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ORIGINAL ARTICLE

Early Histone Modifications in the Ventromedial Hypothalamus and Preoptic Area Following Oestradiol Administration

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Expression of the primary female sex behaviour, lordosis, in laboratory animals depends on oestrogen-induced expression of progesterone receptor (PgR) within a defined cell group in the ventrolateral portion of the ventromedial nucleus of the hypothalamus (VMH). The minimal latency from oestradiol administration to lordosis is 18 h. During that time, ligand-bound oestrogen receptors (ER), members of a nuclear receptor superfamily, recruit transcriptional coregulators, which induce covalent modifications of histone proteins, thus leading to transcriptional activation or repression of target genes. The present study aimed to investigate the early molecular epigenetic events underlying oestrogen-regulated transcriptional activation of the *Pgr* gene in the VMH of female mice. Oestradiol (E_2) administration induced rapid and transient global histone modifications in the VMH of ovariectomised female mice. Histone H3 N-terminus phosphorylation (H3S10phK14Ac), acetylation (H3Ac) and methylation (H3K4me3) exhibited distinct temporal patterns facilitative to the induction of transcription. A transcriptional repressive (H3K9me3) modification showed a different temporal pattern. Collectively, this should create a permissive environment for the transcriptional activity necessary for lordosis, within 3–6 h after E_2 treatment. In the VMH, changes in the H3Ac and H3K4me3 levels of histone H3 were also detected at the promoter region of the *Pgr* gene within the same time window, although they were delayed in the preoptic area. Moreover, examination of histone modifications associated with the promoter of another ER-target gene, oxytocin receptor (*Oxtr*), revealed gene- and brain-region specific effects of E_2 treatment. In the VMH of female mice, E_2 treatment resulted in the recruitment of ERα to the oestrogen-response-elements-containing putative enhancer site of *Pgr* gene, approximately 200 kb upstream of the transcription start site, although it failed to increase ERα association with the more proximal promoter region. Finally, E_2 administration led to significant changes in the mRNA expression of several ER coregulators in a brain-region dependent manner. Taken together, these data indicate that, in the hypothalamus and preoptic area of female mice, early responses to E_2 treatment involve highly specific changes in chromatin structure, dependent on cell group, gene, histone modification studied, promoter/enhancer site and time following E_2.

Key words: oestrogens, oestrogen receptor, progesterone receptor, hypothalamus, transcriptional regulation, histone modification

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The ovarian steroids, oestrogens, regulate a great variety of physiological processes, from reproduction to bone formation, cardioprotection and neurotrophic effects on the central nervous system (CNS) (1). Moreover, oestrogens have been the focus of intense study for decades regarding their role in endometrial and breast tumours. A primary role of oestrogens in the mammalian brain is on the hypothalamic–pituitary axis, where they act as homeostatic feedback messengers between the gonads and the hypothalamus to regulate the levels of gonadotrophins and the reproductive cycle of the female (2). In addition, oestrogens impact reproduction through the facilitation of an entire chain of behaviours essential for reproduction, from courtship to maternal behaviours. The primary sex

behaviour of female rodents, lordosis, is known to critically depend on the actions of oestrogens within the defined neuronal group in the hypothalamus, ventromedial nucleus of hypothalamus (VMH) (3–5).

The mechanism by which oestrogens modulate reproductive behaviour is through the transcriptional induction of target genes and the coordinated actions of these gene products promote different aspects of lordosis (6). Oestrogens regulate transcription through binding to their cognate receptors, oestrogen receptor (ER) α (7) and ERβ (8), which are both members of the nuclear receptor superfamily of transcription factors. Upon ligand binding, ERs dimerise and translocate to the nucleus where they bind to specific DNA sequences called oestrogen response elements (EREs) within the regulatory regions of target genes (9–11). Studies using genetically modified mice with disrupted ERα, as well as temporally restricted knockdown of this receptor, specifically in the VMH, confirm that signalling through ERα is critical for reproduction and the expression of sexual behaviour in response to oestrogens (12–14).

One of the most important regulatory steps in the transcriptional activity of hormone receptors is the interaction with cofactors, which link them to the basal transcriptional machinery. Furthermore, these cofactors are able to regulate transcription by modifying chromatin structure through covalent histone modifications (15). To date, a large number of cofactors involved in ER-mediated transcription have been identified. Among them are coactivators with histone acetyltransferase (HATs) activity, members of the p160 subfamily (steroid-receptor coactivators; SRCs) (16) and the integrator complex p300/CBP (17); histone methyltransferases (HMTs) such as CARM1, PRMT1 and MLLs (18–20); and corepressors, NCOR and SMRT, which recruit histone deacetylases to target gene promoters (21,22). Because of the central role of coregulators in ER-mediated transcriptional regulation, they have long been regarded as the critical determining factors of specificity of oestrogen action.

Among the ER target genes, the induction of the *Pgr* gene by oestradiol (E_2)-activated ERα within the VMH, is one of the earliest and most essential steps in the sequence of events that lead to the expression of female sex behaviours and successful reproduction in rodents (23). *Pgr* is considered one of the classic ERα target genes and its induction by E_2 has been extensively studied in a number of E_2-responsive tissues, most notably in mammary epithelial and breast cancer cells, as well as in the brain. However, as the data accumulate, it has become increasingly clear that the regulation of *Pgr* by ligand-activated ERα is quite complex and displays tissue and age-dependent patterns (24–26). The requirement of different SRC isoforms for the regulation of *Pgr* gene expression by E_2-activated ER is one clear example of a tissue-specific hormonal effect. In the human MCF-7 breast cancer cell line, the expression of the *Pgr* gene in response to E_2 stimulation requires SRC3, but not SRC1 and SRC2 (27,28). By contrast, in the brains of rats and mice, SRC1 and SRC2 are necessary for the induction of the *Pgr* gene, as well as the expression of reproductive behaviour, whereas SRC3 is dispensable for both (29,30).

The *Pgr* gene produces two protein isoforms, PgR-A (94 kDa) and PgR-B (120 kDa), through two alternate transcription start sites (TSS). Moreover, two functional promoters that direct the transcription of each *Pgr* isoforms have been characterised for human and rat *Pgr* genes (31–33). Although the transcription of the *Pgr* gene is directly regulated by ERα, there are no consensus ERE motifs near the transcription start sites. There is, however, an ERE half site within the *Pgr-A* promoter, which is conserved in the mouse, rat and human genome (31–33). Recently, several ERα binding sites have been identified between 48 and 311 kb upstream of the human *PGR-B* TSS (34,35). Three of these sites have been shown to associate with the TSS in an E_2-dependent manner, suggesting that they may act as enhancers responsible for *PGR* gene transcription (35). Despite these recent advances, little is known about the molecular mechanisms involved in the regulation of *Pgr* gene expression by ligand-activated ERα in the mouse nervous tissue.

For decades, the human breast cancer cell system has been the primary source of information about the effects of oestrogens and transcriptional activity of ERs. However, the important physiological role of oestrogens on nervous system functions creates a need to understand the molecular actions of these steroid hormones in nervous tissue. In the present study, we investigated the early molecular events associated with E_2-mediated regulation of *Pgr* gene transcription in the mouse brain. Total levels of histone modifications in the VMH of female mice, as well as acetylation and K4 methylation of histone H3 at the promoter of the *Pgr* gene, were examined to determine the effects of E_2 on chromatin architecture and E_2-target gene expression. We also performed comparative analyses of histone modifications at the promoters of two of E_2-target genes, *Pgr* and *Oxtr*, in two E_2-responsive hypothalamic nuclei, VMH and preoptic area (POA), aiming to investigate the molecular mechanisms of gene- and region-specific effects of E_2 in the brain. Chromatin immunoprecipitation (ChIP) was performed on the VMH tissue of female mice to detect the recruitment of ERα to the putative regulatory sequences of the *Pgr* gene and led to the identification of an E_2-regulated site approximately 200 kb upstream of TSS of the mouse *Pgr* gene. In addition, mRNA expression analysis in the VMH and POA of female mice revealed that E_2 regulates the transcript levels of several histone-modifying factors also in a region-specific manner, providing mechanistic clues as to how the exposure to oestrogens leads to changes in the status of histone proteins.

Materials and methods

Treatment and tissue collection

Animals were handled in accordance with the National Institutes of Health and Rockefeller University Institutional Animal Care and Use Committee guidelines. Six- to 8-week-old female Swiss-Webster mice were ovariectomised (OVX). After recovery for 1 week, mice were injected i.p. with 20 μg of 17β-oestradiol (E_2) (Sigma, St Louis, MO, USA) diluted in 100 μl of vehicle solution (10% ethanol/saline). Control mice were injected only with vehicle (Veh) solution. Mice were sacrificed 1, 3, 6 and 9 h after E_2 injections, the brains removed and immediately frozen. VMH and POA were dissected from each brain on a dry-ice bed and kept frozen until processed. For dissections, frozen brain was placed on dry ice ventral side up and slices containing areas of interest were cut using brain mold and sterile blades. For POA, the optic chiasm and olfactory tubercle were used as landmarks to

isolate an approximately 0.5-mm slice between 0.5 and 0.10 relative to bregma; and, for VMH, the pituitary stalk and mammary bodies were used as landmarks to isolate an approximately 1-mm slice between −1.22 and −2.06 relative to bregma. Subsequently, areas containing POA and VMH were dissected from coronal slices according to the mouse brain atlas (36).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

VMH tissue was thawed in lysis buffer containing protease, phosphatase and deacetylase inhibitors. Tissue was homogenised in 2% SDS buffer to ensure total disruption of the plasma membrane, as well as the nuclear envelope. For the SDS-PAGE and Western blot analysis, VMH tissue from two mice was pooled. The total protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) and 30 μg of protein was ran on 4–20% gradient Tris-glycine Ready gels (Bio-Rad, Hercules, CA, USA). Protein was transferred onto polyvinylidene fluoride membrane (Bio-Rad) and blocked for 1 h at room temperature (RT) in 5% TBS-milk. Primary antibodies that recognise specific modified residues on H3 and H4 histone tails were all purchased from Millipore (Bedford, MA, USA). Primary antibody incubation was carried out at 4 °C overnight, followed by washes and incubation with a secondary horseradish peroxidase–labelled goat anti-rabbit antibody (GE Healthcare, Piscataway, NJ, USA) for 1 h at RT. Membranes were washed and immunolabelled proteins were visualised with enhanced chemiluminescence detection reagent (GE Healthcare). Quantitative densitometric analysis was performed using NIH IMAGE (Bethesda, MD, USA). All modified histone levels were normalised to that of total H3 histone (Millipore) to account for the loading variation. Statistical significance was calculated by one-way ANOVA followed by Dunnett's multiple comparison test using GRAPHPAD PRISM (GraphPad Software Inc., San Diego, CA, USA). Progesterone receptor protein was detected in the same extracts using rabbit monoclonal antibody that recognises both PgR-A and PgR-B isoforms (clone SP2; Thermo Fisher Scientific). PgR levels were normalised to that of β-actin (Santa Cruz Biotechnology; Santa Cruz, CA, USA).

ChIP and quantitative polymerase chain reaction (qPCR)

The ChIP assay was performed using EZ-Magna ChIP kit (Millipore) in accordance with the manufacturer's instructions, with some modifications. Briefly, VMH and POA tissue collected from Veh and E_2-treated mice was cut into approximatey 1-mm pieces and crosslinked in 1% formaldehyde at RT for 15 min. To perform ChIP with ERα antibody (Clone: TE111.5D11; Thermo Fisher Scientific), an additional cross-linking step with 2 mM ethylene glycol-bis(succinimidylsuccinate) (Thermo Scientific) was introduced, followed by 30 min with 1% formaldehyde (37). Tissue from two to four mice was pooled for each sample. After quenching and washing out the formaldehyde, tissue was homogenised in 1% SDS nuclear lysis buffer containing protease inhibitors. Chromatin was sheared using Bioruptor sonicator (Diagenode Inc., Denville, NJ, USA), cleared by centrifugation and the protein concentration was determined using BCA protein assay kit (Thermo Fisher Scientific). An equal amount of chromatin (300 μg) was used from each sample to immunoprecipitate H3Acetyl and H3K4me3 using antibodies that recognise pan-acetylated H3 (06-599) and specific trimethylated H3K4 residue (07-473) (both Millipore). In total, 10% of each sample amount (30 μg) was removed and saved as input. For immunoprecipitating ERα, 1 mg of chromatin was used for each sample and 5% of sample amount was saved as input. The specificity and efficiency of ERα antibody is shown in Figure S2. After several washes, pulled-down complexes were reverse cross-linked and genomic DNA was purified. Quantitative PCR was used to measure the amount of immunoprecipitated DNA and C_t values obtained were normalised to those from input. Statistical significance was calculated by one-way ANOVA followed by Dunnett's multiple comparison test using GRAPHPAD PRISM. The sequences and positions of primers used to detect binding of modified H3 histones to *Pgr* and *Oxtr* promoters are listed in Tables 1 and 2, respectively, and are based on sequences of the mouse *Pgr* (accession number U12644) and *Oxtr* gene (accession number D86631). The sequences and positions of primers used to detect binding of ERα to the upstream putative enhancer regions of the mouse *Pgr* gene are listed in Table 3. Primers were designed using either PRIMER EXPRESS (Applied Biosystems, Carlsbad, CA, USA) or PRIMERQUEST (Integrated DNA Technologies, Coralville, IA, USA).

Total RNA isolation and gene expression

OVX female mice were injected with 20 μg of E_2 or Veh solution (i.p) and sacrificed 3 or 5 h later. Brains were removed and immediately frozen. Total RNA was isolated from VMH and POA using Trizol reagent (Life Technologies, Grand Island, NY, USA) in accordance with the manufacturer's instructions. An equal amount of total RNA (100 ng) was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies) and the resultant cDNA was used as a template for qPCR. TaqMan Gene Expression Assays were used to amplify mRNA for genes of interest: *Src1*, ID: Mm00447958_m1; *Src2*, ID:Mm00500749_m1; *Cbp*, ID:Mm01342452_m1; *p300*, ID:Mm00625535_m1; *Mll1*, ID:Mm01179235; *Mll3*, ID: Mm01156942_m1; *Ash1*, ID:Mm00467322_m1; *Wdr5*, Mm01332446_m1; *Lsd1*, ID:Mm01181033_m1; *Jarid1A*, ID:Mm00524457. Eukaryotic 18S rRNA (4352930E) was used as internal control (all from Life Technologies). The relative ratio of the expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$

Table 1. Nucleotide Sequences and Locations of Primers Used to Amplify Mouse *Pgr* Promoter Regions in Chromatin Immunoprecipitation Experiments

Name	Position	Primer sequence
Prom1	−721/−650	Forward 5′ -CATCC TTA ACTGC TAAG TCACATTGG -3′
		Reverse 5′-GCCTT CCT CTA ACACGCTCAA -3′
Prom2	+261/+332	Forward 5′-AGCGGG AGTC CTTTTTT CAG-3′
		Reverse 5′-TTTTTCGT GTTT CTTC TTGA ATGG-3′
Prom3	+621/+683	Forward 5′-GGAG CTTGG GT CG TCATGA -3′
		Reverse 5′-GCCAGA CGT GTGGAGA AC CT-3′
Prom4	+841/+915	Forward 5′ -AGCTCC CAG ACGGAAAG ACA-3′
		Reverse 5′-GTG GC TTCTACCC CAGAGAAAG -3′

Table 2. Nucleotide Sequences and Locations of Primers Used to Amplify *Oxtr* 5′ UTR regions in Chromatin Immunoprecipitation Experiments

Name	Position	Primer sequence
Prom1	−721/−650	Forward 5′-CATCC TTA ACTGC TAAG TCACATTGG -3′
		Reverse 5′ -GCCTT CCT CTA ACACGCTCAA -3′
Prom2	+261/+332	Forward 5′-AGCGGG AGTC CTTTTTTT CAG-3′
		Reverse 5′ -TTTTT CGT GTTT CTTC TTGA ATGG -3′
Prom3	+621/+683	Forward 5′-GGAG CTTGG GT CG TCATGA-3′
		Reverse 5′-GCCAGA CGT GTGGAGA AC CT-3′
Prom4	+841/+915	Forward 5′ -AGCTCC CAG ACGGAAAG ACA-3′
		Reverse 5′ -GTG GC TTCTACC CCAGAGAAAG -3′

Table 3. Nucleotide Sequences and Locations of Primers Used to Amplify Putative Enhancer Regions of Mouse *Pgr* Gene in Chromatin Immunoprecipitation Experiments.

Name	Position in Mouse genome	Position in Human genome*	Conserved ERE*	Primer sequence
Enh1	−81 kb	−168 kb	ERE1	Forward 5′ -AGCA ACA AGT TGGG TTAGT -3′
				Reverse 5′-CTCT CCAGGA TTGGT ACATTT-3′
			ERE3	Forward 5′ -CTGG TTG TTCTG ACA TT CTTAT TC-3′
				Reverse 5′-CACTC TG TCATG CTTG ATG TA-3′
Enh2	−147 kb	−205 kb	ERE1	Forward 5′ -CCTATT AGAGA ACAAGGAG ATG AC-3′
				Reverse 5′-GG TCACA TTA ACCC TTGGAA -3′
			ERE2	Forward 5′ -TCAAGAT GGG CAGGT ACAA-3′
				Reverse 5′-TAGA GCA GTG TTCC CAGAAA -3′
Enh3	−166 kb	−221 kb	ERE1	Forward 5′ -AT GCTCAA GTCACCAA GC-3′
				Reverse 5′-AATT GCC TCT CTGCACTTT-3′
Enh4	−200 kb	−306 kb	ERE3	Forward 5′ -TGCAAGA ATATA ATG GCAGAGA -3′
				Reverse 5′-CTA ACTTCGC TGC AGATAG AC-3′
Enh5	−207 kb	−311 kb	ERE1	Forward 5′ -ATGAGG GTTCTGT TT CCTTTAT -3′
				Reverse 5′-TAG GCTT CA CCACTTC CT-3′

*Boney-Montoya *et al.* (35). ERE, oestrogen response element.

method (38). Statistical significance was calculated by one-way ANOVA followed by Dunnett's multiple comparison test using GRAPHPAD PRISM.

Results

Oestradiol induces global changes in histone modifications in the VMH

To determine the effects of E_2 treatment on chemical modifications of histone proteins in the VMH of female mice, we first investigated the changes and the time course of total histone modifications in this brain region following E_2 administration. Ovariectomised (OVX) female mice were injected with E_2 (20 μg) or Veh and the brains were analysed 1, 3, 6 and 9 h later. The dose of E_2 was chosen to correlate the molecular and biochemical effects of the hormone with the expression of lordosis behaviour, which, in our experience, requires higher doses of E_2 when used independently of progesterone priming (39). Because the sustained dose-response range for E_2 is extremely wide (40), a dose of 20 μg is considered to be within the physiological range. Total extracts of VMH tissue were isolated from E_2 and Veh-treated mice and analysed by Western blotting using antibodies that recognise specific chemically modified histone residues. We measured global acetylation levels of histone H3 and H4 (H3Ac and H4Ac), trimethylation levels of H3K4, H3K36 and H3K9 (H3K4me3, H3K36me3 and H3K9me3), as well as double phosphorylation/acetylation marks on histone H3 (H3S10phK14Ac) (Fig. 1). Acetylation of Lys residues on histone H3 and H4 is a well-characterised modification that leads to an open chromatin structure and promotes transcription (41). Methylation of H3K4 and H3K36 residues is also associated with transcriptional activation, whereas methylation of H3K9 is generally regarded as a repressive histone mark (41). Moreover, as an example of cross-regulation of histone modifications, H3S10 phosphorylation has been shown to promote H3K14 acetylation in several experimental systems and is linked to transcriptional activation (42–44).

In the VMH of female mice, levels of total histone modifications were highly regulated following E_2 administration. Antibody that recognises double modification H3S10phK14Ac detected significant changes in the levels of this combinatorial histone mark. One-way ANOVA revealed a significant main effect of time of treatment ($F_{4,93} = 2.482$, $P = 0.049$) and a post-hoc Dunnett's multiple comparison test showed a significant increase 1 h after E_2 administration. However, H3S10phK14Ac appeared to be transient and, by 6 h following E_2 injection, the levels returned to baseline (Fig. 1A). Antibody that recognises H3 pan-acetylation detected significant changes following E2 injection (one-way ANOVA: $F_{4,110} = 4.813$, $P = 0.001$). In contrast to H3S10phK14Ac, total H3 acetylation detected by H3 pan-acetyl antibody increased with slower kinetics and reached significantly higher levels only 6 h after E_2 treatment according to a Dunnett's multiple comparison test (Fig. 1B). Another activating histone modification, H3K4me3, is also regulated in the VMH of female mice following E_2 administration. One-way ANOVA revealed a significant main effect of time of treatment ($F_{4,100} = 3.944$, $P = 0.005$) and a post-hoc Dunnett's multiple comparison test showed significantly higher levels 3 and 6 h following E_2 administration (Fig. 1C). An interesting pattern of changes was observed in the level of repressive H3 histone modification, H3K9me3. One-way ANOVA revealed a significant main effect of time of treatment ($F_{4,49} = 15.40$, $P < 0.0001$) and a post-hoc Dunnett's multiple comparison test demonstrated significant changes at all time points examined. Specifically, levels of H3K9me3 were significantly increased at the beginning (1 h) and towards the end (9 h) of the investigated E_2 time course and significantly decreased 3 and 6 h after injection. This decrease of H3K9me3 coincides with the significant up-regulation of H3K4 trimethylation (Fig. 1D). Interestingly, these data are in line with previous studies indicating that these two methylation events are mutually exclusive (42, 45). We failed to detect any changes in the levels of H3K36me3, an active chromatin mark on histone H3 (one-way ANOVA: $F_{4,63} = 1.699$, $P = 0.161$) (Fig. 1E). Finally, analysis of total acetylation levels of

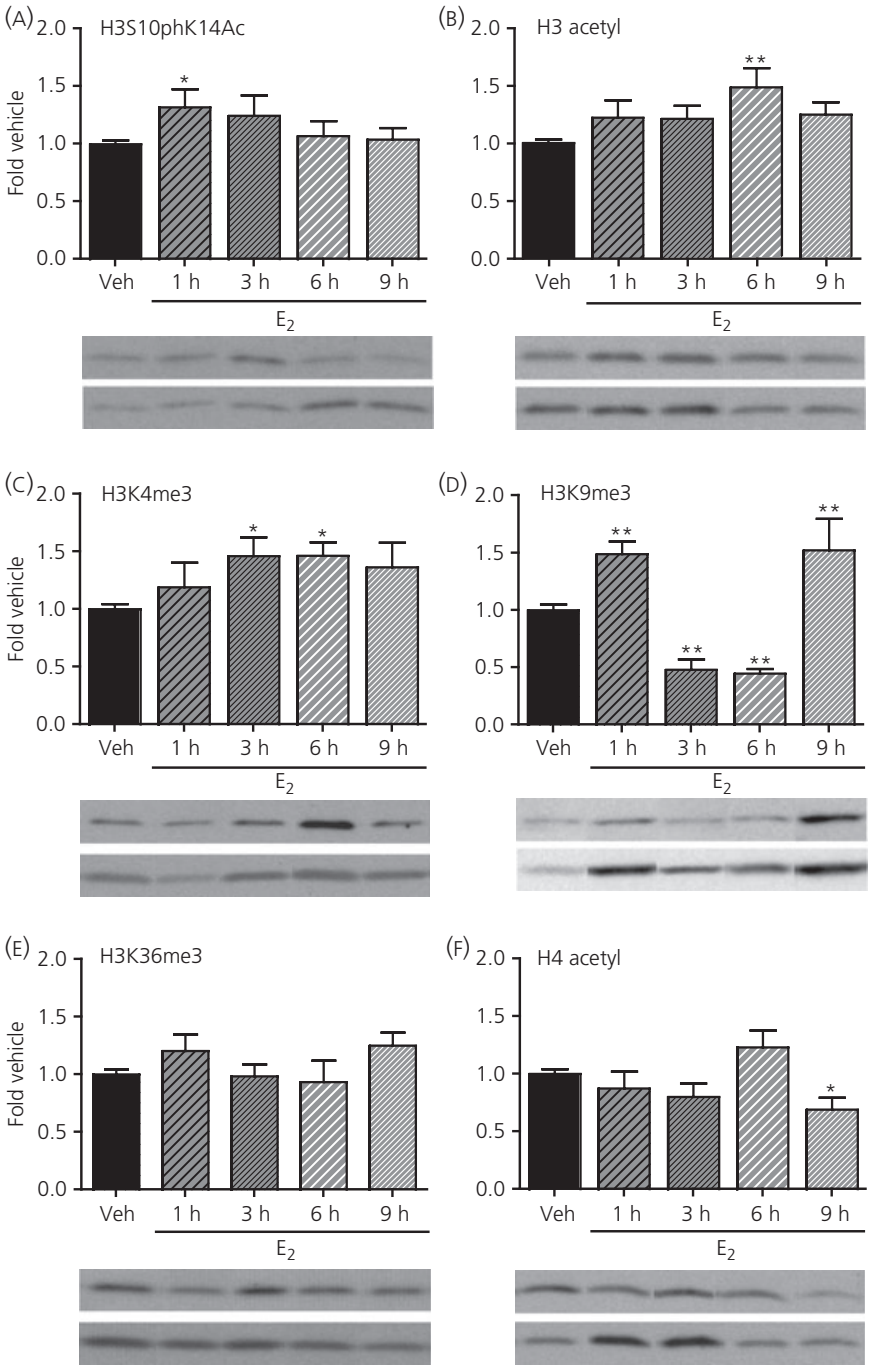

Fig. 1. Oestradiol (E_2) effects on the levels of histone modifications in the ventromedial nucleus of the hypothalamus (VMH) of female mice. Ovariectomised female mice were injected with E_2 or vehicle (Veh) and their brains were collected for the analyses 1, 3, 6 or 9 h later. The levels of histone modifications in VMH tissue were measured by Western blotting using antibodies that recognise specific modified residues: H3S10phK14Ac (A), H3 Acetyl (B), H3K4me3 (C), H3K9me3 (D), H3K36me3 (E) and H4 acetyl (F). Levels of each histone modifications were normalised to that of total histone H3. A representative Western blot for each histone modification examined is shown. Data reported are the mean of three to four independent experiments performed in triplicate. Data are expressed as the fold change (mean ± SEM). Statistical differences were calculated using one-way ANOVA with a post-hoc Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$.

histone H4 revealed a significant main effect of time of treatment (one-way ANOVA: $F_{4,117} = 4.051$, $P = 0.0041$) and a post-hoc Dunnett's multiple comparison test showed that H4 acetylation was significantly decreased 9 h following E_2 injection (Fig. 1F).

These data revealed that, in the female mouse, VMH E_2 treatment induces rapid changes in the covalent modifications of histone H3 tail residues. Moreover, specific modifications appear to be sequential and follow distinct temporal patterns. Collectively, these cova-

lent histone modifications establish a favourable environment for the transcriptional activity in the VMH of female mice within 3–6 h of E_2 treatment.

E_2-induced histone modifications are accompanied by ERα target gene up-regulation

The *Pgr* gene is a classic ERα target gene, which is regulated by E_2 in several tissues (46–48). Thus, we tested whether the E_2-induced 'permissive chromatin' state in VMH leads to the up-regulation of PgR expression. As expected, protein levels of both PgR isoforms, PgR-A and PgR-B, were significantly increased following E_2 administration (Fig. 2B,C). One-way ANOVA revealed a significant main effect of treatment ($F_{4,70} = 5.866$, $P = 0.0004$ for PgR-A and $F_{4,68} = 10.88$, $P < 0.0001$ for PgR-B) and a post-hoc Dunnett's multiple comparison test showed significantly higher levels of both isoforms 6 h after hormone injection, coinciding with the E_2-induced H3 acetylation and H3K4 methylation events.

To confirm that our chosen dose of E_2 was within the physiological range, we performed a control experiment in which PgR protein levels were also examined in VMH tissue of OVX female mice injected with 2 μg of E_2. A low dose of E_2, similar to the effect seen with a higher dose, led to significant induction of PgR protein 6 h after injection (see Supporting information, Fig. S1).

These data reveal that, in the VMH of female mice, an 'active' chromatin state brought about by E_2 exposure coincides with the transcriptional activation of an ERα target gene, *Pgr*, and may contribute to its regulation.

E_2 treatment induces association of activating histone marks with *Pgr* promoter in the VMH

To investigate how E_2-induced histone modifications contribute to the expression of the *Pgr* gene in the VMH of female mice, we examined the association of acetylated (H3Ac) and methylated (H3K4me3) histone H3 with the promoter of this gene using a ChIP assay. OVX female mice were injected with E_2 and Veh and brains collected 3 and 5 h later. The time-points to detect 'activating' histone marks at the regulatory sequences of the *Pgr* gene were selected to precede E_2-induce PgR protein up-regulation. Chromatin isolated from VMH tissue of E_2 and Veh-treated mice was immunoprecipitated using antibodies specific for H3Ac and H3K4me3. It has been shown that H3 histone acetylation and H3K4 methylation most often take place near the TSS of actively transcribed genes (49,50). Although the 5′ sequence of mouse *Pgr* gene has been reported (33), the functional analysis of mouse *Pgr* promoter has not been performed. On the other hand, the existence of two functional promoters for *PGR-A* and *PGR-B* isoforms has been described for rat and human *PGR* genes (31,32). Using sequence alignment, we mapped two regions within an approximately 2-kb sequence around the mouse *Pgr* TSS, which display close to 83% sequence identity with the rat *Pgr* gene promoters (Fig. 3A). Thus, four primer pairs (designated Prom1–4; Table 1), spanning the region between −720 and +915 bp relative to the TSS, were designed to analyse association of H3Ac and H3K4me3 with mouse *Pgr* gene promoter. Both H3Ac and H3K4me3 were enriched at the promoter sequences of the *Pgr* gene in the VMH of E_2-treated female mice compared to control mice. One-way ANOVA analysis revealed significant main effect of treatment on H3Ac at three promoter sites of the *Pgr*

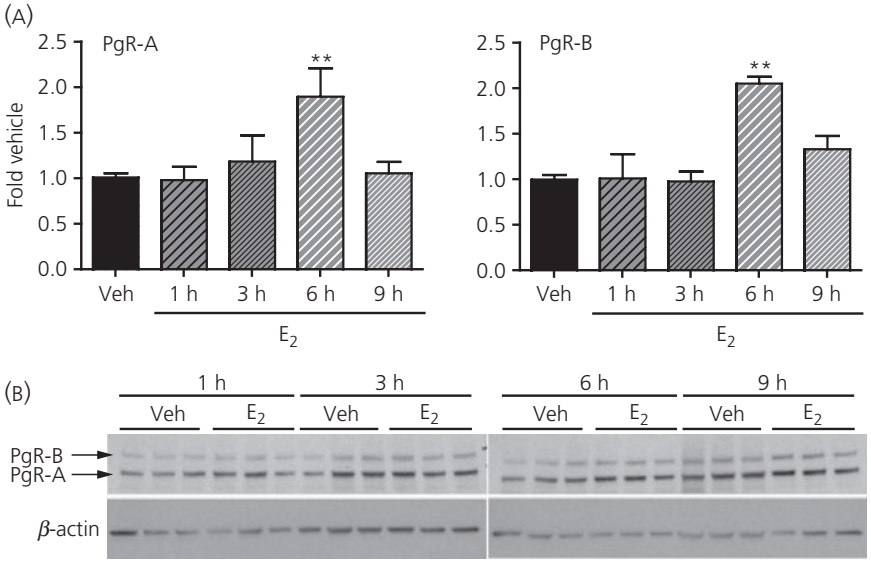

Fig. 2. Oestradiol (E_2)-induced up-regulation of progesterone receptor (PgR)-A and PgR-B in the ventromedial nucleus of the hypothalamus (VMH) of female mice. (A) Whole cell extracts isolated from the VMH of E_2 and vehicle (Veh)-treated mice were subjected to Western blotting using antibody that recognises both isoforms of progesterone receptor, PgR-A and PgR-B. PgR protein levels were normalised to that of β-actin and are expressed as the fold change (mean ± SEM). Data represent the mean of three independent experiments performed in triplicate. Statistical differences were calculated using one-way ANOVA with a post-hoc Dunnett's multiple comparison test. **$P < 0.01$. (B) Representative Western blot analysis of PgR protein expression in the VMH of female mice. Bands corresponding to PgR-A and PgR-B isoforms are indicated.

gene (Prom2, $F_{2,32} = 7.071$, $P = 0.003$; Prom3, $F_{2,32} = 4.599$, $P = 0.018$; Prom4, $F_{2,29} = 4.642$, $P = 0.018$). A post-hoc Dunnett's multiple comparison test revealed significant hyperacetylation of H3 at the Prom2 site, located between *Pgr-B* and *Pgr-A* promoters, 3 h after E_2 administration and at the Prom3 and Prom4 sites, located upstream of *Pgr-B* promoter region, at the 5-h time point (Fig. 3B).

Another histone mark leading to an open chromatin confirmation, H3K4me3, was also significantly up-regulated at *Pgr* gene promoter in the VMH of E_2-treated female mice at two promoter sites (Prom1, $F_{2,31} = 5.776$, $P = 0.007$; Prom4, $F_{2,31} = 5.611$, $P = 0.008$). A post-hoc Dunnett's multiple comparison test revealed a significant increase in H3K4me3 associated with the Prom1 and Prom4 sites, which span the *Pgr-B* and *Pgr-A* promoters, respectively, 5 h after E_2 injection (Fig. 3C). These data suggest that the mechanism by which E_2-activated ERα induces the transcription of the *Pgr* gene in mouse brain involves histone modifications and changes to chromatin structure at the promoter of this target gene.

E_2 affects histone modifications at the *Pgr* promoter in the POA

It has been well documented that E_2 regulates PgR expression in some brain regions but not others (37). One of the brain regions responsive to E_2 treatment is the POA. Thus, we examined histone modification status at *Pgr* gene promoter in the POA of female mice using a ChIP assay. One-way ANOVA analyses revealed significant effects of E_2 treatment on the associations of H3Acetyl with two promoter sites of the *Pgr* gene (Prom2, $F_{2,29} = 3.370$, $P = 0.048$; Prom4, $F_{2,32} = 4.4$, $P = 0.021$). We also observed a significant increase in the H3K4me3 levels at the most distal promoter site of the *Pgr* gene (Prom1, $F_{2,30} = 15.24$, $P < 0.0001$) (Fig. 4A,B). A post-hoc Dunnett's multiple comparison test revealed that both histone modifications (H3Acetyl and H3K4me3) were up-regulated in the VMH of female mice only 5 h after E_2 injection with no changes detected at the 3-h time point (Fig. 4A,B). These results demonstrate that the time course and pattern of E_2-induced histone modifications at the promoter of the *Pgr* gene is brain-region specific and may reflect the differences in the expression of PgR in these nuclei.

E_2 differentially affects histone modifications at *Oxtr* promoter in the POA and VMH

One of the gene products regulated by E_2 that facilitate the expression of lordosis behaviour is oxytocin receptor (51,52). Thus, we examined how E_2 treatment affects histone modifications at the promoter of the *Oxtr* gene in two brain regions, the VMH and POA of female mice. Three primer pairs (*Oxtr* prom1–3; Table 2) that span an approximately 600-bp sequence in the 5′ untranslated region (UTR) of the mouse *Oxtr* gene were designed and used to detect the association of acetylated and methylated histone H3 with the regulatory region of this gene (Fig. 5A). We observed different patterns and time-courses of E_2-mediated regulation of H3Ac and H3K4me3 at the promoter of *Oxtr* gene in the two brain regions (Fig. 5B). In the mouse VMH, one-way ANOVA analysis revealed a significant effect of E_2 treatment on both 'activating' histone marks, H3Acetyl and H3K4me3, albeit to a different degree. Histone H3 hyperacetylation was detected at two promoter sites of

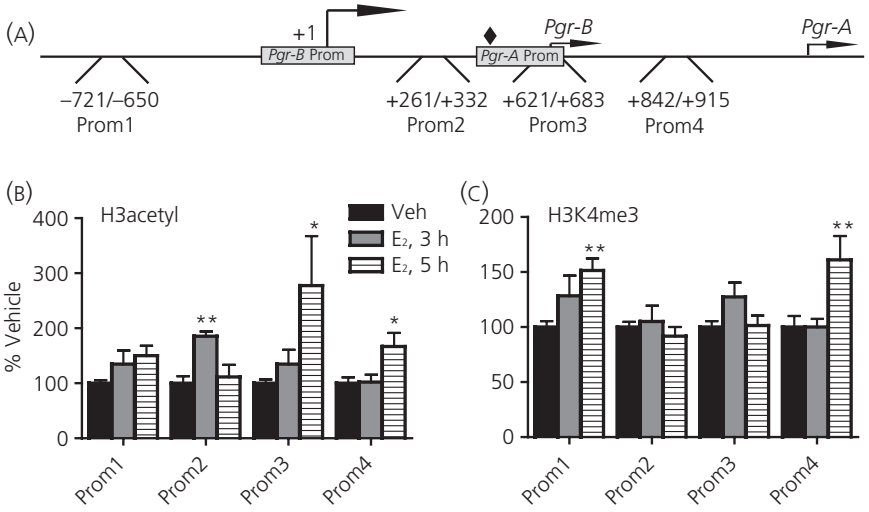

Fig. 3. Oestradiol (E_2) effects on histone modifications at the promoter of the *Pgr* gene in the ventromedial nucleus of the hypothalamus (VMH). (A) Schematic representation of mouse *Pgr* promoter and primer locations relative to the transcription start site (+1). Small arrows indicate translation start sites for *Pgr-B* and *Pgr-A* isoforms. Gray boxes denote sequences corresponding to two functional promoters of the rat *Pgr* gene. Black diamond indicates the location of the oestrogen response element (ERE) half site. Chromatin isolated from the VMH tissue of E_2 and vehicle (Veh)-treated female mice were immunoprecipitated with antibodies specific to (B) H3Acetyl and (C) H3K4me3. A quantitative polymerase chain reaction was used to amplify *Pgr* promoter regions associated with acetylated and methylated histone H3. Data reported are the mean of three independent experiments performed in triplicate. Data are expressed as percentage Veh (mean ± SEM). Statistical differences were calculated using one-way ANOVA with a post-hoc Dunnett's multiple comparison test. *$P < 0.05$; **$P < 0.01$.

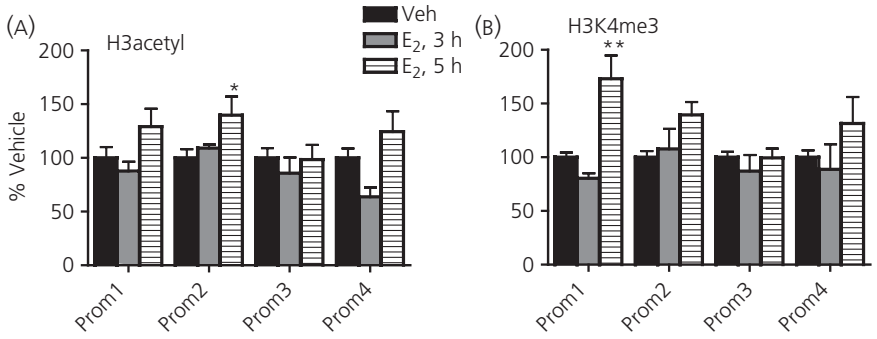

Fig. 4. Oestradiol (E_2) effects on histone modifications at the promoter of the *Pgr* gene in the preoptic area (POA). (A) Histone H3 acetylation and (B) H3K4 trimethylation at the promoter of the *Pgr* gene in the POA of female mice were determined using a chromatin immunoprecipitation assay. Data reported are the mean of three independent experiments performed in triplicate. Data are expressed as percentage vehicle (Veh) (mean ± SEM). Statistical differences were calculated using one-way ANOVA with a post-hoc Dunnett's multiple comparison test. *P < 0.05; **P < 0.01.

Oxtr gene (*Oxtr* prom2, $F_{2,27} = 3.770$, $P = 0.036$; *Oxtr* prom3, $F_{2,31} = 5.118$, $P = 0.0120$) and H3K4 trimethylation was increased at single promoter site (*Oxtr* prom2, $F_{2,32} = 3.575$, $P = 0.04$) (Fig. 5B). Furthermore, a post-hoc Dunnett's multiple comparison test failed to detect significant changes in H3Ac or H3K4me3 at any one of the specific sites of 5′ UTR of *Oxtr* gene 3 h after E_2 administration but revealed significant up-regulation of H3Acetyl and H3K4me3 at the 5-h time point (Fig. 5B).

In the mouse POA, one-way ANOVA analysis revealed the effect of E_2 treatment on H3Ac association with all three *Oxtr* promoter sites (*Oxtr* prom1, $F_{2,32} = 8.565$, $P = 0.001$; *Oxtr* prom2, $F_{2,32} = 6.471$, $P = 0.004$; *Oxtr* prom3, $F_{2,33} = 3.846$, $P = 0.032$) and a post-hoc Dunnett's multiple comparison test demonstrated significant hyperacetylation taking place at *Oxtr* prom1 and *Oxtr* prom2 only 5 h after E_2 administration (Fig. 5C**).** No changes were detected in the POA in the methylation status of H3K4 at any *Oxtr* promoter sites following E_2 administration. These results suggest that E_2 treatment induces distinct covalent modifications of histone H3 in the brains of OVX female mice in a gene- (*Oxtr* ≠ *Pgr*) and region- (POA ≠ VMH) dependent manner.

Effects of E_2 on the recruitment of ERα to the putative regulatory sequences on *Pgr* gene

E_2 regulates the transcriptional activity of target genes by binding to one of the two nuclear ERs, ERα and ERβ. Overwhelming evidence indicates that induction of the *Pgr* gene in the VMH of female mice and subsequent expression of lordosis behaviour critically requires ERα (12,14). Thus, we set out to examine whether E_2 treatment of OVX female mice results in the direct recruitment of ERα to the promoter of *Pgr* gene. For this, OVX female mice were injected with Veh or E_2 and chromatin isolated from the VMH 5 h later was analysed using a ChIP assay. The time-point to analyse the association of ERα with the regulatory sequences of the *Pgr* gene was selected to precede E_2-induce PgR protein up-regulation.

Reportedly, there are no consensus ERE sites within the promoter region of mouse *Pgr* gene; however there is a half ERE site within the *Pgr-A* promoter (Fig. 3A). Furthermore, recent studies suggest that several sequences approximately 150–300 kb upstream of human *PGR* gene contain ERE sites and act as enhancers to regulate *PGR* gene expression (35). Examination of approximately 200-kb genomic sequences upstream of mouse *Pgr* gene led to identification of seven ERE sites, which displayed high homology to the ERE sites characterised by Boney-Montoya *et al.* (35) in human as well as in mouse genomes (Dr Ann Nardulli, personal communication). Thus, we examined the direct association of ERα with these putative regulatory sequences of *Pgr* gene: four sites within the proximal promoter region (Fig. 3A and Table 1) and seven ERE-containing sites within approximately 200 kb upstream of the TSS (Fig. 6A and Table 3). In concert with previous studies, we did not observe E_2-regulated recruitment of ERα to the promoter region of the *Pgr* gene (28). We did, however, detect E_2-induced binding of ERα at two putative enhancer regions located approximately 81 and 200 kb upstream of the *Pgr* gene TSS (Fig. 6B). Statistical analysis using Student's t-test revealed a significant increase in the binding of ERα 5 h after E_2 administration only at enhancer 4 site ($P = 0.043$). This is the first evidence that the ERE-containing site 200 kb upstream of *Pgr* TSS may be involved in the regulation of the *Pgr* gene by ligand-activated ERα in the mouse brain *in vivo*. Surprisingly, little or no E_2-induced recruitment of ERα was observed at any other ERE sites following E_2 administration (Fig. 6B), suggesting that, contrary to data obtained in human breast cancer cells (28,35) these ERE-containing sites are not involved in the regulation of *Pgr* gene expression in the mouse brain. These data suggest that different regulatory sequences contribute to *Pgr* gene activation in different species.

Effects of E_2 on the expression of ERα cofactors in the VMH of female mice

Histone modifications are induced by specialised enzymes or enzyme complexes, which are recruited by transcription factors to promote or repress transcription of target genes though alteration of the chromatin structure. One of the ways to affect the chemical status of histone proteins is by regulating the expression and avail-

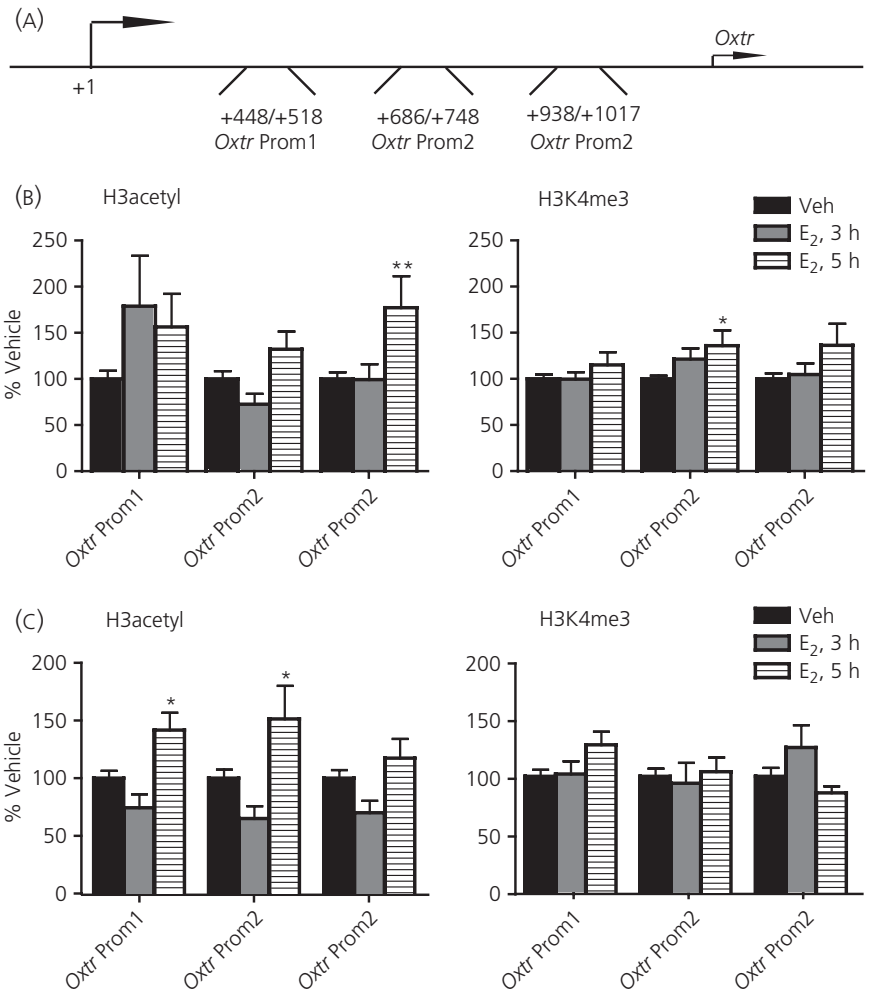

Fig. 5. Oestradiol (E_2) effects on histone modifications at the promoter of *Oxtr* gene in the ventromedial nucleus of the hypothalamus (VMH) and the preoptic area (POA). (A) Schematic representation of mouse *Oxtr* gene 5′ UTR and primer locations relative to the transcription start site (+1). A small arrow indicates the translation start site. Histone H3 acetylation and H3K4 trimethylation at the promoter of mouse *Oxtr* gene in (B) the VMH and (C) the POA of female mice were determined using a chromatin immunoprecipitation assay. Data reported are the mean of three independent experiments performed in triplicate. Data are expressed as percentage vehicle (Veh) (mean ± SEM). Statistical differences were calculated using one-way ANOVA with a post-hoc Dunnett's multiple comparison test. *$P < 0.05$; **$P < 0.01$.

ability of histone-modifying enzymes (53). Although a large number of ERα cofactors with HAT or HMT activity has been identified in human cancer cells, little or no information exists regarding which of these cofactors regulates the transcriptional activity of ERα in the nervous tissue. Thus, we examined the mRNA expression levels of some of transcriptional coregulators, which are able to bring about histone modifications that we detected in the VMH following E_2 treatment. For this, OVX female mice were injected with E_2 and Veh and the brains were analysed 3 or 5 h later. Total RNA was isolated from the VMH and POA tissues, reverse transcribed and the resultant cDNA was used as a template for qPCR. Expression of each gene of interest was normalised to that of a housekeeping gene (ribosomal 18S gene) and the relative ratio between the Veh and E_2-treated samples was calculated as described in the Materials and methods. Among the examined factors were SRC1, SRC2, CBP and p300, comprising well-characterised ERα coactivators with HAT activity, which are involved in ER-mediated transcriptional activation in various tissues, including the brain (15,30); MLL1 and MLL3, two important HMTs, which specifically modify H3K4 residue and are involved in the E_2-regulated expression of various *HOX* genes in human MCF-7 cells (20); WDR5, an essential DNA-binding subunit of MLL complexes (54,55); ASH1L, a H3K4 methyltransferase, not yet characterised as an ERα coregulator (55); LSD1, a coregulator with the activity to demethylase both H3K4 and H3K9, and thus either repress or promote nuclear receptor-mediated transcription (56,57); and JARID1A, which has been shown to regulate E_2-induced *PGR* expression in human MCF-7 cells through H3K4 demethylation (58).

We found that E_2 treatment induced significant changes in the levels of histone-acetylating cofactor mRNAs in the VMH of female mice (Fig. 7). The expression of two *Src* isoforms, *Src1* and *Src2*, was highly regulated by E_2-exposure as revealed by one-way ANOVA

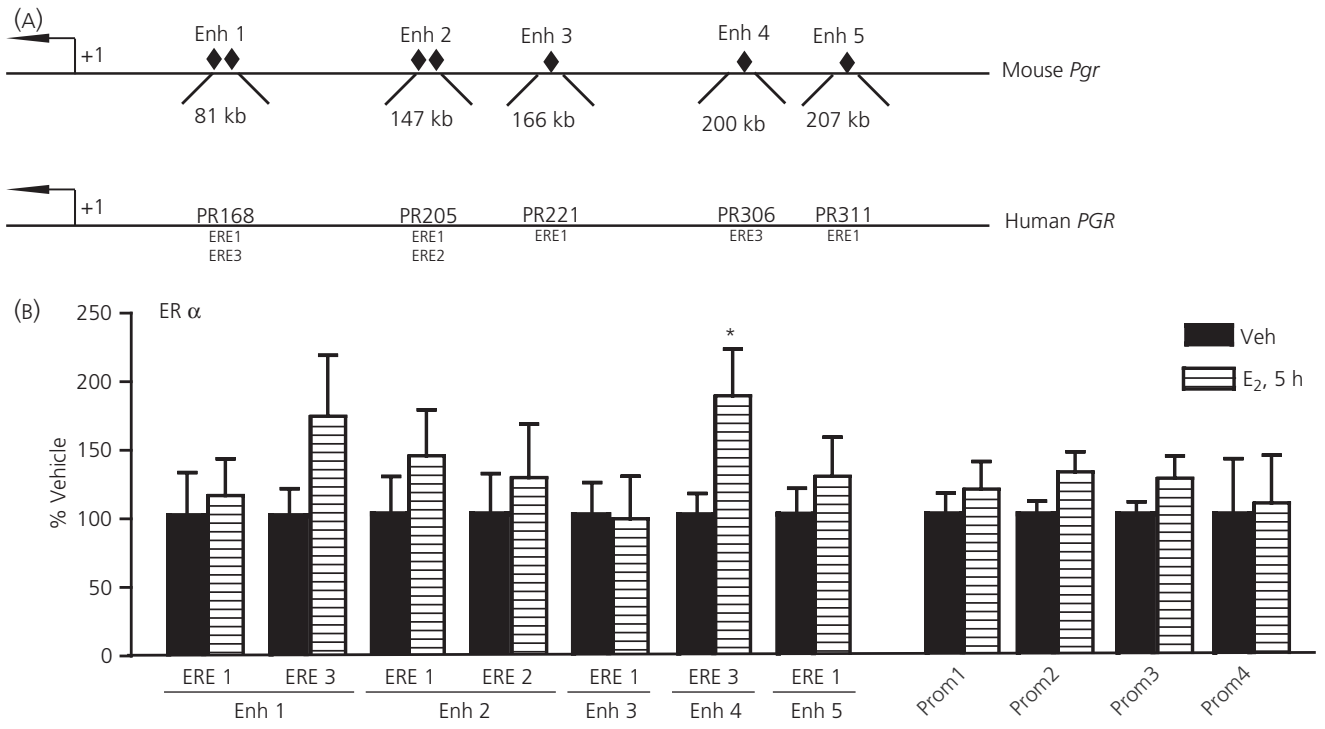

Fig. 6. Recruitment of oestrogen receptor (ER)α to the putative promoter and enhancer sites of mouse *Pgr* gene in the ventromedial nucleus of the hypothalamus (VMH). (A) Location of primers used to amplify putative enhancer sequences upstream of the transcription start site (+1) of mouse *Pgr* gene and corresponding enhancer sites in the human *PGR* gene (35). Black diamonds denote oestrogen response element (ERE) sites that are conserved between human and mouse enhancer sequences. Locations of primers used to amplify sequences within promoter region (Prom1–4) are as shown in Fig. 3(A). (B) Chromatin isolated from oestradiol (E_2) and vehicle (Veh)-treated mice was immunoprecipitated with antibody specific for ERα and associated genomic DNA was quantified by a quantitative polymerase chain reaction. Data are expressed as percentage Veh (mean ± SEM) and represent the mean of two independent experiments performed in triplicate. Statistical differences between E_2 and Veh-treated samples were calculated using Students's t-test. *$P < 0.05$.

(*Src1*, $F_{2,29} = 4.255$ $P = 0.024$; *Src2*, $F_{2,28} = 11.04$ $P = 0.0003$). A post-hoc Dunnett's multiple comparison test revealed significant up-regulation of both *Src* isoforms at the 3-h time point; however, 5 h after E_2 administration, *Src1* stayed elevated, whereas *Src2* mRNA levels returned to baseline. The other two homologous ER cofactors with HAT activity, CBP and p300, also displayed somewhat different patterns of changes. One-way ANOVA revealed a significant main effect of treatment on the expression of *p300* ($F_{2,28} = 22.07$, $P < 0.0001$) but not on the expression of *Cbp* ($F_{2,29} = 2.247$, $P = 0.124$). A post-hoc Dunnett's multiple comparison test demonstrated a transient pattern of *p300* up-regulation, which reached significantly high levels at 3 h and returned to baseline 5 h after E_2 injection (Fig. 7A).

The mRNA expression of two HMTs, *Mll3* and *Ash1*, as well as the accessory protein, *Wdr5*, was also regulated by E_2 in the VMH of OVX female mice (Fig. 7B). One-way ANOVA revealed a significant effect of treatment on the expression of *Mll3* ($F_{2,31} = 4.204$, $P = 0.0242$), *Ash1* ($F_{2,31} = 8.141$, $P = 0.0014$) and *Wdr5* ($F_{2,30} = 8.726$, $P = 0.001$). Moreover, a post-hoc Dunnett's multiple comparison test revealed differential temporal patterns of induction: the increase in the levels of *Ash1* and *Wdr5* mRNA levels was transient, with these being detected at the 3-h time point and returning to baseline after 5 h; *Mll3* mRNA up-regulation was delayed and only reached significant levels 5 h following E_2 administration (Fig. 7B). Interestingly, no changes were detected in the mRNA levels of *Mll1* (Fig. 7B).

The mRNA expression of the two examined histone demethylases (HDMs) was also regulated by E_2 exposure in the VMH of female mice (Fig. 7C). One-way ANOVA revealed a significant effect of treatment on the expression of *Lsd1* ($F_{2,28} = 3.715$, $P = 0.0371$) and *Jarid1A* ($F_{2,32} = 4.59$, $P = 0.0177$). However, a post-hoc Dunnett's multiple comparison test only detected significant down-regulation of *Lsd1* at 3 h, whereas the increase in *Jarid1A* mRNA detected at the same time point was not significant (Fig. 7C).

Effects of E_2 on the expression of ERα cofactors in the POA of female mice

Because (i) we detected differential acetylation and methylation events occurring at the promoters of ERα target genes in the two brain regions, VMH and POA and (ii) we hypothesise that differential regulation and expression levels of ERα cofactors may underlie these effects, we therefore examined the mRNA expression levels of given cofactors in the POA of OVX female mice following E_2 administration. Interestingly, we found that in POA

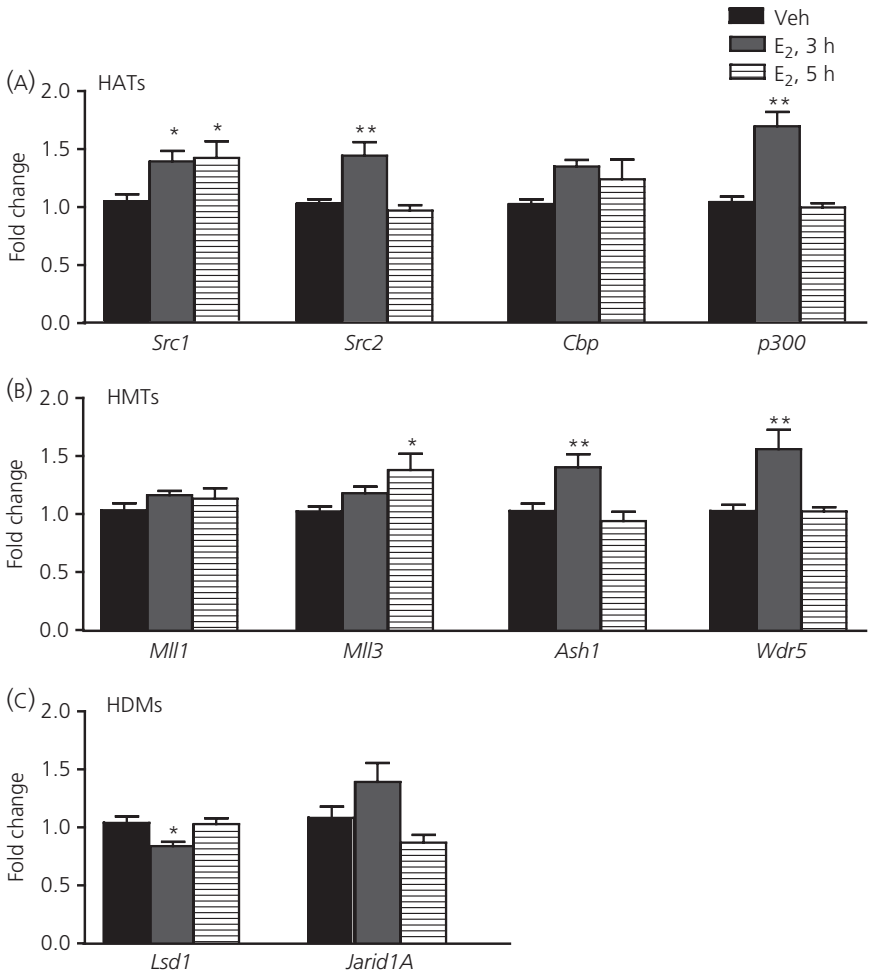

Fig. 7. Oestradiol (E_2) effects on the mRNA expression levels of histone-modifying factors in the VMH. Total RNA isolated from E_2 and vehicle (Veh)-treated mice was reverse transcribed and the expression levels of oestrogen receptor (ER)α cofactors, histone acetyltransferases (A), histone methyltransferases (B) and histone demethylases (C), were analysed by a quantitative polymerase chain reaction. Data are expressed as the fold change (mean ± SEM) and represent the mean of two independent experiments (n = 5 for each). Statistical differences were calculated using one-way ANOVA followed by a post-hoc Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$. HATs, histone acetyltransferases; HDMs, histone demethylases; HMTs, histone methyltransferases.

mRNA levels of examined HATs, HMTs and HDMs were more stable and less dynamically regulated by E_2-exposure compared to the VMH.

Specifically, we found that, in the POA of female mice, *Src1* and *Src2* mRNA levels do not change following E_2 administration (Fig. 8A). By contrast, there was a significant effect of treatment on the mRNA expression of *Cbp* ($F_{2,31} = 8.139$, $P = 0.0014$) and p300 ($F_{2,34} = 4.786$, $P = 0.0147$) as revealed by one-way ANOVA. Furthermore, a post-hoc Dunnett's multiple comparison test showed a significant down-regulation of *Cbp* 5 h after E_2 treatment and a similar trend for *p300*, although this failed to reach statistical significance (Fig. 8A).

Among the factors involved in H3K4 methylation, the effect of E_2 on mRNA expression was only observed for *Ash1* ($F_{2,31} = 4.196$, $P = 0.0244$) 5 h after E_2 administration, as revealed by one-way ANOVA. In contrast to the data obtained from the VMH, the expression of *Jarid1A* but not *Lsd1* was regulated by E_2 administration in the POA ($F_{2,32} = 4.222$, $P = 0.0236$) (Fig. 8C).

Taken together, these data demonstrate that one of the mechanisms by which E_2 induces changes in the chemical makeup of histone proteins in the mouse brain includes the regulation of histone-modifying cofactor mRNA expression. These data also support the hypothesis that the tissue-specific effects of E_2-activated ERα may be mediated by the availability of transcriptional cofactors.

Discussion

The present study shows that, in a specific hypothalamic region important for lordosis behaviour, exposure to oestradiol induces a specific set of histone modifications with distinct temporal characteristics. Moreover, E_2-induced histone modifications are detected at the level of global chromatin, as well as at the promoters of ERα target genes, and provide mechanistic insight into the early molecular events underlying hormonal effects on nervous tissue.

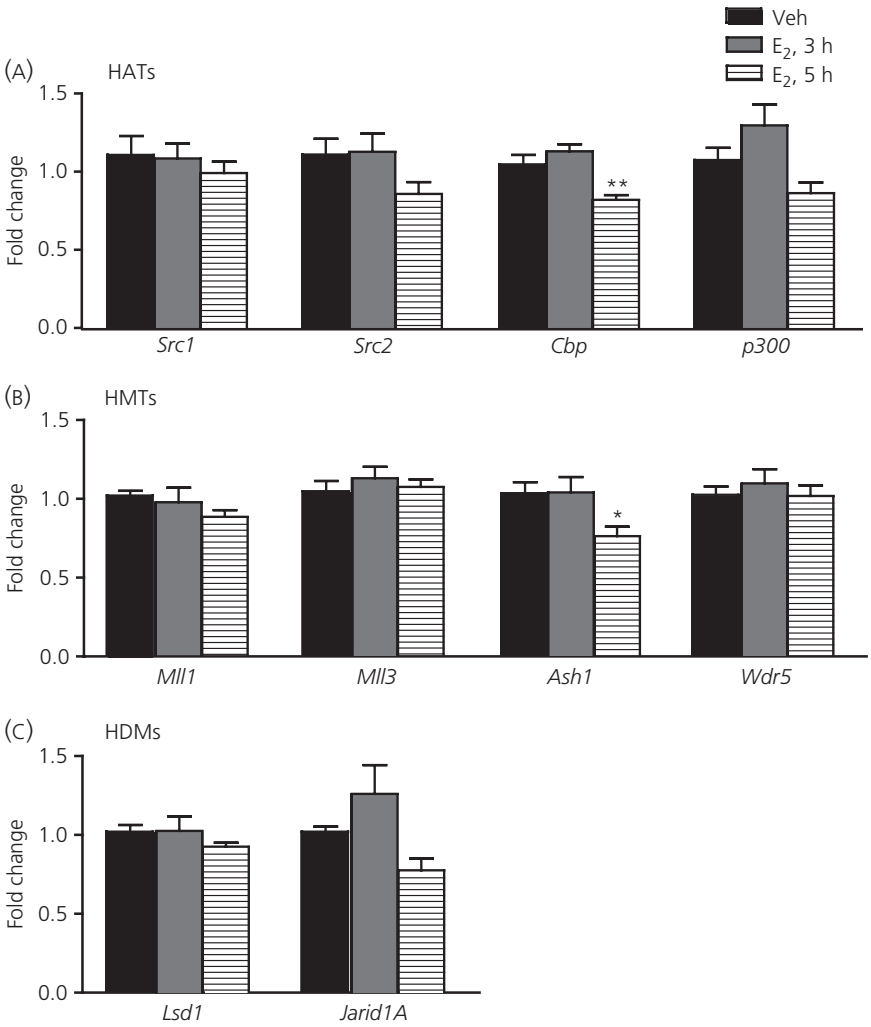

Fig. 8. Oestradiol (E_2) effects on the mRNA expression levels of histone modifying factors in the preoptic area (POA). Total RNA isolated from E_2 and vehicle (Veh)-treated mice was reverse transcribed and the expression levels of oestrogen receptor (ER)α cofactors, histone acetyl transferases (A), histone methyl transferases (B) and histone demethylases (C), were analysed by a quantitative polymerase chain reaction. Data are expressed as the fold change (mean ± SEM) and represent the mean of two independent experiments (n = 5 for each). Statistical differences were calculated using one-way ANOVA followed by a post-hoc Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$. HATs, histone acetyltransferases; HDMs, histone demethylases; HMTs, histone methyltransferases.

In the VMH of female mice, E_2 induces specific set of histone modifications

Acetylation of histone proteins has long been known to promote transcriptional activity and contribute to E_2-induced gene expression in human MCF-7 cells (50,59). Moreover, evidence supports the importance of histone acetylation in E_2-mediated behavioural and physiological processes, such as the masculinisation of sexual behaviour and formation of hippocampus-dependent memory in mice (60,61). Our preliminary data also suggest that histone hyperacetylation induced by histone deacetylase inhibitor, trichostatin A treatment, is able to facilitate E_2-induced sexual behaviour in female mice (see Supporting information, Fig. S3). Thus, it was not surprising that we observed an increase in the acetylation of histone H3 in the VMH of E_2-treated mice. By contrast, we did not find an increase in the levels of histone H4Ac, but rather detected a slight albeit significant decrease at the 9-h time point. Such differences in the patterns of H3 and H4 acetylation appear to be a characteristic early response to E_2 action specifically in the mouse brain. For example, in another brain region, the hippocampus, direct infusion of E_2 also resulted in the acetylation of H3 but not of H4 histone, whereas E_2 treatment of human MCF-7 cells does lead to H4 acetylation at the level of total chromatin, as well as at ERα target gene promoters demonstrating tissue-specific epigenetic effects of E_2 (60,62).

In addition, we have found that, in the VMH of female mice, E_2 administration induces a rapid phosphorylation/acetylation event on histone H3, H3S10ph/H3K14Ac. These two *cis* modifications on histone H3 have been shown to occur in a cooperative fashion, with phosphorylation of H3S10 promoting subsequent acetylation of

H3K14 (45,46). Interestingly, E_2-induced acetylation of H3 (on H3K14) in the mouse hippocampus has been shown to critically depend on ERK activation (60), whereas, in human MCF-7 cells, E_2 also induced H3S10 phosphorylation in a MEK1 kinase-dependent manner (62). Based on this evidence, we can infer that a phosphorylation event, possibly of H3S10, by mitogen-activated protein kinase may be a prerequisite for H3K14 acetylation and a common mechanism for E_2-induced epigenetic effects.

In the VMH of female mice, E_2 administration also led to a significant increase in the levels of H3K4me3, another 'permissive' histone modification, which has been shown to be important for the transcriptional activity of ERα in human breast cancer cell model (28). In addition, our data support the previous finding that H3K4 and H3K9 methylation marks are mutually exclusive (45,48): at 3 and 6 h after E_2 administration, the levels of H3K4me3 in the VMH are significantly increased, whereas levels of H3K9me3 are dramatically decreased. These data, in conjunction with the marked elevation of H3 histone acetylation 6 h following E_2 injection, suggest that, in this behaviourally critical hypothalamic nucleus, hormone treatment induces rapid changes in the make-up of histone proteins. As a result, the combinatorial effect of these changes creates a permissive environment for the transcriptional activity within a distinct time window.

Our analyses also revealed some unexpected results. Notably, in the VMH of female mice, we detected a significant up-regulation of repressive H3K9 methylation within 1 h of E_2 administration, as well as 9 h after E_2 injection, just when the effects of hormones on histone H3 acetylation and H3K4me have worn off. It is well established that E_2 treatment leads to the down-regulation of a number of genes in various mouse tissues (63,64). Moreover, microarray analysis of E_2-regulated genes in mouse uterine tissue had revealed distinct temporal patterns of target gene activation as well as repression (64). Specifically, approximately 17% of total genes identified by Watanabe *et al.* (64) were down-regulated by E_2 treatment within the first hour. Among these were genes coding for signal transduction molecules and transcription factors, indicating that their rapid down-regulation may lead to further amplification of the effects of E_2. Our data suggest that similar mechanisms may be in place in the VMH as well; however, further studies are needed to examine the precise role of ERα repressed genes on the CNS function and physiological outcomes of E_2 treatment. On the other hand, the marked increase in the levels of H3K9me3, together with significant down-regulation in the basal levels of H4 acetylation, 9 h after E_2-exposure, may be indicative of termination of the effects of E_2 on VMH cells.

The pattern of E_2-induced histone modifications at the promoters of target genes is brain-region and gene-specific

We used the ChIP assay to extend our analyses and examine E_2-effects on the histone modifications at the promoters of specific ERα-target genes, *Pgr* and *Oxtr*, in two hormone-responsive brain regions, the VMH and POA. Our data revealed interesting brain-region and gene-specific effects of E_2 treatment (Table 4). Specifically, we found differences in the pattern and the time-course of histone H3 acetylation associated with the *Pgr* gene promoter in the two nuclei: rapid and sustained hyperacetylation (H3Ac) in the VMH and delayed and less pronounced H3Ac in the POA. These data are in agreement with a study demonstrating anatomic specificity of PgR regulation by E_2 in the rodent brain (25). By contrast, the degree and time-course of E_2-induced H3K4 methylation at *Pgr* the promoter was found to be similar in both the VMH and the POA, indicating the importance of this specific histone modification in the regulation of the *Pgr* gene. Moreover, methylation of H3K4 in response to E_2 appears to be a common requirement for the expression of the *Pgr* gene across tissues because knockdown of *Mll1*, the methyltransferase responsible for this modification, leads to a thwarted effect of E_2 in human MCF-7 cells as well (28).

Table 4. Summary of Oestradiol-Induced Histone Modifications at the Promoters of *Pgr* and *Oxtr* Genes in the ventromedial Nucleus of the Hypothalamus (VMH) and Preoptic Area (POA) of Female Mice.

	Pgr				Oxtr			
	H3Ac		H3K4me3		H3Ac		H3K4me3	
	VMH	POA	VMH	POA	VMH	POA	VMH	POA
3 h	↑	–	–	–	–	–	–	–
5 h	↑↑	↑	↑↑	↑	↑	↑↑	↑	–

Each arrow denotes a significant change at single promoter site. ↑$P < 0.05$; ↑$P < 0.01$.

Interesting gene-specific effects were observed when we analysed histone modifications associated with the *Oxtr* promoter (Table 4). In contrast to *Pgr*, H3 histone acetylation appeared to be more prominently regulated by E_2-exposure at the promoter of this specific ERα-target gene, whereas little or no changes in the levels of H3K4me were detected. Moreover, an increase in the levels of H3Ac at the *Oxtr* promoter was only detected 5 h following treatment and this effect was evident in both hypothalamic nuclei examined, the POA and VMH. Such delayed kinetics of 'activating' histone modifications associated with *Oxtr* promoter may reflect the time course of E_2-induced OXTR expression, which only becomes detectable in the VMH after 6 h and reaches significant levels at 24 h (65). These data suggest that the temporal regulation of early epigenetic events, such as covalent histone modifications induced by E_2 treatment, may be the determining factor of the time course of gene induction.

E_2 regulates the expression of transcription cofactors in a brain-region specific manner

Transcription factors, such as nuclear receptors, recruit coregulators with intrinsic histone acetyltransferase and histone methyltransferase activity to induce specific histone modifications at the regulatory sequences of target genes (15). It has been postulated that regulation of cofactor expression levels and availability is one of the mechanisms that impacts histone modifications and chromatin structure (52,53). Our data, demonstrating that E_2-exposure significantly up-regulates mRNA levels of several ERα cofactors with HAT

and HMT activity in the VMH of female mice, suggest that similar mechanisms may underlie E_2-induced changes in the status of histone proteins in the brain. To date, a vast number of ERα cofactors have been identified in reproductive tissues and cell culture systems, which are important for the regulation of ERα-target genes, as well as the physiological and pathological effects of E_2 (15). However, only few of these have been functionally tested in the nervous system. Among them, two SRC isoforms, SRC1 and SRC2, were shown to be co-expressed with ERα in hormone responsive brain regions, physically interact with the receptor and be required for E_2-dependent expression of the *Pgr* gene and reproductive behaviours (29,30). The important role of SRC1, as well as another ERα cofactor with HAT activity, CBP, has also been demonstrated in the development of sexually dimorphic behaviours during the perinatal period in female rats (66). In the present study, we found that, following E_2 administration, mRNA levels of two *Src* isoforms (1 and 2), as well as *p300*, are increased in the VMH of female mice. Interestingly, the observed up-regulation of cofactor mRNA either coincided or preceded H3 histone acetylation events detected at the level of total chromatin and at the promoters of ERα-target genes. Although we have not explored the causal relationship between HAT mRNA induction and histone acetylation detected in the VMH of female mice, the time-courses of these two events lead us to suggest a cause-effect connection (Fig. 7). Among the examined HATs, the E_2-induced increase in mRNA levels of *Src2* and *p300* was very rapid and transient, detected only at 3 h and gone by the 5-h time point. *Src1* was the only co-activator that was persistently elevated following E_2 treatment, suggesting possible functional specificity of this isoform. These results also emphasise the importance of the time-scale of experimental design. When studying the effects of E_2 on the brain, molecular mechanisms and correlates are often investigated at the same time-scale as behavioural outcomes, 24 or 48 h after E_2 treatment. Our study was specifically designed to investigate early molecular events, which take place over the scale of a few hours. These differences may be the bases for the discrepancies between our findings showing that *Src*1 and *Src2* mRNA levels in VMH are regulated by E_2, and previous reports that failed to detect such regulation 24 h after E_2 treatment (67).

Although several histone methyltransferases have been shown to contribute to ERα transcriptional activity in MCF-7 cells, the importance of H3K4me and its modifying enzymes in E_2-mediated effects on the CNS have not been addressed. In the present study, we detected a significant increase of mRNA levels of two HMTs, *Mll3* and *Ash1*, as well as an essential subunit of the MLL complex, *Wdr5*, in the VMH, following E_2 administration. The time course of the increase in HMT mRNA expression coincided with the H3K4 methylation events at the promoters of *Pgr* and *Oxtr* in the VMH of female mice, once again suggesting a causal relationship. Interestingly, MLL1 was shown to be one of the factors required for the expression of the *PGR* gene in human MCF-7 cell (28); however, we did not observe any changes in the levels of this specific HMT. These data suggest that the effect of MLL1 on *Pgr* expression may be tissue specific and that another isoform, MLL3, which is persistently up-regulated in VMH by E_2, is important for *Pgr* regulation in the brain. Alternatively, in the brain, E_2 may affect the recruitment of MLL1 to the promoter of the *Pgr* gene without altering its levels, and further studies will aim to address this possibility. Nonetheless, significant up-regulation of *Wdr5*, a common DNA-binding subunit of both MLL1 and MLL3 complexes, in the VMH of female mice, indicates the important role of these HMT complexes in E_2-induced transcriptional regulation. Interestingly, it has been reported that genetically-modified female mice, which express catalytically inactive MLL3 (*Mll3*) were infertile and males with the same mutation were hypofertile (68). Although no detailed characterisation of this phenotype was provided, given the critical requirement of ERα transcriptional activity for the female reproduction, both at the level of reproductive tissues as well as at the level of the hypothalamic behavioural circuit, we can hypothesise that the infertility observed in these mutant mice was a result of disruptions in E_2-signalling. Moreover, MLL complexes have been previously implicated in the development and various functions of the rodent nervous system, such as nerve cell differentiation, adult neurogenesis and memory formation (69–71), which emphasises the importance of studying the role of these epigenetic factors in the brain.

It has been suggested that tissue- and gene-specific effects induced by ligand-activated ERs may be mediated through differential recruitment or expression of coregulator proteins. In the present study, we tested whether brain-region specific histone modifications (H3 acetylation and H3K4 methylation), which we detected at the promoters of *Pgr* and *Oxtr* genes in the VMH and the POA, may be the result of differential expression of ERα cofactors. Our results, showing vast differences in the mRNA expression of several histone-modifying cofactors between these nuclei, confirmed this hypothesis. Although mRNA expression of most cofactors with HAT or HMT activity was significantly up-regulated in the VMH of female mice, we observed either no change or, in a few cases, a decrease in the levels of these factors in the POA (Figs 7 and 8). These data indicate that, in the POA, E_2-induced histone acetylation and H3K4 methylation at the promoters of *Pgr* and *Oxtr* genes does not require the activity of given coregulators. Alternatively, E_2-induced histone modifications in these brain regions may be the result of the differential recruitment of these factors to the regulatory sequences of the *Pgr* and *Oxtr* genes. In conclusion, based on our results, we propose that E_2-activated ERα may employ differential epigenetic mechanisms to induce brain-region specific transcription of target genes in the VMH and the POA of female mice.

E_2-regulated recruitment of ERα to the putative regulatory-sequences is different between human and mouse

Although it is well established that *Pgr* gene expression is directly regulated by ligand-activated ERα, we failed to convincingly demonstrate the recruitment of ERα to the regulatory sequences on the *Pgr* gene. There is no ERE site within the promoter region of either mouse *Pgr* or human *PGR* genes. Alternatively, it was suggested that half-ERE and proximal Sp1 or AP-1 sites may be sufficient for ERα recruitment to human *PGR* gene promoter (72,73). More recent

studies have shown that ERα does not bind within the promoter region of human *PGR* gene but is rather recruited to the remote enhancer sequences as far as 300 kb upstream of the TSS. In concert with these data, we did not observe E_2-regulated binding of ERα to the proximal promoter sequences of mouse *Pgr*. We did, however identify two putative enhancer sites approximately 81 and 200 kb upstream of the mouse *Pgr* gene TSS, which displayed E_2-regulated recruitment of ERα (Fig. 6). These were two out of seven ERE-containing sites homologous to the enhancer regions described by Boney-Montoya *et al.* (35) in human MCF-7 cells. Lack of E_2-regulated binding at other five ERE-containing sites may be the result of sequence differences between species known to affect ERα binding (74). Alternatively, other transcription factors may be required for the binding of ERα to the chromatin. Indeed, genome-wide analysis of ERα binding sites revealed that one such factor, Forkhead (FoxA1), binds in the close proximity of ERα and is required for ERα association with chromatin in human cell line (34,75). Moreover, Fox1A has been identified as one of only four coregulators essential for the expression of the *Pgr* gene in MCF-7 cells (28). Thus, further analyses of the role of this pioneer factor in the transcriptional activity of ERα in the mouse brain are warranted. Such studies will provide valuable information about the molecular mechanisms of E_2-regulated gene expression in the brain and lead to a better understanding of the effects of hormones on CNS functions.

Conclusions

Although oestrogens exert a considerable influence on the variety of functions of the nervous system not only in females, but also in males, the studies of such influences have mostly focused on behavioural, physiological or pathological effect. Studies on molecular mechanisms that underlie the actions of oestrogens and the analyses of gene networks regulated by hormones are most often performed using more easily accessible and manipulated cell culture systems. This is understandable, considering the challenging technical issues that neuroscientists have to overcome when studying molecular events induced by ligand-activated hormone receptor in the brains of living animals: individual variability, as well as a small amount and heterogeneity of starting material (presence of glial cells, neurones that do not express ERα, and not all ERα expressing neurones being identical). As a result, molecular and biochemical changes in response to hormonal treatments are always more subtle in the brain than in the homologous cell culture systems. However, given the vastly diverse physiological, cellular and transcriptional effects that oestrogens exert on different tissues and cell types, it is of utmost importance to address the questions of molecular transcriptional mechanisms underlying oestrogens actions in a relevant system. Moreover, the prevalent use of hormone replacement therapy in postmenopausal women, as well as the use of different selective oestrogen receptor modulators for the treatment of breast and endometrial cancers or osteoporosis, highlights the immediate benefits of such studies aiming to understand the effects of hormones on women's health and disease

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Supporting Information

The following supplementary material is available:

Fig. S1. Induction of progesterone receptor protein levels in female mice ventromedial nucleus of the hypothalamus (VMH) after administration of low dose oestradiol (E_2).

Fig. S2. Immunoprecipitation of chromatin-bound oestrogen receptor (ER)α by anti-ERα antibody.

Fig. S3. Facilitation of lordosis behaviour by histone deacetylase inhibitor.