Differential Responses of Progesterone Receptor Membrane Component-1 (Pgrmc1) and the Classical Progesterone Receptor (Pgr) to 17β-Estradiol and Progesterone in Hippocampal Subregions that Support Synaptic Remodeling and Neurogenesis

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Progesterone (P4) and estradiol (E2) modulate neurogenesis and synaptic remodeling in the hippocampus during the rat estrous cycle and in response to deafferenting lesions, but little is known about the steroidal regulation of hippocampal progesterone receptors associated with these processes. We examined the neuronal expression of progesterone receptor membrane component-1 (Pgrmc1) and the classical progesterone receptor (Pgr), by in situ hybridization and immunohistochemistry. Pgr, a transcription factor, has been associated with synaptic remodeling and other major actions of P4, whereas Pgrmc1 is implicated in P4-dependent proliferation of adult neuroprogenitor cells and with rapid P4 effects on membranes. Ovariectomized adult rats were given E2, P4, or E2+P4 on two schedules: a 4-d model of the rodent estrous cycle and a 30-d model of postmenopausal hormone therapy. Pgr was hormonally responsive only in CA1 pyramidal neurons, and the induction of Pgr by E2 was partly antagonized by P4 only on the 30-d schedule. In CA3 pyramidal and dentate gyrus (DG) neurons, Pgr was largely unresponsive to all hormone treatments. In contrast to Pgr, Pgrmc1 was generally induced by E2 and/or P4 throughout the hippocampus in CA1, CA3, and DG neurons. In neuroprogenitor cells of the DG (immunopositive for bromodeoxyuridine and doublecortin), both Pgrmc1 and Pgr were detected. The differential regulation of hippocampal Pgrmc1 and Pgr by E2 and P4 may guide drug development in hormonal therapy for support of neurogenesis and synaptic regeneration. (Endocrinology 153: 759–769, 2012)
attenuated E2-induced synaptic growth. In the OVX macaque, the induction by E2 of the synaptic proteins synaptophysin, spinophilin, and pinch in CA1 neurons was attenuated by coadministration of P4 (6). Similarly, in the rat entorhinal cortex lesion model of Alzheimer disease, we showed that cotreatment with P4 attenuated E2-induced neurite outgrowth in the dentate gyrus (DG) (7). In contrast to many examples of P4-E2 cross talk throughout the reproductive system, the proliferation of neural progenitor cells (NPC) derived from adult rat DG was induced by both E2 and P4, in vitro (8) and in vivo (9).

Because combined concurrent E2+P4 is commonly used for menopausal hormone therapy (HT) (10–12), we sought to clarify E2-P4 interactions in neuronal expression of Pgr, a transcription factor, and in progesterone receptor membrane component-1 (Pgrmc1), a putative progesterone receptor (13, 14). Both Pgr and Pgrmc1 have high-affinity P4 binding: Pgr, dissociation constant (Kd) = 0.38 nM (15); Pgrmc1, Kd = 11 nM (16).

Neuronal responses to P4 have been associated with both Pgrmc1 and Pgr. The decline of hippocampal CA1 spines by P4 in OVX rats described above was blocked by RU486, a specific antagonist of Pgr (4). We also observed antagonism of neurite outgrowth by RU486 in an in vitro model (7). However, Pgrmc1 mediated in vitro proliferation of rat NPC, in which Pgr was not detected (8). Based on these findings, we hypothesized that Pgrmc1 will be more responsive than Pgr to ovarian steroids in DG neurons, whereas Pgr regulation will be more responsive in CA1 neurons than CA3 and DG neurons.

Pgrmc1 has been associated with diverse functions across the reproductive system that are less understood relative to Pgr. In rat ovarian granulosa cells, which lack Pgr, Pgrmc1 mediates the antiapoptotic effects of P4 (17). Rapid membrane effects of P4 are also mediated by Pgrmc1, independently of Pgr, e.g. in the rapid P4-induced Ca2+ influx of the acrosome reaction (18, 19). Pgrmc1 sequences are associated with a remarkable variety of cell functions under yet other names (13, 20, 21). During development, Pgrmc1 mediates neuronal guidance under the names: Vema (mouse) and VEM-1 (nematode) (22). We extended to the cellular level prior findings of steroid regulation of Pgrmc1 and Pgr. In whole hippocampal extracts and hypothalamic subregions, Pgr was induced by E2 (25, 26). Whereas some studies have shown P4 antagomism of E2 induction of Pgr, the P4 antagonism may be only transient (27). Moreover, in hypothalamus and posterior pituitary from chick embryos, P4 can induce Pgr (28). Thus, P4 regulation of Pgr is physiologically complex and may vary widely between cell types. Less is known about Pgrmc1, which showed induction by both E2 and P4 in hypothalamic subregions (sexually dimorphic nucleus of the preoptic area and ventromedial nucleus) (25). The hippocampal regulation of Pgrmc1 and Pgr by E2 and P4 is undefined.

Two hormone treatment schedules were used: a 4-d model of rodent ovulatory cycles (4, 26, 29) and a 30-d model of the KEEPS trial of postmenopausal HT (10, 30).

We show differential regulation of Pgrmc1 and Pgr in hippocampal neurons by E2 and P4 and discuss the potential relevance to optimization of postmenopausal HT for maintaining cognitive functions (31–33).

Materials and Methods

Animals and steroid replacement

Experiments conformed with standards of humane animal care in the National Institutes of Health Ethical Guidelines. Adult female Sprague Dawley rats (3 months old, 250–300 g; nulliparous; 44 rats total — six rats per group for the 4-d replacement schedule and five rats per group for 30-d schedule) were used throughout. All animal procedures were performed under anesthesia with ketamine (80 mg/kg) plus xylazine (10 mg/kg). Experiments for the two hormone replacement schedules were run separately with different cohorts of animals. Nonetheless, the in situ hybridization (ISH) grain densities for both receptors in control OVX tissues were very similar in each experiment (Table 1).

Four-day replacement (Fig. 1A). Rats were bilaterally ovariectomized (OVX) 2 wk before hormone replacement and treated in four groups (n = 6 per group): 1) Vehicle, 2) E2 alone, 3) P4 alone, and 4) E2 + P4. The E2 alone and E2+P4 groups received two injections of E2 benzoate (10 μg, sc in 100 μl sesame oil) 24 h apart; other groups received only vehicle (100 μl sesame oil) injections. On d 3, P4 alone and E2+P4 groups received P4 to simulate the luteal phase P4 elevation (single injection, 4 mg/kg, sc in 100 μl sesame oil), the remaining two groups (E2 and vehicle) received vehicle injections. For evaluation of NPC, all groups were given a single injection of bromodeoxyuridine (Brdu) (100 mg/kg, iv) 1 h after last steroid injection, and killed 30 h after Brdu injection (Fig. 1A).

Thirty-day replacement (Fig. 1B). Fourteen days after OVX, rats were implanted sc with E2 pellets (0.72 mg/30 d release; Innovative Research of America, Sarasota, FL) or sham pellets (Innovative Research of America) for a total of 30 d. P4 pellets were administered to P4 alone and E2+P4 groups (50 mg/15 d release; Innovative Research of America) starting on d 21 for a total of 10 d. The E2 alone group received E2 pellet, then sham implant for the last 10 d. The P4-only group received a sham implant for the last 10 d.
pellet for the first 20 d, followed by the P4 pellet for the last 10 d. OVX controls received sham implants (Fig. 1B). Uterine weights showed expected doubling of wet weight in response to E2; the P4-only group was equivalent to OVX (data not shown). The 30-d hormone schedule with these implants yielded physiological levels of plasma E2 and P4 in our prior study (7).

### Tissue collection

After lethal anesthesia, rats were cardiac perfused with 0.9% saline, and brains were removed from the skull. One brain hemisphere was frozen on dry ice for ISH; the other was fixed in 4% paraformaldehyde, followed by sucrose cryoprotection (30% sucrose in 0.1 M phosphate buffer, pH 7.4) for immunohistochemistry (IHC).

### Quantitative RT-PCR

Hippocampal tissue was obtained from intact female Sprague Dawley rats at defined stages of the estrous cycle (estrus; proestrus) and from OVX females. Total cellular RNA was extracted (Tri Reagent), and cDNA was prepared (2 μg RNA; Superscript III kit, Invitrogen, Carlsbad, CA). RT-PCR was performed with SYBR Green I and used the following primers: rPgrmc1 (forward, 5'-GCCTCAAGGGCGGTGACTTC-3'; reverse, 5'-CTGGGCAGGAGTGTCAG-3'); rPgr (forward, 5'-GTCAGTGGACAGATGCTA-3'; reverse, 5'-AGCTTGGTTTCACAAGA-3'). Standard curves were constructed from serial dilutions of Pgrmc1 and Pgr plasmid controls and used the same primers.

### ISH

Frozen brain hemispheres were sectioned sagittally (18-μm) on a cryostat and stored at −80 C until use. For ISH, 1× [35S]UTP-labeled sense- and antisense riboprobes were generated by in vitro transcription using 1 μg linearized plasmid from the following sequences: for Pgrmc1, nucleotides 1012–1374 of rat Pgrmc1 mRNA (34); for Pgr, nucleotides 1–548 of the steroid-binding domain of both rat progesterone receptor isoforms (PR-A and PR-B) [kindly provided by Dr. S.L. Petersen (35)]. Both Pgrmc1 and Pgr cRNA probes had the same specific activity (4.9 × 10⁶ cpm/μl) and concentration in hybridization buffer (0.3 ng/μl/kb). Labeled probe (1 ng) was used per slide and hybridized at 55 C. Posthybridization washes were performed in 50% formamide/2× saline–sodium citrate (SSC), 0.5× SSC, and 0.1× SSC at 60 C. Slides were then dehydrated in graded 0.3 M ammonium acetate-alcohol series and exposed to x-ray film for

### Table 1. Pgrmc1 and Pgr ISH grain densities in hippocampal neurons on 4-d and 30-d hormone schedules

<table>
<thead>
<tr>
<th></th>
<th>E2</th>
<th>P4</th>
<th>E2 + P4</th>
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<tr>
<td>CA1</td>
<td></td>
<td></td>
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<tr>
<td>4-d</td>
<td>8.7 ± 0.6</td>
<td>12.5 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.9 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>30-d</td>
<td>10.7 ± 1.4</td>
<td>13.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>CA3</td>
<td></td>
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<tr>
<td>4-d</td>
<td>9.7 ± 0.6</td>
<td>13.5 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.5 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30-d</td>
<td>10.1 ± 1.0</td>
<td>13.1 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.4 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>DG</td>
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<tr>
<td>4-d</td>
<td>5.1 ± 0.3</td>
<td>7.7 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>30-d</td>
<td>5.1 ± 0.5</td>
<td>6.6 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.4 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> P < 0.05 vs. respective OVX group. Pgr: <sup>a</sup> P < 0.05, vs. respective OVX group.
18–48 h. Slides were emulsion dipped (NTB2, Eastman Kodak, Rochester, NY), developed, and counterstained in Harris modified hematoxylin according to standard procedures (36). Based on x-ray film density, the emulsion-dipped slides were exposed for different times to reach equivalent grain densities needed for accurate comparison: Pgrmc1, 4 d; Pgr, 21 d. Grain density in emulsion-dipped, developed slides was analyzed from brightfield images (Supplemental Figs. 1 and 2 published on The Endocrine Society’s Journals Online web site at http://end. endojournals.org) and counted manually around individual neurons (perikarya) that did not overlap. For CA1 and CA3 neurons, 100 cells were analyzed across six to eight images per brain; for DG neurons, 120 cells across 10 images per brain. Cells for analyses were chosen randomly to ensure uniform sampling. The frequency distributions of grain densities showed negligible overlap of sense and antisense strand grain densities (Supplemental Fig. 2). Cells with at least two grains per cell were classified as positive for mRNA with antisense probes; for sense-strand background probes, few cells (<3%) had at least two grains per cell. The data shown here were calculated from cells with at least two grains per cell. Pgr grain cluster development took 5 times longer than for Pgrmc1. Based on frequency distributions of grain densities for antisense and sense cRNA probes (Supplemental Figs. 2–6), the sense-strand background controls for all probes and regions showed no grains in most cells and few cells showed more than two grains, yielding an average background, 0.3 ± 0.03 grains per cell. We only analyzed perikarya showing at least two grains per cell with antisense cRNA probes.

Antibodies

Primary antibodies used were: polyclonal rabbit anti-Pgrmc1 (1:300, HPA002877, Sigma-Aldrich, St. Louis, MO); polyclonal rabbit anti-Pgr (1:50, sc-538, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); polyclonal goat anti-doublecortin (1:100, sc-8066, Santa Cruz Biotechnology; Refs. 37 and 38); monoclonal rat anti-BrdU (1:100, MCA2060, AbDSerotec, Raleigh, NC; Refs. 39–41). Secondary antibodies used were goat antirabbit biotinylated antibody (1:200, Vector Laboratories,), goat antirat biotinylated antibody (1:100, sc-538P, Santa Cruz Biotechnology), which depleted the Pgr bands on Western blots (Supplemental Fig. 7B) and parikaryal staining after IHC (Supplemental Fig. 7, C and D). We did not detect cellular staining with the rabbit PR antibody (1:100, A0098, DAKO Corp., Carpinteria, CA) in adult rat hippocampus, confirming the results of Waters et al. (44).

Immunohistochemistry (IHC)

Perfused brain hemispheres were fixed in 4% paraformaldehyde at 4 C for 24 h followed by cryoprotection in 30% sucrose/PB before sagittal sectioning as above. IHC was performed on whole hemisphere sagittal sections according to Morgan et al. (36), and the dorsal hippocampus was analyzed. Briefly, sections were fixed in 4% paraformaldehyde and permeabilized in 1% Nonidet P-40, followed by blocking in 5% normal serum. Sections were then incubated in primary antibodies overnight at room temperature for Pgrmc1 and Pgr and at 4 C for others. Secondary antibody incubation was performed for 1 h at room temperature. The Pgrmc1 epitope was visualized by fluorescence using goat antirabbit secondary antibody conjugated to Alexa Fluor 488 (1:400, Molecular Probes). For Pgr, sections were treated with biotinylated secondary antibody, followed by incubation with ABC reagent (Avidin-Biotin-horseradish peroxidase complex, Vector Laboratories). Pgr signal was visualized after signal amplification using tyramide-fluorescein as the horseradish peroxidase substrate (Tyramide Signal Amplification-Fluorescein, PerkinElmer). For BrdU IHC, sections were treated with 2 N HCl (to denature DNA) at 37 C for 45 min followed by neutralization in 0.1 M boric acid for 10 min before blocking in normal serum. BrdU+ cells were quantified in seven sections per animal.

Data analysis

Data are shown as means ± SEM. Grain densities in Fig. 3 were calculated as the percentage of OVX controls. Statistical comparisons are based on ANOVA followed by Fisher post hoc analysis, with significance at P < 0.05.

Results

Distribution of progesterone receptors in hippocampal subregions

First, we determined the relative prevalence of Pgrmc1 and Pgr mRNA in whole hippocampus. By RT-PCR, Pgrmc1 mRNA prevalence was 4-fold above Pgr mRNA in OVX rats. At proestrous and estrus stages, Pgrmc1 was 2-fold above Pgr (Supplemental Fig. 8). The ISH exposure times to reach equivalent grain density differed correspondingly for cRNA probes of the same concentration and specific activity (see Materials and Methods). Hippocampal regions differed in neuronal expression of Pgrmc1 and Pgr. Pgrmc1 mRNA per cell was 2-fold higher in CA1 and CA3 pyramidal neurons than in DG neurons (Fig. 2, A and B, and Table 1). However, CA1 and CA3 neurons had similar levels of Pgrmc1 mRNA. Within hippocampal regions, neuronal Pgr mRNA prevalence was highest in CA3, followed by CA1 and DG, in ratios of 4:2:1 (Fig. 2, A and B, and Table 1). These regional differences in Pgrmc1 and Pgr expression extend semiquantitative analyses of Intlekofer and Petersen (23).

A minority of DG neurons expressed Pgr mRNA (27 ± 4.2% cells had ≥2 grains per cell for Pgr), whereas, Pgrmc1 was detected in most DG neurons (82 ± 5.1% cells had ≥2 grains per cell; P < 0.0001) (Fig. 2B). Thus, 3-fold more DG neurons had significant signal for Pgrmc1 than for Pgr, in
contrast to CA1 and CA3 pyramidal neurons, in which both receptors were detected in more than 80% neurons (Fig. 2B). Immunostaining for cell body protein levels of \textit{Pgrmc1} and \textit{Pgr} (Fig. 2C) corresponded to RNA levels by ISH grain densities (Fig. 2A). Again, \textit{Pgr} protein was detected in a minority of DG neurons. A caveat is that protein levels of \textit{Pgrmc1} and \textit{Pgr} cannot be directly compared due to the different techniques used to visualize the signals from both receptors (Methods; \textit{Pgr} detection required two rounds of signal amplification, whereas \textit{Pgrmc1} did not require amplification). Whereas \textit{Pgrmc1} immunostaining had similar intensity throughout the hippocampal cell layers (Fig. 2C), \textit{Pgr} immunostaining was stronger in CA3 than in CA1 and DG neurons (Fig. 2C).

**E2 and P4 differentially regulate \textit{Pgrmc1} and \textit{Pgr} mRNA in hippocampal subregions**

Two schedules of steroid replacements (E2, P4, and E2+P4) of OVX young rats were compared: a 4-d model
expression in the endogenous NPC of the DG subgranular zone. Double IHC colocalized both Pgrmc1 and Pgr in more than 90% of the BrdU-labeled progenitor cells (Fig. 4, A and B). Furthermore, the Doublecortin marker for NPC destined to be neurons also colocalized with both Pgrmc1 and Pgr (Fig. 4, C and D). The 4-d steroid treatments increased BrdU-labeled cells in the DG subgranular zone by 50–100% (Fig. 4E), consistent with the findings of Liu et al. (9). Separately, E2 or P4 treatment doubled the number of proliferating NPC (P < 0.03), whereas the combination of E2 + P4 was not significant (Fig. 4E).

Discussion

We show that Pgrmc1 and Pgr differ markedly in expression and in regulation by E2 and P4 in neurons of the adult rat hippocampus, and discuss implications for hormonal regulation of synaptic plasticity and NPC proliferation. Overall, Pgrmc1 was widely expressed throughout hippocampal neurons and was induced by both E2 and P4. In contrast, Pgr showed limited expression and regulation by steroids that differed widely among neuronal subpopulations. In those responding neurons, both acute and chronic E2 and P4 replacements gave equivalent induction of Pgrmc1 and Pgr, with little or no mutual antagonism. We hypothesize that Pgrmc1 and Pgr mediate selective hippocampal P4 effects in different neuron subtypes based on their different regional expression and responses to E2 and P4. These findings are relevant to cognitive functions in postmenopausal HT.

Differential expression of Pgrmc1 and Pgr in hippocampal subregions

First we discuss the neuroanatomical differences in expression of these receptors in the hippocampus, a key site of learning and memory. Pgrmc1 was equally prevalent in CA1 and CA3 neurons, with lower expression in DG neurons. Pgr, on the other hand, was most prevalent in CA3 neurons, on the other hand, was most prevalent in CA3 neurons, whereas very little or none was expressed in DG neurons. The absence of induction by E2 + P4 suggests antagonism. In CA3 neurons, only the 4-d schedule induced Pgr mRNA by at least 50% in CA1 neurons. However, the 30-d schedule showed more modest effects, with Pgr induction in CA1 only by E2 or P4 alone (~35%, P < 0.05). The absence of induction by E2 + P4 suggests antagonism. In CA3 neurons, only the 4-d schedule induced Pgr mRNA, and very modestly (22% by P4, P < 0.05; 24% by E2 + P4, P < 0.04; no response to E2 alone), whereas the 30-d schedule had no effect. Moreover, Pgr in DG neurons was unresponsive to all hormone schedules.

Progesterone receptor expression in NPC

Because of conflicting reports on the expression of Pgr in NPC (see Introduction), we evaluated Pgrmc1 and Pgr of the ovulatory cycle and a 30-d model of HT (Fig. 1). By ISH, Pgrmc1 mRNA was broadly induced above OVX controls by 44–92% with E2 and/or P4 on both schedules in all neuronal layers of the hippocampus (Fig. 3A and Table 1). In contrast, Pgr induction was regionally restricted on both hormone schedules. Pgr was induced only in CA1 neurons, with minimal response in CA3 and DG neurons (Fig. 3B and Table 1). All steroid treatments in the 4-d schedule induced Pgr mRNA by at least 50% in CA1 neurons. However, the 30-d schedule showed more modest effects, with Pgr induction in CA1 only by E2 or P4 alone (~35%, P < 0.05). The absence of induction by E2 + P4 suggests antagonism. In CA3 neurons, only the 4-d schedule induced Pgr mRNA, and very modestly (22% by P4, P < 0.05; 24% by E2 + P4, P < 0.04; no response to E2 alone), whereas the 30-d schedule had no effect. Moreover, Pgr in DG neurons was unresponsive to all hormone schedules.

FIG. 3. Regulation of Pgrmc1 and Pgr mRNA by E2 and P4. A, Pgrmc1 mRNA was increased by E2, P4, and E2 + P4 in CA1, CA3, and DG neurons after both 4-d (n = 6 rats/group) and 30-d hormone replacement schedules (n = 5 rats per group). *, P < 0.03 compared with respective OVX. B, In CA1 neurons Pgr mRNA was increased by E2, P4, and E2 + P4 on the 4-d schedule, and by E2 or P4 alone on the 30-d schedule. Modest increase in Pgr mRNA was also seen in CA3 neurons by P4 (P4 alone and E2 + P4 group) only on the 4-d schedule. Pgr mRNA did not respond to either the 4-d or 30-d schedule in DG neurons. **, P < 0.01 vs. OVX in CA1. *, P < 0.05 compared with 4-d schedule in CA1. *, P < 0.05, vs. OVX in CA3 neurons.
microscopy, Waters et al. (44) localized extranuclear Pgr in axons of hippocampal neurons, with greater detection in CA3 axons than in other hippocampal neurons. However, detection of Pgr protein by immunohistochemistry required secondary signal amplification in the present study. We confirmed the neuronal cell type-restricted expression of Pgr by RNA and protein content (in situ hybridization and immunohistochemistry, respectively). In contrast, Pgrmc1 was expressed throughout the hippocampal neuron layers. Expression in the hippocampal hilus of Pgrmc1 and Pgr (Fig. 2C) could represent interneurons, as well as glia. Although this study was focused on Pgrmc1 and Pgr expression in different hippocampal neurons, we also observed glial expression of both receptors both in vivo, and in cultured primary glia (data not shown; our unpublished results, Bali N., T. E. Morgan, C. E. Finch).

**Differential regulation of Pgrmc1 and Pgr mRNA**

In addition to the regional differences in levels of expression between hippocampal neuron types, both...

Pgmc1 and Pgr responded differently to E2 and P4. Two hormone replacement schedules, 4-d and 30-d (Fig. 1) induced similar responses in expression. The 4-day schedule models the normal rodent ovulatory cycle and has been widely used in studies of hippocampal sprouting (4, 29), as well as in a few studies of neurogenesis (45). The 30-d schedule of continual E2 with P4 in the last 10 d is a model of the ongoing KEEPS trial of postmenopausal HT (10). We have used this 30-d replacement schedule to show P4 antagonism of E2-dependent synaptic sprouting in response to deafferenting lesions of the hippocampus (7). Clinical trials for postmenopausal HT for age-associated cognitive decline and Alzheimer disease are controversial. The Women’s Health Initiative study, one of the largest randomized controlled clinical trial of HT, found increased breast cancer incidence in the group receiving equine estrogens plus a progestin, and no cognitive benefits (11, 12). Thus, it is imperative to study the combined effects of E2 and P4 on cognition, cardiovascular disease, and other health outcomes. The KEEPS trial addresses the effects of delayed initiation of HT (critical window hypothesis); KEEPS also evaluates cyclic vs. continuous P4. In a triple transgenic mouse model of Alzheimer disease, three consecutive 30-d cycles of E2 and P4 (present model) were neuroprotective (30). Most animal models of long-term HT have evaluated E2 alone (46–48). The present studies also used a 30-d hormone schedule, as well as a 4-d schedule to model the normal estrous cycle.

On both the 4- and 30-d replacement schedules, Pgr was induced by both E2 and P4, but only in CA1 neurons and not in CA3 or DG neurons. In contrast, Pgmc1 was more broadly responsive to E2 and P4, across all neurons examined. About 40–80% induction was seen after both 4- and 30-d schedules, with similar responses in the CA1, CA3, and DG neurons. The similar induction in CA1 neurons of both Pgmc1 and Pgr by E2 and P4 is relevant to synaptic remodeling. CA1 neurons are notable for synaptic remodeling during the rodent estrous cycle, not observed in CA3 or DG neurons (Introduction). Moreover, in OVX macaques, E2 replacement for 28 d alone induced synaptogenesis in CA1 neurons (6). Introduction of P4 to the last 14 d of E2 treatment antagonized the E2-mediated increase in pre- and postsynaptic proteins, syntaxin, synaptophysin, and spinophilin, whereas P4 alone treatment increased synaptophysin in CA1 neurons. This report gives an unusual example in which P4 alone can induce synaptic proteins and with mutual antagonism of E2 + P4. We found similar responses of Pgr in CA1 neurons during the 30-d schedule. However, on a 4-d schedule, there was no indication of mutual antagonism. The differences in P4 actions when acting alone vs. E2 + P4 on a 30-d schedule are relevant to HT strategies.

The equal induction of Pgmc1 and Pgr by E2 and P4 raises interesting questions about transcriptional regulation, particularly their autoinduction by P4. We did not find full consensus progesterone response elements (PRE) in the Pgr gene. However, Pgr has multiple half-PRE sites, which, in other genes, can bind the Pgr peptides in synergy with other cofactors (49, 50). The autoinduction by P4 is consistent with the elevation of Pgr protein in the hypothalamus during pregnancy, which peaks at d 19 of rat pregnancy when plasma P4 is maximal, with much lower E2 (51). The transcriptional regulation of Pgmc1 has not been studied. We did not find consensus PRE in the Pgmc1 upstream promoter, but did find multiple half-PRE sites. Thus, the P4 autoinduction of Pgmc1 observed in the hippocampus here and in the hypothalamus (25) could be mediated indirectly by Pgr through binding of Pgr peptides to half-PRE sites.

The observed E2 regulation of Pgmc1 and Pgr could be mediated by estrogen receptors (ER). We can dismiss a mechanism of cross talk at the ligand-binding level, because E2 does not compete with P4 for binding to either Pgmc1 (16) or Pgr (53); nor does P4 compete with E2 at physiological levels for binding to ER (54). The Pgr promoter contains multiple ERE (estrogen response element) sequences that could mediate induction by E2 (55, 56, 57). Although we did not find consensus ERE in the Pgmc1 upstream promoter, there are multiple half-ERE sites. Both ERα and ERβ are expressed in hippocampal neurons (58–60), though ERβ was more prevalent in CA1 and CA3 pyramidal neurons than in DG neurons (59, 60), resembling the expression of Pgmc1 and Pgr (Fig. 2). The importance of Erα to Pgr regulation is shown by the absence of induction by E2 in the hippocampus of ERKoα mice (61).

The pharmacological specificity of Pgmc1 and Pgr also needs further study. The antiprogestin RU486 blocked the decrease in CA1 dendritic spines during the estrus (4) and also blocked P4 antagonism of E2-induced neurite outgrowth in an in vitro lesion model (7). Although RU486 does act on recombinant Pgr (62, 63), it is not known whether it also acts on Pgmc1. In addition to Pgrmc1, three other membrane PR are recognized: the G protein-coupled receptors mPRα, mPRβ, and mPRγ. Little is known of their expression patterns in brain cell types, P4 binding characteristics, and steroidal regulation, or P4-specific functions (23, 25, 64–67).

CA1 neurons were the only hippocampal subregion that showed similar hormonal responses of both Pgmc1 and Pgr (Fig. 3). This regional restriction is interesting because CA1 neurons are more vulnerable to Alzheimer disease (68, 69), posts ischemic damage (70), and hypoxia-induced seizure activity, relative to CA3 and DG neurons.
The responsiveness of progesterone receptors to E2 and P4 is relevant to stroke because CA1 neurons are protected in rodent models of ischemia by both E2 (72, 73) and P4 (74). The P4 neuroprotection of CA1 could be mediated by either Pgrmc1 or Pgr. The receptor involved in CA1 neuroprotection could be identified using Pgr knockout mice (PRKO). However, Pgrmc1 knockout mice have not been reported so far.

**Pgrmc1 and Pgr expression in NPC of the subgranular zone of DG**

The DG was examined for expression of Pgrmc1 and Pgr in its P4-sensitive NPC, which have not been characterized in detail. In adult female rats (9) and male mice (75), P4 promoted the generation of nascent neurons in the DG subgranular zone. Both E2 and P4 stimulated NPC proliferation in cells obtained by whole hippocampus cell sorting (9) and in an established adult rat NPC line derived from the DG subgranular zone (8, 76), as confirmed in vivo here. The *in vitro* proliferative effects of P4 involve Pgrmc1 through P4-induced kinase signaling (8). The present study detected both Pgrmc1 and Pgr in newly formed immature neurons (Doublecortin immunopositive) in the subgranular zone. However, most DG mature neurons lack Pgr (Fig. 2B), which may be consequent to DG neuronal maturation, e.g. Pgr expression in the DG peaked by postnatal d 7 and was undetectable by d 28 (77, 78). Lastly, we note the divergence in Pgr expression between NPC lines. Whereas a rat NPC line derived from the DG subgranular zone did not have Pgr by PCR (8), Pgr protein was detected in NPC originated from the subventricular zone, another site of adult neurogenesis (52). This difference could be outcomes of continued *in vitro* propagation of the DG subgranular cell line or to the different sites of NPC origination.

The responses of Pgrmc1 and Pgr to P4 ± E2 were very similar for both 4-d and 30-d hormone replacement schedules. Pgrmc1 was increased in all hipocampal neuronal layers on both schedules in which E2 alone, P4 alone, or combined gave similar induction. Whereas Pgr responses were restricted to CA1 neurons, responses to E2 and P4 alone or together were again equivalent on the 4-d schedule. However, the weaker increase of Pgr in CA1 on the 30-d schedule of combined E2 + P4 suggests possible antagonism. Others have reported selective induction. In the hypothalamus (25) only select nuclei showed Pgrmc1 elevations with P4 alone or E2 + P4; unlike hippocampal responses, there was no effect of E2 alone. In contrast, Pgrmc1 (cited as 25-Dx) was increased in the hypothalamus of E2-primed OVX females, but this increase was attenuated by P4 (34). Reports on Pgr are also divergent. As we observed in CA1 neurons with both hormone schedules, P4 alone increased Pgr in chick embryo hypothalamus and posterior pituitary (28). However, Intlekofer and Petersen (25) observed that P4 alone did not increase hypothalamic Pgr mRNA. A more detailed study of the time course is warranted because Turgeon et al. (27) showed the transience of P4 down-regulation of Pgr, with receptor levels returning to the level of E2-treated controls by 12 h after treatment.

**Conclusions**

Pgrmc1 is widely expressed in neuronal layers of all hippocampal regions and is induced by both E2 and P4. In contrast, Pgr shows restricted regional expression and regulation by E2 and P4. The shared induction of both Pgrmc1 and Pgr by E2-P4 in CA1 neurons may be relevant to their capacity for E2-dependent synaptic remodeling and to CA1 sensitivity to neurodegeneration from Alzheimer disease and ischemia. The differential regulation of hippocampal Pgrmc1 and Pgr gives a rationale for development of drugs in hormonal therapy to target multiple receptors in the support of neurogenesis, neuroprotection, and synaptic regeneration.

**Acknowledgements**

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This work was supported by National Institute on Aging Grants 1PO1 AG026572 (to R.D.B.); Project 4 (to C.E.F. and T.E.M.), Animal Core A (to T.E.M.), and Analytic Core C (to L.Z.).

Disclosure Summary: All authors declare no conflicts.

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