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Hormonal regulation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus

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Abstract

Clinical and preclinical studies indicate that women and men differ in relapse vulnerability to drug-seeking behavior during abstinence periods. As relapse is frequently triggered by exposure of the recovered addict to objects previously associated with drug use and the formation of these associations requires memory systems engaged by the hippocampal formation (HF), studies exploring ovarian hormone modulation of hippocampal function are warranted. Previous studies revealed that ovarian steroids alter endogenous opioid peptide levels and trafficking of mu opioid receptors in the HF, suggesting cooperative interaction between opioids and estrogens in modulating hippocampal excitability. However, whether ovarian steroids affect the levels or trafficking of delta opioid receptors (DORs) in the HF is unknown. Here, hippocampal sections of adult male and normal cycling female Sprague-Dawley rats were processed for quantitative immunoperoxidase light microscopy and dual label fluorescence or immunoelectron microscopy using antisera directed against the DOR and neuropeptide Y (NPY). Consistent with previous studies in males, DOR-immunoreactivity (-ir) localized to select interneurons and principal cells in the female HF. In comparison to males, females, regardless of estrous cycle phase, show reduced DOR-ir in the granule cell layer of the dentate gyrus and proestrus (high estrogen) females, in particular, display reduced DOR-ir in the CA1 pyramidal cell layer. Ultrastructural analysis of DOR-labeled profiles in CA1 revealed that while females generally show fewer DORs in the distal apical dendrites of pyramidal cells, proestrus females, in particular, exhibit DOR internalization and trafficking towards the soma. Dual label studies revealed that DORs are found in NPY-labeled interneurons in the hilus, CA3, and CA1. While DOR colocalization frequency in NPY-labeled neuron somata was similar between animals in the hilus, proestrus females had fewer NPY-labeled neurons that co-labeled with DOR in stratum oriens of CA1 and CA3 when compared to males. Ultrastructural analysis of NPY-labeled axon terminals within stratum radiatum of CA1 revealed that NPY-labeled axon terminals contain DORs that are frequently found at or near the plasma membrane. As no differences were noted by sex or estrous cycle phase, DOR activation on NPY-

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labeled axon terminals would inhibit GABA release probability equally in males and females. Taken together, these findings suggest that ovarian steroids can impact hippocampal function through *direct* effects on DOR levels and trafficking in principal cells and broad *indirect* effects through reductions in DOR-ir in NPY-labeled interneurons, particularly in CA1.

Keywords

Opioids; Hippocampus; Hormones; Estrogen; Neuropeptide Y; Sex differences

1. Introduction

Despite lower rates of use and abuse, clinical evidence suggests that women may be more susceptible to several aspects of addiction than men. For example, women report shorter drug-free periods and higher levels of craving and dysphoria during drug withdrawal (Griffin et al., 1989; Kosten et al., 1996; Elman et al., 2001). Women are also more likely to experience craving following exposure to drug-related cues (Robbins et al., 1999) and relapse to drug-seeking behavior (Rubin et al., 1996; McKay et al., 1996; Elman et al., 2001). Animal models that mimic different phases of the addiction process have been useful in determining whether a biological basis exists for observed sex differences in abuse vulnerability, as these studies control for sociocultural and other factors that may occlude sex differences in clinical studies of drug abuse. In studies of drug relapse or reinstatement, female rats show more extinction responding on the drug-associated lever after drug removal and greater reinstatement after a priming injection than males. Additionally, reinstatement occurs after a lower priming dose in females than in males (Comer et al., 1996; Klein et al., 1997; Lynch & Carroll, 2000). Recent studies using intact and ovariectomized (OVX) rats replaced with estradiol propose that ovarian hormones, particularly estrogen, contribute to the aforementioned sex differences during reinstatement (Fuchs et al., 2005; Kippin et al., 2005; Larson et al., 2005). Taken together, clinical and preclinical studies indicate that women and men differ in relapse vulnerability during abstinence periods. Relapse is frequently triggered by exposure of the recovered addict to objects previously associated with drug use (White, 1996). As the formation of these associations requires the episodic and declarative memory systems engaged by the hippocampal formation (HF) (Nestler, 2001; Hyman & Malenka, 2001; Holden, 2001; Nestler, 2002), studies exploring ovarian hormone modulation of hippocampal function are warranted.

Hippocampal output is regulated through a series of principal cell and inhibitory interneuron connections that are susceptible to modulation by endogenous opioids and exogenous opiates. Ovarian steroid hormones have been shown to influence levels of the endogenous hippocampal opioid peptides, enkephalin and dynorphin, which directly modulate hippocampal excitability (Roman et al., 2006; Drake et al., 2007; Torres-Reveron et al., 2008; Torres-Reveron et al., 2009a; Williams et al., 2010). For example, leu-enkephalin levels are increased in sub-regions of the dentate gyrus (DG) and CA3 of young adult females when estrogen levels are relatively high (Torres-Reveron et al., 2008). Dynorphin levels are similarly increased in the DG and select CA3 lamina 24 hours following estrogen exposure (Torres-Reveron et al., 2009a). In addition, ovarian steroid hormones modulate mu opioid receptor (MOR) trafficking in hippocampal interneurons. Specifically, elevated levels of estrogens, either in proestrus females or after 72 hrs of pulsatile estradiol replacement in OVX females, increased the availability of MORs on the plasma membrane of subpopulations of GABAergic basket cells in the hilar region of the DG (Torres-Reveron et al., 2009b). Such altered trafficking of MORs could potentially alter the disinhibitory effects of endogenous or exogenous opiates, given that MOR activation in the DG produces

excitation by inhibiting GABAergic transmission (Morris & Johnston, 1995; Xie & Lewis, 1995a; Bramham & Sarvey, 1996; Drake et al., 2007). Activation of the delta opioid receptor (DOR) at the circuit level also affects excitatory transmission and the induction of synaptic plasticity in principal cells of the DG (Piguet & North, 1993; Bramham & Sarvey, 1996; Bausch & Chavkin, 1997) and CA1 (Bao et al., 2007) through both direct effects on principal cells and indirect effects via inhibition of interneurons. In addition, recent reports suggest that opioid peptides acting primarily on DORs play an important role in mediating cue- and context-induced drug-seeking behavior (Marinelli et al., 2009). Thus, ovarian steroid modulation of DOR immunoreactivity (ir) levels and trafficking in hippocampal neurons merits further investigation.

Prior studies in male rats demonstrated that the highest DOR mRNA levels were found in GABAergic neurons in the principal cell layers, stratum oriens, and dentate hilus (Stumm et al., 2004). DOR-ir, moreover, was commonly found in somatostatin (SOM) / neuropeptide Y (NPY) containing GABAergic interneurons in hippocampal lamina (Commons & Milner, 1996; Commons & Milner, 1997). Moreover, in CA1 specifically, DORs were found also on pyramidal cell dendrites and somata and could therefore directly modulate pyramidal cell activity (Commons & Milner, 1997). The present study sought to extend these observations, where applicable, to the female hippocampus. Thus, single and dual immunolabeling approaches were used to assess ovarian hormone influences on DOR-ir and distribution within hippocampal principal cells and interneurons. Quantitative densitometric immunocytochemistry and immunoelectron microscopy were employed to measure altered DOR-ir and trafficking in principal cells of normal cycling proestrus (high estrogen) and diestrus (low estrogen) female rats in comparison to male rats. Dual label immunofluorescence and immunoelectron microscopy was used to examine DOR localization to NPY labeled interneurons and axon terminals in select hippocampal lamina of males and normal cycling females. The current study focused on findings in the dorsal hippocampus, where estrogen-induced morphological changes have been consistently reported (Cooke & Woolley, 2005).

2. Material and methods

2.1 Animals and estrous cycle determination

Adult male (275 - 325 g; approximately 60 days old) and female (225 - 250 g; approximately 60 days old) Sprague Dawley rats from Charles River Laboratories (Wilmington, MA) were pair-housed with *ad libitum* access to food and water and with 12:12 light/dark cycles (lights on 0600 - 1800). All procedures were approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health guidelines. Female rats were allowed to acclimate for one week after which estrous cycle phase was determined using vaginal smear cytology (Turner & Bagnara, 1971; Marcondes et al., 2002). Only female rats that showed two consecutive, regular, 4-5 day estrous cycles were included in the study. Animals in proestrus and diestrus 2 phases of the estrous cycle were analyzed in comparison to males. Diestrus 2 rather than metestrus (diestrus 1) was chosen to be certain that the animal was completely out of the estrus phase. For simplicity, the term “diestrus” will refer specifically to diestrus 2 in this report. While vaginal smear cytology was the main method used to determine estrous cycle phase, phases were further verified by measuring uterine weights and plasma estradiol levels from blood samples collected from the heart immediately prior to the perfusion procedure. Plasma serum levels of estradiol were determined by radioimmunoassay using a Coat-A-Count kit from Diagnostics Products Corporation (Los Angeles, CA) with a sensitivity of 8 pg/ml for estradiol. Two cohorts of normal cycling female rats were used in the present study. The first cohort of proestrus and diestrus female rats has been used in prior studies by our laboratory with previously reported estradiol levels

and uterine weights (Torres-Reveron et al., 2008). Select animals from a second cohort of proestrus, estrus, and diestrus female rats were used for the first time in the current report.

2.2 Antisera

A rabbit polyclonal antibody raised against N-terminal amino acids 3-17 of the DOR (Chemicon, Temecula, CA) was used in single label studies. This antibody has been previously used for Western blot, confocal, as well as ultrastructural studies and preimmune sera and preadsorption controls resulted in no detectable labeling (Commons & Milner, 1996; Commons & Milner, 1997; Persson et al., 2000; Persson et al., 2005; Saland et al., 2005). A guinea pig polyclonal antiserum raised against amino acids 34-48 of the DOR was used in dual labeling studies, with previously characterized specificity by immunoblot, preadsorption, and immunocytochemical controls (Cheng et al., 1995; Commons & Milner, 1996). A rabbit polyclonal antiserum raised against NPY was commercially obtained (Peninsula Laboratories, San Carlos, CA). It has been previously characterized for specificity using immunocytochemical and immunoblot approaches (Milner & Veznedaroglu, 1992; Drake & Milner, 2002; Ledoux et al., 2009).

2.3 Section preparation

Rats were deeply anesthetized with pentobarbital (150 mg/kg) in the morning (between 9:30 and 11:30 am) and their brains fixed by aortic arch perfusion with 3.75% acrolein and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.6) (Milner & Veznedaroglu, 1992; Milner et al., 2001). The brains were removed from the skull and cut into 5 mm coronal blocks using a brain mold (Activational Systems, Inc.), and postfixed for 30 minutes in 2% paraformaldehyde in 0.1M phosphate buffer. The brains were sectioned on a Leica Vibratome (40 μ m thick) and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1M phosphate buffer (PB)) until immunocytochemical processing. Prior to immunocytochemistry, coronal sections of all groups were rinsed in PB, coded with hole-punches and pooled into single crucibles to insure identical exposure to immunoreagents (Pierce et al., 1999). Sections then were treated with 1% sodium borohydride in PB for 30 minutes to neutralize free aldehydes and rinsed in PB.

2.4 Light microscopic immunocytochemistry and analysis

To examine changes in DOR-ir or numbers of DOR neurons between male and female rats, sections from each group were rinsed in Tris-buffered saline (TS; pH 7.6) and incubated in 0.5% bovine serum albumin (BSA) in TS for 30 min. Sections then were processed for immunocytochemistry using the avidin-biotin complex (ABC)- peroxidase technique (Hsu et al., 1981). For this, sections were incubated in rabbit polyclonal DOR antisera (1:2000) in 0.1% BSA in TS for 24 hrs at room temperature and 24 hrs at 4 C and then processed through 1) a 1:400 dilution of biotinylated donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 30 min; 2) a 1:100 dilution of peroxidase-avidin complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA), 30 min and 3) 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) and H₂O₂ in TS for 4-6 minutes. All incubations were separated by washes in TS. Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped from xylene with DPX mounting media (Aldrich, Milwaukee, WI).

For quantitative densitometry, images of regions of interest (R.O.I) in the dentate gyrus and CA1 were captured from 5 proestrus females, 5 diestrus females, and 4 male rats using a Dage MTI CCD-72 camera and NIH Image 1.50 software on a Nikon Eclipse 80i microscope (Pierce et al., 1999). The mean gray value (of 256 gray levels) for each selected R.O.I was determined as previously described (Pierce et al., 1999; Torres-Reveron et al., 2008). To compensate for background staining and control for variations in illumination

level between images, the average pixel density for three regions lacking labeling was subtracted. A single hippocampus from each animal with the best morphology and consistent immunoperoxidase labeling was included in the analysis. Optical density values were measured using Image J and net optical density values obtained after subtracting background values were converted to a percentage scale of 256 preset gray values ranging from 0 to 100%.

The number of DOR-labeled neurons in 50,000 m² areas of the dorsal hilus and CA1 stratum oriens (AP -3.6 to -4.0 caudal to Bregma (Swanson, 2000)) was counted in 5 proestrus females, 5 diestrus females, and 4 male rats. Cell counts were summed from 2 hippocampal sections (> 900 m interval) per animal and presented as mean $\pm$ SEM of DOR-labeled neurons per group. Since the purpose of counting cells was not to obtain absolute numbers but to provide a relative estimate between groups, no correction factors were applied to compensate for error of overestimation (Nakamura & McEwen, 2005).

2.5 Immunofluorescence labeling and analysis

Sections of each group were rinsed in 0.05M phosphate-buffered saline (PBS; pH 7.4) and incubated in: 1) 0.1% triton (TX) / 10% normal goat serum (NGS) in PBS for 1hr; 2) a combination of guinea pig polyclonal DOR (1:2000) with rabbit polyclonal NPY (1:2000) antisera in 0.1% TX / 3% NGS in PBS for 48 hrs at 4°C; and 3) a cocktail of goat anti-guinea pig Cy5 IgG (1:400; Jackson ImmunoResearch Labs) and goat anti-rabbit FITC IgG (1:600; Invitrogen, Carlsbad, CA) in 0.1% NGS in PBS for 1hr at room temperature. All incubations were separated by washes in PBS. Sections were mounted on gelatin-coated slides, dehydrated in ascending concentrations of alcohol and xylene, and cover-slipped with Krystalon Mounting Medium (EMD Harelco). As controls, these immunocytochemical procedures were utilized on sections with the omission of the primary or secondary antisera. Immunofluorescence images were acquired sequentially using a Nikon H550L microscope equipped with a Nikon Eclipse 90i camera. Z-stack analysis of select cells using a confocal laser-scanning microscope (Leica, Nussloch, Germany) was used to verify if neurons were dually labeled for DOR and NPY. Alexa Fluor 488 (NPY) was pseudocolored green while Cy5 (DOR) was pseudocolored red. The number of DOR-, NPY-, and dual-labeled neurons in the dorsal hilus, CA3 stratum oriens, and CA1 stratum oriens (between AP -3.6 - -4.0 from Bregma (Swanson, 2000)) was counted in 5 proestrus female and 4 male rats. Cell counts were obtained from 1 hippocampal section per animal and presented as mean $\pm$ SEM of labeled neurons per group. The average percentage of NPY-immunoreactive neurons showing colocalization with DOR and the average percentage of DOR-immunoreactive neurons showing colocalization with NPY also was obtained for each animal group.

2.6 Electron microscopic immunocytochemistry

For single label electron microscopic localization of DOR, sections were labeled for DOR using immunogold. For dual label electron microscopic localization of DOR with NPY, sections were labeled for NPY using immunoperoxidase and DOR using immunogold. Both single and dual label methods have been described previously (Towart et al., 2003; Torres-Reveron et al., 2009b). To enhance reagent penetration, sections were soaked in a cryoprotectant solution (25% sucrose and 3.5% glycerol in 0.05M PB) for 15 min, and rapidly freeze-thawed by sequential immersion in liquid chlorodifluoromethane (Freon, Refron Inc., NY), liquid nitrogen, and PB at room temperature. Sections were incubated in 0.5% BSA for 30 min followed by an antisera cocktail of rabbit polyclonal DOR (1:2000) alone or guinea pig polyclonal DOR (1:1500) with rabbit polyclonal NPY (1:5000) in 0.1% BSA in TS for 72 hrs at 4°C. For dual label studies, NPY was visualized using the ABC method (Hsu et al., 1981) described above. To visualize DOR in single and dual label studies, sections next were processed with the silver-enhanced immunogold technique (Chan

et al., 1990). For this, sections were rinsed in TS and incubated in a 1:50 dilution of donkey anti-rabbit or goat anti-guinea pig IgG conjugated to 1-nm gold particles (Electron Microscopy Sciences, EMS, Washington, PA) in 0.001% gelatin and 0.08% BSA in PBS overnight at 4°C. Sections were rinsed in PBS, postfixed in 1.25% glutaraldehyde in PBS for 10 min, rinsed again in PBS followed by 1.2% sodium citrate buffer, pH 7.4. The conjugated gold particles were enhanced by incubation in silver solution (IntenSE; Amersham) for 5-7 min. Sections were fixed 1 hr in 2% osmium tetroxide, dehydrated in ascending concentrations of ethanols and propylene oxide, and embedded in EMBed 812 (EMS) between two sheets of Aclar plastic (Honeywell, Pottsville, PA). Ultra-thin sections (70-72 nm thick) through the dorsal CA1 (AP -3.6 to -4.0 from Bregma (Swanson, 2000)) were cut on a Leica UCT ultratome. Sections were counterstained with uranyl acetate and Reynold's lead citrate and examined with a Philips CM10 transmission electron microscope equipped with an Advanced Microscopy Techniques digital camera (Danvers, MA).

2.7 Ultrastructural analysis

For DOR single label studies, ultra-thin sections from 5 rats in proestrus, 4 rats in diestrus, and 4 male rats were analyzed. All of the sections from each of the 3 groups were co-processed and identical sampling methods used, thus eliminating variables that could affect between-group comparisons. To circumvent problems due to differences in antibody penetration, images were taken from the plastic-tissue interface (i.e., the surface of the tissue). Silver-intensified immunogold (SIG) labeling for DOR appeared as intense black electron-dense particles located within cytoplasmic compartments or on the plasma membrane. Micrographs containing DOR-ir at magnifications of 10,500x to 19,000x were analyzed in a total tissue area of 3,025 m² per animal in stratum radiatum of the CA1. Profiles containing DOR-ir were classified as neuronal processes (dendrites, dendritic spines, axon terminals) or astrocytes based on criteria described in (Peters et al., 1991). Profiles were considered to be selectively immunogold-labeled when they contained at least one gold particle to avoid false negative labeling of smaller profiles, as background labeling in other morphologically similar profiles in the neuropil was negligible. Moreover, as sections from all groups were processed together, non-specific attachment of gold particles would not differ between groups. This approach of immunogold quantification has been previously validated (Hara et al., 2006; Hara & Pickel, 2007). As DOR-labeling was particularly abundant in dendritic profiles, further quantitative analysis of DOR labeling in dendrites was carried out to determine the distribution and density of DOR-SIG particles in plasmalemmal and cytoplasmic compartments. For this, DOR-immunogold particle localization was recorded as either cytoplasmic, plasmalemmal or "near plasma membrane" (i.e., particles within 50 nm, but not touching, the plasma membrane). Several ratios were calculated based on these designations: 1) plasma membrane SIG particles to total number of SIG particles (**PL:Total**); 2) near plasma membrane SIG particles to total number of SIG particles (**Near:Total**); and 3) cytoplasmic SIG particles to total number of SIG particles (**CY:Total**). Morphological parameters that were used as indirect measures of dendrite or terminal size, including surface area (perimeter), cross-sectional area, and average diameter, were measured using Microcomputer Imaging Device software (MCID, Imaging Research Inc, Ontario, Canada). These measurements were used to determine the number of plasmalemmal DOR-SIG particles in a profile / profile perimeter (**PL: m**) and the number of cytoplasmic DOR-SIG particles in a profile / profile cross-sectional area (**CY: m²**). To compare similar structures across groups, cluster analysis by dendritic size was performed to statistically divide DOR-labeled dendrites into small and large subcategories using SPSS for Windows V. 11 (SPSS Inc., Chicago, IL). In general, small and large dendrites in stratum radiatum of CA1 correspond respectively to distal and proximal portions on the dendritic tree.

For dual label studies, ultra-thin sections from 4 rats in proestrus, 4 rats in diestrus, and 4 male rats were analyzed. As before, all of the sections from each of the 3 groups were co-processed and identical sampling methods used, thus eliminating variables that could affect between-group comparisons. Immunoperoxidase labeling for NPY was distinguished as an electron-dense reaction product precipitate. Micrographs containing NPY-ir in axon terminals at magnifications of 10,500x to 19,000x were analyzed in a total tissue area of 12,100 m² per animal in stratum radiatum of the CA1 and quantitatively assessed for single and dual label frequency with DOR-SIG particles. To avoid false negative labeling of smaller profiles, profiles were considered as dual-labeled if they contained electron-dense reaction product and at least one gold particle. The frequency with which NPY immunoreactive axon terminals made contact with DOR-labeled dendritic profiles was also assessed between groups.

2.8 Statistical Analysis and data presentation

Estradiol levels, uterine weights, cell count, densitometry, and colocalization data were analyzed by Student's t-test or one-way ANOVA followed by Bonferroni post hoc testing using SPSS for Windows V. 11.0 (SPSS). Ultrastructural DOR-SIG particle density and distribution in dendritic and axon terminal profiles were analyzed by Kruskal-Wallis and Mann-Whitney U nonparametric statistical tests as these data were not normally distributed (Shapiro-Wilk $p < 0.05$). Pictures were formatted and adjusted for sharpness, brightness and contrast using Adobe Photoshop CS4 software (Adobe Systems, Inc., San Jose CA) and prepared in Microsoft Office Powerpoint 2007. Graphs were prepared with Graph Pad Prism 5.03 (Graph Pad Software, Inc., San Diego CA). All data in bar graphs are presented as mean $\pm$ SEM.

3. Results

3.1 Ovarian hormones influence DOR-ir in the DG and CA1

In the dentate gyrus, consistent with previously reported DOR-labeling patterns (Commons & Milner, 1996), proestrus and diestrus females and males display diffuse DOR-ir in the granule cell layer as well as more intensely labeled hilar neurons varying in size and shape (Fig. 1A). To determine whether sex or hormonal status influenced DOR-labeling in the DG, we compared DOR-ir levels in granule cells and numbers of DOR-labeled neurons in the hilus between groups. Quantitative densitometric analysis revealed that females had significantly less DOR-ir in the granule cell layer of the DG than male rats when proestrus and diestrus females were analyzed together ($t=2.604$, d.f.=12, $p=0.023$; Fig. 1C). When proestrus and diestrus females were analyzed separately, no significant differences in DOR-ir in the GCL was observed between groups ($F(2,11) = 3.932$, $p = 0.051$; not shown). Females displayed similar numbers of DOR-labeled hilar neurons in comparison to males ($t=1.043$, d.f.=12, $p=0.318$; Fig. 1D). When proestrus and diestrus females were analyzed separately, however, a significant difference in number of DOR-labeled hilar neurons was found between groups ($F(2,11) = 7.114$, $p = 0.010$), emphasizing the importance of separating females by hormonal status to avoid masking effects. Post-hoc analyses revealed that proestrus females showed increased numbers of DOR-labeled hilar neurons in comparison to diestrus females and males ($p<0.05$, both comparisons; Fig. 1E). As these results demonstrated differences in DOR-labeling in some cases by sex (when males and females were compared regardless of estrous cycle phase) and in other cases by hormonal status (when males were compared to proestrus and diestrus females), we next assessed differences in DOR-labeling by sex and hormonal status in the CA1.

In the CA1, consistent with previously reported DOR-labeling patterns (Commons & Milner, 1997), DOR-labeling in males and females is found in interneurons in stratum oriens

(SO) as well as within pyramidal cell somata in the pyramidal cell layer (PCL) and proximal dendrites in stratum radiatum (SR) (Fig. 1B). In contrast to the DG, quantitative densitometric analysis in the CA1 revealed that females had comparable levels of DOR-ir in the pyramidal cell layer to male rats when proestrus and diestrus females were analyzed together ($t=1.464$, $d.f.=12$, $p=0.169$; not shown). When females were analyzed separately, however, proestrus females displayed decreased DOR-ir in the PCL in comparison to males ($t=4.252$, $d.f.=7$, $p=0.004$; Fig. 1F). Females displayed similar numbers of DOR-labeled neurons in SO of the CA1 in comparison to males ($t=1.445$, $d.f.=12$, $p=0.175$; not shown). Similarly, when proestrus and diestrus females were analyzed separately, no significant difference in number of DOR-labeled neurons in SO was found between groups ($F(2,11) = 0.966$, $p = 0.411$; not shown).

3.2 Proestrus females display fewer NPY-labeled neurons with DORs in CA1 and CA3

To determine whether ovarian hormones influence DOR-labeling of interneurons, we compared localization of DOR-ir to subpopulations of GABAergic interneurons containing NPY in males and proestrus females. Consistent with prior reports indicating DOR expression in NPY-containing GABAergic interneurons (Commons & Milner, 1996; Stumm et al., 2004), DOR-ir colocalized with NPY-ir in hippocampal lamina of males and females. No labeling was observed in control sections for which primary antisera were omitted (not shown). In the DG, DOR-ir was found in NPY-labeled neurons primarily located centrally in the hilus (Fig. 2A-C). Both males and proestrus females displayed DOR-labeling on roughly half of the NPY-immunoreactive neurons in the hilar region (Table 1). A substantial percentage of DOR-positive neurons in the hilus also contained NPY (40.66% in proestrus females and 46.66% in males), but no differences were observed between males and proestrus females ($t=0.532$, $d.f.=8$, $p=0.609$; not shown). In the CA3, DOR-ir was found in NPY-labeled neurons located in stratum oriens (Fig. 2D-F). However, proestrus females had significantly fewer NPY-immunoreactive neurons that co-labeled with DOR-ir in comparison to males ($t=3.073$, $d.f.=8$, $p=0.015$; Table 1). Similarly, proestrus females displayed fewer DOR-immunoreactive neurons co-labeled with NPY-ir ($t=2.386$, $d.f.=8$, $p=0.044$; not shown). In the CA1, DOR-ir was found in NPY-labeled neurons located in stratum oriens (Fig. 2G-I). Like the CA3 and in contrast to the DG, proestrus females had fewer dual-labeled neurons than males ($t=3.381$, $d.f.=8$, $p=0.010$; Table 1). Specifically, proestrus females displayed fewer NPY-immunoreactive neurons that co-labeled with DOR-ir ($t=2.859$, $d.f.=8$, $p=0.021$; Table 1) and fewer DOR-immunoreactive neurons co-labeled with NPY-ir ($t=4.080$, $d.f.=8$, $p=0.004$; not shown) in comparison to males.

3.3 Ovarian hormones influence DOR-labeled profile distribution in SR of CA1

In agreement with previous studies in male rats (Commons & Milner, 1997), DOR-ir was present in somata, pyramidal cell dendrites and dendritic spines, axon terminals, and astrocytic processes. In stratum radiatum of CA1, DOR-labeling was particularly abundant in dendritic profiles (Table 2). DOR-labeled pyramidal cell-like dendritic profiles received few synapses on the dendritic shaft and were often visibly spiny (Fig. 3A,B), consistent in morphology with dendrites of pyramidal cells rather than interneurons (Harris et al., 1992). Shafts of DOR-labeled dendrites were small (0.05-1.0 μ m) to large (>1.0 μ m) in stratum radiatum and usually oriented perpendicular to the pyramidal cell layer. DOR-labeling also was frequently observed in axon terminals (Table 2), often near small clear synaptic vesicles within the cytoplasmic compartment or on the plasma membrane (Fig 3C-E). DOR-labeling was less commonly observed in dendritic spines and astrocytic processes (Table 2). Statistical analyses revealed trends for differences in DOR-labeled profile distribution between proestrus females, diestrus females, and males. Further analyses revealed that proestrus females displayed significantly fewer DOR-labeled dendrites than males ($t=2.651$, $d.f.=7$, $p=0.033$; Table 2) and a tendency for more DOR-labeled axon terminals than males.

3.4 Ovarian hormones influence DOR subcellular distribution in pyramidal cell dendrites but not axon terminals in SR of CA1

To determine whether sex or hormonal status influenced the intracellular density or trafficking of DORs, we measured the subcellular distribution of DORs in dendritic and axon terminal profiles in stratum radiatum of CA1. In CA1 stratum radiatum dendrites, most DOR-labeling was in the cytoplasmic compartment with less than 20% of DOR-SIG particles directly associated with the plasma membrane in males and females, regardless of estrous cycle phase. Quantitative ultrastructural analysis revealed that females had fewer DOR-SIG particles per dendrite than males (Mann-Whitney U Test, $z=4.030$, $p=0.000$) when diestrus and proestrus females were analyzed together (Fig. 4A). Moreover, females displayed decreased density and number of DOR-SIG particles on the plasma membrane in comparison to males (PL: m $z=2.312$, $p=0.021$; PL:Total $z=2.220$, $p=0.026$; Fig. 4C, 4E). When proestrus and diestrus females were analyzed separately, a significant difference in number of DOR-SIG particles per dendrite was found between groups (Kruskal-Wallis Test, $H=16.238$, 2d.f., $p=0.000$). Post-hoc analyses revealed that both proestrus and diestrus females had fewer DOR-SIG particles per dendrite than males ($p<0.05$, both comparisons; Fig. 4B). Separate analysis of proestrus and diestrus females also demonstrated significant differences in density of DOR-SIG particles on the plasma membrane ($H=6.195$, 2d.f., $p=0.045$) and in the cytoplasm ($H=6.957$, 2d.f., $p=0.031$). Post-hoc analyses revealed that proestrus females exhibited reduced plasma membrane density of DOR-SIG particles in comparison to males and increased cytoplasmic density of DOR-SIG particles in comparison to diestrus females ($p<0.05$, both comparisons; Fig. 4D). A significant difference was noted in the number of DOR-SIG particles in the cytoplasm ($H=7.490$, 2d.f., $p=0.024$) and a trend for differences in the number of DOR-SIG particles on the plasma membrane was observed between groups. Post-hoc analysis demonstrated that the number of DOR-SIG particles was increased in the cytoplasm of proestrus females in comparison to males ($p<0.05$; Fig. 4F).

To determine whether hormonal influences on DOR subcellular distribution is dependent on soma proximity, cluster analysis by dendritic size was performed to statistically divide DOR-labeled dendrites into small and large subcategories. In general, small (average diameter <1.03 μ m) and large (average diameter $1.03 - 2.86$ μ m) dendrites in stratum radiatum of CA1 correspond respectively to distal and proximal portions on the dendritic tree (Rall, 1967; Vetter et al., 2001). Data was re-analyzed by cluster size with diestrus and proestrus females separated to more accurately compare similar structures across groups. In small dendrites, a significant difference in number of DOR-SIG particles per dendrite was found between groups ($H=14.407$, 2d.f., $p=0.001$). Post-hoc analyses revealed that both proestrus and diestrus females had fewer DOR-SIG particles per dendrite than males ($p<0.05$, both comparisons; Fig. 5B). A significant difference also was noted in the number of DOR-SIG particles in the cytoplasm of small dendrites ($H=8.852$, 2d.f., $p=0.012$). Post-hoc analysis demonstrated that the number of DOR-SIG particles was increased in the cytoplasm of proestrus females in comparison to males ($p<0.05$; Fig. 5F). No significant differences were noted in density or number of DOR-SIG particles on the plasma membrane of small dendrites between groups (PL: m $H=4.219$, 2d.f., $p=0.127$; PL:Total $H=3.913$, 2d.f., $p=0.141$; Fig. 5C, 5E).

In large dendrites, a significant difference in number of DOR-SIG particles per dendrite was found between groups ($H=13.541$, 2d.f., $p=0.001$). Post-hoc analyses revealed that diestrus females had fewer DOR-SIG particles per dendrite than both proestrus females and males ($p<0.05$, both comparisons; Fig. 5B). A significant difference was also noted in the density of DOR-SIG particles in the cytoplasm of large dendrites ($H=31.890$, 2d.f., $p=0.000$). Post-hoc analysis demonstrated that the density of DOR-SIG particles was decreased in the cytoplasm of diestrus females in comparison to both proestrus females and males ($p<0.05$,

both comparisons; Fig. 5D). No significant differences were noted in density or number of DOR-SIG particles on the plasma membrane of large dendrites between groups (PL: m $H=2.736$, 2d.f., $p=0.255$; PL:Total $H=2.142$, 2d.f., $p=0.343$; Fig. 5C, 5E).

In CA1 stratum radiatum axon terminals, most DOR-labeling was in the cytoplasmic compartment with less than 20% of DOR-SIG particles directly associated with the plasma membrane in males and females, regardless of estrous cycle phase. Quantitative ultrastructural analysis revealed no differences in DOR-SIG particles per axon terminal between males and females when diestrus and proestrus females were analyzed together ($z=0.670$ $p=0.503$; not shown) or separately ($H=0.493$, 2d.f., $p=0.781$, not shown). No significant differences were observed in density of DOR-SIG particles in axon terminals of females in comparison to males (PL: m $z=0.142$, $p=0.887$; CY: m^2 : $z=1.711$, $p=0.0872$; not shown). Similarly, when proestrus and diestrus females were analyzed separately, no significant differences were observed in density of DOR-SIG particles in axon terminals between groups (PL: m $H=1.834$, 2d.f., $p=0.400$; CY: m^2 $H=2.937$, 2d.f., $p=0.230$; not shown).

3.5 DOR colocalization with NPY-labeled axon terminals is similar in males and females

To determine whether ovarian hormones influence DOR-labeling of interneuron axon terminals, we compared localization of DOR-ir to interneuron axon terminals containing NPY in males and females. At the electron microscope level, NPY-ir was prominent in axon terminals within SR of CA1 (Fig. 6). Roughly 10-15% of NPY-labeled axon terminals contained DOR-SIG particles in both males and females (Table 3). In dual labeled axonal profiles, DOR-SIG particles were frequently located on or near the plasma membrane (Fig. 6). No significant differences in frequency of NPY- or dual-labeled axon terminals were found between males and females (NPY-labeled: $t=0.764$, d.f.=10, $p=0.462$; Dual-labeled: $t=1.717$, d.f.=10, $p=0.117$). Similarly, no significant differences in frequency of NPY- or dual-labeled axon terminals were found between groups when proestrus and diestrus females were separated (NPY-labeled: $F(2,9)=0.990$, $p=0.409$; Dual-labeled: $F(2,9)=1.615$, $p=0.252$; Table 3). In addition, roughly 30% of NPY-labeled axon terminals were found either forming symmetric synapses with or adjacent to DOR-labeled dendrites in both males and females (Table 3; Fig. 6). No significant differences in frequency of NPY-labeled axon terminals contacting DOR-labeled dendrites were found between males and females ($t=1.098$, d.f.=10, $p=0.298$). Similarly, no significant differences in frequency of NPY-labeled axon terminals contacting DOR-labeled dendrites were found between groups when proestrus and diestrus females were separated ($F(2,9)=0.901$, $p=0.440$; Table 3).

3.6 Uterine weights and serum estradiol levels

Although estrous cycle phase was determined mainly via vaginal smear cytology, we also determined uterine weights and plasma serum estradiol levels at the time of sacrifice to corroborate the observed cytology. Uterine weights and plasma serum estradiol levels have been reported previously for the first cohort of cycling females used in DOR single-label studies (Torres-Reveron et al., 2008). Proestrus and diestrus female rats from a second cohort of cycling females were used in comparison to male rats to assess DOR colocalization with NPY. For the second cycling female cohort, average uterine weights were: proestrus – 0.66 ± 0.1 g; diestrus – 0.26 ± 0.07 g; and estrus – 0.58 ± 0.04 g. Uterine weights were on average 60.6% and 12.1% heavier in the proestrus group compared to the diestrus and estrus groups respectively. A significant difference in uterine weights was found between groups ($F(2,12)=8.096$, $p=0.006$). Post-hoc analyses revealed that the average uterine weights of the proestrus and estrus groups were higher than that of the diestrus group ($p < 0.05$, both comparisons). Plasma estradiol levels in the second cycling female cohort were measured by radioimmunoassay. Average blood levels for estradiol

were: proestrus – 19.1 ± 3.4 pg/ml; diestrus – 10.7 ± 1.0 pg/ml; and estrus – 10.9 ± 1.3 pg/ml. Average blood levels for estradiol were 44% and 42.9% higher in proestrus females in comparison to diestrus and estrus females respectively. A significant difference in serum estradiol levels was found between groups ($F(2,12)=4.935$, $p=0.027$). Post-hoc analyses revealed that proestrus females displayed trends for higher plasma estradiol levels than diestrus females ($p=0.053$) and estrus females ($p=0.059$). Estradiol levels in cycling animals were all within previously reported ranges for normal cycling female rats (Belanger et al., 1981).

4. Discussion

This study is the first to demonstrate that ovarian hormones present in normal cycling females modulate DOR levels and trafficking in hippocampal principal cells. Specifically, in comparison to males: (1) DOR levels are reduced in granule cell somata and distal dendrites of CA1 pyramidal cells of females, regardless of estrous cycle phase, and (2) proestrus (high estrogen) females, in particular, display trafficking of DORs away from the plasma membrane and into the cytoplasm of distal dendrites of CA1 pyramidal cells as well as trafficking of DORs towards the pyramidal cell soma (Fig. 7). In NPY-labeled subpopulations of GABAergic interneurons, ovarian hormones present in normal cycling females alter the number of somata containing DOR-ir in SO of CA1 and CA3 but do not influence the number of axon terminals containing DOR-ir in SR of CA1 (Fig. 7). Taken together, these findings suggest that ovarian hormones can impact hippocampal function through direct effects on DOR levels and trafficking in principal cells and indirect effects on DOR-ir in interneurons, particularly in the CA1.

4.1 Methodological considerations

The pre-embedding immunogold method was chosen to localize DOR immunoreactivity at the ultrastructural level as it maintains morphological preservation while providing discrete subcellular localization of the antigen of interest (Leranth & Pickel, 1989). This method is more appropriate than post-embedding methods for localization of immunoreactivity at extrasynaptic sites, and thus is suitable for quantifying the regional distribution of DORs (Lujan et al., 1996). Although pre-embedding immunogold labeling can produce lower estimates of receptor number than immunoperoxidase labeling, due to reduced reagent penetration (Leranth & Pickel, 1989), this limitation was not likely to affect comparisons of DOR density between groups as (a) tissue was pooled and processed together to facilitate relative comparisons (Pierce et al., 1999), and (b) ultra-thin sections were collected from the plastic-tissue interface where immunoreagent access is maximal. Furthermore, we chose to assess DOR-ir levels using quantitative densitometric approaches as light microscopic optical density measurements correlate linearly with ultrastructural observations, allow for sub-region specific analysis to avoid masking effects, and have been used previously to investigate hormone effects on endogenous opioid peptides (Pierce et al., 1999; Croll et al., 1999; Torres-Reveron et al., 2008; Torres-Reveron et al., 2009a).

Although the roles of ovarian hormones have often been studied using different ovariectomy models (for example: (McEwen, 2001; Adams et al., 2001)), each model has different strengths and weaknesses as estradiol effects depend strongly on hormone dose, time examined after steroid administration, and interval following ovariectomy (Adams et al., 2001; Tanapat et al., 2005). Thus, the current experiments were conducted in normal cycling females in comparison to males. The cycling females were studied at the proestrus and diestrus phases of the rodent estrous cycle; circulating estrogen levels are highest in proestrus and circulating ovarian hormone levels are lowest in diestrus (Belanger et al., 1981). Prior studies in our laboratory and others, particularly regarding ovarian steroid modulation of opioids and opioid receptors in the hippocampus, confirm that morphological

changes induced by exogenously supplied estradiol reflect changes observed during proestrus in normal cycling females while ovariectomized females resemble normal cycling females in diestrus (Woolley & McEwen, 1992; Wilson et al., 2002; Torres-Reveron et al., 2008; Torres-Reveron et al., 2009a; Torres-Reveron et al., 2009b).

4.2 Ovarian hormones influence DOR-ir in NPY-labeled interneurons

Consistent with prior reports indicating DOR expression in NPY-containing GABAergic interneurons (Commons & Milner, 1996; Stumm et al., 2004), DOR-ir colocalized with NPY-ir in all hippocampal lamina of males and proestrus females. Both groups displayed DOR-labeling on roughly half of the NPY-immunoreactive neurons in the hilar region. In contrast, proestrus females had significantly fewer NPY-immunoreactive neurons that co-labeled with DOR-ir in SO of CA3 and CA1 in comparison to males. No significant differences between males and proestrus females were noted for numbers of cells singly labeled with NPY or DOR, suggesting a selective reduction in dual-labeled cells, particularly in the CA1 (Table 1). Moreover, ongoing studies find no significant differences in DOR co-labeling of somatostatin-immunoreactive neurons in SO of CA3 and CA1 between groups (T.J. Williams, unpublished observations). Thus, reduced co-labeling in high estrogen females appears to be limited to NPY-immunoreactive subpopulations of interneurons, which extensive work characterizing the morphological and neurochemical properties of GABAergic interneurons in the HF (reviewed by (Freund & Buzsaki, 1996) would suggest are largely horizontal or OLM cells. These interneurons typically have their soma and dendrites in SO and send axon projections to the distal apical dendrites of pyramidal neurons in stratum lacunosum-moleculare (SLM) and SR. As such, they are positioned to regulate inputs from excitatory terminals from the entorhinal cortex and thalamus and are considered recurrent inhibitory cells as they are activated following CA1 pyramidal neuron discharge (Sik et al., 1995; Yanovsky et al., 1997). Electrophysiological approaches reveal that OLM cells express DORs and demonstrate DOR agonist-induced hyperpolarization (Svoboda et al., 1999), resulting in a reduction of the dendritic inhibition of excitatory inputs from the entorhinal cortex. Proestrus females, with fewer DORs in NPY-immunoreactive OLM cells, are more likely to experience activation of OLM cells, resulting in a reduction in excitatory drive from the entorhinal cortex and increased flow of information through the CA3 (Maccaferri & McBain, 1996; Ali & Thomson, 1998). Conversely, males are better able to facilitate information flow through the entorhinal cortex.

However, intracellular recordings have demonstrated that increases in excitatory synaptic activation of interneurons can overcome the inhibition of action potential generation caused by opioid hyperpolarization of interneuron somata (Madison & Nicoll, 1988). As DORs were also localized to axon terminals in SR of CA1 in single label studies, we further examined DOR-ir in NPY-labeled axon terminals in this region as a site of OLM cell projection. In contrast to findings at the soma of OLM cells in SO of CA1, no differences between males, diestrus, or proestrus females were observed for DOR co-labeling of NPY-immunoreactive axon terminals in SR of CA1. Opioid receptor activation at the presynaptic site, like the axon terminal, has been shown to influence neurotransmitter release (Drake et al., 2007). Enkephalin activation of opioid receptors, in particular, has been shown to reduce GABA release from interneuron terminals in the CA1 (Cohen et al., 1992). Thus, two levels of regulation of synaptic inhibition by opioid receptors are apparent: (1) hyperpolarization of the soma to reduce the influence of the entire neuron by preventing action potential generation and signal propagation in all axonal branches and (2) reduction in GABA release from the axon terminal enabling focal control of specific subsets of inhibitory synapses (Cohen et al., 1992). As current study findings would support an equal probability of GABA release in males and females at both phases of the estrous cycle, ovarian hormones would

appear to play a role in modulating DORs on somata and not terminals to exert broad, rather than fine, control on interneuron function and ultimately hippocampal synaptic transmission. Future ultrastructural studies will address whether DORs, like MORs (Torres-Reveron et al., 2009b), also undergo hormone-modulated trafficking in hippocampal interneurons, particularly in SO of CA1.

4.3 Ovarian hormones alter DOR-ir in principal cells

Prior studies have demonstrated hormonal modulation of DOR-ir and density in the amygdala and frontal cortex respectively (Vathy et al., 2000; Wilson et al., 2002). We report here, for the first time, modulation of DOR-ir in the hippocampus by ovarian hormones present in intact cycling females. Quantitative densitometric analysis revealed that females had significantly less DOR-ir in the granule cell layer of the DG than male rats. Proestrus females, in particular, also displayed reduced DOR-ir in the pyramidal cell layer in comparison to males. Ultrastructural studies demonstrated reduced DORs in distal pyramidal cell dendrites of all females, and selective internalization and trafficking of DORs from distal to proximal pyramidal cell dendrites in proestrus females (Fig. 7). Several studies exploring estrogen modulation of MORs suggest that estrogen-induced MOR internalization is dependent on ligand-receptor binding (Eckersell et al., 1998; Sinchak & Micevych, 2001; Micevych et al., 2003). A similar mechanism may explain the observations of DOR internalization in proestrus females and reduced DOR-ir in all females as internalized DORs are preferentially targeted to protein degradation pathways rather than recycling pathways (Afify et al., 1998; Tsao & von Zastrow, 2000; Scherrer et al., 2006; Pradhan et al., 2009). We have previously shown that levels of enkephalin, the endogenous ligand for DORs (Corbett et al., 1984), are elevated when estrogen levels are high (either in proestrus females or in OVX females replaced with estradiol) likely via genomic and non-genomic actions of estrogen receptors (Torres-Reveron et al., 2008). In the hippocampus, enkephalins are found in mossy fibers in CA3, the lateral portion of the perforant path in the DG, in scattered interneurons along the border of SR and SLM as well as in the temporal-ammonic tract in CA1 (Commons & Milner, 1995; Drake et al., 2007). Hence, we posit that elevated estrogen levels increase the amount of enkephalin available for release and binding to DORs following high frequency stimulation, resulting in receptor internalization and degradation.

A second possibility may involve NPY, as roughly 30% of NPY-immunoreactive axon terminals were found either forming symmetric synapses with or adjacent to DOR-labeled dendrites in SR of CA1. Studies in the hypothalamus suggest that estrogen-induced release of NPY and activation of the NPY receptor results in opioid release to activate and internalize MORs (Mills et al., 2004). A similar mechanism involving sequential sex steroid activation of NPY and DOR circuits, resulting in DOR internalization and degradation may be at work in the hippocampus. As the present study measured frequency of NPY-axon terminals but not NPY content within axon terminals, future studies in normal cycling females and males ascertaining NPY levels in axon terminals or synaptic boutons adjacent to the distal dendrites of CA1 pyramidal cells would shed light on this interesting possibility given substantial anatomical and physiological evidence of estrogen regulation of NPY expression and release in the hippocampus of OVX rodents replaced with estradiol (Nakamura & McEwen, 2005; Nakamura et al., 2007; Ledoux et al., 2009).

Reduced DOR-ir in granule cells of the DG and distal dendrites of CA1 pyramidal cells in females, regardless of estrous cycle phase, could have different physiological outcomes. Namely, females, with fewer DORs available for activation in granule cells would be more likely to transmit perforant path excitatory inputs than male counterparts, as DOR activation *directly* hyperpolarizes granule cells to modulate output (Piguet & North, 1993; Bausch & Chavkin, 1997). In spite of intense electrophysiological investigation and in contrast to the DG (Xie & Lewis, 1995b) and CA3 (Moore et al., 1994), direct opiate receptor effects have

rarely been detected on CA1 pyramidal cells. Instead, as aforementioned, DOR activation is thought to increase the excitability of CA1 pyramidal cells by reducing presynaptic inhibition (Madison & Nicoll, 1988; Neumaier et al., 1988; Svoboda & Lupica, 1998; Svoboda et al., 1999). Therefore, reduced DOR availability for activation on CA1 pyramidal cells would not be expected to *directly* increase pyramidal cell excitation in females, as DOR activation produces little to no effect on CA1 pyramidal cell membrane potential (Neumaier et al., 1988; Madamba et al., 1999). However, as most of the information on DOR effects on CA1 pyramidal cell excitation has historically been gleaned from *in vitro* electrophysiological studies of the drug-naïve male hippocampal slice using agonists of sometimes questionable selectivity, additional studies are warranted to assess DOR effects on CA1 pyramidal cell excitation in the normal cycling female using both *in vitro* and *in vivo* preparations.

Indeed, studies using *in vitro* and *in vivo* electrophysiological approaches to examine the effects of chronic opiate treatment on long-term potentiation (LTP), a leading experimental model of activity-dependent synaptic plasticity and a potential neural mechanism for learning and memory (Bliss & Collingridge, 1993; Malenka & Nicoll, 1999), demonstrate opioid receptor effects on CA1 LTP that are independent of GABAergic activity (Pu et al., 2002). Instead, MOR and DOR agonists restore LTP following withdrawal from chronic opiate treatment via a mechanism involving cAMP-dependent protein kinase A activity (Bao et al., 2007). Regulation of CA1 pyramidal cell excitation may therefore differ in the opioid-dependent and drug naïve animal and involve diverse signal transduction mechanisms. Given the current findings of reduced DOR-ir and plasma membrane availability in CA1 pyramidal cells of females, particularly proestrus females, in comparison to males and evidence that chronic opiate treatment promotes movement of intracellular DOR to the plasma membrane (Cahill et al., 2001; Morinville et al., 2003; Morgan et al., 2009), opioid-dependent trafficking of DORs to the plasma membrane may be altered or impaired in females when estrogen levels are high. Thus, further electrophysiological studies addressing the effect of DOR-selective agonists on CA1 LTP and related cognitive tasks in drug naïve and opioid-dependent cycling females are needed.

4.4 Clinical implications

Our findings provide new anatomical evidence of hormonal regulation of DORs in the HF, adding to the growing body of literature supporting cooperative interaction between opioid systems and estrogens in modulating hippocampal excitability. Such interactions between opioid receptors and hormonal state in the hippocampal formation may influence a variety of cognitive functions, including but not limited to addiction as hippocampal-derived contextual associations formed with select drug-abuse experiences often contribute to the maintenance of addictive processes (Robbins & Everitt, 1999; Berke & Hyman, 2000). Previous reports have established the role of DORs in the development of tolerance, behavioral sensitization, and the conditioning of opiate reward as well as the proven efficacy of DOR antagonists in blocking the reinstatement of drug-seeking behavior in male animal models of opiate, cocaine, and alcohol addiction (Ciccocioppo et al., 2002; Marinelli et al., 2009; Shippenberg et al., 2009; Chefer & Shippenberg, 2009; Moron et al., 2010; Kotlinska et al., 2010). Given these reports, DOR antagonists offer new opportunities for the treatment of dependence and the prevention of relapse. The present study highlights differences in DOR levels and trafficking between drug naïve females and males that, if also apparent in drug-dependent animals, may contribute to observed sex differences in relapse proclivity given the essential role of the hippocampus in context-induced relapse (Fuchs et al., 2007; Crombag et al., 2008). Thus, further research focusing on the impact of circulating hormones and DOR activation in animal models of relapse is warranted in an effort to

identify unique mechanisms in females that may be targeted by novel treatments for addiction.

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Fig. 1.

Light microscopic distribution of delta opioid receptor (DOR) immunoreactivity (-ir) in the rat hippocampal formation. (A) Representative light photomicrograph from a male showing DOR labeling in hilar neurons and diffuse DOR-ir visible over the granule cell layer (GCL). (B) In CA1 of a proestrus female, DOR labeling is found in interneurons (arrows) in stratum oriens (SO) as well as within pyramidal cell somata in the pyramidal cell layer (PCL) and proximal dendrites in stratum radiatum (SR). (C) Quantitative densitometric analysis revealed that females had significantly less DOR-ir in the granule cell layer of the dentate gyrus (DG) than male rats (* $p < 0.05$). (D) When proestrus and diestrus females were grouped together, females showed similar numbers of DOR-labeled hilar neurons in comparison to males ($p > 0.05$). (E) When proestrus and diestrus females were analyzed separately, however, proestrus females displayed a significantly increased number of DOR-labeled hilar neurons in comparison to diestrus females and males (* $p < 0.05$, both comparisons). (F) In the CA1, proestrus females displayed decreased DOR-ir in the pyramidal cell layer in comparison to males (* $p < 0.05$). Scale bars = 100 μm .

Fig. 2.

Immunofluorescent images illustrating co-localization of DOR (A, D, G) with NPY (B, E, H) in the hippocampus. (A-C) Dual-labeled cells in the hilus (Hil) of the dentate gyrus (DG), where A and B are unmerged images of C. (D-F) Dual-labeled cells in stratum oriens (SO) of CA3, where D and E are unmerged images of F. (G-I) Dual-labeled cells in stratum oriens of CA1, where G and H are unmerged images of I. DOR-labeling is red. NPY-labeling is green. Dual-labeled cells appear yellow and are indicated by arrows. PCL = pyramidal cell layer. Scale bars = 100 m.

Fig. 3.

DOR-labeling is found in dendrites and axon terminals in stratum radiatum of the CA1. Representative electron micrographs from a male (A) and proestrus female (B) demonstrate DOR-labeling in both cytoplasmic and plasmalemmal sites in spiny dendrites similar in morphology to pyramidal cells using the immunogold-silver technique. DOR silver-intensified-gold (SIG) particles are found along the plasma membrane (blue circles), near the plasma membrane (red arrows), or in the cytoplasm (blue arrows). Representative electron micrographs from a proestrus female (C) and male (D-E) show DOR-labeling along the plasma membrane (blue circles), near the plasma membrane (red arrows), or affiliated with small clear synaptic vesicles in the cytoplasm (blue arrows) of axon terminals. den = dendrite, ter = DOR-labeled axon terminal, ut = unlabeled axon terminal. Scale bars = 500 nm.

Fig. 4.

Ovarian hormones influence the distribution of DOR-SIG particles in CA1 stratum radiatum dendrites. (A) Females display fewer DOR-SIG particles per dendrite than males (* $p < 0.05$). (B) Both proestrus and diestrus females show fewer DOR-SIG particles per dendrite than males (* $p < 0.05$, both comparisons). (C) Females have reduced plasma membrane density of DOR-SIG particles in comparison to males (* $p < 0.05$). (D) Proestrus females exhibit reduced plasma membrane density of DOR-SIG particles in comparison to males as well as increased cytoplasmic density of DOR-SIG particles in comparison to diestrus females (* $p < 0.05$, all comparisons). (E) The number of DOR-SIG particles is decreased on the plasma membrane of females in comparison to males (* $p < 0.05$). (F) The number of DOR-SIG particles is increased in the cytoplasm of proestrus females in comparison to males (* $p < 0.05$).

Fig. 5.

Ovarian hormones influence DOR-SIG particle distribution in small (average diameter <1.03 m) and large (average diameter 1.03 – 2.86 m) dendrites in SR of CA1. (A) Females display fewer DOR-SIG particles per dendrite than males in small (* $p < 0.05$) but not large dendrites. (B) Both proestrus and diestrus females show fewer DOR-SIG particles per dendrite than males in small dendrites (* $p < 0.05$, both comparisons). Diestrus females show fewer DOR-SIG particles per dendrite than proestrus females and males in large dendrites (* $p < 0.05$, both comparisons). (C) No significant differences were found in plasma membrane density of DOR-SIG particles between groups in either small or large dendrites. (D) Diestrus females exhibit decreased cytoplasmic density of DOR-SIG particles in comparison to proestrus females and males in large (* $p < 0.05$, both comparisons) but not small dendrites. (E) No significant differences were found in the number of DOR-SIG particles on the plasma membrane of either small or large dendrites between groups. (F) The number of DOR-SIG particles is increased in the cytoplasm of small but not large dendrites of proestrus females in comparison to males (* $p < 0.05$).

Fig. 6. DOR-labeling is found in NPY-immunoreactive axon terminals in stratum radiatum of CA1. (A, B) Representative electron micrographs demonstrate NPY-ir in single and dual-labeled axon terminal profiles. In dual-labeled axon terminals, DOR-SIG particles are frequently found along the plasma membrane (blue circles) or near the plasma membrane (red arrows). DOR-labeling is often affiliated with small clear synaptic vesicles in the cytoplasm of axon terminals. Moreover, NPY-labeled axon terminals also found in contact with DOR-labeled dendrites. As aforementioned, DOR-labeling within dendrites is frequently cytoplasmic (blue arrows). den = dendrite, ter = NPY-labeled axon terminal, ut = unlabeled axon terminal. Scale bars = 500 nm.

Fig. 7.

Schematic diagram summarizing ovarian hormone influences on DOR levels and distribution in dendrites and axon terminals within stratum radiatum of CA1. (A) In males, DORs are found on (or near) the plasma membrane and in the cytoplasm of small and large dendrites. DORs are also found in the cytoplasm and infrequently on the plasma membrane of axon terminals. NPY-labeled axon terminals sometimes contact DOR-labeled dendrites and occasionally contain DORs that are frequently found at or near the plasma membrane. (B) In comparison to males, diestrus (low estrogen) females have fewer DORs in both small and large dendrites. In addition, the cytoplasmic density of DORs is selectively reduced in large dendrites. No differences from males are observed in DOR-ir within NPY-labeled or unlabeled axon terminals. (C) In comparison to males, proestrus (high estrogen) females display fewer DORs as well as trafficking of DORs away from the plasma membrane and into the cytoplasm of small dendrites. Although proestrus females also have more DOR-labeled axon terminals, no differences from males or diestrus females are observed in DOR-ir within NPY-labeled or unlabeled axon terminals. In comparison with diestrus females, proestrus females exhibit increased DORs in large dendrites. Taken together, these results suggest that (1) females generally have fewer DORs in pyramidal cell dendrites and (2) when estrogen levels are high, DORs are internalized from the plasma membrane of distal dendrites and trafficked towards the pyramidal cell soma.

Table 1

Average number and percentage of DOR- and NPY-labeled neurons in the hippocampus of males and proestrus females

Region	Animal	Dual-labeled neurons	NPY-labeled neurons	NPY neurons with DOR (%)
Hilus	Pro (n=5)	9.0 0.9	18.6 2.5	49.60
	Male (n=5)	13.6 4.0	25.4 5.2	51.11
CA3 SO	Pro	2.8 0.9	6.6 1.7	40.56*
	Male	4.4 0.9	7.4 1.4	58.11
CA1 SO	Pro	1.8 0.4*	6.8 1.2	30.82*
	Male	6.2 1.0	10.0 2.3	66.12

Neuron counts presented as Mean SEM.

*
p< 0.05

Table 2

Profile distribution of DOR labeling in stratum radiatum of the CA1

Group	Number sampled	Type of profile		
		Pyramidal dendrites	Dendritic spines	Axon terminals
Proestrus (n=5)	220.6	130 (59%) *	17 (8%)	66 (30%)
Diestrus (n=4)	177.5	118 (66%)	18 (10%)	35 (20%)
Male (n=4)	205.3	144 (70%)	19 (9%)	39 (19%)

Average number of DOR-labeled profiles presented per group.

* p<0.05 in comparison to males

Table 3

DOR colocalization in NPY-labeled axon terminals in the hilar region

Group	Total NPY-labeled	Single-labeled with NPY	Dual-labeled	Contacting DOR-labeled dendrite
Proestrus (n=4)	16 4.2	14 2.5 (85%)	2 0.9 (15%)	4 1.4 (27%)
Diestrus (n=4)	26 4.6	25 4.9 (93%)	2 0.3 (7%)	8 3.7 (26%)
Male (n=4)	27 9.1	24 8.3 (86%)	4 1.0 (14%)	10 4.2 (36%)

Average number of axon terminals per group presented as Mean SEM.