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Cannabinoid-1 (CB1) receptor gene deletion has a compartment-specific affect on the dendritic and axonal availability of mu-opioid receptors and on dopamine axons in the mouse nucleus accumbens

Diane A. Lane¹, June Chan¹, Carl R. Lupica², and Virginia M. Pickel¹

¹ Department of Neurology and Neuroscience, Weill Med. Col. of Cornell University, New York, NY 10021

² Electrophysiology Research Section, Cellular Neurobiology Branch, National Institute on Drug Abuse Intramural Research Program, Baltimore MD 21224

Abstract

Cannabinoid-type 1 (CB1) receptors are implicated in mu-opioid receptor (mu-OR)-dependent reward ascribed partially to mesolimbic dopamine release in the nucleus accumbens (Acb) shell. Thus, CB1 receptor gene deletion may preferentially alter the availability of mu-ORs and/or dopamine innervation in this brain region, which is functionally distinct from the motor-associated Acb core. To test this hypothesis, we examined the electron microscopic immunolabeling of the mu-OR and the dopamine-synthesizing enzyme, tyrosine hydroxylase (TH) in Acb shell and core of adult C57BL/6J wild-type (WT) and CB1-knock-out (KO) mice. The mu-OR-immunogold particles were observed in the cytoplasm and on the plasmalemma in dendrites, dendritic spines, and axon terminals throughout the Acb. Compared with WT, the Acb shell of CB1-KO mice showed a lower cytoplasmic density of mu-ORs in dendrites, and fewer mu-OR labeled, but not unlabeled, dendritic spines. In this region, the CB1-KO's had a significantly enhanced plasmalemmal density of mu-OR-immunogold in axon terminals, 70% of which formed excitatory-type synapses. However, the number of both mu-OR-labeled terminals and TH-labeled small varicosities was significantly reduced in the Acb shell of CB1-KO's. These adaptations were not seen in the Acb core, where CB1-KO's had a preferentially lower dendritic plasmalemmal and total spine density of mu-OR immunogold. Our results indicate that constitutive deletion of the CB1 receptor gene has a major impact on the pre- and post-synaptic availability of mu-ORs at axospinous synapses and on the dopamine innervation of the Acb shell, as well as the dendritic surface expression of mu-ORs in Acb core of mature rodents.

Keywords

development; mouse; reward; drug addiction; immunolabeling

Introduction

Endocannabinoids and the primary psychoactive constituent of marijuana, Delta⁹-tetrahydrocannabinol (THC), activate cannabinoid-1 (CB1) receptors that are present in many limbic brain regions including the nucleus accumbens (Acb) shell. This structure is

*Correspondence to: Virginia M. Pickel, Department of Neurology and Neuroscience, Cornell University Medical College, 407 East 61st St., New York, NY 10065, Tel: (646) 962-8275, Fax: (646) 962-0535, vpickel@med.cornell.edu.

critically involved in the motivational effects of opiates and other drugs as well as natural (food) reinforcers (Gardner and Vorel, 1998; Duarte et al., 2004). Enhanced release of dopamine in the Acb, and particularly the Acb shell is essential for reward and reinforcement as well as locomotor activation (Li et al., 2009). Compared with the Acb shell, the Acb core receives less dopamine input from the VTA (Zahm, 1999) and plays a greater role in motor performance (Heimer et al., 1997; Zahm, 2000).

The CB1 receptors are expressed in inhibitory- and excitatory-type axon terminals, the latter of which are enriched in the Acb shell of normal rodents (Pickel et al., 2004). Activation of presynaptic CB1 receptors is essential for long-term depression (LTD) of synaptic activity (Robbe et al., 2002). However, THC can block retrograde signaling in the Acb when administered either acutely (Mato et al., 2004) or chronically (Hoffman et al., 2003) thus significantly affecting the Acb output neurons (Hoffman and Lupica, 2001; Robbe et al., 2001; 2002). The mu-opioid receptor (mu-OR) in the Acb is also present in structurally and chemically diverse axon terminals and projection neurons (Mansour et al., 1995; Svingos et al., 1997) inclusive of some of those that contain CB1 receptors (Pickel et al., 2004). In contrast with the CB1 receptor, however, the mu-OR plays a more decisive role in the induction of long-term potentiation (LTP) ascribed to suppression of GABAergic inhibition (Bramham and Sarvey, 1996; Nugent et al., 2007).

In both the Acb shell and dorsal striatum, a brain region having more anatomical and functionally similarity to the Acb core, CB1 receptors are not only co-expressed with mu-ORs, but also re present in axon terminals providing synaptic input to neurons that contain mu-ORs (Rodriguez et al., 2001; Pickel et al., 2004). These observations suggest important functional links between CB1 and mu-opioid receptors, which are supported by *in vivo* cannabinoid-induced displacement of the [³H]diprenorphine binding to opioid receptors in mouse brain (Valverde et al., 2001). Moreover, blockade of CB1 receptors by acute administration of SR141716A prevents opiate self-administration in rats and mice (Navarro et al., 2001) as does constitutive deletion of the CB1 receptor gene in CB1 knock-out (KO) mice (Martin et al., 2002).

CB1-KO mice have increased expression of striatal endogenous opioid (dynorphin and enkephalin) peptides (Steiner et al., 1999; Zimmer et al., 1999), and enkephalin can induce the internalization of mu-ORs (Arden et al., 1995; Sternini et al., 2000). Thus, the enhanced striatal expression of opioid peptides may significantly reduce the surface/synaptic availability of mu-ORs. However, there is no structural evidence indicating that the subcellular distribution of mu-opioid receptors in the Acb is affected by constitutive deletion of the CB1 receptor gene. Furthermore, the behavioral phenotype of CB1-KO mice is at least partially strain dependent. The CB1-KO mice of the C57BL/6J, but not the CD-1 strains are hypoactive, even though both strains show a reduction in positive reinforcement (Ledent et al., 1999; Steiner et al., 1999; Zimmer et al., 1999; Li et al., 2009). The cellular adaptations that contribute to this complex behavioral phenotype in the CB1-KO mice of the C57BL/6J are not known, but may include changes in both the endogenous opioid and mesolimbic dopamine reward systems. The hypomotor phenotype in CB1-KO mice of the C57BL/6J strain might also be ascribed to the availability of mu-OR and/or dopamine in the motor-associated Acb core (Heimer et al., 1997; Zahm, 2000). We tested the hypothesis that CB1-KO mice of the C57BL/6J strain differ from wild-type (WT) mice in the compartment-specific neuronal targeting of mu-OR and dopamine inputs to the Acb. For this, we quantitatively compared C57BL/6J WT and CB1-KO mice with respect to their neuronal distribution of mu-OR immunogold and immunoperoxidase labeling for the catecholamine-synthesizing enzyme, tyrosine hydroxylase (TH) in the Acb shell and core.

Materials and Methods

Animals

Experiments in adult age-matched C57BL/6J wild-type and CB1 (−/−) mutant mice were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committees (IACUC) at Weill Medical College of Cornell University and the NIH/NIDA Intramural Research Program. The CB1 (−/−) mice were generated by mutation of the CB1 gene in MP12 embryonic stem cells by using standard techniques (Zimmer et al., 1999). Genotyping of the mice was performed by Charles River Laboratories (Raleigh, NC, USA).

Fixation and sectioning

The mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg), and subject to perfusion fixation by the sequential vascular perfusion through the left ventricle of the heart with (1) 5 ml heparin saline (2) 30 ml of 0.1M phosphate buffer (PB) containing 3.75% acrolein and 2% paraformaldehyde, and (3) 100 ml of 2% paraformaldehyde in PB. The brains were then removed from the cranium and post-fixed for 30 min in 2% paraformaldehyde in PB. Coronal sections of 40 μm were cut using a Leica Vibratome (Leica Microsystems, Bannockburn, IL) through the rostrocaudal extent of the Acb, extending from 0.98 to 1.54 mm caudal to Bregma as determined from a mouse brain atlas (Franklin and Paxinos, 1997). The cut sections were collected in 0.1 M PB, and processed for dual electron microscopic immunolabeling for antisera raised against mu-OR and TH.

Antisera

A polyclonal antiserum was raised in rabbits against a synthetic 15-residue peptide sequence corresponding to C-terminus amino acids 384–398 predicted from the cloned rat mu-opioid receptor (Chen et al., 1993) (Catalog number 24216, Immunostar, Inc., Hudson, W.I.). Prior adsorption of the antiserum with mu-OR peptide (384–398) at 10 μg/ml eliminates labeling. Moreover, Western blots using this antiserum shows labeling in mu-OR transfected, but not in non-transfected COS-7 and Neuro2a cells (Immunostar and (Arvidsson et al., 1995). The antiserum has been used extensively for light and electron microscopic immunolabeling studies in the Acb (Gracy et al., 1997; Pickel and Colago, 1999; Svingos et al., 2001). The neuronal mu-OR immunolabeling with this antiserum is comparable to that of mu-OR mRNA as seen by *in situ* hybridization (Mansour et al., 1994; Mansour et al., 1995).

A monoclonal mouse antibody against TH (mol WT, 60kDa) was obtained commercially from Immunostar, Inc. (Catalog number 22941), who have shown that this antibody identifies TH in TH-transfected, but not non-transfected cells. This antibody also specifically recognizes TH and not other catecholamine synthesizing enzymes, and has been localized in dopaminergic as well as other catecholaminergic cell groups throughout brain (Nirenberg et al., 1996; Yang et al., 1997; Lessard and Pickel, 2005).

Electron Microscopic Immunolabeling

The prepared tissue sections from CB1-KO and WT mice were co-incubated overnight (4 °C) plus one day (room temperature) in a 0.1 M Tris-buffered saline (TS) solution containing mouse anti-TH (1:10,000) and rabbit anti-mu-OR (1:1,000). Subsequently, the tissue was passed sequentially through several washes in TS followed by a 30 min incubation in a TS solution containing 0.1% bovine serum albumin (BSA) and horse anti-mouse biotinylated IgG (1:400; Jackson) for identification of the mouse anti-TH. The biotinylated IgG was then detected by incubation of the tissue in Vector Stain ABC Elite kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction product was visualized by

reaction for 6 min in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide.

The peroxidase-reacted tissue sections were returned to the primary antisera solution for one additional day, a modification of our originally developed dual labeling method Chan et al., (1990) that improved the mu-OR immunogold labeling. These sections then were washed in TS, and incubated for 2 hr in a 1:50 dilution of Ultrasmall gold (Electron Microscopy Sciences, Hatfield, PA) conjugated to donkey anti-rabbit IgG. The sections were rinsed in 0.1M PBS, and post-fixed with 2% glutaraldehyde in 0.1M PB for 10 min, then rinsed in 0.1M PB followed by rinses in 0.2 M citric acid. The gold particles were visualized by reaction for 7 min at room temperature using the Silver IntensEM kit (GE Healthcare, Piscataway, NJ). The silver-intensified sections were rinsed in 0.2 M citrate buffer followed by a second rinse in 0.1 M PB, and a one hr incubation in 2% osmium tetroxide in 0.1M PB before embedding in epoxy resin between sheets of Aclar plastic using conventional electron microscopic methods (Leranth and Pickel, 1989) and the Embed 812 kit (Electron Microscopy Sciences, Hatfield, Pa).

Data Analysis

The regions of the ventromedial Acb shell and dorsomedial core (Franklin and Paxinos, 1997) were chosen for electron microscopic analysis. Ultrathin sections were cut from the surface of the tissue with a Leica ultramicrotome, counterstained using uranyl acetate and lead citrate (Reynolds, 1963), and examined with a FEI Tecnai 12 transmission electron microscope. The thin sections were initially examined at low (8–9K) magnification to identify the surface of the tissue, and those regions showing immunolabeling of both the mu-OR and TH. These were then magnified and captured as digital images by an investigator, who was unaware of whether the tissue was derived from CB1 (–/–) or wild-type (WT) mice.

Immunoperoxidase labeling was regarded as positive when the electron dense precipitate in individual profiles was greater than that seen in other morphologically similar profiles in the neuropil. Immunogold-labeled structures were identified as those containing one or more gold particles. This method was validated by determining in immunogold labeled tissue, the number of spurious gold-silver deposits overlying myelin and other structures, not known to express mu-opioid receptors.

Quantitative regional comparisons of the distribution of mu-OR and TH labeling patterns in Acb shell and core were conducted independently in tissue defined as being from CB1 (–/–) and wild-type mice only after completion of the data acquisition. For this, we examined thin sections that were collected equally from the surface of two vibratome sections from the Acb shell or core of 4 CB1-KO and 5 WT mice. In each region, 35–60 electron microscopic images were obtained from the tissue-plastic interface showing immunolabeling for both antisera. In these images, the immunolabeled and unlabeled neuronal profiles were defined using the guidelines provided by Peters et al., (1991). These neuronal profiles included varying sized dendrites and dendritic spines, small (<0.15 μ m cross-sectional diameter) axons typically containing neurotubules, but not synaptic vesicles, and large (>0.15 μ m cross-sectional) vesicle-filled axon terminals that were without recognizable synapses or formed either asymmetric or symmetric synapses with defined targets. Morphometric image analysis was performed on labeled profiles from at least 30 micrographs from each block of tissue. Micrographs were tallied for number of TH or Mu-OR labeled profiles in a given neuropil area and for the subcellular location of individual immunogold-silver deposits identifying the mu-OR immunoreactivity in dendritic or axonal compartments. Numbers of unlabeled profiles were also tallied where necessary for comparison with the number of labeled structures in the neuropil. In both WT and CB1-KO mice, we examined

approximately 15,000 μm^2 of tissue from the Acb shell and 8,000 μm^2 from the Acb core. Statistical comparisons were made using ANOVA and Chi-Square analysis for categorical data generated by JMP v.5 software (SAS Institute, Cary, NC) and graphed using Excel software. Microscopic illustrations were prepared by importing digital images into Adobe Photoshop 7.0 and Powerpoint, Microsoft Office, 2007. Software of these programs was used to enhance contrast, prepare composite plates, and add lettering.

Results

Mu-OR immunogold labeling was observed in many dendrites and dendritic spines as well as small axons and axon terminals within the Acb shell and core of both wild-type (WT) and CB1 receptor ($-/-$) knock out (KO) mice. There were, however, region-specific differences between the Acb shell and core in the extent to which CB1 gene deletion affected the mu-OR immunogold distribution and numbers of mu-OR labeled dendritic and axonal profiles. In addition to the notable differences between CB1-KO and WT mice in the Acb neuronal distribution of mu-OR immunogold, the CB1-deficient mice also showed a significant region-specific reduction in the number/area of small axonal varicosities containing immunoperoxidase labeling for TH in the Acb shell.

Respective decrease in cytoplasmic and plasmalemmal mu-ORs in medium dendrites within the Acb shell and core of CB1-KO mice

In both the Acb shell and core, the CB1-KO and WT mice differed in mu-OR immunogold distributions in medium dendritic profiles, whose minor axis measured 0.7–1.0 μm in transverse sections. Similar differences were not seen in either the small (0–0.7 μm) or large (>1.1 μm) diameter dendrites in either of these regions.

In the Acb shell, immunogold labeling for the mu-OR was often located within the cytoplasm distal to, or in contact with the plasma membrane in medium dendrites (Fig. 1A, B). The mu-OR immunogold particles on the plasma membrane were largely located at extrasynaptic sites near appositional contacts with small axons (Fig., 1A, B) or filamentous glial processes. As compared with WT, the CB1-KO mice showed a qualitative (Fig. 1A, B) and statistically significant quantitative (Fig. 1C) reduction in the cytoplasmic ($F(1,986) = 5.22$, $p < 0.05$) and total (cytoplasmic plus plasmalemmal; $F(1,986) = 7.45$, $p < 0.05$) density of mu-OR immunogold particles in medium dendrites of the Acb shell. However, there was no significant effect of CB1 deletion on number of either mu-OR labeled ($F(1,17) = 0.38$, $p > 0.05$; Fig. 1D) or unlabeled ($F(1,17) = 0.17$, $p > 0.05$; Fig. 1E) dendrites in this region.

As compared with the Acb shell, the mu-OR immunogold labeling in the Acb core appeared more prevalent on the plasma membrane and was more rarely observed in the cytoplasm of medium dendrites in either WT (Fig. 2A) or CB1-KO (Fig. 2B) mice. Moreover, in the Acb core, the plasmalemmal ($F(1,316) = 7.74$, $p < 0.05$), but not cytoplasmic ($F(1,316) = 1.31$, $p > 0.05$) or total (plasmalemmal and cytoplasmic; $F(1,316) = 4.98$, $p > 0.05$) density of mu-OR immunogold particles in dendrites was significantly reduced in CB1-KO compared with WT mice (Fig. 2C). The CB1-KO mice also had a significant reduction in density (number/area) of mu-OR labeled dendritic profiles ($F(1,16) = 8.48$, $p < 0.05$; Fig. 2D) and a small, but not significant, increase in the density of unlabeled dendritic profiles ($F(1,16) = 2.18$, $p > 0.05$; Fig. 2E) in the Acb core. Only transversely sectioned dendritic profiles were included in the analysis, suggesting that the reduction in number of mu-OR labeled dendrites in the Acb core is directly ascribed to the more sparsely distributed plasmalemmal immunogold particles in the CB1-KO compared with WT mice.

Region-specific decrease in the number of Mu-OR labeled dendritic spines in Acb shell and in the density of mu-OR immunogold in spines of the Acb core

Mu-OR immunogold particles were located in the cytoplasm and on subsynaptic and perisynaptic plasma membranes near asymmetric excitatory-type synapses on dendritic spines in the Acb shell of both WT (Fig. 3A) and CB1-KO (Fig. 3B). This distribution was similar to that observed in the Acb core, where the mu-OR immunogold particles also were located in the cytoplasm on mainly non-synaptic plasma membrane of dendritic spines in WT (Fig. 3C) and CB1-KO mice (Fig. 3D). However, the deletion of the CB1 receptor produced notable differences between the Acb shell and core in mu-OR immunolabeling in dendritic spines. In the Acb shell, the CB1-KO mice compared with WT had significantly fewer mu-OR labeled ($F(1,17) = 3.40, p < 0.05$; Fig. 3E), but not unlabeled ($F(1,17) = 0.20, p > 0.05$; Fig. 3F) dendritic spines. There was also a small, but non-significant increase in plasmalemmal ($F(1,653) = 0.37, p > 0.05$; Fig. 3G) and decrease in cytoplasmic ($F(1,653) = 0.79, p > 0.05$; Fig. 3H) and total ($F(1,653) = 0.35, p > 0.05$; Fig. 3I) density of mu-OR immunogold in dendritic spines of the Acb shell in CB1-KO compared with WT mice. In contrast with the Acb shell, we observed no significant difference between CB1-KO and WT mice in the number of mu-OR labeled dendrites in Acb core ($F(1,16) = 2.07, p > 0.05$). In this region, however, the total (plasmalemmal and cytoplasmic) density of mu-OR immunogold particles in dendritic spines was significantly reduced in CB1-KO compared with WT mice ($F(1,644) = 5.68, p < 0.05$; Fig. 3I).

Increased plasmalemmal Mu-OR in largely excitatory-type axon terminals that are less abundant in the Acb shell of CB1-KO mice

Mu-OR immunogold particles were located on the plasmalemmal surface and within the cytoplasm of many axon terminals defined by their accumulation of synaptic vesicles in the Acb shell of both WT (Fig. 4A) and CB1-KO (Fig. 4B) mice. In this region, approximately the same percentage of mu-OR labeled terminals formed asymmetric, excitatory-type synapses in the WT (69% 430/625) and CB1-KO mice (70% 347/494). The majority of these asymmetric synapses were on dendritic spines without detectable mu-OR labeling (Fig. 4A, B). In both CB1-KO and control mice, approximately 30% of the mu-OR immunogold labeled terminals in the Acb shell formed symmetric, inhibitory-type synapses.

In comparison with WT (Fig. 4A), the CB1-KO mice (Fig. 4B) showed a qualitative and significant quantitative ($F(1,660) = 4.45, p < 0.05$; Fig. 4E) increase in the presynaptic plasmalemmal density of mu-OR immunogold particles in axon terminals of the Acb shell. The increased presynaptic plasmalemmal density of mu-OR immunogold was paralleled by a small, but not statistically significant increase in number of synaptic contacts formed by individual axon terminals in the Acb shell of CB1-KO and WT mice ($\chi^2 = 3.7, p > 0.05$). However, significantly fewer mu-OR containing axon terminals/neuropil area were observed in the Acb shell of CB1-KO compared with WT mice ($\chi^2 = 36.07, p < 0.05$; 494 vs. 625 terminals, respectively).

In the Acb core, the mu-OR immunogold was somewhat more prevalent in the cytoplasm than on the plasma membrane of axon terminals in both WT (Fig. 4C) and CB1-KO (Fig. 4D) mice. These labeled terminals formed asymmetric axospinous or more rarely symmetric axodendritic synapses on neurons that usually did not contain mu-OR immunogold. In this region, we observed no significant differences between CB1-KO and WT mice in either the plasmalemmal density of mu-OR immunogold ($F(1,1077) = 0.17, p > 0.05$; Fig. 4E) or prevalence (number/area) of mu-OR labeled axon terminals ($F(1,16) = 2.07, p > 0.05$; Fig. 4F).

Decreased prevalence of small TH-labeled varicosities in Acb shell of CB1-KO mice

Immunoperoxidase labeling for TH was seen in small axons having a cross-sectional area of $<0.1 \mu\text{m}^2$, and in either small ($0.1\text{--}0.28 \mu\text{m}^2$) or large ($> 0.28 \mu\text{m}^2$) vesicle-filled varicosities in the Acb shell. In the Acb shell of WT mice, the small TH-containing varicosities frequently contacted the necks or heads of dendritic spines receiving excitatory-type synapses (Fig. 5A), whereas the larger varicosities more commonly apposed or formed symmetric synapses with dendritic shafts. The small, but not the large TH-labeled axonal varicosities appeared less abundant in the Acb shell of the CB1-KO mice (Fig. 5B). These large TH-containing varicosities sometimes converged with mu-OR labeled terminals on a common dendrite (Fig. 5B). The qualitative assessment of reduced prevalence of small TH-labeled axonal profiles in the Acb shell of CB1-KO compared with WT mice was quantitatively confirmed and shown to be statistically significant ($F(1,17) = 28.11$, $p < 0.05$; Fig. 5E). Similar types of TH-immunoreactive axonal varicosities were seen in the Acb core, but in this region there was no apparent difference between the WT (Fig. 5C) and CB1-KO (Fig. 5D) in the amount of large or small TH-labeled varicosities. No significant difference between CB1-KO and WT mice was seen in the number/neuropil area of either the small ($F(1,16) = 0.16$, $p > 0.05$) or large ($F(1,16) = 0.26$, $p > 0.05$) varicosities in the Acb core (Fig. 5F).

Discussion

Our results are summarized in Fig. 6, which is a schematic representation of the region-specific postsynaptic (dendritic and spinous) as well as the presynaptic (axonal) adaptations in the distributions of mu-ORs and in the number of TH-containing varicosities in the Acb of CB1-KO compared with WT mice. These postsynaptic adaptations include (1) a decrease in the dendritic cytoplasmic reserve of mu-ORs in medium dendrites of the Acb shell and in the plasmalemmal density of these receptors in the Acb core; and (2) a decrease in the number of mu-OR containing dendritic spines in the Acb shell and in the prevalence of these receptors in dendritic spines in the Acb core. The only presynaptic differences between CB1-KO and WT were seen in the Acb shell. These include (1) an increase in the mu-OR plasmalemmal density, but a reduction in the number of mainly excitatory-type mu-OR-labeled axon terminals; and (2) a decrease in the number of small-TH containing varicosities. Together, these results suggest that CB1 receptors are critical for expression and/or dendritic plasmalemmal targeting of mu-ORs in the Acb shell and core, and preferentially affect mu-OR dependent inhibition of transmitter release and mesolimbic dopamine innervation of the Acb shell. These results are discussed with respect to their implications for understanding the role of CB1 receptors in positive reinforcement and in motor performance.

CB1 receptor gene deletion reduces the dendritic cytoplasmic reserve of mu-OR and the density of mu-OR containing dendritic spines in Acb shell: comparison with Acb core

The observed reduction in the cytoplasmic density of mu-OR immunogold particles in medium dendrites of the Acb shell in CB1-KO mice, suggests that the expression and/or trafficking of mu-ORs in these dendritic segments is suppressed by the absence of CB1 receptors throughout ontogeny or in the mature brain. The affected dendrites may be among those that normally express the mu-OR and receive synaptic input from axon terminals that contain the CB1 receptor (Pickel et al., 2004). The selectivity of the down-regulation of the cytoplasmic reserve of mu-ORs in the medium dendrites of CB1-KO is consistent with a disruption in CB1 receptor-mediated endocannabinoid signaling that inhibits the release of GABA and co-stored enkephalin-type opioid peptides (Kalivas et al., 1993; Svingos et al., 1997).

Fewer mu-OR labeled dendritic spines were seen in the Acb shell of CB1-KO compared with WT mice. Thus, mu-OR expression in dendritic spines may be suppressed below detectable immunolabeling possibly through mechanisms comparable to those occurring in the medium dendrites. However, there was no significant difference between CB1-KO and WT mice in density of unlabeled dendritic spines in the Acb shell, where a preferential reduction in mu-OR levels in existing spines would necessitate a concomitant increase in the area density of unlabeled spines. A more tenable alternative interpretation of our results is a preferential reduction in the formation and/or maintenance of mu-OR containing dendritic spines in the Acb shell of mice deprived of CB1 receptor-mediated endocannabinoid signaling during postnatal development. Synaptogenesis is one of the many developmental processes that is dependent on CB1 receptor-mediated endocannabinoid signaling (Basavarajappa et al., 2009; Fride et al., 2009). Moreover, the expression of mu-OR in striatal dendritic spines parallels that of the formation of excitatory synapses (Wang et al., 2003) suggesting that the presently observed reduction in mu-OR labeled dendritic spines in the Acb shell of CB1-KO mice may be ascribed not only to high levels of endogenous opioid peptides (Liao et al., 2005), but also altered cortico-striatal transmission.

The adaptations in the circuitry expressing mu-OR in the Acb shell was not observed in the Acb core, where instead there was a significant reduction in mu-OR plasmalemmal density in medium dendrites and in both the plasmalemma and cytoplasmic density of these receptors in dendritic spines of CB1-KO mice. Together, these observations suggest that the constitutive expression of the CB1 receptor gene is a major determinant of the plasmalemmal targeting of mu-ORs in both dendrites and dendritic spines of the Acb core. The reduction in the plasmalemmal density of mu-OR receptors in dendrites of the Acb core may be the primary basis for the observed reduction in number of mu-OR labeled dendrites, since only those transverse sections passing through portions of the plasma membrane expressing mu-OR would be identified as labeled (Fig. 6). The decreased plasmalemmal availability of dendritic mu-OR in the Acb core of CB1-KO mice is consistent with the enhanced expression of endogenous opioid peptides in the anatomically and functionally similar dorsal striatum (Steiner et al., 1999; Zahm, 1999). Enkephalin-type opioid peptides reduce the dendritic surface expression of both mu- and delta opioid receptors (Afify et al., 1998; Zaki et al., 2000). The functional importance of enkephalin over-production in the CB1-KO mice is evidenced by the fact that prevention of enkephalin degradation eliminates their differences from WT in locomotor activity, anxiety and antidepressant-like effects of opiates (Jardinaud et al., 2005).

CB1-KO reduces the number of mu-OR containing terminals, but increases their plasmalemmal density in the Acb shell: comparison with Acb core

We observed significantly fewer mu-OR containing axon terminals in the Acb shell of CB1-KO compared with WT mice. This reduction was equally applicable to the terminals forming asymmetric, excitatory-type or symmetric inhibitory-type synapses, the latter of which were far less abundant than the asymmetric synapses in both CB1-KO and WT mice. In the Acb shell of WT mice, the mu-OR-containing terminals formed asymmetric, often perforated postsynaptic membrane specializations with dendritic spines that were commonly without detectable mu-OR. These observed perforations are comparable to those described for excitatory-type terminals expressing CB1 receptors and/or mu-OR in the rat Acb shell (Pickel et al., 2004). Perforated excitatory-type synapses are frequently associated with synaptic plasticity (Calverley and Jones, 1990; Jones and Harris, 1995) that is dependent on presynaptic CB1 receptors in glutamatergic cortico-striatal inputs to GABAergic neurons in the Acb shell (Robbe et al., 2001). Thus, the presently observed reduction in mu-OR containing terminals, 70% of which form excitatory-type synapses on dendritic spines in Acb shell may partially reflect arrest in the plasticity of cortico-striatal inputs in absence of

CB1 receptors (Robbe et al., 2001; 2002). This mechanism would be expected to be effective in those terminals that co-express CB1 and mu-OR, which we have shown to include both excitatory- and inhibitory-type terminals in the rat Acb (Pickel et al., 2004).

Although reduced in number, the mu-OR containing axon terminals of CB1-KO mice expressed a significantly greater plasmalemmal density of mu-OR immunogold particles and also showed a trend toward increased numbers of synaptic contacts (Fig. 6). Together, these observations suggest increased capacity for mu-OR active opiates to inhibit the release of glutamate or other transmitters onto GABAergic spiny neurons, and particularly those that do not express the mu-OR in the Acb (Svingos et al., 1997). These target neurons likely include those that contain dopamine D2 receptors present in many Acb neurons expressing proenkephalin-derived opioids that are associated with positive reinforcement (Aubert et al., 2000).

In contrast with the Acb shell, the Acb core of CB1-KO and WT mice did not significantly differ in either number of mu-OR labeled terminals or in their content of mu-OR immunogold particles. This observation, together with the lower abundance of CB1-containing excitatory-type terminals in the Acb core versus shell (Pickel et al., 2004) suggest that CB1 receptor-dependent plasticity has a relatively minor role in determining the presynaptic efficacy of mu-OR mediated inhibition of transmitter release.

Fewer small TH-containing varicosities are present in the Acb shell of CB1-KO mice

We observed a significant reduction in small TH-labeled varicosities resembling those previously shown to express the plasmalemmal dopamine transporter (DAT) (Nirenberg et al., 1997) in the Acb shell of CB1-KO compared with WT mice. This reduction may reflect decreased expression and/or delivery of TH in mesolimbic dopaminergic projection neurons terminating preferentially in the Acb shell, a hypothesis supported by known cannabinoid-induced enhancement of TH mRNA expression during early brain development (Bonnin et al., 1995). The results of the present study are consistent with evidence that as compared with wild-type, CB1-KO ($-/-$) mice of the C57BL/6J strain are deficient in mesolimbic dopamine release affecting locomotor activation (Li et al., 2009).

Implications

Our results suggest that the presence of CB1 receptors is essential for the full expression of dendritic as well as axonal targeting of mu-opioid receptors the Acb shell, where these receptors are key substrates for reward function (Ledent et al., 1999; Valverde et al., 2000). Furthermore, in this region, we show a regionally selective decrease in mu-OR containing axon terminals and in small TH-expressing axonal varicosities, whose morphologies are similar to those of mesolimbic dopamine inputs in the CB1-KO mice. This region plays an essential role in dopamine-dependent positive reinforcement (Di Chiara et al., 2004; Di Chiara and Bassareo, 2007). Together, these observations suggest that the defective reward system in CB1-KO mice is ascribed not only to compartment-specific adaptations in mu-OR expression, but also decreased mesolimbic dopamine inputs in the Acb shell. The behavioral phenotype of the CB1-KO mice and responsivity to opiates, however, also may reflect the decreased plasmalemmal availability of mu-OR in dendritic shaft and/or dendritic spines in the Acb core (Fernandez-Ruiz et al., 2004; Nikoshkov et al., 2008). The changes observed in mu-OR distribution in the Acb core of the CB1-KO C57BL/6J mice are consistent with their hypoactivity, but may not be typical of other strains (Ledent et al., 1999; Steiner et al., 1999; Zimmer et al., 1999; Li et al., 2009).

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Fig. 1.

As compared with WT, CB1-KO mice show a qualitative (A, B) and quantitative (C) reduction in the cytoplasmic density of mu-OR immunogold particles in medium-diameter dendrites (C), but no differences from WT in number of mu-labeled (D), or unlabeled dendrites (E) in the Acb shell. In A and B, electron microscopic images show mu-OR immunogold particles within the cytoplasm (small arrows) and on the plasma membrane (arrowhead) in dendrites (Mu-OR-de). In A, one of the dendritic profiles contains a multivesicular body (mvb) and shows a single mu-OR immunogold particle (arrowhead) on portions of the plasma membrane contacted by a small unlabeled axonal profile (ax). In B, there is a similar location of the mu-OR immunogold on the dendritic plasma membrane opposed to a small axon. In this dendrite, the plasmalemmal mu-OR labeling is also in contact with a coated vesicle (cv), an organelle associated with internalization of G-protein coupled receptors. Scale bar = 0.5 μ m in A and B. In bar graph of C, the mean number of mu-OR immunogold particles is expressed per unit plasma membrane length of cytoplasmic area. In D and E, the respective bar graphs show lack of statistically significant (*, $p > 0.05$) difference between WT and CB1-KO mice in the numbers of mu-OR labeled or unlabeled dendrites in 1,000 μ m² area of tissue collected from the Acb shell of 4 CB1-KO and 5 WT mice.

Fig. 2.

Compared with WT, CB1-KO mice have a qualitative (A, B) and quantitative reduction in the plasma membrane density of mu-OR immunogold particles (arrowheads) in dendrites (C), and in the number of mu-OR-labeled (D), but not unlabeled (E) dendrites in equally sized (1,000 μm^2) Acb core neuropil. In the electron microscopic images of A and B, mu-OR labeled dendrites (Mu-OR-de) are opposed by axon terminals (Mu-OR-te) showing plasmalemmal (arrowheads) Mu-OR-immunogold labeling. These terminals form asymmetric synapses (white block arrows) with unlabeled dendritic spines and also have one immunogold particle located on portions of the membrane contacted by a small unlabeled axonal profile (ax). Scale bar= 0.5 μm . Bar graphs in C, show that as compared with WT, CB1-KO mice have a significantly (*, $p < 0.05$) lower plasma membrane, but not cytoplasmic or total density of mu-OR immunogold particles in dendritic profiles of the Acb core. Bar graph in D shows that as compared with WT, CB1-KO mice have a statistically significant (*, $p < 0.05$) reduction in mu-OR labeled dendrites in the Acb core. Within this area, there is a non-significant trend toward increased numbers of unlabeled dendrites in the CB1-KO compared with WT mice as shown in bar graph of E. The data in the bar graphs was taken from a neuropil area of 1,000 μm^2 that was collected in thin sections of the Acb core in 4 CB1-KO and 5 WT mice.

Fig. 3.

The mu-OR immunogold is seen on the plasma membrane and/or within the cytoplasm (small straight arrows) of dendritic spines (Mu-OR-s) receiving asymmetric (white block arrows) synapses that appears to be perforated (double white arrow heads) from unlabeled axon terminals in the Acb shell (A,B) and core (C,D) of both WT and CB1-KO mice. The TH-labeled axonal varicosities of small (TH-sv) and large (TH-lv) sizes are seen in the respective neuropil of the Acb shell in WT (A) and CB1-KO (B) mice. No TH-labeled terminals are evident in the Acb core of C and D, the latter of which shows plasmalemmal mu-OR immunogold (arrowhead) within a dendritic spine (mu-OR-s) and in an axon terminal (mu-OR-t) forming a synapse with an unlabeled spine (us). Scale bars = 0.5 μm . In E, the bar graphs indicate that as compared with WT, the CB1-KO mice have a statistically significant (*, $p < 0.05$) reduction in the area density of mu-OR labeled dendritic spines in the Acb shell, and approximately the same number of these dendritic profiles in the Acb core. In F, the bar graphs show a small, but non-significant (*, $p > 0.05$) reduction in numbers of unlabeled dendritic spines/neuropil area (ie., profile density) in Acb shell and core of the CB1-KO mice. The data in these bar graphs was taken from 1,000 μm^2 of tissue in Acb shell or core of 4 CB1-KO and 5 WT mice. Bar graphs in G, H, and I, respectively, show that the CB1-KO and WT mice do not significantly (*, $p > 0.05$) differ in plasmalemmal, cytoplasmic, or total density of mu-OR immunogold particles in dendritic spines of the Acb shell, although the total (cytoplasmic and plasmalemmal) density of these particles is significantly (*, $p < 0.05$) reduced in dendrites of the Acb core of the CB1-KO mice.

Fig. 4.

Electron microscopic images taken from the Acb shell of WT (A) and CB1-KO (B) show presynaptic mu-OR immunogold particles located respectively within the cytoplasm (small straight arrows) or on the plasma membrane (arrowheads) of axon terminals (mu-OR-te) that form asymmetric (white block arrow) synapses with two unlabeled dendritic spines. In the Acb core of both WT (C) and CB1-KO (D) mice, the presynaptic mu-OR immunogold is located in the cytoplasm of mu-OR-te, and these terminals form asymmetric synapses (block white arrow) with dendritic spines (mu-OR-s) showing plasmalemmal (arrowhead, A) or cytoplasmic (small black arrow, B) mu-OR immunogold. In C, a coated vesicle (cv) is also present in the mu-OR labeled spine on which the synaptic specialization appears perforated (double white arrow heads). TH-immunoperoxidase labeling is seen in axonal varicosities having large (TH-lv) or small (TH-sv) sizes in panels C and D, respectively. Scale bars = 0.5 μm . In E, bar graphs show a region-specific and statistically significant (* $p < 0.05$) increase in the plasmalemmal density of mu-OR immunogold in axon terminals of the Acb shell, not the Acb core, of CB1-KO compared with WT mice. In F, bar graphs demonstrate a statistically significant (*, $p < 0.05$) reduction in number of mu-OR labeled vesicle-filled axonal profiles occurring only in the Acb shell, not the core of CB1-KO mice. The analysis was conducted in equally sized ($1,000 \mu\text{m}^2$) areas of neuropil in CB1-KO ($n=4$) and WT ($n=5$) mice.

Fig. 5.

Compared with WT, CB1-KO mice show a region-specific reduction in the abundance of small TH-labeled axonal varicosities in Acb shell (A,B), but not the Acb core (C,D), both of which are quantitatively confirmed in E and F, respectively. In A the immunoperoxidase labeling for TH is seen in small (TH-sv) and large (TH-lv) varicosities. The TH-sv is in contact with the neck of an unlabeled dendritic spine (us). In B, the TH-lv and a mu-OR immunogold labeled terminal (mu-OR-te) form symmetric synapse (black block arrow) with an unlabeled dendrite (U-den). The immunogold (arrowhead) within this terminal is located on the plasma membrane. In the Acb core (C, D), cytoplasmic (small arrows) and/or plasmalemmal (arrowheads) mu-OR immunogold particles are seen in axon terminals (mu-OR-te) forming asymmetric (white block arrow) synapses with unlabeled spines (us). Scale bars = 0.5 μm. Bar graphs show that as compared with WT, the CB1-KO mice have a statistically significant ($p < 0.05$) reduction in the number of small, but not large TH-immunolabeled varicosities in the Acb shell (E), and no differences from WT in prevalence of either small or large TH-labeled varicosities in the Acb core (F). The values in the bar graphs are expressed as profile density (ie., number of labeled varicosities/ 1,000 μm² tissue). The images were obtained from 4 CB1-KO and 5 WT mice.

Fig 6.

Schematic diagram showing the differential effect of CB1-KO on mu-OR distribution and dopamine innervation on the Acb shell and core, respectively. Compared with WT, the Acb shell of CB1-KO mice has (A-1) fewer mu-OR labeled (blue shade) excitatory-type (+) axon terminals characterized by an increased plasmalemmal density of mu-OR immunogold (yellow spheres) and a reduction in number of TH-containing small axonal varicosities (red) convergent on unlabeled dendritic spines; (A-2) fewer mu-OR labeled dendritic (blue shade) spines without change in plasmalemmal (yellow spheres) density of mu-OR immunogold; (A-3) a reduction in the cytoplasmic (light blue), but not plasmalemmal (yellow spheres) density of mu-OR immunogold. In contrast, the Acb core of CB1-KO compared WT mice shows (B-1) no change in the number of mu-OR labeled terminals, the cytoplasmic (blue shading) or plasmalemmal (yellow spheres) density of mu-OR in these terminals, or the number of small TH-labeled (red) varicosities convergent on unlabeled dendritic spines; (B-2) no change in number of mu-OR labeled (blue) spines, but a decrease in both the cytoplasmic (dark to light blue) and plasmalemmal (yellow spheres) mu-OR immunogold in these spines; (B-3) a decrease in the plasmalemmal (yellow spheres), but not cytoplasmic (blue) density of mu-immunogold labeling with an apparent decrease in numbers of mu-OR labeled dendrites possibly reflecting a decrease in number of transverse sections (dashed line) intersecting the sparsely distributed plasmalemmal gold particles.