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Delta opioid receptors colocalize with corticotropin releasing factor in hippocampal interneurons

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Abstract

The hippocampal formation (HF) is an important site at which stress circuits and endogenous opioid systems intersect, likely playing a critical role in the interaction between stress and drug addiction. Prior study findings suggest that the stress-related neuropeptide corticotropin releasing factor (CRF) and the delta opioid receptor (DOR) may localize to similar neuronal populations within HF lamina. Here, hippocampal sections of male and cycling female adult Sprague-Dawley rats were processed for immunolabeling using antisera directed against the DOR and CRF peptide, as well as interneuron subtype markers somatostatin or parvalbumin, and analyzed by fluorescence and electron microscopy. Both DOR- and CRF-labeling was observed in interneurons in the CA1, CA3, and dentate hilus. Males and normal cycling females displayed a similar number of CRF immunoreactive neurons co-labeled with DOR and a similar average number of CRF-labeled neurons in the dentate hilus and stratum oriens of CA1 and CA3. In addition, 70% of DOR/CRF dual-labeled neurons in the hilar region co-labeled with somatostatin, suggesting a role for these interneurons in regulating perforant path input to dentate granule cells. Ultrastructural analysis of CRF-labeled axon terminals within the hilar region revealed that proestrus females have a similar number of CRF-labeled axon terminals that contain DORs compared to males but an increased number of CRF-labeled axon terminals without DORs. Taken together, these findings suggest that while DORs are anatomically positioned to modulate CRF immunoreactive interneuron activity and CRF peptide release, their ability to exert such regulatory activity may be compromised in females when estrogen levels are high.

Keywords

Opioids; Stress; Hormones; Estrogen; Parvalbumin; Somatostatin

A wealth of evidence demonstrates that stress interacts with addictive processes to increase drug use, drug seeking, and relapse (Erb et al., 1996; Shaham et al., 2000; Stewart, 2003;

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Saal et al., 2003; Kauer, 2003; Sinha et al., 2004). Interestingly, the relationship between stress and relapse to drug-seeking behavior is particularly pronounced in females (Rubin et al., 1996; McKay et al., 1996; Elman et al., 2001). While the underlying mechanisms involve many brain areas traditionally associated with drug reward circuitry, the hippocampal formation (HF) also plays a critical role. In example, hippocampal learning mechanisms are engaged by addictive drugs and drug-associated memories may be encoded in the hippocampal formation (White, 1996; Koob et al., 1998; Robbins & Everitt, 1999; Berke & Hyman, 2000; Hyman & Malenka, 2001; Nestler, 2001; Nestler, 2002). Furthermore, both synaptic plasticity and behavioral studies support hippocampal involvement in drug, particularly opiate, addiction (Mansouri et al., 1999; Fan et al., 1999; Lu et al., 2000; Pu et al., 2002; Bao et al., 2007). As the HF also regulates stress effects on synaptic plasticity and learning and memory (Pavlidis et al., 1996; de Quervain et al., 1998; McEwen, 1999; Kim & Diamond, 2002), the role played by the HF in the interaction between stress and drug addiction, particularly in females, requires further inquiry.

While several studies investigating the impact of stress on relapse vulnerability have focused on interactions between endogenous opioid systems and the stress neurohormone corticotropin releasing factor (CRF) in the locus coeruleus (Curtis et al., 2006; Valentino & Van Bockstaele, 2008; Reyes et al., 2008; Van Bockstaele et al., 2010), few studies have explored the relationship between these two systems in the HF. Prior studies in male rats demonstrate that delta opioid receptor (DOR) mRNA and immunoreactivity (ir) is commonly found in somatostatin (SOM) / neuropeptide Y containing GABAergic interneurons in hippocampal lamina (Commons & Milner, 1996; Commons & Milner, 1997; Stumm et al., 2004). Similarly, studies in immature male rats indicate that CRF is synthesized in interneurons and released by stress to activate CRF receptors on principal cell dendrites (Chen et al., 2000; Chen et al., 2001; Chen et al., 2004b). Functionally, both DORs (Piguet & North, 1993; Bramham & Sarvey, 1996; Drake et al., 2007; Bao et al., 2007) and CRF (Wang et al., 1998; Blank et al., 2002; Schierloh et al., 2007) affect excitatory transmission and the induction of synaptic plasticity in the hippocampus. Reports also indicate that CRF and DORs play a role in reinstatement of drug-seeking behavior in animal models of addiction (Shaham et al., 1997; Marinelli et al., 2007; Marinelli et al., 2009; Brown et al., 2009; Shalev et al., 2010) and are susceptible to modulation by ovarian hormones (Vamvakopoulos & Chrousos, 1993; Vamvakopoulos & Chrousos, 1994; Vathy et al., 2000; Wilson et al., 2002; Miller et al., 2004; Chen et al., 2008a; Williams et al., 2011). Thus, the relationship between DORs and the CRF system in the HF merits direct study.

As prior study findings suggest that CRF and the DOR may localize to similar neuronal populations and subcompartments within HF lamina, the present study sought to confirm these observations in males and extend them, where applicable, to the female hippocampus. Our laboratory has previously demonstrated ovarian hormone influences on DOR-ir levels and trafficking in hippocampal principal cells (Williams et al., 2011) while others have reported estrogen modulation of CRF peptide levels (Chen et al., 2008a), warranting the use of females selected at different phases of the rodent estrous cycle to reflect different hormonal profiles in the current study. Hence, dual immunolabeling approaches were used to assess ovarian hormone influences on DOR-ir and CRF-ir within hippocampal interneurons of normal cycling proestrus (high estrogen), estrus (high progesterone) and diestrus (low estrogen and progesterone) female rats in comparison to male rats. Immunofluorescence and immunoelectron microscopy were used to examine DOR localization to CRF-labeled interneurons and axon terminals in select hippocampal lamina in 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).

EXPERIMENTAL PROCEDURES

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, estrus, 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). Two cohorts of normal cycling female rats were used in the present study. The first cohort of proestrus, estrus, and diestrus female rats has been used in prior studies by our laboratory with previously reported estradiol and progesterone levels and uterine weights (Torres-Reveron et al., 2008). Proestrus animals also were selected from a second cohort of proestrus, estrus, and diestrus female rats that has been used in prior studies by our laboratory with previously reported estradiol levels and uterine weights (Williams et al., 2011).

Antisera

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; Svingos et al., 1995; Commons & Milner, 1996) as well as comparable immunolabeling to a commercially available rabbit polyclonal DOR antisera (Chemicon)(Commons & Milner, 1996; Commons & Milner, 1997). A rabbit polyclonal antiserum raised against human/rat CRF (PBL rC70) was generously supplied by Dr. Wylie Vale from the Salk Institute for Biological Studies (San Diego)(Justice et al., 2008). This antisera was found to specifically recognize CRF via radioimmunoassay and competition studies with CRF or structurally related peptides (Vale et al., 1983; Sawchenko, 1987). A mouse monoclonal antibody against parvalbumin (PARV) was purchased from Sigma (St. Louis, MO). This antibody has been previously characterized by radioimmunoassay, immunoblots and the ability to recognize PARV in brain tissue (Celio et al., 1988). A mouse monoclonal antibody (“S8”) raised against SOM 14 was generously supplied by Dr. Andrew Malcolm of the MRC Regulatory Group (Vancouver, British Columbia, Canada). This antibody was previously shown to be specific (Sloviter & Nilaver, 1987) and labeled the same pattern of somata in immunolabeling studies as a commercially available rabbit polyclonal SOM antisera (Diasorin, Stillwater, MN)(Drake & Milner, 2002).

Section preparation

Rats were deeply anesthetized with sodium 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 &

Vezenadaroglu, 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.

Immunofluorescence labeling and analysis

For dual label studies, 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 CRF (1:1000) antisera in 0.1% TX / 3% NGS in PBS for 48 hrs at 4°C; and 3) a cocktail of goat antiguinea pig Cy5 immunoglobulin (IgG) (1:600; Jackson ImmunoResearch Labs, Inc., West Grove, PA) 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 Harellco). For triple label studies, primary antisera cocktails also contained mouse monoclonal SOM (1:400) or PARV (1:1500) and secondary antisera cocktails included goat anti-mouse Cy3 IgG (1:400; Jackson). 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 CRF or triply labeled with SOM or PARV. Alexa Fluor 488 (CRF) was pseudocolored green, Cy5 (DOR) was pseudocolored red, and Cy3 (SOM or PARV) was pseudocolored orange.

For dual label studies, the number of DOR- and CRF-labeled neurons in 50,000 μ m² areas of the dorsal hilus and stratum oriens of CA3 and CA1 (AP -3.6 to -4.0 caudal to Bregma (Swanson, 2000)) was counted in 5 proestrus females, 5 estrus females, 5 diestrus females, and 5 male rats. Cell counts were summed from 2 hippocampal sections (> 900 μ m interval) per animal and presented as mean $\pm$ SEM of DOR- and CRF-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). The percentage of DOR-immunoreactive neurons showing colocalization with CRF and the percentage of CRF-immunoreactive neurons showing colocalization with DOR was obtained for each animal and the average percentage $\pm$ SEM was calculated per group. For triple label studies, the number of DOR/CRF dual-labeled neurons co-labeled for SOM or PARV in the dorsal hilus was counted in 5 proestrus female and 5 male rats. Cell counts were obtained from 1 hippocampal section per animal and the average percentage $\pm$ SEM of DOR/CRF-immunoreactive neurons showing colocalization with SOM or PARV was obtained for each animal group.

Electron microscopic immunocytochemistry

For dual label electron microscopic localization of DOR with CRF, sections were labeled for CRF using immunoperoxidase and DOR using immunogold through methods that 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 then were processed for immunocytochemistry using the avidin-biotin complex (ABC)-peroxidase technique (Hsu et al., 1981). For this, 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 were then incubated in an antisera cocktail of guinea pig polyclonal DOR (1:1500) with rabbit polyclonal CRF (1:4000) in 0.1% BSA in TS for 72 hrs at 4°C and then processed through 1) a 1:400 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), 30 min; 2) a 1:100 dilution of peroxidase-avidin complex (Vectastain Elite Kit, Vector Labs), 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. To visualize DOR, 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 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 dentate gyrus (AP –3.6 to –4.0 from Bregma (Swanson, 2000)) were cut on a Leica UCT ultratome and collected on 400 mesh copper grids. 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).

Ultrastructural analysis

Single ultra-thin sections taken from the surface of coronal vibratome sections of 5 rats in proestrus and 5 male rats were analyzed. Immunoperoxidase labeling for CRF was distinguished as an electron-dense reaction product precipitate. Silver-intensified immunogold (SIG) labeling for DOR appeared as intense black electron-dense particles located within cytoplasmic compartments or on the plasma membrane. 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. Criteria for field selection included good morphological preservation, the presence of immunolabeling in the field, and proximity to the plastic-tissue interface (i.e., the surface of the tissue) to circumvent problems due to differences in antibody penetration. Micrographs containing CRF-ir in axon terminals at magnifications of 10,500× to 19,000× were analyzed in four 55 × 55 μm fields (corresponding to four squares of the copper grid) for a total scanned area of 12,100 μm² per animal in the hilar region of the dentate gyrus. All axon terminal profiles containing CRF-ir in the selected fields were quantitatively assessed for single and dual label frequency with DOR-SIG particles. The frequency with which CRF immunoreactive axon terminals made contact with DOR-labeled dendritic profiles was also assessed between groups.

Statistical analysis and data presentation

Data were analyzed by Student's t-test or one-way ANOVA followed by Tukey post hoc analyses using SPSS for Windows V. 11.0 (SPSS). 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.

RESULTS

DOR-ir colocalizes with CRF-ir in the dentate hilus, CA3, and CA1

Consistent with prior reports (Swanson et al., 1983; Commons & Milner, 1996; Commons & Milner, 1997; Yan et al., 1998; Chen et al., 2004b), DOR immunoreactive neurons and CRF immunoreactive neurons were found in the hilar region of the dentate gyrus (DG) (Fig. 1) and in stratum oriens (SO) and the pyramidal cell layer (PCL) of CA3 and CA1 (Fig. 2) of male and female rats. No labeling was observed in control sections for which primary antisera were omitted (not shown). In the DG, DOR-ir was found in CRF-labeled neurons primarily located centrally in the hilus and occasionally in the immediate infragranular hilus, directly apposed to cells in the granule cell layer (Fig. 1). In the CA3 and CA1, DOR-ir was found in CRF-labeled neurons primarily located in stratum oriens, particularly at the border between SO and the alveus (Fig. 2).

Quantitative analysis of immunolabeling in the hilar region between males and normal cycling females revealed differences in numbers of immunoreactive neurons. Specifically, females displayed a trend for increased numbers of DOR-labeled hilar neurons in comparison to males ($t=1.895$, d.f.=18, $p=0.071$). When cycling females were analyzed separately, however, no significant difference in number of DOR-labeled hilar neurons was found between groups ($F(3,16) = 1.751$, $p = 0.197$; Table 1). No significant differences were noted between groups in number of CRF-labeled hilar neurons when cycling females were analyzed together ($t=0.206$, d.f.=18, $p=0.839$) or separately ($F(3,16) = 0.100$, $p = 0.959$; Table 1).

We next assessed whether ovarian hormones influenced the percentage of DOR-labeled neurons containing CRF-ir or the percentage of CRF-labeled neurons containing DOR-ir in the hilar region of the dentate gyrus. Females displayed a smaller percentage of DOR immunoreactive neurons that co-labeled with CRF-ir ($t=2.852$, d.f.=18, $p=0.011$) but no difference in the percentage of CRF immunoreactive neurons co-labeled with DOR-ir ($t=0.306$, d.f.=18, $p=0.763$) in comparison to males. When cycling females were analyzed separately, a significant difference in the percentage of DOR immunoreactive neurons that co-labeled with CRF-ir was observed between groups ($F(3,16) = 3.358$, $p = 0.045$; Table 1). Post-hoc analysis revealed that proestrus and estrus females showed non-significant trends for reduced percentages of DOR-labeled neurons containing CRF peptide in comparison to males (proestrus: $p=0.054$; estrus: $p=0.077$). No difference in the percentage of CRF immunoreactive neurons that co-labeled with DOR-ir was observed between groups ($F(3,16) = 1.135$, $p = 0.365$; Table 1).

In stratum oriens of CA3 and CA1, quantitative analysis of immunolabeling between males and normal cycling females revealed no differences in numbers of DOR or CRF immunoreactive neurons. Specifically, females displayed similar numbers of DOR-labeled neurons in comparison to males when analyzed together (CA3: $t=1.920$, d.f.=18, $p=0.370$; CA1: $t=1.686$, d.f.=18, $p=0.109$) or separately (CA3: $F(3,16) = 0.715$, $p = 0.557$; CA1: $F(3,16) = 2.278$, $p = 0.119$; Table 1). Similarly, no significant differences were noted between groups in number of CRF-labeled neurons when cycling females were analyzed together (CA3: $t=0.247$, d.f.=18, $p=0.808$; CA1: $t=0.951$, d.f.=18, $p=0.356$) or separately (CA3: $F(3,16) = 0.510$, $p = 0.681$; CA1: $F(3,16) = 0.349$, $p = 0.791$; Table 1).

We next assessed whether ovarian hormones influenced the percentage of DOR-labeled neurons containing CRF-ir or the percentage of CRF-labeled neurons containing DOR-ir in

SO of CA3 and CA1. Females displayed no differences in the percentage of DOR immunoreactive neurons that co-labeled with CRF-ir (CA3: $t=0.202$, d.f.=18, $p=0.842$; CA1: $t=1.657$, d.f.=18, $p=0.115$) or the percentage of CRF immunoreactive neurons co-labeled with DOR-ir (CA3: $t=1.443$, d.f.=18, $p=0.166$; CA1: $t=0.350$, d.f.=18, $p=0.730$) in comparison to males. Analysis by estrous cycle phase demonstrated no differences in the percentage of DOR immunoreactive neurons that co-labeled with CRF-ir (CA3: $F(3,16) = 0.253$, $p = 0.858$; CA1: $F(3,16) = 0.856$, $p = 0.484$; Table 1) or the percentage of CRF immunoreactive neurons co-labeled with DOR-ir (CA3: $F(3,16) = 1.409$, $p = 0.277$; CA1: $F(3,16) = 0.973$, $p = 0.430$; Table 1) in comparison to males.

DOR colocalization with CRF peptide most frequent in hilar interneurons containing SOM rather than PARV

The distributions of interneurons containing SOM-ir and PARV-ir in the DG were consistent with previous descriptions (reviewed by Freund & Buzsaki, 1996). Namely, SOM immunoreactive somata were numerous in the deep or central hilus, the subgranular zone, and in areas bordering CA3c (Fig. 3C). PARV immunoreactive somata, in contrast, were frequently observed in the granule cell layer, slightly less in the hilus, and rarely in stratum moleculare (Fig. 3G). As aforementioned, DOR colocalization with CRF peptide was frequently observed in the central hilus. Triple label studies revealed that these DOR/CRF dual-labeled somata also contained SOM or PARV (Fig. 3). Quantitative analysis of proestrus females in comparison to males revealed that greater than 70% of DOR/CRF dual-labeled somata also displayed SOM-ir whereas 30% or fewer DOR/CRF dual-labeled somata also displayed PARV-ir (Fig. 4). No significant differences in triple label frequency were observed between groups (SOM: $t=0.083$, d.f.=7, $p=0.936$; PARV: $t=0.702$, d.f.=8, $p=0.503$; Fig. 4).

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

As our laboratory and others have previously demonstrated extensive overlap of endogenous opioid peptides with opioid receptors in the DG (reviewed by (Drake et al., 2007)), we further explored DOR colocalization with CRF peptide in this region at the ultrastructural level using electron microscopy. We noted that CRF-ir was prominent in axon terminals within the central hilus of the DG (Fig. 5). Roughly 6% of CRF-labeled axon terminals in proestrus females contained DOR-SIG particles whereas 15% of CRF-labeled axon terminals in males contained DOR-SIG particles (Table 2). In dual-labeled axonal profiles, DOR-SIG particles were frequently located on or near the plasma membrane (Fig. 5B–D). While no significant difference in frequency of dual-labeled axon terminals was found between males and proestrus females ($t=1.480$, d.f.=8, $p=0.212$; Table 2), proestrus females had an increased total number of CRF-labeled axon terminals (both single and dual-labeled) in comparison to males ($t=4.196$, d.f.=8, $p=0.003$; Table 2) as well as an increased number of single CRF-labeled axon terminals in comparison to males ($t=3.801$, d.f.=8, $p=0.005$; Table 2). In addition, roughly 15–20% of CRF-labeled axon terminals were found either forming synapses with or adjacent to DOR-labeled dendrites in both males and proestrus females (Table 2; Fig. 5C–F). No significant difference in frequency of CRF-labeled axon terminals contacting DOR-labeled dendrites was found between males and proestrus females ($t=0.517$, d.f.=8, $p=0.619$; Table 2).

DISCUSSION

The current study demonstrated that DORs are found on CRF immunoreactive interneurons in the hilar region, CA3, and CA1 of males and normal cycling females (Fig. 6). In the hilar region, specifically, DOR-ir and CRF-ir colocalized preferentially in SOM immunoreactive GABAergic interneurons. At the ultrastructural level, proestrus (high estrogen) females

displayed similar numbers of CRF-labeled axon terminals with DOR-ir but increased numbers of CRF-labeled axon terminals without DOR-ir in comparison to males. These findings suggest that while DORs are anatomically positioned to modulate CRF immunoreactive interneuron activity and CRF peptide release, their ability to exert such regulatory activity may be compromised in females when estrogen levels are high.

Methodological considerations

Although the roles of ovarian hormones have often been studied using 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 to reduce the influence of hormone replacement variables. The cycling females were studied at the proestrus, estrus, and diestrus phases of the rodent estrous cycle; circulating estrogen levels are highest in proestrus, circulating progesterone levels are highest in estrus, 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). Future studies will (1) explore whether the impact of ovarian hormones on CRF-labeled axon terminals persists with age following reproductive senescence and (2) elucidate the precise role of estrogens and progestins using hormone replacement paradigms in ovariectomized rodents.

DORs colocalize with CRF peptide in hippocampal interneurons

Consistent with prior reports in males (Swanson et al., 1983; Commons & Milner, 1996; Commons & Milner, 1997; Yan et al., 1998; Chen et al., 2004b; Williams et al., 2011), DOR immunoreactive neurons and CRF immunoreactive neurons were found in the dentate hilar region and stratum oriens of CA3 and CA1 in females at all phases of the rodent estrous cycle. In all regions examined in the current study, DOR-ir was found in over 50 % of CRF immunoreactive neurons and no significant differences in DOR-labeling of CRF immunoreactive neurons was observed between groups. Triple-label studies with interneuron subtype markers somatostatin and parvalbumin in the hilar region of the DG revealed that a substantial proportion of interneurons containing DOR- and CRF-ir also demonstrated SOM-ir whereas a smaller percentage of dual labeled interneurons also displayed PARV-ir. Extensive work characterizing the morphological and neurochemical properties of GABAergic interneurons in the HF (reviewed by (Freund & Buzsaki, 1996) and interneurons labeled with DOR-ir or CRF-ir in particular (Commons & Milner, 1996; Smith et al., 1997; Drake et al., 2007; Williams et al., 2011) would suggest that the majority of DOR/CRF – containing interneurons are hilar perforant path (HIPP) associated cells. HIPP cells are characterized by somata and dendritic arbors largely restricted to the hilar region as well as extensive axonal arborization in the outer two-thirds of the molecular layer. Notably, HIPP cells contain both somatostatin and neuropeptide Y (NPY) (Freund & Buzsaki, 1996). Thus, the findings of the present study of DOR colocalization with CRF peptide in SOM immunoreactive neurons (1) are in agreement with previous reports in adult animals indicating DOR (Commons & Milner, 1996; Williams et al., 2011) and CRF peptide (Smith et al., 1997) localization to interneurons with SOM- or NPY-ir and (2) suggest that these interneurons project to the outer molecular layer for termination postsynaptically on granule cells or presynaptically on perforant path fibers to ultimately regulate granule cell input. As somatostatin-containing interneurons provide feedback inhibition to distal granule

cell dendrites to limit sustained firing, DOR/CRF labeled interneurons likely regulate late-persistent inhibition of granule cells (Spruston, 2008). However, a small percentage of DOR/CRF labeled cells also contained PARV-ir, a feature prominent in basket or chandelier cells which project to the somata and initial axon segments of granule cells (Freund & Buzsaki, 1996) to inhibit the generation of action potentials (Stumm et al., 2004). Thus, a role for these dual labeled interneurons in also regulating granule cell output by modulating feedforward inhibition cannot be excluded. Prior ultrastructural studies noting CRF-labeled presynaptic terminals forming synapses on axon initial segments of principal cells in the immature male rat would support this possibility (Yan et al., 1998). Interestingly, PARV immunoreactive interneurons in the hilar region preferentially express mu opioid receptors (MORs) and MOR trafficking in these interneurons is modulated by estrogen (Drake & Milner, 2006; Torres-Reveron et al., 2009b), suggesting the additional prospect of differential CRF immunoreactive interneuron regulation by MORs in males and females. Future studies will further explore the relationship between MORs and CRF peptide in the HF and address whether opioid receptor colocalization with CRF peptide in the dorsal hippocampus also extends to the ventral hippocampus.

Electrophysiological studies in the DG reveal roles for both the DOR and CRF peptide in modulating long-term potentiation (LTP), a candidate mechanism of memory storage in the mammalian brain (Squire, 1992; Bliss & Collingridge, 1993), at the perforant path – granule cell synapse (Bramham et al., 1991; Wang et al., 1998). For example, DOR antagonist administration blocked LTP induction and lateral perforant path synaptic transmission (Bramham et al., 1991). Importantly, this effect was largely attributed to DOR blockade on interneurons projecting to lateral perforant path synapses on the distal dendrites of granule cells rather than DOR inhibition on granule cells themselves as granule cells also demonstrate DOR-ir (Piguet & North, 1993; Bramham & Sarvey, 1996; Commons & Milner, 1996; Williams et al., 2011). In contrast, CRF injection produced a slow onset, long-lasting excitation of granule cell neurons that added to the effect of tetany, suggesting a role for CRF in LTP maintenance (Wang et al., 1998; Wang et al., 2000). Thus, the DOR and CRF peptide appear to regulate different phases of LTP generation at the perforant path-granule cell synapse, namely the early induction phase (DOR) and the late maintenance phase (CRF) (Bliss & Collingridge, 1993; Huang & Kandel, 1994; Huang et al., 1994). The appearance of both DORs and CRF peptide in the same hilar interneuron population therefore poses interesting functional consequences for LTP modulation. DOR activation, through its principal endogenous ligand enkephalin, has been shown to broadly hyperpolarize the interneuron soma to prevent the action potential generation and signal propagation necessary for neurotransmitter release as well as focally control neurotransmitter release from the axon terminal at inhibitory synapses (Cohen et al., 1992). The findings of the present study suggest that, in both males and females, DORs are well positioned to exert influences on CRF immunoreactive somata and, though less frequently, axon terminals. Whether opioid receptor activation causes presynaptic inhibition of CRF peptide release in the hippocampus, as it does for release of GABA, serotonin, norepinephrine, and acetylcholine (Drake et al., 2007), will need to be experimentally addressed in future studies.

Ovarian hormones influence the number of CRF immunoreactive axon terminals in the hilar region

Although no significant differences between normal cycling females and males were noted for DOR localization to CRF immunoreactive somata or axon terminals, proestrus (high estrogen) females revealed an increased number of CRF-labeled axon terminals without DOR-ir in the hilar region of the DG. Such increased sampling of CRF-labeled axon terminals in proestrus females would suggest that CRF-labeled axon terminals either

increase in size or number. While future studies will investigate whether ovarian hormones influence CRF-labeled axon terminal size using ultrastructural approaches given the established role of CRF in dendritic growth and pruning (Chen et al., 2004a; Chen et al., 2008b; Chen et al., 2010), current evidence would instead support ovarian hormone modulation of the number of axon terminals producing CRF peptide. Such ovarian hormone regulation of CRF levels is consistent with previous reports of estrogen modulation of CRF peptide levels in other areas of the brain (Vamvakopoulos & Chrousos, 1993; Vamvakopoulos & Chrousos, 1994). Specifically, *in vitro* and *in vivo* studies demonstrate that estradiol, via estrogen receptors (ERs), increases CRF transcription and peptide levels in hypothalamic, and potentially extrahypothalamic, regions (Patchev et al., 1995; Miller et al., 2004; Lalmansingh & Uht, 2008). Moreover, CRF mRNA is selectively elevated in the hypothalamus of proestrus females, but not during other phases of the rodent estrous cycle (Bohler, Jr. et al., 1990). The CRF gene contains two half-palindromic estrogen response elements, AP-1 sites, and cAMP response elements, all of which are mediators of ER stimulated transcription (Miller et al., 2004; Chen et al., 2008a). In the hippocampus, ERs are found at both nuclear and extranuclear sites on interneuron somata as well as terminals (Milner et al., 2001; Milner et al., 2005), suggesting the potential for increased CRF transcription and peptide translation when estrogen levels are high and accounting for the increased number of CRF immunoreactive terminals observed in proestrus females. Importantly, as these axon terminals lack DOR-ir, the increased amount of CRF peptide available for release in females when estrogen levels are high would be unsusceptible to DOR regulation.

CRF immunoreactive axon terminals in the hilar region often formed synaptic contacts with or were immediately adjacent to non-spiny dendrites that occasionally contained DOR-ir but no frequency differences were observed between males and proestrus females. These non-spiny dendrites were contacted by numerous terminals, some of which contained DOR-ir, in the plane of section analyzed. As these are ultrastructural features previously reported for dendritic profiles of NPY- and SOM-containing interneurons (Milner & Bacon, 1989; Deller & Leranth, 1990; Milner & Veznedaroglu, 1992), it is likely that the observed CRF immunoreactive axon terminals are synapsing on the same HIPP cells that demonstrated DOR/CRF/SOM-ir in the aforementioned immunofluorescence studies. While the possibility of CRF activation of GABAergic interneurons has been hinted at (Smith et al., 1997; Wang et al., 1998) but not experimentally addressed, the current ultrastructural findings would support the feasibility of such modulation. Future dual labeling immunoelectron microscopy studies will specifically address (1) whether the CRF receptor is present on this GABAergic interneuron population to add support to potential CRF regulation of interneuron activity and (2) where the CRF positive axon terminals found in the central hilus originate as they are unlikely to arise from HIPP or basket cells but may instead project from sites outside the hippocampus.

Clinical implications

Our findings provide new anatomical evidence of DOR colocalization with CRF peptide in hippocampal interneurons, supporting and extending the growing body of literature investigating the interaction between endogenous opioid systems and CRF. Specifically, they suggest that DORs are well positioned to influence CRF release from interneurons of males and normal cycling females. Such modulation is particularly feasible in somatostatin-containing interneurons that preferentially regulate perforant path inputs to granule cells. In addition, the number of CRF immunoreactive axon terminals with releasable CRF peptide upon appropriate stimulation, like stress (Chen et al., 2004b), is greater in high estrogen females than in males. Such ovarian hormone modulation of CRF peptide levels in hilar axon terminals is consistent with compelling evidence of sexual dimorphism in stress

reactivity and the increased prevalence of stress-related psychiatric disorders in women (Kessler et al., 1993; Kessler et al., 1995; Marcus et al., 2005; Marcus et al., 2008). Reports indicate that CRF precipitates reinstatement of drug-seeking behavior in animal models of addiction (Shaham et al., 1997; Brown et al., 2009; Shalev et al., 2010) and excessive elevation of hippocampal CRF peptide levels may contribute to abnormal neuronal excitation (i.e., seizures) (Baram & Hatalski, 1998). Thus, tight regulation of CRF levels in and release from hippocampal neurons may be important for both normal and pathological activation of the hippocampal circuit. Further research focused on opioid regulation of hippocampal CRF activity, particularly by DORs, may therefore highlight new pharmacological targets to counteract heightened CRF sensitivity in women.

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Fig. 1.

Immunofluorescent images illustrating colocalization of DOR (A, D, G, J) with CRF peptide (B, E, H, K) in the hilar (Hil) region of the dentate gyrus (DG). Dual-labeled cells in the hilus of the DG appear yellow and are indicated by white arrows in representative merged images taken from a male (C), diestrus female (F), proestrus female (I), and estrus female (L). DOR-labeling is red. CRF-labeling is green. Scale bars = 100 μ m.

Fig. 2.

Immunofluorescent images illustrating colocalization of DOR (A, D, G, J) with CRF peptide (B, E, H, K) in stratum oriens (SO) of CA1. Dual-labeled cells in SO of CA1 appear yellow and are indicated by white arrows in representative merged images taken from a male (C), diestrus female (F), proestrus female (I), and estrus female (L). DOR-labeling is red. CRF-labeling is green. PCL = pyramidal cell layer. Scale bars = 100 μ m.

Fig. 3.

Immunofluorescent images illustrating colocalization of DOR (A, E) and CRF peptide (B, F) with SOM (C) or PARV (G) in the hilar (Hil) region of the dentate gyrus. (D) Triple-labeled cells with DOR, CRF, and SOM in the hilus are indicated by white arrows in representative merged images taken from a proestrus female, where A–C are unmerged images of D. (H) Triple-labeled cells with DOR, CRF, and PARV in the hilus are indicated by white arrows in representative merged images taken from a proestrus female, where E–G are unmerged images of H. DOR-labeling is red. CRF-labeling is green. SOM- and PARV-labeling is orange. Scale bars = 100 μ m.

Fig. 4. Percent of DOR/CRF dual-labeled neurons co-labeled with SOM and PARV in the hilar region of the DG. (A) Over 70% of DOR/CRF dual-labeled neurons co-labeled with SOM in both proestrus females and males. (B) 30% or fewer of DOR/CRF dual-labeled neurons co-labeled with PARV in both proestrus females and males.

Fig. 5. DOR-labeling is found in CRF immunoreactive axon terminals in the hilar region of the DG. (A–C) Representative electron micrographs taken from a female demonstrate CRF-ir in single and dual-labeled axon terminal profiles. In axon terminals, DOR-SIG particles (red arrowheads) are found along or near the plasma membrane or affiliated with small clear synaptic vesicles in the cytoplasm. (D–E) CRF-labeled axon terminals, dual and single-labeled, in a proestrus female are also found in contact with DOR-labeled dendrites (blue arrows indicate DOR-SIG particles in profiles without CRF-ir). (F–G) CRF-labeled axon terminals in a male are similarly found in contact with DOR-labeled dendrites that in some

cases also receive contacts from DOR-labeled axon terminals. den = dendrite, ter = CRF-labeled axon terminal, ut = unlabeled axon terminal. Scale bars = 500 nm.

Fig. 6.

DORs colocalize with CRF-labeled interneuron somata and axon terminals in the dentate hilus. In both males and proestrus females, the majority of dual-labeled interneurons contain somatostatin and project to the distal dendrites of granule cells to regulate perforant path input. A smaller fraction of dual-labeled neurons contain parvalbumin and project to granule cell somata to regulate granule cell output. Notably, PARV-containing interneurons also contain mu opioid receptors (MORs). CRF-labeled axon terminals also make contact with DOR/CRF dual labeled interneurons and occasionally contain DORs. In contrast to males, proestrus females (schematized in the figure) have an increased number of CRF-labeled axon terminals in the hilar region that do not contain DORs. Thus, while DORs are

anatomically positioned to modulate CRF immunoreactive interneuron activity and CRF peptide release from axon terminals in males, their ability to exert such regulatory activity at axon terminals may be compromised in females when estrogen levels are high. GCL = granule cell layer.

Table 1

Average number and percentage of DOR- and CRF-labeled neurons in the hippocampus of males and normal cycling females

Region	Animal	Dual-labeled neurons	DOR-labeled neurons	DOR neurons with CRF (%)	CRF-labeled neurons	CRF neurons with DOR (%)
Hilus	Pro (n=5)	3.4±0.6	10.6±1.1	30.80±3.0 [^]	6.8±1.3	50.88±5.6
	Est (n=5)	3.0±0.2	9.4±0.5	32.04±2.8 [^]	6.1±0.7	50.31±3.3
	Di (n=5)	3.5±0.5	9.1±1.0	39.15±5.4	6.1±1.4	63.46±9.3
	Male (n=5)	3.7±0.3	7.9±0.6	48.98±6.1	6.6±0.5	57.00±2.2
CA3 SO	Pro	1.5±0.4	2.7±0.4	52.75±6.2	2.6±0.5	54.47±4.7
	Est	1.6±0.4	2.6±0.5	59.23±3.5	2.5±0.4	61.74±7.2
	Di	1.1±0.4	2.0±0.5	53.84±7.9	2.0±0.4	49.21±6.0
	Male	1.6±0.2	2.9±0.5	56.57±4.6	2.4±0.6	65.33±6.2
CA1 SO	Pro	2.4±0.4	4.5±0.5	52.51±3.6	3.3±0.6	75.67±5.8
	Est	2.0±0.4	3.5±0.6	55.59±8.3	2.7±0.5	72.24±4.7
	Di	2.0±0.5	3.5±0.4	53.89±8.9	3.0±0.6	64.04±6.4
	Male	1.9±0.1	3.0±0.2	65.79±3.1	2.7±0.1	71.94±1.6

Neuron counts presented as Mean ± SEM.

Pro=proestrus, Est=estrus, Di=diestrus

[^] p < 0.10 in comparison to males

Table 2

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

Group	Total CRF-labeled	Single-labeled with CRF	Dual-labeled	Contacting DOR-labeled dendrite
Proestrus (n=5)	28.0±1.9 *	26.4±4.0 * (94%)	1.6±0.2 (6%)	3.4±1.2 (14%)
Male (n=5)	15.0±2.4	12.8±3.1 (85%)	2.2±0.9 (15%)	2.8±1.2 (18%)

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

* p < 0.05