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)

Interactions of estradiol (E2) and progesterone (P4) drive reproductive organ remodeling during ovulatory cycles (1, 2). In anticipation of implantation, uterine cell growth is stimulated by blood elevations of E2 during the follicular phase. During the luteal phase in the absence of implantation, endometrial cell death (apoptosis) is promoted by the cyclic elevation and decrease of P4 (1, 2). Moreover, during rodent ovulatory cycles, there is cyclic synaptic remodeling in the hippocampus (3, 4), a brain region critical for memory. In the follicular phase, hippocampal CA1 pyramidal neurons grow additional dendritic spines and synapses, which then regress rapidly after ovulation when E2 falls and P4 rises (3–5). These ovarian-driven processes have been further resolved in ovariectomized (OVX) rats as independent actions of E2 and P4: after induction by E2 treatment, the decline of CA1 spines is dependent on the presence of elevated plasma P4 (4).

In these examples, E2 and P4 appear to act independently at different phases of cyclical remodeling processes. However, in several animal models, cotreatment with P4

Abbreviations: BrdU, Bromodeoxyuridine; DG, dentate gyrus; E2, estradiol; ER, estrogen receptor; ERE, estrogen response element; HT, hormone therapy; ISH, *in situ* hybridization; NPC, neural progenitor cell; OVX, ovariectomized; P4, progesterone; SSC, saline-sodium citrate.

attenuated E2-induced synaptic growth. In the OVX macaque, the induction by E2 of the synaptic proteins syntaxin, synaptophysin, and spinophilin 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 (K_d) = 0.38 nM (15); *Pgrmc1*, K_d = 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 Ca^{2+} 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 verified that Vema and *Pgrmc1* in GenBank share amino acid sequences. In adult rodents, *Pgrmc1* was detected in the hippocampus, hypothalamus, and cerebellum (23, 24). Both E2 and P4 induced *Pgrmc1* in the sexually dimorphic nucleus of the preoptic area and the ventromedial nucleus of the hypothalamus (25). However, these reports did not describe its cell level expression.

We extend 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 antag-

onism 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, ip) 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

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

	OVX	E2	P4	E2+P4
Pgrmc1				
CA1				
4−d	8.7 ± 0.6	12.5 ± 1.6[a]	14.9 ± 1.8[a]	13.3 ± 1.3[a]
30−d	10.7 ± 1.4	13.8 ± 0.7[a]	16.6 ± 0.6[a]	14.4 ± 0.4[a]
CA3				
4−d	9.7 ± 0.6	13.5 ± 1.1[a]	15.5 ± 1.3[a]	15.9 ± 1.7[a]
30−d	10.1 ± 1.0	13.1 ± 1.1[a]	13.4 ± 0.5[a]	13.4 ± 0.5[a]
DG				
4−d	5.1 ± 0.3	7.7 ± 0.9[a]	9.3 ± 0.8[a]	8.7 ± 0.8[a]
30−d	5.1 ± 0.5	6.6 ± 0.6[a]	7.4 ± 0.6[a]	7.1 ± 0.1[a]
Pgr				
CA1				
4−d	9.2 ± 1.0	14.4 ± 1.9[a]	17.6 ± 0.7[a]	16.5 ± 1.1[a]
30−d	11.6 ± 1.4	16.2 ± 1.3[a]	15.7 ± 1.3[a]	13.2 ± 1.0
CA3				
4−d	23.2 ± 2.0	25.8 ± 2.1	28.3 ± 0.7[a]	28.9 ± 1.8[a]
30−d	23.9 ± 0.9	27.0 ± 2.5	27.3 ± 0.8	26.3 ± 1.1
DG				
4−d	4.1 ± 0.1	4.2 ± 0.2	4.4 ± 0.2	4.6 ± 0.2
30−d	3.0 ± 0.4	3.1 ± 0.4	3.5 ± 0.2	3.2 ± 0.4

Pgrmc1 and *Pgr* grain densities after hormone treatment. Emulsion-dipped slides for *Pgrmc1* and *Pgr* were exposed for different times to achieve similar grain densities. Statistical comparisons were made within treatment groups for each receptor. Grain densities are means ± SEM of six rats per hormone group for 4-d and five rats per group for 30-d schedules; 100 cells per brain in CA1 and CA3, and 120 cells per brain in DG. *Pgrmc1*: [a], $P < 0.03$ *vs.* respective OVX group. *Pgr*: [a], $P < 0.05$, *vs.* respective OVX group.

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 hemi-

A

4-day hormone replacement

B

30-day hormone replacement

FIG. 1. Two hormone replacement schedules were studied. A, For 4-d hormone replacement, rats (n = 6 rats per group) were given two injections of E2 (10 μg, sc), 24 h apart, followed by single P4 injection (4 mg/kg, sc) 24 h after last E2 injection. A single injection of BrdU (100 mg/kg, ip) was given 1 h after P4; tissue collection, 30 h after BrdU. B, For 30-d hormone replacement, rats (n = 5 rats per group) were implanted with E2 pellet (0.72 mg/30 d release) 2 wk after OVX, followed by P4 pellet (50 mg/15 d release) in the last 10 d; tissue collection on d 30.

sphere 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 ug RNA; Superscript III kit, Invitrogen, Carlsbad, CA). RT-PCR was performed with SYBR Green I and used the following primers: r*Pgrmc1* (forward, 5′-GCCTCAAGCCGCGTGACTTC-3′; reverse, 5′-CT GGGCAGGAGTGAGGTCAG-3′);r*Pgr*(forward,5′-GTCAGT GGACAGATGCTA-3′; reverse, 5′-AGCTGTTTCACAAGA TCA-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 sagitally (18-μm) on a cryostat and stored at −80 C until use. For ISH, [^{35}S]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 (4.9×10^6 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
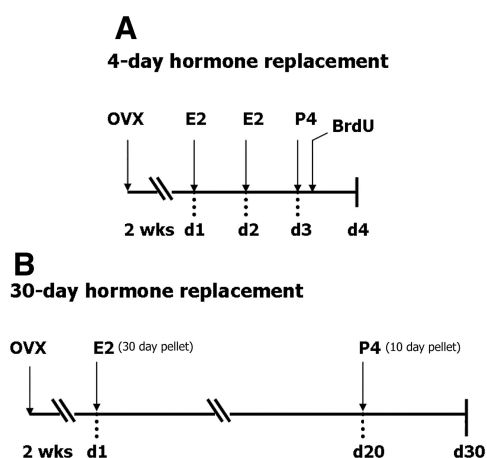
18–48 h. Slides were emulsion dipped (NTB2, Eastman Kodak, Rochester, NY), developed, and counterstained in Harris modified hematoxylin according to standard procedures (36). Based on x-ray film density, the emulsion-dipped slides were exposed for different times to reach equivalent grain densities needed 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 conjugated to Alexa Fluor 594 (1:400, Molecular Probes, Inc., Eugene, OR; Invitrogen), donkey antigoat conjugated to Alexa Fluor 594 (1:400, Molecular Probes, Invitrogen). The specificity of the *Pgrmc1* antibody was confirmed by Western blotting of rat whole hippocampal and cortical lysates. Two bands were detected, a strong band at 25 kDa and a weak band at 50 kDa corresponding to *Pgrmc1* monomer and dimmer, respectively (42, 43). The Santa Cruz *Pgr* antibody detected both PR-A and B isoforms. Western blot bands corresponded to PR-A and B at approximately 95 kDa and 110 kDa (Supplemental Fig. 7A). Specificity of the antibody was confirmed by preadsorbing the antibody with 10-fold excess of immunizing peptide (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% su-

crose/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 $\pm$ 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 proestrus 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 $\pm$ 4.2% cells had $\geq$2 grains per cell for *Pgr*), whereas, *Pgrmc1* was detected in most DG neurons (82 $\pm$ 5.1% cells had $\geq$2 grains per cell; $P < 0.0001$) (Fig. 2B). Thus, 3-fold more DG neurons had significant signal for *Pgrmc1* than for *Pgr*, in
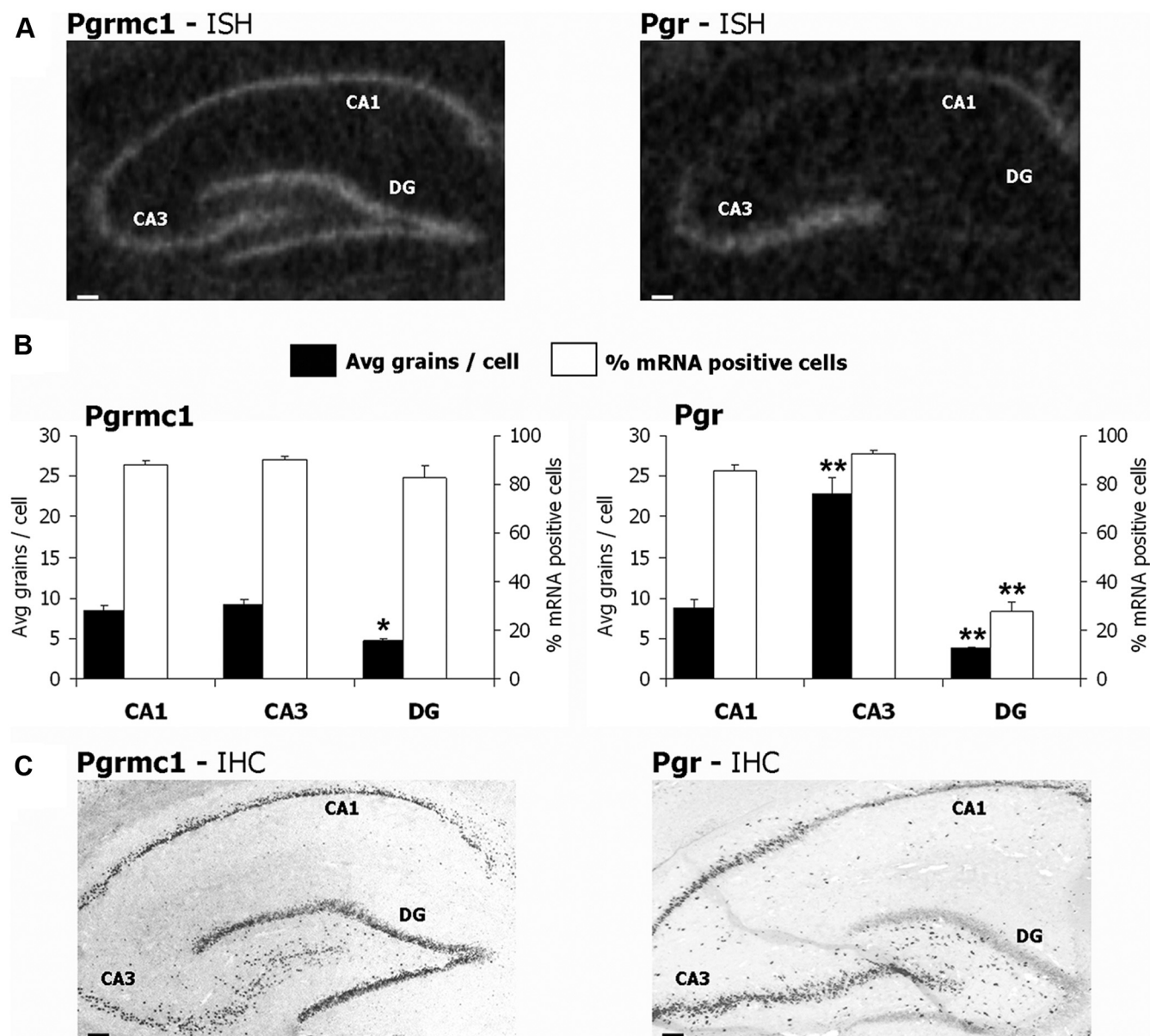
FIG. 2. Expression of *Pgrmc1* and *Pgr* in hippocampal neuronal layers. A, Autoradiographic film images showed differential distribution of mRNA for *Pgrmc1* (*left*) and *Pgr* (*right*) in hippocampal neuronal layers. *Pgrmc1* was prevalent across all three neuronal layers. By contrast, *Pgr* mRNA was prevalent in CA1 and CA3 pyramidal neurons, but it was barely detected in adjacent DG neurons. B, Average grain density for *Pgrmc1* and *Pgr* in individual neurons of CA1 (100 cells per rat), CA3 (100 cells per rat), and DG (120 cells per rat). *Pgrmc1* grain density was similar in CA1 and CA3 neuronal perikarya and far below in DG neurons. *, $P < 0.01$ *vs.* CA1 and CA3. More than 80% neurons in CA1, CA3, and DG were positive for *Pgrmc1* mRNA. CA3 neurons had 2-fold more *Pgr* mRNA/perikaryon than CA1 and DG neurons. CA1 neurons had 2-fold *Pgr* over DG neurons. More than 80% CA1 and CA3 neurons, but only approximately 25% DG neurons were positive for *Pgr* mRNA. **, $P < 0.0001$ *vs.* other groups. C, Immunohistochemistry for *Pgrmc1* and *Pgr* showed similar protein expression of both receptors as mRNA. *Scale bars*, 100 μm. Avg., Average.

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 *Pgrmc1* and *Pgr* (Fig. 2C) corresponded to RNA levels by ISH grain densities (Fig. 2A). Again, Pgr protein was detected in a minority of DG neurons. A caveat is that protein levels of *Pgrmc1* and *Pgr* cannot be directly compared due to the different techniques used to visualize the signals from both receptors (Methods; *Pgr* detection required two rounds of signal amplification, whereas

Pgrmc1 did not require amplification). Whereas *Pgrmc1* immunostaining had similar intensity throughout the hippocampal cell layers (Fig. 2C), *Pgr* immunostaining was stronger in CA3 than in CA1 and DG neurons (Fig. 2C).

E2 and P4 differentially regulate *Pgrmc1* and *Pgr* mRNA in hippocampal subregions

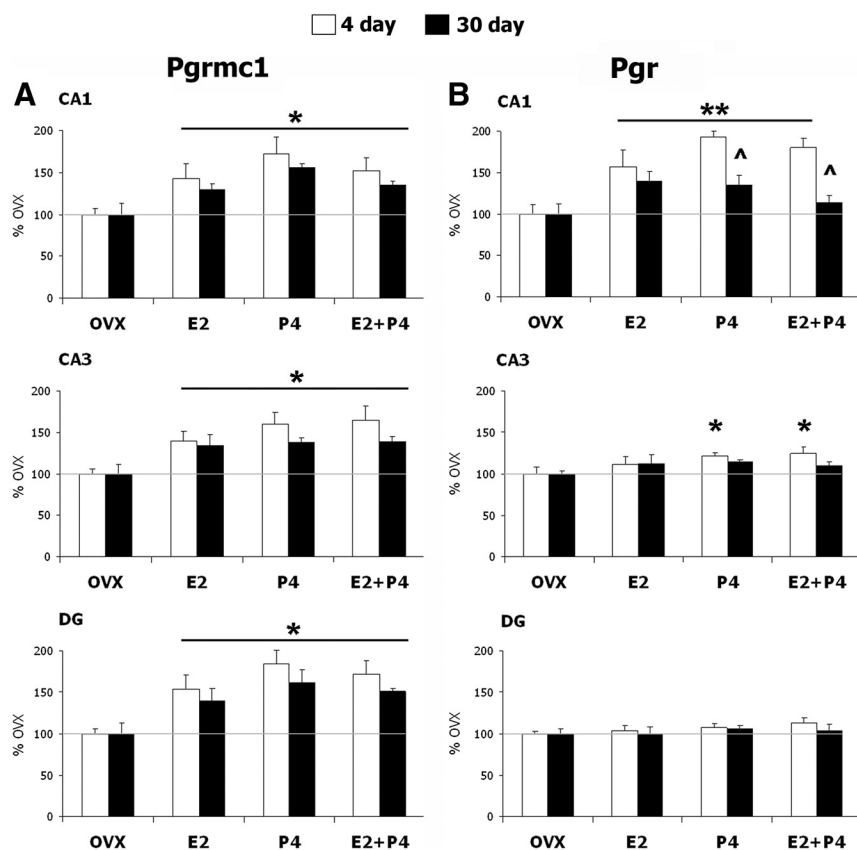Two schedules of steroid replacements (E2, P4, and E2+P4) of OVX young rats were compared: a 4-d model

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. ^, $P < 0.01$ compared with 4-d schedule in CA1. *, $P < 0.05$, *vs.* OVX in CA3 neurons.

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.

Progesterone receptor expression in NPC

Because of conflicting reports on the expression of *Pgr* in NPC (see *Introduction*), we evaluated *Pgrmc1* and *Pgr*

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 ($P < 0.03$), wherease 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 and much less in DG neurons. CA1 neurons had intermediate levels of *Pgr* mRNA. These findings at the cellular level confirm the regional distributions of *Pgrmc1* and *Pgr* reported by Intlekofer *et al.* (23). With electron
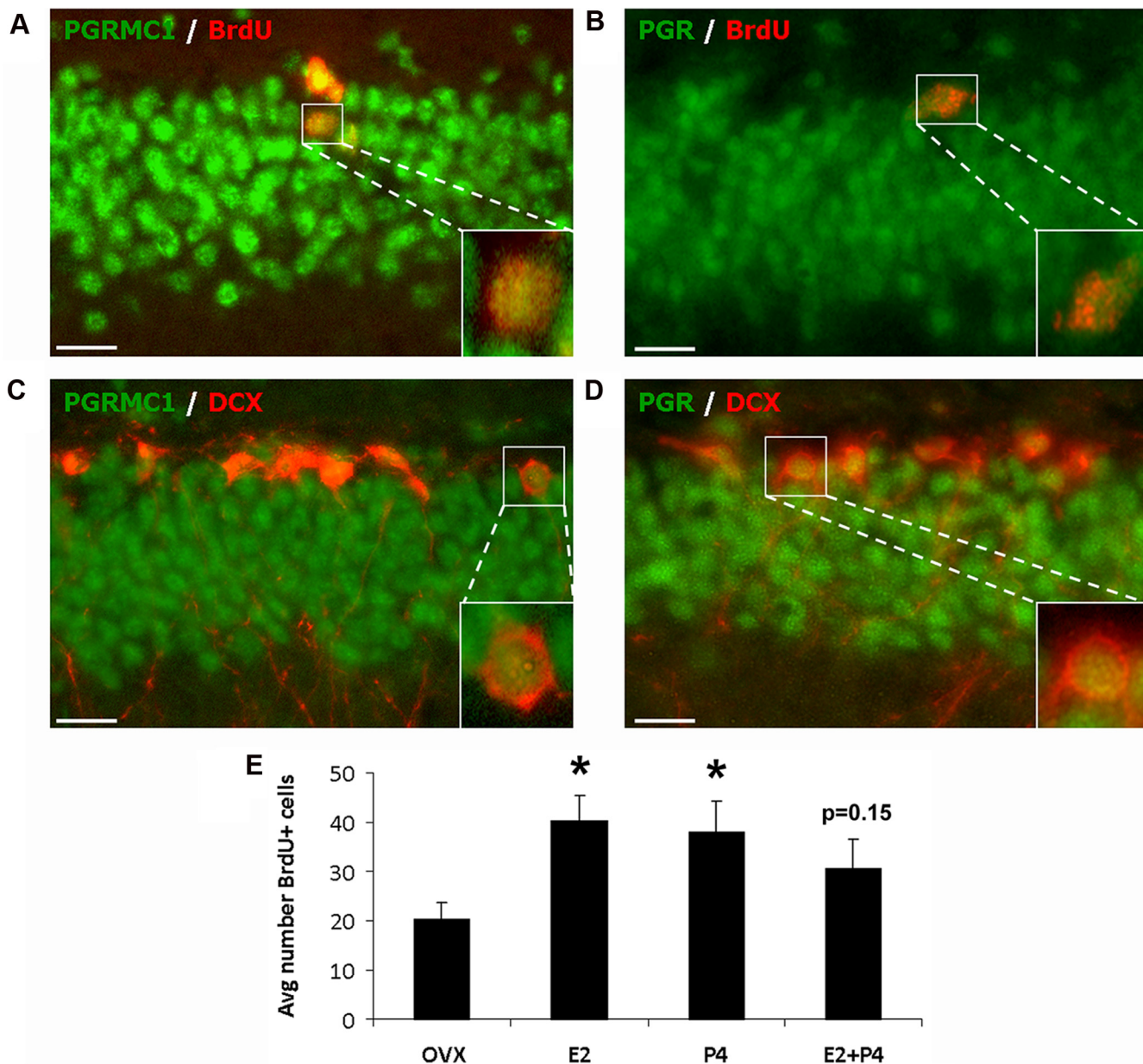
FIG. 4. Expression of *Pgrmc1* and *Pgr* in NPC. A and B, Colocalization of *Pgrmc1* and *Pgr* in replicating (BrdU) NPC by double immunohistochemistry for *Pgrmc1* with BrdU (A) and *Pgr* with BrdU (B); individual colabeled cell magnified in *inset*. C and D, Double immunohistochemistry for *Pgrmc1* (C) with Doublecortin (DCX), a marker of newly generated neurons in SGZ of DG and Pgr (D) with DCX showed colocalization of both in DCX-positive newly generated neurons; individual colabeled cell magnified in *inset*. *Scale bars*, 20 μm. E, Hormonal regulation of neurogenesis by E2 and P4 in the DG subgranular zone. Both E2 and P4 (4-d schedule; n = 6 rats per group) increased the number of BrdU$^+$ cells in SGZ; response to E2+P4 was slightly lower ($P = 0.15$; not significant). *, $P < 0.03$, *vs.* OVX. Avg., Average.

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

Pgrmc1 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, *Pgrmc1* 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 *Pgrmc1* 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 *Pgrmc1* 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 *Pgrmc1* has not been studied. We did not find consensus PRE in the *Pgrmc1* upstream promoter, but did find multiple half-PRE sites. Thus, the P4 autoinduction of *Pgrmc1* 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 *Pgrmc1* 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 *Pgrmc1* (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 *Pgrmc1* 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 *Pgrmc1* 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 *Pgrmc1* 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 Pgrmc1. 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 *Pgrmc1* and *Pgr* (Fig. 3). This regional restriction is interesting because CA1 neurons are more vulnerable to Alzheimer disease (68, 69), postischemic damage (70). and hypoxia-induced seizure activity, relative to CA3 and DG neurons

(71). 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 hippocampal 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 sched-

ules, 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 transiency 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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