Published in final edited form as:

Synapse. 2011 August ; 65(8): 827–831. doi:10.1002/syn.20911.

Decreased parvalbumin immunoreactivity in the cortex and striatum of mice lacking the CB1 receptor

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Abstract

Cortical and striatal regions of the brain contain high levels of the cannabinoid-1 (CB1) receptor, the central neuronal mediator of activity-dependent synaptic plasticity evoked by endocannabinoids. The expression levels of parvalbumin, a calcium-binding protein found in fast-spiking interneurons of both regions, may be controlled in part by synaptic activity during critical periods of development. However, there is presently no evidence that CB1 receptor expression affects parvalbumin levels in either cortical or striatal interneurons. To assess this possibility, we examined parvalbumin immunoreactivity in the dorsolateral striatum, primary motor cortex (M1), and prefrontal cortex (PFC) of CB1 knockout and wild-type C57/BL6 mice. Quantitative densitometry showed a significant decrease in parvalbumin immunoreactivity within individual neurons in each of these regions of CB1 knockout mice relative to the controls. A significantly lower density (number of cells per unit area) of parvalbumin-labeled neurons was observed in the striatum, but not the cortical regions of CB1 knockout mice. These findings suggest that CB1 receptor deletion may elicit a compensatory mechanism for network homeostasis affecting parvalbumin-containing cortical and striatal interneurons.

Keywords

CB1 knockout; caudate putamen; primary motor cortex; medial prefrontal cortex

The cannabinoid CB1 receptor (CB1R) binds the endogenous cannabinoids (eCBs) 2-arachidonyl glycerol and anandamide as well as Δ^9 -tetrahydrocannabinol, the primary psychoactive ingredient in marijuana. The CB1R is one of the most widely-expressed G-protein coupled receptors in the mammalian brain (Herkenham et al., 1991). This receptor is especially abundant in GABAergic terminals of the cerebral cortex (Bodor et al., 2005; Katona et al., 1999; Tsou et al., 1998), but is also present in inhibitory and excitatory terminals and postsynaptic dendrites of the striatum (Köfalvi et al., 2005; Rodriguez et al., 2001). Presynaptic activation of CB1Rs in these regions triggers both short- and long-term forms of synaptic depression at individual glutamatergic and GABAergic synapses (Ohno-Shosaku et al., 2002; Uchigashima et al., 2007), but the *in vivo* role of CB1Rs on the activity within larger-scale network is uncertain.

Activity-dependent plasticity during critical periods of development may alter the neurochemical composition of cortical GABAergic interneurons (Marty et al., 1997).

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Parvalbumin (PV) is a high-affinity calcium-binding protein found in fast-spiking GABAergic interneurons of the cerebral cortex and striatum (Kita et al., 1990; Tamás et al., 2000). Expression levels of both PV and CB1R in the cerebral cortex concurrently peak at a developmentally sensitive period around postnatal days 21-25 (Alcántara et al., 1993; Heng et al., 2010; Schlösser et al., 1999), and both have been implicated in critical period maturation of neural networks (Cellerino et al., 1992; Li et al., 2009a). Deficits in cortical PV immunoreactivity similar to those reported herein have been observed in response to sensory deprivation during critical periods (Balmer et al., 2009; Cellerino et al., 1992) when CB1R activation is required for correct cortical mapping (Li et al., 2009a). Moreover, both the CB1 receptor and PV protein are implicated in neurodevelopmental psychiatric disorders such as schizophrenia. Schizophrenic subjects show a decrease in CB1R mRNA and protein levels in the prefrontal cortex (PFC) (Eggan et al., 2008, 2010) as well as diminished PV mRNA in the same region (Hashimoto et al., 2003). There is also evidence for involvement of striatal CB1Rs and PV in neurodegenerative diseases such as Parkinson's and Huntington's disease (Fernández-Ruiz, 2000). It is not known, however, whether eCB signaling plays a role in the establishment and/or maintenance of PV interneuron networks in either the cortex or striatum. We addressed this critical question by quantitatively comparing neuronal PV immunolabeling in mice lacking the CB1R with wild-type controls in the dorsolateral striatum and two cortical regions-- the prefrontal cortex (PFC) and primary motor cortex (M1).

The animal protocols in this study followed NIH guidelines concerning the Care and Use of Laboratory Animals in Research and were approved by the Animal Care Committee at Weill Cornell Medical College. Adult male wild type (CB1^{+/+}) and CB1 knockout (CB1^{-/-}) mice were bred onto a C57BL/6J genetic background. Genotyping was performed by Charles River (Germantown, MD). CB1^{-/-} and CB1^{+/+} mice were housed together in common litters of 3-5 mice, and the genotypes were matched according to age (20 to 32 weeks).

Four CB1^{-/-} and five CB1^{+/+} were compared in this study. Prior to sacrifice, mice were deeply anesthetized with sodium pentobarbitol (100 mg/kg, i.p.). To fix brain tissue, mice underwent rapid vascular perfusion through the left ventricle of the heart with sequential delivery of (1) 5mL of heparin-saline, (2) 30 mL of 3.75% acrolein and 2% paraformaldehyde (PF) in 0.1M phosphate buffer (PB, pH 7.4), and (3) 100 mL of 2% PF in 0.1M PB. Brains were removed, post-fixed in 2% PF for 30 min, placed into 0.1M PB, and cut coronally into 40 µm sections on a Vibratome (Leica, Deerfield, IL).

The PFC, M1, and striatal coronal sections were selected according to the mouse brain atlas by Paxinos and Franklin (2001). Layers II-VI through the prelimbic and infralimbic regions of the PFC, layers II-VIa of M1, and the dorsolateral striatum were sampled for analysis. Four coronal sections from each region were selected per mouse. Immunolabeling of the PV protein was achieved using a well-characterized (Celio and Heizmann, 1981; Karson et al., 2010) mouse monoclonal antibody (Sigma, St. Louis, MO). The pattern of parvalbumin labeling observed was similar to previously reported studies (Alcántara et al., 1993; Kita et al., 1990; Tamás et al., 2000).

To ensure identical labeling conditions between CB1^{+/+} and CB1^{-/-} mice, sections were marked with identifying punches and pooled for processing for each region. To examine changes in PV expression in response to CB1R deletion, the PV protein was labeled with immunoperoxidase and visualized using avidin-biotin complex (ABC) peroxidase (Hsu and Raine, 1981). Free-floating sections were incubated in 1% sodium borohydride in 0.1M PB for 30 min, washed in PB, rinsed in 0.1M Tris-saline (TS, pH 7.6), and blocked in 0.1% bovine serum albumin (BSA) in 0.1M TS for 30 min before being placed in primary antisera solution. Sections were incubated for 48h at 4°C in a 1:10,000 dilution of PV antisera

prepared in 0.1M TS containing 0.1% Triton and 0.05% BSA. The sections were then rinsed in 0.1M TBS and placed in a 1:400 dilution of biotinylated horse anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 30 min. Following washes in TS, tissue was incubated in avidin-biotin peroxidase complex (VectaStain Elite Kit, Vector Laboratories, Burlingame, CA) for 30 min, washed in 0.1M TS, and reacted in 0.022% 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO) and 0.003% hydrogen peroxide in 0.1M TBS for 7 min, 30 sec. Matched sections from CB1^{+/+} and CB1^{-/-} mice were mounted onto gelatin-coated slides in 0.05M PB. The tissue-mounted slides were placed into a dessicator overnight, dehydrated through ascending concentrations of alcohol followed by three 10-min changes of xylene. The xylene-cleared tissue was placed beneath a glass coverslip and adhered with DPX mountant for histology (Sigma, St. Louis, MO).

Each slide contained matched coronal sections from CB1^{+/+} and CB1^{-/-} mice through cortical or striatal regions of the brains. White balance adjustments were conducted on specimen slides prior to photomicrograph capture. Quantitative densitometry was performed on individual PV-labeled cell bodies (Pierce et al., 1999). The tissue was imaged using a Dage CCD C72 camera on a Nikon (Tokyo, Japan) E800 light microscope interfaced with MCID analysis software (Imaging Research, St. Catharines, Ontario, Canada) on a PC. Four photomicrographs from four sections per region of interest were collected at 20X magnification for each animal. Micrographs were imaged at the highest focal plane in the section to control for apparent differences in labeling due to changes in focal depth. The average pixel intensity (of 256 grey values) of each cell body was determined using ImageJ computer software (NIH, USA). These values were subtracted from the average pixel intensity of three non-labeled areas to compensate for differences in background staining in each section. Validity of hardware and software components of this approach was confirmed by calculating a linear regression of measured pixel intensities against a set of neutral density gelatin filters (Eastman Kodak Co., Rochester, NY) with defined transmittances ranging from 10% to 80% ($r^2 = 1.00$, $P < 0.0001$). Average intensity of PV expression was calculated for each animal from approximately 30 labeled cells per region out of a total of 120-150 cells per region per group. PV expression in CB1^{+/+} and CB1^{-/-} animals were compared using a two-tailed unpaired t-test. Cells were counted using a reticule that positioned a 10 × 10 grid over a 0.5 mm² area of each region of interest using a 20X objective lens. Counts per region per animal were calculated from four sections from each animal as an average number of cells per 0.5 mm² area. Cell counts in CB1^{-/-} mice and CB1^{+/+} controls were then compared using a two-tailed unpaired t-test. Images were prepared for publication using Adobe Photoshop CS4 and Microsoft PowerPoint 2008 for Mac.

In M1 of CB1^{-/-} mice, there was a significant reduction in the intensity of PV immunolabeling ($t_{(7)} = 5.80$, $p < 0.001$) relative to CB1^{+/+} controls. The knockout mice also showed a decreased density (number of PV-labeled cells per unit area) that did not reach statistical significance ($t_{(7)} = 2.12$, $p = 0.07$) (Fig. **1A-1D**). Likewise, in the PFC, we observed a significant decrease in PV expression in individual cells ($t_{(7)} = 3.68$, $p < 0.01$) and a non-significant trend towards fewer PV-labeled cells ($t_{(3)} = 2.25$, Welch's correction for unequal variances, $p = 0.11$) (Fig. **1E-1H**). In the dorsolateral striatum, however, deletion of the CB1R induced both a significant decrease in PV expression in individual cells ($t_{(7)} = 3.58$, $p < 0.01$) and a decrease in PV-expressing cells ($t_{(7)} = 2.56$, $p < 0.05$) (Fig. **1I-1L**).

In both cortical regions examined, we observed a decrease in PV immunoreactivity in individual cells with a non-statistically significant trend toward decreased density of labeled cells. Average PV optical density in the PFC cortical region in CB1^{+/+} mice is lower than in M1 (M1: 37.31 ± 0.93 , PFC: 28.56 ± 2.33 , $t_{(7)} = 3.49$, $p < 0.01$). The increased variability in number of PV-labeled cells/0.5 mm² in the PFC relative to M1 in CB1^{-/-} mice may reflect a

population of lightly-labeled PV-expressing neurons, such as the chandelier interneurons (Woodruff et al., 2009), that dropped below reliably detectable threshold limits in this region. This may indicate increased vulnerability of PV interneurons in the PFC to CB1-mediated effects relative to other cortical areas.

Loss of PV immunoreactivity has been shown to indicate hypofunctionality of PV interneurons in the cortex as well as the striatum. Reduced PV in the mPFC is a concomitant of both a reduction in coordinated neural activity and behavioral impairment in a latent inhibition task (Lodge et al., 2009). In the striatum, a reduction in PV-positive interneurons is correlated with a disinhibition of striatal GABAergic medium spiny projection neurons (Gernert et al., 2000).

We report a similar depression of PV immunoreactivity within neurons of the PFC, M1, and dorsolateral striatum of CB1^{-/-} mice despite striking differences in CB1R and PV expression in cortical and striatal regions. In the cortex, CB1Rs are primarily localized to presynaptic terminals of basket interneurons expressing the neuropeptide cholecystinin (CCK) and are not observed in interneurons immunolabeled for PV (Bodor et al., 2005). Cortical PV and CCK/CB1 interneurons also display marked differences in electrophysiological properties: PV interneurons are fast-firing with high probability of synchronous GABA vesicle release and generate phasic output, while CCK interneurons have an increased probability of long-lasting asynchronous release (Hefft and Jonas, 2005).

Although cortical PV and CCK/CB1R basket interneurons are non-overlapping populations, they are not functionally dichotomous. Terminals from both PV and CCK basket interneurons may target the same excitatory pyramidal projection neuron (Bodor et al., 2005). Moreover, these two groups of basket interneurons display some interconnectivity, perhaps serving to maintain network homeostasis. There is recent ultrastructural as well as electrophysiological evidence of mutual synaptic transmission between PV and CCK/CB1R interneurons in the hippocampus (Karson et al., 2009). In this region, CCK release concurrently triggers an increase in firing rates of PV-containing neurons with a CB1R activation-dependent decrease in GABA release from CCK fibers (Földy et al., 2007). These same mechanisms of reciprocated CCK/PV connectivity may underlie the decreased PV expression and/or interneuron functionality in the PFC and M1 regions of CB1 knockout mice.

Striatal expression of both the CB1R and PV, however, differs greatly from cortical distribution patterns (Bodor et al., 2005; Cowan et al., 1990; Fusco et al., 2004; Kita et al., 1990). In this region, we observed a significant decrease in both PV immunolabeling and the density (number/area) of labeled cells in CB1^{-/-} mice relative to CB1^{+/+} mice. In the striatum, CB1 receptors are expressed in many corticostriatal excitatory-type terminals presynaptic to dendritic spines of GABAergic neurons providing local collaterals to PV-containing interneurons (Rodriguez et al., 2001). These PV-positive GABAergic interneurons constitute ~2% of the neuronal population in the striatum and a majority of these cells also co-express the CB1R (Cowan et al., 1990; Fusco et al., 2004; Gerfen et al., 1985). Despite their small quantity, these interneurons are a powerful source of inhibition. Axon terminals of striatal PV interneurons preferentially target the soma of medium spiny GABAergic projection neurons, which comprise ~95% of striatal neurons and are the most potent source of inhibitory output in the basal ganglia (Cowan et al., 1990; Gerfen, 2004; Kita et al., 1990).

The loss of PV neurons in the dorsolateral striatum of CB1^{-/-} mice is likely to result in decreased inhibition of medium spiny projection neurons targeting the globus pallidus. This principal output region of the basal ganglia, responsible for the planning and initiation of

movement, receives GABAergic afferents from both direct and indirect striatal projection neurons (Gerfen, 2004). A deficit in striatal PV interneurons would therefore decrease the inhibition of GABAergic axon collaterals (thus resulting in net increased inhibition of the globus pallidus), and may manifest behaviorally as locomotor hypoactivity. This phenotype has, in fact, been observed in CB1^{-/-} mice of the same strain (Li et al., 2009b; Zimmer et al., 1999), and is consistent with observations of locomotor hypoactivity in rodents lacking striatal PV interneurons (Gernert et al, 2000).

Whereas striatal PV interneurons have been implicated in movement disorders and rodent models thereof (Fernández-Ruiz, 2000; Giampà et al., 2009), deficits in cortical PV have been observed in schizophrenic subjects (Hashimoto et al., 2003). Mice modeling this cortical PV deficit replicate aspects of a psychotic phenotype, including decreased prepulse inhibition to acoustic startle, increased stereotypic behavior, spatial working memory impairment, and decreased latent inhibition (Belforte et al. 2010, Lodge et al., 2009; Wen et al., 2009). While CB1^{-/-} mice do not natively display the same phenotype, they show an increased vulnerability to phencyclidine (PCP)-induced stereotypy and ataxia (Haller et al., 2005). This may reflect enhanced PCP toxicity to PV cortical interneurons in CB1^{-/-} mice (Wang et al., 2008).

The full behavioral phenotype of either decreased striatal or cortical PV may not be recapitulated in CB1^{-/-} mice. These models demonstrate extremely region-specific effects of reduced PV, whereas in CB1^{-/-} mice both cortical and striatal PV are reduced. Furthermore, the behavioral phenotype resulting from decreased PV in CB1^{-/-} mice is likely tempered by absence of the CB1R and its effects. The decrease in PV in the absence of CB1Rs may in fact represent a compensatory mechanism by which interneurons regulate network homeostasis. Our work indicates that these two seemingly disparate proteins might be far more intricately related than previously thought.

Acknowledgments

This work was supported by the National Institutes of Health (1P01 HL096571, MH40342, and DA04600 to VMP, T32 DA 7274 to MLF), and the National Institute on Drug Abuse Intramural Research Program (CRL).

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Fig. 1.

Light microscopic comparison of PV immunoreactivity in CB1 $+/+$ and CB1 $-/-$ mice as seen in the M1, PFC, and DL striatum (boxed regions indicated in the three drawings of coronal sections through the mouse brain). In each region, PV cell bodies (black arrows) and processes are evident in both CB1 $+/+$ and CB1 $-/-$ mice. Arrowheads indicate cell bodies in deeper tissue layers, which were not analyzed for densitometry but were included in cell counts. As compared with the wild-type control (**A**), a photomicrograph through the M1 region of a CB1 $-/-$ mouse (**B**) shows an apparent reduction in PV immunoreactivity. Bar graphs indicate a statistically significant reduction in optical density of PV-labeled soma (**C**) and a trend towards fewer PV-labeled cells (**D**) in the M1 region of the CB1 $-/-$ compared with CB1 $+/+$ mice. A similar qualitative (**E**, **F**) and quantitative (**G**, **H**) reduction in the PV immunoreactivity is seen in the PFC of the CB1 $-/-$ mice. In the dorsolateral (DL) striatum, the apparent reduction in PV-immunoreactivity as seen in representative photomicrographs of CB1 $+/+$ (**I**) and CB1 $-/-$ mice (**J**) is quantitatively confirmed as a significant reduction in both the optical density of PV immunoreactivity (**K**) and number of PV-labeled cells (**L**). Statistical comparisons are based on four replicated samples from each region in 5 CB1 $+/+$ and 4 CB1 $-/-$ mice. Scale bar = 100 μ m.