Aromatase promoter I.f is regulated by progesterone receptor in mouse hypothalamic neuronal cell lines

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Abstract

Aromatase catalyzes the conversion of C_{19} steroids to estrogens. Aromatase and progesterone, both of which function at different steps of steroidogenesis, are crucial for the sexually dimorphic development of the fetal brain and the regulation of gonadotropin secretion and sexual interest in adults. The aromatase gene (*Cyp19a1*) is selectively expressed in distinct neurons of the mouse hypothalamus through a distal brain-specific promoter, I.f, located ~40 kb upstream of the coding region. However, the regulation of aromatase expression in the brain is not well understood. In this study, we investigated a short feedback effect of progesterone analogues on aromatase mRNA expression and enzyme activity in estrogen receptor α (Esr1)-positive or -negative mouse embryonic hypothalamic neuronal cell lines that express aromatase via promoter I.f. In a hypothalamic neuronal cell line that highly expresses aromatase, progesterone receptor (Pgr), and Esr1, a progesterone agonist, R5020, inhibited aromatase mRNA level and enzyme activity. The inhibitory effect of R5020 was reversed by its antagonist, RU486. Deletion mutants of promoter I.f suggested that inhibition of aromatase expression by progesterone is conferred by the nt −1000/−500 region, and R5020 enhanced binding of Pgr to the nt −800/−600 region of promoter I.f. Small interfering RNA knockdown of *Pgr* eliminated progesterone-dependent inhibition of aromatase mRNA and enzyme activity. Taken together, progesterone enhances recruitment of Pgr to specific regions of the promoter I.f of *Cyp19a1* and regulates aromatase expression in hypothalamic neurons.

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Introduction

Sex steroids influence most aspects of cellular organization and mammalian development. They play important roles in shaping neural functions and reproductive behavior throughout all stages of life. One of the major sex steroids is progesterone, which has a wide spectrum of functions in many organs, including the brain. Progesterone is primarily synthesized by the corpus luteum in females and by the testes and adrenal cortex in males, but it can also be locally synthesized by other tissues such as the brain (Keefe 2002, Wagner 2006).

Besides its well-established organizational and activational effects on reproductive neuroendocrine function and recovery after brain injury, progesterone also exerts a wide variety of actions on regions of the developing and adult brain that influence higher cognitive functions, pain mechanisms, fine motor skills, susceptibility to seizures, mood, temperature regulation, and sleep (Levine *et al.* 2001, Pluchino *et al.* 2006, Wagner 2006). Estrogen biosynthesis is dependent on the entry of cholesterol into mitochondria, followed by six enzymatic steps. Aromatase is the key enzyme that catalyzes the final step, the conversion of C_{19} steroids to biologically active estrogen, estradiol (E_2; Simpson & Davis 2001, Bulun *et al.* 2005).

A single-copy gene (*Cyp19a1*) encodes aromatase, the inhibition of which effectively eliminates estrogen production in the entire body (Simpson & Davis 2001). The mouse *Cyp19a1* gene, which spans a 30 kb coding region and a 75 kb regulatory region (~105 kb in total length), is located on the long arm of chromosome 9 (Zhao *et al.* 2009). The downstream 30 kb coding region comprises nine coding exons (II–X; Golovine *et al.* 2003). The upstream 75 kb portion of the gene contains multiple promoters that direct transcription of alternative first exons, giving rise to aromatase mRNA species with unique 5′-untranslated regions (Chow *et al.* 2009). The upstream regulatory region contains two major promoters regulated in a tissue-specific manner: the proximal gonad-specific promoter and the distal brain-specific promoter, which lies ~40 kb upstream of the ATG translational start site in coding exon II (Honda *et al.* 1996; http://www.ncbi.nlm.nih.gov/BLAST/).

In the vertebrate brain, aromatase is primarily expressed in the hypothalamus, hippocampus, and

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amygdala via a highly conserved promoter, I.f (Lephart 1997, Honda *et al.* 1999). Aromatase expression in the hypothalamus is primarily localized in the medial preoptic area and the ventromedial nucleus of the hypothalamus, which are the centers that govern reproductive functions of both sexes of different species (Lephart 1996, Sharma *et al.* 2004, Peterson *et al.* 2005, Voigt *et al.* 2007, Zhao *et al.* 2007).

The physiological roles of aromatase in the brain became obvious after examining mice with a disrupted aromatase gene (aromatase knockout (ArKO)) and men with inactivating mutations of the aromatase gene (Fisher *et al.* 1998, Jones *et al.* 2006). In both cases, aromatase deficiency was associated with increased testosterone and gonadotropin levels, indicating that aromatase and E_2 were essential for regulating gonadotropin secretion. In addition, libido was significantly decreased in aromatase-deficient male mice and men and could be restored by E_2 treatment, thus revealing essential roles for aromatase and E_2 in regulating sexual behavior (Fisher *et al.* 1998, Bakker *et al.* 2004*a*,*b*, Simpson 2004*b*).

The signaling pathways or molecular mechanisms that regulate the brain-specific aromatase promoter I.f are not well understood. Several groups of investigators have found that protein kinases A and C and cAMP regulate aromatase expression and activity in the brain (Balthazart *et al.* 2001, Lavaque *et al.* 2006). Other groups showed that testosterone also upregulates aromatase mRNA and enzyme activity in the brain (Abdelgadir *et al.* 1994, Roselli *et al.* 1997, Negri-Cesi *et al.* 2001). Moreover, E_2 was found to upregulate and downregulate hypothalamic aromatase mRNA and enzyme activity under both *in vivo* and *in vitro* circumstances (Iivonen *et al.* 2006, Zhao *et al.* 2007). However, progesterone has been thought to suppress aromatase mRNA levels and enzyme activity in some tissues (Fortune & Vincent 1983) but induce it in others, implying that it may have divergent effects on aromatase (Lephart *et al.* 1998, Trainor *et al.* 2003, Pluchino *et al.* 2006, Wagner 2006). However, none of these studies provided a connection between aromatase expression and the regulation of promoter I.f in brain tissue. One group proposed that the transcription factor stress response element regulates the aromatase promoter via binding to its upstream region (Honda *et al.* 1999). Progesterone, a steroid hormone primarily secreted by the ovary and also locally synthesized in the brain, exerts its functions mostly via progesterone receptor (Pgr). Previously, progesterone has been proposed to regulate aromatase expression in the brain (Rhoda *et al.* 1987, Ing & Tornesi 1997, Prange-Kiel *et al.* 2001). In this regard, we conducted this study to elucidate the mechanisms regulating hypothalamic aromatase activity by progesterone via the brain-specific aromatase promoter I.f in mouse hypothalamic neuronal cell lines.

Materials and methods

Cell culture

Mouse embryonic hypothalamic neuronal cell lines were purchased from Cellutions Biosystems, Inc. (Toronto, Canada) and were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (Gibco) at 37 °C in 95% air and 5% CO_2. The cells were studied at passages 15–16. After 24 h of serum starvation, cells were treated with various doses of the progesterone agonist, R5020 (Promegestone, Sigma–Aldrich), for varying lengths of time.

RNA isolation and real-time RT-PCR

Hypothalamic neurons were treated with either vehicle (ethanol) or 10^{-7}, 10^{-8}, or 10^{-9} M R5020, and total RNA was extracted after 6, 12, and 24 h of treatment using TRI reagent (Sigma) as per the manufacturer's instructions. On-column DNAse digestion was carried out using a DNAse I kit (Qiagen). The integrity of the isolated RNA was verified by running 5 μg total RNA on a 1% formaldehyde gel. Total RNA (5 μg) from vehicle, R5020 or progesterone antagonist, RU486 (mifepristone)-treated hypothalamic neurons was used for reverse transcription in a final volume of 20 μl using the Superscript III First Strand RT synthesis kit (Invitrogen) according to the manufacturer's protocol. cDNA (5 μl) was used for real-time PCR (the Taqman real-time primers and probe for aromatase were designed using ABI primer express Software 3.0) on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The reactions were carried out using the ABI Taqman assay system for aromatase promoter I.f and power SYBR green for the aromatase coding region according to the manufacturer's protocol. The primers and probes used are as follows: Taqman assay primers for aromatase promoter I.f; forward 5′-AACTCACCATCTTCAAGAGTCCA-3′, reverse 5′-GAGTGGCATGGCACTGACAGT-3′; probe 5′-AGGTCCGGTTTA-3′. Each contained a 6-carboxy-fluorescein phosphoramidite (FAM dye) label at the 5′-end and a minor groove binder and nonfluorescent quencher at the 3′-end and were designed to hybridize to the junction between promoter I.f and exon II. SYBR green assay primers for aromatase are as follows: forward 5′-TGTGTTGACCCTCATGAGACA-3′, reverse 5′-CTTGACGGATCGTTCATACTTC-3′; for estrogen receptor α (*Esr1*): forward 5′-ATGAAAGGCGGCATACGGAAAG-3′, reverse 5′-CACCCATTTCATTTCGGCCTTC-3′; and for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*): forward 5′-ACCACAGTCCATGCCATCAC-3′, reverse 5′-TCCACCACCCTGTTGCTGTA-3′.

In order to elucidate which *Pgr* isoform is predominantly expressed in hypothalamic neuronal cell lines, we amplified all three isoforms employing real-time PCR. For *Pgr-B*: forward 5′-GTGGAGGGCGCTTTCTCTG-3′, reverse 5′-TCTGCCTCCCTCCCTATGAGT-3′; *Pgr-AB*: forward 5′-CTGGAGACCGAGGGCTCT-3′, reverse 5′-CCAGTGCTCGAGGTTTGCTC-3′; *Pgr-ABC*: forward 5′-GACACTGGCTGTGGAATTTCC-3′, reverse 5′-CCAGGATCTTGGGCAACTG-3′. Conventional PCR primers for *Esr1*: forward 5′-TCCTTCTAGACCCTTCAGTGAAGCC-3′, reverse 5′-ACATGTCAAAGATCTCCACCATGCC-3′.

PCR was carried out in triplicate in a 50 μl reaction volume using SYBR green PCR mix. The reactions were incubated at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. TaqMan C_T values were converted into absolute copy numbers. All RNA samples were normalized to the expression levels of *Gapdh* endogenous control. 'No template' and 'no RT' controls were used to ensure the absence of genomic DNA and reaction specificity. Totally, three independent experiments were performed to demonstrate reproducibility. Real-time RT-PCR product specificity was confirmed by melt curve analysis, gel electrophoresis, and product sequencing.

Aromatase enzyme activity assay

Aromatase enzyme activity within hypothalamic neurons was measured by a [^{3}H] water release assay, which is used routinely in our laboratory (Shozu *et al.* 2003). In each well, 60 pmol [^{3}H]androstenedione (PerkinElmer Life Sciences, Waltham, MA, USA) and 240 pmol cold androstenedione (Sigma) were added to 2 ml serum-free DMEM covering hypothalamic neurons in culture dishes. In order to study the effect of R5020, hypothalamic neurons at 80–90% confluency were serum starved for 24 h and then treated with vehicle or R5020 for 6, 12, and 24 h at 37 °C in 95% air and 5% CO_2. Each treatment was performed in triplicate. A mixture of labeled and cold androstenedione was added to each well 4 h before the end of each time point and incubated until the completion of each treatment period. [^{3}H]Androstenedione conversion to [^{3}H]E_2 was stopped by adding 10% (weight/volume) trichloroacetic acid. Steroidal compounds containing unconverted [^{3}H]androstenedione were removed from the mixture by first mixing with 4 ml chloroform followed by centrifugation at 900 ***g***. The upper aqueous layer was removed and mixed with dextran-coated charcoal (1% weight/volume). Charcoal was precipitated by centrifugation. From each tube, 2 ml clear solution was placed into a scintillation vial containing 10 ml scintillation fluid and counted in a scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA, USA).

Immunoblotting

Hypothalamic neurons were cultured in 150 mm dishes in DMEM media containing 10% FBS until reaching 80% confluency. Total protein was extracted from neurons using the M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Protein concentration was determined using the BCA protein assay kit (Pierce). Cell lysate (50 μg) was mixed with 2×Laemmli Sample Buffer (Bio-Rad Laboratories) and fractionated in 4–15% SDS–PAGE. Proteins from the gel were transferred to a nitrocellulose membrane. The membranes were then incubated with anti-Esr1 or anti-Pgr antibody (1:1000 dilution; Upstate, Charlottesville, VA, USA) in 5% milk solution overnight at 4 °C. The membrane was washed for 30 min and then incubated with anti-rabbit IgG-peroxidase conjugate (Upstate) as a secondary antibody (1:3000 dilution). Incubation with the secondary antibody was performed at room temperature for 1 h. The signal was detected using SuperSignal West Femto maximum sensitivity substrate chemiluminescence kit (Pierce) according to the manufacturer's instructions followed by exposure to BioMax ML X-ray film (Eastman Kodak) for 1–5 min. Then, the membranes were stripped of the first antibody and probed with anti-actin followed by the same signal detection procedure.

Transient transfection and luciferase assays

Transient transfection of N42 hypothalamic neurons was carried out in 12-well plates with Fugene HD transfection reagent (Roche Diagnostics) with the following plasmids: 1) 1 μg pGL3-basic luciferase reporter plasmid containing the promoter I.f nt −1000/−1 fragment or progressively truncated promoter I.f fragments (−700, −500, −200, and −50) and 2) 50 ng pRL-TK plasmid as an internal control (Promega). Neurons were serum deprived 1 day before transfection. After 24 h of transfection, N42 hypothalamic neurons were treated either with vehicle (ethanol) or with 10^{-7} M R5020 for 24 h. At the end of the treatment period, transfected neurons were washed twice in PBS and lysed in 250 μl lysis buffer (0·1 M potassium phosphate (pH 7·8), 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA). Luciferase assays were performed with 20 μl cell lysate employing a Dual-Luciferase Reporter Assay System kit (Promega). Luminescence was measured with a LUMAT LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Results are presented as the average of

luciferase activity from triplicate experiments and expressed as the ratio to the internal standard Renilla luciferase. Plasmids used in transfection experiments were purified using an EndoFree Plasmid Isolation Kit (Qiagen), and their purity was verified by spectrophotometry and agarose gel electrophoresis. All transfection assays were performed using equimolar amounts of plasmids.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed with the Acetyl-Histone H4 Immunoprecipitation Assay Kit (Upstate) following the manufacturer's instructions with minor modifications. Briefly, after reaching 80–90% confluency in 150 mm culture dishes ($\sim 2\times10^7$ cells), hypothalamic neurons were serum starved for 24 h, then treated with either vehicle (ethanol) or 10^{-7} M R5020 for 6 h. Neurons were then cross-linked with 1% formaldehyde for 10 min at room temperature on an orbital shaker followed by the addition of 1 M glycine for 5 min. Neurons were washed with cold PBS containing protease inhibitors and were lysed using a lysis buffer (50 mM Tris–HCl, pH 8·1, containing 1% SDS and 10 mM EDTA). The cells were sheared using a Sonic Dismembrator Model 100 (Fisher Scientific, Hampton, NH, USA) at 70% maximum amplitude (15 s on, 60 s off) for five pulses to obtain DNA fragments in the range of 200–1000 bp. The sonicated cell supernatant was diluted tenfold in ChIP dilution buffer and 40 µl was used as input DNA (positive control). Pre-cleared chromatin was used for immunoprecipitation reactions with 5 µg of the Pgr antibody or nonspecific (NS) rabbit IgG (Upstate). The reactions were incubated on a rotator overnight at 4 °C. The immune complexes were collected using protein A-agarose/DNA beads and washed five times using low salt, high salt, and lithium chloride and twice with Tris–EDTA buffers. The absorbed immune complexes were recovered by incubation with elution buffer (1% SDS and 0·1 M $NaHCO_3$). Part of the eluate was saved and checked for targeted protein immunoprecipitation by immunoblotting. After reverse cross-linking at 65 °C for 4 h, the genomic DNA was purified using a DNA purification kit (Qiagen). The promoter I.f region was scanned for Pgr recruitment by PCR amplification using seven different pairs of primer sets representing the nt −1000/−1 region in ~150 bp intervals. PCRs were optimized according to each primer set, with the best amplification conditions for all primer sets being the following: 35 cycles (30 s denaturation at 94 °C, 1 min annealing at 57·5 °C, and 1 min elongation at 72 °C). ChIP primers were designed using the Primer3 Software (http://frodo.wi.mit.edu/primer3/input.htm) and were as follows: forward 5′-GGCTTCTCTTGGTACGCTGA-3′ and reverse 5′-TTGTTGCTAAGAGATCAGTTGCTT-3′, which amplified the nt −190/−40 region of promoter I.f to yield a 150 bp amplicon; forward 5′-ACCACAGAGAGTGAAAGTTTGAG-3′ and reverse 5′-GCGTACCAAGAGAAGCCAAT-3′, which amplified the nt −334/−173 region of promoter I.f to yield a 162 bp amplicon; forward 5′-GCATTCAAGTTTGCTCAGAGG-3′ and reverse 5′-TTCTTTTGATGGGGTTGCAC-3′, which amplified the nt −431/−281 region of promoter I.f to yield a 151 bp amplicon; forward 5′-CCTGCCTAAAGGCTAAGATCC-3′ and reverse 5′-ACACACATACACCTCTGAGCAAA-3′, which amplified the nt −549/−399 region of promoter I.f to yield a 151 bp amplicon; forward 5′-CCCAAACCTTATCAACTTAGCC-3′ and reverse 5′-GGATCTTAGCCTTTAGGCAGGT-3′, which amplified the nt −692/−528 region of promoter I.f to yield a 165 bp amplicon; forward 5′-TGAGATGTAAACATTATGTGTGTGCT-3′ and reverse 5′-GGTTTGGGTTTAGGGGAAT-3′, which amplified the nt −793/−685 region of promoter I.f to yield a 109 bp amplicon; and forward 5′-AGCAGAGATGGCTTGTGGTT-3′ and reverse 5′-GCACACACATAATGTTTACATCTCA-3′, which amplified the nt −925/−768 region of promoter I.f to yield a 158 bp amplicon. All ChIP buffers used in this protocol contained a 1x protease inhibitor cocktail (Sigma).

Small interfering RNA

NS small interfering RNA (siRNA) and *Pgr* siRNA were purchased from Dharmacon (Chicago, IL, USA). *Pgr* knockdown was verified by real-time RT-PCR and immunoblotting. Before 1 day of transfection, hypothalamic neurons were plated in 6-well plates to achieve 50% confluency at the time of transfection and were then transfected with 100 nM NS siRNA or *Pgr* siRNA in triplicate using Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies, Inc.) according to the manufacturer's protocol. After 48 h of transfection, neurons were serum-starved for 24 h, followed by treatment with 10^{-7} M R5020 for 6 h. Total mRNA was extracted for real-time RT-PCR assay, and total protein was isolated using M-PER reagent for immunoblot analysis.

Statistical analysis

Statistical analyses were performed by Welch's paired *t* test and one-way analysis of variance followed by a Tukey multiple comparisons test using the StatView 5.0 Statistical Software package (SAS Institute, Cary, NC, USA). Significance was determined at $\alpha=0{\cdot}05$ and $\beta=0{\cdot}20$. The values for mRNA and aromatase activity were provided as mean ± S.E.M.

Results

Aromatase, Esr1, and Pgr expression levels in hypothalamic neuronal cell lines

To verify estrogen production and estrogen and progesterone responsiveness of the study cell lines, conventional RT-PCR, real-time RT-PCR, and immunoblot analyses were performed to measure aromatase, *Esr1*, and *Pgr* mRNA or protein in N38 and N42 hypothalamic neurons. Promoter I.f-driven aromatase expression (Fig. 1A), Pgr expression (Fig. 1B and C), and Esr1 expression (Fig. 1D and E) were lower in N38 hypothalamic neurons compared with N42 hypothalamic neurons. These results were reproducibly observed in three independent experiments.

Effect of the progesterone agonist, R5020, on aromatase mRNA expression and enzyme activity in N38 and N42 cell lines

Real-time RT-PCR and aromatase enzyme activity assays were performed to determine whether the progesterone agonist R5020 had an effect on aromatase mRNA expression and enzyme activity. We treated N38 and N42 hypothalamic neurons with 10^{-7} M R5020 for 6, 12, and 24 h. We observed that R5020 suppressed aromatase mRNA expression (Fig. 2A) and enzyme activity (Fig. 2B) at the 6 h time point but not at other time points in N42 hypothalamic neurons. However, aromatase RNA expression and enzyme activity at the 6 h time point in N38 hypothalamic neurons were induced by R5020 treatment (data not shown). Aromatase enzyme activity in each neuronal line treated with the aromatase enzyme inhibitor letrozole (LET) was undetectable.

The progesterone antagonist, RU486, inhibits R5020-dependent suppression of aromatase mRNA expression and enzyme activity in N42 hypothalamic neuronal cell lines

To clarify whether the effect of R5020 on aromatase mRNA expression and enzyme activity was Pgr dependent, we treated N42 hypothalamic neurons with the progesterone antagonist, RU486 (mifepristone). RU486 inhibited R5020-dependent suppression of aromatase mRNA expression and enzyme activity as assessed by real-time RT-PCR and aromatase enzyme activity assays (Fig. 3A and B). Aromatase activity in N42 neuronal line treated with the aromatase enzyme inhibitor LET was undetectable.

The nt −1000/−500 region of aromatase promoter I.f confers responsiveness to a progestin

To identify progestin-responsive cis-regulatory elements within the nt −1000/−1 region of aromatase promoter I.f, we generated a series of truncated reporter constructs and transiently transfected them into N38 and N42 hypothalamic neurons. In N42 hypothalamic neurons, the nt −1000/−1 and the nt −700/−1 promoter-luciferase reporter constructs were responsive to R5020 (threefold repression; Fig. 4).

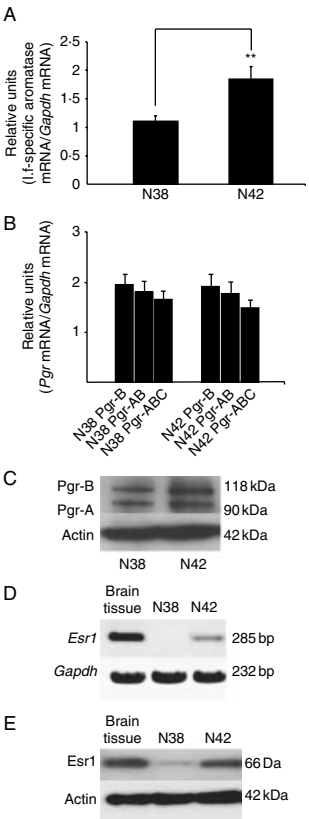

Figure 1 Promoter I.f-driven aromatase expression and Pgr and Esr1 expression in N38 and N42 hypothalamic neurons. (A) Real-time RT-PCR was performed with a probe complementary to the promoter I.f to exon II junction to measure promoter I.f-driven aromatase mRNA expression. Relative units are shown as the ratio of aromatase mRNA to *Gapdh* mRNA. Results are expressed as mean ± S.E.M. of three independent experiments (**$P<0{\cdot}01$, paired *t* test). (B) Conventional RT-PCR and (C) immunoblotting were performed to measure Pgr expression in brain tissue (positive control) and N38 or N42 hypothalamic neurons. (D) Conventional RT-PCR and (E) immunoblotting were performed to measure Esr1 expression in brain tissue (positive control) and N38 or N42 hypothalamic neurons. Gapdh and actin were used as loading controls. Figures represent one of the three independently performed experiments.

Pgr interacts with aromatase promoter I.f

We performed ChIP assays to demonstrate that Pgr is recruited to aromatase promoter I.f in N42 hypothalamic neurons. We used seven different sets of overlapping primer pairs representing the nt −1000/−1 region of promoter I.f in ∼150 bp

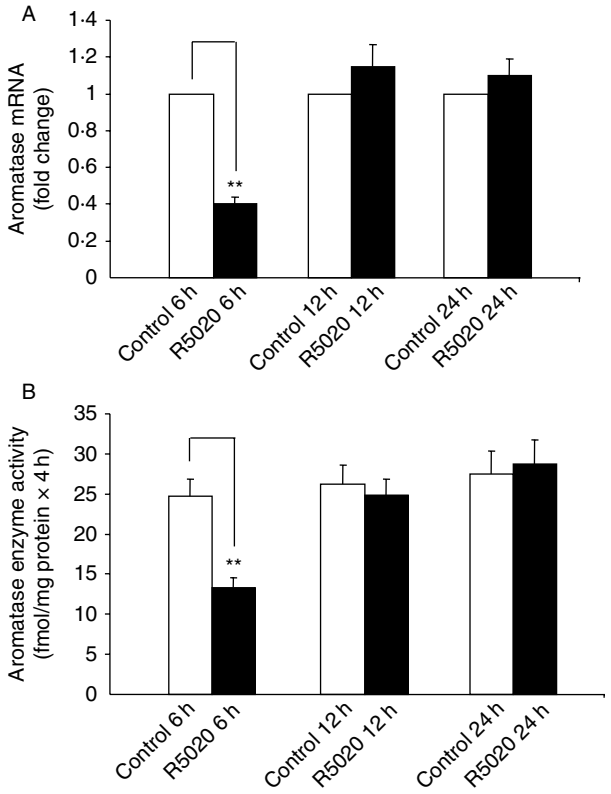

Figure 2 The progesterone agonist, R5020, regulates aromatase mRNA expression and enzyme activity in N42, but not N38, hypothalamic neurons. (A) Real-time RT-PCR was performed to measure aromatase mRNA expression after 6, 12, and 24 h of R5020 (10^{-7} M) treatment. Aromatase mRNA levels were normalized to *Gapdh* mRNA levels. Results are expressed as mean±S.E.M. from three independent experiments (**$P<0{\cdot}01$, paired *t* test). Aromatase activity assays were performed in (B) N42 hypothalamic neurons after 6, 12, and 24 h of R5020 (10^{-7} M) treatment. The results are expressed as mean±S.E.M. from three independent experiments (**$P<0{\cdot}01$, paired *t* test).

intervals. The most consistent and intense Pgr recruitment occurred in the nt −800/−600 region (Fig. 5A). Under serum-starved conditions, we did not observe Pgr recruitment to the nt −800/−600 region of promoter I.f in N42 hypothalamic neurons; however, recruitment to this region was enhanced by R5020 treatment (10^{-7} M, 6 h; Fig. 5B). We applied densitometry to PCR gels to calculate fold enrichment as a ratio of recruitment of Pgr antibody-bound chromatin to that of nonspecific IgG-bound chromatin. We demonstrated that R5020 induced recruitment of Pgr by 2·1-fold to this progestin-responsive region (Fig. 5B).

Pgr mediates R5020-dependent suppression of aromatase mRNA expression and enzyme activity

siRNA was used to determine whether endogenous Pgr in N42 hypothalamic neurons mediates R5020-dependent suppression of aromatase mRNA expression and enzyme activity. As shown in Fig. 6A and B, siRNA knockdown of *Pgr* in N42 hypothalamic neurons abolished suppression of aromatase mRNA levels and enzyme activity by R5020 (10^{-7} M) treatment. The knockdown of *Pgr* was confirmed by real-time RT-PCR and immunoblotting (Fig. 6C and D).

Relative Pgr and Esr1 expression is critical for E_2- and progestin-dependent regulation of aromatase mRNA expression and enzyme activity

We have previously shown that E_2 induces aromatase mRNA expression and enzyme activity in N42 but not in N38 hypothalamic neurons, which express lower Esr1 and Pgr levels compared with N42 hypothalamic neurons (Fig. 1C and D). To determine whether different levels of Esr1 or Pgr expression affect estrogen or progestin regulation of aromatase mRNA levels and enzyme activity, we treated N38 and N42 hypothalamic neurons with 10^{-7} M R5020, 10^{-7} M E_2, or both for 6 h. R5020 treatment induced aromatase mRNA expression (Fig. 7A) and enzyme activity (Fig. 7B) in N38 hypothalamic neurons, but suppressed them in N42 hypothalamic neurons. In addition, E_2 treatment did not change aromatase mRNA expression and enzyme activity in N38 hypothalamic neurons but induced both

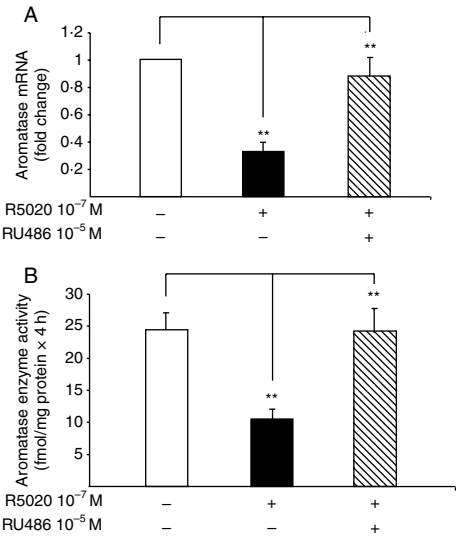

Figure 3 The progesterone antagonist, RU486, reverses R5020-dependent suppression of aromatase mRNA expression and enzyme activity in N42 hypothalamic neurons. (A) Real-time RT-PCR was performed in the presence or absence of 10^{-7} M R5020 plus or minus 10^{-5} M RU486 for 6 h. Aromatase mRNA levels were normalized to *Gapdh* mRNA levels. The results are expressed as mean±S.E.M. from three independent experiments (**$P<0{\cdot}01$, ANOVA). (B) Aromatase activity assays were performed to measure enzyme activity in the presence or absence of 10^{-7} M R5020 plus or minus 10^{-5} M RU486 for 6 h. The results are expressed as mean±S.E.M. ($n=3$; **$P<0{\cdot}01$, ANOVA).

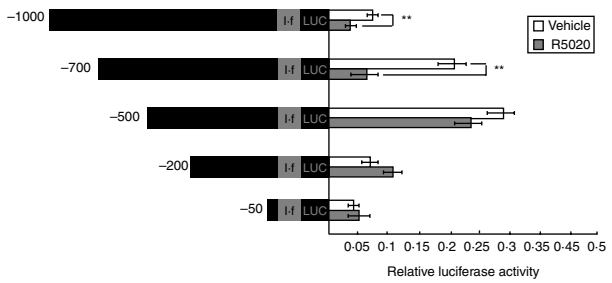

Figure 4 Cis-regulatory elements within the nt −1000/−500 region of aromatase promoter I.f are essential for R5020-dependent suppression of aromatase expression in N42 hypothalamic neurons. Serial deletion analysis was performed to localize the R5020-responsive regions of aromatase promoter I.f. The promoter I.f-luciferase reporter constructs were named according to the nucleotide positions of their 5′-termini. All constructs were transiently transfected into N42 hypothalamic neurons. Luciferase assays were performed a minimum of three times in the presence or absence of 10^{-7} M R5020. Mean luciferase activity of each construct is given relative to the nt −1000/−1 promoter-luciferase reporter construct. Summary data for three independent experiments are shown. Results are expressed as mean±S.E.M. (**$P<0{\cdot}01$, paired *t* test).

aromatase expression and activity in N42 hypothalamic neurons. Moreover, when treated with R5020 and E_2 together, aromatase mRNA expression and enzyme activity were induced in both hypothalamic neuronal lines (Fig. 7A and B). Aromatase enzyme activity in each neuronal line treated with the aromatase enzyme inhibitor LET was undetectable.

Discussion

Aromatase deficiency in humans disrupts normal gonadotropin regulation and libido (Jones *et al.* 2006). Likewise, ArKO mice exhibit disrupted reproductive behavior, including defective lordosis (females), low frequency of copulatory behavior (males; Bakker *et al.* 2003), reduced sexual interest in the opposite sex, and deficiency in pup care (Bakker *et al.* 2002, 2003, Matsumoto *et al.* 2003*a*,*b*). The gonadotropin secretion profile is also disrupted in ArKO mice, which have increased LH, FSH, testosterone, and prolactin levels, and decreased E_2, but unchanged dihydrotestosterone and progesterone (Fisher *et al.* 1998, Jones *et al.* 2006).

Aromatase is primarily expressed in gonads and the brain (Simpson 2004*a*, Bulun *et al.* 2005). Loss of local E_2 synthesis in the brain likely leads to the hormonal and behavioral phenotypes observed in aromatase-deficient humans and mice. In fact, alterations in brain aromatase expression have been directly linked to changes in sexual behavior in birds, rodents, sheep, and humans (Balthazart *et al.* 2003, Matsumoto *et al.* 2003*a*,*b*, Jones *et al.* 2006, Perkins & Roselli 2007).

The role of progesterone in females appears similar in a variety of species, particularly for maternal and reproductive behavior. However, progesterone is capable of protecting females from the masculinizing and defeminizing effects of testosterone (Sodersten & Hansen 1977, Hansen & Sodersten 1978). However, progesterone treatment suppresses mounting and intromissive behavior in males (Michael *et al.* 1968, DeBold *et al.* 1978, Witt *et al.* 1995).

Gonadectomy reduces aromatase expression and enzyme activity in the brain in parallel with a recession in gender-specific sexual behavior of both sexes (Jakab *et al.* 1993, Iivonen *et al.* 2006). However, treatment of gonadectomized animals with progesterone markedly restores female-specific but not male-specific sexual behavior (Sodersten & Hansen 1977, Hansen & Sodersten 1978, Witt *et al.* 1995). Moreover, treatments of intact animals with the progesterone antagonist, RU486, or antisense oligonucleotides to *Pgr* increased the number of mounts and intromissions in males but

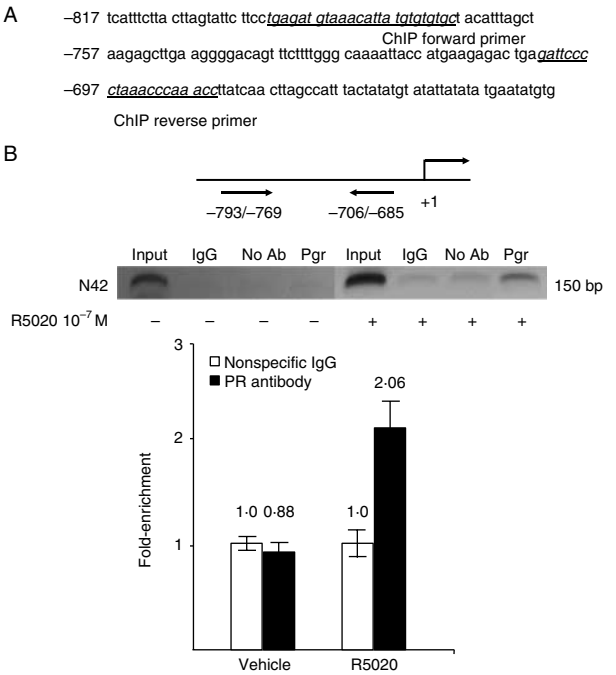

Figure 5 ChIP analysis reveals Pgr recruitment to aromatase promoter I.f in N42 hypothalamic neurons. We performed ChIP assays using N42 hypothalamic neuron extracts treated with vehicle or 10^{-7} M R5020 for 6 h employing different sets of primers; the most consistent recruitment was observed with the primers (underlined) in (A). Sonicated cell supernatant was used as input DNA (positive control). Pre-cleared chromatin was used for immunoprecipitation reactions with a rabbit polyclonal antibody directed against human PGR and normal rabbit IgG. The images in (B) are from one of the five independently performed experiments. The bars represent densitometric measurements of the ratio of enrichment of DNA precipitated using an antibody against Pgr versus nonspecific IgG. The bars were generated from repeated experiments.

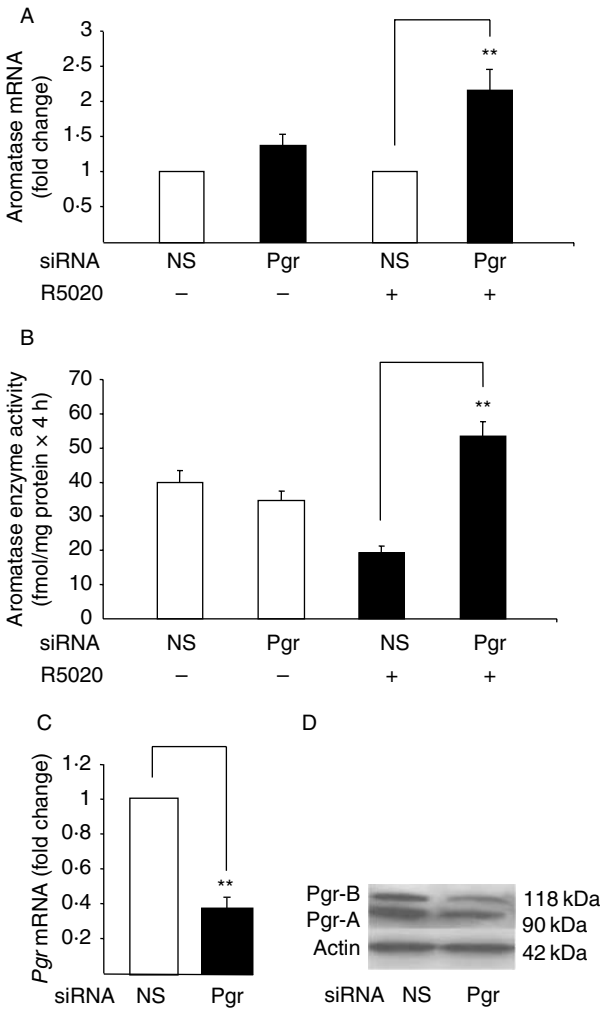

Figure 6 Pgr mediates R5020-dependent suppression of aromatase mRNA expression in N42 hypothalamic neurons. N42 hypothalamic neurons were transfected with *Pgr* siRNA or with non-specific siRNA as a negative control (NS) and cultured for an additional 48 h. (A) Aromatase mRNA levels were analyzed by real-time RT-PCR in the presence or absence of R5020 (10^{-7} M, 6 h). Aromatase mRNA levels were normalized to *Gapdh* mRNA levels. The results are expressed as mean ± S.E.M. from three independent experiments (**$P<0{\cdot}01$, ANOVA). (B) Aromatase activity assays were performed in the presence or absence of R5020 (10^{-7} M, 6 h). The results are expressed as mean ± S.E.M. from three independent experiments (**$P<0{\cdot}01$, ANOVA). Aromatase enzyme activity in N42 neuronal line treated with the aromatase enzyme inhibitor letrozole was undetectable (data not shown). (C) Real-time RT-PCR and (D) immunoblotting were performed to measure *Pgr* mRNA and protein levels respectively. Actin was used as a loading control. The immunoblot represents one of the three independently performed experiments. Real-time RT-PCR results are expressed as mean ± S.E.M. from three independent experiments (**$P<0{\cdot}01$, ANOVA) and Gapdh was used as a loading control. Representative results from three independent experiments are shown.

decreased the frequency of lordosis in females (Pollio *et al.* 1993, Mani *et al.* 1994). Therefore, the actions of progesterone are necessary for the proceptive components of female sexual behavior, although the mechanism of progesterone action is not fully understood.

The reproductive phenotypes manifested in ArKO male mice are opposite to those observed in progesterone receptor knockout (PRKO) male mice, in which most measures of male sexual behavior are enhanced, suggesting that aromatase and Pgr are important in mediating opposite aspects of reproductive behavior controlled by the brain, in particular, by the hypothalamus in males (Matsumoto *et al.* 2003*a*, Schneider *et al.* 2005). However, PRKO female mice are sterile with impaired maternal and reproductive behavior and neuroendocrine and gonadotropin regulation, suggesting a prominent role of Pgr in feminization rather than masculinization process (Conneely *et al.* 2001).

Taken together, the roles that are attributed to progesterone in gender-specific sexual behavior mostly favors the feminization process both in males and in females, which is in contrast to what has been observed in male mice with inactivated *Esr1* or *Cyp19a1* (aromatase), suggesting that Pgr and Esr1/aromatase may regulate opposite aspects of reproductive behavior, particularly in males.

In the hypothalamic tissue of neonatal males, progesterone does not affect the accumulation of testosterone, but completely prevents the formation of E_2, suggesting that progesterone may inhibit hypothalamic aromatase expression and/or enzyme activity in the neonatal brain (Rhoda *et al.* 1987). Consistent with these findings, we found that progesterone treatment suppressed aromatase mRNA expression and enzyme activity (Fig. 2).

The varying intracellular concentration of progesterone and possible alterations in the composition of the transcriptional complex at promoter I.f over time may explain the mechanism of biphasic response observed in this study. These considerations will be investigated as future directions.

Progesterone can also inhibit *Esr1* expression and E_2 can induce the expression of both Pgr isoforms (Evans & Leavitt 1980, Smanik *et al.* 1983). We observed a slight decrease in *Esr1*, but not *Esr2* (also known as ERβ) expression levels upon R5020 treatment in hypothalamic cells (data not shown). This may represent an alternative mechanism whereby progesterone regulates aromatase expression in hypothalamic neurons.

In parallel, it is known that E_2 induces Pgr expression and inhibition of aromatase suppresses Pgr expression in the brain, suggesting a negative feedback mechanism between progesterone and aromatase (Ing & Tornesi

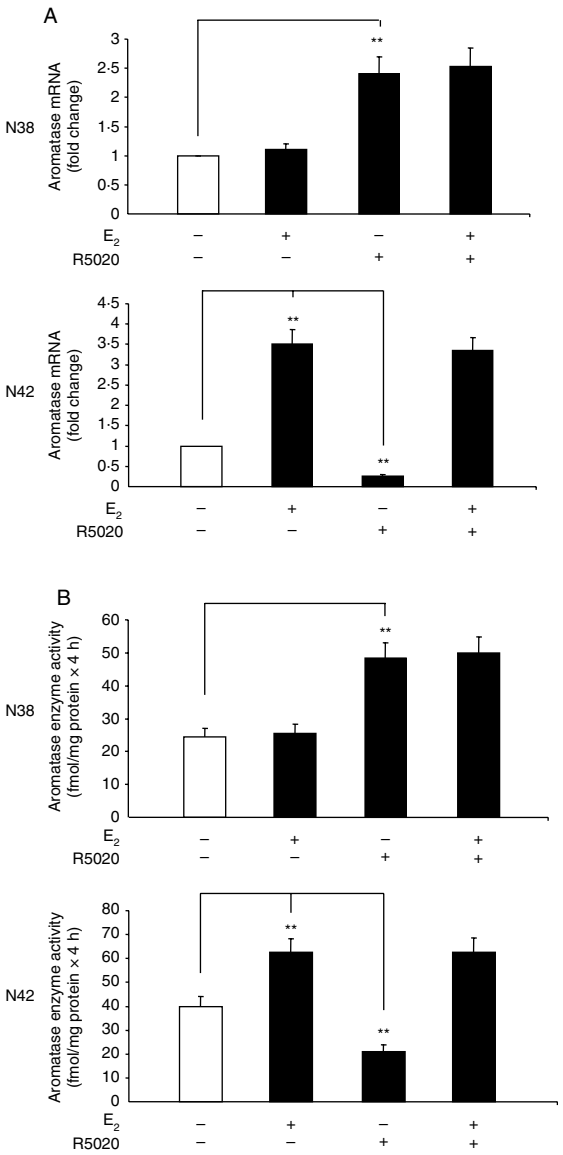

Figure 7 Combinatory effects of E_2 and R5020 on aromatase are cell-type specific. (A) Real-time RT-PCR was performed to measure aromatase mRNA expression after 6 h of R5020 (10^{-7} M) or E_2 (10^{-7} M) or R5020 (10^{-7} M)+E_2 (10^{-7} M) treatment in both N38 and N42 hypothalamic neurons. Aromatase mRNA levels were normalized to *Gapdh* mRNA levels. Results are expressed as mean±S.E.M. from three independent experiments (**$P<0{\cdot}01$, paired *t* test). (B) Aromatase activity assays were performed in both N38 and N42 hypothalamic neurons following the described treatment procedure in (A). The results are expressed as mean±S.E.M. from three independent experiments (**$P<0{\cdot}01$, paired *t* test).

1997, Prange-Kiel *et al.* 2001). Consistent with this hypothesis, we found that Pgr interacts with the aromatase I.f promoter in the presence but not in the absence of R5020, with the highest intensity at the nt −800/−600 region of promoter I.f (Fig. 4).

The perinatal rodent brain expresses all the enzymes required for the *de novo* synthesis of progesterone from cholesterol, potentially producing locally high concentrations of this steroid. E_2 concentration in certain brain areas, such as the hypothalamus, preoptic area, and the hippocampus, is higher than the plasma E_2 levels (Hojo *et al.* 2004, Rune *et al.* 2006). Even though the total amount of E_2 synthesized by these brain sites seems to be low, the achieved local tissue concentrations are probably quite high and exert significant biologic influence locally (Simpson *et al.* 1999, Prange–Kiel *et al.* 2003). The same phenomenon could also be true for progesterone adding another layer of complexity to the phenomenally complex mechanism involving estrogen and progesterone production and action in various regions.

Various studies investigating the effects of progesterone on reproductive behavior used supraphysiological levels of progesterone that would greatly exceed its levels accounted for by ovarian secretion (Erpino 1975, Connolly & Resko 1989). However, the endogenous levels of progesterone can be locally regulated in specific tissues or regions of the brain (Le Goascogne *et al.* 2000, Tagawa *et al.* 2006). In our experiments, aromatase regulation by 10^{-7} M R5020, which may represent the cellular progesterone concentration in a particular brain region, cannot be generalized to the effects of this steroid to the entire brain tissue. As a further twist, both the Pgr and the Esr1 levels in N42 neurons are higher than those in N38 hypothalamic neurons, which are possibly accounted for differential responsiveness of these cells to progesterone. Differential responsiveness of N42 and N38 cells to R5020 with respect to aromatase expression was a function of Pgr levels and consistent across various experiments because knockdown of Pgr in N42 cells converted an inhibitory response to a stimulatory one (Figs 1, 6 and 7). We speculate that, *in vivo*, the varying levels of Pgr and Esr1 in specific brain cells would be key determinants of progesterone responsiveness.

The developmental and tissue-, region-, and neuron-specific expression of aromatase in the brain has been reported to be transcriptionally regulated through promoter I.f (Honda *et al.* 1999, Nausch *et al.* 2007). There are several potential cis-regulatory elements, which may be important for promoter regulation, residing in the 5′-flanking region of promoter I.f including PRE half sites. In our experiments, R5020 significantly suppressed luciferase activity of the nt −1000/−1 and nt −700/−1 promoter-reporter constructs but not other promoter-reporter luciferase constructs in N42 hypothalamic neurons (Fig. 4). We did not observe suppression of luciferase activity of the promoter-luciferase reporter constructs on R5020 treatment in N38 hypothalamic neurons, due possibly

to higher Pgr expression in N42 hypothalamic neurons compared with N38 hypothalamic neurons.

Aromatase in the fetal brain is also proposed to be regulated via sex hormones, specifically E_2 that has both stimulatory and inhibitory effects on aromatase expression and enzyme activity in the brain (Iivonen *et al.* 2006). Our group has recently showed that Esr1 mediates E_2 actions on aromatase expression and enzyme activity through its brain-specific promoter in embryonic hypothalamic neuronal cell lines (Yilmaz *et al.* 2009). Progesterone has also been implicated in the regulation aromatase expression and enzyme activity (Rhoda *et al.* 1987, Ing & Tornesi 1997, Prange-Kiel *et al.* 2001, Trainor *et al.* 2003). Interestingly, Brock *et al.* (2010) has recently showed that Pgr and aromatase expression is conversely related in the mouse hypothalamus, suggesting a feedback loop between progesterone and aromatase. In this study, we showed that progesterone enhances recruitment of Pgr to specific regions of the promoter I.f of *Cyp19a1* and regulates aromatase expression in hypothalamic neurons.

We identified several progesterone response elements (half sites) in both nt −1000/−700 and nt −700/−500, with nt −800/−600 having the highest intensity of Pgr recruitment sites as assessed by ChIP. Furthermore, the effect of E_2 and progesterone on aromatase mRNA expression and enzyme activity is Esr1/Pgr expression dependent (Fig. 7), with higher receptor expression favoring induction of aromatase on E_2 treatment and suppression on R5020 treatment, and with lower receptor expression favoring induction on R5020 treatment and no alteration on E_2 treatment.

Taken together, we demonstrated the mechanism of a Pgr action in mediating progesterone-dependent regulation of aromatase expression and enzyme activity in hypothalamic neurons in a time-, concentration-, and Esr1/Pgr expression-dependent manner. Our future directions include verification of these findings using *in vivo* models. We also plan to further investigate the *in vitro* and *in vivo* effects of other hormones and metabolites on aromatase expression and activity to elucidate the molecular mechanisms mediating local E_2 synthesis in the hypothalamus and other regions of the brain.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Dr M B Y performed the key experiments and wrote the draft manuscript. Dr H Z and C B performed other important experiments. Drs A W and S E B significantly contributed to the experimental design.

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