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Early histone modifications in the ventromedial hypothalamus and preoptic area following oestradiol administration

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Abstract

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 hours. 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 aim of this study was to investigate early molecular epigenetic events underlying oestrogen-regulated transcriptional activation of the *Pgr* gene in the VMH of female mice. Oestradiol (E2) 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; and a transcriptional repressive (H3K9me3) modification showed a different temporal pattern. Collectively these should create a permissive environment for the transcriptional activity necessary for lordosis, within 3-6 hours after E2-treatment. In the VMH, changes in the H3Ac and H3K4me3 levels of histone H3 were also detected at the promoter region of *Pgr* gene within the same time window, but 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 E2 treatment. In the VMH of female mice, E2-treatment resulted in the recruitment of ER α to the oestrogen-response-elements-containing putative enhancer site of *Pgr* gene ~200kb upstream of the transcription start site (TSS), but failed to increase ER α association with the more proximal promoter region. Finally, E2 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 E2-treatment involve highly specific changes in chromatin structure, dependent on cell group, gene, histone modification studied, promoter/enhancer site and time following E2.

Keywords

oestrogens; oestrogen receptor; progesterone receptor; hypothalamus; transcriptional regulation; histone modification

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Introduction

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 for 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 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 alpha (ER α) [7], and oestrogen receptor beta (ER β) [8], 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 (ERE) within the regulatory regions of target genes [9-11]. Studies using genetically modified mice with disrupted ER α as well as temporally restricted knockdown of this receptor, specifically in the VMH, confirm that signalling through ER α 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 ER target genes, the induction of the *Pgr* gene by oestradiol (E2)-activated ER α 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 ER α target genes and its induction by E2 has been extensively studied in a number of E2-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 ER α is quite complex and displays tissue and age-dependent patterns [24-26]. Requirement of different SRC isoforms for the regulation of *Pgr* gene expression by E2-activated ER is one clear example of a tissue-specific hormonal effect. In human MCF-7 breast cancer cell line, the expression of the *Pgr* gene in response to E2 stimulation requires SRC3, but not SRC1 and SRC2 [27,28]. In contrast, in the brains of rats and mice, SRC1 and SRC2 are necessary for the induction of the *Pgr* gene as well as expression of reproductive behaviour, while 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 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 ER α , there are no consensus ERE motifs near the transcription start sites. There is, however, an ERE half site within the *Pgr-A* promoter, that is conserved in the mouse, rat and human genome [31-33]. Recently, several ER α binding sites have been identified between 48 and 311kb upstream of the human *PGR-B* TSS [34,35]. Three of these sites have been shown to associate with the TSS in an E2-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 ER α 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 current study we investigated early molecular events associated with E2-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 *Pgr* gene were examined to determine the effects of E2 on chromatin architecture and E2-target gene expression. We also performed comparative analyses of histone modifications at the promoters of two of E2-target genes, *Pgr* and *Oxtr*, in two E2-responsive hypothalamic nuclei, VMH and POA, to investigate the molecular mechanisms of gene- and region-specific effects of E2 in the brain. Chromatin immunoprecipitation (ChIP) was performed on the VMH tissue of female mice to detect the recruitment of ER α to the putative regulatory sequences of *Pgr* gene and led to the identification of an E2-regulated site ~200kb upstream of TSS of the mouse *Pgr* gene. In addition, mRNA expression analysis in the VMH and POA of female mice revealed that E2 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 eight-week-old female Swiss-Webster mice were ovariectomised (OVX). After a weeklong recovery period mice were injected intraperitoneally (i.p) with 20 μ g 17 β -oestradiol (E2)(Sigma, St. Louis, MO) diluted in 100 μ l vehicle solution (10% ethanol/saline). Control mice were injected only with vehicle (Veh) solution. Mice were sacrificed 1, 3, 6, and 9 hours after E2 injections, 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 bad ventral side up and slices containing areas of interest were cut using brain mold and sterile blades. For POA, optic chiasm and olfactory tubercle were used as landmarks to isolate ~0.5mm slice between 0.5 to -0.10 relative to bregma; and for VMH, pituitary stalk and mammary bodies were used as landmarks to isolate ~1mm slice between -1.22 to -2.06 relative to bregma. Subsequently areas containing POA and VMH were dissected from coronal slices according to the mouse brain atlas [36].

SDS-PAGE and Western Blot

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 were pooled. Total protein concentration was determined using BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL) and 30µg of protein was ran on 4-20% gradient Tris-glycine Ready gels (BioRad, Hercules, CA). Protein was transferred on PVDF membrane (BioRad, Hercules, CA) and blocked for 1h 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). Primary antibody incubation was done at 4°C overnight followed by washes and incubation with a secondary horseradish peroxidase-labelled goat anti-rabbit antibody (GE Healthcare, Piscataway, NJ) for 1 h at RT. Membranes were washed and immunolabelled proteins were visualised with enhanced chemiluminescence (ECL) detection reagent (GE Healthcare). Quantitative densitometric analysis was performed using NIH Image software. 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 software. 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 (SCBT, Santa Cruz, CA).

Chromatin immunoprecipitation and qPCR

ChIP assay was performed using EZ-Magna ChIP kit (Millipore) according to manufacturer's instructions with some modifications. Briefly, VMH and POA tissue collected from Veh and E2-treated mice was cut into ~1mm pieces and crosslinked in 1% formaldehyde at RT for 15min. To perform ChIP with ER α antibody (Clone: TE111.5D11, Thermo Fisher Scientific) an additional cross-linking step with 2 mM Ethylene Glycol-bis (Succinimidylsuccinate) (EGS, Thermo Scientific) was introduced, followed by 30min 1% formaldehyde [37]. Tissue from 2-4 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), cleared by centrifugation and the protein concentration was determined using BCA protein assay kit (Thermo Fisher Scientific). Equal amount of chromatin (300µg) were 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). 10% of each sample amount (30µg) was removed and saved as input. For immunoprecipitating ER α 1mg of chromatin was used for each sample and 5% of sample amount was saved as input. The specificity and efficiency of ER α antibody is shown in Figure 2S. 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 Ct 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 software. 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 #U12644) and *Oxtr* gene (accession #D86631). Sequences and positions of primers used to detect binding of ER α to the upstream putative enhancer regions of the mouse *Pgr* gene are listed in Table 3. Primers were designed using either Primer Express software (Applied Biosystems, Carlsbad, CA) or PrimerQuest (Integrated DNA Technologies, Coralville, IA).

Total RNA isolation and gene expression

OVX female mice were injected with 20µg E2 or Vehicle solution (i.p) and sacrificed 3 or 5 hours after. Brains were removed and immediately frozen. Total RNA was isolated from VMH and POA using Trizol® reagent (Life Technologies, Grand Island, NY) according to manufacturer's instructions. Equal amount of total RNA (100ng) was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies) and the resultant cDNA was used as a template for qPCR. Following 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). Relative ratio of the expression of each gene was calculated using the $2^{-\Delta\Delta C_t}$ method [38]. Statistical significance was calculated by one-way ANOVA followed by Dunnett's Multiple Comparison Test using GraphPad Prism software.

Results

Oestradiol induces global changes in histone modifications in the VMH

In order to determine the effects of E2-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 E2 administration. Ovariectomised (OVX) female mice were injected with E2 (20µg) or vehicle (Veh) and the brains were analysed 1, 3, 6 and 9 hours later. The dose of E2 was chosen to correlate molecular and biochemical effects of the hormone with the expression of lordosis behaviour, which in our experience requires higher doses of E2 when used independent of progesterone priming [39]. Since sustained dose-response range for E2 is extremely wide [40], 20µg dose is believed to be within the physiological range. Total extracts of VMH tissue were isolated from E2 and Veh-treated mice and analysed by Western blot 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 mark 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 a transcriptional activation [42-44].

In the VMH of female mice, levels of total histone modifications were highly regulated following E2-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 the treatment ($F(4,93)=2.482$, $p=0.049$) and *post-hoc* Dunnett's multiple comparison test showed significant increase 1 hour after E2 administration. However, H3S10phK14Ac appeared to be transient and by 6 hours following E2 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 hours after E2 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 E2 administration. One-way ANOVA revealed a significant main effect of time of the treatment ($F(4,100)=3.944$, $p=0.005$) and *post-hoc* Dunnett's multiple comparison test showed significantly higher levels 3 and 6 hours following E2 administration (Fig. 1C). 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 the treatment ($F(4,49)=15.40$, $p<0.0001$) and *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 hour) and towards the end (9 hours) of the investigated E2 time course and significantly decreased 3 and 6 hours after injection. This decrease of H3K9me3 coincides with the significant upregulation of H3K4 trimethylation (Fig. 1D). Interestingly, these data are in line with previous reports 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 histone H4 revealed a significant main effect of time of treatment (one-way ANOVA, $F(4,117)=4.051$, $p=0.0041$) and *post-hoc* Dunnett's multiple comparison test showed that H4 acetylation was significantly decreased 9 hours following E2 injection (Fig. 1F).

These data revealed that in the female mouse VMH E2 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 covalent histone modifications establish favourable environment for the transcriptional activity in the VMH of female mice within 3-6 hours of E2-treatment.

E2-induced histone modifications are accompanied by ER α target gene upregulation

The *Pgr* gene is a classic ER α target gene, which is regulated by E2 in several tissues [46-48]. Thus, we tested whether E2-induced "permissive chromatin" state in VMH leads to the upregulation of PgR expression. As expected, protein levels of both PgR isoforms, PgR-A and PgR-B, were significantly increased following E2 administration (Fig. 2B and 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 *post-hoc* Dunnett's multiple comparison test showed significantly higher levels of both isoforms 6 hours after hormone injection, coinciding with the E2 induced H3 acetylation and H3K4 methylation events.

To ascertain that our chosen dose of E2 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 μ g of E2. Low dose of E2, similar to the effect seen with higher dose, led to significant induction of PgR protein 6 hours after injection (Fig. 1S).

These data reveal that in the VMH of female mice, an "active" chromatin state brought about by E2 exposure coincides with the transcriptional activation of an ER α target gene, *Pgr*, and may contribute to its regulation.

E2 treatment induces association of activating histone marks with *Pgr* promoter in the VMH

In order to investigate how E2-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 ChIP analysis. OVX female mice were injected with E2 and Veh and brains collected 3 and 5 hours later. The time-points to detect "activating" histone marks at the regulatory sequences of *Pgr* gene was selected to precede E2-induce PgR protein upregulation. Chromatin isolated from VMH

tissue of E2 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 published [33], the functional analysis of mouse *Pgr* promoter has not been reported. On the other hand, the existence of two functional promoters for *PGR-A* and *PGR-B* isoforms has been described for rat as well as for human *PGR* genes [31,32]. Using sequence alignment, we mapped two regions within ~2kb sequence around the mouse *Pgr* TSS, which display approximately 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 to +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 *Pgr* gene in the VMH of E2-treated female mice compared to control mice. One-way ANOVA analysis revealed significant main effect of treatment on H3Ac at three promoter sites of *Pgr* gene (Prom2, $F(2,32)=7.071$, $p=0.003$; Prom3, $F(2,32)=4.599$, $p=0.018$; Prom4, $F(2,29)=4.642$, $p=0.018$). *Post-hoc* Dunnett's multiple comparison test revealed significant hyperacetylation of H3 at Prom2 site, located between *Pgr-B* and *Pgr-A* promoters, 3 hours after E2 administration and at Prom3 and Prom4 sites, located upstream of *Pgr-B* promoter region, at 5 hour time point (Fig. 3B).

Another histone mark leading to an open chromatin confirmation, H3K4me3, was also significantly upregulated at *Pgr* gene promoter in the VMH of E2-treated female mice at two promoter sites (Prom1, $F(2,31)=5.776$, $p=0.007$; and Prom4, $F(2,31)=5.611$, $p=0.008$). *Post-hoc* Dunnett's multiple comparison test revealed a significant increase in H3K4me3 associated with Prom1 and Prom4 sites, which span *Pgr-B* and *Pgr-A* promoters, respectively, 5 hours after E2 injection (Fig. 3C). These data suggest that the mechanism by which E2-activated ER α induces the transcription of *Pgr* gene in mouse brain involves histone modifications and changes to chromatin structure at the promoter of this target gene.

E2 affects histone modifications at *Pgr* promoter in the POA

It has been well documented that E2 regulates PgR expression in some brain regions but not the others [46]. One of the brain regions responsive to E2 treatment is the POA. Thus, we examined histone modification status at *Pgr* gene promoter in the POA of female mice using ChIP assay. One-way ANOVA analyses revealed significant effects of E2 treatment on the associations of H3Acetyl with two promoter sites of *Pgr* gene (Prom2, $F(2,29)=3.370$, $p=0.048$; and 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 *Pgr* gene (Prom1, $F(2,30)=15.24$, $p<0.0001$) (Fig. 4A and B). *Pos-hoc* Dunnett's multiple comparison test revealed that both histone modifications (H3Acetyl and H3K4me3) were upregulated in the VMH of female mice only 5 hours after E2 injection with no changes detected at 3h time point (Fig. 4A and B). These results demonstrate that the time course and the pattern of E2-induced histone modifications at the promoter of *Pgr* gene is brain-region specific and may reflect the differences in the expression of PgR in these nuclei.

E2 differentially affects histone modifications at *Oxtr* promoter in the POA and VMH

One of the gene products regulated by E2 that facilitate the expression of lordosis behaviour is oxytocin receptor [51,52]. Thus we examined how E2-treatment affects histone modifications at the promoter of the *Oxtr* gene in two brain regions, VMH and POA of female mice. Three primer pairs (*Oxtr* prom1-3, Table 2.) that span ~600bp sequence in the 5'UTR of the mouse *Oxtr* gene were designed and used to detect association of acetylated and methylated histone H3 with the regulatory region of this gene (Fig. 5A). We observed different patterns and time-course of E2-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 E2 treatment on both “activating” histone marks, H3Acetyl and H3K4me3, albeit to a different degree. Histone H3 hyperacetylation was detected at two promoter sites of *Oxtr* gene (*Oxtr* prom2, $F(2,27)=3.770$, $p=0.036$; and *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, *Pos-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 hours after E2 administration but revealed significant upregulation of H3Acetyl and H3K4me3 at 5 hour time point (Fig. 5B).

In the mouse POA, one-way ANOVA analysis revealed the effect of E2 treatment on H3Ac association with all three *Oxtr* promoter sites (*Oxtr* prom1, $F(2,32)=8.565$, $p=0.001$; *Oxtr* prom2, $F(2,32)=6.471$, $p=0.004$; and *Oxtr* prom3, $F(2,33)=3.846$, $p=0.032$) and *Pos-hoc* Dunnett’s multiple comparison test demonstrated significant hyperacetylation taking place at *Oxtr* prom1 and *Oxtr* prom2 only 5 hours after E2 administration (Fig. 5C). No changes were detected in the POA in the methylation status of H3K4 at any *Oxtr* promoter sites following E2 administration. These results suggest that E2-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.

E2 effects on the recruitment of ER α to the putative regulatory sequences on *Pgr* gene

E2 regulates the transcriptional activity of target genes by binding to one of the two nuclear ERs, ER α and ER β . Overwhelming evidence indicates that induction of *Pgr* gene in the VMH of female mice and subsequent expression of lordosis behaviour critically requires ER α [12,14]. Thus, we set out to examine whether E2-treatment of OVX female mice results in the direct recruitment of ER α to the promoter of *Pgr* gene. For this, OVX female mice were injected with Veh or E2 and chromatin isolated from the VMH 5 hours after was analysed using ChIP assay. The time-point to analyse the association of ER α with the regulatory sequences of *Pgr* gene was selected to precede E2-induce *Pgr* protein upregulation.

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 reports suggest that several sequences ~150-300kb upstream of human *PGR* gene contain ERE sites and act as enhancers to regulate *PGR* gene expression [35]. Examination of ~200kb genomic sequences upstream of mouse *Pgr* gene led to identification of seven ERE sites, which displayed high homology to ERE sites characterised by Boney-Montoya and colleagues in human as well as in mouse genomes (Dr. Ann Nardulli, personal communication). Thus, we examined direct association of ER α with these putative regulatory sequences of *Pgr* gene: four sites within the proximal promoter region (Table 1 and Fig. 3A) and seven ERE-containing sites within ~200kb upstream of the TSS (Table 3 and Fig. 6A). In concert with previous reports we did not observe E2-regulated recruitment of ER α to the promoter region of *Pgr* gene [28]. We did, however, detect E2-induced binding of ER α at two putative enhancer regions located ~81kb and 200kb upstream of the *Pgr* gene TSS (Fig. 6B). Statistical analysis using *Student’s t-test* revealed a significant increase in the binding of ER α 5 hours after E2 administration only at Enhancer 4 site ($p=0.043$). This is the first evidence that the ERE-containing site 200kb upstream of *Pgr* TSS may be involved in the regulation of *Pgr* gene by ligand-activated ER α in the mouse brain *in vivo*. Surprisingly, little or no E2-induced recruitment of ER α was observed at any other ERE sites following E2 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.

E2 effects on the expression of ER α 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 through alteration of the chromatin structure. One way to affect the chemical status of histone proteins is by regulating the expression and availability of histone modifying enzymes [53]. Although a large number of ER α cofactors with HAT or HMT activity has been identified in human cancer cells, little or no information exists as to which of these cofactors regulates the transcriptional activity of ER α in the nervous tissue. Thus, we examined the mRNA expression levels of some of transcriptional coregulators, which are able to bring about histone modifications we detected in the VMH following E2-treatment. For this, OVX female mice were injected with E2 and Veh and the brains were analysed 3 or 5 hours later. Total RNA was isolated from the VMH and POA tissues, reverse transcribed and 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 E2-treated samples were calculated as described in *Materials and Methods*. Among the examined factors were SRC1, SRC2, CBP and p300, well-characterised ER α 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 E2-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 a ER α coregulator [55]; LSD1, a coregulator with the activity to demethylase both H3K4 and H3K9, thus either repress or promote nuclear receptor-mediated transcription [56,57]; and JARID1A, which has been shown to regulate E2-induced *PGR* expression in human MCF-7 cells through H3K4 demethylation [58].

We found that E2 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*, were highly regulated by E2-exposure as revealed by one-way ANOVA (*Src1*, $F(2,29)=4.255$ $p=0.024$ and *Src2*, $F(2,28)=11.04$ $p=0.0003$). *Post-hoc* Dunnett's multiple comparison test revealed significant upregulation of both *Src* isoforms at the 3 hour time point, however, 5 hours after E2-administration *Src1* stayed elevated while *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 the treatment on the expression of *p300* ($F(2,28)=22.07$, $p<0.0001$) but not on the expression of *Cbp* ($F(2,29)=2.247$, $p=0.124$). *Post-hoc* Dunnett's multiple comparison test demonstrated transient pattern of *p300* upregulation, which reached significantly high levels at 3 hours and returned to baseline 5 hours after E2 injection (Fig. 7A).

The mRNA expression of two HMTs, *Mll3* and *Ash1*, as well as the accessory protein, *Wdr5*, were also regulated by E2 in the VMH of OVX female mice (Fig. 7B). One-way ANOVA revealed a significant effect of the 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, *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, were detected at 3 hour time point and returned to baseline after 5 hours; *Mll3* mRNA upregulation was delayed and only reached significant levels 5 hours following E2

administration (Fig. 7B). Interestingly, no changes were detected in the mRNA levels of *Mlll* (Fig. 7B).

The mRNA expression of the two examined HDMs were also regulated by E2 exposure in the VMH of female mice (Fig. 7C). One-way ANOVA revealed a significant effect of the 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, *post-hoc* Dunnett's multiple comparison test only detected significant downregulation of *Lsd1* at 3 hours while *Jarid1A* mRNA increase detected at the same time point was not significant (Fig. 7C).

E2 effects on the expression of ER α cofactors in the POA of female mice

Since (a) we detected differential acetylation and methylation events occurring at the promoters of ER α target genes in the two brain regions, VMH and POA; and (b) we hypothesise that differential regulation and expression levels of ER α cofactors may underlie these effects; we therefore examined the mRNA expression levels of given cofactors in the POA of OVX female mice following E2-administration. Interestingly, we found that in POA mRNA levels of examined HATs, HMTs and HDMs were more stable and less dynamically regulated by E2-exposure compared to the VMH.

Specifically, we found that, in the POA of female mice *Src1* and *Src2* mRNA levels do not change following E2 administration (Fig. 8A). In 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, *post-hoc* Dunnett's multiple comparison test showed a significant downregulation of *Cbp* 5 hours after E2 treatment and a similar trend for *p300*, albeit it failed to reach statistically significant level (Fig. 8A).

Among the factors involved in H3K4 methylation, E2 effect on mRNA expression was only observed for *Ash1* ($F(2,31)=4.196$, $p=0.0244$) 5 hours after E2 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 E2-administration in the POA ($F(2,32)=4.222$, $p=0.0236$) (Fig. 8C).

Together these data demonstrate that one of the mechanisms by which E2 induces changes in the chemical makeup of histone proteins in the mouse brain includes regulation of histone modifying cofactor mRNA expression. These data also support the hypothesis that tissue-specific effects of E2-activated ER α may be mediated by 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, E2-induced histone modifications are detected at the level of global chromatin as well as at the promoters of ER α target genes, and provide mechanistic insight into the early molecular events underlying hormonal effects on nervous tissue.

In the VMH of female mice E2 induces specific set of histone modifications

Acetylation of histone proteins has long been known to promote transcriptional activity and contribute to E2-induced gene expression in human MCF-7 cells [50,59]. Moreover, evidence supports the importance of histone acetylation in E2-mediated behavioural and physiological processes, such as 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 (HDAC) inhibitor, trichostatin A (TSA) treatment, is able to facilitate E2-induced sexual behaviour in female mice (Figure 3S). Thus, it was not surprising that we observed an increase in the acetylation of histone H3 in the VMH of E2-treated mice. In contrast, we did not find an increase in the levels of histone H4Ac, but rather detected a slight albeit significant decrease at the 9-hour time point. Such differences in the patterns of H3 and H4 acetylation seem to be characteristic early response to E2 action specifically in the mouse brain. For example, in another brain region, the hippocampus, direct infusion of E2 also resulted in the acetylation of H3 but not of H4 histone, whereas E2-treatment of human MCF-7 cells does lead to H4 acetylation at the level of total chromatin as well as at ER α target gene promoters demonstrating tissue-specific epigenetic effects of E2 [60,62].

In addition, we have found that in the VMH of female mice E2 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 [42,43]. Interestingly, E2-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, E2 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 MAP kinase may be a prerequisite for H3K14 acetylation and a common mechanism for E2-induced epigenetic effects.

In the VMH of female mice, E2-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 ER α in human breast cancer cell model [28]. In addition, our data support the previous finding that H3K4 and H3K9 methylation marks are mutually exclusive [42,45]: at 3 and 6 hours after E2-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 hours following E2 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 revealed some unexpected results as well. Notably, in the VMH of female mice, we detected a significant upregulation of repressive H3K9 methylation within 1 hour of E2 administration, as well as 9 hours after E2 injection, just when hormone effects on histone H3 acetylation and H3K4me have worn off. It is well established that E2-treatment leads to the downregulation of a number of genes in various mouse tissues [63,64]. Moreover, microarray analysis of E2 regulated genes in mouse uterine tissue had revealed distinct temporal patterns of target gene activation as well as repression [64]. Specifically, ~17% of total genes identified by Watanabe and colleagues were downregulated by E2-treatment within the first hour. Among these were genes coding for signal transduction molecules and transcription factors, indicating that their rapid downregulation may lead to further amplification of E2 effects. 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 ER α repressed genes on the CNS function and physiological outcomes of E2-treatment. On the other hand the marked increase in the levels of H3K9me3 together with significant downregulation in the basal levels of H4 acetylation, 9 hours after E2-exposure, may be indicative of termination of E2 effects on VMH cells.

The pattern of E2-induced histone modifications at the promoters of target genes is brain-region and gene-specific

We used ChIP assay to extend our analyses and examine E2-effects on the histone modifications at the promoters of specific ER α -target genes, *Pgr* and *Oxtr*, in two hormone-responsive brain regions, VMH and POA. Our data revealed interesting brain-region and gene-specific effects of E2 treatment (Table 4). Specifically, we found differences in the pattern and the time-course of histone H3 acetylation associated with *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 the report that demonstrates anatomic specificity of PgR regulation by E2 in rodent brain [25]. In contrast, the degree and the time-course of E2-induced H3K4 methylation at *Pgr* promoter were found to be similar in both, the VMH and the POA, indicating the importance of this specific histone modification in the regulation of *Pgr* gene. Moreover, methylation of H3K4 in response to E2 seems to be a common requirement for the expression of *Pgr* gene across tissues, as knockdown of *Mll1*, the methyltransferase responsible for this modification leads to a thwarted effect of E2 in human MCF-7 cells as well [28].

Interesting gene-specific effects were observed when we analysed histone modifications associated with the *Oxtr* promoter (Table 4). Contrary to *Pgr*, H3 histone acetylation appeared to be more prominently regulated by E2-exposure at the promoter of this specific ER α -target gene, whereas little or no changes in the levels of H3K4me were detected. Moreover, the increase in the levels of H3Ac at the *Oxtr* promoter was only detected 5 hours following treatment and this effect was evident in both hypothalamic nuclei examined, POA and VMH. Such delayed kinetics of “activating” histone modifications associated with *Oxtr* promoter may in fact reflect the time course of E2-induced OXTR expression, which only becomes detectable in VMH after 6h and reaches significant levels at 24h [65]. These data suggest that the temporal regulation of early epigenetic events, such as covalent histone modifications induced by E2-treatment may be the determining factor of the time course of gene induction.

E2 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 E2-exposure significantly upregulates mRNA levels of several ER α cofactors with HAT and HMT activity in the VMH of female mice, suggest that similar mechanisms may underlie E2-induced changes in the status of histone proteins in the brain. To date, a vast number of ER α cofactors have been identified in reproductive tissues and cell culture systems, which are important for the regulation of ER α -target genes as well as physiological and pathological effects of E2 [15]. However, only few of them have been functionally tested in the nervous system. Among them, two SRC isoforms, SRC1 and SRC2, have been shown to be co-expressed with ER α in hormone responsive brain regions, physically interact with the receptor and are required for the E2-dependent expression of *Pgr* gene and reproductive behaviours [29,30]. The important role of SRC1, as well as another ER α 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 current study, we found that following E2-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 upregulation of cofactor mRNA either coincided or preceded H3 histone acetylation events detected at the level of total chromatin and at the promoters of ER α -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, E2-induced increase in mRNA levels of *Src2* and *p300* was very rapid and transient, detected only at 3 hours and gone by 5 hour time point. *Src1* was the only co-activator that was persistently elevated following E2-treatment, suggesting possible functional specificity of this isoform. These results also underscore the importance of the time scale of experimental design. When studying E2 effects on the brain, molecular mechanisms and correlates are often investigated at the same time scale as behavioural outcomes, 24 or 48 hours after E2-treatment. Our study was specifically designed to investigate early molecular events, which take place on the scale of a few hours. These differences may be the bases for the discrepancies between our findings that *Src1* and *Src2* mRNA levels in VMH are regulated by E2, and previous reports which failed to detect such regulation 24 hours after E2 treatment [67].

Although several histone methyltransferases have been shown to contribute to ER α transcriptional activity in MCF-7 cells, the importance of H3K4me and its modifying enzymes in E2-mediated effects on the CNS have not been addressed. In our current study, we detected 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 E2-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 has been 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 upregulated in VMH by E2, is important for *Pgr* regulation in the brain. Alternatively, in the brain E2 may affect the recruitment of MLL1 to the promoter of *Pgr* gene without altering its levels, and further studies will address this possibility. Nonetheless, significant upregulation of *Wdr5*, a common DNA-binding subunit of both MLL1 and MLL3 complexes, in the VMH of female mice, points to the important role of these HMT complexes in the E2-induced transcriptional regulation. Interestingly, it has been reported that genetically modified female mice, which express catalytically inactive MLL3 (*Mll3* ^{Δ/Δ}) were infertile and males with the same mutation were hypofertile [68]. Although no detailed characterization of this phenotype has been provided in the article, given the critical requirement of ER α 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 infertility observed in these mutant mice was due to disruptions in E2-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 underlies 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 current 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 ER α cofactors. Our results, showing vast differences in the mRNA expression of several histone-modifying cofactors between these nuclei, confirmed this hypothesis. While mRNA expression of most cofactors with HAT or HMT activity was significantly upregulated 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 (Fig. 7 and 8). These data indicate that in the POA, E2-induced histone acetylation and H3K4 methylation at the promoters of *Pgr* and *Oxtr* genes

does not require the activity of given coregulators. Alternatively, E2 induced histone modifications in these brain regions may be the result of differential recruitment of these factors to the regulatory sequences of the *Pgr* and *Oxtr* genes. In conclusion, based on our results, we propose that E2-activated ER α may employ differential epigenetic mechanisms to induce brain-region specific transcription of target genes in the VMH and the POA of female mice.

E2-regulated recruitment of ER α 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 ER α , we failed to convincingly demonstrate the recruitment of ER α 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 ER α recruitment to human *Pgr* gene promoter [72,73]. More recent studies have shown that, in fact, ER α does not bind within the promoter region of human *PGR* gene but is rather recruited to the remote enhancer sequences as far as 300kb upstream of the TSS. In concert with these data, we did not observe E2-regulated binding of ER α to the proximal promoter sequences of mouse *Pgr*. We did, however identify two putative enhancer sites ~81kb and ~200kb upstream of the mouse *Pgr* gene TSS, which displayed E2-regulated recruitment of ER α (Fig. 6). These were two out of seven ERE-containing sites homologous to the enhancer regions described by Boney-Montoya and colleagues in human MCF-7 cells. Lack of E2-regulated binding at other five ERE-containing sites may be the result of sequence differences between species known to affect ER α binding [74]. Alternatively, other transcription factors may be required for the binding of ER α to the chromatin. In fact, genome-wide analysis of ER α binding sites revealed that one such factor, Forkhead (FoxA1), binds in the close proximity of ER α and is in fact required for ER α 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 *Pgr* gene in MCF-7 cells [28]. Thus, further analyses of the role of this pioneer factor in the transcriptional activity of ER α in the mouse brain are warranted. Such studies will provide valuable information about the molecular mechanisms of E2 regulated gene expression in the brain and lead to better understanding of hormone effects on CNS functions.

Conclusions

Although oestrogens exert 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 oestrogens' actions 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 neuroscientists have to overcome when studying molecular events induced by ligand-activated hormone receptor in the brains of living animals: individual variability, small amount of starting material, heterogeneity of starting material (presence of glial cells, neurones that do not express ER α , and not all ER α expressing neurones are 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, prevalent use of hormone replacement therapy (HRT) in postmenopausal women as well as the use of different selective oestrogen receptor modulators (SERMS) for the

treatment of breast and endometrial cancers or osteoporosis, highlight immediate benefits of such studies for the understanding of hormone effects on women's health and disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. E2 effects on the levels of histone modifications in the VMH of female mice
Ovariectomised female mice were injected with E2 or vehicle and their brains were collected for the analyses 1, 3, 6, or 9 hours later. The levels of histone modifications in VMH tissue were measured by Western blot 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. Representative Western blot for each histone modification examined is shown. Data reported are the average of three to four independent experiments done in triplicate. Data are expressed as fold change $\pm$ SEM. Statistical differences were calculated

using one-way ANOVA with *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$, **, $p < 0.01$.

Fig. 2. E2-induced upregulation of PgR-A and PgR-B in the VMH of female mice

(A) Whole cell extracts isolated from the VMH of E2 and Veh-treated mice were subjected to Western blot 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 fold change $\pm$ SEM. Data represent average of three independent experiments done in triplicate. Statistical differences were calculated using one-way ANOVA with *post-hoc* Dunnett's Multiple Comparison Test. **, $p < 0.01$. (B) Representative Western blot analysis of PgR protein expression in VMH of female mice. Bands corresponding to PgR-A and PgR-B isoforms are indicated.

Fig. 3. E2 effects on histone modifications at the promoter of *Pgr* gene in the VMH
 (A) Schematic representation of mouse *Pgr* promoter and primer locations relative to the TSS (+1). Small arrows indicate translation start sites for *Pgr-B* and *Pgr-A* isoforms. Yellow boxes denote sequences corresponding to two functional promoters of rat *Pgr* gene. Red diamond indicates the location of ERE half site. Chromatin isolated from the VMH tissue of E2 and Veh-treated female mice were immunoprecipitated with antibodies specific to (B) H3Acetyl and (C) H3K4me3. Quantitative PCR was used to amplify *Pgr* promoter regions associated with acetylated and methylated histone H3. Data reported are the average of three independent experiments done in triplicate. Data are expressed as % Vehicle $\pm$ SEM. Statistical differences were calculated using one-way ANOVA with *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$; **, $p < 0.01$.

Fig. 4. E2 effects on histone modifications at the promoter of *Pgr* gene in the POA
 (A) Histone H3 acetylation and (B) H3K4 trimethylation at the promoter of *Pgr* gene in the POA of female mice were determined using ChIP assay. Data reported are the average of three independent experiments done in triplicate. Data are expressed as % Vehicle $\pm$ SEM. Statistical differences were calculated using one-way ANOVA with *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$; **, $p < 0.01$.

Fig. 5. E2 effects on histone modifications at the promoter of *Oxt* gene in the VMH and the POA
 (A) Schematic representation of mouse *Oxt* gene 5'UTR and primer locations relative to the TSS (+1). Small arrow indicates translation start site. Histone H3 acetylation and H3K4 trimethylation at the promoter of mouse *Oxt* gene in (B) the VMH and (C) the POA of female mice were determined using ChIP assay. Data reported are the average of three independent experiments done in triplicate. Data are expressed as % Vehicle $\pm$ SEM. Statistical differences were calculated using one-way ANOVA with *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$; **, $p < 0.01$

Fig. 6. Recruitment of ER α to the putative promoter and enhancer sites of mouse *Pgr* gene in the VMH

(A) Location of primers used to amplify putative enhancer sequences upstream of the TSS (+1) of mouse *Pgr* gene and corresponding enhancer sites in the human *PGR* gene [35]. Red diamonds denote ERE sites which are conserved between human and mouse enhancer sequences. Locations of primers used to amplify sequences within promoter region (Prom 1-4) are as in Fig. 3A. (B) Chromatin isolated from E2 and Veh-treated mice was immunoprecipitated with antibody specific for ER α and associated genomic DNA was quantified by qPCR. Data are expressed as % Vehicle $\pm$ SEM and represent the average of two independent experiments done in triplicate. Statistical differences between E2 and Veh-treated samples were calculated using *Student's t-test*. *, $p < 0.05$.

Fig.7. E2 effects on the mRNA expression levels of histone modifying factors in the VMH
Total RNA isolated from E2 and Veh-treated mice was reverse transcribed and the expression levels of ER α cofactors, histone acetyltransferases (A), histone methyltransferases (B), and histone demethylases (C) were analyzed by qPCR. Data are expressed as fold change $\pm$ SEM and represent the average of two independent experiments each with n=5. Statistical differences were calculated using one-way ANOVA followed by *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$, **, $p < 0.01$.

Fig.8. E2 effects on the mRNA expression levels of histone modifying factors in the POA
Total RNA isolated from E2 and Veh-treated mice was reverse transcribed and the expression levels of ER α cofactors, histone acetyl transferases (A), histone methyl transferases (B) and histone demethylases (C) were analyzed by qPCR. Data are expressed as fold change $\pm$ SEM and represent the average of two independent experiments each with n=5. Statistical differences were calculated using one-way ANOVA with a *post-hoc* Dunnett's Multiple Comparison Test. *, $p < 0.05$, **, $p < 0.01$.

Table 1
Nucleotide sequences and locations of primers used to amplify mouse *Pgr* promoter regions in ChIP experiments

Name	Position	Primer sequence
Pro m 1	-721/-650	Forw 5' -CATCC TTA ACTGC TAAG TCACATTGG -3' Rev 5'-GCCTT CCT CTA ACACGCTCAA -3'
Pro m 2	+261/+332	Forw 5' -AGCGGG AGTCCTTTTTTTCAG -3' Rev 5'-TTTTTCGT GTTT CTTC TTGA ATGG-3'
Pro m 3	+621/+683	Forw 5' -GGAG CTTGG GTCG TCATGA -3' Rev 5'-GCCAGA CGTGTGGAGA AC CT-3'
Pro m 4	+841/+915	Forw 5' -AGCTCC CAG ACGGAAAG ACA -3' Rev 5'-GTG GC TTCTACCC CAGAGAAAG -3'

Table 2
Nucleotide sequences and locations of primers used to amplify OTR 5' UTR regions in ChIP experiments

Name	Position	Primer Sequence
<i>Oxtr</i> prom1	+448/+518	Forw 5'-CGGCGTTGGGAAGTTGTG-3' Rev 5'-CCCCAAAGCCCATCCTTCTT-3'
<i>Oxtr</i> prom2	+686/+748	Forw 5'-GGTTAATCGCAAGCTTCCTCTCT-3' Rev 5'- GCGCTTAGGTCCCAGGAAAG-3'
<i>Oxtr</i> prom3	+938/+1017	Forw 5'- CGGTTCTCCTGGTTTGTTC-3' Rev 5'- GCGCTCAGTCCAGGTGTCTAT-3'

Table 3
Nucleotide sequences and locations of primers used to amplify putative enhancer regions of mouse *Pgr* gene in ChIP experiments

Name	Position in Mouse genome	Position in Human genome*	Conserved ERE*	Primer sequence
Enh 1	-81kb	-168kb	ERE1	Forw 5' -AGCA ACA AGT TGGG TTAGT -3' Rev 5'-CTCT CCAGGA TTGGT ACATTT-3'
			ERE3	Forw 5' -CTGG TTGTTCTGACATT CTTAT TC-3' Rev 5'-CACTC TGTCATGCTTGATG TA-3'
Enh 2	-147kb	-205kb	ERE1	Forw 5' -CCTATT AGAGA ACAAGGAG ATG AC-3' Rev 5'-GG TCACATTAACCC TTGGAA -3'
			ERE2	Forw 5' -TCAAGAT GGG CAGGT ACAA -3' Rev 5'-TAGA GCA GTGTTCC CAGAAA -3'
Enh 3	-166kb	-221kb	ERE1	Forw 5' -AT GCTCAA GTCACCAA GC-3' Rev 5'-AATT GCC TCT CTGCACTTT -3'
Enh 4	-200kb	-306kb	ERE3	Forw 5' -TGCAAGA ATATA ATG GCAGAGA -3' Rev 5'-CTA ACTTCGC TGC AGATAG AC-3'
Enh 5	-207kb	-311kb	ERE1	Forw 5' -ATGAGG GTTCTGTTT CCTTTAT -3' Rev 5'-TAG GCTT CA CCACTTC CT-3'

* Based on: Boney-Montoya et al., 2010.

Table 4
Summary of E2-induced histone modifications at the promoters of *Pgr* and *Oxtr* genes in the VMH and POA of female mice.

	<i>Pgr</i>				<i>Oxtr</i>			
	H3Ac		H3K4 me3		H3Ac		H3K4me3	
	VMH	POA	VMH	POA	VMH	POA	VMH	POA
3h	↑	–	–	–	–	–	–	–
5h	↑↑	↑	↑↑	↑	↑	↑↑	↑	–

Each arrow denotes significant change at single promoter site.

↑
p<0.05,

↑
p<0.01.