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Subcellular plasticity of the corticotropin-releasing factor (CRF) receptor in dendrites of the mouse bed nucleus of the stria terminalis following chronic opiate exposure

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Abstract

Chronic opiate administration alters the expression levels of the stress-responsive peptide, corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis (BNST). This brain region contains CRF receptors that drive drug-seeking behavior exacerbated by stress. We used electron microscopy to quantitatively compare immunolabeling of the CRF receptor (CRFr) and CRF in the anterolateral BNST (BSTal) of mice injected with saline or morphine in escalating doses for 14 days. We also compared the results with those in non-injected control mice. The tissue was processed for CRFr immunogold and CRF immunoperoxidase labeling. The non-injected controls had a significantly lower plasmalemmal density of CRFr immunogold particles in dendrites compared with mice receiving saline, but not those receiving morphine, injections. Compared with saline, however, mice receiving chronic morphine showed a significantly lower plasmalemmal, and greater cytoplasmic, density of CRFr immunogold in dendrites. Within the cytoplasmic compartment of somata and dendrites of the BSTal, the proportion of CRFr gold particles associated with mitochondria was three times as great in mice receiving morphine compared with saline. This subcellular distribution is consistent with morphine-, and CRFr-associated modulation of intracellular calcium release or oxidative stress. The between-group changes occurred without effect on the total number of dendritic CRFr immunogold particles, suggesting that chronic morphine enhances internalization or decreases delivery of the CRFr to the plasma membrane, a trafficking effect that is also affected by the stress of daily injections. In contrast, saline and morphine treatment groups showed no significant differences in the total number of CRF-immunoreactive axon terminals, or the frequency with which these terminals contacted CRFr-containing dendrites. This suggests that morphine does not influence axonal availability of CRF in the BSTal. The results have important implications for drug-associated adaptations in brain stress systems that may contribute to the motivation to continue drug use during dependence.

Keywords

stress; trafficking; morphine; addiction; relapse; mitochondria

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Opiate dependence increases vulnerability to stress (Kreek & Koob, 1998; Blatchford et al, 2005; Koob et al, 2008). Opiate-induced adaptations in the activity of neurohormonal systems involved in the stress response may, in part, underlie this vulnerability (Buckingham & Cooper, 1984; el Daly, 1996; Kreek and Koob, 1998; Houshyar et al, 2004). Far less, however, is known regarding the effects of opiates on the brain corticotropin-releasing factor (CRF) system, which significantly contributes to negative affective states in normal animals (Heinrichs & Koob, 2004; Sahuque et al, 2006; Jaferi & Bhatnagar, 2007; Keen-Rhinehart et al, 2008; Lee et al, 2008), and in animal models of opiate addiction (Contarino & Papaleo, 2005; Wang et al, 2006; Skelton et al, 2007).

Chronic opiate administration alters the stress-induced elevation of CRF mRNA levels in the bed nucleus of the stria terminalis (BNST) (Shalev et al, 2001), a region where CRF receptor (CRFr) activation elicits relapse to drug-seeking behavior during stress (Erb & Stewart, 1999; Erb et al, 2001; Wang et al, 2006). The BNST is part of a macrostructure of several basal forebrain regions collectively termed the “extended amygdala,” and implicated in many of the motivational aspects of drug dependence (Alheid & Heimer, 1988; Koob, 2003; Walker & Davis, 2008). The lateral region of the BNST is of particular interest with regard to opiate dependence, since morphine withdrawal increases the number of c-Fos-immunoreactive neurons along a lateral to medial decreasing gradient (Veinante et al, 2003). In addition, chronic morphine administration selectively strengthens a population of excitatory fibers entering the lateral region of the anterior BNST (BSTal). These excitatory inputs form synapses on BSTal neurons projecting to the reward-associated ventral tegmental area (VTA) (Dumont et al, 2008), suggesting a subregion-specific effect of morphine within the BNST.

The observed alterations in CRF synthesis and stress responsivity following opiate administration suggest potential opiate-induced changes in the availability of the CRFr in the BSTal (Buckingham & Cooper, 1984; Suemaru et al, 1985; Buckingham & Cooper, 1986; Buckingham & Cooper, 1987; Nikolarakis et al, 1989; el Daly, 1996; Kreek & Koob, 1998). Although CRF has been strongly linked to drug withdrawal (Iredale et al, 2000; Veinante et al, 2003; Contarino & Papaleo, 2005), changes in the BSTal CRF system during morphine exposure are of interest because they may contribute to the motivation to continue drug use during dependence. In the present study, we hypothesized that chronic morphine administration alters the prevalence and/or subcellular distribution of CRFr immunogold in BSTal neurons. To test this hypothesis, we used electron microscopy to quantitatively compare the immunogold-labeling of the CRFr in the BSTal of mice injected intermittently with saline or morphine in escalating doses for 14 days (3 injections/day). Comparisons were also made with control mice that did not receive injections. The tissue for analysis of CRFr gold particle distributions was co-processed for immunoperoxidase labeling of CRF.

Experimental Procedures

Animals

All experiments were performed in accordance with NIH regulations of animal care, and were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. Nine adult, male C57BL6 mice (Jackson Laboratories, Bar Harbor, ME) were group-housed in a quiet facility on a reverse 12-hour light/dark cycle and were given unlimited access to food and water. All efforts were made to minimize suffering and the number of mice used for experiments.

Drug injections

We examined three groups of mice (morphine-injected, saline-injected, and non-injected), with primary comparisons made between morphine- and saline-injected mice. Mice were

administered three daily intraperitoneal injections of saline or morphine sulfate (Sigma, St. Louis, MO) at 10:00 AM, 4:00 PM and 10:00 PM for 14 consecutive days at escalating doses. These doses started at 2.5 mg/kg (cumulative dose per day) and reached 20 mg/kg by day 14, a paradigm based on prior animal self-administration data (Kruzich et al, 2003; Glass et al, 2005) that mimicks human opiate addiction (Kreek et al, 2004). On day 14, mice were perfused one hour following the last injection to avoid withdrawal.

Tissue preparation

Prior to perfusion, mice were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). Blood samples were collected from each mouse, just prior to perfusion, to determine plasma levels of corticosterone. Their brain tissue was fixed by vascular perfusion through the left ventricle of the heart with sequential delivery of (1) 5 ml of heparin-saline (1000 U/ml), (2) 30 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4), and (3) 100 ml of 2% paraformaldehyde in PB. Brains were removed from the cranium, post-fixed with 2% paraformaldehyde in 0.1M PB for 30min, and then transferred to PB. These brains were then cut coronally into 40 μ m sections on a Vibratome (Leica, Deerfield, IL). The coronal sections were collected through the rostrocaudal extent of the BNST based on the mouse brain atlas by Hof et al (2000). Rostrocaudally-matched pairs of sections from morphine- and saline-injected mice were simultaneously processed for immunogold-silver labeling of the CRFr and immunoperoxidase labeling of CRF.

Antisera

For immunolabeling of the CRFr, we used a rabbit polyclonal antiserum raised against the epitope corresponding to amino acids 230–444 mapping at the C-terminus of the CRF-R1 of human origin (CRF-R1/R2 (H-215): sc-5543 Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The specificity of this antiserum for the CRFr has previously been demonstrated by the diminished immunoreactivity in brain tissue processed after prior adsorption with the antigenic peptide (Treweek et al, 2009), and in brain sections of CRFr-1 (–/–) knockout compared with CRFr-1 (+/+) wildtype mice (Reyes et al, 2008; Treweek et al, 2009).

For immunolabeling of CRF, we used a guinea pig polyclonal antiserum raised against human/rat CRF (T-5007, Bachem Peninsula; San Carlos, CA). The specificity of this antiserum for CRF has been shown by the diminished immunoreactivity in brain sections of CRF (–/–) knockout mice compared to CRF (+/+) wildtype mice, as well as in brain sections immunolabeled with antiserum pre-adsorbed with the antigenic peptide (Treweek et al, 2009).

Immunocytochemical Labeling

Brain sections from mice receiving chronic saline or morphine administration were co-processed for dual immunogold-silver and immunoperoxidase labeling of the CRFr and CRF (Chan et al, 1990; Drake & Milner, 2002; Pickel et al, 2004; Hara & Pickel, 2005; Lane et al, 2008; Jaferi & Pickel, 2009). For this dual-labeling, the vibratome sections were placed in 1% sodium borohydride in 0.1 M PB for 30 minutes to remove excess aldehydes, and then washed thoroughly in 0.1 M PB wash. To enhance the penetration of tissue by immunoreagents, the sections were soaked in a cryoprotectant solution (25% sucrose and 3.5% glycerol in 0.05M PB) for 15 minutes, and rapidly freeze-thawed by sequential immersion in liquid chlorodifluoromethane (Freon, Refron Inc., NY), liquid nitrogen, and 0.1 M PB at room temperature. The sections were then rinsed in 0.1 M Tris-buffered saline (TBS). To reduce non-specific binding of the antisera, the sections were incubated in 0.5% bovine serum albumin (BSA) in 0.1 M TBS, pH 7.6, for 30 minutes before placement in the primary antisera solution. The sections were incubated for 48 hours at 4°C in a solution containing both primary antisera (1:100 rabbit anti-CRFr for immunogold-silver labeling and 1:2000 guinea pig anti-CRF for

immunoperoxidase labeling.) All antisera dilutions were prepared in 0.1% BSA in 0.1 M TBS. Incubations with primary as well as secondary antisera were done with shaking on a rotator (OS-500; VWR, West Chester, PA).

For immunoperoxidase labeling of CRF, the sections were rinsed in 0.1 M TBS and then placed for 30 min in a 1:400 dilution of biotinylated anti-guinea pig. These were then washed in 0.1 M TBS and incubated for 30 min in avidin-biotin peroxidase complex (Vectastain Elite Kit, Vector Labs, Burlingame, CA). This incubation was followed by a wash in 0.1 M TBS, and reaction for 6 min in 0.022% 3,3'-diaminobenzidine (DAB, Aldrich, Milwaukee, WI) and 0.003% hydrogen peroxide in 0.1 M TBS.

For dual detection of antigens, the sections were rinsed in 0.01M PBS (pH 7.4) after the DAB reaction, and blocked in 0.8% BSA and 0.1% gelatin in PBS for 10 minutes. The sections were subsequently incubated with a 1:50 dilution of anti-rabbit IgG conjugated with one nm colloidal gold (Amersham, Arlington Heights, IL) for 2 hours at room temperature. The sections were washed several times with 0.1 M TBS. To aid in the binding of gold particles conjugated to IgG, the sections were incubated in 2% glutaraldehyde for 10 minutes. Silver intensification was performed using the IntenS-EM kit (Amersham) with a 6 minute reaction in the silver solution at room temperature. The sections were post-fixed in 2% osmium tetroxide in 0.1 M PB for 60 minutes, washed in PB, and dehydrated by passing through an increasingly concentrated series of ethanol solutions, followed by 100% propylene oxide. The sections were incubated overnight in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences, Fort Washington, PA), and then transferred to 100% Epon for 2–3 hours before flat-embedding between sheets of Aclar plastic (Electron Microscopy Sciences, Fort Washington, PA). These flat-embedded sections were viewed with a light microscope to select immunolabeled portions of the dorsal area of the BSTal at Bregma 0.2 mm for collection of ultra-thin sections (Hof et al, 2000). Ultra-thin sections of approximately 70nm thickness were cut from the superficial surface of the section using a diamond knife (Diatome, Fort Washington, PA) on an ultratome (NOVA LKV-Productor AB, Bromma, Sweden). The thin sections were collected on 400-mesh copper grids (Electron Microscopy Sciences), and counterstained with uranyl acetate and lead citrate (Reynolds, 1963). These grids were rinsed and allowed to air dry before viewing at 60 kV with a Philips CM10 transmission electron microscope (FEI, Hillsboro, OR). Those areas showing immunolabeling of CRFr and/or CRF were then magnified and captured as digital images using AMT Advantage HR/HR-B CCD Camera System (Advanced Microscopy Techniques, Danvers, MA).

Electron Microscopic Analysis

Ultra-thin sections of the BSTal from 15 vibratome sections (six sections from three saline-injected mice, six sections from three morphine-injected mice, and three sections from three non-injected mice) were used for electron microscopic analysis. Micrographs containing CRFr and CRF immunoreactivity at magnifications of 13500X to 25000X were analyzed in a total tissue area of 17,716 μm^2 in the BSTal. We only examined tissue at the surface of vibratome sections at the Epon-tissue interface in order to avoid differences in labeling due to differential penetration of reagents. All electron microscopic images were imported into an IBM computer, where Adobe Photoshop (version 7.0.1, Adobe Systems Inc., Mountain View, CA) was used only to sharpen and enhance contrast as needed. These images were then imported into PowerPoint (Microsoft Office, 2007) which was used to assemble figures and add lettering.

We classified profiles containing CRFr and/or CRF immunoreactivity as neuronal processes (dendrites, dendritic spines, axons, axon terminals) based on criteria described in Peters et al (1991). Immunoperoxidase labeling was regarded as positive when the electron-dense reaction product in individual profiles was greater than that seen in other morphologically similar profiles in the neuropil. Profiles were considered to be immunogold-labeled when they (1)

contained one or more gold particles, and (2) were located in a neuropil in which no presumed extraneous particles were seen in a comparable area of neuropil. This approach was validated by examining spurious gold particles overlying myelin, a structure not known to express receptors. For example, out of a total of 250 myelinated axons observed in 9,643 μm^2 in the BSTal, none displayed CRFr immunogold particles. We subsequently quantified the number of CRFr- and/or CRF-labeled neuronal profiles in the BSTal, as well as the number and types of synaptic associations and appositions/non-synaptic contacts. Asymmetric synapses were recognized by thick postsynaptic densities while symmetric synapses were recognized by thin pre- and postsynaptic specializations. Appositions were recognized by closely spaced, parallel plasma membranes that lacked discernible synaptic densities.

The majority of CRFr-labeling was observed on the plasma membrane or within the cytoplasm of dendritic profiles. Therefore, quantitative analysis of dendritic CRFr labeling was carried out to determine the density of the CRFr gold–silver deposits in the plasmalemmal and cytoplasmic compartments. Gold particles in contact with any portion of the surface membrane were considered to be plasmalemmal while intracellular gold particles not in contact with the surface membrane were classified as cytoplasmic. Out of the cytoplasmic CRFr labeling, we further measured the association of CRFr with particular subcellular organelles, namely mitochondria. CRFr gold particles in contact with any portion of the surface or membranes of mitochondria were classified as mitochondrial labeling.

Morphological parameters that were used as indirect measures of dendritic size, including surface area (perimeter), cross-sectional area, and minor axis length of dendrites were measured using Microcomputer Imaging Device software (MCID, Imaging Research Inc., Ontario, Canada). Our analysis included transversely-cut dendritic profiles, and excluded longitudinally-cut dendrites with high plasma membrane to surface area ratios. The parameters used for statistical comparisons were: 1) the number of plasmalemmal CRFr gold particles in a dendritic profile/dendritic perimeter, 2) the number of cytoplasmic CRFr gold particles in a dendritic profile/dendritic cross-sectional area, 3) the number of total CRFr gold particles in a dendritic profile/dendritic cross-sectional area, and 4) the number of mitochondrial CRFr gold particles on a dendritic profile/total number of cytoplasmic CRFr gold particles on a dendritic profile. In order to compare similar structures across groups, cluster analysis by dendritic size was performed to statistically divide CRFr-labeled dendrites from morphine and saline groups into small, medium and large subcategories using JMP 5.0.1 statistical software (SAS Institute Inc., NC, USA). In general, small, medium and large dendrites in the BSTal correspond respectively to distal, intermediate, and proximal portions on the dendritic tree, although there also may be some size variations in dendrites belonging to neurotransmitter- and/or function-specific neurons. Data were analyzed by Analysis of Variance, and differences in means were analyzed by Tukey's post hoc test.

Corticosterone Radioimmunoassay

All blood samples were collected near the start of the light (inactive) phase, corresponding to the trough of the diurnal rhythm in corticosterone secretion (Dallman et al, 1987; Barriga et al, 2001; Kim et al, 2008). Blood was collected from the heart in EDTA-coated microcentrifuge tubes immediately prior to perfusion, and one hour following the last saline or morphine injection. Blood samples were kept on ice until centrifuged. After centrifugation, the plasma was aliquoted and kept frozen at -20°C until assay. Plasma corticosterone was measured using a rat/mouse corticosterone ^{125}I kit (MP Biomedicals, Irvine, CA).

Results

Distribution of CRFr and CRF in neuronal profiles

Many somatodendritic and axonal profiles of the mouse BSTal contained immunogold labeling of the CRFr. CRFr immunogold particles in dendritic profiles were observed along the plasma membrane as well as in the cytoplasm near intracellular organelles such as mitochondria, and endomembranes, including rough or smooth endoplasmic reticulum (Fig. 2A, 2B). Out of all CRFr-labeled neuronal (dendritic and axonal) profiles, 78% (849/1089) were dendritic and 22% (240/1089) were axonal. CRFr labeling was observed in 353 small (0.01 to 0.67 μm), 386 medium (0.68 to 1.21 μm), and 110 large (1.22 to 2.38 μm) dendrites within the BSTal.

Immunoperoxidase labeling of CRF was widely distributed in somatodendritic and axonal profiles throughout the BSTal (Fig. 2). CRF peroxidase was typically diffusely distributed in the cytoplasm and along the plasma membrane in dendritic and axonal profiles, and was predominantly localized in axon terminals (Fig. 2A–2C). Out of all CRF-containing neuronal profiles, 39% (343/883) were dendritic and 61% (540/883) were axonal. CRF-labeled axon terminals were often apposed to dendrites that were immunogold-labeled for the CRFr ($n=124$ appositions) (Fig. 2A, 2B).

A small population of neuronal profiles in the BSTal contained both CRF and the CRFr. Specifically, 6% (47/849) of all CRFr-labeled dendrites, and 8% (18/240) of all CRFr-containing terminals, co-expressed CRF within the BSTal.

Equal prevalence of dendritic CRFr and association with CRF in saline and morphine groups

Mice receiving chronic administration of morphine or saline did not significantly differ in the number of CRFr-expressing dendrites (140 ± 13 for saline, 143 ± 8 for morphine) or CRF-immunoreactive axon terminals (96 ± 2 for saline; 84 ± 23 for morphine). In the BSTal of either saline- (Fig. 2A) or morphine-administered mice (Fig. 2B), dendrites that were immunogold-labeled for the CRFr were apposed to CRF-immunoreactive axon terminals. Similar numbers of CRF-immunoreactive terminals were apposed to CRFr-containing dendrites in saline (21 ± 3) and morphine (20 ± 5) treatment groups.

As compared with saline, chronic administration of morphine did not significantly affect the frequency with which the CRFr was co-distributed with CRF (Fig. 2D). Immunoperoxidase labeling of CRF was seen in $4\% \pm 0.03$ (5/143) of the CRFr-containing dendritic profiles in the morphine-injected group, and in $7\% \pm 0.01$ (10/140) of those in the saline-injected group (Fig. 2D). CRFr-labeled dendrites in the BSTal of either saline- or morphine-administered groups received symmetric, inhibitory-type (Fig. 2C) or asymmetric, excitatory-type synapses from unlabeled terminals (Fig. 3A, 3D).

Differential effect of saline and morphine on distribution of dendritic CRFr

The relatively small numbers of dendritic profiles co-expressing CRF and the CRFr in the BSTal precluded between-group quantitative analyses of CRFr-containing dendrites with CRF separately from those without CRF. Therefore, the following analysis refers to all CRFr-labeled dendritic profiles with *and* without CRF immunoreactivity. In BSTal dendrites of mice injected with saline, CRFr immunogold particles were commonly observed contacting the plasma membrane (Fig. 3A, 3C), while in dendrites of morphine-injected mice, CRFr immunogold was often seen in the cytoplasm (Fig. 3B, 3D). These cytoplasmic CRFr immunogold particles were associated with dendritic endomembranes (Fig. 3B). Quantitative analysis confirmed this difference in that mice injected with morphine had a significantly lower number of CRFr immunogold particles on the dendritic plasma membrane per unit perimeter compared with saline-injected mice (Fig. 3E). The lower dendritic plasmalemmal CRFr labeling in mice

receiving morphine compared with saline was observed for all dendritic size categories- small ($F_{(1,350)} = 13.8$; $p < 0.001$), medium ($F_{(1,384)} = 17.9$; $p < .001$) and large ($F_{(1,108)} = 4.8$; $p < 0.05$). Interestingly, non-injected mice also showed significantly lower plasmalemmal CRFr labeling in small dendrites compared to saline-injected mice ($F_{(2,390)} = 13.99$; $p < 0.001$) (Fig. 3E).

Compared with saline, the morphine-injected mice showed a significantly larger number of CRFr immunogold particles in the cytoplasm per unit cross-sectional area in small ($F_{(1,350)} = 6.3$; $p < 0.01$), medium ($F_{(1,384)} = 10.6$; $p < 0.001$), and large dendrites ($F_{(1,108)} = 4.1$; $p < 0.05$) (Fig. 3F). The significant increase in cytoplasmic CRFr labeling in small dendrites of morphine-injected mice was also seen when compared with non-injected mice ($F_{(2,390)} = 7.69$; $p < 0.001$) (Fig. 3F).

Within cytoplasm, CRFr-immunogold particles were often seen contacting the membranes of mitochondria, particularly in dendrites of morphine-administered mice (Fig. 4A, 4B, 4C). In morphine- compared to saline-injected mice, a greater proportion of dendritic CRFr immunogold particles in the cytoplasm was associated with mitochondria in all sized dendrites- small ($F_{(1,195)} = 17.8$; $p < 0.001$), medium ($F_{(1,293)} = 7.3$; $p < 0.01$), and large ($F_{(1,83)} = 4.4$; $p < 0.05$) (Fig. 4D). The significant increase in CRFr association with mitochondria in morphine-injected mice was also seen in comparison with non-injected control mice in small ($F_{(2,226)} = 12.57$; $p < 0.001$), medium ($F_{(2,354)} = 6.90$; $p < 0.001$), and large dendrites ($F_{(2,158)} = 11.78$; $p < 0.001$) (Fig. 4D). There were no significant between-group differences in the total number of dendritic CRFr immunogold particles per unit cross-sectional area.

Hypothalamic-pituitary-adrenal (HPA) activity

One hour following the last morphine injection, mice displayed mean plasma corticosterone levels (53.01 ± 26.7 ng/ml) that were lower, but not significantly different, from those of mice that received saline (72.93 ± 27.5 ng/ml). These levels in saline and morphine groups are within the basal range of plasma corticosterone levels in mice (Barriga et al, 2001; Kim et al, 2008).

Discussion

Our results provide the first ultrastructural evidence that the cytoplasmic, and particularly mitochondria-associated, CRFr density is increased in dendrites of the BSTal of mice receiving chronic injections of morphine compared with saline. Furthermore, daily injections of saline enhance plasmalemmal CRFr density, an effect that is opposed by chronic morphine injections. These treatment-specific differences occur in all size classes of dendritic profiles without affecting the total number of dendritic CRFr immunogold particles. In contrast, mice injected with morphine or saline do not differ in frequencies of the association of CRFr-containing dendrites with CRF-immunoreactive terminals, or the total number of terminals containing CRF. The relocation of the CRFr in BSTal dendrites, and subsequent change in CRFr availability for ligand-binding, may contribute to the known alterations in the stress response following long-term exposure to opiates. In addition, however, observed differences in CRFr labeling in the BSTal of saline- or morphine-injected versus control non-injected groups also suggests that morphine administration ameliorates the CRFr trafficking in BSTal dendrites induced by the stress of daily injections.

Methodological Considerations

Both the CRFr and CRF antisera meet important criteria of specificity including immunoreactivity that is absent or diminished in brain sections of the respective CRFr-1 and CRF knockout mice as well as in brain sections immunolabeled with antisera pre-adsorbed with the antigenic peptide (Reyes et al, 2008; Treweek et al, 2009). We cannot totally rule out

the possibility that the CRFr antiserum may partially recognize the CRFr-2 subtype (Treweek et al., 2009). Profiles were considered as CRFr-positive when they contained one or more gold particles. This is a valid approach in samples of brain tissue in which structures such as myelin, which are not known to express the antigen detected by immunolabeling, show few, if any, spurious gold particles (Wang & Pickel, 2001; Wang & Pickel, 2002; Hara & Pickel, 2005; Jaferi & Pickel, 2009). The pattern of labeling obtained with these antisera is consistent with previous light and electron microscopic localization of the CRFr and CRF in neurons of the BSTal (Moga et al, 1989; Phelix & Paull, 1990; Jaferi & Pickel, 2009).

The quantification methods used for between-group comparisons of immunogold labeling were similar to those used in other studies (Glass et al, 2005; Lessard and Pickel, 2005; Hara & Pickel, 2007; Lane et al, 2008). To avoid artificial between-group treatment differences, sections of the BNST were (1) selected at the same anatomical level from saline and morphine groups, (2) co-processed for immunolabeling, and (3) sampled in electron microscopic thin-sections collected exclusively from the surface of tissue where immunoreagents have the greatest access to antigens. The reliability and reproducibility of this method is evidenced by the absence of substantial inter-animal variability in immunolabeling.

Dendritic CRFr distribution

Many dendritic profiles of the mouse BSTal contained immunogold labeling of the CRFr. CRFr immunogold particles were observed in all size classes of dendrites. The highest density of CRFr labeling in the BSTal was seen in small- and medium-sized dendritic profiles. This suggests that CRFr-mediated signaling within the BSTal may primarily occur at distal (small) and intermediate (medium), compared to more proximal (large), portions of the dendritic tree (Hausser et al, 2000; Vetter et al, 2001). However, chronic morphine- compared with saline-injected mice showed a significant shift in the distribution of the CRFr from plasmalemmal to cytoplasmic compartments within BSTal dendrites of all sizes. Therefore, the treatment-specific differences between morphine and saline groups discussed below apply to the CRFr in all dendritic size classes.

The observed lower density of plasmalemmal, and higher density of cytoplasmic CRFr in BSTal dendrites may reflect agonist-induced receptor trafficking, a common mechanism for modulating cellular sensitivity of G protein-coupled receptors, such as CRFr, to their endogenous ligands (Gainetdinov et al, 2004; Reyes et al, 2006). A morphine-induced increase in CRF release from axon terminals in the BSTal is suggested by the fact that many of these terminals arise from the central amygdala, whose own CRF system is potently activated by chronic morphine administration (Maj et al, 2003). However, neither the total number of CRF-immunoreactive axon terminals in the BSTal nor their association with CRFr-containing dendrites was significantly altered by the 14-day morphine compared with saline administration. This suggests that chronic morphine administration does not influence the axonal availability of CRF in the BSTal, but does not exclude the possibility that saline and morphine differentially affect CRF release in this region. Changes in CRF release can affect excitatory transmission since CRF acts via the CRFr-1 to enhance glutamate release in the BSTal (Kash et al, 2008). Little is known about the release of CRF, mediated by chronic morphine, in brain sites outside of the hypothalamus. In the hypothalamus, acute administration of morphine stimulates CRF release in non-stressed animals (Buckingham & Cooper, 1984; Suemaru et al, 1985; Buckingham & Cooper, 1986; Buckingham & Cooper, 1987; Nikolarakis et al, 1989). It is possible that after thrice daily injections of morphine for 14 days, the last injection of morphine remains effective in inducing the release of CRF in the BSTal above that of mice receiving saline-injections. This is in line with previous suggestions that down-regulation of mu-opioid receptor signaling during chronic opiate administration may lead to increased transcriptional activation of CRF (McNally & Akil, 2002).

The observed lower plasmalemmal density of CRFr immunogold particles in dendrites of non-injected compared with saline-injected mice indicate that the stress of repeated i.p. injections may influence trafficking of CRF receptors. Our results are consistent with prior evidence that saline injections influence the performance of rodents in tests of depressive behavior (Drago et al, 2001). Thus, the lower density of plasmalemmal CRFr particles in the morphine group, which is paralleled by an increase in cytoplasmic CRFr density, may reflect opiate-opposition to the reinsertion of CRF receptors that were internalized following CRF release induced by daily saline injections.

We have recently shown that mu-opioid and CRF receptors are present in many of the same dendrites of the BSTal and central amygdala (Jaferi & Pickel, 2009). The molecular basis for a potential interaction between these receptors within the same neurons has not yet been identified. It is possible that the mu-opioid receptor forms heterodimers with the CRFr, which could modulate trafficking of either or both receptors (Pfeiffer et al, 2003). The trafficking effects are supported by the fact that we observed no significant differences between morphine and saline groups in their total numbers of dendritic CRFr immunogold particles. Collectively, these findings suggest that, as compared with saline, chronic morphine administration by thrice daily injections over a 14-day period results in enhanced internalization or decreased delivery of the CRFr to the plasma membrane, but does not affect CRFr synthesis in BSTal neurons (Fig. 5).

Increases in association of CRFr with mitochondria

The present results are the first to show that, in dendrites of the mouse BSTal, chronic morphine results in two- to three-fold increases in the association of cytoplasmic CRFr with mitochondrial membranes relative to saline. Mitochondria are a major site of production of cytotoxic free radicals such as reactive oxygen species (Trushina & McMurray, 2007). The observed morphine-induced increase in the association of the CRFr with mitochondria is particularly interesting in the context of recent evidence that the effects of opiate exposure on the brain and spinal tissues may involve oxidative stress (Rambhia et al, 2005; Guzman et al, 2006; Lin et al, 2007; Cunha-Oliveira et al, 2008; Feng et al, 2008), and that acute activation of the CRFr is associated with protection of neurons from oxidative stress (Lezoualc'h et al, 2000; Pedersen et al, 2001; Pedersen et al, 2002; Bayatti & Behl, 2005; Izzo et al, 2005; Martin et al, 2005). Given the differences in expression pattern and function of the CRFr-1 and CRFr-2 subtypes (Steckler & Holsboer, 1999), it is possible that the observed CRFr immunoreactivity on the mitochondrial membrane versus the plasma membrane may reflect differential distribution of CRFr subtypes in dendritic compartments. The present results suggest the unique possibility that chronic morphine may influence oxidative stress via one or both CRFr subtypes in BSTal neurons.

Alternatively, the potential functional significance of the CRFr localization on mitochondria in morphine-injected mice may be related to intracellular calcium release (Pickel et al, 1997). This is supported by the observation that, in some instances, the outer mitochondrial membranes in BSTal dendrites were in continuity with smooth endoplasmic reticulum, both of which are known to store calcium (Lenaz et al, 1998; Baumann & Walz, 2001). Increased intracellular calcium release in neurons has been individually associated with morphine tolerance (Smith et al, 1999), and CRF in conjunction with ryanodine receptor calcium-release channels (Riegel & Williams, 2008). Thus, chronic morphine and the CRFr may play a collaborative role in cytoplasmic release of calcium from mitochondria and/or smooth endoplasmic reticulum in BSTal dendrites.

Implications

Our findings of a CRF_r re-distribution in the BSTal following chronic morphine administration is in line with previous evidence that chronic exposure to drugs of abuse can induce adaptations in stress-related neurotransmitter or neuropeptide systems in the extended amygdala that are not linked to the acute reinforcing effects of the drug, but that are nonetheless recruited during the drug administration due to intrinsic neuroplasticity of the brain (Zhou et al, 1996; Koob & Le Moal, 1997; Koob & Kreek, 2007; Koob, 2008). Chronic morphine-induced reductions in the availability of the CRF_r in BSTal dendrites may lead to decreased CRF_r activation by CRF, and subsequent alterations in stress responsivity. The re-distribution of the dendritic CRF_r may underlie related functional observations that chronic morphine-administered animals fail to mount a normal neuroendocrine response to acute stress (Buckingham & Cooper, 1984). In turn, drug-associated adaptations in brain stress systems may further contribute to the motivation to continue drug use during dependence (Koob et al, 2008).

The brain CRF system is markedly upregulated during withdrawal from morphine and other drugs of abuse (Heinrichs et al, 1995; Iredale et al, 2000; McNally & Akil, 2002; Skelton et al, 2007). However, it is likely that the present findings are associated with the effects of morphine itself, rather than withdrawal from morphine. Animals injected chronically with escalating doses of morphine and perfused one hour following the last morphine injection, as done in the present study, do not yet exhibit behavioral indices of withdrawal (Lane & Pickel, unpublished observations). Furthermore, morphine-injected animals in the present study display lower corticosterone levels compared to saline-injected animals. Although this difference is not statistically significant, it is more reminiscent of the tolerance or blunting of corticosterone levels observed during chronic opiate exposure (Buckingham & Cooper, 1984; Milanes et al, 1997; Fuentres et al, 2000) than it is with elevations in corticosterone secretion seen in opiate withdrawal (McNally & Akil, 2002; Zhou et al, 2006; Nunez et al, 2007). Collectively, this evidence suggests that, at the time of brain fixation, our animals were not yet in withdrawal, and morphine levels were still present in the system. However, whether or not the morphine-induced reduction of the CRF_r on the cell surface persists during other stages of morphine addiction such as withdrawal or protracted abstinence remain open questions.

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Abbreviations

BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
BSTal	anterolateral bed nucleus of the stria terminalis
CeA	central amygdala
CeL	lateral central amygdala

CRF	corticotropin-releasing-factor
CRFr	corticotropin-releasing-factor receptor
CRFr-1	corticotropin-releasing-factor receptor 1
i.p.	intraperitoneal
PB	phosphate buffer
PBS	phosphate-buffered saline
TBS	Tris-buffered saline
VTA	ventral tegental area

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Fig. 1.
The BNST subregion sampled for electron microscopy is shown by the trapazoid in a plastic-embedded coronal hemisection through the region of the anterior commissure (ac) in a mouse brain. This vibratome section was collected 0.2 mm anterior to Bregma (Hof et al, 2000). Lv = lateral ventricle, cp = caudate putamen nucleus. Scale bar = 100 μ m.

Fig. 2.

Electron microscopic evidence that the numbers of dendrites containing the CRFr and their contacts with CRF-immunoreactive axon terminals in the BSTal are unaffected by chronic morphine compared with saline injections. Electron micrographs show immunogold-silver labeling of CRF receptors (arrows) in dendrites (CRFr-De) in contact with axonal profiles showing immunoperoxidase labeling of CRF within the BSTal of mice that received chronic injections of saline (A) or morphine (B, C). These axonal profiles include vesicle-filled axon terminals (CRF-Te) and small axons (CRF-Ax). In both (A) and (B), the CRFr-immunogold labeling (small arrows) is near mitochondria (M), whose outer membranes appear to be in continuity with endomembranes (em), presumably including smooth endoplasmic reticulum

(ER). All of the dendrites are also contacted by unlabeled terminals (ut), but a symmetric, inhibitory-type synapse (curved arrow) is recognizable only in (C), where the CRFr-immunogold labeling is distant from the mitochondria and located near the base of a dendritic spine.. Scale bars= 500nm. In (D), the bar graph quantitatively confirms that almost all CRFr-containing dendrites are without CRF-immunoreactivity and that the percentage of these dendrites, or those containing CRF, are not affected by chronic morphine compared with saline administration.

Fig. 3.

Chronic morphine compared with saline administration decreases plasmalemmal, and increases cytoplasmic, CRFr-immunogold labeling in dendrites of the BSTal. Electron micrographs show CRF receptor immunogold particles in dendritic profiles (CRFr-De) in the BSTal of mice that received chronic injections of saline (A, C) or morphine (B, D). In (A), a CRFr immunogold particle (circled) contacts the plasma membrane of a dendrite that contains a mitochondrion (M) and numerous unlabeled endomembranes (em), and also receives a symmetric synapse (curved arrow) from an axon terminal (Te). In (B), CRFr immunogold particles (arrow) contact endomembranes (em) in the cytoplasm of a dendrite. In (C), the plasmalemmal CRFr (circled) is in proximity to a nearby mitochondrion (M). The CRFr-immunogold labeling in (D) is seen near a mitochondrion (M) and distant from synaptic (curved

arrow) and non synaptic plasma membranes. Scale bars = 500 nm. Bar graphs in (E) and (F) show significant differences between non-injected, saline- and morphine-injected mice in the plasmalemmal (number/perimeter length) and cytoplasmic (number/area) density of CRF receptor (CRFr) immunogold particles in dendrites of varying sizes (small, medium, and large) in the BSTal.

Fig. 4.

Chronic morphine compared with saline administration results in enhanced association of CRF-immunogold with mitochondria (M) in dendrites of the BSTal. Electron micrographs show cytoplasmic CRF immunogold particles (arrow) that contact outer membranes of mitochondria (M) in dendrites (CRFr-De) of the BSTal in mice that received chronic injections of morphine. In (B), a dendrite with mitochondrial CRF immunogold labeling receives an asymmetric, excitatory-type synapse (arrowhead) from an unlabeled axon terminal (ut). Em = endomembrane. Scale bars = 500 nm. In (D), a bar graph shows the percentage of cytoplasmic CRF receptor (CRFr) immunogold particles that contact the membranes of mitochondria in dendritic profiles of the BSTal in non-injected, saline- and morphine-injected

mice. The percentage of CRFr immunogold particles that contact mitochondria is significantly increased in the chronic morphine group compared to saline and non-injected groups in all size classes of dendrites.

Fig. 5.

Schematic diagram showing the somatic and dendritic distribution of CRFr (black triangles) in the BSTal of mice injected chronically with saline (on left) or morphine (on right). The relative plasmalemmal/cytoplasmic densities of CRFr gold particles in dendritic compartments (small, medium and large dendrites) are shown. In small, medium and large dendrites, the significantly lower density of plasmalemmal CRFr particles in the morphine group is shown to be paralleled by an increase in cytoplasmic CRFr density, without a between-group difference in the total number of dendritic CRF receptors. Also illustrated is the greater proportion of cytoplasmic CRFr associated with mitochondria (M) in small, medium and large dendrites of morphine- compared with saline-injected mice. In both saline and morphine groups, CRFr-containing dendrites are seen as apposed by CRF-immunoreactive axon terminals (CRF-Te). CRFr-containing dendrites in the BSTal of both saline and morphine groups are also seen receiving symmetric, inhibitory-type (–) or asymmetric, excitatory-type (+) synapses from unlabeled terminals (UT).