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Cannabinoid-1 (CB1) receptors in the mouse ventral pallidum are targeted to axonal profiles expressing functionally opposed opioid peptides and contacting NAPE-PLD terminals

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Abstract

The ventral pallidum (VP) is a major recipient of inhibitory projections from nucleus accumbens (Acb) neurons that differentially express the reward (enkephalin) and aversion (dynorphin) associated opioid peptides. The cannabinoid-1 receptor (CB1R) is present in Acb neurons expressing each of these peptides, but its location in the VP is not known. To address this question, we used electron microscopic dual immunolabeling of the CB1R and either dynorphin 1-8 (Dyn) or Met⁵-enkephalin (ME) in the VP of C57BL/6J mice, a species in which CB1R gene deletion produces a reward deficit. We also used similar methods to determine the relationship between the CB1R and N-acylphosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD), an anandamide-synthesizing enzyme located presynaptically in other limbic brain regions. CB1R-immunogold was principally localized to cytoplasmic endomembranes and synaptic or extrasynaptic plasma membranes of axonal profiles, but was also affiliated with postsynaptic membrane specializations in dendrites. The axonal profiles included many single CB1R-labeled axon terminals as well as terminals containing CB1R-immunogold and either Dyn or ME immunoreactivity. Dually labeled terminals comprised 26% of all Dyn- and 17% of all ME-labeled axon terminals. Both single and dual labeled terminals formed mainly inhibitory-type synapses, but almost 16% of these terminals formed excitatory synapses. Approximately 60% of the CB1R-labeled axonal profiles opposed or converged with axon terminals containing NAPE-PLD immunoreactivity. We conclude that CB1Rs in the mouse VP have subcellular distributions consistent with on demand activation by endocannabinoids that can regulate the release of functionally opposed opioid peptides and also modulate inhibitory and excitatory transmission.

Keywords

Enkephalin; dynorphin; electron microscopic immunolabeling; addiction; reward; aversion

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Introduction

The ventral pallidum (VP) is a key portion of the mesocorticolimbic neural circuit that separates wanting, liking, and prediction components of the same reward (Smith et al., 2011), which can be differentially affected by enkephalin and dynorphin-type opioid peptides (Smith and Berridge, 2005; Frankel et al., 2008). The conserved expression of these opioids in the VP of many different species including human suggests their biological relevance (Neal and Newman, 1989; Hurd, 1996; Harkany et al., 2003). Moreover, there are multiple sources of opioid peptides in the VP which include not only the nucleus accumbens (Acb) and olfactory tubercle (Zhou et al., 2003), but also VP neurons that express prodynorphin and proenkephalin messenger RNAs (Hurd, 1996; Voorn, 2010).

Anandamide and other endocannabinoids active at the cannabinoid-1 receptor (CB1R) modulate the strength of incoming signals by inhibition of the release of γ -aminobutyric acid (GABA) and other neurotransmitters (Hashimoto et al., 2007). GABA is co-expressed with dynorphin and enkephalin in many brain regions including those that provide input to the VP and to the anatomically linked ventral tegmental area (VTA; Kalivas et al., 1993; Lu et al., 1998; Besson et al., 1990; Ma et al., 2003). In the limbic-associated VTA and Acb, the involvement of the CB1R in the presynaptic regulation of GABAergic and glutamatergic transmission is well established (Riegel and Lupica, 2004; Robbe et al., 2001). Electron microscopic immunolabeling in these regions has shown the subcellular distribution of the CB1R in axon terminals forming either inhibitory- or excitatory-type synapses distinguished by the respective symmetry and asymmetry of their synaptic membrane specializations (Pickel et al., 2006; Matyas et al., 2008). While the CB1R is also expressed in the VP (Glass et al., 1997; Harkany et al., 2003), neither its subcellular localization nor affiliation with neurons of defined transmitter phenotypes has been examined in this important brain region. It seems likely however that the CB1R in the VP is present in axon terminals that contain enkephalin (Zahm et al., 1985) and/or dynorphin (Zhou et al., 2003). This is suggested not only by the involvement of cannabinoids in opiate reward (Cossu et al., 2001), but also by the similarities between the VP and the globus pallidus (Pang et al., 1998), a region that receives input from dorsal striatal projection neurons that contain CB1 receptors and proenkephalin or prodynorphin mRNA (Hohmann and Herkenham, 2000).

We used electron microscopic immunolabeling to examine the subcellular localization of the CB1R in the VP and to specifically test the hypothesis that the CB1R in this region is present in morphologically heterogeneous axon terminals inclusive of those containing dynorphin and/or enkephalin. In conducting this study, we also processed sections through the VP for dual labeling of (1) ME and Dyn to ascertain the recognition of different subsets of opioid terminals and (2) CB1R and N-acylphosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (NAPE-PLD), an enzyme that can synthesize the endocannabinoid, anandamide and other N-acyl ethanolamines (NEAs; Ueda et al., 2010; Tsuboi et al., 2011). The studies were conducted in C57BL/6 mice, a species in which CB1R gene deletion affects both reward (Valverde et al., 2001) and expression of μ -opioid receptors (Lane et al., 2010). Together, the results provide the first ultrastructural evidence that CB1Rs are strategically positioned for anandamide activation and involvement in the release of functionally complementary opioid peptides found in axon terminals that share some of the same dendritic targets in the mouse VP.

Materials and Methods

Animals

The experiments were conducted in adult male C57BL/6J mice (20–25 g; Jackson Laboratory, Bar Harbor, ME). The procedures 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 Indiana University.

Fixation and Sectioning

The mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). For electron microscopy, brain tissue was fixed by vascular perfusion with 30 ml of a solution containing 3.75% acrolein and 2.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) followed by 150 ml of 2% paraformaldehyde in PB. The acrolein-infused brains were removed from the cranium and post-fixed for 30 min in 2% paraformaldehyde in PB. For immunofluorescence, brain tissue was fixed by vascular perfusion with 4% paraformaldehyde in PB. The brains were then removed from the cranium and placed into a solution of 4% paraformaldehyde in PB for approximately one hour. A Leica vibratome (Leica Microsystems, Bannockburn, IL) was used to cut coronal sections of 40 μ m through the VP at Bregma + 0.62 mm in the brain atlas of (Franklin and Paxinos, 1997). The aldehyde-fixed tissue sections were collected in 0.1 M PB. Acrolein fixed tissue was then placed for 30 minutes in a solution of 1% sodium borohydride in 0.1 M PB to remove excess active aldehydes. The tissue was rinsed with 0.1 M Tris saline (TS) and placed in primary antisera.

Antisera

The primary antisera used in this study include a guinea pig anti-CB1R, rabbit anti-Met⁵ enkephalin (ME); rabbit and guinea pig anti-dynorphin (Dyn), and rabbit anti-NAPE-PLD. The guinea pig polyclonal CB1R antibody was raised against the full C-terminus of the rat CB1R (Wager-Miller et al., 2002) in the laboratory of the co-author, Dr. Mackie. The antiserum shows a selective immunolabeling pattern in mouse forebrain, which is absent in CB1R knockout (KO) mice (Lane et al., 2012).

The ME antiserum generated in rabbits was obtained from Cambridge Research Biochemicals (Billingham Cleveland, UK). Immunolabeling using this antiserum has a region-specific brain distribution and is absent after prior adsorption with the antigenic peptide. At dilutions above 1:2,000, immunodot blots show that the rabbit anti-ME antiserum has little if any cross-reaction with Leu⁵-enkephalin or any of the three (1-13, 1-7, and 1-8) fragments of dynorphin A, which is seen at a 1:2,000 dilution (Velley et al., 1991).

The rabbit and guinea pig anti-dynorphin antisera were obtained from Peninsula Laboratories Europe LTD, a company that is currently Bachem, Torrance, California. The rabbit antiserum was raised against dynorphin A (1-8) and shows no detectable cross-reactivity with dynorphin B, β -endorphin or ME (Zhang and Houser, 1999). The guinea pig antiserum was raised against dynorphin B and shown to specifically recognize the antigenic peptide in absorption control studies (Svingos et al., 1999). Each of these Dyn antisera show a similar labeling pattern in brain (Torres-Reveron et al., 2009), and this pattern resembles that reported previously using other antisera (Fallon and Leslie, 1986).

The rabbit anti-NAPE-PLD antiserum was raised against residues 38–53 of NAPE-PLD, a biosynthetic enzyme of anandamide and its related bioactive congeners, the N-acylethanolamines (Fu et al., 2007). This antiserum has been shown to be specific by

absence of labeling in sections through the hippocampal formation of NAPE-PLD KO mice (Nyilas et al., 2008).

Immunofluorescence

Selected tissue slices were washed in 0.1 M PB and TS and then blocked in 0.1 M TS containing 0.5% bovine serum albumin (BSA) for 30 minutes. The tissue slices were then incubated for 24 hours at room temperature in a mixture of 0.1 M TS containing 0.1% BSA, 0.25% Triton, a guinea pig anti-CB1R (1:500 dilution) and either a rabbit anti-Dyn (1:15,000) or rabbit anti-ME (1:30,000). Following incubation, tissue slices were washed with TS and then incubated in TS with 0.1% BSA, 0.25% Triton, and Alexa Fluor 488-conjugated goat anti-guinea pig IgG (Immunoglobulin G; Invitrogen, Carlsbad, CA; 1:400 dilution) and Cyanin 3-conjugated (Cy3) goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:400 dilution) for 1 hour at room temperature. Tissue slices were then washed repeatedly with TS and PB, mounted on subbed slides, and coverslipped using FluorSave Reagent (Calbiochem, La Jolla, CA).

Slides were then imaged using a Leica TCS SP5 confocal microscope with LAS AF Version 2.0.2 (Leica Microsystems, Wetzlar, Germany) for a PC computer. Individual channels were sequentially captured using a monochrome CCD camera and were pseudocolored using the LAS AF software. Images were collected digitally in a 2048 × 2048 pixel frame using x10 and x40 objective lenses. Images from individual channels and the compilation of the two channels are shown.

Electron Microscopic Dual Labeling

The dual labeling protocol used for electron microscopy was modified from that originally described by Chan et al., (1990). For this, the prepared sections from the aldehyde-fixed tissue were incubated overnight at room temperature in 1) a mixture of guinea pig anti-CB1R antiserum (1:1,000) and rabbit anti-ME (1:60,000) or Dyn (1:30,000) in a solution of 0.1M TS containing 0.1% BSA, 2) guinea pig anti-CB1R antiserum (1:500) and rabbit anti-NAPE-PLD (1:500) in 0.1% BSA, or 3) guinea pig anti-Dyn (1:50) and rabbit anti-ME (1:60,000) in 0.1% BSA. For immunoperoxidase labeling of the rabbit ME, Dyn, and NAPE-PLD, sections previously incubated with both primary antisera were washed and placed for 30 min in biotinylated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). These sections were then incubated for 30 min in Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA). The product was visualized by reaction in 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide. Subsequently, for immunogold labeling of the guinea pig CB1R or Dyn, the tissue was washed and placed in a solution of Ultrasmall gold (Electron Microscopy Sciences, Hatfield, PA) conjugated to donkey anti-rabbit IgG. The particles were visualized by using the Silver IntensEM kit (GE Healthcare). The immunolabeled sections of tissue were post-fixed in 2% osmium tetroxide and embedded in plastic using conventional methods (Leranth et al., 1989).

Electron Microscopic Data Analysis

A Leica ultramicrotome (Leica Microsystems, Wetzlar, Germany) was used to collect ultrathin sections for electron microscopic analysis in a total of ten vibratome sections through the VP of three mice. These sections were mounted on copper grids, counterstained using uranyl acetate and lead citrate, and examined with a Phillips Electronics CM-10 electron microscope (FEI, Hillsboro, OR). Immunoperoxidase labeling was regarded as positive when an electron dense precipitate indicative of peroxidase reaction product was seen in selective profiles, but absent in adjacent profiles having otherwise similar ultrastructural features. Immunogold-labeled structures were identified as those containing

one or more gold particles. This method was validated in immunogold labeled tissue by ascertaining the absence of gold-silver deposits overlying myelin and other structures not known to express the target of interest. In addition, we compared the CB1 immunogold density over axon terminals and other neuronal and glial structures with the area density of the gold particles in microscopic images.

The immunolabeled structures were separated into categories of dendrites (dendritic shafts and spines), axon terminals, small neuronal profiles (mainly unmyelinated axons and spine necks) or glial processes according to the nomenclature of (Peters et al., 1991). Labeled terminals were further defined with respect to the type of synaptic specialization and immunolabeling in the targeted neuron. Chi square, ANOVA and paired t-test analysis were done using SPSS software (IBM Corporation, Armonk, NY).

Acquired digital images from the confocal and electron microscope were adjusted for brightness and contrast using Adobe Photoshop CS4 and these images were trimmed and assembled into figures using Microsoft Office PowerPoint 2007 (Microsoft Corporation, Redmond, WA).

Results

Dyn and ME

Confocal fluorescence microscopy showed Dyn and ME-immunoreactivities in the dorsal portion of the VP, which contains many myelinated and unmyelinated axons (Riedel et al., 2002). The ME labeling was present, but less abundant in the ventral VP. Electron microscopic analysis of tissue processed for dual labeling of Dyn and ME showed that these peptides were located in many, largely separate, small, unmyelinated axons (Fig. 1A) and axon terminals (Fig. 1B-D). Separate Dyn and ME-labeled axons often opposed each other when located in bundles containing many other unmyelinated and lightly myelinated axons without detectable immunoreactivity. The terminals containing Dyn and/or ME sometimes converged on a single postsynaptic dendrite (Fig. 1B), or opposed each other while contacting separate somatodendritic profiles (Fig. 1C). Dyn-immunoreactivity was also seen in some of the somata and dendrites receiving input from terminals containing one or both opioid peptides. Dyn-immunogold was located near cytoplasmic endomembranes and plasmalemmal surfaces contacted by other axon terminals. The opposing axon terminals were largely unlabeled and were either without recognizable synapses or formed asymmetric or, less commonly, symmetric synapses (Fig. 1D).

CB1R and Dyn

Confocal immunofluorescence demonstrated a partially overlapping, but more prominent differential distribution of CB1R and Dyn in the VP (Fig. 2). Within the area of greatest overlap, isolated varicosities showed dual labeling for CB1R and Dyn (Fig. 2). Electron microscopy analysis of the VP confirmed the presence of CB1R in Dyn containing axon terminals as well as in many small axons and axon terminals without immunoperoxidase labeling for Dyn (Figs 3-4, Table 1). In these profiles, the CB1R immunolabeling was located within the cytoplasm and on the presynaptic plasma membrane as well as on non-synaptic membranes opposing other unlabeled axon terminals (Fig. 3 A-C). The majority of the CB1R-labeled profiles formed symmetric synapses (Fig. 3A), or showed undefined dendritic contacts (Fig 2B). However, a small percentage of the terminals containing CB1R formed asymmetric excitatory-type synapses typical of glutamatergic terminals (Fig. 3C and Table 2). The CB1R labeling was also affiliated with the postsynaptic density at excitatory synapses formed by terminals without detectable levels of CB1R or Dyn (Fig. 3D).

In dual Dyn-CB1R labeled terminals, the CB1R had a distribution similar to that seen in terminals without Dyn-immunoreactivity, and identified on both presynaptic and non-synaptic portions of the plasma membrane (Fig. 4). The dually labeled terminals comprised about 25% of the Dyn terminals and approximately 50% of the CB1R-labeled terminals (Table 1). The terminals dually labeled for CB1R and Dyn formed symmetric and asymmetric synapses with a frequency similar to that of the terminals containing only the CB1R (Table 2). In contrast, the single Dyn-labeled terminals formed asymmetric synapses much less frequently than those terminals containing CB1R or CB1R and Dyn (Table 2). The dendritic profiles receiving input from CB1R and/or Dyn-labeled terminals were either unlabeled or also showed Dyn-immunoreactivity (Fig. 4A-C). These dendrites received inputs from many other unlabeled terminals, including those forming asymmetric, excitatory-type junctions (Fig. 4 B and D).

CB1R and ME

Confocal immunofluorescence revealed a dense distribution of ME in the dorsal VP, where there is relatively sparse CB1R labeling and only occasional apparent co-localization of ME and CB1R in dot-like processes presumed to be axon terminals (Fig 5). Electron microscopy analysis of the VP region further established that CB1R and ME are located in many separate (Fig. 6 A, B), as well as some of the same axon terminals (Fig. 6 C-D). A smaller percentage of the ME-, compared with Dyn-, labeled terminals contained CB1R, but these differences were not statistically significant (Chi Square test, Table 1). Axon terminals separately labeled for ME and CB1R converged with each other and with other axon terminals forming excitatory or inhibitory-type synapses on shared dendritic targets (Fig. 6A and B).

As in the CB1R and Dyn labeling study, the CB1R was located mainly on the presynaptic membrane specialization (Fig. 5A) and opposed plasmalemmal surfaces (Fig. 5 B) of axon terminals, whether these terminals contained CB1R alone or together with ME. The vast majority of the single (CB1R) and dual (ME-CB1R) labeled terminals showed symmetric or undefined (Fig. 6D) synaptic specializations (Table 2). However, these terminals also sometimes formed synapses with thickened postsynaptic membrane specializations typical of excitatory-type synapses. These junctions were more often seen on small dendritic profiles. ME-labeled terminals showed presynaptic and axonal distributions of CB1R comparable to those seen in terminals without detectable ME (Fig. 6B and E).

CB1R and NAPE-PLD

NAPE-PLD immunoreactivity was diffusely distributed throughout the cytoplasm of many axon terminals. The NAPE-PLD containing terminals formed symmetric (Fig. 7A) or asymmetric (Fig. 7B) synapses on largely unlabeled dendrites. In contrast with the predominant axonal distribution of NAPE-PLD and CB1R, dendrites less frequently showed NAPE-PLD and/or CB1R labeling (Fig. 7C, Table 3). Of the total NAPE-PLD labeled axonal profiles (460 in an area of $4,377\mu\text{m}^2$, 16% opposed and 18% converged with an axon terminal containing CB1R. Of the CB1R-labeled axonal profiles, however, 30% opposed and 34% converged with NAPE-PLD containing terminals (Fig 7 D-F). Co-localization was seen in only 9.5% of the total labeled , and these were exclusively axon terminals (Table 3).

CB1R immunogold distribution in axon terminals

Of 1,275 CB1R immunogold particles identified in $16,500\mu\text{m}^2$ area of tissue, 88.71% were located in axons and axon terminals; 9.33% labeled in somatodendritic profiles, and the remaining 1.96% in glia. The labeling density (gold particles/ μm^2) was significantly higher in axon terminals ($2.06/\mu\text{m}^2 \pm 0.06$) as compared to all other labeled profiles in the micrograph field [$0.027/\mu\text{m}^2 \pm 0.001$, $t(870) = 34.26$, $p < 0.0001$].

The CB1R immunogold distribution was quantitatively examined in 849 axon terminals from the combined dual labeling experiments. From a total of 1,131 gold particles identified in these axonal profiles, 6.3 % were located on presynaptic and 23.7% on non-synaptic plasma membranes of axon terminals, whereas 70% were located in the cytoplasmic compartment and frequently overlaid synaptic vesicles in these terminals. In contrast with the CB1R-labeled axonal profiles in which there was an average of $2.06/\mu\text{m}^2$ gold-silver particles per axon terminal, only $0.02/\mu\text{m}^2$ immunogold particles were seen in the remaining micrograph field and only one particle was seen overlying myelin of myelinated axons.

Discussion

Our results provide the first ultrastructural evidence that the CB1R is expressed alone or together with either Dyn or ME in axon terminals forming mainly, but not exclusively, inhibitory type synapses on dendritic profiles in mouse VP. We have also shown that in this brain region the CB1R is localized to pre- and postsynaptic as well as non-synaptic plasmalemmal surfaces of axon terminals inclusive of those that contain Dyn and ME. Moreover, these CB1R terminals were sometimes located near axon terminals expressing NAPE-PLD thus suggesting that other axons are one of the potential sources of anandamide. These results are discussed specifically as related to on demand retrograde and transaxonal endocannabinoid signaling, which locally modulates the release of opioid peptides as well as both inhibitory and excitatory transmitters that serve to regulate the behavioral response to biologically salient (rewarding or aversive) stimuli.

Methodological Considerations

Our confocal images show only a sparing distribution of CB1R immunolabeling in the many varicosities that contain either Dyn or ME, whereas the ultrastructural analysis reveals a far more prevalent localization of the CB1R in axon terminals containing each of these opioid peptides in the VP. This apparent dichotomy is most likely a direct reflection of the greater resolution of the electron microscopic compared with light microscopic immunolabeling methods for detection of low levels of antigens in small axonal profiles. The ultrastructural analysis often showed only 1-2 CB1R immunogold particles in small axonal profiles, many of which were below the resolution of the light microscope. However, the significantly greater immunogold density seen over axon terminals compared with the total area of tissue exclusive of these terminals indicates that the axonal CB1R gold labeling is not a product of randomly scattered particles. The specificity of the labeling is further established by the extensive characterization of the CB1R antiserum and by the almost complete absence of gold particles over myelin, a structure not known to express CB1 receptors.

Synaptic CB1R distribution and affiliation with Dyn and ME terminals

Electron microscopy showed that the CB1R immunogold particles in axon terminals were mainly located in the cytoplasm overlying synaptic vesicles, which suggests a potentially large reserve pool of CB1 receptors that can be mobilized to the surface in accordance with demand of incoming signals. The cytoplasmic abundance of CB1R immunogold is consistent with the known rapid recycling of cell surface CB1 receptors in presynaptic GABAergic terminals (Irving et al., 2000) whose symmetric synaptic specializations resemble many of the CB1R-labeled terminals in the present study.

A subpopulation of CB1R immunogold particles were strategically located on presynaptic and non-synaptic portions of the plasma membrane in axon terminals. These locations are ideally suited for CB1R mediation of retrograde and trans axonal endocannabinoid signaling. Retrograde endocannabinoid signaling that decreases the release of GABA in the

VP, may have behavioral consequences that resemble those produced by a decreased in dopamine levels in the Acb (Farrar et al., 2008).

Axon terminals forming symmetric, inhibitory-type synapses were those that most frequently showed co-expression of CB1R and either Dyn or ME, two peptides that were shown to be present in separate axon terminals forming synapses on single dendritic targets in the VP. Together, these observations suggests that the CB1R plays a dual role in modulating the release of aversion and reward-associated opioid peptides onto many of the same output neurons. These output neurons may include the cholinergic neurons in the VP (Zaborszky and Cullinan, 1992) that project extensively to the prefrontal cortex (De Souza Silva et al., 2002), basolateral amygdala (Nickerson et al., 2006), and thalamus (Hreib et al., 1988). This anatomical circuitry is consistent with the involvement of these cholinergic neurons in the brain processes underlying the conditioned place preference and place aversion respectively associated with the enkephalin and dynorphin peptides (Smith et al., 2004).

Our demonstration that the CB1R has a pre- as well as a postsynaptic distribution at asymmetric, excitatory-type synapses in the VP is consistent with the known expression of the CB1R in some of the glutamatergic as well as GABAergic neurons in the basal forebrain (Hrabovszky et al., 2012) and in the ventral tegmental area (Matyas et al., 2008). This subcellular location of the CB1R suggests that endocannabinoid signaling in the VP may suppress both the presynaptic release and postsynaptic response to glutamate as has been shown in other limbic structures (Robbe et al., 2001; Katona et al., 2006). Local glutamatergic neurons as well as glutamatergic projection neurons in the hippocampus, amygdaloid and midline thalamic nuclei are potential sources of CB1R containing terminals forming excitatory-type synapses in the VP (Fuller et al., 1987; Harkany et al., 2003). However, to our knowledge none of these glutamatergic neurons express opioid peptides. Conceivably, opioid containing CB1R-labeled terminals may derive from brainstem catecholaminergic neurons, whose terminals can form either symmetric or asymmetric synapses (Miller and Pickel, 1980; Van Bockstaele et al., 2000).

The presence of CB1R in Dyn and Enk containing terminals in the VP is consistent with evidence showing that cannabinoids can either enhance or oppose anxiety and other behaviors requiring the striatal outflow through the VP (Haller et al., 2004; Kessler et al., 2012). The expression of the CB1R in Dyn and ME terminals that converged with other terminals forming excitatory or inhibitory-type synapses on a common target in the VP is an arrangement that would facilitate the participation of the CB1R in the heterosynaptic plasticity as has been described in other limbic brain regions (Katona et al., 2006). Such plasticity may contribute to the development of tolerance and dependence on opiates and other abused drugs (Nikoshkov et al., 2005; Parolaro et al., 2005). Moreover, the activation of presynaptic CB1Rs specifically in ME containing terminals may attenuate GABAergic or glutamatergic transmission indirectly through mechanisms involving the ME-activated μ -opioid receptors (Mitrovic and Napier, 2002; Napier and Mitrovic, 1999).

Non-synaptic CB1R distribution in axonal profiles opposed to NAPE-PLD or opioid terminals

The present demonstration that, in mouse VP, the CB1R is targeted to axonal plasma membranes opposing those that contain NAPE-PLD suggests that NAPE generated anandamide is involved in signaling between axons. In contrast, NAPE-PLD was infrequently seen in dendrites, suggesting that anandamide is less likely to play a role in the retrograde signaling from dendrites in this brain region. Furthermore, we have shown that the NAPE-PLD is present in axon terminals forming either symmetric or asymmetric synapses in the VP. In the hippocampus, however, NAPE-PLD is expressed exclusively in

excitatory-type terminals, none of which are found near CB1R labeled profiles (Nyilas et al., 2008). This suggests important region specific differences in the extent to which anandamide affects synaptic transmission. The localization of NAPE-PLD in axon terminals of the VP is consistent with the high expression levels of NAPE-PLD in the amygdala and periaqueductal grey (PAG), brain regions that project to the VP and play a critical role in determining the response to certain anxiety producing stressors (Sutt et al., 2008; Tsuboi et al., 2011) that also affect the output from the VP (Hasenohrl et al., 2000; Nikolaus et al., 2000).

The non-synaptic plasmalemmal distribution of CB1R was evident in some of the Dyn and ME-containing terminals opposing other axonal profiles in the VP. This distribution suggests that the release of both these opioid peptides is at least partially regulated by endocannabinoids released from neighboring axons. The axon terminals opposing the CB1R and Dyn-labeled terminals included those forming excitatory-type synapses, thus providing a substrate whereby endocannabinoids may indirectly modulate glutamate release through activation of kappa opioid receptors known to be expressed in glutamatergic terminals in multiple brain regions (Drake et al., 1996; Svingos et al., 1999; Kreibich et al., 2008). Similarly, the observed location of CB1R immunogold on non-synaptic plasma membranes of ME containing axonal profiles opposed to other axons provides a cellular substrate for cannabinoid modulation of ME release and thereby the activation of presynaptic μ -opioid receptors that can affect the release of dopamine, GABA, or other neurotransmitters (Hubner and Koob, 1990; Mitrovic and Napier, 2002; Tang et al., 2005).

Functional Implications

We have shown that CB1R is located within many opioid and non-opioid containing axon terminals in the VP, a region that projects extensively to the ventral tegmental area (VTA; Groenewegen et al., 1993; Kalivas et al., 1993) and to the mediodorsal thalamus (Kalivas et al., 2001). Endocannabinoids active at CB1 receptors are thought to be downstream effectors of the GABAergic inhibition produced by dopamine D2 receptor activation in the ventral striatum (Centonze et al., 2004). The multiple interactive sites between CB1R and Dyn or ME opioids in the VP suggests that this brain region may be one of the sites for dynamic interactions between the mesocorticolimbic endocannabinoid and opioid systems that have direct relevance for locomotor activity and reward (Zimmer et al., 1999; Ellgren et al., 2008).

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Abbreviations

Acb	nucleus accumbens
BSA	bovine serum albumin
CB1R	cannabinoid-1 receptor
Cy3	Cyanin 3-conjugated
DAB	3,3'-diaminobenzadine
Dyn	dynorphin 1-8
er	endoplasmic reticulum

GABA	γ -aminobutyric acid
IACUC	Institutional Animal Care and Use Committees
IgG	immunoglobulin
KO	knockout
ME	Met ⁵ -enkephalin
NEAs	N-acylethanolamines
NAPE-PLD	N-acylphosphatidylethanolamine-hydrolyzing phospholipase
D PAG	periaqueductal grey
PB	phosphate buffer
TS	Tris-saline
VP	ventral pallidum
VTa	ventral tegmental area

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Highlights (for review)

- CB1 receptors are present in both enkephalin and dynorphin terminals.
- Presynaptic and non-synaptic axonal membranes express CB1 receptors.
- CB1 receptors have distributions enabling retrograde and transaxonal signaling.

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Fig. 1.

Electron micrographs showing immunogold labeling (arrows) for dynorphin (Dyn) and immunoperoxidase labeling for Met⁵-enkephalin (ME) in small axons (a) and terminals (te) in mouse VP. **A**, Transverse sections through pairs of small unmyelinated axons containing either Dyn (Dyn-a) or ME (ME-a) immunoreactivity. **B**, ME and Dyn-labeled terminals converge with other unlabeled terminals (U-te) on a common dendrite also containing dynorphin immunogold (Dyn-De). **C**, Two terminals differentially expressing ME or Dyn are opposed to each other but differentially contact an unlabeled dendrite (U-De) and a dynorphin-labeled somata (Dyn-So), respectively. **D**, Unlabeled terminals (U-te) form synapses with a dendrite (Dyn-de) showing a postsynaptic distribution of Dyn immunogold. White block arrows = symmetric synapse; Black block arrows = asymmetric synapse; Scale bars = 500 nm.

Fig. 2.

Confocal images showing the dual immunofluorescence labeling for the CB1 receptor (CB1R green) and dynorphin (Dyn red) in mouse VP. **A**, Low magnification merged image reveals the intense Dyn distribution in the dorsal VP located beneath the anterior commissure (ac) and the more sparse Dyn labeling in the ventral VP, where there is more overlap with CB1 labeled axons (boxed region). **B-D**, High magnification images in the area of the boxed region in A shows the respective distribution of CB1R and Dyn alone or together as evidenced by the yellow dot-like structures in the merged image (white arrows). Corner arrows point dorsal (d) and medial (m). Scale bars = 500 μ m.

Fig. 3.

CB1R immunogold (small arrows) distribution in axon terminals (CB1-te) and dendrites (CB1-den) without dynorphin immunoperoxidase reaction, which is seen as the black precipitate in nearby axons (Dyn-a) in panels C and D. **A**, CB1 immunogold is located on the presynaptic plasma membrane of an axon terminal (CB1-te) that forms a symmetric synapse with a CB1R-immunogold labeled dendrite (CB1-den). **B**, CB1R immunogold is located on a non-synaptic portion of the plasma membrane of an axon terminal (CB1-te) that is opposed to another unlabeled terminal (U-te) and also contacts an unlabeled dendrite (U-den). **C, D**, Respective pre- and postsynaptic distribution of CB1R immunogold at asymmetric synapses (black block arrows) between an axon terminal (CB1-te) and a dendrite (CB1-den). White block arrow indicates a symmetric synapse from a dynorphin terminal (Dyn-te) on the dendritic target in D. Scale bars = 500 nm.

Fig. 4.

Localization of CB1R immunogold (arrows) in axon terminals that contain dynorphin immunoperoxidase labeling (Dyn-CB1-te) in the mouse VP. The neuropil also contains other axon terminals that are immunolabeled for CB1R alone (CB1-te), dynorphin alone (Dyn-te) as well as those without immunoreactivity (U-te). **A-B**, Presynaptic plasmalemmal distribution of CB1R immunogold is seen in axon terminals that converges with other unlabeled and/or dynorphin labeled terminals in contact with medium sized unlabeled dendrites (U-den.) respectively cut in a longitudinal and coronal plane of section. **C**, A dual labeled terminal is presynaptic to a small unlabeled dendrite (U-den) seen in a coronal section. **D, E**, CB1 immunogold is seen on non-synaptic plasma membranes of dual labeled terminals that respectively oppose an unlabeled and a single CB1R-labeled terminal. The dually labeled terminal in **D** is also presynaptic to a small unlabeled dendrite (U-den). White block arrows = symmetric synapse; Black block arrows = asymmetric synapse; Scale bars = 500 nm.

Fig. 5.

Confocal images showing the dual immunofluorescence labeling for the CB1 receptor (CB1R green) and enkephalin (ME red) in mouse VP. **A**, Low magnification merged image shows the prominent ME labeling in the dorsal VP and the more sparse labeling in the ventral VP, where there is some overlap with CB1R immunoreactivity (boxed region). **B-D**, High magnification images in the area of the boxed region in **A** shows the respective distribution of CB1R and ME alone or together as evidence by the yellow dot-like structures in the merged image (white arrows). Corner arrows point dorsal (d) and medial (m). Scale bars = 500 μm.

Fig. 6. Subcellular localization of CB1R immunogold (arrows) in axon terminals without (CB1R-te, A and B) or with (ME/CB1-te, C-D) immunoperoxidase labeling for enkephalin in mouse VP. **A**, the CB1R gold particle is located on the presynaptic plasma membrane of an axon terminal that converges with other unlabeled (U-te) or enkephalin labeled (ME-te) on a common dendrite containing numerous smooth endoplasmic reticulum (er). **B**, the CB1R immunogold particle is located on a non-synaptic plasma membrane of an axon terminal (CB1-te) that opposes an enkephalin-labeled terminal (ME-te). Both these terminals and other ME-labeled and unlabeled terminals contact a single unlabeled dendrite (U-den). **C, D**, CB1R immunogold is located near the presynaptic plasma membrane in axon terminals respectively contacting medium and small diameter unlabeled dendrites (U-den). **E**, CB1 immunogold is located on the non-synaptic plasmalemmal surface of a dually labeled terminal that opposes an unlabeled axon terminal (U-te). The dually labeled terminal also encloses a putative small axon (U-a). White block arrows = symmetric synapse; Black block arrows = asymmetric synapse; Scale bars = 500 nm.

Fig. 7.

Immunoperoxidase labeling for NAPE-PLD in neuronal profiles, some of which are located in proximity to CB1R immunogold-labeled axons (CB1-a) and axon terminals (CB1-te). **A**, **B**, NAPE-labeled axon terminals respectively form a symmetric (white block arrow) or asymmetric (black block arrow) synapses with an unlabeled dendrite (U-den). NAPE-PLD peroxidase reaction product is seen in a dendrite (NAPE-den) that is postsynaptic to an unlabeled terminal, but located within the neuropil containing a CB1R-labeled terminal. **D**, NAPE-PLD labeled terminal is opposed to a CB1R-labeled terminal and an unlabeled axon (U-a). The two labeled terminals converge on a common dendrite. **E**, CB1R immunogold is located on the plasma membrane of a small axon (CB1-a). **F**, A NAPE-PLD labeled terminal is opposed on two sides by CB1R-labeled terminals. White block arrows = symmetric synapse; Black block arrows = asymmetric synapse; Scale bars = 500 nm.

Table 1

Percentage of terminals containing CB1R and/or Dyn or ME in mouse ventral pallidum.

	CB1R-Dyn Dual Labeling		CB1R-ME Dual Labeling	
Terminals	CB1R	Dyn	CB1R	ME
Single	53%	76%	62%	83%
Dual	47%	24%	38%	17%
* Total	378	748	351	790

* Number of CB1R and/or Dyn or ME-labeled axon terminals seen in thin sections from the surface of ten vibratome sections, which included 1-3 sections through the ventral pallidum in each of three mice. The CB1/Dyn and CB1/ME encompassed a respective tissue area of 6,839 and 7,881 μm^2 .

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Table 2

Synaptic specializations formed by axon terminals immunolabeled for CB1R and/or Dyn or ME in mouse ventral pallidum.

	CB1R-Dyn Dual Labeling		CB1R-ME Dual Labeling		
	CB1R	CB1+Dyn	Dyn	CB1R	CB1+ ME
Terminals					ME
Symmetric	38%	51%	45%	53%	58%
Asymmetric	13%	16%	08%	13%	14%
Undefined	50%	34%	42%	33%	28%
* Total	200	178	570	217	134
					656

* Total number of CB1R and/or Dyn- or ME-labeled axon terminals seen in thin sections from the surface of ten vibratome sections, which included 1-3 sections through the ventral pallidum in each of three mice. The two sets of dual labeling represented a tissue area of 6,839 and 7,881 μm^2 respectively.

Table 3

Percentage of axonal and dendritic profiles containing NAPE-PLD and/or CB1R in mouse ventral pallidum.

Profiles	NAPE	NAPE/CB1R	CB1R
Axonal	88%	100%	92%
Dendritic	12%	0%	08%
* Total	525	83	265

* Total number of labeled profiles seen in a tissue area of 4,377 μm^2 using immunogold for NAPE-PLD and immunoperoxidase for CB1R or the reverse markers. The tissue sample was from one vibratome section through the ventral pallidum of 3 mice.