Gene Expression Profiles of Intracellular and Membrane Progesterone Receptor Isoforms in the Mediobasal Hypothalamus During Pro-Oestrus

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Progesterone action is mediated by its binding to specific receptors. Two progesterone receptor (PR) isoforms (PRA and PRB), three membrane progesterone receptor (mPR) subtypes (mPRα, mPRβ and mPRγ) and at least one progesterone membrane-binding protein [PR membrane component 1 (PRmc1)] have been identified in reproductive tissues and brain of various species. In the present study, we examined gene expression patterns for PR isoforms, mPR subtypes and PRmc1 in the rat mediobasal hypothalamus (MBH) during pro-oestrus. The mRNA level for each receptor subtype was quantified by a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) at the time points: 13.00 h on dioestrous day 2; 09.00, 13.00, 17.00 and 22.00 h on pro-oestrus; and 13.00 h on oestrus. For PR, one primer set amplified PRA+PRB, whereas a second primer set amplified PRB. As expected, PRA+PRB mRNA expression was greater than PRB in MBH tissue. PRB mRNA levels increased throughout the day on pro-oestrus, with the highest levels being observed at 17.00 h. PRB mRNA levels in the MBH were increased by 2.4- and 3.0-fold at 13.00 and 17.00 h, respectively, on pro-oestrus compared to 13.00 h on dioestrous day 2. There were differential mRNA expression levels for mPRs and PRmc1 in the MBH, with the highest expression for PRmc1 and the lowest for mPRγ. The mPRα mRNA contents at 13.00 and 17.00 h on pro-oestrus were increased by 1.5-fold compared to that at 13.00 h on dioestrous day 2. The mPRβ mRNA levels at 13.00 and 17.00 h on pro-oestrus were 2.5- and 2.4-fold higher compared to that at 13.00 h on dioestrous day 2, respectively. PRA+PRB, mPRγ and PRmc1 mRNA levels did not vary on pro-oestrus. These findings suggest that the higher expression of PRB, mPRα and mPRβ in the MBH on pro-oestrous afternoon may influence both genomic and nongenomic mechanisms of progesterone action during the critical pre-ovulatory period.

Key words: oestradiol, oestrous cycle, arcuate nucleus, dopamine, prolactin.

doi: 10.1111/j.1365-2826.2009.01920.x

Journal of Neuroendocrinology
increased attention in the past decade (24–26). The first mPR, designated mPRa, was cloned from spotted seatrout ovary and its protein met the designation as a steroid membrane receptor (27). Subsequently, mPR subtypes, mPRa, mPRβ and mPRγ, were identified in tissues including ovary, hypothalamus and pituitary gland (28–30). The mPR protein has an approximately 40 kDa molecular mass and seven transmembrane domains (28–30). In addition to the mPRs, a small progesterone-binding protein (25 kDa) may also mediate progesterone action (31). This progesterone-binding protein was initially identified from porcine vascular smooth muscle cells and liver microsomes (32, 33). This protein was referred to as PR membrane component 1 (PRmc1) and has one single transmembrane domain (34). PRmc1 was detected in the reproductive system (35, 36) and basal forebrain (37).

The present study explored PRs, mPRs and PRmc1 gene expression patterns in the MBH during oestrous cycle, especially on pro-oestrus. The study provides important information for understanding progesterone regulation of hypothalamic neuronal activities during the critical pre-ovulatory phase of the reproductive cycle.

Materials and methods

Animals

Adult female Sprague–Dawley rats (200–250 g) were obtained from Charles River (Raleigh, NC, USA). Rats were housed under a 14 : 10 h light/dark cycle (lights on 07.00 h) and controlled temperature. Food and water were supplied ad lib. The stage of the oestrous cycle was monitored by performing a daily vaginal lavage at 10.00–12.00 h. Only rats exhibiting three consecutive 4-day oestrous cycles were used in the study. Rats were killed by decapitation at 13.00 h on dioestrus 2, 09.00, 13.00, 17.00 and 22.00 h on pro-oestrus, or 13.00 h on oestrus. The brain was removed rapidly and frozen in Fisherbrand Super Friendly Freeze It solution (Fisher Scientific, Pittsburgh, PA, USA) and stored at −80 °C. MBH tissue was punched with a 2-mm diameter sample corer (Fine Science Tools, Foster City, CA, USA) from frozen brain equilibrated to 4 °C for 15 min. RNA was isolated from the MBH tissue using RNA Bee method (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer’s instructions. Briefly, MBH tissue was homogenised in samples in 0.8 ml RNA Bee solution using a glass homogeniser. Chloroform (80 µl) was added for phase separation and samples were centrifuged at 12 000 g (4 °C) for 15 min. RNA in the aqueous phase was precipitated with 0.4 ml isopropanol. After centrifugation, RNA precipitates were washed with 0.8 ml of 75% ethanol and RNA pellet was solubilised in water. RNA quantity was determined spectrophotometrically. RNA samples were stored at −80 °C until further use.

cDNA synthesis

Complementary DNA (cDNA) was synthesised using SuperScript™ First-strand synthesis system (Invitrogen, Carlsbad, CA, USA). A 12-µl reaction, including 5 µl of RNA, 1 µl of oligo(dT)12-18 (25 µg/µl) and diethylpyrocarbonate-treated water was incubated at 70 °C for 10 min, followed by at least 1 min of incubation on ice. The cDNA synthesis mix, including 2 µl of (10X) reverse transcriptase buffer, 2 µl of 25 mM magnesium chloride, 1 µl of 10 mM deoxynucleotide triphosphates and 2 µl of 0.1 mM dithiothreitol, was added to the above reaction tube. The reaction mixture was then incubated at 42 °C for 5 min. After addition of 1 µl (200 units) SuperScript™ III reverse transcriptase, the reaction mixture was incubated at 42 °C for 50 min. The reaction was terminated by incubation at 70 °C for 15 min. RNAase H (1 µl) was added to each tube and the reaction mixture was incubated at 37 °C for 20 min. The cDNA samples were stored at −20 °C.

Real-time polymerase chain (PCR) for PRs, mPRs and PRmc1

Real-time PCR was performed in the SmartCycler System (Cepheid, Sunnyvale, CA, USA) by utilising the SYBR® Green JumpStart™ Taq ReadyMix Kit (S 4438; Sigma, St Louis, MO, USA). The 25-µl reactions contained 12.5 µl of SYBR Green JumpStart Taq ReadyMix. 0.5 µl of each primer (0.2 µm final concentration), 1 µl of cDNA template and 10.5 µl of water. Primer sequences were designed using MacVector 9.5 software (MacVector Inc., Cary, NC, USA) and are presented in Table 1. After the initial denaturation step at 95 °C for 2 min, amplification was performed for 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. The specificity and purity of the amplification reaction was determined by performing a melting curve analysis at 60 °C up to 95 °C at 0.5 °C increments. One distinct peak was observed, indicating that a single DNA sequence was amplified during PCR. In addition, end reaction products were visualised on ethidium bromide-stained 2% agarose gels and the appearance of a sole band of the correct molecular size was confirmed. Each PCR assay included two negative controls, without reverse transcription or without template in the PCR reaction. The data were analysed with SmartCycler Software. The comparative threshold cycle number (Ct) method (2−DDCt) was used to quantify the results obtained by real-time RT-PCR (39). The gene expression patterns of PRA+PRB, PRB, mPRα, mPRβ, mPRγ and PRmc1 were normalised to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH mRNA levels did not change during the oestrous cycle in the MBH as determined from the mean Ct value for each group. The ΔCt in each sample was calculated by subtracting CtGAPDH from CtPRB, mPRα or PRmc1. The gene expression changes were determined by subtracting ΔCt experimental groups (pro-oestrus and oestrus) from the ΔCt control group (13.00 h dioestrus 2). Fold induction was determined by calculating 2ΔΔCt. The real-time PCR efficiency for each primer pair was calculated using the equation E = 10−1/s, where ‘s’ is the slope derived from the SmartCycler REST-MCS software.

Statistical analysis

Data are presented as the mean ± SEM. Statistical significance was evaluated by one-way ANOVA and multiple comparisons were made with Fisher’s least significant procedures. P < 0.05 was considered statistically significant.

Results

Standard curves for PRs, mPRs, PRmc1 and GAPDH cDNA

To establish standard curves for PRs, mPRs, PRmc1 and GAPDH gene expression in the MBH, we tested cDNA concentrations of 0.5,
Table 1. Primer Sequences for Quantitative Real-time Polymerase Chain Reaction.

| Gene name | GenBank accession no. | Primer sequence | Amplicon size (bp) |
|-----------|-----------------------|-----------------|-------------------|
| PRA+PRB   | NM_022847             | Forward: GGT CTA AGT CTC TGC CAG GTT TCC | 182               |
|           |                       | Reverse: CAA CTC CIT CAT CCT CTG CTC ATT C |                  |
| PRB       | NM_022847             | Forward: GCA TCG TCT GTA TGC TCG CCA ATA C | 176               |
|           |                       | Reverse: GCT CTG GGA TTT GTG GTA CCA CAG C |                  |
| PRα       | NM_001034081          | Forward: CAC ACT GTT CCA GCA GCA CAA C   | 143               |
|           |                       | Reverse: CAA TGA TGA AGA GGG GCA CAG C   |                  |
| PRβ       | NM_001014099          | Forward: CTC TTT CAG AAC CAC AAC GAG GTG | 168               |
|           |                       | Reverse: GGT GAG GTA AGT AGT GTA CAG C    |                  |
| PRγ       | NM_001014092          | Forward: CTC GTG GAC GCT TTG ACT ACA TTG | 198               |
|           |                       | Reverse: TGC TGA GGC TGA AGA TGA TGC     |                  |
| PRmc1     | NM_021766             | Forward: ACT TCA CCC CTG CCG AAC TAA G   | 199               |
|           |                       | Reverse: TCA TCC TTC AGT CGC TCA GAC C   |                  |
| GAPDH     | DQ403053              | Forward: AAC GAC CCC TTC ATG GAC C        | 182               |
|           |                       | Reverse: TCC ACG ACA TAC TCA GCA C        |                  |

1, 2, 5, 10, 100 and 1000 ng from mixed MBH cDNA samples, which included MBH tissues from dioestrus 2, pro-oestrus and oestrus. This study was repeated three times with different mixed cDNA samples. As shown in Fig. 1, a concentration-dependent change of Ct value was observed for PRA+PRB, PRB, mPRα, mPRβ, mPRγ, PRmc1 and GAPDH in MBH cDNA samples. The real-time PCR efficiencies of the primer sets for GAPDH, PRA+PRB, PRB, mPRα, mPRβ, mPRγ and PRmc1 were 1.89, 1.95, 1.93, 1.85, 1.81, 1.94 and 1.87, respectively. GAPDH, the housekeeping gene, and PRmc1 mRNA levels in the MBH were expressed at the highest levels compared to PRs and mPRs mRNA levels. As expected, because PRA+PRB primer set recognises a cDNA sequence common to both PRA and PRB, the expression of PRA+PRB was higher than PRB alone. The mRNA expression levels for mPRα and mPRβ were higher in MBH than mPRγ mRNA levels (Fig. 1).

Expression of PR genes in the MBH

Gene expression patterns for PRA+PRB and PRB in the MBH were evaluated at 13.00 h on dioestrus 2; 09.00 13.00, 17.00 and 22.00 h on pro-oestrus; and 13.00 h on oestrus. PRA+PRB mRNA contents in the MBH were similar on dioestrus 2, pro-oestrus and oestrus (Fig. 2A). By contrast, PRB mRNA contents were increased 1.5-, 2.4- and 3.0-fold at 09.00, 13.00 and 17.00 h, respectively, on pro-oestrus, compared to 13.00 h on dioestrus 2. PRB mRNA content at 22.00 h on pro-oestrus declined to 2.0-fold dioestrous levels, and was further reduced by 13.00 h on oestrus to 43% of dioestrous levels (F5,39 = 4.202, P < 0.01; Fig. 2B).

Expression of mPR genes in the MBH

Gene expression of mPRs, including mPRα, mPRβ, mPRγ and PRmc1, in the MBH were also determined at 13.00 h on dioestrus 2; 09.00, 13.00, 17.00 and 22.00 h on pro-oestrus; and 13.00 h on oestrus. Analysis using one-way ANOVA revealed significant changes in mRNA contents for mPRα (F5,38 = 2.494, P < 0.05; Fig. 3A) and mPRγ (F5,39 = 2.884, P < 0.05; Fig. 3A) in the MBH, but no changes were found for mPRβ (Fig. 3C) and PRmc1 (Fig. 3D) expression. Specifically, mPRα mRNA contents at 13.00 and 17.00 h on pro-oestrus were significantly higher than that at 13.00 h on dioestrus 2,
22.00 h on pro-oestrus and 13.00 h on oestrus, with a 1.5-fold increase at 13.00 and 17.00 h on pro-oestrus compared to that at 13.00 h on dioestrus 2. The mRNA contents for mPR\(b\) at 13.00 and 17.00 h on pro-oestrus were higher than the values at all the other time points during the oestrous cycle. The mPR\(b\) mRNA contents at 13.00 and 17.00 h were 2.5- and 2.4-fold higher than that at 13.00 h on dioestrus 2, respectively. PRmc1 and mPR\(c\) mRNA levels were not altered on pro-oestrus.

**Discussion**

The present study characterised gene expression patterns of PR isoforms (PRA+PRB and PRB), three mPR subtypes (mPR\(a\), mPR\(b\) and mPR\(c\)) and PRmc1 in the rat MBH during the oestrous cycle, especially on pro-oestrus. The data obtained indicate that PRA, PRB, mPR\(a\), mPR\(b\), mPR\(c\) and PRmc1 are expressed in MBH tissue, albeit at different levels. PRB, mPR\(a\) and mPR\(b\) expression in the MBH varied during the oestrous cycle with higher mRNA contents on pro-oestrous afternoon compared to that on pro-oestrous morning, early afternoon of dioestrus 2 and early afternoon of oestrus. This basic profile for PRs gene expression in the MBH during the reproductive cycle may be beneficial for understanding the mechanism(s)
of steroid hormone regulating neuronal activity, hormone secretion and reproductive behaviours.

Of the classical intracellular PRs, PRβ mRNA contents in the MBH fluctuated during the oestrous cycle, especially on pro-oestrous. The highest level of PRβ mRNA expression occurred during pro-oestrous afternoon, but PRβ mRNA contents declined on pro-oestrous evening and were markedly reduced by oestrous afternoon. These data are in general agreement with the previous study by Guerra-Araiza et al. (17) with respect to differential mRNA expression of PR isoforms during the oestrous cycle. These investigators examined PRβ expression at 12.00 h on each day of the oestrous cycle and reported highest PRβ mRNA levels in the hypothalamus on pro-oestrous compared to other days of the oestrous cycle. Our data expand these data to indicate that PRβ expression continues to increase during pro-oestrous afternoon and reaches the highest levels coincident with the timing of LH and prolactin surges. It is not clear how these changes in PRβ mRNA translate to functional PRβ protein on pro-oestrous afternoon. Guerra-Araiza et al. (41) reported no change in PRβ isoforms protein content in the hypothalamus at 12.00 h on pro-oestrous compared to 12.00 h on other days of the oestrous cycle. However, future studies should examine protein expression for PR and mPR isoforms throughout pro-oestrous day because elevated levels in the later afternoon might provide maximum sensitivity at the time of the pre-ovulatory progesterone rise. Early studies indicated that PR binding in the hypothalamus is highest on pro-oestrous and lowest on dioestrous (42). Because the entire PRA sequence is contained within the PRβ sequence, primers were designed to amplify PRA+PRβ. It is noteworthy that PRA+PRβ mRNA contents were not significantly altered during pro-oestrous day or on different days of the oestrous cycle. These data suggest that PRA isoforms expression did not increase in a similar manner to PRβ on pro-oestrous or potentially offset PRβ expression changes. In either case, the data obtained in the present study suggest that the ratio of PRA : PRβ might be altered during pro-oestrous and allow for distinct progesterone-dependent transcriptional changes in the MBH during the pre-ovulatory period.

The data obtained in the present study indicate that PRβ may be more sensitive to endogenous steroid hormone variance. A number of studies reported that PR expression in the hypothalamus is dependent on both oestriol priming (2, 21, 22, 43). Oestriol treatment of ovarioctomised rats results in increased PR expression (22, 43, 44) and a high co-expression of PR with tyrosine hydroxylase (TH) in the arcuate nucleus (3, 4, 45). Oestrogen receptor-α primarily mediates oestriol-mediated PR expression in specific hypothalamic nuclei, but other oestriol receptor(s) or splice variants may also contribute in some nuclei (46, 47). The action of oestriol with respect to promoting PR expression was observed at 24 h post-injection in the arcuate and ventromedial nuclei of rat hypothalamus (22). In addition, a higher PRβ mRNA content, as compared to PRA+PRβ mRNA contents, was obtained in the hypothalamus/preoptic area within 12 h after oestriol treatment (43). These data taken together with our data suggest that increased PRβ expression on pro-oestrous afternoon may be a result of the high serum oestriol that rises from late on dioestrous 2 (48) and is maintained until 19.00 h on pro-oestrous (49). Indeed, oestriol enhances PRβ gene expression in the preoptic area and hypothalamus (23, 43, 50). Two functionally distinct promoters have been identified in the rat PR gene with a degree of promoter specificity for oestrogen responsiveness (12, 14). These characteristics of the rat PR promoter may account for the ability of oestriol to preferentially induce PRβ expression in the MBH. By contrast to the action of oestrogen, progesterone inhibits PR gene expression in the hypothalamus (23). The pre-ovulatory progesterone rise that occurs from 17.00 h through 22.00 h (49) may contribute to the reduction of PRB mRNA levels in the MBH at 22.00 h on pro-oestrous. At 3 h after treatment, progesterone diminishes the protein content of PR isoforms in the hypothalamus of oestriol-primed ovariectomised rats (41).

This is the first study to describe the gene expression patterns of mPRs and PRmc1 in the MBH. Our data show that mPRα and mPRβ gene expressions varied on pro-oestrous afternoon. The mPRα and mPRβ mRNA levels were higher at 13.00 and 17.00 h on pro-oestrous than at other time points tested during the oestrous cycle. Interestingly, the pro-oestrous changes in mPRα and mPRβ expression levels were similar to that of PRβ and the profile of the changes is consistent with up-regulation by oestriol and subsequent down-regulation by progesterone. Indeed, oestriol increases mPRα and mPRβ mRNA expression in human myometrium, albeit with dissimilar timing and magnitude, whereas progesterone differentially modulates expression of these receptors (51). The identification and characterisation of mPRs is a recent development in our understanding of rapid progesterone signalling. The mPRs have characteristics of seven transmembrane cell surface receptors. Recently, mPR was demonstrated to directly couple to G protein to down-regulate membrane-bound adenyl cyclase activity (52). Moreover, mPRα and mPRβ protein are found in the plasma membrane fraction of GT1-7 cells (53). Activity changes of protein kinase C and calcium and calmodulin-dependent protein kinase II in the ventromedial nucleus and preoptic area of the rat hypothalamus can be initiated by progesterone (54, 55). These studies imply that mPR-regulated intracellular signalling pathways may be sites for progesterone action in the MBH. Further studies are required before we fully understand the subcellular localisation, importance and regulation mPRα and mPRβ in the MBH during the reproductive cycle. Although there was a high expression of PRmc1 mRNA and a relatively low level of mPRα mRNA in the MBH, their mRNA levels were not altered at the times examined during the rat oestrous cycle. However, PRmc1 expression in the ventromedial nucleus of the rat hypothalamus is up-regulated by oestriol and down-regulated by progesterone (37). The MBH fragment in the present study included the arcuate nucleus as well as the ventromedial nucleus and the modest, albeit nonsignificant, changes in PRmc1 during pro-oestrous in the present study may represent a contribution from the ventromedial nucleus.

The data obtained in the present study indicate progressively increasing expression levels for PRβ, mPRα and mPRβ throughout pro-oestrous afternoon. Peak expression occurs at the time of the onset of the pre-ovulatory rise in circulating progesterone levels and suggests that increased progesterone responsiveness might be
bestowed to cells in the MBH by this subset of progesterone receptors. It is notable that oestradiol also increases 3β-hydroxy-steroid dehydrogenase/Δ5-Δ4 isomerase in the hypothalamus/preoptic area of ovariectomised-adrenalectomised rats and stimulates de novo progesterone synthesis in hypothalamic astrocyte cultures (43, 56, 57). The ovarian steroid hormones, oestradiol and progesterone, are essential for the expression and amplification/extension of LH and prolactin surges on pro-oestrous. The intracellular PRs are critically involved in LH surge in rats, which is blocked or attenuated by the intracellular PR antagonist, RU486 (50, 51). Blockade of local progesterone synthesis in the brain also results in decreased LH surge (57). The LH surge is absent in PR knockout mice on pro-oestrous day and in ovariectomised PR-knockout mice treated with steroid hormones (58). Recently, mPRs have been implicated in the essential for the expression and amplification/amplification of GnRH release (53). Given the timing of PRA⁄ steroid hormones (58). Recently, mPRs have been implicated in the oestrous day and in ovariectomised PR-knockout mice treated with steroid hormones (58). Currently, mPRs have been implicated in the PRA/PRB-independent rapid negative-feedback mechanisms responsible for the suppression of GnRH release (53). Given the timing of increases in mPRx and mPRβ expression just prior to the onset of the LH surge, our data point to the importance of examining the role of the mPRs in positive feedback-mechanisms responsible for LH secretion on pro-oestrous in future studies.

Our laboratory has an interest in understanding the regulation of the pre-ovulatory prolactin surge. Although the prolactin surge is absolutely dependent on oestradiol (48), progesterone is responsible for amplifying the magnitude or extending the duration of the surge by decreasing TH activity and TH phosphorylation state in tuberoinfundibular dopamine (TIDA) neurones of MBH (8, 9, 49). The progesterone-induced decrease in TIDA neuronal activity can be blocked by antisense oligonucleotides to progesterone receptor (59), suggesting that progesterone is acting via specific PR. The high co-expression of PR with TH in the MBH supports that the neuroendocrine dopaminergic neurones in the arcuate nucleus are the targets of progesterone action (3–5, 45, 60, 61). Noteworthy, the MBH tissue dissected in the present study included arcuate nucleus and median eminence, where dopaminergic neuronal body or terminals are located. Additionally, the colocalisation of PR neuropeptide Y (S), neuropeptides (6) and dynorphin (62) in the arcuate nucleus indicates the potential importance of this study in a wide range of physiological functions.

Differential expression patterns for PRs and mPRs during oestrous cycle, especially on pro-oestrous, provide an important profile for understanding progesterone-mediated activity in the MBH. Whether these changes in PR and mPR mRNA levels translate into differences in functional protein levels for PR and mPR isoforms in the MBH remains to be explored in future studies. The present study suggests that increased expression of PRB, mPRx and mPRβ may enhance progesterone responsiveness in the MBH on pro-oestrous afternoon and contribute to genomic and/or nongenomic mechanisms underlying the progesterone action with respect to regulating pituitary hormone secretion or other reproductive processes occurring during the pre-ovulatory period.

Acknowledgements
This work was support by NIH grants HD045805 and HD048925 to LAA.

Received: 6 April 2009, revised 29 August 2009, accepted 14 September 2009

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