547 of human PR (GLPQVYPPYLNYLRP)	PR-AT 4.14	GeneTex	Mouse; monoclonal	1:1,000
pMAPK			Cell Signaling,	Rabbit; polyclonal	1:1,000
Total MAPK			Cell Signaling,	Rabbit; polyclonal	1:1,000
mPRα	Amino acids 1–75 of the N-terminus of mouse mPRα	M-75	Santa Cruz, sc-134816	Rabbit; poloyclonal	1:1,000
mPRβ	Peptide mapping near N-terminus of human mPRβ	T-15	Santa Cruz, sc-50110	Goat; polyclonal	1;1,000
GFAP	GFAP isolated from cow spinal cord	Z 0334	Dako; Z0334	Rabbit; polyclonal	1:5,000
GAPDH	Rabbit glyceraldehyde-3-phosphate dehydrogenase	6C5	Millipore, MAB374	Mouse; monoclonal	1:10,000
Flotillin-1	Synthetic peptide derived from within residues 1 – 100 of Human Flotillin 1	ab41927	Abcam, ab41927	Rabbit; polyclonal	1:1,000

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Table 2

Steroids and Drugs

Steroid/Drug	Action	Company, Cat #	Vehicle	Concentration	Reference(s)
Estradiol	Estrogen Receptor Agonist	Sigma, E8875	DMSO	1 nM	[32,36,54,61]
Progesterone (cyclodextrin encapsulated)	Progesterone Receptor Agonist	Sigma, P7556	Water	1 nM	[29]
R5020	PR agonist	PerkinElmer, NLP004005MG	DMSO	10 nM	[53,62,63]
Src	Src family activator	Santa Cruz, sc-3052	Water	1 μM	[64,65]
PP2	Src inhibitor	Tocris, 1407	DMSO	10 μM	[66]
AGT	P450scc/CYP11A1 inhibitor	Sigma, A9657	DMSO	10 μM	[67]

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Table 3

PCR Primers

Target	FW Primer	RV Primer	Primer Concentration (nM)	Annealing, Extension Temp (°C)	Reference
mPRα (284 bp)	GGC CTC CTT CAC CTA CCT CT	CAG ACC CGG CTT CTG GCT GT	400	56, 72	[68]
mPRβ (217 bp)	GTG GCT CTC TGC CAC CTG GC	CAG CTG GGA GAG CGT GCA GA	400	56, 72	[68]
3βHSD1 (250 bp)	TGG ACA AGG TAT TCC GAC CAG A	GGC ACA CTT GCT TGA ACA CAG	200	57, 72	[69]
Kisspeptin (132 bp)	TGC TGC TTC TCC TCT GT	ACC GCG ATT CCT TTT CC	500	60	[70]
GAPDH (150 bp)	GCA CAG TCA AGG CCG AGA AT	GCC TTC TCC ATG GTG GTG AA	200	57	[29,71]