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Cellular and subcellular localization of estrogen and progesterin receptor immunoreactivities in the mouse hippocampus

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Abstract

Estrogen receptor- α (ER α), - β (ER β) and progesterin receptor (PR) immunoreactivities are localized to extranuclear sites in the rat hippocampal formation. Since rats and mice respond differently to estradiol treatment at a cellular level, the present study examined the distribution of ovarian hormone receptors in the dorsal hippocampal formation of mice. For this, antibodies to ER α , ER β , and PR were localized by light and electron immunomicroscopy in male and female mice across the estrous cycle. Light microscopic examination of the mouse hippocampal formation showed sparse nuclear ER α - and PR-immunoreactivity (-ir) most prominent in the CA1 region and diffuse ER β -ir primarily in the CA1 pyramidal cell layer as well as in a few interneurons. Ultrastructural analysis additionally revealed discrete extranuclear ER α -, ER β - and PR-ir in neuronal and glial profiles throughout the hippocampal formation. While extranuclear profiles were detected in all animal groups examined, the amount and types of profiles varied with sex and estrous cycle phase. ER α -ir was highest in diestrus females, particularly in dendritic spines, axons and glia. Similarly, ER β -ir was highest in estrus and diestrus females, mainly in dendritic spines and glia. Conversely, PR-ir was highest during proestrus, and mostly in axons. Except for very low levels of extranuclear ER β -ir in mossy fiber terminals in mice, the labeling patterns in the mice for all three antibodies were similar to the ultrastructural labeling found previously in rats, suggesting that regulation of these receptors is well conserved across the two species.

Keywords

electron microscopy; estrogen receptor alpha; estrogen receptor beta; extranuclear steroid receptors; axons; dendrites

Introduction

The estrogen and progesterin steroid hormone families affect cellular functions in many cell types in the brain and throughout the periphery. In the brain, these hormones act genomically, on nuclear receptors that act as transcription factors through estrogen and progesterin response elements in the DNA (Becker and Hu, 2008). Estrogens and progestins in the brain also act non-genomically, on extranuclear receptors affiliated with the plasma membrane or membranous organelles to rapidly activate signaling pathways (Kelly and

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Levin, 2001; Spencer et al., 2007). The estrogen receptors ER α and ER β , and the progesterone receptor (PR), may initiate both genomic and non-genomic actions (Razandi et al., 1999; Hammes and Levin, 2007). Thus, the effects of these steroid hormones in different brain regions likely depend on the cellular location of their receptors. Estrogens bind with nearly equal affinity to ER α and β (Shughrue and Merchenthaler, 2000; McEwen et al., 2001; Levin, 2001). In the rat hippocampus, ER α , ER β and PR are differentially located at nuclear and extranuclear sites. Specifically, nuclei with ER α -immunoreactivity (ir) are scarce and are found in inhibitory interneurons (Weiland et al., 1997; Milner et al., 2001; Nakamura and McEwen, 2005; McEwen and Milner, 2007), whereas nuclei with ER β - or PR-labeling are not detected in either principal cells or interneurons in the rat hippocampus (Milner et al., 2005; Waters et al., 2008). However, extranuclear ER α -, ER β - and PR-immunoreactivities are abundant in the rat hippocampus (Milner et al., 2001; Milner et al., 2005; Herrick et al., 2006; Waters et al., 2008). Furthermore, we have recently found that the amount of extranuclear ER α -ir and extranuclear PR-ir in the rat hippocampus is sensitive to fluctuating hormone levels (Romeo et al., 2005; Waters et al., 2008). This finding is consistent with previous studies in a number of brain areas, where the expression of nuclear ER α and nuclear PR was regulated by fluctuating hormone levels whereas ER β was not (Haywood et al., 1999; Milner et al., 2008).

Several investigators have found that estrogens and progestins affect hippocampal-dependent learning and memory processes in both rats and mice. In rats, ER α , ER β and PR agonists can enhance spatial learning (Luine et al., 1998; Korol and Kolo, 2002; Korol et al., 2004; Frye et al., 2007). The extensive extranuclear location of ERs and PRs in the rat hippocampus and their sensitivity to fluctuating steroid levels suggests that non-genomic actions may be responsible for the effects of ovarian steroid hormones on hippocampal function in this species. Although most studies of the effects of ovarian steroid hormones on the hippocampus have been conducted in rats, the ease of genetic manipulation in mice makes this rodent species an attractive model for follow-up studies. Behavioral studies using ER knockout mice have begun to demonstrate an important role for ER α and ER β in hippocampal function. Knockout of the ER α gene (ERKO) reduces estrogen responsiveness and hippocampal related memory which can be restored following ER α administration (Foster et al., 2008). ER β knock out (BERKO) mice show deficits in long-term potentiation LTP and hippocampal related-memory (Day et al., 2005), and administration of an ER β agonist improves cognitive performance in wild-type but not BERKO mice (Walf et al., 2008). Progestins may be important as well, as administration of progesterone to ovariectomized (OVX) mice enhances cognitive behavior in some learning tasks but not others (Frye and Walf, 2008).

Despite the behavioral similarities, several lines of evidence indicate that the cellular mechanisms by which ovarian hormones, particularly estrogens, affect hippocampal function may differ between rats and mice (Spencer et al., 2007). For example, elevated levels of estrogens either in proestrus or following estrogen administration to OVX rats increases the total number of dendritic spines in stratum radiatum of the CA1 region (Woolley et al., 1996; Woolley, 1998). In contrast, estradiol administration to OVX mice increases the number of spines with mushroom shapes and certain synaptic properties measured by electron microscopy, but does not increase the overall number of spines (Li et al., 2004; Xu and Zhang, 2006). Estrogens increase the expression of synaptic proteins in the hippocampus of both rats and mice; however, these changes are mostly limited to CA1 in rats whereas they occur in many hippocampal regions in mice (Brake et al., 2001; Waters et al., 2009). On the basis of these differences, we predicted that rats and mice differ in the anatomical location of ERs and PRs and/or their sensitivity to circulating hormones. These differences would alter the mechanisms of estrogen actions in the mouse hippocampus, explaining some of the species differences in hormone sensitivity. To address this

hypothesis, in this study we determined the ultrastructural localization of ER α , ER β , and PR in the hippocampal formation of male mice and female mice at different stages of the estrous cycle.

METHODS

Animals

All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Weill Cornell Medical College and Rockefeller University Institutional Animal Care and Use Committees. Female (n = 18) and male (n = 6) adult (aged between 2 and 3 months) C57BL/6 mice from Jackson Laboratory (Bar Harbor, ME) were used. All mice were housed with 12:12-hr light/dark cycles (lights on 0600–1800). Vaginal smear cytology (Turner and Bagnara, 1971) was used to determine estrous cycle stage and females were assessed for at least three full weeks. Female mice were perfused at the diestrus (N = 6), proestrus (N = 6), and estrus (N = 6) stages of the estrous cycle.

Mice were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and were perfused through the ascending aorta sequentially with: 1) 5–10 ml saline (0.9%) containing 1,000 units of heparin; 2) 40 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4) (Milner et al., 2001). Each brain was removed from the skull and post-fixed in 2% acrolein and 2% paraformaldehyde in PB for 30 minutes. Following the post-fix, the brains were cut into 40 μ m thick coronal sections using a vibrating microtome (Vibratome, Leica) and collected into PB. Sections then were stored at -20°C in cryoprotectant (30% sucrose, 30% ethylene glycol in PB) until immunocytochemical processing. For this, sections from each mouse were marked with punches in the cortex and pooled into sets, with each set containing a male, a diestrus female, a proestrus female, and an estrus female (n = 4/set). To minimize differences in immunocytochemical labeling, tissue from animals in the same set were processed together (Pierce et al., 1999; Spencer et al., 2008).

Immunocytochemistry

Antibodies—ER α immunolabeling was performed using a rabbit polyclonal antiserum (AS409) against the near full-length peptide of the native rat ER α (aa 61 through the carboxyl terminus) (produced and generously supplied by S. Hayashi's laboratory, Japan). Binding of ^3H -estradiol to ER α from the rat uterus was inhibited by the AS409 antibody in a dose-dependent manner. Moreover, the antibody recognized the ER α occupied by ^3H -estradiol (Okamura et al., 1992; Alves et al., 1998). The specificity of this ER α antibody has been previously demonstrated (Milner et al., 2001). Specifically, on immunoblots of uterine lysates from female rats the AS409 antiserum recognized one major band migrating at $\sim 67\text{kD}$ (the molecular weight of ER α). Additionally when tested on immunoblots of ER α fusion protein, the AS409 antibody recognized minor bands migrating at $\sim 110\text{kD}$ (likely the ER α /fusion protein complex), one major band migrating at $\sim 67\text{kD}$ and minor bands migrating at $\sim 41\text{--}45\text{kD}$ (the degradation products of ER α , resulting from the purification process of ER α from the fusion protein). When the antibody was preadsorbed with purified ER α , no bands were detected in any of these locations. Additionally, immunolabeling of nuclei in the hypothalamus of rats whose brains had been fixed using identical fixation conditions as the present study was not observed when the AS409 antiserum was preadsorbed with purified ER α or when the sections were incubated in preimmune serum (AS401).

ER β immunolabeling was performed using a rabbit polyclonal antibody (485; produced and generously supplied by Merck Research Laboratories, Rahway, NJ) against a conserved sequence (rat aa 64–82) of the mouse, human, and rat ER β that is located within the A/B domain of ER β (exons 2–3) and is not present in ER α (Mitra et al., 2003). Specificity of this antibody has been demonstrated previously (Mitra et al., 2003). Briefly, immunoblots of total extract of SF9 cells transfected with cloned rat and mouse or human ER β identified a band migrating at ~60kDa, whereas no staining was detected in extracts of cells transfected with either rat or human ER α . Western blots showed, a band migrating at ~55kDa in extracts of human ovary and human testes; the latter tissue also showed a band migrating at ~70kDa. Additionally, Western blots of whole brain extracts from rat and mouse showed a single band of ~70kDa. No bands were seen in brain extracts or extracts of SF9 cells expressing the cloned mouse, rat, or human ER β when 80424 was preincubated with the immunogenic peptide. COS-7 cells transfected with human ER β showed strong nuclear labeling with the 80424 antibody. This labeling was eliminated with preadsorption of the immunogenic peptide. Moreover, no staining was detected in COS-7 cells transfected with ER α or an empty vector. In hippocampal sections perfusion fixed with acrolein and paraformaldehyde, no immunoreactivity was detected following incubation without the primary antibody or with antisera preadsorbed with cognate peptide (Mitra et al., 2003; Milner et al., 2005).

PR immunolabeling was performed using a rabbit polyclonal antibody (Dako (A0098; Carpinteria, CA against a peptide corresponding to amino acids 533–547 (NYLRPDSEASQY) which is contained in both the A and B isoforms of human PR (Traish and Wotiz, 1990). Specificity of this antibody has been previously demonstrated (Traish and Wotiz, 1990). Briefly, in sucrose density gradients of PR prepared and labeled in the presence of proteolysis inhibitors and sodium molybdate, the PR antibody bound to a site on the intact undenatured PR, but failed to bind to partially degraded steroid-binding form of the receptor, suggesting that the antibody-binding domain is at or near a site sensitive to proteolysis. The antibody did not react with ER α , glucocorticoid, or androgen receptors, but recognized PR from human breast cancer and uteri from calf, rabbit, mouse and rat. No labeling was detected in brain tissues following preadsorption with the immunizing peptide (Haywood et al., 1999; Quadros et al., 2002; Quadros et al., 2007), and in thymus, uterus, and brain of PR knockout mice (Kurita et al., 1998; Tibbetts et al., 1999; Quadros et al., 2007; Waters et al., 2008).

Immunolabeling—Free-floating sections were processed for immunocytochemical localization using a modification (Milner et al., 2001) of the avidin-biotin complex (ABC) protocol (Hsu et al., 1981). Briefly, hippocampal sections were washed in 1) PB to remove cryoprotectant, three 10-minute washes; 2) 1% sodium borohydride in PB for 30 minutes to remove active aldehydes; 3) PB to remove sodium borohydride. At this point, the sections for PR labeling were put through an additional freeze-thaw procedure. Briefly, sections were 1) incubated in cryoprotectant solution (25% sucrose, 1% glycerol in 0.05M PB) for 15 minutes; 2) laid flat on thin-mesh grids in cryoprotectant; 3) blotted dry on filter paper; 4) quickly submerged in Freon; 5) quickly submerged in liquid nitrogen; and 6) washed in PB. All sections were then incubated in 1) 0.5% bovine serum albumin (BSA) in Tris-saline solution (TS; 0.9% saline in 0.1M Tris, pH 7.6) to block non-specific binding; 2) primary antisera in 0.1% BSA in TS (ER α - 1:10,000; ER β - 1:500; PR - 1:1500) for 1 day at room temperature (~23°C), followed by 3–4 days cold (~4°C); 3) 1:400 of anti-rabbit biotinylated IgG, 30 minutes; 4) 1:100 peroxidase-avidin complex (Vectastain Elite Kit), 30 minutes; 5) 3,3'-diaminobenzidine (DAB; Aldrich, Milwaukee, WI) and H₂O₂ in TS, 6–8 minutes; all incubations were separated by washes in TS.

For light microscopy, 0.25% Triton-X was included in the primary antibody diluents for ER α and PR. Following the DAB procedure, sections were rinsed in PB and mounted onto

gelatin-coated glass slides. Sections were dehydrated through a graded alcohol series to xylene and coverslipped with DPX mounting media (Aldrich).

For electron microscopy, sections were postfixated for 1 hour in 2% osmium tetroxide in PB, dehydrated through a series of alcohols and propylene oxide, and embedded in EMBED 812 (EMS) between two sheets of plastic (Milner et al., 2001). Sections from the midseptotemporal level of the dorsal hippocampus [between AP -2.10 and -2.40 from Bregma (Hof et al., 2000)] were selected, mounted on EMBED chucks and trimmed to 1–1.5 mm trapezoids. Ultrathin sections (70 nm thick) close to the plastic-tissue interface (within $0.1 - 0.2 \mu\text{m}$) were cut on a Leica UTC ultratome, collected on grids, and counterstained with uranyl acetate and Reynold's lead citrate. Final preparations were analyzed on a FEI Tecnai Biotwin transmission electron microscope and images were acquired with a digital camera system (Advanced Microscopy Techniques, v. 3.2). For the figures, digital images were adjusted for levels, brightness, contrast, and sharpness (using the unsharp mask function) in Adobe PhotoShop 7.0. Final figures were assembled in Quark Xpress 6.1.

Quantitative Analysis—Electron microscopic examination of immunoreactivity was performed on one hippocampal section from each animal (3 proestrus, 3 diestrus or 3 estrus females or 3 males; $N = 12$). From each section, the CA1 region, CA3 region, and dentate gyrus were examined. In the CA1, stratum radiatum was divided into a proximal field (closer to the stratum pyramidale) and a distal field (closer to the stratum lacunosum-moleculare). In each section, ten random, but not overlapping, micrographs ($36 \mu\text{m}^2$ /micrograph) per lamina per brain region were taken. Immunolabeled profiles were classified using the nomenclature of Peters et al. (Peters et al., 1991). Dendritic profiles contained regular microtubular arrays and were usually postsynaptic to axon terminal profiles. Unmyelinated axons were profiles smaller than $0.2 \mu\text{m}$ that contained a few small synaptic vesicles and lacked a synaptic junction in the plane of section. Axon terminal profiles had numerous small synaptic vesicles and had a cross-sectional diameter greater than $0.2 \mu\text{m}$. Astrocytic profiles were distinguished by their tendency to conform to the boundaries of surrounding profiles, by the absence of microtubules, and/or by the presence of glial filaments. "Unknown profiles" contained immunoperoxidase reaction product but could not definitely be placed in one of the above categories.

Data was analyzed three ways. First, the relative distribution of each type of profile per lamina regardless of cycle/sex was calculated. For this, the mean and standard error of the mean (SEM) for each type of profile in each lamina was calculated by combining data from all 12 animals; these values are shown in the tables. Second, all profiles from each animal were pooled by type (dendritic shafts, dendritic spines, terminals, axons and glia) and compared across groups using a two way ANOVA. The data for each profile type are described as a percentage of the total number of profiles per group. Third, a lamina analysis limited to the CA1 stratum radiatum (distal) and the CA3 stratum lucidum was performed because of their known sensitivity to estrogens (McEwen and Milner, 2007; Torres-Reveron et al., 2008; Torres-Reveron et al., 2009) and because pTrkB-labeled axons fluctuate across the estrous cycle in these regions in the same mice used for the present studies (J.L. Spencer et al. unpublished). For this, profile type and group were compared by two-way ANOVA. Data is expressed as the percentage of each profile type to total profiles in each lamina. To test significant effects found in the two-way ANOVA analysis, Fisher's PLSD post hoc tests were run. Data analyzed using StatView 5.0. Significance was considered greater than 0.05.

RESULTS

Light microscopic localization of ER α –, ER β –, and PR-immunoreactivities

Consistent with studies in the rat (Weiland et al., 1997; Milner et al., 2001), a few scattered cell nuclei with immunoreactivity for ER α were detected in the mouse hippocampus. These nuclei were primarily in stratum oriens (Fig. 1A) and the border of stratum radiatum and lacunosum-moleculare of CA1 and in the subgranular zone of the dentate gyrus. Like rats (Milner et al., 2005), ER β -ir was primarily found in the cytoplasm of pyramidal cell soma and dendrites. Unlike rats, where ER β -ir is most prominent in CA3, ER β -ir in mice was most noticeable in CA1 (Fig. 1B). At the light microscopic level, PR-ir was found in some cell nuclei in the mouse hippocampus that were most prominent in stratum radiatum of CA1 (Fig. 1C). Unlike the estrogen sensitive PR-ir discussed below and reported in other brain areas, these PR-ir nuclei were present regardless of circulating estrogen levels. This was in contrast to the rat hippocampus (Waters et al., 2008), in which no PR-ir nuclei were detected.

Electron microscopic localization of ER α –, ER β –, and PR-immunoreactivities

At the electron microscopic level, ER α , ER β , and PR labeling was found in all lamina of the dorsal hippocampus examined at all stages of the estrous cycle and in male mice. Extranuclear labeling for all three antibodies was found in dendrites, dendritic spines, axons, terminals, and glia; however, the proportion of the types of labeled profiles varied across both lamina and with sex/cycle. (For distribution of labeled profiles per lamina for each antibody, see tables 1, 2, and 3.)

The subcellular localization of ER α –, ER β –, and PR-ir was similar to what we have observed previously in rats (Milner et al., 2001; Milner et al., 2005; Waters et al., 2008). In general, peroxidase reaction product appeared as discrete patches in dendritic spine heads (see Figs. 2B; 3B; 4A, C) or near the base of dendritic spines (Fig. 2A), in axons and terminals (see Figs. 2C, D, F; 3E; 4D) and in glial processes (Fig. 3D, F). Most of the dendritic shafts with ER α –, ER β – and PR-ir were in principal cells as reflected by the presence of associated spines and their location in CA1 stratum radiatum and the dentate molecular layer (Tables 1–3). However, some dendritic shafts lacked spines, received numerous contacts and were found in the dentate hilus, suggesting that they arose from interneurons. Compared to ER α and PR, ER β -ir was more often detected on or near the plasma membrane of soma (Fig. 3A) and dendrites (Fig. 3C), or endomembranes near mitochondria (Fig. 3A). Terminals with ER α –, ER β –, and PR-ir were small (0.4 – 0.6 μ m in diameter), contained numerous small clear vesicles and, rarely, one dense-core vesicle (Fig. 2C; 3E; 4B). Terminals with ER α –, ER β –, and PR-ir often formed asymmetric synapses on dendritic spines (Figs. 2C, 3E, 4B). Unlike rats (Milner et al., 2005), ER β was rarely detected in large mossy fiber terminals in CA3 or the dentate gyrus.

ER α

When data were pooled for all lamina, two-way ANOVA analysis showed a significant effect of sex/cycle phase ($F(3,40)=3.42$, $p=0.0261$). Diestrus animals had the highest number of ER α -labeled profiles for every profile type, except dendritic shafts. Post hoc analysis revealed that diestrus females had significantly more labeled glia (Fig. 2G) than proestrus females or males, and more ER α -labeled axons (Fig. 2D, E) than males ($p<0.05$ for all). In the diestrus females, ER α -labeled axons and glia represented the majority of labeled profiles (28.46% and 22.75% of total, respectively), while dendrites (Fig. 2A) represented only a small fraction (3.32%) of total labeling. Although not significant, abundant ER α labeling was seen in synaptic profiles in the diestrus females, with 12.05% of all labeling was seen in spines (Fig. 2B) and 12.17% was seen in terminals (Fig. 2C).

In the CA1 stratum radiatum (proximal), diestrus females had significantly more ER α -labeled profiles ($F(4,40)=3.23$, $p=0.0292$). Post hoc analysis revealed that diestrus females had more ER α -labeled profiles than males, driven mainly by increases in dendritic spines and glia (6.25% and 6.25% of labeling, respectively). Similarly, in the CA3 stratum lucidum, two-way ANOVA analysis showed a significant effect of sex/cycle phase ($F(3,40)=3.77$, $p=0.0178$). Post hoc analysis revealed that diestrus females had more ER α -labeled profiles in the CA3 stratum lucidum than the other three groups, driven by increased labeling in axons during this cycle phase ($p<0.05$). In the stratum lucidum of diestrus females, axons represented 24.31% of labeling in the CA3. There was also a non-significant trend for an interaction effect between sex/cycle phase and profile type ($F(3,40)=1.79$, $p=0.0833$). The majority of the ER α -labeled axons in stratum lucidum were found in bundles suggesting that they are mossy fiber pre-terminal axons (Torres-Reveron et al., 2008).

ER β

When data were pooled for all lamina, a significant effect of sex/cycle phase was found using two-way ANOVA analysis ($F(3, 40)=0.87$, $p=0.0035$). Diestrus and estrus females had more ER β -labeled profiles than proestrus females and males. Post hoc analysis revealed that diestrus and estrus females had more ER β -labeling in dendritic spines (Fig. 3B) and glia (Fig. 3D, F) compared to proestrus females and males ($p<0.05$). In diestrus females, 15.30% and 30.93% of ER β immunoreactivity was in dendritic spines and glia, respectively. In estrus females, ER β -labeled dendritic spines and glia represented 15.01% and 32.23%, respectively. ER β -labeling was also seen in the other cellular profiles measured during these two cycle phases. In diestrus females, dendritic shafts (Fig. 3C) and axons constituted 33.23% and 20.97% of all labeling, respectively, while 6.77% was found in axon terminals (Fig. 3E). In estrus females, dendritic shafts and axons represented 17.20% and 19.40% of total labeling, respectively, while 12.13% of labeling was found in axon terminals.

There were no significant effects on the numbers of ER β -labeled profiles in the CA1 stratum radiatum (distal). However, in the CA3 stratum lucidum, there was a significant effect of sex/cycle phase on ER β -labeling ($F(3,40)=3.25$, $p=0.0317$). Post hoc analysis revealed that diestrus females had more ER β -labeled profiles compared to estrus and proestrus females, due mostly to increased labeling in dendritic shafts, dendritic spines and axons ($p<0.05$ for all). In the CA3 stratum lucidum of diestrus females, ER β -labeled dendrites, spines, and axons represented 7.87%, 4.13%, and 13.27%, respectively, while terminals and glia represented only 0.53% and 5.40%, respectively, of labeling in the CA3.

PR

When data were pooled for all lamina, a significant effect of sex/cycle phase and a significant interaction effect between sex/cycle phase and PR-labeled profile type were found ($F(3,40)=14.872$, $p<0.0001$; and $F(3,40)=3.184$, $p=0.0029$, respectively). Proestrus females had more PR-labeled profiles of every type, except for dendritic shafts. Post hoc analysis revealed that proestrus females had significantly more PR-labeled axons (Fig. 4D) than the other three groups ($p<0.05$). In proestrus females, PR-labeled axons represented 46.43% of all labeling, while PR-labeled dendritic shafts and glia (Fig. 4C) represented only 3.10% and 24.90%, respectively. While not significant, PR-labeling also was seen in synaptic profiles in proestrus females, with 30.33% of all labeling was found in spines (Fig. 4A) and 9.07% was found in terminals (Fig. 4B).

In the CA1 stratum radiatum (distal) and CA3 stratum lucidum, there were a non-significant trends of proestrus females having more labeling than the other three groups, specifically with more axons being labeled than other profiles ($F(3,40)=2.14$, $p=0.11$ and $F(3,40)=2.22$, $p=0.10$, respectively). In the CA1 stratum radiatum (distal) of proestrus females, axons

represented 12.47% of total labeling in the CA1, while in the CA3 stratum lucidum of the same cycle phase, 9.03% of all labeling the CA3 stratum lucidum was found in axons.

Discussion

In this study, we described the cellular and subcellular localization of ER α -, ER β -, and PR-immunoreactivities in the mouse hippocampal formation. Our studies confirmed previous reports that nuclear ER α and PR is detected at the light microscopic level in the mouse hippocampus (Alves et al., 2000). Additionally, our electron microscopic studies revealed abundant extranuclear labeling for all three antibodies (ER α , ER β , and PR) throughout the mouse hippocampus. In particular, we found extranuclear receptors in neurons and glia well positioned to influence hippocampal functions through local actions via extranuclear steroid signaling. In addition to this localization, we demonstrated novel but predictable fluctuations in receptor expression across the cycle that may have important implications for the sensitivity of hippocampal function to circulating ovarian steroids. Combined with our previous ultrastructural localization of these receptors in the rat hippocampal formation (Milner et al., 2001; Milner et al., 2005; Waters et al., 2008), this study is an important step towards understanding the mechanisms by which circulating ovarian steroids influence hippocampal-dependent behaviors such as mood and cognition in mammals.

Methodological considerations

The antibodies to ER α , ER β , and PR used in the present studies have been well characterized and used in our previous studies in the rat hippocampus under identical labeling conditions (Milner et al., 2001; Milner et al., 2005; Waters et al., 2008). The antibodies used in this study allowed localization of specific ERs and PRs, but in some cases may not distinguish between different receptor isoforms. For example, the Dako antibody used in this study recognizes both PR isoforms A and B. These receptors have distinct cellular effects, and their relative distribution in the hippocampal formation may have important consequences for the effects of progestins on hippocampal function. In addition, several splice variants of the ER β have been described; not all of these splice variants may be recognized by the ER antibodies used in these studies [see (Milner et al., 2005)]. Moreover, steroid hormones may affect hippocampal function via other ERs and PRs encoded by different genes and not examined in this study.

The primary goal of this study was to localize steroid receptors that may carry out extranuclear steroid signaling previously described in hippocampal neurons (Spencer et al., 2007; Spencer et al., 2008). Because of this, we labeled the tissue processed for electron microscopy without Triton, allowing for the best preservation of cellular morphology. In our experience these labeling conditions allow for minimal detection of nuclear steroid receptor labeling, even when it is present at the light microscopic level (Waters et al., 2008). However, when the tissue is permeabilized, nuclear staining of ERs and PRs is seen in the mouse hippocampal formation (Alves et al., 2000).

ERs and PRs are positioned for local modulation of hippocampal function

We previously showed that the entire dorsal mouse hippocampal formation responds to changes in circulating ovarian steroids, measured as signaling pathway activation or synaptic protein expression (Li et al., 2004; Spencer et al., 2008). The widespread presence of ER α -, ER β - and PR-ir throughout the hippocampal formation suggests that these changes may occur via the direct action of ovarian steroids on estrogen and progesterone receptors, in every hippocampal subregion. The localization of these receptors far from the nucleus, in synapses and neuronal process, suggests that they act via extranuclear estradiol signaling rather than classical nucleus-initiated hormone signaling (Kelly and Levin, 2001). By acting

on these receptors, estrogen and progesterone may locally activate kinases, such as the Akt or ERK kinases, to direct the formation or maturation of synapses at specific sites (Spencer et al., 2007; Spencer et al., 2008; Harburger et al., 2009).

The abundance of ER- and PR-ir in glial cells suggests that these cells may have distinct roles in the hippocampal response to ovarian steroids. Little attention has been paid to the role of glial cells in this process. In female rats, conflicting reports have shown increases or decreases in astrocytic volume during proestrus (Klintsova et al., 1995; Arias et al., 2009). While glial cells have been shown to mediate the neuroprotective effects of estradiol (Sortino et al., 2004), the mechanisms behind glial-neuronal actions in response to ovarian steroids are still unclear. The findings of the current study suggest that this may be a fruitful area for future investigation.

We previously described the distribution of extranuclear ERs and PRs in the hippocampal formation of the female rat. In localizing the same three receptors in the mouse, we expected to find differences between the species that might explain previously described differences in estradiol sensitivity between the two species. Surprisingly, we found that the distribution of extranuclear ovarian steroid receptors in the mouse and rat hippocampus was strikingly similar. In particular, the two ER isoforms, α and β , had distinct distributions in neuronal processes, with ER α favoring axons over dendrites, and ER β present about equally in both types of processes. Because ER α and ER β activate distinct signaling pathways as homo- or hetero-dimers (Matthews and Gustafsson, 2003), the ratio of ERs available likely allows for distinct effects of estradiol in axons and dendrites.

While the distribution of extranuclear ERs in the mouse hippocampus is similar to labeling in the rat, very little ER β was detected in the mouse mossy fiber pathway, whereas abundant ER β -ir is seen in the rat mossy fiber pathway (Milner et al., 2005; Torres-Reveron et al., 2008). The lack of ER β along with the lack of ER α in the mouse mossy fiber pathway suggests that estrogen does not directly affect this neural circuit in the mouse. Additionally, the mouse hippocampus, unlike the rat, contained some nuclear PR labeling, consistent with previous reports (Alves et al., 2000). However, except for such subtle differences, any species differences in estradiol sensitivity between mice and rats therefore cannot be explained by differential expression or localization of the ER α , ER β , or PR.

Although estrogen enhances hippocampal function in both rats and mice, in rats estradiol increases dendritic spine density (Woolley and McEwen, 1993), “mushroom” shaped spines believed to be more mature spines (Gonzalez-Burgos et al., 2005), and multi-synaptic boutons (Woolley et al., 1996). In contrast, estradiol increases in the density of “mushroom” shaped spines but not the overall number of spines in mice (Li et al., 2004). The outcome of estrogen-induced synapse formation in both rats and mice is likely to increase synaptic strength; however, species differences in the mechanisms regulating spine dynamics exist. In particular, estradiol regulation of synaptic proteins in rats is limited to the CA1 region (Brake et al., 2001; Lee et al., 2004; Waters et al., 2009) while in mice changes occur throughout the hippocampal formation (Li et al., 2004; Spencer et al., 2008). The current findings that the numbers of profiles expressing extranuclear ERs and PRs are altered over the estrous cycle throughout the hippocampus support the notion that ERs and PRs are involved in the synaptic changes in mice. Additionally, the species may differ in other, downstream components of ER and PR signaling, or in the expression of other steroid receptors or receptor isoforms.

Estrous cycle affects the expression of extranuclear hormone receptors

The expression of extranuclear ERs and PRs fluctuated differentially across the estrous cycle. Extranuclear ER α and ER β labeling was low during proestrus, when circulating

estradiol is high, and highest during estrus or diestrus, when circulating estradiol is low. This suggests that circulating estradiol downregulates the expression of extranuclear ERs similar to nuclear receptors (Milner et al., 2008). In the brain, estradiol decreases ER β -ir via an ER α -dependent mechanism (Nomura et al., 2003). A similar mechanism could explain the downregulation of ER α expression seen here during proestrus and in rats (Milner et al., 2008).

In contrast to ERs, extranuclear PR labeling increased during proestrus, when circulating estradiol is high. Estradiol is known to induce cytosolic PR in the rat hippocampus (Parsons and McEwen, 1982; Waters et al., 2008) and nuclear PR in reproductive tissues and the hypothalamus (Kudwa et al., 2004). Interestingly, the numbers of detectable extranuclear PR profiles were greater in males compared to diestrus females suggesting that the presence of circulating androgens and/or low levels of estrogens converted from androgens are sufficient to allow low levels of extranuclear PR expression. Hippocampal PR expression is unique in that its estrogen regulation occurs via extranuclear ERs. The mechanism of PR induction by extranuclear ERs is not yet clear. Several second messenger pathways are activated by estrogen's actions at extranuclear ER (Akama and McEwen, 2003; Mannella and Brinton, 2006). In both the rat and mouse hippocampus, estrogen can regulate the expression of Akt (protein kinase B), a serine/threonine kinase that mediates the downstream effects of phosphatidylinositol 3-kinase signaling and LIM-kinase which is important for actin polymerization (Znamensky et al., 2003; Spencer et al. 2008; Yildirim et al., 2008). Activation of these second messenger pathways may mediate estrogen effects in the hippocampus by signaling back to the nucleus to induce the expression of extranuclear PRs observed in this study.

The abundance of extranuclear ERs and PRs in the mouse hippocampus across the estrous cycle suggests estradiol and progesterone actions may be coordinated. Estradiol, in particular, both induces PR and downregulates ERs, thus increasing PR expression may facilitate the same signaling pathways as estrogen or may activate complimentary mechanisms. For example, both estradiol and progesterone have been reported to be neuroprotective albeit via different mechanisms of action. Cyclicity of steroid receptor levels may also contribute to variations in cognitive ability across the estrous cycle, as they have also been shown to regulate dendritic spine formation and synaptogenesis (Woolley and McEwen, 1992; Woolley and McEwen, 1993; Li et al., 2004).

Subcellular distribution of hormone receptors fluctuates across the estrous cycle

Extranuclear staining of ERs and PRs was found in most subcellular profiles across all stages of the estrous cycle. Similar to previous results, ER α - and ER β -ir peaked during diestrus, while PR-ir was highest during the proestrus stage. Examination of receptor labeling in each profile, suggests possible roles for extranuclear ERs and PRs. In particular the prominent localization of ER α to axons during periods of low estrogens (diestrus phase) coincides with greater ER α -ir in axon terminals, suggesting that some of the ER α -ir seen in the axons could represent receptors that are being transported to axon terminals. Increased PR-ir in axons during periods of high estrogen levels may also represent PR movement between the cell body and axon terminals. Within axons extranuclear ERs and PRs are often localized to endomembranes further suggesting that they may be involved in cell signaling during retrograde transport (Cosker et al., 2008).

Unlike the staining patterns for ER α and PR in the pre-synaptic profiles, ER α - and PR-ir are higher in dendritic spines compared to dendritic shafts, suggesting that ER α and PR either exert most of their effects at the synapse itself and play a smaller role in trafficking along dendrites or undergo more rapid trafficking in the dendritic shafts. Levels of ER β -ir also increase in dendrites and dendritic spines as estrogens decrease, peaking during diestrus.

This suggests that some of the ER β labeling seen in dendrites represents receptors being transported from the cell body to the dendritic spines. Dendritic spines are known to mediate excitatory neurotransmission (Peters et al., 1991) and are believed to be important in the induction of LTP (Yuste and Bonhoeffer, 2004). Ovarian hormones, particularly estrogens, can enhance the magnitude of LTP, and regulate glutamatergic NMDA receptor mediated neurotransmission as well as expression and distribution within dendritic spines (Woolley et al., 1997; Gazzaley et al., 2002; Smith and McMahon, 2006). Thus, the present findings implicate cyclic fluctuations in extranuclear ERs and PRs in estrogen-mediated changes in LTP and glutamatergic transmission.

Conclusion

In this study, we described the localization of ovarian steroid receptors ER α - ER β - and PR-immunoreactivities in the mouse hippocampal formation. We found abundant extranuclear expression, with all three receptors well placed to regulate local spine synapse dynamics, neurite outgrowth, and glial cell function via extranuclear steroid signaling. The striking similarity between the distribution of these receptors in the mouse and rat hippocampus suggests that these receptors have a conserved and important role in the maintenance of hippocampal function. We also identified a novel fluctuation of hippocampal extranuclear ER and PR expression across the estrous cycle that may participate in cyclic changes in hippocampal function.

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Abbreviations

A	axon
ABC	avidin-biotin complex
BSA	bovine serum albumin
bv	blood vessel
D	dendrite
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
g	glial process
ir	immunoreactivity
LTP	long term potentiation
PB	phosphate buffer
PR	progesterin receptor
PRE	progesterin response element
pTrkB	phosphorylated tropomyosin-related kinase B receptor
S	dendritic spine
sp	stratum pyramidale

T	terminal
TS	Tris saline solution
uD	unlabeled dendrite
uT	unlabeled terminal

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Figure 1.

By light microscopy, ER α -, ER β -, and PR-immunoreactivities are detected in the dorsal mouse hippocampal formation. **A.** Schematic diagram showing the hippocampal regions sampled for light and electron microscopy [adapted from (Hof et al., 2000)]. **B–D.** Examples of light microscopic localization of ER α -, ER β -, and PR-immunoreactivities in the mouse CA1 region are shown. When the tissue is permeabilized with triton, sparse nuclear labeling for ER α is detected in stratum oriens (SO, arrows, B) and for PR in stratum radiatum (SR, arrows, D). Diffuse immunoreactivity for ER β is detected in the pyramidal cell layer (pcl) and in a few interneurons (arrows, C). Bar 50 μ m

Figure 2.

ER α immunoreactivity is found in select dendrites, axons, terminals, and glia in males and females at all stages of the estrous cycle. **A.** ER α labeling is seen in a dendritic shaft at the base of a spine (ERa-S; arrowhead). (male mouse, DG mml) **B.** An ER α -labeled dendritic spine (ERa-S) receives a synapse (curved arrow) from an unlabeled terminal (uT). An unlabeled dendritic spine (uS) is shown for comparison. (diestrus, CA1 sr) **C.** An ER α -labeled terminal (ERa-T; arrowhead pointing at patch of peroxidase labeling) forms a synapse (curved arrow) with an unlabeled dendritic spine (uS). An unlabeled terminal (uT) and unlabeled dendritic shaft (uD) are nearby. (proestrus, CA3 so) **D.** In this bundle of axons, ER α labeling is found in a discrete patch within an unmyelinated axon (ERa-A) cut longitudinally. An unlabeled axon (uA) is also shown for comparison. (male, CA3 slu) **E.** ER α labeling is found in an unmyelinated pre-terminal axon leading to a terminal (T). (male, CA3 sr) **F.** ER α labeling was also found in a myelinated axon (ERa-mA; arrowhead). (Di, CA3 so) **G.** An ER α -labeled glial processes (ERa-g) apposes an unlabeled dendritic spine (uS) and an unlabeled terminal (uT). (pro, CA1 sr) Bar 500nm.

Figure 3.

ER β immunoreactivity is found in select dendrites, axons, terminals, and glia in males and females at all stages of the estrous cycle. **A.** ER β labeling is in discrete patches (examples, arrowheads) throughout the cytoplasm of a perikaryon. The higher magnification (inset) shows a dense patch of ER β -labeling associated at an endomembrane near the mitochondrion (bar 250 nm). (CA1 sr) **B.** An ER β -labeled spine (ERb-S) arising from a dendritic shaft lacking labeling (D) is contacted (curved arrow) by an unlabeled terminal (uT). (CA1 so) **C.** ER β labeling is found in a dendrite (ERb-D), both in the cytoplasm (black arrowhead) and on a mitochondrion (white arrowhead). (CA1 sr) **D.** ER β labeling is found in a glia process (ERb-g) that conforms to the boundaries of the neuropil. (CA1 sr) **E.** An ER β -labeled terminal (ERb-T; arrowhead points to cluster of peroxidase labeling) forms a synapse (curved arrow) with an unlabeled dendritic spine (uS). (CA1 sr) **F.** ER β -labeling is found in an astrocytic profile abutting the basement membrane (bm) of a blood vessel (bv). (CA1 sr near) Bar A–C and F 500nm; D and F 250nm

Figure 4.

PR immunoreactivity is found in select dendrites, axons, terminals, and glia primarily in proestrus mice. **A.** A PR-labeled dendritic spine (PR-S) receives a synapse (curved arrow) from an unlabeled terminal (uT). (proestrus, DG sgz) **B.** Two PR-labeled terminals (PR-T; arrowhead) synapse (curved arrows) on unlabeled dendritic spines (uS). A PR-labeled unmyelinated axon (PR-A) apposes to one of the unlabeled dendritic spines. (Pro, CA1 sr dist) **C.** A PR-labeled spine (PR-S) is contacted (curved arrows) by an unlabeled mossy fiber terminal (u-mfT). An unlabeled axon (uA) apposes a PR-labeled glial process (PR-g). (pro, dg hil) **D.** Two PR-labeled unmyelinated axons (PR-A) are shown in a field of unlabeled unmyelinated axons. An unmyelinated axon (uA) is marked for comparison. (pro, dg hil) Bar 500nm

Figure 5.

Summary diagram showing the levels of ER α -, ER β -, and PR-ir in male mice and across the estrous cycle in female mice in comparison to the known fluctuations of circulating estradiol and progesterone.

TABLE 1

Distribution of ER α -Immunoreactive Profiles in the Hippocampal Formation

Hippocampal subregion	Dendritic shafts	Dendritic spines	Axons	Terminals	Glia	Unknown
CA1						
SO	0.92 $\pm$ 0.45	1.42 $\pm$ 0.47	2.08 $\pm$ 0.58	1.25 $\pm$ 0.37	2.00 $\pm$ 0.56	0.75 $\pm$ 0.18
SR (proximal)	0.42 $\pm$ 0.19	2.17 $\pm$ 0.42	1.83 $\pm$ 0.51	0.92 $\pm$ 0.29	1.92 $\pm$ 0.36	0.50 $\pm$ 0.15
SR (distal)	0.50 $\pm$ 0.34	1.50 $\pm$ 0.38	2.08 $\pm$ 0.43	0.92 $\pm$ 0.19	1.92 $\pm$ 0.54	0.50 $\pm$ 0.23
SLM	0.17 $\pm$ 0.11	0.67 $\pm$ 0.26	1.75 $\pm$ 0.37	0.50 $\pm$ 0.23	2.25 $\pm$ 0.49	0.25 $\pm$ 0.18
CA3						
SO	0.50 $\pm$ 0.19	2.17 $\pm$ 0.44	2.33 $\pm$ 0.59	1.33 $\pm$ 0.45	1.08 $\pm$ 0.29	0.00 $\pm$ 0.00
SR	0.25 $\pm$ 0.13	1.33 $\pm$ 0.38	1.83 $\pm$ 0.49	1.08 $\pm$ 0.38	0.75 $\pm$ 0.22	0.00 $\pm$ 0.00
Slu	0.25 $\pm$ 0.13	0.75 $\pm$ 0.28	3.58 $\pm$ 0.92	0.50 $\pm$ 0.19	1.00 $\pm$ 0.33	0.08 $\pm$ 0.08
DG						
OML	0.08 $\pm$ 0.08	1.08 $\pm$ 0.36	1.50 $\pm$ 0.38	1.00 $\pm$ 0.39	1.08 $\pm$ 0.26	0.42 $\pm$ 0.15
MML	0.58 $\pm$ 0.23	0.83 $\pm$ 0.24	0.75 $\pm$ 0.22	1.00 $\pm$ 0.28	1.92 $\pm$ 0.68	0.17 $\pm$ 0.11
IML	0.25 $\pm$ 0.13	0.58 $\pm$ 0.23	1.00 $\pm$ 0.25	0.33 $\pm$ 0.26	1.50 $\pm$ 0.29	0.17 $\pm$ 0.11
HIL	0.17 $\pm$ 0.11	0.67 $\pm$ 0.19	2.75 $\pm$ 0.89	0.67 $\pm$ 0.19	1.83 $\pm$ 0.47	0.08 $\pm$ 0.08
SGZ	0.33 $\pm$ 0.14	0.50 $\pm$ 0.19	1.83 $\pm$ 0.51	0.83 $\pm$ 0.24	1.83 $\pm$ 0.52	0.17 $\pm$ 0.11

Ten random, but not overlapping, micrographs per lamina (360 μm^2 /lamina/block) were analyzed for labeling. On average, 73.92 $\pm$ 7.66 immunoreactive profiles were labeled per section per animal. The mean and standard error of the mean (SEM) were calculated by averaging counts from all animals (males and proestrus, diestrus and estrus females; N = 12) for each type of ER α -labeled profile in each lamina of each hippocampal section.

TABLE 2

Distribution of ER β -Immunoreactive Profiles in the Hippocampal Formation

Hippocampal subregion	Dendritic shafts	Dendritic spines	Axons	Terminals	Glia	Unknown
CA1						
SO	3.33 $\pm$ 0.59	5.00 $\pm$ 0.82	4.17 $\pm$ 0.67	2.33 $\pm$ 0.58	5.25 $\pm$ 0.48	0.75 $\pm$ 0.22
SR (proximal)	3.92 $\pm$ 0.84	2.92 $\pm$ 0.58	3.33 $\pm$ 0.48	1.42 $\pm$ 0.45	4.42 $\pm$ 0.61	0.92 $\pm$ 0.31
SR (distal)	3.58 $\pm$ 0.62	3.58 $\pm$ 0.82	3.00 $\pm$ 0.72	1.58 $\pm$ 0.45	4.92 $\pm$ 0.73	0.42 $\pm$ 0.19
SLM	3.00 $\pm$ 0.69	1.25 $\pm$ 0.35	2.42 $\pm$ 0.67	1.33 $\pm$ 0.45	5.17 $\pm$ 0.65	0.92 $\pm$ 0.19
CA3						
SO	2.83 $\pm$ 0.60	2.42 $\pm$ 0.43	3.33 $\pm$ 0.78	0.67 $\pm$ 0.31	6.00 $\pm$ 0.96	0.08 $\pm$ 0.08
SR	2.08 $\pm$ 0.36	2.33 $\pm$ 0.75	3.25 $\pm$ 0.74	0.83 $\pm$ 0.21	4.17 $\pm$ 0.59	0.00 $\pm$ 0.00
Slu	2.17 $\pm$ 0.56	1.25 $\pm$ 0.37	4.08 $\pm$ 1.00	0.67 $\pm$ 0.19	2.25 $\pm$ 0.39	0.00 $\pm$ 0.00
DG						
OML	2.50 $\pm$ 0.58	1.42 $\pm$ 0.40	2.75 $\pm$ 0.92	1.00 $\pm$ 0.35	4.25 $\pm$ 0.88	0.58 $\pm$ 0.29
MML	2.58 $\pm$ 0.50	1.00 $\pm$ 0.33	2.33 $\pm$ 0.61	1.25 $\pm$ 0.41	5.00 $\pm$ 0.98	0.58 $\pm$ 0.36
IML	3.08 $\pm$ 0.63	1.08 $\pm$ 0.48	2.92 $\pm$ 0.58	0.83 $\pm$ 0.32	4.33 $\pm$ 0.73	0.67 $\pm$ 0.22
HIL	1.50 $\pm$ 0.44	0.50 $\pm$ 0.26	3.08 $\pm$ 0.56	1.75 $\pm$ 0.43	3.67 $\pm$ 0.64	0.58 $\pm$ 0.26
SGZ	1.83 $\pm$ 0.39	0.83 $\pm$ 0.21	2.42 $\pm$ 0.42	1.17 $\pm$ 0.30	3.75 $\pm$ 0.52	1.08 $\pm$ 0.29

Ten random, but not overlapping, micrographs per lamina (360 μm^2 /lamina/block) were analyzed for labeling. On average, 167.67 $\pm$ 11.50 immunoreactive profiles were labeled per section per animal. The mean and standard error of the mean (SEM) were calculated by averaging counts from all animals (males and proestrus, diestrus and estrus females; N = 12) for each type of ER β -labeled profile in each lamina of each hippocampal section.

TABLE 3

Distribution of PR-Immunoreactive Profiles in the Hippocampal Formation

Hippocampal subregion	Dendritic shafts	Dendritic spines	Axons	Terminals	Glia	Unknown
CA1						
SO	1.08 ± 0.38	1.75 ± 0.35	6.17 ± 0.88	1.58 ± 0.50	4.00 ± 0.44	0.58 ± 0.19
SR (proximal)	0.33 ± 0.14	1.83 ± 0.49	4.58 ± 1.08	1.17 ± 0.34	4.25 ± 0.62	0.17 ± 0.11
SR (distal)	0.25 ± 0.13	1.75 ± 0.49	5.75 ± 1.24	1.08 ± 0.45	4.67 ± 0.91	0.42 ± 0.19
SLM	0.42 ± 0.23	1.08 ± 0.23	4.67 ± 0.79	0.50 ± 0.19	4.00 ± 0.46	0.17 ± 0.11
CA3						
SO	0.42 ± 0.26	1.25 ± 0.35	5.33 ± 0.89	2.17 ± 0.52	4.08 ± 0.56	0.08 ± 0.08
SR	0.75 ± 0.25	1.00 ± 0.30	3.17 ± 0.80	1.08 ± 0.40	3.08 ± 0.53	0.08 ± 0.08
Slu	0.25 ± 0.13	0.33 ± 0.22	1.75 ± 0.51	0.83 ± 0.30	1.75 ± 0.41	0.00 ± 0.00
DG						
OML	0.42 ± 0.26	1.17 ± 0.30	4.83 ± 0.98	0.75 ± 0.33	2.83 ± 0.63	0.25 ± 0.13
MML	0.50 ± 0.19	1.75 ± 0.41	3.08 ± 0.63	0.75 ± 0.33	3.25 ± 0.46	0.08 ± 0.08
IML	0.33 ± 0.14	1.00 ± 0.30	4.58 ± 0.78	1.00 ± 0.39	3.50 ± 0.71	0.08 ± 0.08
HIL	0.17 ± 0.11	0.42 ± 0.34	5.17 ± 1.24	1.00 ± 0.39	1.92 ± 0.60	0.25 ± 0.18
SGZ	0.33 ± 0.19	0.42 ± 0.42	3.25 ± 0.59	1.00 ± 0.46	2.17 ± 0.41	0.08 ± 0.08

Ten random, but not overlapping, micrographs per lamina (360 µm²/lamina/block) were analyzed for labeling. On average, 126.00 ± 12.41 immunoreactive profiles were labeled per section per animal. The mean and standard error of the mean (SEM) were calculated by averaging counts from all animals (males and proestrus, diestrus and estrus females; N = 12) for each type of PR-labeled profile in each lamina of each hippocampal section.