GABAergic and Cortical and Subcortical Glutamatergic Axon Terminals Contain CB₁ Cannabinoid Receptors in the Ventromedial Nucleus of the Hypothalamus

Leire Reguero¹, Nagore Puente¹, Izaskun Elezgarai¹, Juan Mendizabal-Zubiaga¹, Miren Josune Canduela¹, Ianire Buceta¹, Almudena Ramos¹, Juan Suárez², Fernando Rodríguez de Fonseca², Giovanni Marsicano³, Pedro Grandes¹*

¹ Department of Neurosciences, Faculty of Medicine and Dentistry, Basque Country University, Leioa, Spain, ² Fundación IMABIS, Málaga, Spain, ³ "Endocannabinoids and Neuroadaptation", INSERM U862 NeuroCentre Magendie, Université Bordeaux 2, Bordeaux, France

Abstract

Background: Type-1 cannabinoid receptors (CB₁R) are enriched in the hypothalamus, particularly in the ventromedial hypothalamic nucleus (VMH) that participates in homeostatic and behavioral functions including food intake. Although CB₁R activation modulates excitatory and inhibitory synaptic transmission in the brain, CB₁R contribution to the molecular architecture of the excitatory and inhibitory synaptic terminals in the VMH is not known. Therefore, the aim of this study was to investigate the precise subcellular distribution of CB₁R in the VMH to better understand the modulation exerted by the endocannabinoid system on the complex brain circuits converging into this nucleus.

Methodology/Principal Findings: Light and electron microscopy techniques were used to analyze CB₁R distribution in the VMH of CB₁R-WT, CB₁R-KO and conditional mutant mice bearing a selective deletion of CB₁R in cortical glutamatergic (Glu-CB₁R-WT and Glu-CB₁R-KO) or GABAergic neurons (GABA-CB₁R-WT and GABA-CB₁R-KO). At light microscopy, CB₁R immunolabeling was observed in the VMH of CB₁R-WT and Glu-CB₁R-WT and Glu-CB₁R-KO animals, being remarkably reduced in GABA-CB₁R-WT and GABA-CB₁R-KO mice. In the electron microscope, CB₁R appeared in membranes of both glutamatergic and GABAergic terminals/preterminals. There was no significant difference in the percentage of CB₁R immunopositive profiles and CB₁R density in terminals making asymmetric or symmetric synapses in CB₁R-WT mice. Furthermore, the proportion of CB₁R immunopositive terminals/preterminals in CB₁R-WT and Glu-CB₁R-WT and Glu-CB₁R-KO mice was reduced in GABA-CB₁R-WT and GABA-CB₁R-KO mutants. CB₁R density was similar in all animal conditions. Finally, the percentage of CB₁R labeled boutons making asymmetric synapses slightly decreased in Glu-CB₁R-WT and Glu-CB₁R-KO mutants relative to CB₁R-WT mice, indicating that CB₁R was distributed in cortical and subcortical excitatory synaptic terminals.

Conclusions/Significance: Our anatomical results support the idea that the VMH is a relevant hub candidate in the endocannabinoid-mediated modulation of the excitatory and inhibitory neurotransmission of cortical and subcortical pathways regulating essential hypothalamic functions for the individual's survival such as the feeding behavior.

Introduction

The hypothalamus plays a crucial role in regulating energy balance and food intake [1]. The ventromedial nucleus (VMH) is placed in the tuberal region of the hypothalamus and is associated with several homeostatic and behavioral functions, including regulation of appetite, energy balance, sexual behavior, anxiety, thermogenesis, cardiovascular functions and pain [2,3]. Functionally, the dorsomedial VMH participates in the regulation of energy homeostasis, whereas the ventrolateral VMH controls female reproduction [2,4].

While the large majority of VMH neurons expresses abundant vesicular glutamate transporter VGluT2 mRNA [5–7], only weak GAD65 mRNA and GAD67 mRNA signals are observed in this nucleus [6,7]. The VMH is surrounded by hypothalamic GABAergic neurons [8] and about 12% of the ventrolateral VMH neurons are GABAergic [6].

The VMH has been proposed as a satiety nucleus that provides a strong excitatory input to arcuate neurons, contributing to the activation of anorexigenic neuronal pathways [9,10]. The endocannabinoid system is implicated in endocrine regulation
and energy balance. The derivatives of Cannabis sativa are well known to regulate food intake and the endocannabinoid system controls neuronal signalling in hypothalamic networks [11]. Although low levels of cannabinoid receptors are present in the hypothalamic nuclei [12,13], their efficiency is higher than in other brain regions [14]. Moreover, specific cannabinoid receptor binding is found in several hypothalamic areas, including the VMH, which also expresses high levels of CB1R mRNA [13]. Although the overall CB1R immunolabeling intensity is much lower in the hypothalamus than in other brain regions, the VMH, in particular, exhibits a moderate CB1R immunostaining [15].

The levels of the two main endocannabinoids, anandamide and 2-arachidonoylgllycerol (2-AG), in the hypothalamus are higher during fasting and lower following food intake reaching a critical point that favors a motivational state for food intake [11,16–18]. The administration of anandamide into the VMH also stimulates appetite in rats [19]. In contrast, both chronically-treated animals with CB1R antagonists [11,20,21] and CB1R null mice [11,20,22] display an anorexic phenotype. Furthermore, activation of presynaptic CB1R inhibits the excitatory and inhibitory neurotransmission in neuronal circuits involved in eating behaviors [11,18,23–25]. Indeed, Glu-CB1R-KO conditional mice that do not express CB1R in neurons of cortical origin exhibit a hypophagic phenotype after food deprivation very similar to the full CB1R-KO. On the contrary, GABA-CB1R-KO mutants that lack CB1R in forebrain GABAergic neurons are hyperphagic under the same experimental conditions [26].

Taken together, it is well established that the endocannabinoid system exerts a neuronal modulation through the activation of presynaptic CB1R localized on both excitatory and inhibitory pathways in distinct brain networks regulating homeostatic and behavioral functions including food intake. In view of the described observations that both the endocannabinoid system and the VMH play a role in ingestive behaviors, the aim of this study was to analyze the CB1R contribution to the molecular architecture of the excitatory and inhibitory synaptic terminals in the mouse VMH. For this purpose, preembedding immunocytochemical techniques for light and high resolution electron microscopy were used. Highly specific CB1R antibodies were applied to the VMH of conditional mutant mice with a selective deletion of CB1R mainly from cortical glutamatergic (Glu-CB1R-KO) and mainly from forebrain GABAergic neurons (GABA-CB1R-KO) [27,28]. Mutants with the lack of CB1R in all the cells of the body (CB1R-KO mice) were also examined [29].

Results

Immunolocalization of CB1R in the VMH

In the light microscope, the CB1R immunoreactivity was uniformly distributed throughout the entire VMH of CB1R-WT (Fig. 1A) with a somehow similar appearance in the Glu-CB1R-KO mice (Fig. 1B). At higher magnification, the pattern consisted of abundant small immunoreactive dots densely packed within the oval-shaped VMH (Fig. 1A, B). However, CB1R staining decreased drastically in the VMH of GABA-CB1R-KO mice (Fig. 1C), particularly in the dorsomedial part (Fig. 1C'), suggestive of the presence of CB1R in GABAergic profiles. The immunolabelling fully disappeared in the VMH of CB1R-KO mice (Fig. 1D, D').

Then, we analyzed the ultrastructural distribution of CB1R in the dorsomedial region of the VMH using a preembedding immunogold method for electron microscopy (Fig. 2). CB1R immunoparticles were typically localized away from the active zones on preterminal or synaptic terminal membranes making synapses with dendrites or dendritic spines. They showed characteristic features of excitatory (asymmetric synapses with obvious postsynaptic densities, abundant clear and spherical synaptic vesicles) and inhibitory (symmetric synapses with more pleomorphic synaptic vesicles) synapses (Fig. 2A, B). 24.0±2.9% and 28.9±7.5% of the synaptic terminals making asymmetric and symmetric synapses, respectively, were CB1R immunopositive in the VMH of CB1R-WT mice (Fig. 3A). In this case, CB1R density was 0.42 immunoparticles/μm membrane in terminals making asymmetric synapses and 0.47 immunoparticles/μm in terminals making symmetric synapses (Fig. 3B). There were no statistically significant differences in these parameters between terminals with asymmetric or symmetric synapses in the CB1R-WT mice.

To define the contribution of cortical glutamatergic and GABAergic synaptic terminals to the intrinsic CB1R pattern in the VMH, conditional CB1R null mice lacking the receptor either in cortical glutamatergic (Glu-CB1R-KO) or in forebrain GABAergic neurons (GABA-CB1R-KO) were used. CB1R was still observed in VMH axon terminals making synapses with dendritic and spiny elements of both mutant strains (Fig. 2C-H). In Glu-CB1R-KO mice, CB1R immunopositive terminals made asymmetric (Fig. 2C, D) and symmetric synapses (Fig. 2E). Also, CB1R immunonegative asymmetric synaptic terminals were found in the Glu-CB1R-KO mutants (Fig. 2C, E), suggesting the presence of CB1R in cortically-derived axonal terminals. In GABA-CB1R-KO tissue, CB1R immunoparticles decorated presynaptic membrane profiles forming asymmetric (Fig. 2F, G) but not symmetric synapses (Fig. 2H). The immunolabeling was specific as the CB1R pattern disappeared in the VMH of CB1R-KO mice (Fig. 2I, J).

The proportion of CB1R immunopositive synaptic terminals/presynaptic terminals in CB1R-WT (20.5%) was maintained in Glu-CB1R-KO mice (20.8%) and reduced in the VMH of GABA-CB1R-KO mutants (12.4%) (Fig. 4A). CB1R immunoparticles virtually disappeared in the VMH of CB1R-KO mice (Fig. 4A). Furthermore, CB1R density in WT and both mutant animals was estimated to be rather low (between 0.40–0.50 immunoparticles/μm membrane, differences not statistically significant) (Fig. 4B).

We next semiquantified the CB1R immunolabeled excitatory axonal boutons to determine the contribution of cortical axons to the pattern of CB1R in the VMH. For this purpose, only typical excitatory terminals with abundant clear and spherical vesicles, forming asymmetric synapses with thick postsynaptic densities were taken into account. In this case, 21.3±2.5% and 27.2±0.7% of the asymmetric synapses were CB1R immunopositive in the VMH of Glu-CB1R-KO and CB1R-WT mice, respectively (Fig. 4C). However, this difference was not statistically significant (χ² = 0.4189, p = 0.5175). Finally, the percentage of CB1R immunolabeled asymmetric synapses was very low in CB1R-KO mice (Fig. 4C).

Taken together, these observations indicate that CB1R is localized in GABAergic as well as in cortical and subcortical glutamatergic inputs to the VMH.

Discussion

CB1R is localized in excitatory and inhibitory presynaptic boutons in the VMH

The main finding of this study was the localization of CB1R in VMH presynaptic terminals impinging on postsynaptic dendrites and spines of CB1R-WT, Glu-CB1R-KO and GABA-CB1R-KO mice. Furthermore, an extensive analysis of the proportion of immunolabeled profiles identified the contribution of CB1R to GABAergic and cortical and subcortical glutamatergic inputs to the VMH.
The dense network of synaptic connections constitutes the anatomical basis for the neuroendocrine and vegetative functions regulated by the hypothalamus. The proportion of CB₁R immunolabeled synaptic terminals in the VMH of mice lacking CB₁R in neurons of cortical origin (Glu-CB₁R-KO) was identical to WT animals (~20%), indicating that CB₁R probably was in excitatory synaptic terminals of intrinsic hypothalamic neurons. However, although the difference was not statistically significant, the analysis of synaptic terminals forming asymmetric synapses showed a slight decrease of glutamatergic synaptic profiles with CB₁R in Glu-CB₁R-KO compared to CB₁R-WT mice. Altogether, these results indicate that CB₁R localizes mostly in subcortical excitatory axon terminals [8,22,30,31] and to a lesser extent in excitatory synaptic boutons of cortical origin [1,8,27,31].

The absence of CB₁R in forebrain GABAergic neurons (GABA-CB₁R-KO) caused a reduction of the CB₁R immunolabeled synaptic terminals (12.4%) indicating that CB₁ receptors are also a molecular component of the GABAergic axon boutons in the VMH. For GABA-CB₁R-KO mutants, DLX mice lead also to recombination in hypothalamic dopaminergic neurons [32]. However, it is unlikely the presence of CB₁R in dopaminergic synaptic terminals in the VMH of the GABA-CB₁R-KO mutants as there is no tyrosine hydroxylase immunoreactivity in the VMH [32]. Overall, our findings can be interpreted as for the presence of CB₁R in VMH GABAergic and Glutamatergic Synapses
CB1R in GABAergic presynaptic terminals of both VMH and intrinsic hypothalamic inhibitory pathways.

**Functional significance**

This investigation has demonstrated that CB1 receptors in GABAergic and glutamatergic afferents explain the CB1R pattern in the VMH. The density of CB1R immunoparticles was rather low in GABAergic and glutamatergic boutons in the VMH (~0.40–0.50 particles/μm) as compared to the density found in other brain regions [33,34], particularly in inhibitory synaptic terminals [35]. However, CB1R efficiency in the activation of GTP-binding proteins appears to be much higher in the hypothalamus than in other brain regions [14], which may have a functional significance. Physiologically, the identification of CB1R in glutamatergic and GABAergic synaptic terminals in the VMH could be regarded as a potential neuronal substrate for the
effects of cannabinoids on eating behaviors. Actually, Glu-CB1R-KO conditional mice exhibit a hypophagic behavior after food deprivation very similar to the full CB1R-KO. On the contrary, GABA-CB1R-KO mutants are hyperphagic under the same experimental conditions [26]. As a conclusion, the VMH may be a good hub candidate in the endocannabinoid-mediated modulation of the excitatory and inhibitory neurotransmission regulating food intake behaviors. These anatomical data contribute to the understanding of the complex regulation of energy balance by the endocannabinoid system.

### Materials and Methods

#### Ethics Statement

The protocols for animal care and use were approved by the appropriate Committee at the Basque Country University (CEBA/93/2010/GRANDESMORENO). Furthermore, the animal experimental procedures were carried out in accordance with the European Communities Council Directive of 22 July 2003 (2003/65/CE) and current Spanish regulations (Real Decreto 1201/2005, BOE 21–10–2005). Great efforts were made in order to minimize the number and suffering of the animals used.

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Figure 3. Statistical analysis of CB1R in terminals forming asymmetric and symmetric synapses in the VMH of CB1R-WT mice processed by a preembedding immunogold method. A: 24.0 ± 2.9% of synaptic terminals making asymmetric and 28.9 ± 7.5% of terminals with symmetric synapses are CB1R immunopositive. No statistically significant difference is detected ($\chi^2 = 0.5946$, p = 0.4466, analyzed area: 2,376 $\mu$m$^2$). B: CB1R density after subtraction of background labeling (0.015 ± 0.003 particles/ $\mu$m in the VMH of CB1R-KO) is pretty similar in synaptic terminals making asymmetric or symmetric synapses (0.42 ± 0.03 and 0.47 ± 0.09 immunoparticles/ $\mu$m respectively, p = 0.6553).

doi:10.1371/journal.pone.0026167.g003

Figure 4. Statistical analysis of CB1R in the mouse VMH processed by a preembedding immunogold method. A: 20.5 ± 1.3% of the synaptic terminals/preterminals are CB1R immunopositive in CB1R-WT mice. Similar proportion is in Glu-CB1R-KO (20.8 ± 0.5%, $\chi^2 = 0.00024$, p = 0.9876), lower in GABA-CB1R-KO (12.4 ± 1.2%, $\chi^2 = 8.593$, p = 0.0034) and virtually disappears in CB1R-KO mice (3.9 ± 0.6%, $\chi^2 = 48.61$, p < 0.0001). A similar area was analyzed for each animal condition (1,467 $\mu$m$^2$ in CB1R-WT; 1,562 $\mu$m$^2$ in Glu-CB1R-KO; 1,646 $\mu$m$^2$ in GABA-CB1R-KO and 1,519 $\mu$m$^2$ in CB1R-KO mice). B: CB1R immunoparticle density after subtraction of background labeling (0.015 ± 0.003 particles/ $\mu$m in the VMH of CB1R-KO) is very close in CB1R-WT (0.49 ± 0.07), Glu-CB1R-KO (0.42 ± 0.02, P = 0.7000) and GABA-CB1R-KO (0.45 ± 0.03, P = 0.7000) mice. C: There is no statistically significant difference between the percentage of CB1R immunopositive asymmetric synapses in the VMH of CB1R-WT (27.2 ± 0.7%) and Glu-CB1R-KO (21.3 ± 2.5%, $\chi^2 = 0.4189$, p = 0.5175) mice. This value practically disappears in CB1R-KO mice (2.9 ± 2.9%, $\chi^2 = 15.47$, p < 0.0001). A similar area was analyzed for each animal condition (1,352 $\mu$m$^2$ in CB1R-WT; 1,547 $\mu$m$^2$ in Glu-CB1R-KO and 1,274 $\mu$m$^2$ in CB1R-KO mice).

doi:10.1371/journal.pone.0026167.g004
CBR mutant lines

Mutant animals were obtained and genotyped as previously described [26,27,29]. CBR-KO mice were generated by crossing the respective Cre-expressing mouse line with CB1f/f mice [36], using a three-step breeding protocol [27].

Generation of CB1f/f, NEX-Cre mice (here Glu-CB1R-KO). CB1f/f, NEX-Cre mice were obtained by crossing CB1f/f with NEX-Cre mice [37,30]. The helix-loop-helix transcription factor NEX is a marker of embryonic neuronal progenitors, which will develop into mature cortical glutamatergic neurons [39], whereas, in the adult brain, NEX is expressed in mature glutamatergic cortical neurons, but not in cortical GABAergic interneurons and to a much lesser extent in subcortical regions [40]. Cre expression under the control of the regulatory sequences of NEX in transgenic mice (NEX-Cre mice) as generated by knock-in into the NEX locus, leads to the specific deletion of “floxed” alleles in forebrain neurons [37].

Generation of CB1f/f, Dlx5/6-Cre mice (here GABA-CB1R-KO). Transgenic mice (Dlx5/6-Cre) were produced as previously described [41]. Dlx5/6-Cre mice were crossed with CB1f/f mice to obtain CB1f/f, Dlx5/6-Cre mice [27]. Dlx5/6-Dlx6 genes are homeobox genes that are expressed in differentiating and migrating forebrain GABAergic neurons during embryonic development [42]. Thus, expression of Cre recombinase under the control of the regulatory sequences of Dlx5/Dlx6 genes is expected to drive recombination ofloxP sites in GABAergic neurons [27].

Animal treatment

12 wild-type, Glu-CBR-KO, GABA-CBR-KO and CBR-KO mice (3 of each condition) were used in this study. Mice were deeply anesthetized by intraperitoneal injection of ketamine/xylazine (80/10 mg/kg body weight) and were transcardially perfused at room temperature (RT, 20–25°C) with phosphate-buffered saline (PBS 0.1M, pH 7.4) for 20 seconds, followed by the fixative solution made up of 4% formaldehyde (freshly depolymerized from paraformaldehyde), 0.2% picric acid and 0.1% glutaraldehyde in phosphate buffer (PB 0.1 M, pH 7.4) for 10–15 minutes. Then, brains were removed from the skull and postfixed in the fixative solution for approximately one week at 4°C. Afterwards, brains were stored at 4°C in 1:10 diluted fixative solution until used.

CBR immunocytochemistry for light microscopy

Coronal hypothalamic vibrisections were cut at 50 μm in a vibratome and collected in 0.1 M PB at RT. Sections were preincubated in a blocking solution of 10% bovine serum albumin (BSA), 0.1% sodium azide and 0.5% triton X-100 prepared in Tris-HCl buffered saline (TBS 1X, pH 7.4) for 30 minutes at RT. Then, they were incubated in a primary polyclonal goat anti-CBR antibody (2 μg/ml, Frontier Science co. Ltd, 1–777–12, Shinko-nishi, Ishikari, Hokkaido, Japan) prepared in the blocking solution but with 0.04% saponin, on a shaker for 2 days at 4°C. After several washes with 1% BSA in TBS, tissue sections were incubated in a secondary 1.4 nm nano-gold anti-goat antibody (1:100, Fab’ fragment, Nanoprobe Inc., Yaphank, NY, USA) prepared in the same solution as the primary antibody for 3 hours on a shaker at RT. Then, tissue was washed overnight at 4°C and postfixed in 1% glutaraldehyde for 10 minutes. After several washes in double distilled water, gold particles were silver-intensified with a HQ Silver Kit (Nanoprobe Inc., Yaphank, NY, USA) for 12 minutes in the dark. Then, tissue was extensively washed in double distilled water and in 0.1 M PB and osmicated in 1% osmium tetroxide for 20 minutes. After washing in 0.1 M PB, sections were dehydrated in graded alcohols (50–70–96%–100%) to propylene oxide and embedded in Epon resin. 80 nm ultrathin sections were collected on mesh nickel grids, stained with lead citrate for 2 minutes and examined in a Phillips EM200S electron microscope. Tissue preparations were photographed by using a digital camera coupled to the electron microscope. Figure compositions were made using Adobe Photoshop (CS, Adobe Systems, San Jose, CA, USA).

Specificity of the immunostainings was assessed by incubation of the CBR antibody in CBR-KO VMH tissue in the same conditions as above.

Statistical analysis of CBR in the VMH

Coronal hypothalamic vibrisections from each animal condition (n = 3 each) showing good and reproducible silver-intensified gold particles were cut at 80 nm. Image-J (version 1.43 μ, NIH, USA) was used to measure the membrane length. Electron micrographs (18,000–28,000X) were taken from grids (132 μm side) containing silver-intensified gold particles; all of them showed a similar labeling intensity indicating that selected areas were at the same depth. Furthermore, to avoid false negatives, only ultrathin sections in the first 1.5 μm from the surface of the tissue block were examined. Positive labeling was considered if at least one immunoparticle was within approximately 30 nm from the plasmalemma. Metal particles on synaptic membranes were visualized and counted.

Percentages of CBR positive profiles and density of immunoparticles were analyzed and displayed as mean ± S.E.M. using a statistical software package (GraphPad Prism 4, GraphPad Software Inc, San Diego, USA). Group differences were compared by chi-square test, p<0.05 (percentages of CBR positive profiles) and Mann Whitney test, p<0.05 (CBR density).
Author Contributions
Conceived and designed the experiments: PG GM LR NP. Performed the experiments: LR NP IE JM-Z MJC IB AR. Analyzed the data: LR NP PG. Wrote the paper: PG LR NP. Supervised part of the work: JS FRF.

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