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## Enhanced dendritic availability of mu-opioid receptors in inhibitory neurons of the extended amygdala in mice deficient in the corticotropin-releasing factor-1 receptor

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### Abstract

Activation of the corticotropin-releasing factor-1 (CRF-1) receptor in the anterolateral BNST (BSTal), a key subdivision of the extended amygdala, elicits opiate-seeking behavior exacerbated by stress. However, it is unknown whether the presence of CRF-1 affects expression of the mu-opioid receptor ( $\mu$ -OR) in the many GABAergic BSTal neurons implicated in the stress response. We hypothesized that deletion of the CRF-1 receptor gene would alter the density and/or subcellular distribution of  $\mu$ -ORs in GABAergic neurons of the BSTal. We used electron microscopy to quantitatively examine  $\mu$ -OR immunogold and GABA immunoperoxidase labeling in the BSTal of CRF-1 knockout (KO) compared to wildtype (WT) mice. To assess regional specificity, we examined  $\mu$ -OR distribution in dorsal striatum. The  $\mu$ -ORs in each region were predominantly localized in dendrites, many of which were GABA-immunoreactive. Significantly more cytoplasmic  $\mu$ -OR gold particles per dendritic area were observed selectively in GABA-containing dendrites of the BSTal, but not of the dorsal striatum, in KO compared to WT mice. In both regions, however, significantly fewer GABA-immunoreactive axon terminals were present in KO compared to WT mice. Our results suggest that absence of CRF-1 results in enhanced expression and/or dendritic trafficking of  $\mu$ -ORs in inhibitory BSTal neurons. They also suggest that expression of CRF-1 is a critical determinant of the availability of GABA in functionally diverse brain regions. These findings underscore the complex interplay between CRF, opioid and GABA systems in limbic and striatal regions, and have implications for the role of CRF-1 in influencing the pharmacological effects of opiates active at  $\mu$ -ORs.

### Keywords

stress; BNST; striatum; opiate; addiction; GABA; trafficking; immunocytochemistry

### Introduction

Stress is a potent motivator of opiate seeking and relapse (Krueger, 1981; Kosten et al, 1986; Shaham & Stewart, 1995; Erb et al, 1996; Piazza & Le Moal, 1998; Shaham et al, 2000; Mayer et al, 2002; Grusser et al, 2007). These effects are mediated, in part, by the corticotropin-releasing factor-1 (CRF-1) receptor in the anterolateral bed nucleus of the stria terminalis (BSTal; Lu et al, 2000; Wang et al, 2006). The BSTal, comprised within the extended amygdala, is a complex limbic brain region implicated in many of the motivational aspects of opiate dependence (Alheid & Heimer, 1988; Koob, 2003; Veinante et al, 2003;

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Kash et al, 2008; Walker & Davis, 2008). In postsynaptic dendrites of the BSTal, the CRF-1 receptor is extensively co-expressed with the mu-opioid receptor ( $\mu$ -OR; Jaferi & Pickel, 2009), the major receptor mediating the analgesic and rewarding effects of the opiates, morphine and heroin (Kieffer, 1999; Contet et al, 2004). However, it is not yet known whether the expression of the CRF-1 receptor is a determinant of the availability of the  $\mu$ -OR in BSTal neurons, many of which contain gamma-aminobutyric acid (GABA; Sun & Cassell, 1993).

Intact GABAergic transmission in the extended amygdala is critical for normal inhibition of affective behaviors and conditioned associations, which, if over-expressed, could appear as pathological states such as anxiety disorders and addiction (Sanders and Shekhar, 1995; Fendt et al, 2003; Quirk & Gehlert, 2003; Sajdyk et al, 2008). Neurons containing the  $\mu$ -OR express GABA in reward-associated limbic (Svingos et al, 1997; Steffensen et al, 2006) and antinociceptive brainstem regions (Kalyuzhny & Wessendorf, 1998). In line with this are findings that opiate activation of the  $\mu$ -OR modulates GABA neurotransmission within the amygdala (Finnegan et al, 2005) and the mesocorticolimbic dopamine reward system (Steffensen et al, 2006). Opiate activation of the  $\mu$ -OR also enhances the effect of GABA<sub>A</sub> receptor agonists on anxiolytic behavior (Sasaki et al, 2002). In turn, modulators of GABAergic transmission can influence the pharmacological and behavioral effects of opiates (Tejwani et al, 1993; Rattan and Sribanditmongkol, 1994; Sasaki et al, 2002; Cabral et al, 2009). Alterations in  $\mu$ -OR expression is therefore of particular interest with regard to opiate addiction and affective behavior when occurring specifically in GABAergic neurons.

Given the anatomical and functional associations between the  $\mu$ -OR and the CRF-1 receptor in the BSTal, we hypothesize that the availability of the  $\mu$ -OR within subcellular compartments of GABAergic neurons of this region is CRF-1 receptor-dependent. To test this hypothesis, we used electron microscopy to quantitatively compare the immunogold labeling of the  $\mu$ -OR in the BSTal of CRF-1 receptor ( $-/-$ ) knockout (KO) and CRF-1 receptor ( $+/+$ ) wildtype (WT) mice. The tissue used for analysis of  $\mu$ -OR gold particle distributions was co-processed for immunoperoxidase labeling of GABA. To assess regional specificity, we conducted the equivalent analysis in the dorsal striatum (caudate-putamen nucleus) which, like the BSTal, is comprised mainly of GABAergic neurons that express the  $\mu$ -OR (Aoki & Pickel, 1989; Wang et al, 1997), and of neurochemically-unidentified neurons that express the CRF-1 receptor (Justice et al, 2008). The dorsal striatum is essential for motor function, but is also prominently involved in learning of motor habits associated with addictive diseases (Reading et al, 1991; Everitt & Robbins, 2005; Grahn et al, 2008; White, 2009). The results have implications for the role of the CRF-1 receptor in regulation of  $\mu$ -OR availability in inhibitory neurons of limbic and striatal brain regions.

## Materials and Methods

### Animals

All experiments were performed in accordance with the NIH regulations of animal care and were reviewed by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. We used a total of eight adult, male mice of which four were CRF-1 receptor ( $-/-$ ) KO mice and the rest were WT ( $+/+$ ) littermates. CRF-1 receptor KO mice were purchased from the Jackson Laboratories (Bar Harbor, ME). To generate homozygous mice, the heterozygous littermates were crossed, and the litters were genotyped by standard polymerase chain reaction (PCR) procedures with tail DNA. Mice 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.

## Tissue Preparation

Prior to perfusion, mice were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.). 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 um sections on a Vibratome (Leica, Deerfield, IL). The coronal sections were collected through the rostrocaudal extent of the BNST and striatum based on the mouse brain atlas by Hof et al (2000). Rostrocaudally-matched pairs of sections from CRF-1 receptor KO and WT mice were simultaneously processed for immunogold–silver labeling of the  $\mu$ -OR and immunoperoxidase labeling of GABA.

## Antisera

For immunogold-silver labeling of the  $\mu$ -OR, we used a polyclonal guinea pig antiserum directed against a synthetic peptide corresponding to amino acids 384-398 of the C-terminus of the cloned rat  $\mu$ -OR (AB1774; Chemicon, Temecula, CA, USA). The specificity of this antiserum has previously been demonstrated by the absence of immunoreactivity in adsorption controls (Rodriguez et al., 2001; Drake and Milner, 2002; Chemicon, 2005). The guinea-pig  $\mu$ -OR antiserum has also been shown to recognize proteins that are attenuated in  $\mu$ -OR KO mice with deletions at distinct exons of the  $\mu$ -OR gene including exon 1, exons 2/3 and exon 11 (Jaferi & Pickel, 2009).

For immunoperoxidase labeling of GABA, we used a polyclonal rat antiserum that was raised against GABA-glutaraldehyde-hemocyanin conjugates (Bayer & Pickel, 1991). Immunolabeling of this antiserum shows a concentration-dependent reduction following prior adsorption with GABA-bovine serum albumin (BSA) conjugates (Lauder et al, 1986). The cellular distribution of this antiserum in rat brain is comparable to that of the GABA-synthesizing enzyme, glutamic acid decarboxylase (GAD; Oertel et al, 1983; Ottersen & Storm-Mathisen, 1984).

## Immunocytochemical Labeling

Brain sections from CRF-1 receptor KO and WT mice were co-processed for dual immunogold-silver and immunoperoxidase labeling of the  $\mu$ -OR and GABA (Chan et al, 1990; Drake & Milner, 2002; Lane et al, 2008; Jaferi & Pickel, 2009; Jaferi et al, 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. 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 nonspecific binding of the antisera, the sections were incubated in 0.5% 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 (1:500 guinea pig anti- $\mu$ -OR for immunogold-silver labeling and 1:10,000 rat anti-GABA 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 GABA, the sections were rinsed in 0.1 M TBS and then placed for 30 min in a 1:400 dilution of biotinylated anti-rat immunoglobulin (IgG). 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 then 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-guinea pig 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 placed 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 kept 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 (Fig. 1A) and the dorsolateral striatum (Fig. 1B). These regions were identified in coronal sections of forebrain located at Bregma 0.2 mm in the mouse stereotaxic atlas (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 sections on grids were rinsed and allowed to air dry before viewing at 60 kV with a Philips CM10 transmission electron microscope (FEL, Hillsboro, OR). Those areas showing immunolabeling of the  $\mu$ -OR and/or GABA 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 16 vibratome sections (eight sections from four CRF-1 receptor KO mice, eight sections from four WT mice) were used for electron microscopic analysis. Micrographs containing  $\mu$ -OR and GABA immunoreactivity at magnifications of 13,500 to 25,000 were analyzed in a total tissue area of 17,893  $\mu\text{m}^2$  in the BSTal, of which equal tissue areas were examined from each mouse from WT and KO groups. This analysis was repeated in ultra-thin sections of the striatum from four KO and four WT mice. 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  $\mu$ -OR and/or GABA immunoreactivity as neuronal processes (dendrites, dendritic spines, axons and 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 an equally large area of the surrounding tissue.

This approach was validated by examining spurious gold particles overlying myelin, a structure not known to express  $\mu$ -ORs. For example, out of a total of 222 myelinated axons observed in 8,208  $\mu\text{m}^2$  in the BSTal, none displayed  $\mu$ -OR immunogold particles. We subsequently quantified the number of  $\mu$ -OR- and/or GABA-labeled neuronal profiles as well as the density of the  $\mu$ -OR gold–silver deposits in the plasmalemmal and cytoplasmic compartments of neurons. 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.

The quantification methods used for between-group comparisons of immunogold labeling were similar to those used in other studies (Glass et al, 2005; Lane et al, 2008; Jaferi et al, 2009). 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). The parameters used for statistical comparisons in the BSTal and striatum were: (1) the number of plasmalemmal  $\mu$ -OR gold particles in a dendritic profile/dendritic perimeter, (2) the number of cytoplasmic  $\mu$ -OR gold particles in a dendritic profile/dendritic cross-sectional area, (3) the number of total  $\mu$ -OR gold particles in a dendritic profile/dendritic cross-sectional area, (4) the number of GABA immunoperoxidase-labeled dendritic profiles per neuropil area, (5) the number of GABA immunoperoxidase-labeled axonal profiles per neuropil area. Analyses #1-3 were conducted for all  $\mu$ -OR-containing dendrites, and additionally for only those  $\mu$ -OR-containing dendrites that were GABA-immunoreactive. Quantification of immunolabeled profiles was performed by an investigator who was blind to experimental groups (i.e., KO or WT.)

In order to compare similar structures across groups, cluster analysis by dendritic size was performed to statistically divide  $\mu$ -OR-labeled dendrites from KO and WT groups using JMP 5.0.1 statistical software (SAS Institute Inc., NC, USA). This analysis was conducted because dendritic size is an indirect indicator of the distance of dendrites from somata, with the larger dendrites being more proximal and the smaller dendrites more distal from the somatic origins (Peters et al, 1991). There is, however, variation in dendritic size that may be attributed to their origin from neurons of different sizes and/or dendritic branching patterns.

Dendrites from mice within the same group can also display variations in immunoperoxidase labeling intensities (Bayer & Pickel, 1991; Lane et al, 2008). Dendritic populations that display distinct intensities in immunolabeling are also known to differ in their afferent inputs (Bayer & Pickel, 1991), and are differentially affected by drug administration (Lane et al, 2008), suggesting that these neurons may be transmitter- and/or function-specific. Data for immunolabeling were analyzed by individual dendrites, a method of analysis that has previously been used in electron microscopic studies of receptor or neurotransmitter distribution (Hara & Pickel, 2007; Lane et al, 2008). A within-groups analysis was carried out to assess whether significant variability exists between mice within each group. We found no significant intra-animal differences in the number of intensely- and lightly-labeled GABA dendrites or in the numbers of  $\mu$ -OR gold particles. Data were analyzed by one-way Analysis of Variance (ANOVA), and differences in means were analyzed by Tukey's post hoc test.

## Results

### Commonalities in $\mu$ -OR subcellular location and association with GABA in CRF-1 KO and WT

CRF-1 receptor KO and WT mice were similar in their neuronal distributions of  $\mu$ -ORs and associations with GABA-immunoreactive neurons. In WT (Fig. 2A) and CRF-1 receptor KO (Fig. 2B) mice,  $\mu$ -OR immunogold labeling in the BSTal was observed in many dendritic profiles (dendrites and dendritic spines). In addition,  $\mu$ -OR immunogold was sparsely distributed in axonal profiles (axons and axon terminals) of both WT (Fig. 2A) and KO (Fig. 2B). Quantification confirmed that, out of all  $\mu$ -OR-labeled neuronal (dendritic and axonal) profiles in the BSTal of WT mice, 79% (893/1131) were dendritic and 21% (238/1131) were axonal. Similarly, in CRF-1 receptor KO mice, 74% (809/1088) of all  $\mu$ -OR-labeled neuronal profiles in the BSTal were dendritic and 26% (279/1088) were axonal. Cluster analysis revealed  $\mu$ -OR labeling in three major size groups of dendrites- small (0.70-1.17  $\mu$ m), medium (1.20-1.40  $\mu$ m), and large (2.0-3.8  $\mu$ m). The  $\mu$ -OR labeling was observed in 401 small, 294 medium, and 104 large dendrites within the BSTal of WT mice. Similar patterns were observed in the BSTal of CRF-1 receptor KO mice where there were 319 small, 331 medium, and 90 large dendrites.

The  $\mu$ -OR immunogold particles were commonly distributed within the cytoplasm and along the plasma membrane of dendritic profiles that also contained GABA immunoperoxidase in both WT (Fig. 2A) and CRF-1 receptor KO mice (Fig. 2B). Unlike the predominantly dendritic location of the  $\mu$ -OR, the distribution of GABA immunoreactivity was almost equally prevalent in dendritic (53%; 980/1859) and axonal (47%; 879/1859) profiles of WT mice as well as in dendritic (50%; 702/1408) and axonal (50%; 706/1408) profiles of KO mice. A subset of neurons that were immunogold-labeled for the  $\mu$ -OR also contained GABA immunoperoxidase reaction product. Forty percent (354/893) of dendritic, and 33% (78/238) of axonal profiles containing  $\mu$ -OR immunogold particles were immunoperoxidase-labeled for GABA in WT mice. In CRF-1 receptor KO mice, 26% (210/809) of dendritic, and 22% (60/279) of axonal profiles contained both  $\mu$ -OR immunogold and GABA immunoperoxidase labeling.

**Synaptic associations of  $\mu$ -OR-labeled neurons**—The types of synaptic associations between axon terminals and  $\mu$ -OR-labeled neurons in the BSTal of WT mice were similar to those observed in CRF-1 receptor KO mice. Out of all synaptic contacts seen on  $\mu$ -OR-labeled dendrites with or without GABA immunoreactivity in the BSTal of WT mice ( $n=322$ ), the most common were from unlabeled axon terminals (272/322). These contacts were characterized by asymmetric, excitatory-type (180/322) or less often, symmetric, inhibitory-type (92/322) synapses (Fig. 2A, 3A). Interestingly, the  $\mu$ -OR immunogold particles were preferentially located near asymmetric, excitatory-type synapses (Fig. 3A). The  $\mu$ -OR-labeled dendrites also received inhibitory-type synapses (43/322) from, or were apposed ( $n=97$ ) to, GABA-immunoreactive axon terminals (Fig. 2A). There were rarely recognizable affiliations of dendritic  $\mu$ -OR immunogold particles with symmetric synapses.

### Preferential enhancement of the cytoplasmic density of $\mu$ -OR immunogold in GABA-containing dendrites of the BSTal in CRF-1 KO

Quantitative comparisons of  $\mu$ -OR immunogold labeling between KO and WT groups were first carried out for only those  $\mu$ -OR-labeled dendritic profiles with detectable GABA peroxidase (Fig. 2A, 2B), as described below. Compared with WT mice, CRF-1 receptor KO mice showed similar plasmalemmal (Fig. 2C), but significantly more cytoplasmic  $\mu$ -OR immunogold particles per unit cross-sectional area of large GABA-immunoreactive dendrites ( $F_{(1,87)} = 4.1$ ;  $p < 0.05$ ); (Fig. 2C). In contrast with large dendrites, there was an

apparent decrease in the cytoplasmic density of  $\mu$ -OR immunogold in medium dendrites of CRF-1 receptor KO mice (Fig. 2C). However, neither medium nor small dendrites significantly differed in dendritic  $\mu$ -OR immunogold in CRF-1 receptor KO compared with WT mice. In GABA-immunoreactive dendrites regardless of size, the percentage of  $\mu$ -OR immunogold particles that were associated with synapses was significantly less in CRF-1 receptor KO ( $1.4\% \pm 0.01$ ) compared with WT ( $6.2\% \pm 0.01$ ) mice ( $F_{(1,510)} = 6.3$ ;  $p < 0.01$ ). There were no significant between-group differences in the cross-sectional area or perimeter of dendrites in the BSTal.

When quantitative comparisons of  $\mu$ -OR immunogold labeling between KO and WT groups were carried out for the total population of  $\mu$ -OR-labeled dendritic profiles (with and without detectable GABA peroxidase; Fig. 3A, 3B), KO and WT mice did not significantly differ in the plasmalemmal, cytoplasmic or total number of  $\mu$ -OR immunogold particles for any dendritic size class (Fig. 3C). However, the percentage of synapse-associated  $\mu$ -OR immunogold was significantly less in dendrites of CRF-1 receptor KO ( $3.4\% \pm 0.01$ ) compared with WT ( $6.1\% \pm 0.01$ ) mice ( $F_{(1,1537)} = 7.7$ ;  $p < 0.01$ ). This difference was also observed specifically in small dendrites of CRF-1 receptor KO ( $3.2\% \pm 0.01$ ) compared with WT ( $6.7\% \pm 0.01$ ) mice ( $F_{(1,718)} = 9.0$ ;  $p < 0.01$ ). No significant between-group differences were seen for the number of  $\mu$ -OR-expressing dendrites in the BSTal.

As a comparison for the BSTal, quantitative analysis of  $\mu$ -OR immunogold labeling between WT and KO groups was carried out in the dorsal striatum. Quantitative analyses were conducted for  $\mu$ -OR-labeled dendritic profiles with detectable GABA peroxidase (Fig. 4A) separately from the total  $\mu$ -OR-labeled dendritic population (Fig. 4B). For either population of dendritic profiles, no significant between-group differences were observed for small (0.70-1.18  $\mu$ m), medium (1.2-1.3  $\mu$ m) or large (2.0-5.0  $\mu$ m) dendrites (Fig. 4A, 4B).

### Reduction in numbers of GABA-immunoreactive dendritic and axonal profiles in BSTal and striatum of CRF-1 KO mice

In the BSTal of both CRF-1 receptor KO and WT mice, numerous dendritic and axonal profiles containing GABA immunoperoxidase were apposed or formed symmetric synaptic contacts with  $\mu$ -OR-labeled neurons (Fig. 5A, 5B). Quantitative analysis of GABA immunoperoxidase-labeled neuronal profiles in the BSTal showed significant between-group differences in the abundance of labeled profiles. Significantly fewer GABA immunoperoxidase-labeled ( $F_{(1,399)} = 19.5$ ;  $p < 0.01$ ), and significantly more unlabeled ( $F_{(1,350)} = 40.5$ ;  $p < 0.01$ ) dendritic profiles were observed in the BSTal of CRF-1 receptor KO compared with WT mice (Fig. 5C). In the dorsal striatum, there was a trend towards fewer GABA-immunoreactive dendritic profiles in CRF-1 receptor KO compared to WT mice ( $F_{(1,238)} = 2.9$ ;  $p = 0.09$ ) (Fig. 5D). In addition, there were significantly fewer GABA immunoreactive axonal profiles in both the BSTal ( $F_{(1,382)} = 11.9$ ;  $p < 0.01$ ) and striatum ( $F_{(1,203)} = 5.0$ ;  $p < 0.05$ ) of the CRF-1 receptor KO compared with the WT mice (Fig. 5C, 5D).

## Discussion

Our results provide the first ultrastructural evidence that, as compared to WT mice, those deficient in the CRF-1 receptor have a greater cytoplasmic density of  $\mu$ -OR immunogold in large dendrites of GABAergic neurons in the BSTal. We also show a concomitant reduction in synapse-associated  $\mu$ -OR immunogold in small dendrites of CRF-1 receptor KO compared with WT mice. These changes in dendritic  $\mu$ -OR immunogold labeling are specific to the BSTal and not observed in the dorsal striatum of CRF-1 receptor KO mice. The results suggest that the availability of  $\mu$ -ORs in GABAergic dendrites of the BSTal is dictated in part by expression of the CRF-1 receptor. In addition, however, we observed

fewer GABA-immunoreactive dendritic and axonal profiles in both the BSTal and dorsal striatum of CRF-1 receptor KO compared to WT mice. Together, these findings have important implications for understanding the capacity of CRF-1 receptor agonists to modulate the response to opiates active at the  $\mu$ -OR, and to influence GABAergic expression in limbic and striatal neurons.

### Methodological Considerations

In both the rat and mouse brain, GABA immunoreactivity is localized in somatodendritic and axonal profiles within the BSTal (Sun & Cassell, 1993; Bali et al, 2005) and striatum (Delle Donne et al, 1987; Pickel & Heras, 1996; Chen et al, 2003). However, the detection of GABA immunoreactivity is often limited to larger dendrites and axon terminals, and less commonly seen in smaller dendrites and dendritic spines (Pickel & Heras, 1996; Chen et al, 2003). Thus, in the present quantitative analyses, co-localization of the  $\mu$ -OR and GABA may have been particularly underestimated in smaller dendrites. We cannot rule out the possibility that dendrites categorized as unlabeled (i.e., without GABA peroxidase) could arise from GABAergic cell bodies having undetectable dendritic labeling. This limitation, however, is equally applicable to CRF-1 receptor KO and WT mice and is not likely to significantly affect the interpretation of the results in the present study.

### Enhancement of cytoplasmic $\mu$ -OR density in GABA dendrites of the BSTal in CRF-1 KO

In the BSTal of CRF-1 receptor KO mice, the observed significant increase in cytoplasmic  $\mu$ -OR density in large GABAergic dendrites without change in plasmalemmal  $\mu$ -OR suggests that this increase is not related to internalization of surface/synaptic receptors. In contrast, however, the increase in  $\mu$ -OR immunogold density in large dendrites may be attributed in part to a CRF1-dependent shift in the locations of the cytoplasmic reserve of  $\mu$ -ORs from medium to large dendrites of GABA-containing neurons in the BSTal. This is suggested by the decrease in cytoplasmic  $\mu$ -ORs in medium GABAergic dendrites of CRF-1 receptor KO mice. Furthermore, the proportion of  $\mu$ -ORs associated with synapses in small BSTal dendrites was significantly less in CRF-1 receptor KO compared with WT mice. Therefore, the increase in cytoplasmic  $\mu$ -OR immunogold in large dendrites of CRF-1 receptor KO mice may also be attributed to CRF-1 receptor dependent trafficking of  $\mu$ -ORs from synaptic locations in distal dendritic spines to the cytoplasm of proximal dendrites.

The enhanced cytoplasmic density of  $\mu$ -ORs in CRF-1 receptor KO mice may preferentially occur in those BSTal GABAergic neurons in which there is lost expression of CRF-1 receptors. This is suggested by the frequent co-expression of  $\mu$ -ORs and CRF-1 receptors in BSTal dendrites of WT mice (Jaferi & Pickel, 2009). In addition, we have observed, in WT mice, that both  $\mu$ -ORs and CRF-1 receptors within the extended amygdala are frequently associated with excitatory-type synapses (Jaferi & Pickel, 2009; Treweek et al, 2009). It is conceivable that the enhanced accumulation of  $\mu$ -ORs in the cytoplasm of large dendrites is a reflection that these receptors are no longer necessary at excitatory-type synapses devoid of CRF-1 receptors. This would be somewhat analogous to observed CRF-1 receptor trafficking in BSTal dendrites following chronic morphine-induced activation of the  $\mu$ -OR (Jaferi et al, 2009).

Unlike the BSTal, our examination of the dorsal striatum revealed a lack of significant changes in dendritic  $\mu$ -OR immunogold densities in GABAergic dendrites of CRF-1 receptor KO compared to WT mice. The BSTal and dorsal striatum differ in their anatomical connections and specific contributions to drug addiction. The dorsal striatum is implicated in the formation of habits, including those that are drug-motivated (Everitt & Robbins, 2005). Habits and motor functions are strongly dependent on striatal inputs from the substantia nigra, somatosensory cortex and the motor, premotor, supplementary and



cingulate motor cortical areas (Nakano et al, 2000). In contrast, the BSTal, involved in mediating the interaction between stress and drug reward (Shaham et al, 2000; Koob, 2003), is innervated by the insular and prefrontal cortices (Yasui et al, 1991; Radley et al, 2009), the central amygdala (Dong et al, 2001) and the reward-associated ventral tegmental area (Hasue & Shammah-Lagnado, 2002) as well as brainstem regions conveying visceral sensory information through the nucleus of the solitary tract and ventrolateral medulla (Gaykema et al, 2007). The BSTal is also among a select group of regions that receives dense, likely glutamatergic, afferent input from the paraventricular thalamus (Frassoni et al, 1997; Li & Kirouac, 2008), a critical regulator of neuroendocrine responses to repeated stress (Jaferi & Bhatnagar, 2006). Neurons in many of the regions providing input to the BSTal normally express CRF-1 receptors (Justice et al, 2008; Treweek et al, 2009). Thus, the observed increase in  $\mu$ -OR density in large BSTal dendrites of CRF-1 receptor KO mice may be due not only to changes occurring in single BSTal neurons but also to CRF-1 receptor-containing neurons in brain regions that project extensively to the BSTal and minimally innervate the dorsal striatum.

Receptor changes in genetic KO models are often attributed to compensatory adaptations resulting from absence of the gene during development (Clifton et al, 2003; Wittmann et al, 2005; Kralic et al, 2006). However, evidence showing that acute intracerebroventricular administration of an anti-CRF IgG significantly increases brain  $\mu$ -OR density in adult mice suggests continued plasticity into adulthood (Rothman et al, 2002). CRF-1 receptor-mediated regulation of  $\mu$ -OR availability in BSTal neurons may arise not just as a compensatory adaptation in response to CRF-1 receptor deletion, but may also normally exist in association with short-term changes in CRF-1 receptor activation in the adult brain.

The CRF-1 receptor KO and WT mice showed no significant differences in  $\mu$ -OR density in non-GABA-immunoreactive dendrites of either the BSTal or dorsal striatum. Large, neurochemically-identified populations of neurons other than GABAergic neurons have also been localized in the BSTal and striatum. Neurons rich in dopamine (Pickel et al, 1981; Phelix et al, 1992), and opioids such as dynorphin and enkephalin are prevalent in each of these regions (Pickel et al, 1980; Yakovleva et al, 2006; Poulin et al, 2009). In addition, the BSTal has many CRF-containing neurons (Phelix & Paull, 1990; Jaferi et al, 2009). In the present study,  $\mu$ -OR-labeled dendrites without GABA-immunoreactivity may represent any of these neuronal populations and also likely include those of GABAergic neurons with non-detectable GABA levels.

### **Decreased numbers of GABA neurons in BSTal and dorsal striatum of CRF-1 KO**

Fewer GABA-immunoreactive dendrites, and more unlabeled dendrites, were observed in the BSTal of CRF-1 receptor KO compared with WT mice. Furthermore, in both the BSTal and striatum of CRF-1 receptor KO mice, fewer GABA-immunoreactive axon terminals were observed compared to WT mice. Mice deficient in the CRF-1 receptor gene are known to display morphological and functional alterations in developing brain regions including significantly increased dendritic branching in the hippocampus (Chen et al, 2004), attenuated synaptic potentiation in inhibitory hippocampal neurons (Schierloh et al, 2007), and a lost capacity of CRF to enhance GABA<sub>A</sub> inhibitory postsynaptic currents in central amygdala neurons (Nie et al, 2004). The results of the present study suggest that a permanent absence of CRF-1 receptors also induces compensatory changes in the BSTal and dorsal striatum resulting in downregulation of GABAergic expression.

### **Implications**

In the present study, the enhanced cytoplasmic reserve of  $\mu$ -ORs in large dendrites, and the decreased density of synapse-associated  $\mu$ -ORs in small dendrites, of GABAergic neurons in

the BSTal of CRF-1 receptor-deficient mice may profoundly affect the capacity of these neurons to respond to repeated opiate administration. Behavioral studies have revealed a compelling role for CRF, primarily via CRF-1 receptor activation, in modulation of the response to opiates (Heinrichs et al, 1995; Shaham et al, 1997; Shaham et al, 1998; Iredale et al, 2000; Lu et al, 2000; McNally & Akil, 2002; Contarino & Papaleo, 2005; Stinus et al, 2005; Wang et al, 2006). For example, genetic deletion or pharmacological blockade of the CRF-1 receptor attenuates stress-induced relapse to opiate seeking (Shaham et al, 1998, Wang et al, 2006) as well as the somatic and affective symptoms of opiate withdrawal (Iredale et al, 2000; Lu et al, 2000; Contarino & Papaleo, 2005; Stinus et al, 2005). More direct region-specific evidence demonstrates that blockade of CRF-1 receptors within the BNST attenuates stress-induced reinstatement of morphine conditioned place preference (Wang et al, 2006).

A significant decrease in GABA expression was observed in neurons of both the BSTal and dorsal striatum of CRF-1 receptor KO mice. This observation is consistent with evidence showing that CRF enhances GABAergic synaptic transmission by activation of the CRF-1 receptor in several different brain regions including the BNST (Nie et al, 2004; Kash & Winder, 2006; Schierloh et al, 2007; Bagosi et al, 2008; Kirby et al, 2008). GABAergic neurons in the BNST also regulate anxiety-like behavior (Sajdyk et al, 2008) and hypothalamic-pituitary-adrenal responses to acute emotional stressors (Radley et al, 2009). Therefore, a reduction or loss of CRF-1 receptors available for activation in the BSTal and associated brain regions may have a major impact on GABAergic neurotransmission in BSTal neurons and in their outputs controlling behavioral and neuroendocrine responses to stress. Given the involvement of the BNST in stress-induced opiate relapse (Wang et al, 2006), and of the striatum in the compulsive nature of addiction (Everitt & Robbins, 2005), a marked decrease in GABAergic inhibitory tone in both the BSTal and dorsal striatum has important implications for the maintenance of opiate seeking during addiction.

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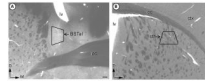
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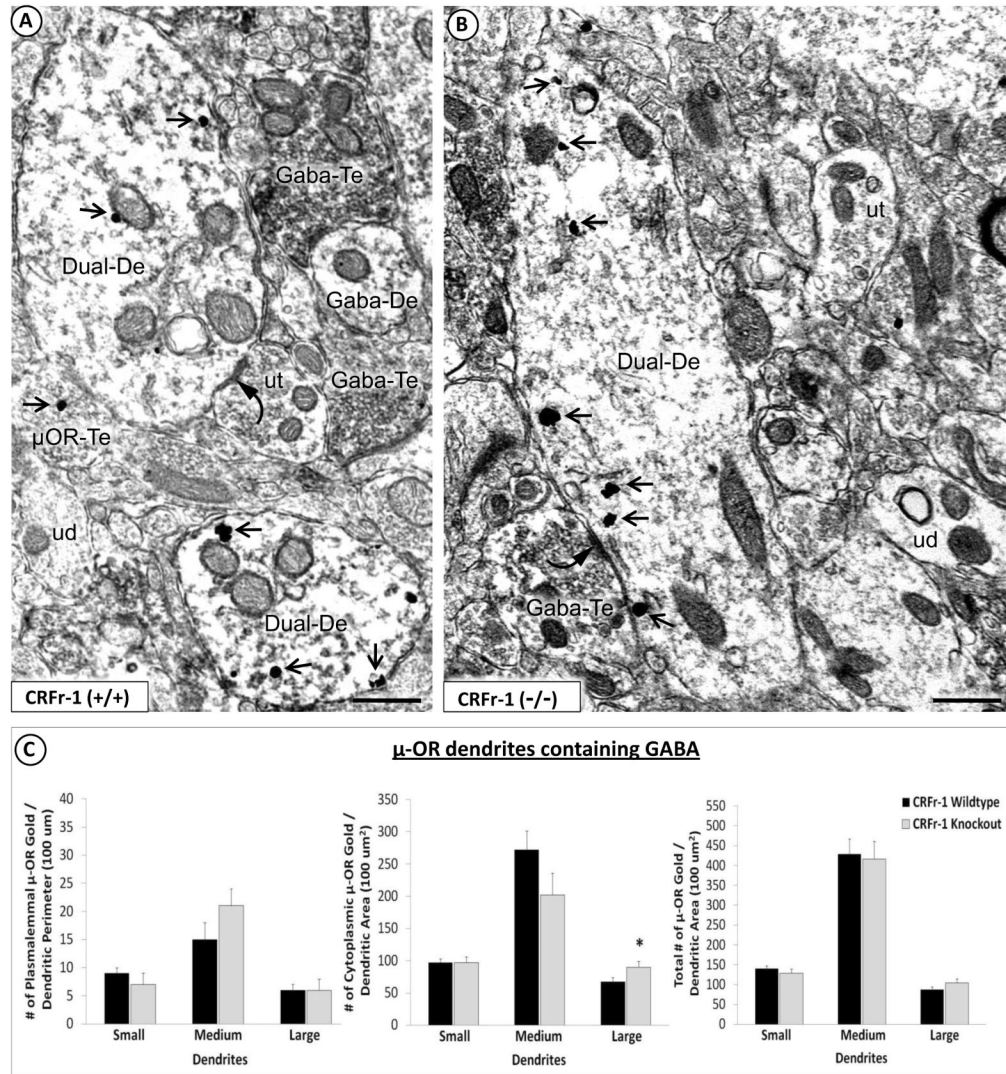
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**Fig. 1.**

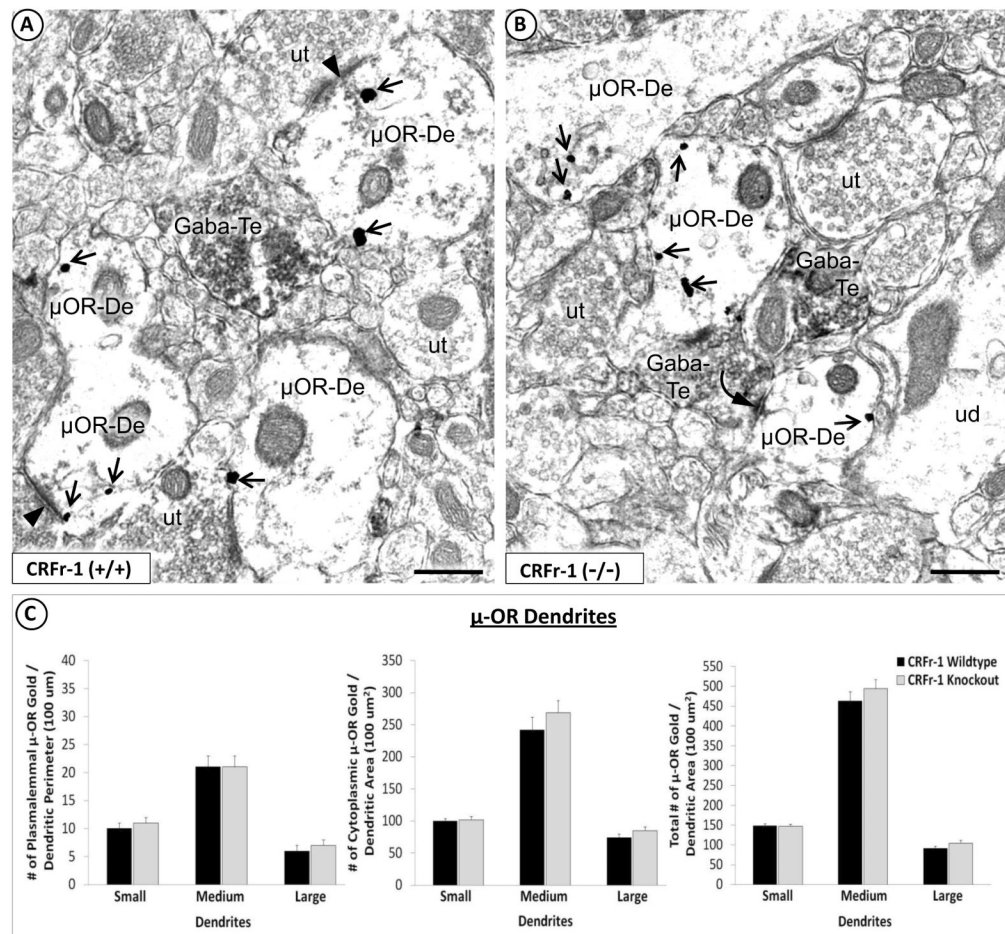
Brain regions sampled for electron microscopy. The BNST (A) and striatal (B) subregions sampled for electron microscopy are shown by the trapezoids in plastic-embedded coronal hemisections through the region of the anterior commissure (ac) in a mouse brain. These coronal sections were collected 0.2 mm anterior to Bregma (Hof et al, 2000). Corner arrows point dorsal (D), medial (M) or lateral (L) with respect to the brain surface. BSTal = anterolateral bed nucleus of the stria terminalis, Lv = lateral ventricle, cc = corpus callosum, str = dorsolateral striatum, ctx = somatosensory cortex. Scale bar = 100  $\mu$ m.





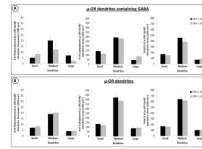
**Fig. 2.** Qualitative and quantitative evidence for enhanced cytoplasmic  $\mu$ -OR immunolabeling in large GABA-containing dendrites of the BSTal in CRF-1 receptor KO compared with WT mice. Electron micrographs show  $\mu$ -OR immunogold particles in dendrites that contain GABA immunoperoxidase in the BSTal of CRF-1 receptor WT (A) and KO mice (B). Shown in (A) are GABA immunoperoxidase-labeled dendrites with (Dual-De) or without (GABA-De)  $\mu$ -OR immunogold particles (arrow) within the cytoplasm and contacting the plasma membrane. Dendrites co-labeled for the  $\mu$ -OR and GABA (Dual-De) in (A) are apposed to, or receive symmetric, inhibitory-type synapses (curved arrow) from, axon terminals that are unlabeled (ut) or that contain  $\mu$ -OR immunogold labeling ( $\mu$ OR-Te). Also seen in the neuropil in (A) is a dendrite that contains GABA peroxidase (GABA-De) but lacks any detectable  $\mu$ -OR immunogold labeling. The dual-labeled dendrite in (B) receives a symmetric synapse (curved arrow) from an axon terminal that contains GABA peroxidase (GABA-Te). In (A) and (B) are unlabeled dendrites that are without either immunoperoxidase or immunogold labeling (ud). Scale bars = 500 nm. Bar graphs in (C) show plasmalemmal (number/perimeter length), cytoplasmic (number/area) and total (number/area) density of  $\mu$ -OR immunogold particles in GABA-immunoreactive dendrites of

varying sizes (small, medium, and large) in 17,893  $\mu\text{m}^2$  of the BSTal in CRF-1 receptor KO and WT mice (n = 4 mice per group). The asterisk (\*) indicates that, as compared with WT, the CRF-1 receptor KO group has a significantly greater cytoplasmic  $\mu$ -OR immunogold particle density. \* p < 0.05. Error bars indicate SEM.

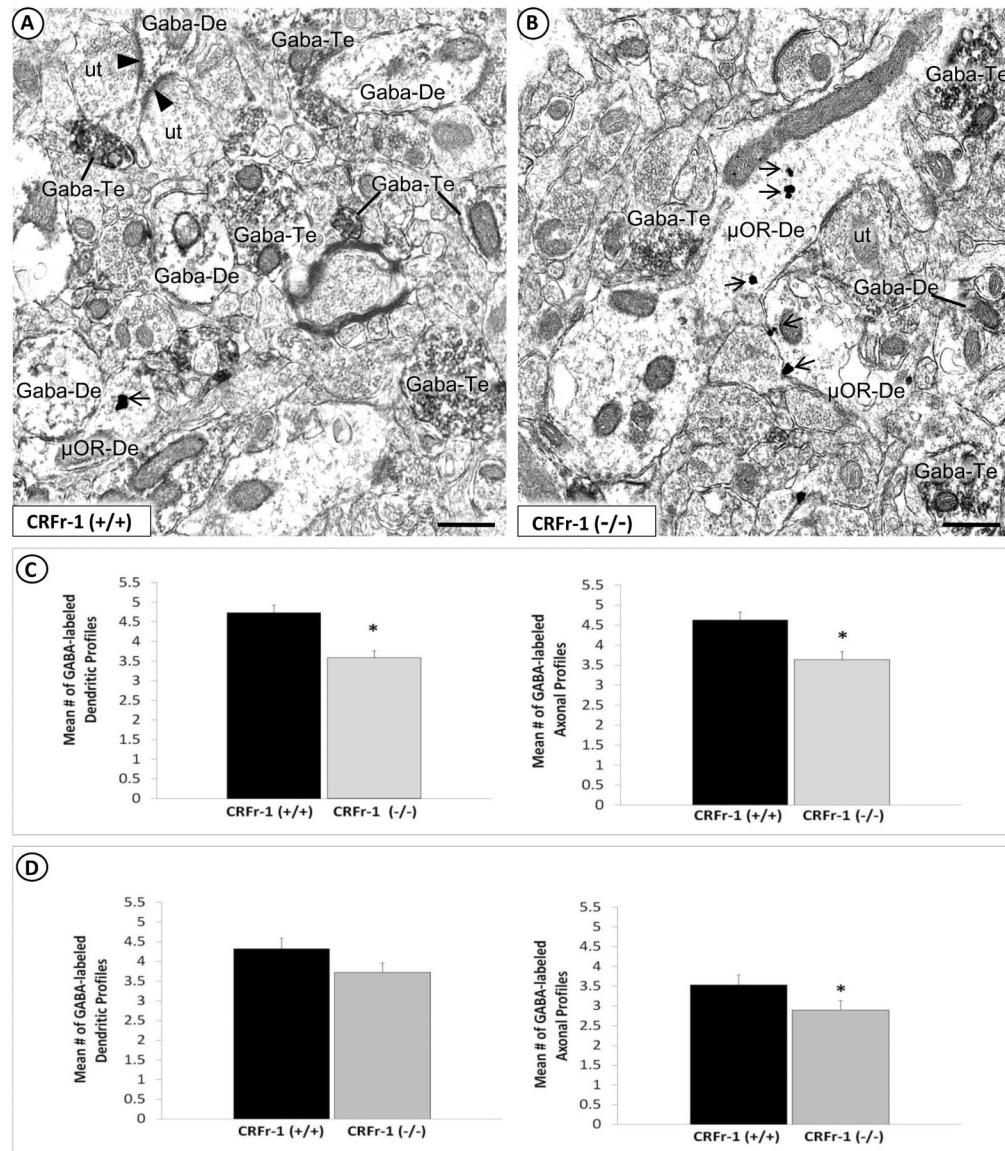


**Fig. 3.**

Absence of a global effect of CRF-1 receptor deletion in the total population of  $\mu$ -OR-containing dendrites. Electron micrographs show the similarity of  $\mu$ -OR immunogold distribution in dendrites that lack detectable GABA peroxidase in the BSTal of CRF-1 receptor WT (A) or KO mice (B). In (A),  $\mu$ -OR immunogold particles (arrow) are distributed in the cytoplasm, on the plasma membrane and near asymmetric, excitatory-type synapses (arrowhead) in dendrites ( $\mu$ OR-De). The  $\mu$ -OR-labeled dendrites in (A) receive asymmetric, excitatory-type synapses (arrowhead) from unlabeled axon terminals (ut).  $\mu$ -OR-labeled dendrites also receive symmetric, inhibitory-type synapses (curved arrow) from axon terminals that contain GABA peroxidase (GABA-Te), as seen in (B). ud = unlabeled dendrite. Scale bars = 500 nm. Bar graphs in (C) show a lack of significant differences between CRF-1 receptor KO and WT mice in the plasmalemmal (number/perimeter length), cytoplasmic (number/area) or total (number/area) density of  $\mu$ -OR immunogold particles in the total population of BSTal dendrites whether or not they contain GABA immunoperoxidase (n = 4 mice per group). Error bars indicate SEM.



**Fig. 4.** Quantitative analyses of  $\mu$ -OR immunogold labeling in dendrites of varying sizes (small, medium, large) are shown for the dorsal striatum, used as a region of comparison for the BSTal. Bar graphs show plasmalemmal (number/perimeter length), cytoplasmic (number/area) and total (number/area) density of  $\mu$ -OR immunogold particles in GABA-immunoreactive dendrites (A) or in all dendrites with and without GABA immunoreactivity (B) in CRF-1 receptor KO and WT mice (n = 4 mice per group). Error bars indicate SEM.



**Fig. 5.** Region-specific differences between CRF-1 receptor KO and WT in the prevalence of GABA-labeled dendrites and axon terminals in the BSTal, but only axon terminals in the dorsal striatum. Electron micrographs show GABA immunoperoxidase-labeled dendrites (GABA-De) and axon terminals (GABA-Te) of CRF-1 receptor WT (A) and KO (B) mice. A GABA-immunoreactive dendrite in (A) receives two asymmetric, excitatory-type synapses (arrowhead) from unlabeled axon terminals (ut). GABA peroxidase-containing dendrites and axon terminals in (A) and (B) are seen in the neuropil with dendrites ( $\mu$ OR-De) that contain  $\mu$ -OR immunogold particles (arrow). Note that many more GABA-labeled dendrites as well as axon terminals are seen in the BSTal of WT (A) compared with CRF-1 receptor KO (B). Scale bars = 500 nm. Bar graphs in (C) show a significant reduction in the mean number of GABA immunoperoxidase-labeled dendrites and axon terminals per neuropil area in the BSTal of CRF-1 receptor KO and WT mice. In the striatum (D), there is also a significant reduction in GABA-labeled axon terminals, but no significant difference in

dendrites of CRF-1 receptor KO compared with WT mice (n = 4 mice per group). \*  $p < 0.05$ . Error bars indicate SEM.