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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.

Key words: opioids, stress, hormones, estrogen, parvalbumin, somatostatin.

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Abbreviations: CRF, corticotropin releasing factor; DG, dentate gyrus; Di, diestrus; DOR, delta opioid receptor; Est, estrus; HF, hippocampal formation; Hil, Hilar; HIPP, hilar perforant path; IgG, immunoglobulin; ir, immunoreactivity; LTP, long-term potentiation; MORs, mu opioid receptors; NPY, neuropeptide Y; PARV, parvalbumin; PB, phosphate buffer; PBS, phosphate-buffered saline; PCL, pyramidal cell layer; Pro, proestrus; SIG, silver-intensified immunogold; SO, stratum oriens; SOM, somatostatin; TS, tris–buffered saline.

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; 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 and Everitt, 1999; Berke and Hyman, 2000; Hyman and Malenka, 2001; Nestler, 2001, 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 (Pavlides et al., 1996; de Quervain et al., 1998; McEwen, 1999; Kim and 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 and 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) are commonly found in somatostatin (SOM)/neuropeptide Y containing GABAergic interneurons in hippocampal lamina (Commons and Milner, 1996, 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, 2001, 2004b). Functionally, both DORs (Piguet and North, 1993; Bramham and 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, 2009; Brown et al., 2009; Shalev et al., 2010) and are susceptible to modulation by ovarian hormones (Vamvakopoulos and Chrousos, 1993, 1994; Vathy et al., 2000; Wilson et al., 2002;

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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 and 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, USA) 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 1 week after which estrous cycle phase was determined using vaginal smear cytology (Turner and 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, USA). 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 have 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 and Milner, 1996) as well as comparable immunolabeling to a commercially available rabbit polyclonal DOR antisera (Chemicon, incorportated into Millipore, Billerica, MA, USA) (Commons and Milner, 1996, 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, USA). 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 and Nilaver, 1987) and labeled the same pattern of somata in immunolabeling studies as a commercially available rabbit polyclonal SOM antisera (Diasorin, Stillwater, MN, USA) (Drake and 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 0.1 M phosphate buffer (PB) (pH 7.6) (Milner and 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., Warren, MI, USA), and postfixed for 30 min in 2% paraformaldehyde in 0.1 M phosphate buffer. The brains were sectioned (40 μm thick) on a Leica Vibratome (Nussloch, Germany) and stored in cryoprotectant (30% sucrose and 30% ethylene glycol in 0.1 M 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 min to neutralize free aldehydes and rinsed in PB.

Immunofluorescence labeling and analysis

For dual label studies, sections of each group were rinsed in 0.05 M phosphate-buffered saline (PBS; pH 7.4) and incubated in: (1) 0.1% triton (TX)/10% normal goat serum (NGS) in PBS for 1 h; (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 h at 4 °C; and (3) a cocktail of goat anti-guinea pig Cy5 immunoglobulin (IgG) (1:600; Jackson ImmunoResearch Labs, Inc., West Grove, PA, USA) and goat anti-rabbit fluorscein isothiocyanate (FITC) IgG (1:600; Invitrogen, Carlsbad, CA, USA) in 0.1% NGS in PBS for 1 h 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 Harleco, Gibbstown, NJ, USA). 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-scan-