eman ta zabal zazu

Universidad del País Vasco
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HIGH RESOLUTION IMAGING TO UNVEIL THE SUBCELLULAR LAYOUT OF THE CANNABINOID TYPE-1 RECEPTOR IN RODENT MODELS OF BRAIN DISEASE

DOCTORAL THESIS
Itziar Bonilla Del Río
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ABBREVIATIONS

- **AA:** Arachidonic acid
- **ABHD6:** α/β-hydrolase domain containing 6
- **ABHD12:** α/β-hydrolase domain containing 12
- **ACHI:** Awake closed head injury
- **AEA:** Arachidonoyl-ethanolamine or anandamide
- **AMPA:** D-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
- **AMT:** Anandamide membrane transporter
- **ANOVA:** Analysis of variance
- **AOB:** Accessory olfactory bulb
- **AON:** Anterior olfactory nucleus
- **as:** Astrocyte
- **ATP:** Adenosine-triphosphate

- **BD:** Binge drinking
- **BEC:** Blood ethanol concentration
- **BLA:** Basolateral amygdaloid nucleus
- **BNST:** Bed nucleus of the stria terminalis
- **BSA:** Bovine serum albumin

- **CA:** Cornu ammonis or Ammon´s horn
- **CA1:** Region 1 of Cornu ammonis
- **CA2:** Region 2 of Cornu ammonis
- **Ca^{2+}:** Calcium
- **CA3:** Region 3 of Cornu ammonis
- **cAMP:** Cyclic adenosine monophosphate
- **Cb:** Cerebellar cortex
- **CB_1:** Type I cannabinoid receptor
- **CB_1-eLTD:** CB_1 receptor-dependent excitatory long-term depression
- ***CB_1*-KO:** Cannabinoid type-1 receptor knock-out mice
- ***CB_1*-WT:** Cannabinoid type-1 receptor wild-type mice
- **CB_2:** Type II cannabinoid receptor
- **CCI:** Controlled cortical impact
- **CCK:** Cholecystokinin
- **Cg:** Cingulate cortex
- **CNS:** Central nervous system
- **COX-2:** Cyclooxigenase-2

- **CPu:** Caudate putamen
- **CRE:** cAMP response element
- **CRIP1a:** Cannabinoid receptor associated protein 1a
- **CSF:** Cerebrospinal fluid

- **DAB:** Diaminobezidine
- **DAG:** Diacylglycerol
- **DAGL:** Diacylglycerol lipase
- **DAI:** Diffuse axonal injury
- **den:** Dendrite
- **DG:** Dentate gyrus
- **DHEA:** N-docosahexaenoyl ethanolamine
- **DML:** Dentate molecular layer
- **DSE:** Depolarization-induced suppression of excitation at glutamatergic synapses
- **DSI:** Depolarization-induced suppression of inhibition at GABAergic synapses
- **DVI:** Diffuse vascular Injury

- **E2:** Estradiol
- **EAAT1:** Excitatory amino acid transporter 1
- **EC:** Entorhinal cortex
- **eCB:** Endocannabinoid
- **eCBS:** Endocannabinoid system
- **eCB-LTD:** Endocannabinoid-mediated long-term depression
- **eCB-STD:** Endocannabinoid-mediated short-term depression
- **EGFP:** Enhanced green fluorescent protein
- **EMCDDA**: European monitoring centre for drugs and drug addiction
- **Ent:** Entorhinal cortex
- **EP:** Entopeduncular nucleus
- **EPEA:** N-eicosapentaenoyl ethanolamine
- **EtOH:** Ethyl alcohol or ethanol
- **Exc:** Excitatory

- **FAAH:** Fatty acid amide hydrolase
- **fEPSP:** Field excitatory postsynaptic potential

- **GABA:** Gamma-aminobutyric acid

- **$GABA_A$:** Type A gamma-aminobutyric acid
- **GABA-*CB_1*-KO:** GABAergic neurons cannabinoid type-1 receptor knock-out mice
- **GABA-*CB_1*-RS:** GABAergic neurons cannabinoid type-1 receptor rescue mice
- **GASP1:** G-protein-associated sorting protein 1
- **GFAP:** Glial fibrillary acidic protein
- **GFAP-*CB_1*-KO:** Astrocyte cannabinoid type-1 receptor knock-out mice
- **GFAP-*CB_1*-RS:** Astrocyte cannabinoid type-1 receptor rescue mice
- **GFAPhrGFP-*CB_1*-KO:** CB_1-KO mice that express hrGFP in astrocytes
- **GFAPhrGFP-*CB_1*-WT:** CB_1-WT mice that express hrGFP in astrocytes
- **GFP:** Green fluorescence protein
- **GL:** Granule layer
- **GLAST:** Glutamate/aspartate transporter
- **Glu-*CB_1*-KO:** Dorsal telencephalic glutamatergic neurons cannabinoid type-1 receptor knock-out mice
- **Glu-*CB_1*-RS:** Dorsal telencephalic neurons cannabinoid type-1 receptor rescue mice
- **GluA1:** Glutamate A1
- **GluA2:** Glutamate A2
- **GluN2B:** Glutamate N2B
- **GP:** Globus pallidus
- **GPCRs:** G-protein-coupled receptors
- **GPR18:** G protein-coupled receptor 18
- **GPR55:** G protein-coupled receptor 55
- **GPR92:** G protein-coupled receptor 92
- **GPR119:** G protein-coupled receptor 119

- **HF:** Hippocampal formation
- **Hi:** Hippocampus
- **HIF-1:** Hypoxia‑induced factor‑1
- **hrGFP:** Humanized renilla green fluorescent protein

- **Iba1:** Ionized calcium binding adaptor molecule 1
- **IL-1b:** Interleukin 1 beta
- **IML:** Inner molecular layer
- **iNOS:** Inducible nitric oxide synthase
- **IRES:** Internal ribosomal entry site

Abbreviations

- **K^+:** Potassium

- **LPP:** Lateral perforant pathway
- **LPS:** Lipopolysaccharide
- **LTD:** Long-term depression
- **LTP:** Long-term potentiation

- **m:** Mitochondria
- **M1:** Primary motor cortex
- **MAGL:** Monoacylglicerol lipase
- **METH:** Methamphetamine
- **mGluR:** Metabotropic glutamate receptors
- **MOB:** Main olfactory bulb
- **MPP:** Medial perforant pathway
- **MRI:** Magnetic resonance imaging
- **mRNA:** Messenger ribonucleic acid
- **MSE:** Metabotropic-induced suppression of excitation
- **MSI:** Metabotropic-induced suppression of inhibition
- **$mtCB_1$:** Mitochondrial CB_1
- **mTBI:** Mild traumatic brain injury

- **Na^+:** Sodium
- **NAAA:** Lysosomal N-acyl ethanolamine cysteine-amidohydrolase
- **NADA:** N-arachidonoyl dopamine
- **NAE:** N-acyl ethanolamine
- **NAPE:** N-acylphosphatidyl ethanolamine
- **NAPE-PLD:** N-acyl phosphatidyl ethanolamine hydrolyzing phospholipase D
- **NArGly:** N-arachidonoyl glicyne
- **NArPE:** N-arachidonoyl phosphatidyl ethanolamine
- **NArS:** N-arachidonoyl serine
- **NAT:** N-acyl transferase
- **NCIPC:** National Center for Injury Prevention and Control
- **NMDA:** N-methyl-D-aspartate
- **NMDARs:** N-Methyl-D-aspartate receptors
- **nNOS:** Neural nitric oxide synthase
- **NOR:** Novel object recognition

- **NOS:** Nitric oxide synthase
- **NR2B:** N-methyl D-aspartate receptor subtype 2B
- **NT:** Neurotransmitters
- **NuAc:** Nucleus accumbens

- **OEA:** N-oleoyl ethanolamine
- **OML:** Outer molecular layer

- **PAG:** Periaqueductal gray
- **Part:** Particles
- **PB:** Phospate buffer
- **PBS:** Phosphate buffered saline
- **PC:** Purkinje cell
- **PE**: Phosphatidyl ethanolamine
- **PEA:** Palmitoyl ethanolamine
- **PF:** Parallel fiber
- **PID:** Post-injury day
- **PKA:** Protein kinases type A
- **PLC:** Phospholipase C
- **Pnd:** Postnatal day
- **PP:** Paired pulse
- **PP:** Perforant pathway
- **PPARs:** Peroxisome proliferator-activated receptors
- **PSD95:** Postsynaptic density protein 95

- **r-mTBI:** Repeat mild traumatic brain injury
- **ROS:** Reactive oxygen species
- **RT:** Room temperature

- **S1:** Primary somatosensory cortex
- **SAMHSA:** Substance abuse mental health services administration
- **SEA**: *N*-stearoyl ethanolamine
- **SEM:** Standard error mean
- **SLM:** Stratum lacunosum moleculare
- **SNR:** Substantia nigra pars reticulata
- **Sp:** Dendritic spine

- **SP:** Stratum pyramidale
- **SR:** Stratum radiatum
- **SR141716A:** N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
- **SSE:** Synaptically-evoked suppression of excitation
- **SSI:** Synaptically-evoked suppression of inhibition
- **STOP-CB_1:** Mice carrying a loxP-flanked stop cassette inserted in the 5'UTR upstream of the CB_1 receptor translational start codon

- **TA:** Temporoammonic pathway
- **TBI:** Traumatic brain injury
- **Ter:** Terminal
- **TNF-α:** Tumoral necrosis factor α
- **TRP:** Transient receptor potential
- **TRPM8:** Transient receptor potential melastatin type 8
- **TRPV1:** Transient potential receptors of vanilloid type 1

- **UTR:** Untranslated

- **V1:** Primary visual cortex
- **VMH:** Ventromedial hypothalamus
- **VTA:** Ventral tegmental area

- **WHO:** World health organization
- **WT:** Wild type

- **2-AG:** 2-arachidonoyl glycerol
- **2-OG:** 2-oleoyl glycerol
- **5xFAD:** 5 familial Azheimer´s disease mutations
- **[^{35}S] GTPγS:** [^{35}S]guanosine-5*-O-(3-thiotriphosphate)
- **Δ9-THC** or **THC:** Delta (9)-tetrahydrocannabinol

1. SUMMARY

The endocannabinoid system (eCBS) is composed of cannabinoid receptors, such as, cannabinoid type-1 (CB_1), endogenous ligands (endocannabinoids, eCBs) and their synthesizing and degrading enzymes. CB_1 receptors are expressed in different cell types (i.e., astrocytes, neurons) and intracellular compartments (i.e., mitochondria). In the brain, the eCBS is a crucial modulator of synaptic transmission and plasticity, regulating several physiological processes. Furthermore, activation of CB_1 receptors by endogenous or exogenous ligands (cannabis derivatives, i.e. THC) has a wide variety of behavioral effects, both positive and negative.

Different histological techniques have been crucial in defining the CB_1 receptor expression and localization at the cellular level. However, it is extremely difficult to identify the subcellular distribution of CB_1 receptors in some cell-types (i.e., astrocytes) due to its low expression level on those cells. Moreover, it remains a key question to know the pattern of the subcellular CB_1 receptor expression and distribution under pathological states. Thus, the present work focuses on the description of new subcellular localizations of CB_1 receptors in normal brain and the study of the CB_1 receptor expression in certain pathophysiological states.

High resolution immunoelectron microscopy has shown to be an excellent approach for the fine detection of CB_1 receptors in the brain. The single pre-embedding immunogold method for electron microscopy based on the use of specific primary CB_1 receptor antibodies and silver-intensified 1.4 nm gold-labeled Fab' fragments, and the combined pre-embedding immunogold and immunoperoxidase method implies the additional use of biotinylated secondary antibodies and avidin-biotin complex for the simultaneous localization of CB_1 receptors and protein markers of specific brain cells or synapses.

CB_1 receptors in astroglial mitochondria.

In order to identify and characterize the presence of mitochondrial CB_1 receptors ($mtCB_1$) in astrocytes we used conditional knock-out mice lacking CB_1 receptor specifically in glial fibrillary acidic protein (GFAP)-containing astrocytes (GFAP-CB_1-KO) mice and rescue mice expressing CB_1 receptors exclusively in astrocytes (GFAP-CB_1-RS). Complementary, to identify astroglial structures by immunoelectron microscopy, an adeno-associated virus expressing humanized renilla green fluorescent protein (hrGFP) under the control of human GFAP promoter was injected in the hippocampus of CB_1 knock-out (CB_1-KO) mice and wild-type (CB_1-WT) littermates to generate GFAPhrGFP-CB_1-KO and -WT mice, respectively.

Double immunogold (for CB_1) and immunoperoxidase (for GFAP, GLAST or hrGFP) revealed the presence of mtCB_1 receptors in hippocampal astrocytes. Altogether, we demonstrated the existence of a precise molecular architecture of the CB_1 receptor in astrocytes that will have to be taken into account in evaluating the functional activity of cannabinergic signaling at the tripartite synapse.

CB_1 receptors in pathophysiological states.

1. Ethanol (EtOH) consumption heavily impacts on the structure and function of the brain, particularly in adolescence. It is currently unknown how CB_1 receptor expression in astrocytes is affected by long-term exposure to EtOH. Here we examined EtOH-exposed adolescent mice to determine its effect on CB_1 receptor localization and density in the hippocampal astrocytes. Our results revealed a significant reduction in CB_1 receptor immunoparticles in astrocytic processes of EtOH-exposed mice when compared with controls. Such a decrease reveals a long lasting effect of EtOH on astrocytic CB_1 receptors. This deficiency may also have negative consequences for synaptic function.

2. Traumatic brain injury (TBI) and concussion (or mild TBI, mTBI) are major worldwide health and socioeconomic concern. Repeated mTBI (r-mTBI) in juvenile populations can result in cumulative neuropathology and learning and memory deficits during adulthood. However, there is scarce preclinical data showing the extent of such these deficits. To examine this issue, we used a model of r-mTBI in juvenile male and female rats. The animals were sacrificed at different time points after injury (1, 10 or 40 days after). Pre-embedding immunoelectron revealed a significant reduction in the proportion of CB_1 receptor immunopositive synaptic terminals in the adult dentate molecular layer (DML) after juvenile concussion. The loss of CB_1 receptor indicate that r-mTBI may induce deficits that are progressive in nature, and that develop over a prolonged period of time following the cessation of injury.

3. Cannabis is the most widespread illicit drug in the world and its main psychotropic ingredient Δ9-tetrahydrocannabinol (THC) exerts psychoactive effects through the activation of CB_1 receptors. Despite the fact that cannabis consumption often begins during adolescence, no much information is available on the fine anatomical changes potentially produced by the drug during this period. Moreover, the impact of adolescent THC consumption on the localization of CB_1 receptor in adult brain remains unknown. To investigate this, adolescent WT mice were injected subcutaneously with THC or vehicle. After 30 min, animals were sacrificed for posterior pre-embedding immunogold technique in the hippocampus.

The acute THC administration affected dendritic morphology and caused a sharp increase in the number of dendritic mitochondria. Also, CB_1 receptor distribution was drastically reduced in inhibitory synapses, astrocytic processes and mitochondria.

Altogether, the present data indicate the existence of changes in CB_1 receptor expression and structural brain adaptations that support the behavioral alterations caused by pathological conditions such as adolescent binge drinking (BD), concussion and cannabis intoxication, consequently representing these changes novel pharmacological targets to palliate the structural, functional and behavioral consequences of these disorders.

2. INTRODUCTION

2.1. DISTRIBUTION OF THE ENDOCANNABINOID COMPONENTS IN THE CENTRAL NERVOUS SYSTEM (CNS)

The endocannabinoid system is a complex endogenous signalling system widely distributed throughout the mammalian organism that participates in multiple metabolic pathways regulating cell physiology. This system is made up of the cannabinoid receptors, endogenous ligands and their synthesizing and degrading enzymes, intracellular signalling pathways as well as transport systems (Piomelli, 2003, 2014; Marsicano & Lutz, 2006; Kano et al., 2009; Katona & Freund, 2012; Lutz et al., 2015; Pertwee, 2015; Lu & Mackie, 2016).

The eCBS, besides from being present in many other organs (Piazza et al., 2017), is widely distributed in the central and peripheral nervous system (Katona & Freund, 2012; Lu & Mackie, 2016), where it regulates brain functions by acting on different cell types and cellular compartments (Bénard et al., 2012; Katona & Freund, 2012; Busquets-Garcia et al., 2018; Gutiérrez-Rodríguez et al., 2017, 2018; Lu & Mackie, 2016).

2.2. CB_1 AND CB_2 RECEPTORS IN THE CNS

Cannabinoid receptors are membrane associated G protein-coupled receptors (Fig. 1) with two subtypes referred as cannabinoid type-1 (CB_1) and cannabinoid type-2 (CB_2) receptors. CB_1 and CB_2 are widely expressed throughout the body but CB_1 receptors are concentrated within the brain, whereas CB_2 receptors are largely expressed by cells of the immune system. The two cannabinoid receptors can be stimulated by eCBs (principal ones are anandamide (AEA) and 2-arachidonoylglycerol (2-AG)) or exogenous cannabinoids (Fig.1).

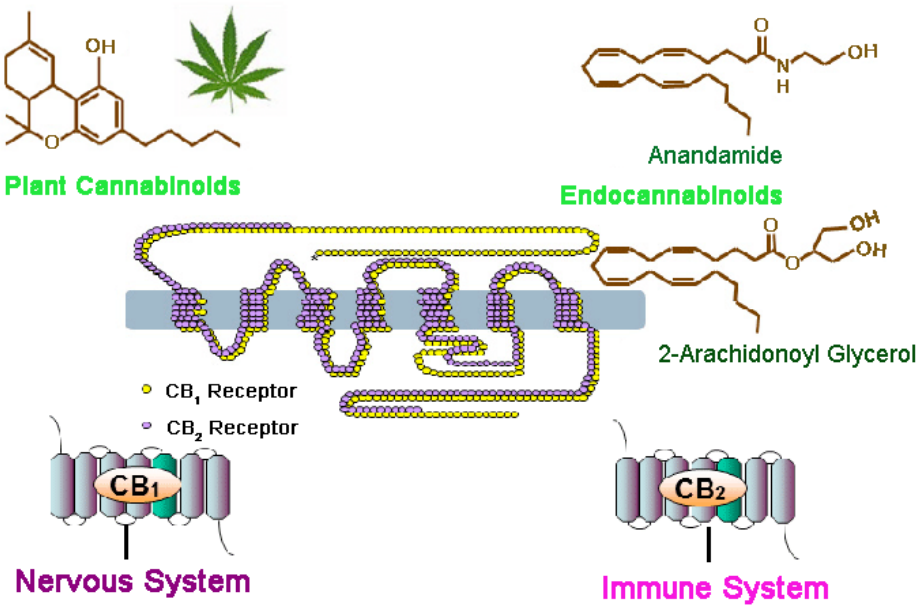


Figure 1. The cannabinoid system. (Modified from Price & Baker, 2012).

The **CB_1 receptor** is one of the most abundant G protein-coupled receptor in the brain (Herkenham et al., 1991; Tsou et al., 1998; Moldrich & Wenger, 2000). Its expression is widespread, heterogeneous and has crucial roles in brain function, dysfunction and cognition (Marsicano et al., 2002; Monory et al., 2006; Marsicano & Kuner, 2008; Bellocchio et al., 2010; Puente et al., 2011; Castillo, 2012; Katona & Freund, 2012; Steindel et al., 2013; Ruehle et al., 2013; Soria-Gómez et al., 2014, 2015; Hu & Mackie, 2015; Katona, 2015; Martín-García et al., 2015; Gutiérrez-Rodríguez et al., 2017, 2018; Bonilla-Del Río et al., 2019).

The CB_1 receptor distribution in the brain closely fits into the deleterious effects of cannabinoids on locomotion, perception, learning, memory or the cannabinoid-positive effects as anti-convulsant or food intake enhancers, and its low amount in the brainstem correlates with the low toxicity and lethality of marihuana (Bellocchio et al., 2010; Han et al., 2012; Katona & Freund, 2012; Hebert-Chatelain et al., 2014a,b, 2016; Soria-Gómez et al., 2014; Martín-García et al., 2015; Lu & Mackie, 2016; Mechoulam, 2016). CB_1 receptors are abundant in the basal ganglia (substantia nigra reticulata, globus pallidus, striatum, entopeduncular nucleus), cortex, nucleus accumbens, cerebellum, hippocampus (Howlett et al., 1990; Tsou et al., 1998; Hu & Mackie, 2015; Martín-García et al., 2015) but they are poorly expressed in the hypothalamus, brainstem and spinal cord (Tsou et al., 1998).

In order to achieve higher resolution on the distribution of the CB_1 receptors, genetically modified animals are a useful model. Mice with CB_1 receptor gene deletion (*CB_1*-KO) lack CB_1 receptor protein expression (Steiner et al., 1999; Zimmer et al., 1999; Marsicano et al., 2002; Zimmer, 2015) and, therefore, only unspecific CB_1 receptor immunolabeling is

observed in these mutants tissue. Conditional mutant mice lacking CB_1 receptor mainly from cortical glutamatergic neurons (Glu-*CB_1*-KO), and mainly from GABAergic neurons (GABA-*CB_1*-KO) (Monory et al., 2006, 2007) show a selective decrease in the brain pattern of CB_1 receptor staining but not in the same degree as in *CB_1*-KO; in particular, the CB_1 receptor immunoreactivity is greatly reduced in the GABA-*CB_1*-KO and less in the Glu-*CB_1*-KO compared with the wild-type (Monory et al., 2006, 2007; Marsicano & Kuner, 2008; Steindel et al., 2013; Martín-García et al., 2015) indicating that CB_1 receptors are more abundantly expressed in GABAergic neurons than in glutamatergic neurons. An exception would be the great reduction in CB_1 receptor staining observed in the granule cell layer of the Glu-*CB_1*-KO olfactory bulb (Soria-Gómez et al., 2014). Substantia nigra pars reticulata lacks CB_1 receptor immunoreactivity in GABA-*CB_1*-KO, and a large decrease in CB_1 receptor staining is observed in the GABA-*CB_1*-KO hippocampus but not at the zone of the glutamatergic commissural/associational synapses in the inner one-third of the DML (Monory et al., 2006, 2007; Marsicano & Kuner, 2008; Martín-García et al., 2015). Conversely, the weak pattern of CB_1 receptor immunostaining in genetic rescue mice expressing CB_1 receptors only in dorsal telencephalic glutamatergic neurons (Glu-*CB_1*-RS) (Ruehle et al., 2013; Soria-Gómez et al., 2014; de Salas-Quiroga et al., 2015; Lange et al., 2017; Gutiérrez-Rodríguez et al., 2017, 2018) relative to the rescue mice expressing CB_1 receptors only in GABAergic neurons (GABA-*CB_1*-RS) (de Salas-Quiroga et al., 2015; Lange et al., 2017; Remmers et al., 2017; Gutiérrez-Rodríguez et al., 2017, 2018) correlates with the low CB_1 receptor distribution in glutamatergic neurons and high in GABAergic cells, respectively. However, a conspicuous CB_1 receptor staining in Glu-*CB_1*-RS is observed in the striatum, cortex, olfactory tubercle, amygdala, hippocampus (strata oriens and radiatum of the hippocampal Ammon's horn) and, remarkably, in the inner one-third of the DML of Glu-*CB_1*-RS (Monory et al., 2006; Ruehle et al., 2013; Gutiérrez-Rodríguez et al., 2017). In GABA-*CB_1*-RS, strong CB_1 receptor immunoreactivity is seen in the cortex, anterior olfactory nucleus, piriform cortex, globus pallidus, entopeduncular nucleus, amygdala, and substantia nigra, and moderate to strong in the striatum (Gutiérrez-Rodríguez et al., 2017). In the hippocampus, heavy CB_1 receptor immunoreaction is present throughout the hippocampus, particularly in the Ammon's horn pyramidal cell layer, at the limit between the strata radiatum and the lacunosum-moleculare and in the inner one-third of the DML (Gutiérrez-Rodríguez et al., 2017; Remmers et al., 2017).

When observed under the electron microscope the distribution of CB_1 receptor correlates with the previously reported results. First, CB_1 receptor expression is very high in inhibitory GABAergic synaptic terminals mostly in cortical and hippocampal cholecystokinin (CCK)-positive GABAergic interneurons (Kawamura et al., 2006; Ludányi

et al., 2008; Marsicano & Kuner, 2008; Katona & Freund, 2012; De-May & Ali, 2013; Steindel et al., 2013; Hu & Mackie, 2015; Lu & Mackie, 2016; Gutiérrez-Rodríguez et al., 2017), low in excitatory glutamatergic synapses (Marsicano et al., 2003; Domenici et al., 2006; Katona et al., 2006; Monory et al., 2006; Takahashi & Castillo, 2006; Kamprath et al., 2009; Bellocchio et al., 2010; Puente et al., 2011; Reguero et al., 2011; Ruehle et al., 2013; Soria-Gómez et al., 2014; Gutiérrez-Rodríguez et al., 2017) and very low in brain astrocytes (Rodríguez et al., 2001; Navarrete & Araque, 2008, 2010; Stella, 2010; Han et al., 2012; Bosier et al., 2013; Metna-Laurent & Marsicano, 2015; Viader et al., 2015; Da Cruz et al., 2016; Kovács et al., 2017; Gutiérrez-Rodríguez et al., 2018). Brain CB_1 receptors are mostly localized in axon terminals and preterminals away from the presynaptic active zones and are also localized at mitochondria in neurons (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016; Koch et al., 2015) and astrocytes (Gutiérrez-Rodríguez et al., 2018).

CB_1 receptors also localize in adipose tissue, muscle, liver, heart, gastrointestinal tract, pancreas, spleen, tonsils, prostate, testicle, uterus, ovary, skin, eye, or presynaptic sympathetic nerve terminals (Galiegue et al., 1995; Ishac et al., 1996; Pertwee, 2001; Macarrone, 2016; Zou & Kumar, 2018). They are also present at mitochondria of skeletal (gastrocnemius and rectus abdominis) and myocardial muscles (Mendizabal-Zubiaga et al., 2016) whose activation by THC reduces mitochondria coupled respiration (Mendizabal-Zubiaga et al., 2016).

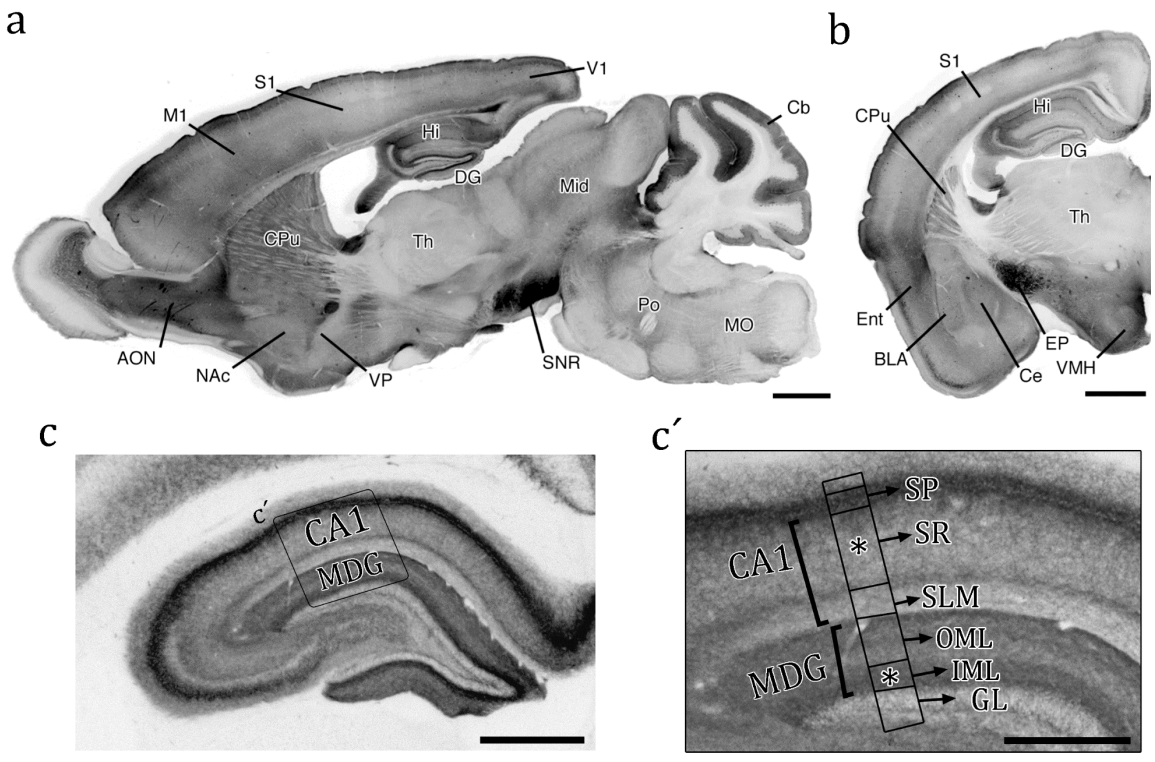


Figure 2. Distribution of CB_1 receptors in the CNS of WT mice. (a) Overall distribution in parasagittal and (b) coronal brain sections of wild-type. CB_1 immunoreactivity is highest along striatal output pathways, including the substantia nigra pars reticulata (SNR), globus pallidus (GP), and entopeduncular nucleus (EP). High levels are also observed in the hippocampus (Hi), dentate gyrus (DG), and cerebral cortex, such as the primary somatosensory cortex (S1), primary motor cortex (M1), primary visual cortex (V1), cingulate cortex (Cg), and entorhinal cortex (Ent). High levels are also noted in the basolateral amygdaloid nucleus (BLA), anterior olfactory nucleus (AON), caudate putamen (CPu), ventromedial hypothalamus (VMH), and cerebellar cortex (Cb) (modified from Kano et al., 2009). (c, c´) High-power views of the hippocampal formation (CA1 and DML). (c´) SP stratum pyramidale, SR stratum radiatum, SLM stratum lacunosum moleculare, OML: outer molecular layer, IML inner molecular layer GL granular layer. Scale bars: 1 mm (a, b), 500 μm (c) and 200 μm (c´). ***** *Areas of interest in this doctoral thesis*.

The **CB_2 receptor** was first described in spleen (Munro et al., 1993) and, in addition to this organ, it was believed to be only present in the immune system (tonsils, B and T lymphocytes, natural killer cells, macrophages and CD8 and CD4 T-lymphocytes) (Galiègue et al., 1995; Ameri, 1999; Cabral et al., 2015). However, CB_2 receptors are also expressed in heart, endothelium, bone, liver, pancreas, testicle (Zou & Kumar, 2018). The localization of CB_2 receptors in the CNS is a controversial issue as not specific CB_2 receptor antibodies are available so far (Atwood & Mackie, 2010; Lu & Mackie, 2016). Therefore, new genetic strategies based on mouse lines expressing enhanced green fluorescent protein (EGFP) under the control of the CB_2 promoter have been developed to circumvent this problem (López et al., 2018).

In this regard, some authors have not found CB_2 receptors in the intact CNS (Derocq et al., 1995; Galiegue et al., 1995; Griffin et al., 1999; Schatz et al., 1997; Sugiura et al., 2000; Carlisle et al., 2002; López et al., 2018), while others have pointed to the CB_2 receptor expression in the brain of several animal species, though at much lower levels than in the immune system (Benito et al., 2003; Maresz et al., 2005; Van Sickle et al., 2005; Gong et al., 2006). Furthermore, some studies have reported neuronal CB_2 receptor expression in healthy brain (Van Sickle et al., 2005; Ashton et al., 2006; Gong et al., 2006; Baek et al., 2008; Onaivi et al., 2008b), but others only find CB_2 receptors that are highly inducible by pathological conditions that elicit neuro-inflammatory responses (Benito et al., 2008; Atwood & Mackie, 2010; Dhopeshwarkar & Mackie, 2014; Di Marzo et al., 2015; Lu & Mackie, 2016; López et al., 2018), particularly, in activated microglia (Carlisle et al., 2002; Maresz et al., 2005; Benito et al., 2008; Cabral et al., 2008; Stella, 2010). Nevertheless, keeping in mind the point about antibody specificity, the presence of CB_2 receptors has

been described in neural progenitors as well as in cortical, hippocampal, pallidal and mesencephalic neurons (Lanciego et al., 2010); in the cerebellar Purkinje and molecular layers (Van Sickle et al., 2005; Ashton et al., 2006; Gong et al., 2006; Baek et al., 2008; Onaivi et al., 2008b); in the brainstem dorsal motor nucleus of the vagus (Van Sickle et al., 2005; Baek et al., 2008), parvocellular portion of the medial vestibular nucleus and dorsal and ventral cochlear nuclei (Baek et al., 2008), and in the hippocampus (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2006, 2008a,b; Brusco et al., 2008b). In addition, CB_2 receptor-like staining was detected in olfactory tubercle, islands of Calleja, frontal cortex, amygdala, striatum, substantia nigra, periaqueductal gray, paratrochlear nucleus, paralemniscal nucleus, red nucleus and inferior colliculus (Gong et al., 2006; Onaivi, 2006; Onaivi et al., 2006, 2008b; Liu et al., 2009; Kim & Li, 2015). Furthermore, the receptor has also been seen in striatal GABAergic neurons of non-human primates (Lanciego et al., 2011; Sierra et al., 2015). Only low levels of CB_2 receptor mRNA, but not of CB_2 receptor protein, was detected in the rat inferior olive and pontine nuclei (Viscomi et al., 2009); CB_2 receptor-like immunoreactivity, but not CB_2 receptor mRNA, was seen in the thalamus (Gong et al., 2006) and CB_2 receptor mRNA was detected in striatum and hypothalamus, but not in olfactory bulb, cortex and spinal cord (Gong et al., 2006; Onaivi et al., 2008b). In human samples, CB_2 receptors are highly expressed in testis, but also in spleen and leukocytes (Liu et al., 2009); it is also expressed in perivascular microglia in control brains (Nuñez et al., 2004) and in the amygdala, caudate, putamen, nucleus accumbens, cortex, hippocampus and cerebellum at low levels (Liu et al., 2009; Zou & Kumar, 2018). Subcellularly, CB_2 receptors seem to be mainly in plasma membranes of pyramidal cell apical dendrites and some interneurons in the hippocampus (Gong et al., 2006; Brusco et al., 2008b; Onaivi et al., 2008b) and also associated to the rough endoplasmic reticulum and Golgi apparatus in cell bodies (Brusco et al., 2008a).

2.3. ENDOCANNABINOIDS IN THE CNS

The eCBs are lipid messengers considered as promiscuous molecules since they activate CB_1 and CB_2 receptors and other receptors such as the transient receptor potential channel V1 (TRPV1), peroxisome proliferator-activated receptors (PPARs), the glycine receptor α1 subunit and the $GABA_A$ receptor β2 subunit, among others (Piomelli, 2003; Kano et al., 2009; Pertwee et al., 2010; Katona & Freund, 2012; Lutz et al., 2015; Lu & Mackie, 2016; Zou & Kumar, 2018). The physiology and pharmacology of the eCBs are complex due to both the vast distribution of the numerous components and the features

of the system. The eCBs exert their influence in a paracrine and autocrine manner, and probably even in endocrine mode, because their lipid nature allows them to diffuse and cross membranes. They are cannabinoid receptor agonists that constitute a family of molecules that are not accumulated in secretory vesicles (though might be stored in adiposomes) but rather synthesized on demand and released right after to the extracellular space following physiological and pathological stimuli (Piomelli, 2003; Kano et al., 2009; Pertwee et al., 2010; Katona & Freund, 2012; Lutz et al., 2015; Lu & Mackie, 2016; Zou & Kumar, 2018).

The two main eCBs are derivatives of polyunsatured fatty acids, AEA (Devane et al., 1992) and 2-AG (Mechoulam et al., 1995). AEA produces the "tetrad" effects of cannabinoids (i.e., catalepsy, antinociception, hypolocomotion, and hypothermia) in rodents (Fride & Mechoulam, 1993) whereas 2-AG plays a key role in most of the CB_1 receptor-dependent modulation of synaptic transmission and plasticity (Kano et al., 2009). 2-AG concentration in brain tissue is about 200-fold higher than AEA (Bisogno et al., 1999) and correlates well with the cannabinoid receptor density in the brain (Sugiura et al., 2006). However, this is not the case for AEA that accumulates in brain regions with high cannabinoid receptor density (hippocampus, cortex, striatum) and also in regions with low receptor expression (thalamus, brainstem) (Felder & Glass, 1998). 2-AG is an agonist with high efficacy on both CB_1 and CB_2 receptors (Lynn & Herkenham, 1994; Slipetz et al., 1995; Gonsiorek et al., 2000; Sugiura et al., 2000), while the AEA efficacy is low at CB_1 (partial agonist) and very low at CB_2 receptors (weak partial agonist/antagonist) (Showalter et al., 1996; Gonsiorek et al., 2000; Sugiura et al., 2000; Luk et al., 2004).

There is a great variety of biochemical pathways for the synthesis, transport, release and degradation of eCBs. Thus, the biosynthetic enzymes phospholipase D selective N-acylphosphatidyl ethanolamine (NAPE-PLD) for AEA and diacylglycerol lipases (DAGL) α and β for 2-AG, as well as the hydrolytic enzymes fatty acid amide hydrolase (FAAH) for AEA inactivation and monoacylglycerol lipase (MAGL) for 2-AG, among others, are responsible for the distinctive physiological and pathophysiological roles of both eCBs (Kano et al, 2009; Fezza et al., 2014; Piomelli, 2014; Lu & Mackie, 2016; Zou & Kumar, 2018).

The production of AEA precursor, N-arachidonoyl phosphatidyl ethanolamine (NArPE), takes place by the arachidonic acid (AA) transfer from sn-1 position of phospholipids to the nitrogen atom of the phosphatidyl ethanolamine (PE) by the Ca^{2+} dependent N-acyl transferase (NAT) (Cadas et al., 1996; Kano et al., 2009; Fezza et al., 2014). Then, AEA is synthezised by the NAPE-PLD that hydrolyses NArPE localized in cell membranes (Okamoto et al., 2004; Kano et al., 2009). An alternative pathway for AEA synthesis is the

N-acylphosphatidyl ethanolamine (NAPE) hydrolysis by phospholipase C to phosphoanandamide followed by the protein tyrosine phosphatase PTPN22-mediated dephosphorylation (Liu et al., 2006). The AEA half-life is very short because of its quick uptake by a high affinity transporter (AMT, *anandamide membrane transporter*) distributed in neurons and glia (Di Marzo et al., 2015). AEA is inactivated by FAAH present in many organs and also in the brain (Dinh et al., 2002; Ueda, 2002; Kano et al., 2009) where its postsynaptic localization meets with presynaptic CB_1 receptors (Egertová et al., 2003; Kano et al., 2009; Hu & Mackie, 2015). FAAH is serine-hydrolase bound to intracellular membranes that catalyzes AEA into AA and ethanolamine (Fezza et al., 2014). It also degrades the bioactive N-acyl ethanolamines, N-palmitoyl ethanolamine (PEA) and N-oleoyl ethanolamine (OEA) (Di Marzo et al., 2001). There are two more hydrolases for AEA degradation: FAAH-2 and the lysosomal *N*-acyl ethanolamine cisteine-amidohydrolase (NAAA).

2-AG participates in the CB_1-dependent retrograde signalling and is an intermediate metabolite for lipid synthesis providing AA for prostaglandin synthesis (Kano et al., 2009; Fezza et al., 2014; Lu & Mackie, 2016). Neuronal membrane depolarization or the activation of Gq-coupled GPCRs triggers the synthesis of 2-AG (Kano et al., 2009). The DAG precursors come from the hydrolysis of membrane phosphatidylinositol by phospholipase C, β or δ. The degradation of these precursors by DAGL-α and DAGL-β drives 2-AG synthesis (Kano et al., 2009; Gao et al., 2010; Tanimura et al., 2010; Lu & Mackie, 2016; Zou & Kumar, 2018). The DAGLα isoform synthesizes the greatest amount of 2-AG; DAGLβ synthesizes 2-AG under certain circumstances yielding the cannabinoid upon an immune response (Di Marzo et al., 2015). MAGL is a serine-hydrolase that catalyzes 2-AG into AA and glycerol (Dinh et al., 2002; Ueda, 2002; Kano et al., 2009); MAGL is mainly found in presynaptic terminals (Kano et al., 2009; Straiker et al., 2009; Hu & Mackie, 2015; Lu & Mackie, 2016). Also, the α/β-hydrolase domain 6 (ABHD6) and domain 12 (ABHD12) degrade 2-AG (Blankman et al., 2007; Kano et al., 2009; Fezza et al., 2014).

AEA and 2-AG are also metabolized by lipooxygenases and cyclooxygenase-2 (COX-2) (Kano et al., 2009; Lu & Mackie, 2016) and AEA is additionally the target of P450 cytocrome. The degradation products obtained from AEA by lipooxygenases (hydroxyanandamides) are CB_1, CB_2, PPARs and TRPV1 receptor ligands and can also interact with enzymes of the eCBS. COX-2 shows more preference for AEA degradation than for other acyl ethanolamines, and generates prostamides (Kano et al., 2009; Iannotti et al., 2016; Lu & Mackie, 2016). The prostaglandine E2–glycerol esther generated by COX-2 potentiates synaptic transmission and plasticity and produces hyperalgesia (Katona &

Freund, 2012).

AEA inhibits presynaptic GABA and glutamate release under certain circumstances and activates TRPV1 which belongs to the transient receptor potential (TRP) channel family activated by noxious heat (>42ºC), low pH (<6.0) and capsaicin, the active component of the hot chilli pepper (Zygmunt et al., 1999; Ross, 2003; Chávez et al., 2010; Puente et al., 2011; Lu & Mackie, 2016; Zou & Kumar, 2018). 2-AG also activates TRPV1, though a higher concentration is needed for reaching similar AEA effect. *In vitro*, AEA antagonizes TRP melastatin type 8 (TRPM8) channel responsible for the cold sensation induced by menthol and temperatures lower than 25ºC (Pertwee, 2015). Interestingly, other N-acyl etanolamines (NAE) such as OEA with appetite suppressant properties (Fu et al., 2003; Wang et al., 2005; Thabuis et al., 2008) and PEA with anti-inflammatory properties (Di Marzo et al., 2001; Costa et al., 2008) exhibit a variety of similar biological activities and share with the main eCBs certain metabolic routes potentiating their effects through competitive hydrolytic inhibition or allosteric modulation of binding to the receptor (Iannotti et al., 2016). The biological activity of these NAEs involves TRPV1 and PPARα activation as well as CB_1 receptors (De Petrocellis & Di Marzo, 2009). OEA, PEA and 2-oleoylglycerol (2-OG) also activate GPR119 receptor expressed mostly in human and rat pancreas; besides, OEA and PEA activate GPR55 at high concentrations. PEA does not act on CB_1 receptors in the CNS. The *N*-stearoyl ethanolamine (SEA) is a NAE that controls cell growing and has anti-inflammatory, immunomodulator and antinociceptive with anorexic properties. There are more lipids such as *N*-arachidonoyl glicyne (NArGly) and *N*-arachidonoyl serine (NArS). NArGly is a high affinity GPR18 ligand, GPR92 partial agonist, potent FAAH inhibitor and with neuroprotective effects through CB_2 receptors and TRPV1 (Pertwee, 2015). Further, the ω-6 fatty acids *N*-dihomo-γ-linolenoyl ethanolamine (weak cannabinoid receptors agonist); 2-AG ether (noladin ether; CB_1 receptor agonist and very weak CB_2 receptor agonist that interferes AEA reuptake); *N*-arachidonoyl dopamine (NADA) and the analogue *N*-oleoyl dopamine (TRPV1 y PPARγ agonists); *O*-arachidonoyl ethanolamine (virodhamine; *in vitro*: partial CB_1 receptor agonist and CB_2 receptor agonist; *in vivo*: CB_1 receptor antagonist and weak AEA reuptake inhibitor). Like AEA, noladin ether and virodhamine interact with PPARα and GPR55. Two ethanolamines of ω-3 (n-3) polyunsaturated fatty acids, *N*-eicosapentaenoyl ethanolamine (EPEA) and *N*-docosahexaenoyl ethanolamine (DHEA), are cannabinoid receptors and PPARγ agonists (Pertwee, 2015). Finally, there are more structurally related endocannabinoid compounds such as *N*-acyl palmitic acid and *N*- and *O*-acyl oleic acid derivatives that are more abundant than AEA but lack affinity for cannabinoid receptors, though they are metabolized by the same synthesizing and degrading enzymes of the most common eCBs

(Fezza et al., 2014; Iannotti et al., 2016).

2.4. THE ACTIVATION OF CB_1 RECEPTORS

CB_1 expression, as already mentioned, is widespread, heterogeneous and has crucial roles in the brain during prenatal and postnatal development and participates outstandingly in many brain functions ranging from food intake to cognition, through the modulation of synaptic transmission and plasticity (Marsicano et al., 2002; Monory et al., 2006; Marsicano & Kuner, 2008; Bellocchio et al., 2010; Puente et al., 2011; Castillo, 2012; Katona & Freund, 2012; Ruehle et al., 2013; Steindel et al., 2013; Soria-Gómez et al., 2014, 2015; Hu & Mackie, 2015; Katona, 2015; Martín-García et al., 2016; Gutiérrez-Rodríguez et al., 2017, 2018; Berger et al., 2018; Monday et al., 2018; Bonilla-Del Río et al., 2019; Terral et al., 2019; Peñasco et al., 2019, 2020). A wide variety of intracellular effects, such as modulation of kinases, ion channels and transcription factors are triggered by the activation of CB_1 receptors (Bosier et al., 2010; Pertwee, 2015). One of the most known and important result of these intracellular events in neurons is the retrograde inhibition of transmitter release (Kano et al., 2009).

There are three basic forms on endocannabinoid-mediated synaptic plasticity involving eCBs as retrograde messengers: (1) depolarization-induced suppression of inhibition (DSI)/depolarization-induced suppression of excitation (DSE), (2) metabotropic-induced suppression of inhibition (MSI)/metabotropic-induced suppression of excitation (MSE) (also known as synaptically-evoked suppression of inhibition/excitation (SSE/SSI) (Safo et al., 2006) or endocannabinoid-mediated short term depression (eCB-STD) (Kano et al., 1992), and (3) endocannabinoid-mediated long term depression (eCB-LTD) (Lafourcade et al., 2007; Puente et al., 2011; Lu & Mackie, 2016; Peñasco et al., 2019). Consequently, the activation of CB_1 receptors modulates the release of several neurotransmitters, such as glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine, serotonin and cholecystokinin (Kano et al., 2009).

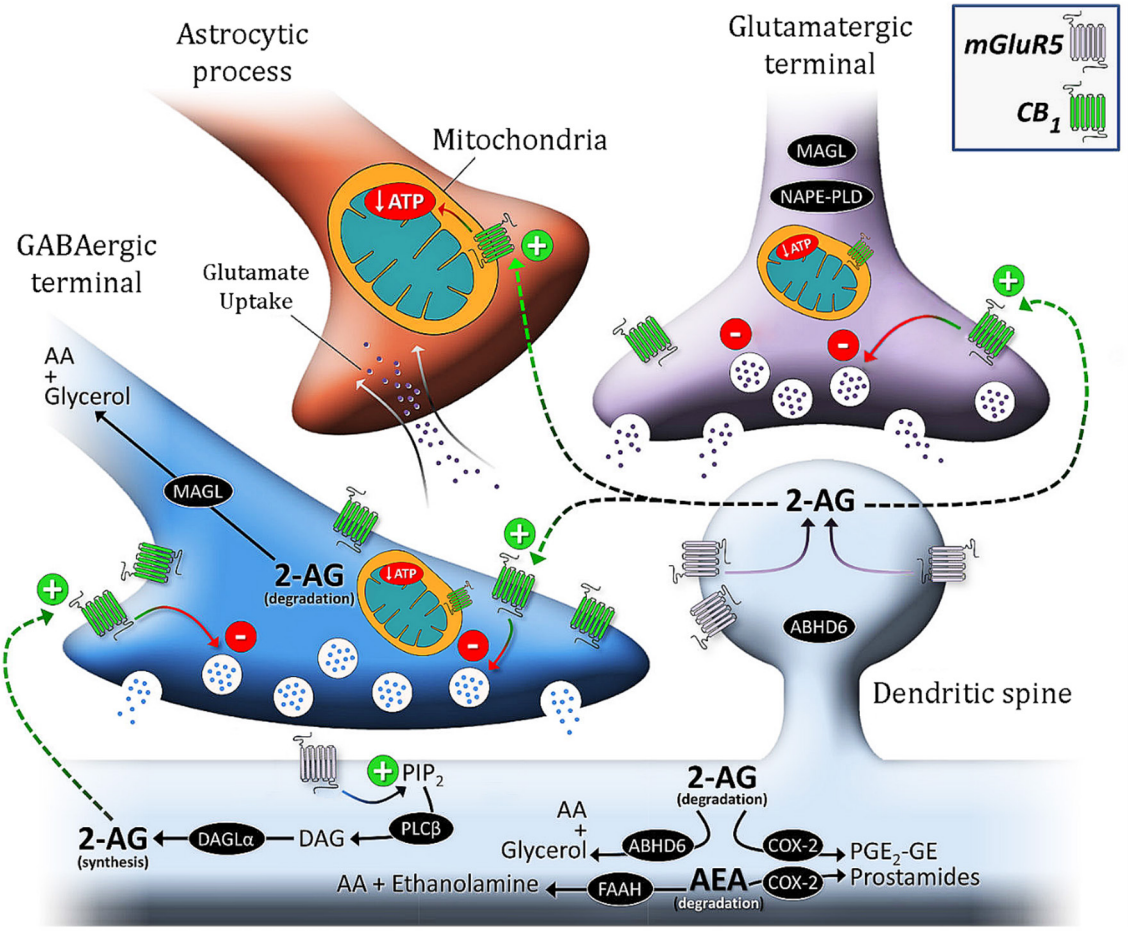


Fig. 3. The localization of the eCBS at the synapse. Schematic representation of an inhibitory and excitatory terminal making synapses with the neuronal dendritic shaft and dendritic spine, respectively. Abbreviations: ABHD6, alpha/beta domain-containing hydrolase 6; CB_1, CB_1 cannabinoid receptor; CCK, cholecystokinin; COX-2, cyclooxygenase-2; DAGLα, diacylglycerol lipase α; MAGL, monoacylglycerol lipase; mGluR5, metabotropic glutamate receptor 5; NAPE-PLD, N-arachidonoyl phosphatidyl ethanolamine-preferring phospholipase D; PLCβ, phospholipase C β. The increased number of CB_1 receptors on the GABAergic terminal represents the higher density of CB_1 receptors found on these axon terminals (modified from Lu & Mackie, 2016).

Nevertheless, the traditionally thought role of eCBS in synaptic function is not strictly limited to retrograde neurotransmission, as their CB_1 receptors can also be found on astrocytes (Gutiérrez-Rodríguez et al., 2017, 2018). Indeed, endocannabinoid signaling has been demonstrated to play a key role in the potentiation of glutamatergic transmission through neuron-glia communication, also known as tripartite synapses, that includes the activation of astroglial CB_1 receptors (Navarrete & Araque, 2008, 2010; Navarrete et al., 2014; Metna-Laurent & Marsicano, 2015; Da Cruz et al., 2016). Finally, intracellular CB_1 receptors have been localized to neuronal mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b; Koch et al., 2015) where they regulate memory through the modulation of energy metabolism (Hebert-Chatelain et al., 2016), and CB_1 receptors are also present in astroglial mitochondria (Gutiérrez-Rodríguez et al., 2018).

Thus, the eCBS acting on different cell types and cellular compartments (Katona & Freund, 2012; Lu & Mackie, 2016; Gutiérrez-Rodríguez et al., 2017; Busquets-Garcia, et al., 2018) through the activation of CB_1 receptors, plays an important role in normal brain function (Herkenham et al., 1990; Tsou et al., 1998; Kano et al., 2009; Castillo, 2012; Katona & Freund, 2012; Lutz et al., 2015; Pertwee, 2015; Lu & Mackie, 2016; Busquets-Garcia et al., 2018; Robin et al., 2018).

2.4.1. Glial cells and astroglial CB_1 receptors

Glial cells constitute the most abundant cell population in the CNS. Amongst glial cell types, astrocytes are excellent players in brain information processing (Volterra & Meldolesi, 2005), due to the bidirectional communication established with neurons (Araque et al., 2001; Bezzi & Volterra, 2011).

Old and recent evidences indicate that astrocytes are crucial in the regulation of brain energy metabolism and brain activity by detecting central and peripheral metabolic changes that affect brain homeostasis (Bélanger et al., 2011; Barros et al., 2018; Magistretti et al., 2018). Their morphology is particularly complex, they are distributed in close apposition to the synaptic structures and contact tens of thousands of synapses (Halassa et al., 2007). Astrocytes interact with neurons at many different levels, ranging from physical support, protection and metabolic sustenance (Hansson et al., 1990; Allen et al., 2009; Bélanger et al., 2011; Oliveira et al., 2015; Bolaños et al., 2016). In particular, CB_1 receptor activation is involved in energy supply to the brain through the control of leptin receptor expression in astrocytes (Bosier et al., 2013). In addition, it is well known that astrocytes produce and release lactate, which is one of the major fuel sources for neurons (Bélanger et al., 2011; Suzuki et al., 2011; Barros et al., 2018; Magistretti et al., 2018). Actually, a large body of studies have shown that astroglial release and neuronal uptake of lactate play fundamental roles in a range of behaviors, such as sleep (Petit et al., 2015; Haydon et al., 2017), learning and memory (Suzuki et al., 2011), between others (Carrard et al., 2016). Now is clear that the modulation of astroglial bioenergetics represents a powerful primary signaling contributor to brain activity, plasticity and behavior (Allen et al., 2009; Bélanger et al., 2011; Suzuki et al., 2011; Oliveira et al., 2015; Magistretti et al., 2018; Robin et al., 2018; García-Cáceres et al., 2019).

Further, astrocytes are forming tripartite synapses, and play important roles in maintaining and regulating synaptic physiology (Araque et al., 2014; Pérez-Alvarez et al., 2014), in metabolic processes (Magistretti & Allaman, 2015; García-Cáceres et al., 2019),

and in processing brain information (Volterra & Meldolesi, 2005).

Although it is generally assumed that the high levels of CB_1 receptor expression in neurons account for most of the cannabinoid-induced brain effects, recent studies have underlined the pivotal importance of astroglial CB_1 receptors in modulating astrocyte-neuron communication at both the synaptic and the behavioural levels (Araque et al., 2017; Martín-Fernandez et al., 2017; Busquets-Garcia et al., 2018; Robin et al., 2018). In fact, low-frequency astrocytic activation, in the absence of presynaptic activity, is sufficient to induce postsynaptic AMPA receptor removal and LTD expression in the CA1 hippocampus (Gómez-Gonzalo et al., 2015; Navarrete et al., 2019). Thus, the activation of CB_1 receptors in astrocytes, in addition to promote astroglial differentiation (Aguado et al., 2006), plays a role in synaptic plasticity, memory and behavior (Navarrete & Araque, 2008, 2010; Han et al., 2012; Araque et al., 2014; Navarrete et al., 2014; Gómez-Gonzalo et al., 2015; Metna-Laurent & Marsicano, 2015; Da Cruz et al., 2016; Robin et al., 2018; Durkee & Araque, 2019). However, the potential impact of pathological conditions on the astroglial CB_1 is currently unknown.

2.4.2. Mitochondria and mitochondrial CB_1 receptors

The brain accounts for 2% of body weight, but it consumes 20% of the energy of the body (Attwell & Lauglin, 2001; MacAskill & Kittler, 2010), which indicates that bioenergy processes play a special role in the brain. Thus, ensuring and regulating cellular energy supplies, mitochondria are key elements of eukaryotic cell functions (Nicholls & Ferguson, 2002; MacAskill & Kittler, 2010) that are crucial for the regulation of brain functions (Mattson et al., 2008; MacAskill & Kittler, 2010). Mitochondrial oxidative phosphorylation converts most of the energy contained in nutrients into ATP, required for cellular reactions. These key organelles also regulate several other important physiological processes, including calcium homeostasis, oxidative stress, apoptosis, and steroidogenesis. Besides, mitochondrial structure and functions are constantly adjusted to maintain cellular metabolic homeostasis (Hebert-Chatelain et al., 2014). The involvement of neuronal energetics in brain physiology and pathology has been the focus of intensive research (Laughlin et al., 1998; Mattson et al., 2008; MacAskill & Kittler, 2010) but the molecular mechanisms linking mitochondrial activity to brain functions are still poorly documented.

G protein–coupled receptors (GPCRs) represent one of the largest protein families controlling neuronal activity. However, there are also consistent evidences that mitochondria contain G proteins (Lyssand & Bajjalieh, 2007; Andreeva et al., 2008).

Moreover, several reports have shown the intramitochondrial localization of potential downstream effectors of G protein signaling, such as soluble adenylyl cyclase (Zippin et al., 2003), phosphodiesterase (Acin-Pérez et al., 2009, 2011) and protein kinase A (PKA) (Ryu et al., 2005). Accordingly, cAMP can be produced in mitochondria (Chen et al., 2004; Helling et al., 2008; Acin-Pérez et al., 2009, 2011). Besides, it has been described that the intra-mitochondrial Gαi protein activation by mtCB_1 receptors leads to the inhibition of soluble adenylyl cyclase and, consequently, to a decrease in intra-mitochondrial PKA activity (Hebert-Chatelain et al., 2016).

Therefore, the presence of functional mtCB_1 receptors in different tissues (Aquila et al., 2010; Benard et al., 2012; Koch et al., 2015; Hebert-Chatelain et al., 2016; Mendizabal-Zubiaga et al., 2016; Gutiérrez-Rodríguez et al., 2018), underlies the contribution of cannabinoid signaling to key bioenergetics processes. Indeed, in mouse hippocampal neurons, the presence of CB_1 receptors in mitochondria regulates the activation of cellular respiration and energy production (Benard et al., 2012; Hebert-Chatelain et al., 2016). Specifically, by modulating the activity of complex I in the electron transport chain, as well as mitochondrial respiration and mobility, brain mtCB_1 receptors affect synaptic transmission, memory formation and behavior (Hebert-Chatelain et al., 2016). However, the upstream mechanisms regulating the intramitochondrial cAMP–PKA signaling cascade in neurons, and the mechanisms coupling mitochondrial activity and neuronal physiology, remain poorly understood (Mattson et al., 2008; MacAskill & Kittler, 2010).

As mitochondria are directly involved in the vast majority of brain functions, mitochondrial dysfunction can cause neurodegenerative diseases, strokes or disorders associated with ageing (Hebert-Chatelain et al., 2016). Thus, determining the mitochondrial effects of cannabinoids would open new hopes for the specific use of the therapeutic potential of cannabinoids in CNS disease. Therefore, it is urgent to know the distribution of CB_1 receptors in the healthy or diseased brain in certain specific neurone compartments, with a view to developing new therapeutic tools based on the most effective and safest cannabinoids in the treatment of certain brain diseases. However, the low CB_1 receptor expression in astrocytes (Rodríguez et al., 2001; Han et al., 2012; Bosier et al., 2013; Kovács et al., 2017; Gutiérrez-Rodríguez et al., 2018) and mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b and 2016) constrains a consolidated picture of the subcellular CB_1 receptor distribution in the astroglial compartments that holds the anatomical substrate for a functional interaction with the nearby synapses under normal or pathological conditions. In this sense, rescue mutant mice are key tools for the detailed anatomical characterization of the subcellular distribution of the receptor

in specific cell types, independently of its level of expression. In an interesting way, the GFAP-*CB_1*-RS mice expressing the CB_1 receptor gene exclusively in the astrocytes and the GFAPhrGFP-*CB_1*-WT mutant mice, which target to express hrGFP only into astroglial cells are ideal genetic tools to study if really intracellular CB_1 receptors are present in astroglial mitochondria as observed in neuronal and muscular mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014 and 2016; Mendizabal-Zubiaga et al., 2016; Gutiérrez-Rodríguez et al., 2018).

2.5. HIGH RESOLUTION ELECTRON MICROSCOPY

CB_1 receptor density is not uniform through the regions expressing the receptor, which makes extremely difficult to identify low CB_1 receptor expression in cell types and/or in subcellular compartments of wild-type brains (Busquets-Garcia et al., 2015).

In the 1990´s, autoradiography of radioligand binding ([3H]CP55,940) was used to assess qualitatively and quantitatively brain cannabinoid receptors (Herkenham et al., 1990). With the cloning of the CB_1 receptor, in situ hybridization techniques were routinely applied to study the CB_1 mRNA distribution in brain somata (Mailleux & Vanderhaeghen, 1992; Matsuda et al., 1993; Marsicano & Lutz, 1999). Later on, the Mackie laboratory raised polyclonal antibodies against a specific N-terminal amino acid sequence of the CB_1 receptor protein (Twitchell et al., 1997) and shortly after a new CB_1 receptor antiserum was raised against a C-terminal amino acid sequence of the rat CB_1 receptor (Egertova et al., 1998). These immunological tools combined with the appropriate histochemical techniques for light microscopy allowed visualizing the pattern of CB_1 receptor-like immunoreactivity in the brain (Egertova et al., 1998; Tsou et al., 1998). This spring from the regional (autoradiography) and cellular distribution (in situ hybridization) to the CB_1 receptor immunohistochemistry was a great advance in the cannabinoid field as new localizations of the receptor were revealed.

Yet, the subcellular distribution of the CB_1 receptor expression was a pending matter until pre-embedding immunocytochemical technique for electron microscopy was applied as very valuable tool for the study of the precise CB_1 receptor localization in brain tissue and peripheral organs. This method helped to demonstrate that CB_1 receptors are not evenly distributed throughout brain cell types and subcellular compartments, since high CB_1 receptor concentrations at inhibitory GABAergic synaptic terminals (Katona et al., 1999, 2000; Kawamura et al., 2006; Ludányi et al., 2008; Marsicano & Kuner, 2008; Katona & Freund, 2012; De-May & Ali, 2013; Steindel et al., 2013; Hu & Mackie, 2015), low at glutamatergic synaptic terminals (Marsicano et al., 2003; Domenici et al., 2006; Katona et

al., 2006; Monory et al., 2006; Takahashi & Castillo, 2006; Kamprath et al., 2009; Bellocchio et al., 2010; Puente et al., 2011; Reguero et al., 2011; Ruehle et al., 2013; Soria-Gómez et al., 2014) and in astrocytes were revealed (Navarrete & Araque, 2008, 2010; Stella, 2010; Han et al., 2012; Bosier et al., 2013; Metna-Laurent & Marsicano, 2015; Viader et al., 2015; Da Cruz et al., 2016). Furthermore, it turned out that the levels of CB_1 receptor expression (Marsicano & Lutz, 1999) do not directly correlate with their importance in a physiological context, as low or very low CB_1 receptor expression passing unaware in brain cell types or subcellular compartments (Katona et al., 1999, 2000; Hájos et al., 2000) even considered as background staining have been demonstrated to hold functional and behavioral relevance (Busquets-Garcia et al., 2018).

There are certainly some limitations inherent to the pre-embedding immunogold method, mostly that labeling does not correlate with the exact localization of the antigen, therefore, is not quantitative (only semi-quantitative). Success of the pre-embedding detection method was improved by the development of ultra-small gold secondary conjugates (Nanogold®) in combination with gold particle enlarging chemistry, making it possible for probes to detect deeper into tissues, thus reducing the inherent limitation of antibody penetration and molecule detection while enhancing the resolution of receptor localization. However, it does not serve to multiple labeling for simultaneous visualization of several synaptic proteins unless other techniques are combined. In combination with immunoperoxidase and 3,3'-diaminobenzidine (DAB) reaction product immunochemistry, pre-embedding procedures can reveal protein co-localizations with cell specificity and at high resolution.

2.6. LIMBIC SYSTEM

The limbic system regulates a number of behaviors that are essential for the survival of all vertebrate species including humans (Sokolowski & Corbin, 2012). Predominantly, the limbic system (Fig. 4) plays a pivotal role in behavior by controlling appropriate responses to stimuli with social, emotional, or motivational salience. Moreover, the intricate functional neuroanatomy of limbic system with its diverse circuits may explain some of the manifestations of neuropsychiatric disorders.

Research has identified the role of the amygdala in various anxiety disorders and emotional memory, the trisynaptic hippocampal circuitry underlying cognitive functioning and the significance of hypothalamus in various neurovegetative functions, facts that suggest the integral role of the limbic system in understanding human behavior and its aberrations (Rajmohan & Mohandas, 2007).

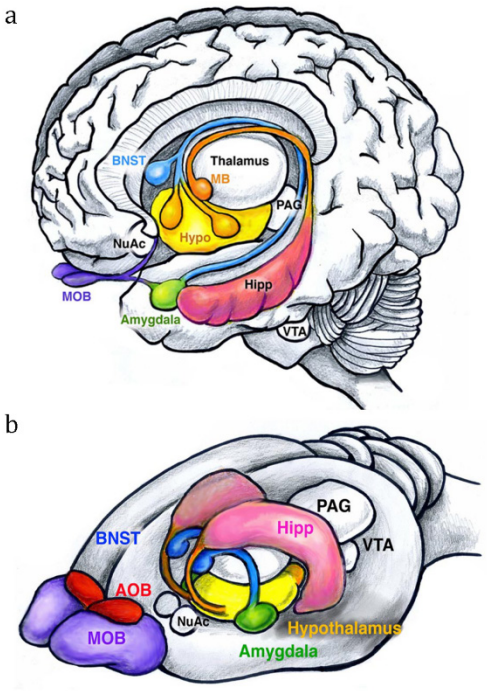


Figure 4. Main structures of the human and rodent limbic system.

(a) Human brain showing the amygdala (green), bed nucleus of stria terminalis (BNST, blue), hypothalamus (yellow), and hippocampus (pink).
The hippocampus (pink) attaches to the mamillary bodies (orange) through the fimbria-fornix. Olfactory inputs are received by the main olfactory bulbs (MOB, purple). Other structures include the nucleus accumbens (NuAc), Ventral tegmental area (VTA), and the periaqueductal gray (PAG).

(b) Similar structures are found in rodents. Note the enlarged olfactory bulbs compared to humans, and the presence of the accessory olfactory bulbs (AOB, red). Together these structures facilitate the execution and reinforcement of innate behaviors (from Sokolowski & Corbin, 2012).

The limbic system in the human brain is compared to the rodent brain; due to the structural and functional similarities between the limbic areas, many studies have been carried out in rodents (Crews et al., 2000; Jones et al., 2008b; Coleman et al., 2011, 2014; Kwon et al., 2011a; Yu et al., 2012; Forbes et al., 2013; Almeida-Suhett et al., 2014; Petraglia et al., 2014b, c; Vetreno et al., 2016; Qin et al., 2018).

2.7. THE HIPPOCAMPAL FORMATION

The hippocampal formation (HF) plays an essential role in spatial and contextual memory, as well as in learning and mood regulation. In addition, disorders such as anxiety, depression, some of the neurodegenerative diseases and addiction, including cannabis and EtOH, are related to alterations in regions of the HF. Thus, for the purposes of this dissertation, I will focus on the hippocampus.

The main flow of information in the hippocampus is the well-known trisynaptic circuit (Fig. 5). Briefly, the axons of layer II neurons in the entorhinal cortex (EC) project to the DG through the perforant pathway (PP) including the lateral perforant pathway (LPP) and medial perforant pathway (MPP). The granule cells of the DG send projections to the

pyramidal cells in CA3 through mossy fibres. CA3 pyramidal neurons relay the information to CA1 pyramidal neurons through ipisilateral Schaffer collaterals. CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC. CA3 also receives direct projections from EC layer II neurons through the PP and CA1 gets a direct input from EC layer III neurons through the temporoammonic pathway (TA). The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells.

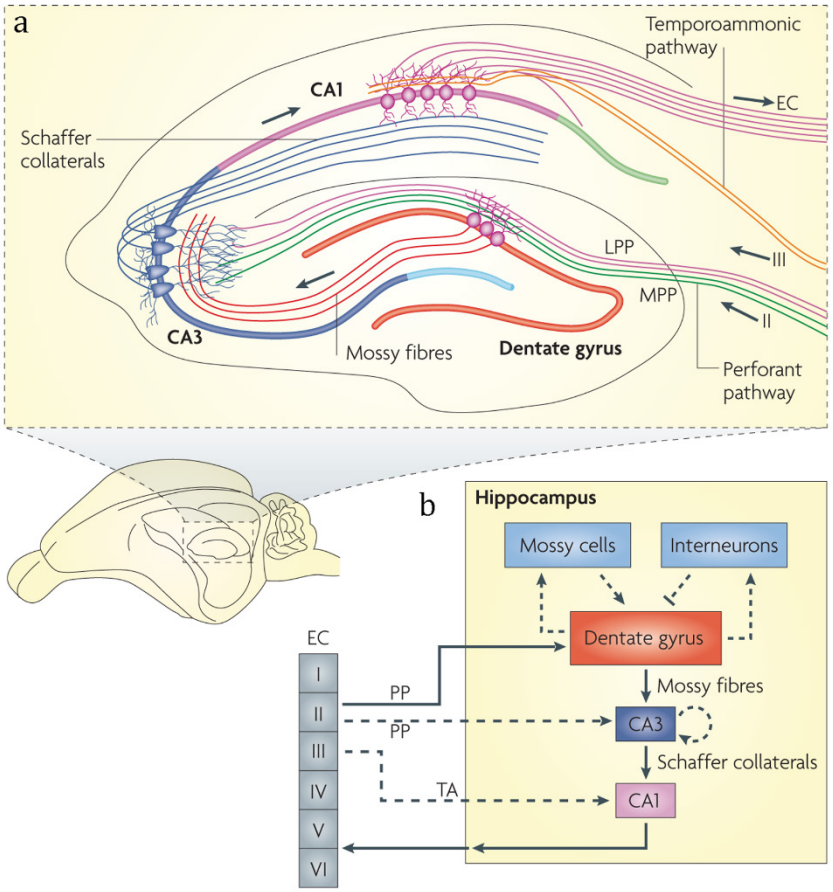


Figure 5. The neural circuitry in the rodent hippocampus. (a) An illustration of the hippocampal circuitry. (b) Diagram of the hippocampal neural network. The excitatory trisynaptic pathway (entorhinal cortex (EC)–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows (from Deng et al., 2010).

2.8. CB_1 RECEPTORS IN NORMAL AND PATHOLOGICAL CONDITIONS

2.8.1. Ethanol

Ethyl alcohol or ethanol (CH3-CH2-OH) is probably the most commonly consumed addictive drug in the world (SAMHSA, 2011) and is an important health and social problem worldwide (WHO, 2019).

Binge drinking is the typical pattern of alcohol consumption in youth. It is characterized by an intermittent consumption of large amounts of EtOH in short periods of time (3 or more drinks in 1-2 hours) followed by a period of abstinence (Courtney & Polich, 2009). This intake pattern causes large and rapid spikes in blood EtOH concentration (BEC) that brings serious consequences in terms of acute toxicity but also leads to vulnerability for later EtOH abuse and dependence (Amodeo et al., 2017).

Despite of being a weak drug (it is needed a quantity of grams to produce a pharmacological effect), EtOH heavily impacts on the structure and function of the brain, particularly during adolescence (Pascual et al., 2007; Clark et al., 2012; Keshavan et al., 2014; Liu & Crews, 2015; Vetreno & Crews, 2015; Adermark & Bowers, 2016; Montesinos et al., 2016; Spear, 2016a). Because EtOH modifies brain maturation, adolescent drinking associates with deficits in attention, learning, memory, intellectual development or visual-spatial functions. (Brown & Tapert, 2004; Nagel et al., 2005; Zeigler et al., 2005; Lacaille et al., 2015). This correlates with loss of hippocampal, prefrontal cortex and cerebellar volumes as well as ventricular enlargement found in young people starting drinking at early age (Shear et al., 1992; De Bellis et al., 2000, 2005; Nagel et al., 2005; Medina et al., 2008; Lisdahl et al., 2014).

EtOH causes a significant loss of hippocampal neurons, astrocytes and microglia (Oliveira et al., 2015) and mitochondrial dysfunction that leads to brain inflammation, synaptic dysfunction and memory loss (Crews et al., 2000). Moreover, all these effects are long lasting (Coleman et al., 2011, 2014; Forbes et al., 2013). Actually, binge drinking alters brain volume in animal models that mimics the alteration found in young drinkers (Crews et al., 2000; Coleman et al., 2011, 2014; Forbes et al., 2013; Vetreno et al., 2016) and EtOH-exposed adolescent animals are more sensitive and show memory and learning dysfunctions (Markwiese et al., 1998; White & Swartzwelder, 2005) which can extend into adulthood (Sircar & Sircar, 2005; Pascual et al., 2007).

Given the incidence of binge drinking in adolescents and young adults and the lesion effects of EtOH in the CNS, it is critical to understand both the long-term consequences of

this exposure and methods by which this damage can be overcome by therapeutic interventions. The persistent behavioral effects of EtOH in adolescence are accompanied by disturbance of synaptic plasticity and neurotransmission. Thus, numerous studies have shown that EtOH alters several neurotransmitter and neuromodulatory systems, in particular, the eCB (Hungund et al., 2003; Basavarajappa, 2007; Mitrirattanakul et al., 2007; Adermark et al., 2011; Talani & Lovinger, 2015; Varodayan et al., 2017), glutamatergic (Tabakoff & Hoffman, 1996; Fadda & Rossetti, 1998; Heinz et al., 2004; Alele & Devaud, 2005; Larsson et al., 2005), GABAergic (Mehta & Ticku, 2005; Fleming et al., 2007, 2012, 2013; Centanni et al., 2014), and dopaminergic (Coleman et al., 2011; Boutros et al., 2015; Shnitko et al., 2014; Vetreno et al., 2014; Spoelder et al., 2015) systems in many brain areas. Moreover, it is well documented that the eCBS regulates the EtOH-induced changes in excitatory and inhibitory transmission and participates in EtOH addictive behaviors of consumption, motivation, reinforcing and dependence (Rimondini et al., 2002; Colombo et al., 2005; Thanos et al., 2005; Economidou et al., 2006; Mitrirattanakul et al., 2007; Basavarajappa et al., 2008; Kelm et al., 2008; Vinod et al., 2008, 2012; Roberto et al., 2010; Pava et al., 2012; Pava & Woodward 2012; Talani & Lovinger, 2015) and, reciprocally, EtOH modulates the behavioral and neural eCB-dependent effects (Pava et al., 2012; Talani & Lovinger, 2015).

However, how binge drinking affects receptor populations in the brain and impacts on the structure of the developing adolescent brain is not well understood. As already mentioned, there is considerable evidence for the involvement of the eCBS in alcohol consumption and motivation, reinforcing properties of EtOH and EtOH dependence (Pava & Woodward, 2012). For instance, CB_1 receptor agonists stimulate a dose-dependent increase in EtOH intake (Colombo et al., 2002), while antagonists reduce voluntary EtOH intake, preference and craving (Economidou et al., 2006). Moreover, CB_1 receptor knock-out mice show a reduced EtOH preference and intake (Hungund et al., 2003). Interestingly, chronic EtOH exposure causes a decrease in CB_1 receptor mRNA expression (Ortiz et al., 2004; Mitrirattanakul et al., 2007) as well as in CB_1 receptor density and functionality (Basavarajappa et al., 1998; Vinod et al., 2006) associated with an eCB increase in the hippocampus (Mitrirattanakul et al., 2007), but not in amygdala or striatum (Rubio et al., 2009), that persists after a long withdrawal period (Mitrirattanakul et al., 2007). Furthermore, an increase in AEA was detected in EtOH animal models (Vinod et al., 2006) and in the ventral striatum of postmortem human alcoholics (Vinod et al., 2010), together with a decrease in the anandamide-degrading enzyme FAAH and CB_1 receptor expression (Vinod et al., 2010). A decrease in CB_1 receptor expression and a reduced G-protein coupling of the receptor was also observed in the striatum, hippocampus, nucleus

accumbens and amygdala of FAAH knock-out mice (Vinod et al., 2008). The changes in hippocampal CB_1 receptor expression have negative consequences on the CB_1 receptor mediated inhibitory synaptic transmission, despite the recovery of the CB_1 receptor expression after a prolonged withdrawal (Rimondini et al., 2002; Mitrirattanakul et al., 2007; Vinod et al., 2012) probably due to a reduction in eCBs (Vinod et al., 2012).

As to the astroglia, prolonged EtOH exposure alters the distribution and content of the glial fibrillary acidic protein (GFAP) that has a negative impact on the astrocytic intermediate filaments and, ultimately, on the astrocyte morphology eventually leading to brain dysfunction (Renau-Piqueras et al., 1989). As mentioned above in previous sections, activation of CB_1 receptors expressed in astrocytes promotes astroglial differentiation, modulates synaptic transmission through the neuron-astrocyte crosstalk (Navarrete & Araque, 2010; Han et al., 2012; Bosier et al., 2013; Araque et al., 2014; Gómez-Gonzalo et al., 2015; Metna-Laurent & Marsicano, 2015; Da Cruz et al., 2016) and regulates leptin receptor expression in cultured cortical and hypothalamic astrocytes (Bosier et al., 2013). To our knowledge, however, there is no direct evidence on the effect of chronic EtOH exposure during adolescence on the CB_1 receptor expression in astrocytes of the adult brain.

2.8.2. Traumatic brain injury

As it has elegantly been written in full detail in the doctoral thesis´ manuscript of Cristina Pinar PhD (supervisor: Dr. Brian Christie), Division of Medical Sciences, University of Victoria, Victoria (Canada), traumatic brain injury (TBI) is a brain damage resulting from impulsive force transmitted to the head by an external mechanical force (NCIPC, 2003; Frieden et al., 2015). This form of trauma is a major worldwide health and socioeconomic concern, as it represents the foremost cause of mortality and disability for individuals 45 years of age and under (Ghajar, 2000; Cole, 2004). Globally, 10 million hospitalizations and/or deaths are the direct result of TBI with an estimate of 57 million people currently living having a history of TBI (Langlois et al., 2006). These prevalent injuries, most commonly caused by falls and motor vehicle crashes (Frieden et al., 2015), can lead to persistent structural and functional damage in the brain that alter behaviour such as learning and memory, emotion, anxiety and decision-making.

A TBI can either be a penetrating injury or a closed-head injury. An injury classified as a penetrating TBI presents with damage to the skull, dura and brain parenchyma, while in a closed-head TBI, the skull and usually these other brain structures remain intact or do

not show evident alterations (Cassidy et al., 2004). The severity of the injury is determined based on: level of consciousness (duration and severity, if lost), memory and neurological deficits and brain imaging. Mild TBI, also referred to with the term "concussion", represents the most common type of TBI.

The clinical symptoms of mTBI may include, but are not limited to: headaches, confusion, nausea, balance problems, attention deficits, sleep disturbances, learning and memory problems, and emotional alterations (Kelly & Rosenberg, 1997; Caine et al., 2014) Symptoms are typically short-lived and resolve spontaneously in a matter of days or weeks; however, in a subset of individuals, mTBI symptoms can persist for over a year for undetermined reasons (McCrea et al., 2003b; Hall et al., 2005a).

While a single mTBI may not cause evident or long-lasting structural or functional deficits, it may render the brain vulnerable to subsequent injuries, creating a window of susceptibility where the accumulation of multiple mild concussive events may lead to more severe cumulative damage and long-term cognitive dysfunction (Guskiewicz et al., 2003; Prins et al., 2012; Fehily & Fitzgerald, 2017). Mild TBI accounts for up to 80% off all head injuries (Faul et al., 2010; Frieden et al., 2015). Based on those medically reported injuries, the incidence of mTBI is currently estimated to be 100 - 300 people per 100,000 (Cassidy et al., 2004; Nguyen et al., 2016). However, mTBI is an under-reported injury as many people who sustain a mTBI do not seek medical care (Setnik & Bazarian, 2007). A more accurate estimate for the incidence of mTBI is likely to be approximately 600 people per 100,000, or roughly 42 million globally (Cassidy et al., 2004).

Understanding how the mechanical energy from the external force is transferred to the brain and the effects of this physical stimulus on the living tissue and neural/glial networks is critical for understanding concussive injuries.

Despite the protection that the skull and cerebrospinal fluid (CSF) provide, head injury—even without skull fracture—can damage fragile brain tissue via acceleration and deceleration forces. Due to its physical properties, the brain tissue shows nonlinear behavior in response to the applied loading rate (Arbogast et al., 1997; Donnelly & Medige, 1997; Miller & Chinzei, 1997; Prange-Kiel et al., 2003; Takhounts et al., 2003). Thus, as brain tissue is mostly composed of water, it is resistant to changing its shape when subjected to pressures. However, it deforms easily in response to shear forces compared with other biologic tissues. Several studies have investigated the impact of shear deformation in comparison to other forces and have led to the idea that shear deformation is the main cause of injury in concussion (Unterharnscheidt & Higgins, 1969; Adams et al., 1982; Gennarelli et al., 1982).

The anatomical location and structure of different brain regions can make certain areas

more susceptible to the shearing forces. For instance, clinical studies using magnetic resonance imaging (MRI) have reported the hippocampus as one of the most vulnerable regions to shearing forces after moderate and severe TBI (Kotapka et al., 1992; Tate & Bigler, 2000; Bigler et al., 2002; Tomaiuolo et al., 2004; Serra-Grabulosa et al., 2005; Bigler, 2018).The biomechanical forces of mTBI generate intracranial pressure gradients that lead to shearing and tearing of neurons, glial cells, and blood vessels in the brain (Blennow et al., 2012; Pekna & Pekny, 2012). Therefore, the disruption of axonal fibers caused by mTBI mechanical forces can lead to synaptic transmission alterations and neuronal circuit dysfunction. In addition, blood vessels are also structures vulnerable to shear forces. Indeed diffuse axonal injury (DAI) is usually accompanied by microbleeds in the same locations which can be referred to as diffuse vascular injury (DVI) (Gentry et al., 1988; Onaya, 2002; Pittella & Gusmão, 2003). Rupture of several capillaries, a phenomenon known as multiple petechial hemorrhages, is commonly observed in TBI patients with different severities (Mckee & Daneshvar, 2015). The hypoxic event caused by this halt in blood flow can contribute to the immediate dysfunctions following mTBI. Moreover, it has been reported that following TBI, the cerebrovascular reactivity (brain ability to elevate blood flow above baseline) is compromised (Adams et al., 2018; Amyot et al., 2018). This means that the brain capacity to modulate metabolic demands caused by neuronal activity is deficient and can alter the normal functioning of the brain. Disruption of the axolemmas increases their permeability (Pettus et al., 1994; Povlishosk & Pettus, 1996), Ca^{2+} influx, and mitochondrial swelling (Maxwell et al., 1997; Mata et al., 1986). Microtubule disorganization post-injury has been identified as a consequence of axon stretching where ultrastructural analysis has shown breakage and folding of microtubules after TBI, triggering microtubule disassembly (Povlishosk & Pettus, 1996; Maxwell et al., 1997; Tang-Schomer et al., 2010). This causes accumulation of organelles in the axon and axonal swelling, with eventual disconnection and axotomy (Christman et al., 1994; Barkhoudarian et al., 2011; Johnson et al., 2013; Giza & Hovda, 2014).

The biomechanical forces induced by the impact also cause pathophysiological changes like the opening of voltage-dependent potassium (K^+) channels (Farkas et al., 2006). This disruption causes an unregulated amount of ion flux, specifically K^+ efflux and sodium (Na^+) influx at the cellular level, and a subsequent dysregulated release of neurotransmitters (NT), particularly the excitatory amino acid glutamate. In order to restore the ionic balance, the Na^+/K^+ ATP-dependent pumps activity is increased, which results in a depletion of the energy stores creating a metabolic crisis. In order to restore the energy reservoir, the system mobilizes intracellular glucose to generate more ATP

causing hyperglycolysis. Impaired oxidative metabolism may result in decreased ATP production, thereby exacerbating the energy crisis, ionic imbalance and further contributing to hyperglycolysis. Following this trauma induced hyperglycolysis, there is an accumulation of lactate, resulting in acidosis, increased membrane permeability and cerebral edema (Kalimo et al., 1981). Studies have shown an increase in glucose metabolism as early as 5 minutes post-TBI and lasting up to 4 hours in rats (Gardiner et al., 1982), and this is followed by a period of hypometabolism of variable duration dependent upon injury severity (Peskind et al., 2011).

In addition to these energy perturbations, excessive extracellular glutamate binds to post-synaptic N-methyl-D-aspartate (NMDA), AMPA and kainate receptors causing further regional depolarization (Faden, 1992). Consequently, activated NMDA receptors (NMDARs) flux Ca^{2+} into the cell. This Ca^{2+} acts as a second messenger triggering numerous pathways. For instance, increased Ca^{2+} following trauma may cause cell death via over activation of calpains (Roberts-Lewis & Siman, 1993; Kampfl et al., 1997), phospholipases (Farooqui & Horrocks, 1991), or protein kinases (Verity, 1992). Moreover, the large influx of Ca^{2+} via NMDARs accumulates in the mitochondria resulting in impaired oxidative metabolism (Xiong et al., 1997). This mitochondrial dysfunction leads to decreased production of ATP, thereby worsening the energy situation (Xiong et al., 1997; Vagnozzi et al., 2007). All these events following the initial insult are believed to be the cause of acute post-injury deficits (Giza & Hovda, 2014b; Barkhoudarian et al., 2016) and in the long run they could cause permanent alterations in the eCBS.

2.8.3. Δ9-THC

Marijuana (Cannabis sativa) used by adolescents has been on the rise since the early 1990's. It is the most commonly used illicit drug among young people in Europe (EMCDDA, 2019). With recent legalization in certain countries and states of USA and decriminalization acts passed, cannabinoid exposure in adolescents will undoubtedly increase even more. The main psychotropic substance of the Cannabis sativa plant is Δ9-tetrahydrocannabinol (Bossong & Niesink, 2010; Klein et al., 2011). As CB_1 receptor is the target of THC, its distribution in the brain closely fits into the deleterious effects of cannabinoids on locomotion, perception, learning, memory or the cannabinoid-positive effects as anti-convulsant or food intake enhancers, and its low amount in the brainstem correlates with the low toxicity and lethality of marijuana (Bellocchio et al., 2010; Han et al., 2012; Katona & Freund, 2012; Hebert-Chatelain et al., 2014a,b, 2016; Soria-Gómez et

al., 2014; Lu & Mackie, 2016; Martín-García et al., 2016; Mechoulam, 2016).

Even though, THC and other cannabinoid receptor agonists (CB_1/CB_2) seem to produce beneficial effects in conditions related to pain, inflammation, anxiety, muscle spasticity, feeding, or nausea (Di Marzo, 2008; Blankman & Cravatt, 2013; Mechoulam & Parker, 2013; Pacher & Kunos, 2013). However, psychomimetic effects, memory-impairing actions, and dependence liabilities dampen enthusiasm for therapeutic development of CB_1 receptor agonists (Schlosburg et al., 2014). These deficits are more evident when this substance is taken during critical developmental periods like adolescence (Viveros et al., 2012). In fact, previous reports have shown that chronic adolescent administration of CB_1 receptor agonists induces alterations of the emotional behaviour, the cognitive function as well as psychotic-like symptomatology in adult rats (Biscaia et al., 2003; Schneider & Koch, 2003, 2007; O'Shea et al., 2004, 2006; Llorente-Berzal et al., 2011; 2013a; Mateos et al., 2011; Zamberletti et al., 2012). The administration of CB_1 receptor agonists during adolescence also induces long-term neurochemical changes in the brain (Rubino et al., 2008; Llorente-Berzal et al., 2013a) and sex-dependent changes in expression and functionality of hippocampal CB_1 receptors (Mateos et al., 2011; López-Gallardo et al., 2012).

Human studies are also consistent with preclinical work implicating a regulatory role of THC in modulating emotional processing and fear learning. Acute THC administration reduces amygdala reactivity to social signs of threat, without affecting activity in primary visual cortex and motor cortex (Phan et al., 2008), and impairs recognition of facial fear and anger, but not sadness or happiness (Ballard et al., 2013).

At the molecular level, activation of CB_1 receptors on axon terminals by plant-derived compounds or synthetic agonists inhibits neurotransmitter release throughout the CNS (Lévénès et al., 1998; Szabo et al., 1998; Katona et al., 1999; Misner & Sullivan, 1999; Hoffman & Lupica, 2000; Gerdeman & Lovinger, 2001; Hoffman & Lupica, 2001). It is known that in the hippocampus, the endocannabinoids are released from hippocampal neurons in an activity-dependent fashion to initiate short- and long-term changes in synaptic efficacy following activation of CB_1 receptors (Wilson & Nicoll, 2001; Alger, 2002; Freund et al., 2003). In the same way, the activation of CB_1 receptors by acute or long-term exposure to THC, disrupts hippocampal function and impairs behaviorally and physiologically defined memory processes (Heyser et al., 1993; Misner & Sullivan, 1999; Ranganathan & D'Souza, 2006; Wise et al., 2009; Hoffman et al., 2010). These observations

were supported by human studies, in which THC impaired working and episodic memory (Curran et al., 2002; Ilan et al., 2004; Squire, 2004). In rats, both systemic and intra-hippocampal injection of THC and synthetic CB_1 agonists including WIN 55,212-2 (WIN-2), HU-210 and CP 55,940 impairs hippocampal-dependent (i.e. short-term) memory across a variety of learning tasks (Lichtman et al., 1995; Lichtman & Martín, 1996; Ferrari et al., 1999; Hampson & Deadwyler, 2000; Hampson et al., 2003; Barna et al., 2007).

In the hippocampus, the selective and dose-dependent effect of THC on synchronous firing is particularly interesting, as it suggests that temporal binding within local networks of CA3 and CA1 principal cells involving GABAergic feed-forward and feedback inhibition (Nitsch et al., 1990; Sargsyan et al., 2001) is more vulnerable to cannabinoids, while interregional synchrony (here possibly issued by the Schaffer collaterals) is more resilient to cannabinoid stimulation. A possible mechanistic underpinning may be the presence of CB_1 and non-CB_1 receptors at cholecystokinin containing GABAergic interneurons (Katona et al., 1999; Freund et al., 2003) with a seemingly higher sensitivity to exogenous cannabinoid than the glutamatergic neurons of Schaffer collaterals. Furthermore, the fact that a low dose of THC selectively de-synchronized principal neurons without having an effect on firing/bursting rates suggests that functional decoupling of principal neurons precedes alterations in their firing/bursting patterns. Interestingly, single cell burst characteristics appear intact as long as CA3→CA1 synchrony was maintained (Lisman, 1997; Harris et al., 2001).

The exact neuroanatomical substrates underlying each effect of THC are, however, not known. For example, mice lacking CB_1 receptors in GABAergic neurons responded to THC similarly as wild-type littermates did, whereas deletion of the receptor in all principal neurons abolished or strongly reduced the behavioral and autonomic responses to the drug (Monory et al., 2007). Moreover, locomotor and hypothermic effects of THC depend on cortical glutamatergic neurons, whereas the deletion of CB_1 from the majority of striatal neurons and a subpopulation of cortical glutamatergic neurons blocked the cataleptic effect of the drug (Monory et al., 2007). These results indicate that one of the important pharmacological actions of THC do not depend on functional expression of CB_1 on GABAergic interneurons, but on other neuronal populations, and pave the way to a refined interpretation of the pharmacological effects of cannabinoids on neuronal functions (Monory et al., 2007).

Regarding the involvement of the eCBS in the control of neuroinflammation, cannabinoid receptors expressed in astrocytes and microglia cells (Stella, 2010) seem to contribute to modulate the inflammatory response. In fact, THC is immunomodulatory, with the

majority of literature demonstrating immune suppressive and anti-inflammatory activity in vivo and in vitro (Croxford and Yamamura, 2005; Karmaus et al., 2011; Karmaus et al., 2013; Katchan et al., 2016). In this sense, also, the synthetic CB_1 agonist, WIN-2, decreased the number of activated microglia after treatment with the pro-inflammatory molecule lipopolysaccharide (LPS), suggesting that the cannabinoid system may play a role in the control of microglia reactivity in response to an insult (Marchalant et al., 2007). Furthermore, THC reduces methamphetamine (METH)-induced brain damage via inhibition of neural nitric oxide synthase (nNOS) expression and astrocyte activation through CB_1-dependent and independent mechanisms, respectively (Castelli et al., 2014). Moreover, THC inhibits astroglial growth in vitro (Tahir et al., 1992) and affects the development of astroglia in vivo (Suárez et al., 2000).

Regarding the mitochondria, early studies suggested that THC could affect mitochondrial functions (Bartova & Birmingham, 1976). Although, with the identification of cannabinoid receptors as typical plasma membrane GPCRs (Matsuda et al., 1990; Piomelli, 2003), mitochondrial effects of lipophilic cannabinoids on neurons were ascribed to nonspecific alterations of membrane properties (Martín, 1986). Later, it has been also described that CB_1 receptor signaling to regulate mitochondrial biogenesis in peripheral non-neural tissues (Aquila et al., 2010; Tedesco et al., 2010). And the lipophilic nature of most cannabinoids (Piomelli, 2003) implies that receptor–ligand interactions might occur not only at plasma membranes, but also inside cells. Indeed, different intracellular compartments contribute to the regulation of endocannabinoid metabolism (Gulyas et al., 2004; Marsicano & Kuner, 2008), and CB_1 receptors have been shown to functionally signal in lysosomal or endosomal intracellular membranes (Rozenfeld & Devi, 2008) and in mitochondria (Hebert-Chatelain et al., 2014). At last, interestingly, Hebert-Chatelain et al. (2016) demonstrated that by genetic elimination of $mtCB_1$ receptors, mice exposed to high doses of THC do not experience mitochondrial damage and were subsequently protected from cannbinoid-induced memory impairment.

Despite all described before about the effects of THC consumption, nevertheless, no much information is available on the fine anatomical changes taken place in neurons and astrocytes after THC consumption during adolescence. In addition, the impact of adolescent THC consumption on the localization of CB_1 receptors in the brain remains unknown. CB_1 receptors are expressed in different cell types and/or in different subcellular compartments where THC is acting. Therefore, it is of special interest to study in detail if an acutely administered low-dose of THC alters the CB_1 receptor localization or

expression in the brain, as well as if THC consumption during the adolescence produces structural adaptations in the brain that could be underlying the behavioural alterations caused by cannabis intoxication.

2.9. WORKING HYPHOTESIS

CB_1 receptor-mediated astrocytic functions are highly dependent on the CB_1 receptor distribution in astrocytes relative to close neuronal compartments, particularly at the synapses. However, little is known about the expression and precise localization of the CB_1 receptor in astrocytes and their mitochondria relative to the synapses. We hypothesized that intracellular CB_1 receptors are present in astroglial mitochondria as it has been previously described by our group in neuronal (Bénard et al., 2012; Hebert-Chatelain et al., 2016) and muscle mitochondria (Mendizabal-Zubiaga et al., 2016). The GFAP-CB_1-RS mice expressing the CB_1 receptor gene exclusively in the astrocytes and the GFAPhrGFP-CB_1-WT mice will be ideal genetic tools to test this hypothesis.

The adolescent brain is characterized by continuous maturation and structural development processes (Kyzar et al., 2016). Alcohol abuse during this critical period causes long-term alterations in neurotransmitter synthesis and release, signaling cascades, neuronal and astroglial morphology, gene expression, axonal outgrowth, dendritic pruning or synaptic transmission and plasticity (Keshavan et al., 2014). Based on this, we hypothesized that excessive EtOH consumption during the adolescence produces anatomical alterations in astrocytes and modifies their CB_1 receptor expression in the CA1 hippocampus of adult mice, disrupting physiological processes in which the eCBS plays a key role, such as synaptic function and memory.

Learning and memory impariments are the most commonly reported cognitive deficiencies following r-mTBI (Tabaddor et al., 1984; van Zomeren & van den Burg, 1985; Baddeley et al., 1987; Dikmen et al., 1987; King et al., 1995; Ylvisaker & Szekeres, 2002). These processes are known to involve the hippocampal formation. Because the adolescent brain is particularly sensitive to experience and damage (Spear, 2000; Andersen, 2003) and taking into consideration the possibility that sex differences influences brain conditions, our working hypothesis is that r-mTBI would alters CB_1 receptors distribution in the juvenile brain of both, male and female rodents. This fact could be due to the potencial physical damage produced by r-mTBI, which compromises the blood-brain barrier and microvasculature (Liu et al., 2014).

Some studies have demonstrated in the hippocampus that the efficacy of THC is different at excitatory and inhibitory synapses. Thus, while THC acts as a partial agonist of CB_1 receptors located in excitatory terminals (Shen & Thayer, 1999; Hoffman et al., 2010), CB_1 receptors in

inhibitory terminals are more sensitive and completely antagonized by THC (Hájos et al., 2000; Robbe et al., 2006). Also, the effects of THC in vivo show an alteration of the CB_1-dependent LTD of the inhibitory synaptic transmission in the hippocampus and of the excitatory synaptic transmission in the nucleus accumbens (Mato et al., 2004), and studies indicate that THC administered acutely produces a mismatch in cellular metabolism through the activation of CB_1 receptors located in the mitochondria and, as a consequence, memory is altered (Hebert-Chatelain et al., 2016). Besides, long-term potentiation (LTP) is facilitated in the hippocampus of Glu-*CB_1*-KO mice, which is accompanied by an increase in spine density and dendritic arborization. In contrast, the decrease in LTP in GABA-*CB_1*-KO mice correlates with a decrease in both spine density and dendritic arborization (Monory et al., 2015). Therefore, while the behavioral and physiological effects of THC are well described, scarce anatomical studies have examined its actions on structural plasticity, i.e., the morphological changes that occur after THC exposure. Because the use of marijuana usually begins in the adolescence, we hypothesized that acute administration of cannabis during this time period produces modifications in the brain ultrastructure and alters the CB_1 receptor expression in the CA1 hippocampus.

3. OBJECTIVES

The general goal of my doctoral thesis was to investigate in the rodent brain by high resolution electron microscopy:

First, the expression and precise localization of the CB_1 receptor in astrocytes and their mitochondria relative to the synapses.

Second, the subcellular pattern of the CB_1 receptor distribution in rodents exposed to pathological insults sharing the common denominator of memory impairment, in order to provide insights of their impact on the CB_1 receptor topography in distinct compartments and organelles of neurons and astrocytes involved in memory formation in the hippocampus.

The specific objectives were to:

1. Determine the CB_1 receptor expression and distribution in the GFAPhrGFP-*CB_1*-WT and GFAPhrGFP-*CB_1*-KO mutant mice, specifically:
   - 1.1. Localization of CB_1 receptors in astrocytes and astroglial mitochondria in the hippocampus.
   - 1.2. Topography of the CB_1 receptors in astroglial mitochondria relative to the synapses in the hippocampus.
   - 1.3. Density of CB_1 receptors in mitochondria of astrocytes and neurons in CA1 stratum radiatum, prefrontal cortex, piriform cortex and accumbens.

2. Study the impact of chronic EtOH intake during the adolescence (binge drinking) on:
   - 2.1. The cellular and subcellular localization and density of the CB_1 receptor in adult CA1 neurons and astrocytes.
   - 2.2. The topography of the CB_1 receptors in the adult CA1 hippocampal astrocytes relative to the synapses.
   - 2.3. The ultrastructure of the CA1 hippocampal astrocytes.

3. Investigate in juvenile male and female rats the effects of mTBI at different time points on:
   - 3.1. The cellular and subcellular localization and density of CB_1 receptors in the molecular layer of the hippocampal dentate gyrus.
   - 3.2. The ultrastructure of the DML astrocytes, neurons and mitochondria.

4. Assess the effects of an acute THC administration in the mature brain on:
   4.1. The cellular and subcellular localization and density of CB_1 receptors in the CA1 hippocampus.
   4.2. The ultrastructure of the CA1 astrocytes, neurons and mitochondria.

4. MATERIALS AND METHODS

4.1. ETHICS STATEMENT

Experiments were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country UPV/EHU (CEEA/M20/2015/093, CEEA/M20/2016/073, CEIAB/M30/2015/094, CEIAB/M30/2016/074) and the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization number, A501350). All mice were used according to the European Community Council Directive of 22nd September 2010 (2010/63/EU) and the Spanish and French legislation (RD 53/2013, BOE 08-02-2013, Ley 6).

The mTBI procedures applied to Long-Evans rats were approved by the Animal Care Committee at the University of Victoria (Victoria, BC, Canada) and were performed in accordance with the guidelines set by the Canadian Council for Animal Care.

Maximal efforts were made in order to minimize the number and the suffering of the animals used.

4.2. RESEARCH ANIMALS

In my doctoral thesis, fifteen *CB_1*-WT mice, nine *CB_1*-KO mice and at least three animals of other conditional mice (GFAP-*CB_1*-KO, GFAP-*CB_1*-RS, *CB_1*-STOP, GFAPhrGFP-*CB_1*-WT and GFAPhrGFP-*CB_1*-KO) were used, as it will be described below. In addition, the brains of twenty-one Long-Evans male and twenty-one female rats (three animals per group) were kindly provided by Dr. Brian R. Christie and Dr. Patrick Nahirney (Division of Medical Sciences, University of Victoria, Victoria BC, Canada).

4.2.1. C57BL/6 mice (hereafter *CB_1*-WT)

C57BL/6 mice (The Jackson Laboratory and Janvier labs) were received at the University of the Basque Country and after being in quarantine for one week, they were available to the experimenter.

4.2.2. *CB1* receptor mutant lines

I. Conventional and conditional *CB1*-KO

CB1-KO mice were generated and genotyped as previously described (Marsicano et al., 2002). In addition, conditional *CB1* receptor mutant mice were obtained by crossing the respective Cre expressing mouse line with *CB1* f/f mice (Marsicano et al., 2003), using a three-step breeding protocol (Monory et al., 2006).

II. Generation of GFAP-*CB1*-KO

Transgenic mice expressing the inducible version of the Cre recombinase CreERT2 under the control of the human glial fibrillary acid protein promoter, i.e. GFAP-CreERT2 mice (Hirrlinger et al., 2006) were crossed with mice carrying *CB1* receptor "floxed" sequence (Marsicano et al., 2003). As a result, transgenic mice *CB1* f/f;GFAPCreERT2 were obtained. This animal model allows the on-demand control of astroglial CB_1 receptor recombination in adult mice (Han et al., 2012).

III. Generation of GFAP-*CB1*-RS

STOP-*CB1* mice were previously generated by inserting a loxP-flanked stop cassette into the 5' untranslated (UTR) of the coding exon of the *CB1* gene, 32 nucleotides upstream of the translational start codon (Ruehle et al., 2013). The STOP-*CB1* mice were crossed with GFAP-CreERT2 mice (Hirrlinger et al., 2006) to obtain *CB1* stop/stop; GFAP-CreERT2 mice.

Seven to nine-week-old *CB1* f/f;GFAP-CreERT2 and *CB1* f/f littermates, as well as *CB1* stop/stop; GFAP-CreERT2 and *CB1* stop/stop littermates were treated daily for 8 consecutive days with 1 mg/kg (i.p.) of either tamoxifen or 4OH-tamoxifen synthesized as previously reported (Detsi et al., 2002; Yu & Forman, 2003) to induce the Cre-dependent astroglial deletion of *CB1* (GFAP-*CB1*-KO and GFAP-*CB1*-WT littermate mice) or its exclusive astroglial reexpression (GFAP-*CB1*-RS and STOP-*CB1* littermates). Mice were used for immunocytochemistry 3 to 5 weeks after the last day of tamoxifen or 4OH-tamoxifen injections.

IV. Generation of GFAPhrGFP-*CB1*-WT and GFAPhrGFP-*CB1*-KO mice

Intrahippocampal injection of a recombinant adeno associated virus expressing humanized renilla green fluorescent protein (hrGFP) under the control of the human GFAP promoter (von Jonquieres et al., 2013) were performed in *CB1*-WT and *CB1*-KO mice to generate GFAPhrGFP-*CB1*-WT and GFAPhrGFP-*CB1*-KO, respectively. The vector

backbone was the pAAV-GFAP-hChR2(H134R)-EYFP kindly provided by Karl Deisseroth (Stanford University, CA, USA). The hChR2(H134R)-EYFP with the cDNA encoding for hrGFP was replaced using standard molecular cloning techniques. The virus production and purification, as well as the injection procedure were performed as previously described (Chiarlone et al., 2014). Coordinates for intrahippocampal injections were: anteroposterior -2.0mm, mediolateral +/- 1.5mm, dorsoventral -2mm relative from bregma. Mice were allowed to recover for at least 4 weeks after surgery before their anatomical characterization.

4.2.3. Long-Evans rats

As mentioned above, brains of Long Evans rats were provided by Dr. Brian R. Christie and Dr. Patrick Nahirney (Division of Medical Sciences, University of Victoria, Victoria BC, Canada).

Long-Evans female rats (Charles River Laboratories, St. Constant, PQ, Canada) were paired with proven male breeders (250-275 grams; post-natal day (pnd) 100-150) and plug checks were performed to confirm pregnancies. Care was taken not disturb the Dam and any new litter of pups for the first 24-36 hours post-partum to facilitate bonding. Pups were monitored to ensure they were thriving. At pnd 2, all litters were culled to 12 pups to facilitate uniformity of maternal care across litters. Pups were weaned at pnd 21 and at this time were re-housed in same-sex groups of 2-3 animals prior to experimental use.

4.3. ANIMAL TREATMENT

4.3.1. Descriptive anatomical characterization

C57BL/6N adult mice: CB_1-WT, CB_1-KO, GFAP-CB_1-KO, CB_1-STOP, GFAP-CB_1-RS, GFAPhrGFP-CB_1-WT and GFAPhrGFP-CB_1-KO mice (at least three animals of each condition) (between 60 and 90 postnatal days) of either sex were habituated in their environment for at least 1 week before experimental procedures were initiated. Animals were maintained at 22°C with a 12:12-hour light:dark cycle and had food and water ad libitum. Then, mice were deeply anesthetized to carry out brain tissue processing, as noted later.

4.3.2. Adolescent ethanol intake

Three-week-old male C57BL/6J mice (three animals per group) were housed in standard Plexiglas cages (17 cm × 14.3 cm × 36.3 cm). Mice were habituated in their environment for at least 1 week before experimental procedures were initiated. Animals were maintained at 22°C with a 12:12-hour light:dark cycle (red light on at 9:00 hours) and had ad libitum access to food throughout all experiments and ad libitum access to water except during EtOH access.

4.3.2.1. Drinking in the dark procedure

Adolescent male mice from postnatal day 32 to 56 (4–8 weeks) were randomly assigned to either the water (control) or EtOH experimental group. Mice were subjected to a 4 day BD in the dark procedure (Rhodes et al., 2007) over a period of 4 weeks. Each week, animals were weighed 1 hour before lights out on days 1–4. During these days, mice were separated and placed individually in standard Plexiglas cages (17 cm × 14.3 cm × 36.3 cm). Three hours into the dark cycle, mice were either exposed to a single bottle of EtOH [20% EtOH (v/v) prepared from 96% EtOH and tap water (Alcoholes Aroca S. L., Madrid, Spain)] or a bottle of tap water (control group) for 2 hours and on day 4 for 4 hours. After 4 days of EtOH or water exposure, EtOH bottles were removed and mice had access to only water for 3 days (food was always available). EtOH intake was calculated throughout treatment as grams of EtOH per kilogram of animal per hour (g/kg/hour). The average amount was 2.50 ± 0.15 g/kg/hour (Fig. 6a). At the end of the treatment, blood samples were collected from the lateral tail veins using a capillary tube (Sarstedt, Germany), and EtOH levels were measured with an EtOH assay kit (Sigma-Aldrich). The average blood EtOH concentration was 58.07 ± 6.04 mg/dl (Fig. 6b;***$p < 0.001$).

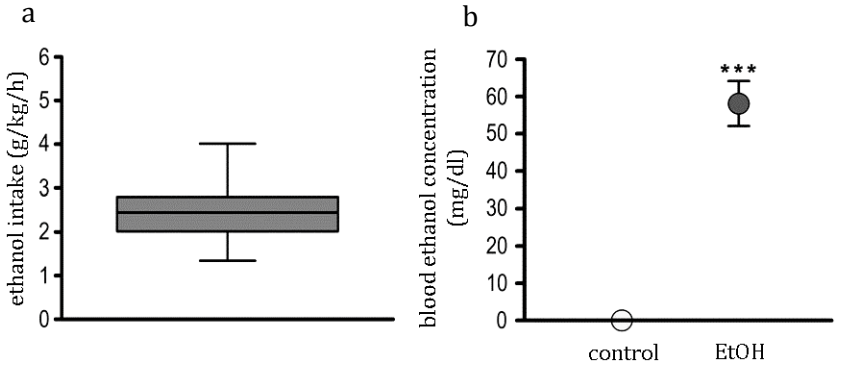


Figure 6. Voluntary oral ethanol consumption and blood ethanol concentration (BEC). (a) Total ethanol intake (g/kg/h) and (b) BEC (mg/dl) in C57BL6 mice exposed to 4-day BD in the dark procedure over a period of 4 weeks during adolescence (pnd 30–58). Data are expressed as mean ± standard error mean (SEM). Data were analyzed by means unpaired t-test; ***$p < 0.001$.

Four weeks after cessation of EtOH exposure, control, EtOH-treated and CB_1-KO mice (n = 3 each group) were deeply anesthetized to carry out brain tissue processing.

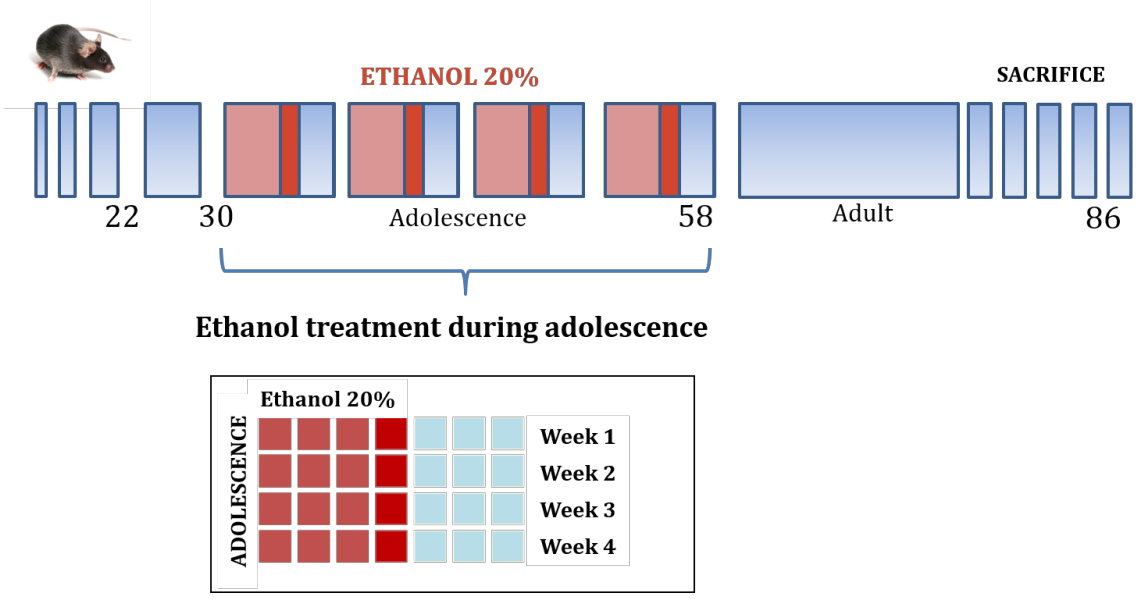


Figure 7. Experimental timeline. EtOH mice had free EtOH access (20% (v / v)) during 4 weeks in the adolescence (pnd 30-58). Each week, the mice were exposed to 2 or 4 hours of free EtOH access. In the remaining 3 days of the week, animals were kept resting in their respective cages. After 4 weeks of withdrawal (adulthood), mice were sacrificed.

4.3.3. R-mTBI model

Long-Evans rats (Charles River Laboratories, St. Constant, PQ, Canada) were housed in sex-specific groups of 2-3 animals and were maintained at 22°C with a 12:12-hour light:dark cycle and had food and water ad libitum prior to experimental use.

4.3.3.1. Awake closed head injury (ACHI)

Repeat mild traumatic brain injuries were induced using the ACHI model (Meconi et al., 2018). This model was designed to produce a mild closed head injury in non anesthetized juvenile rats and its design was based on a similar model (Petraglia et al., 2014b, 2014c).

To produce the injury, rats were immobilized using a soft plastic restraint cone with an opening at the nostril to allow adequate ventilation (Model DC-200, Braintree

Scientific, Braintree, MA). Once the rats were properly positioned in the restraint, a 3D printed helmet was placed over the head and held in place using a rubber elastic band and double sided tape. A circular flat disk on top of the helmet centered the impact site over the left parietal cortex.

The animals were then placed on a soft foam platform (3" thick Super-Cushioning Polyurethane Foam Sheet, McMaster-Carr, OH) below the injury apparatus. The injury was induced using a modified CCI device (Impact One, Leica Biosystems Inc., ON, Canada) mounted on a stereotaxic frame. The impact tip, modified with the addition of a 7 mm diameter flat rubber, was aligned vertically over the impact site on the helmet. Impact parameters were adjusted to an impact speed of 6 m/s, 10 mm of impact depth and 0.1 s of dwell time (or time to retraction). When the subject was motionless and the helmet and impactor were properly aligned, the impact was delivered using the control box. Animals were removed from the restraint bag immediately after the impact.

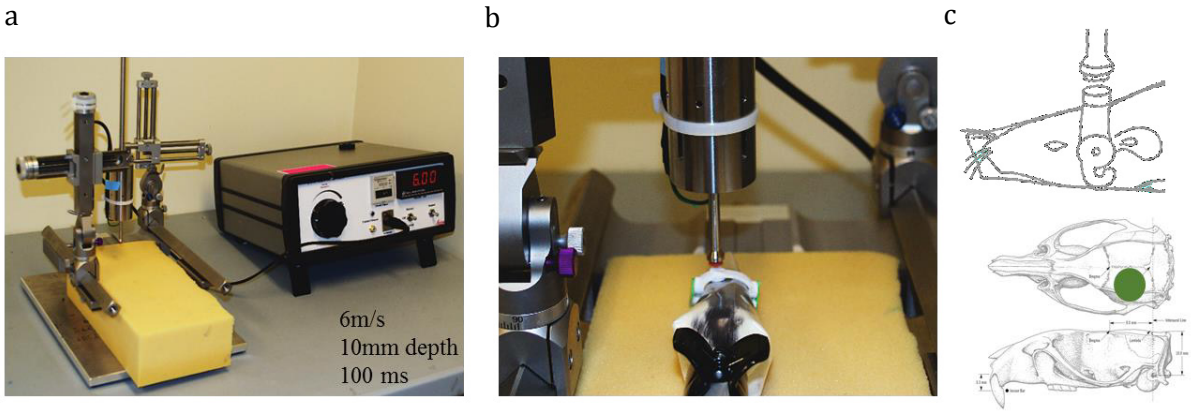


Figure 8. Awake closed head injury model description. (a, b) A modified Leica Impact One controlled cortical impactor is used to produce a closed head injury. The control unit sets the velocity (6 m/s) and dwell time (10 ms) of an electromagnetic piston that is affixed to a stereotaxic frame. The piston drives a customized impact tip with a 7 mm diameter rubber tip. (b, c) The subject is placed in a soft plastic restraint bag, and a 3D printed helmet is placed on the head so the impact target is centred over the left parietal cortex. (c) The restrained subject is placed on a soft foam platform, and the stereotax is adjusted to centre the impact tip over the helmet target. The impact is initiated using the control unit, and then the subject is immediately moved from the platform and from restraint so that assessment can begin. (Figure adapted from Meconi et al., 2018).

Rats were randomly assigned to one of two groups: uninjured or injured. In all experiments both sexes were included. At pnd 25 to 28, animals in the injured group received 2 or 4 impacts per day for 4 days (8 or 16 in total). Sham subjects underwent the exact same procedure as the injured animals, including being placed in the restraint bag and on the injury platform, wearing the helmet and hearing the piston sound, but

without receiving the impact. To assess immediate neurological changes after each ACHI were developed a test battery and standardized scoring criteria: the neurological assessment protocol (NAP) (Shapira et al., 1988; Shohami et al., 1995; Schaar et al., 2010; Ding et al., 2013). Animals were sacrificed at post-injury day (PID) 1, 10 and 40 for immunocytochemistry experiments.

The laboratory of Dr. Brian R. Christie and Dr. Patrick Nahirney (Division of Medical Sciences, University of Victoria, Victoria BC, Canada) performed this protocol and I describe it as a part of this thesis with their permission and approval.

4.3.4. Acute model of Δ9-tetrahydrocannabinol

Nine-week-old male C57BL/6J mice (three animals per group) were housed in standard Plexiglas cages (17 cm × 14.3 cm × 36.3 cm). Mice were habituated in their environment for at least 1 week and maintained at 22°C with a 12:12-hour light:dark cycle and had food and water ad libitum before experimental procedures were initiated.

4.3.4.1. Acute administration of THC

At the time of investigation, six mice had a body weight of 21–26 g. They were just once injected intraperitoneally with Δ9-THC (5 mg/kg) or vehicle (Busquets-Garcia et al., 2017). The solution was prepared with THC purchased from THC-Pharm-GmbH (Frankfurt, Germany) and dissolved in 4% ethanol, 4% Cremophor-EL and 92% saline. The sham group was treated by injection of a matched saline and Cremophor (vehicle) mixture. After half an hour, mice were anesthetized to carry out brain tissue processing.

4.4. PRE-EMBEDDING IMMUNOLABELING FOR ELECTRON MICROSCOPY

The protocol published by our laboratory was applied (Puente et al., 2019):

4.4.1. Preservation of brain tissue

1. Animals were anesthetized (at least n = 3) by intraperitoneal injection of ketamine/xylazine (80 (for rat) or 100 (for mouse)/10 mg/kg body weight).
2. They were transcardially perfused at room temperature (20-25 °C) through the left ventricle with PBS (0.1 M, pH 7.4) for ~20 s at RT, followed by ice-cold fixative solution made up of 4% formaldehyde (freshly depolymerized from paraformaldehyde), 0.2% picric acid, and 0.1% glutaraldehyde in PB (0.1 M, pH 7.4). The fixative solution was 250 ml per mouse and 500 ml per rat and the perfusion time was 15 min per mouse and 30 min per rat.
3. The brains were removed from the skull and post-fixed in the fixative solution for ~1 week at 4 °C. Samples were stored in 0.1 M PB diluted fixative (1:10) containing 0.025% sodium azide at 4 °C until use.

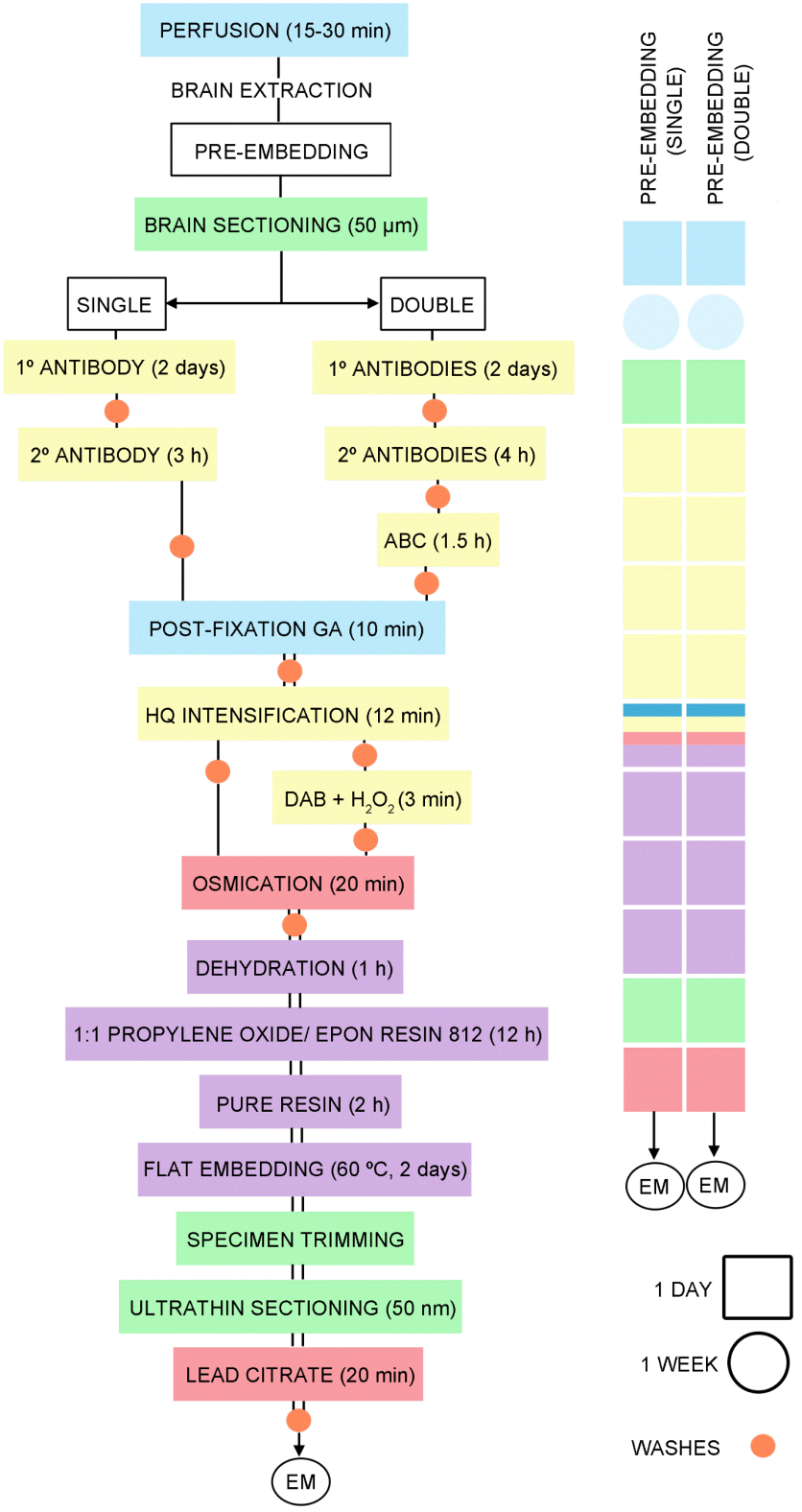


Figure 9. Timeline of the general steps for pre-embedding immunoelectron microscopy techniques. (Figure adapted with permission from Puente et al., 2019).

4.4.2. Single pre-embedding immunogold and double pre-embedding immunogold and immunoperoxidase method for electron microscopy

The protocol published by our laboratory was used (Puente et al., 2019):

1. Brains coronal sections were cut at 50 µm in a vibratome and collected in 12-well cell culture plates in 0.1 M PB (pH 7.4) at RT. Sections from each brain were stored in separate plates for storage at 4 °C in 0.1 M PB (pH 7.4) with 0.025% sodium azide. Then, two or three sections per brain containing the area of interest were selected and placed in a new plate. Total volume per well: 1 ml.
2. Pre-incubation in blocking solution (1 ml/well) containing 10% bovine serum albumin (BSA), 0.02% saponin and 0.1% sodium azide in tris-hydrogen chloride buffered saline (TBS) 1X, pH 7.4 on a shaker (300 rpm) for 30 min at RT.
3. Sections were incubated with goat polyclonal anti-CB_1 receptor antibody or guinea pig polyclonal anti-CB_1 receptor antibody (diluted 1:100, 1 ml/well) alone, or in double immunostaining in combination with either a mouse monoclonal anti-GFAP antibody (1:1,000), rabbit polyclonal anti-GLAST antibody (0.3 µg/ml), rabbit polyclonal anti-hrGFP antibody (1:500) or mouse monoclonal anti-gephyrin antibody (1:250) prepared in 10% BSA/TBS 1X containing 0.004% saponin and 0.1% sodium azide. Dish was placed on an orbital shaker for 2 days at 4 °C.

Note: The guinea pig polyclonal anti-CB_1 antibody was used for double immunolabeling with the rabbit polyclonal anti-GLAST antibody.

4. Several washes in 1% BSA/TBS (3 x 1 min and 2 x 10 min) to remove excess of the antibody.
5. Incubation with 1.4 nm gold-conjugated secondary rabbit anti-goat IgG (Fab' fragment, 1:100, Nanoprobes Inc., Yaphank, NY, USA) or 1.4 nm gold-conjugated secondary goat anti-guinea pig IgG (Fab' fragment, 1:100, Nanoprobes Inc., Yaphank, NY, USA) alone or in the case of double immunostaining along with the corresponding biotinylated secondary antibody (1:200) diluted in 1% BSA/TBS with 0.004% saponin on a shaker for 3 h for single and 4 h for double immunostaining at RT.
6. Several washes in 1% BSA/TBS (3 x 1 min and 2 x 10 min) on a shaker at RT.
7. In the case of double immunostaining, sections were incubated in avidin-biotin-peroxidase complex (ABC) (1:50) prepared in washing solution (1 ml/well) for 1.5 h at RT and washed three times in 1% BSA/TBS (10 min each).
8. The tissue was kept in 1% BSA/TBS on a shaker overnight at 4 °C.
9. Post-fixation with 1% glutaraldehyde in TBS (1 ml/well) for 10 min at RT.

10. Several washes in double distilled water (3 x 10 min each).
11. Sections were transferred to test tubes.
12. Silver intensification of gold particles with the HQ Silver kit (Nanoprobes Inc., Yaphank, NY, USA; 1 ml/tube) in the dark for 12 min.
13. Three washes in double distilled water (1 min each).
14. Three washes in 0.1 M PB (pH 7.4) (10 min each).
15. Sections were transferred to glass vials (15 ml, 3 x 5 cm).
16. In the case of double immunostaining, incubation in 0.05% DAB and 0.01% hydrogen peroxide prepared in 0.1 M PB (1 ml/vial) for 3 min at RT and several washes in 0.1 M PB (pH 7.4) (3 x 1 min and 2 x 10 min).
17. Samples were osmicated (1% osmium tetroxide in 0.1 M PB, pH 7.4; 1 ml/vial) in the dark for 20 min.
18. Several washes in 0.1 M PB (pH 7.4) (3 x 1 min and 2 x 10 min).
19. Dehydration in graded ethanols (50%, 70%, 96%; 5 min/each) followed by three times in 100% ethanol (5 min each) (1 ml/vial).
20. Clearing in propylene oxide (3 x 5 min, 1 ml/vial).
21. Embedding in a 1:1 mixture of propylene oxide and Epon resin 812 (1 ml/vial) on a shaker overnight at RT.
22. Embedding in pure Epon resin 812 (1 ml/vial) for > 2 h at RT.
23. Sections were placed between two glass slides and wrapped in aluminum foil.
24. Polymerization of resin-embedded sections in an oven at 60 °C for 2 days.
25. Blocks with the resin-embedded sections were trimed and then semi-thin sections were cut into 1 µm on ultramicrotome with a histo diamond knife (Diatome USA) and stained with 1 % toluidine blue to perceive the tissue.
26. Ultra-thin sections of 50 nm were cut with a diamond knife and collected on nickel mesh grids.
27. Sections were stained with 2.5% lead citrate (1 drop/grid) for 20 min at RT.
28. Three washes in double distilled water (1 drop/grid) (10 min each).
29. Examination under a Philips EM208S transmission electron microscope and a JEOL JEM-1400 Plus electron microscope (JEOL Canada).
30. Tissue was photographed by using a digital Morada camera (Olympus) and a Gatan SC1000 digital camera (Gatan USA).

Table 1. Antibodies.

ANTIBODY	Manufacturer; species; catalog number; RRID
Anti-cannabinoid receptor type-1 (CB_1) (2 µg/ml)	Frontier Institute Co., ltd; goat polyclonal; CB_1-Go-Af450; AB_2571592, AB-257130
Anti-cannabinoid receptor type-1 (CB_1) (2 µg/ml)	Frontier Institute Co., ltd; guinea pig polyclonal; CB_1-GP-Af530; AB_2571593
Anti-glial fibrillary acidic protein (GFAP) (1:1000)	Sigma-Aldrich; mouse monoclonal; G3893; AB_257130,AB_477010
Anti-gephyrin (1:250)	Synaptic Systems; mouse monoclonal; 147021; AB_2232546
Anti-A522 (EAAT1 [GLAST]) (0.3 µg/ml)	Gifted by Prof. Niels Christian Danbolt University of Oslo; rabbit polyclonal; Ab#314; AB_2314561
Polyclonal humanized Renilla reniformis Green Fluorescence Protein	Stratagene; rabbit polyclonal; #240142-51, AB_10598674
Biotinylated anti-mouse secondary antibody (1:200)	Vector Labs; BA-2000; AB_2313581
Biotinylated anti-rabbit secondary antibody (1:200)	Vector Labs; BA-1000; AB_2313606
1.4 nm gold-conjugated anti-guinea pig IgG (Fab' fragment) secondary antibody (1:100)	Nanoprobes; goat; #2055
1.4 nm gold-conjugated anti-goat IgG (Fab' fragment) antibody (1:100)	Nanoprobes; rabbit; #2004

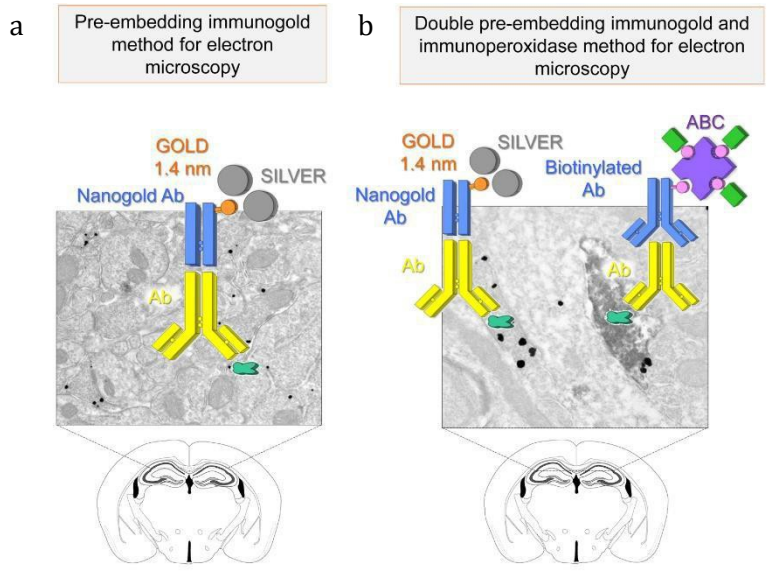


Figure 10. Immunolabeling methods used for high resolution electron microscopy. (Adapted with permission from Puente et al., 2019).

The following images are representative images of the single pre-embedding immunogold and double pre-embedding immunogold and immunoperoxidase methods applied in the present doctoral thesis (Fig. 11), to study the subcellular CB_1 localization in the different neuronal and astroglial compartments in the healthy or diseased brain, such as in GABAergic and glutamatergic synaptic terminals (ter), in astrocytes (as) and in mitochondria (m).

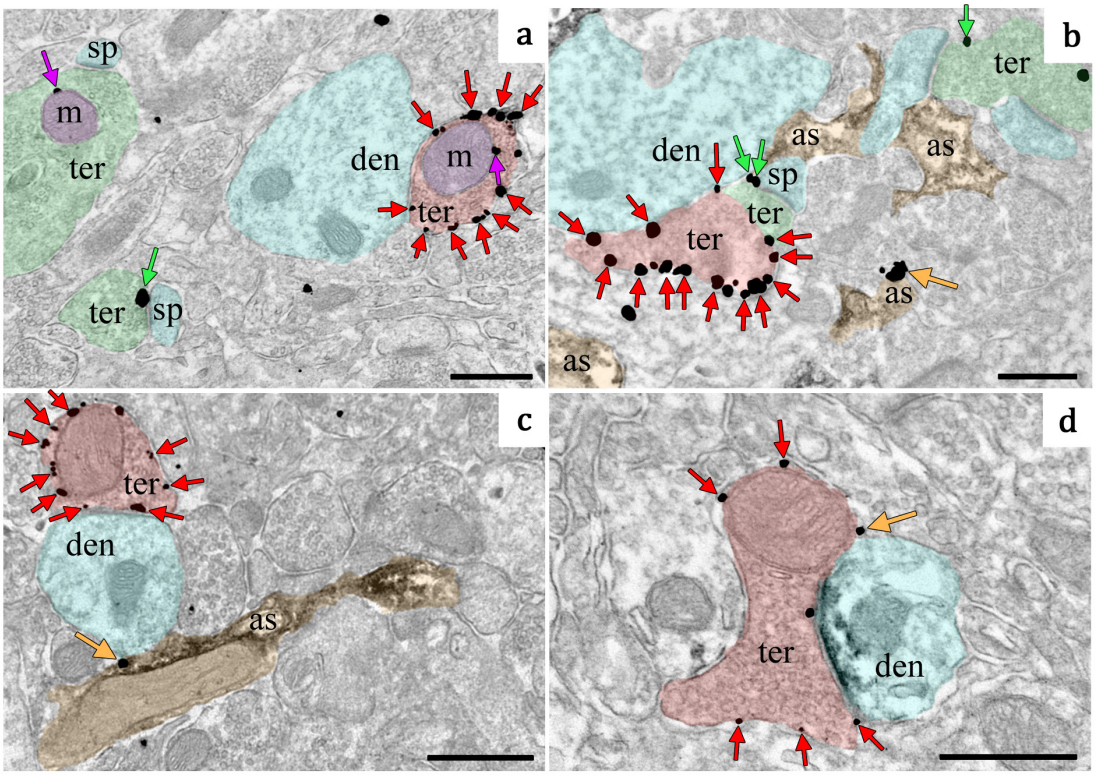


Figure 11. CB_1 receptor immunolocalization in different subcellular compartments of the rodent brain. Single pre-embedding immunogold (a) and double pre-embedding immunogold and immunoperoxidase methods (b, c, d). (a) CB_1 receptor labeling (arrows) at a presynaptic GABAergic terminal (ter, red) adjacent to a dendrite (den, blue). CB_1 receptor particle is localized to a presynaptic glutamatergic terminal (ter, green) associated with a spine (sp, blue). Mitochondria (m, purple) exhibit CB_1 receptor immunolabeling in both glutamatergic (ter, green) and GABAergic (ter, red) presynaptic terminals (CA1 stratum radiatum, adult mouse hippocampus). (b) CB_1 receptor labeling (arrows) at a presynaptic GABAergic terminal (ter, red), glutamatergic terminals (ter, green) and in one astrocyte branch (as, orange) in the mouse piriform cortex. Astrocytes are labeled with anti-GLAST/immunoperoxidase/DAB method (black precipitate in as). (c) CB_1 receptor labeling (arrows) at a presynaptic GABAergic terminal (ter, red) adjacent to a dendrite (den, blue) and in one astrocyte process (as, orange) in the molecular layer of the mouse DML. Astrocytes are labeled with anti-GFAP/immunoperoxidase/DAB method (black precipitate in as). (d) CB_1 receptor labeling (arrows) at a presynaptic terminal (ter, red) combined with anti- gephyrin/immunoperoxidase/DAB method (black precipitate in den, blue) to positively identify the inhibitory postsynaptic membrane of a GABAergic synapse. Orange arrow: CB_1 receptor labeling at a thin astrocytic process filling the intercellular space (rat prelimbic cortex). (Adapted with permission from Puente et al., 2019).

4.5. DATA ANALYSIS

Semi-quantification of the CB_1 receptor labeling obtained with the pre-embedding immunogold method

It was performed according to the protocol published by our laboratory (Puente et al., 2019).

With the aim of maximizing the standard conditions, the pre-embedding immunogold method was applied simultaneously to all the sections obtained from the animals under study (at least n = 3). Three replicated experiments were done for each animal.

Immunogold-labeled resin-embedded vibratome sections were first visualized under the light microscope in order to select portions of the region of interest with reproducible CB_1 receptor immunolabeling. Then, semi-thin sections from resin embedded tissue were cut and the first five ultra-thin sections were collected onto two grids. To further standardize the conditions between the different animals, only the first 1.5 μm from each specimen surface was collected and randomly photographed. Sampling was always performed carefully and in the same way for all the animals studied. To avoid bias, whenever possible, I remained blind when taking and analyzing the electron micrographs.

The excitatory and inhibitory synapses were identified by their ultrastructural features (Fig. 12). In some cases a gephyrin/immunoperoxidase/DAB method was also applied to positively identify the inhibitory postsynaptic membrane of a GABAergic synapse (Puente et

al., 2019; Fig.11):

- **Excitatory synapses** are asymmetrical with postsynaptic densities and presynaptic axon terminals containing abundant, clear and spherical synaptic vesicles.
- **Inhibitory synapses** are symmetrical with slender postsynaptic membranes and axon terminals containing pleomorphic synaptic vesicles. Because of the lack of postsynaptic membrane density, the inhibitory nature of the synapse might be misleading unless serial sections were done. An alternative to circumvent this is to use the antibody against gephyrin, a postsynaptic anchor protein marker of inhibitory synapses which can be used to unequivocally identify inhibitory synapses. CB_1 receptors in **astrocytes** were assessed in astrocytic processes containing hrGFP, GFAP or GLAST DAB immunodeposits.

The proportion of the CB_1 receptor labeling on different compartments identified as described above was then tabulated. Positive labeling was considered when at least one CB_1 receptor immunoparticle was within ~30 nm of the membrane of the specific compartment under study, and ≥ 80 nm from other membranes in the case of mitochondrial labeling. Metal particles were then counted and CB_1 receptor density (particles/µm membrane) in the positive compartments was determined with Image-J software (NIH; RRID:SCR_003070) by measuring their membrane length. I also estimated the proportion of CB_1 receptor immunoparticles in different profiles versus the total CB_1 receptor expression. This gives information about the CB_1 receptor distribution throughout different compartments of a particular brain region (excitatory and inhibitory synapses, astrocytes, mitochondria, other cellular compartments). As for astrocytes, the distance from astrocytic and astrocytic mitochondria CB_1 receptor immunoparticles to the nearest synapse was also calculated to determine how the receptors were distributed in the context of the tripartite synapse. To do this, the nearby synapses surrounding the CB_1 receptor positive astrocytic elements were identified, distances measured (ImageJ software), the nearest synapse to the astrocytic immunoparticle selected, and data from all the nearest synapses tabulated and analyzed.

All values are given as mean ± S.E.M. using a statistical software package (GraphPad Prism 5, GraphPad Software Inc., San Diego, USA). The normality test (Kolmogorov-Smirnov normality test) was always applied before running statistical tests. Data were analyzed using parametric or non-parametric two-tailed Student's t-test or one-way ANOVA with subsequent post-hoc analysis (Bonferroni post-test). A potential variability between mice of the same group of mutant lines was analyzed statistically. Since there were no differences between them, all data from each line were pooled.

Minor adjustments in contrast and brightness were made to the figures using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). Gimp and Adobe Photoshop were used

to blend the electron micrographs into the serial photocomposition.

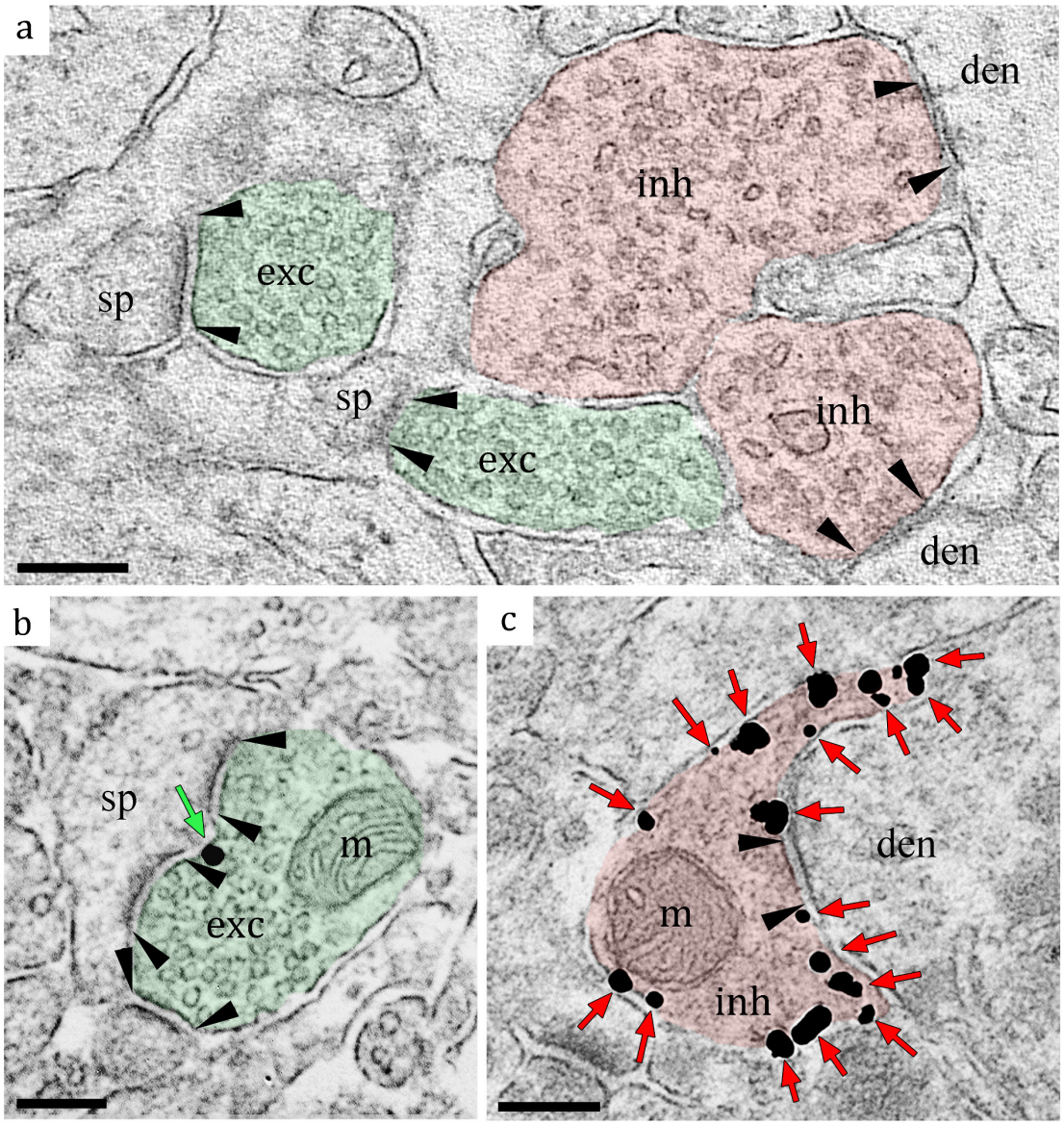


Figure 12. Ultrastructural features of excitatory and inhibitory synapses in the electron microscope. Pre-embedding immunogold method. (a) Typical presynaptic axon terminals of excitatory nature (exc, green) contain abundant, clear and spherical synaptic vesicles and form asymmetric synapses which have postsynaptic densities (black arrowheads) in dendritic spines (sp). Inhibitory (inh) presynaptic terminals (red) contain pleomorphic synaptic vesicles and make symmetric synapses with dendrites (den) which have no postsynaptic densities (black arrowheads). (b) An excitatory terminal (exc) making an asymmetric synapse with a dendritic spine (sp) shows CB_1 labeling (green arrow). (c) A typical inhibitory terminal (inh) making a symmetric synapse (arrowheads) with a dendrite (den) is decorated with numerous CB_1 receptor immunoparticles (red arrows) (m: mitochondria) (Scale bars: 200 μm).

5. RESULTS

5.1. CB_1 RECEPTORS IN ASTROCYTES

Astrocytes and their processes were identified by DAB immunodeposits of GFAP or hrGFP or GLAST and the CB_1 receptor was detected by immunogold labeling. As expected, the CB_1 receptor was mainly localized on neuronal terminals, preterminal membranes and, to a lesser extent, on labeled astrocytes (Gutiérrez-Rodríguez et al., 2018). In the GFAP-*CB_1*-KO hippocampus, the CB_1 receptor particles were only on synaptic terminals but not in astrocytic processes (Figs. 13c, 14c and 15). In addition, CB_1 receptor immunoparticles were found in neuronal mitochondria but not in mitochondria of astrocytes in GFAP-*CB_1*-KO (Fig. 13c and 16). Conversely, in the GFAP-*CB_1*-RS hippocampus, immunolabeling was restricted to the DAB-containing astrocytic elements and astrocytic mitochondria but no labeling was found on axon boutons (Figs. 13d, 14d, 15 and 16) (Gutiérrez-Rodríguez et al., 2018). Finally, the subcellular distribution of the CB_1 receptor on astrocytic mitochondria and in general, on astrocytic elements of the GFAPhrGFP-*CB_1*-WT resembled the *CB_1*-WT hippocampus (Figs. 13f and 14f, 15 and 16). Importantly, this CB_1 receptor staining pattern was absent in *CB_1*-KO, STOP-*CB_1* and GFAPhrGFP-*CB_1*-KO mice (Figs. 13b, e, g; 14b, e, g; 15 and 16).

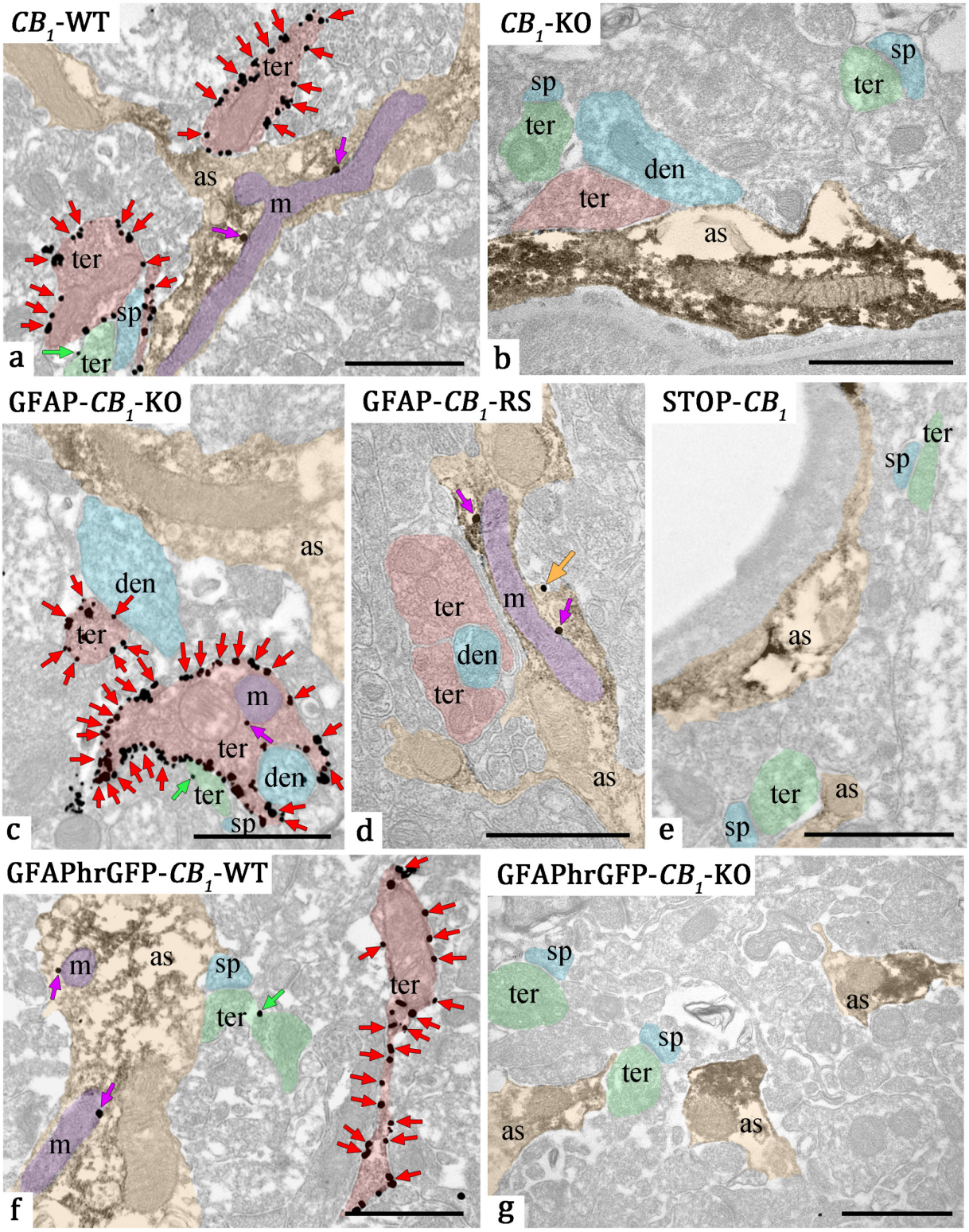


Figure 13. CB_1 receptor localization in identified astrocytes and astrocytic mitochondria in the CA1 stratum radiatum of wild-type and mutant mice. Pre-embedding immunogold and immunoperoxidase method for electron microscopy. (a, d and f) Mitochondrial CB_1 receptor labeling is visualized in identified astrocytes. (a, c, f) As expected, CB_1 receptor immunoparticles are also on membranes of synaptic terminals and preterminals. (b) No CB_1 receptor immunolabeling is detected in *CB_1*-KO confirming the specificity of the CB_1 receptor antibody, (c) CB_1 receptor particles are found in synaptic terminals and neuronal mitochondria, but not in astrocytes and astrocytic

mitochondria of GFAP-*CB₁*-KO. (d) Astrocytic processes and astrocytic mitochondria, but not axon terminals, are CB_1 receptor immunopositive in GFAP-*CB₁*-RS. (e) No CB_1 receptor immunoparticles are observed in STOP-*CB₁*. (f) In GFAPhrGFP-*CB₁*-WT, presynaptic terminals and astrocytic mitochondria are CB_1 receptor positive. (g) No CB_1 receptor immunolabeling is detected in GFAPhrGFP-*CB₁*-KO. CB_1 receptor labeling (arrows), presynaptic GABAergic terminal (ter, red), presynaptic glutamatergic terminal (ter, green), dendrite (den, blue), spine (sp, blue), mitochondria (m, purple) and astrocyte branch (as, orange) Scale bars: 1 µm.

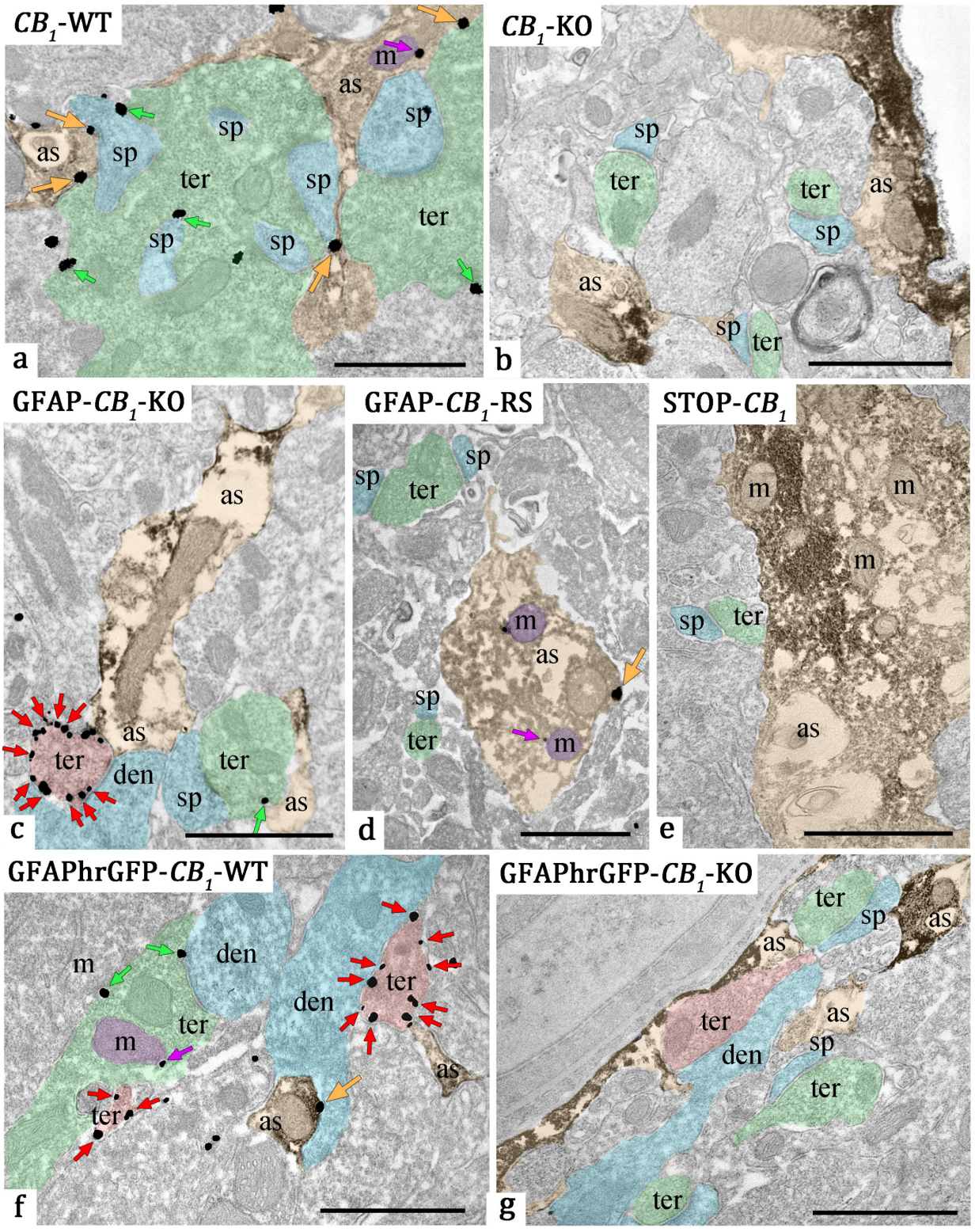


Figure 14. CB_1 receptor localization in identified astrocytes and astrocytic mitochondria in the DML of wild-type and mutant mice. Pre-embedding immunogold and immunoperoxidase method for electron microscopy. (a, d) Mitochondrial CB_1 receptor labeling is visualized in identified astrocytes. (a, c, f) As expected, CB_1 receptor immunoparticles are also on membranes of synaptic terminals and preterminals. (b) No CB_1 receptor immunolabeling is detected in *CB_1*-KO confirming the specificity of the CB_1 receptor antibody, (c) CB_1 receptor particles are found in synaptic terminals but not in astrocytes of GFAP-*CB_1*-KO. (d) Astrocytic mitochondria, but not axon terminals, are CB_1 receptor immunopositive in GFAP-*CB_1*-RS. (e) No CB_1 receptor immunoparticles are observed in STOP-*CB_1*. (f) In GFAPhrGFP-*CB_1*-WT, presynaptic terminals and astrocytic processes are CB_1 receptor positive. (g) No CB_1 receptor immunolabeling is detected in GFAPhrGFP-*CB_1*-KO. CB_1 receptor labeling (arrows), presynaptic GABAergic terminal (ter, red), presynaptic glutamatergic terminal (ter, green), dendrite (den, blue), spine (sp, blue), mitochondria (m, purple) and astrocyte branch (as, orange) Scale bars: 1 μm.

5.1.1. CB_1 receptor immunolabeling assessment in astrocytes of the CA1 stratum radiatum and DML

Regarding the proportion of the astrocytic CB_1 particles in the CA1 stratum radiatum, 5.31 ± 0.84% of the total CB_1 receptor labeling in the *CB_1*-WT, 11.97 ± 2.17% in the GFAPhrGFP-*CB_1*-WT ($p > 0.05$; Fig. 15a) and 95.31 ± 1.87% in the GFAP-*CB_1*-RS were in astrocytic processes (***$p < 0.001$; Fig. 15a). Only background immunoparticles were detected in astrocytic processes of the STOP-*CB_1*, GFAP-*CB_1*-KO, *CB_1*-KO and GFAPhrGFP-*CB_1*-KO (***$p < 0.001$; Fig. 15a).

Similarly, in DML, of the total CB_1 receptor labeling, 5.35 ± 1.00% in the *CB_1*-WT, 13.13 ± 2.60% in the GFAPhrGFP-*CB_1*-WT ($p > 0.05$; Fig. 15b) and 95.61 ± 1.56% in the GFAP-*CB_1*-RS was in astrocytes (***$p < 0.001$; Fig. 15b). Non-specific CB_1 receptor immunoparticles were found on astrocytic processes in the STOP-*CB_1*, GFAP-*CB_1*-KO, *CB_1*-KO and GFAPhrGFP-*CB_1*-KO mice (***$p < 0.001$; Fig. 15b).

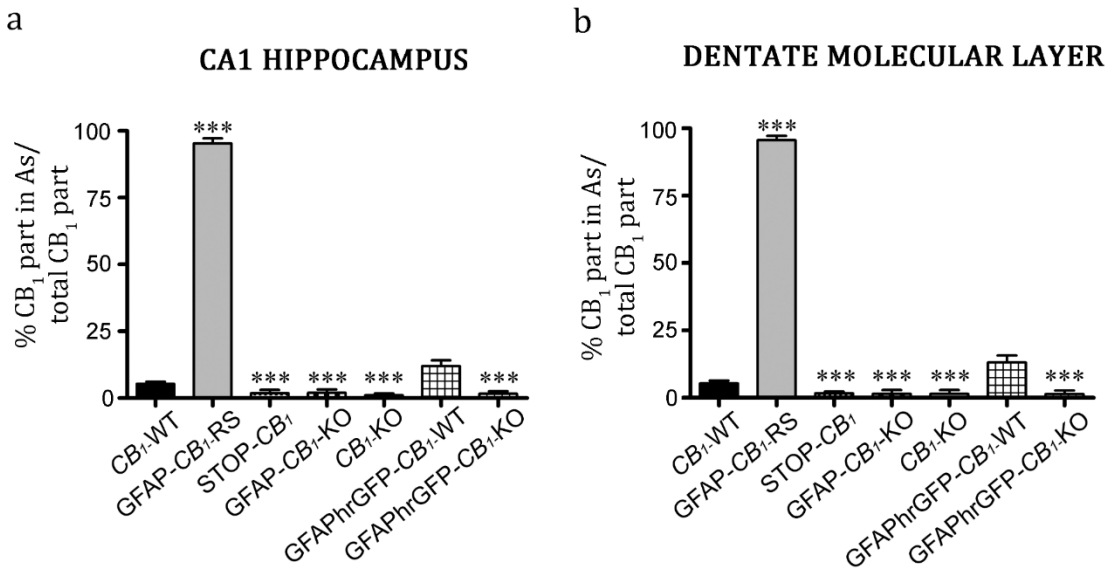


Figure 15. Statistical assessment of the CB_1 receptor distribution on astrocytes in the CA1 stratum radiatum and DML of wild-type and mutant mice. (a) Proportion of CB_1 receptor gold particles on astrocytic membranes versus total CB_1 receptor expression on plasmalemma: 5.31 ± 0.84% of the total CB_1 receptor immunoparticles are located in astrocytes of *CB_1*-WT and 95.31 ± 1.87% in astrocytes of GFAP-*CB_1*-RS. Only residual CB_1 immunoparticles are in astrocytic processes of STOP-*CB_1* (1.76 ± 1.29%), GFAP-*CB_1*-KO (1.96 ± 1.28%), *CB_1*-KO (1.02 ± 0.72%) and GFAPhrGFP-*CB_1*-KO (1.62 ± 0.94%). (b) Proportion of CB_1 receptor immunoparticles on astrocytic membranes versus total CB_1 receptor expression on plasmalemma: 5.35 ± 1.00% (*CB_1*-WT), 95.61 ± 1.56% (GFAP-*CB_1*-RS). Almost null non-specific immunoparticles are found in STOP-*CB_1* (1.65 ± 0.66%), GFAP-*CB_1*-KO (1.45 ± 1.45%), *CB_1*-KO (1.43 ± 1.43%) and GFAPhrGFP-*CB_1*-KO (1.37 ± 1.37%). Data are expressed as mean ± SEM of three different animals. Data were analyzed by means of Kruskal-Wallis test and the Dunn's multiple comparison post-hoc test. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. As: astrocytic processes; part: particles.

5.1.2. CB_1 receptor localization in astroglial mitochondria

CB_1 receptor labeling was observed in mitochondria (mtCB_1 receptors) of astrocytes distributed throughout the CA1 stratum radiatum (Fig. 13a, d, f) and DML (Fig. 14a, d). In *CB_1*-WT mice, 11.12 ± 1.80% of the mitochondrial sections in astrocytes of the CA1 stratum radiatum and 11.56 ± 2.33% of the DML were CB_1 receptor immunopositive (Figs. 16a, b). The percentage was roughly similar in GFAP-*CB_1*-RS (CA1: 12.39 ± 1.81% ($p > 0.05$; Fig. 16a); DML: 11.48 ± 1.76% ($p > 0.05$; Fig. 16b) and GFAPhrGFP-*CB_1*-WT (CA1: 13.12 ± 2.53% ($p > 0.05$; Fig. 16a); DML: 13.74 ± 3.20% ($p > 0.05$; Fig. 16b). Non-specific mitochondrial particles were detected in STOP-*CB_1* (CA1: 4.66 ± 1.55%, **$p < 0.01$; Fig. 16a; DML: 5.38 ± 1.22%, *$p < 0.05$; Fig. 16b), GFAP-*CB_1*-KO (CA1: 3.97 ± 1.70%, **$p < 0.01$; Fig. 16a; DML: 3.04 ± 1.04%, **$p < 0.01$; Fig. 16b), *CB_1*-KO (CA1: 2.97 ± 1.15%, ***$p < 0.001$; Fig. 16a; DML: 2.49 ± 0.80%, ***$p < 0.001$; Fig. 16b) and GFAPhrGFP-*CB_1*-KO mice (CA1: 0.95 ± 0.95%, ***$p < 0.001$; Fig. 16a; DML: 1.98 ± 0.91%, ***$p < 0.001$; Fig. 16b).

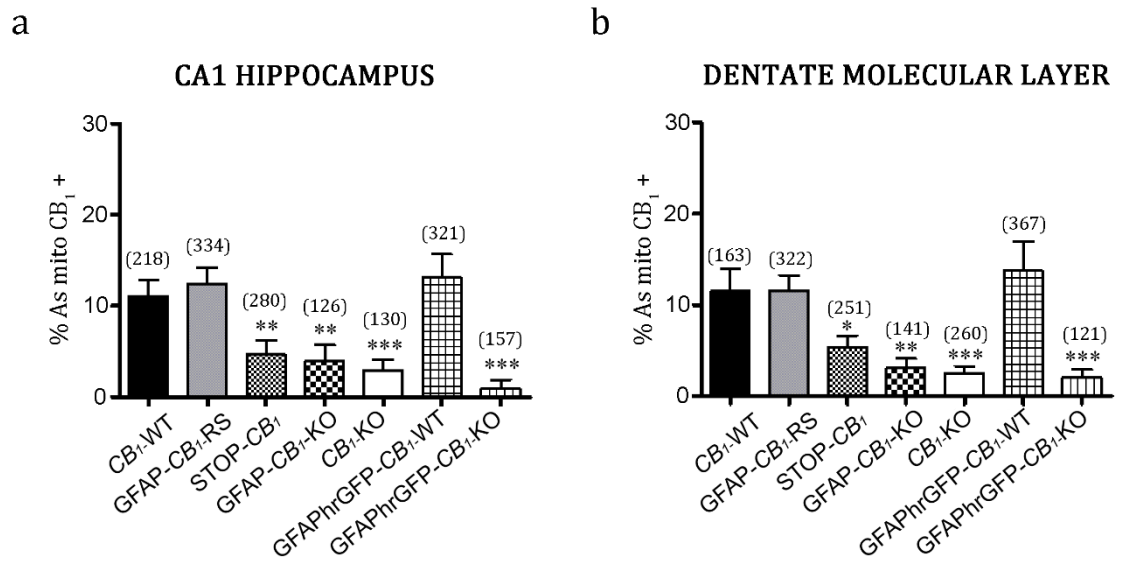


Figure 16. Proportion of CB_1 receptor immunopositive astrocytic mitochondria in the CA1 and DML of wild-type and mutant mice. (a) Values of the CB_1 receptor immunopositive astrocytic mitochondria in GFAP-*CB_1*-RS (12.39 ± 1.81%) and GFAPhrGFP-*CB_1*-WT (13.12 ± 2.53%) are closely similar to *CB_1*-WT (11.12 ± 1.79%) in the CA1 stratum radiatum. The background in astroglial mitochondria is: STOP-*CB_1* (4.66 ± 1.55%), GFAP-*CB_1*-KO (3.97 ± 1.71%), *CB_1*-KO (2.97 ± 1.15%) and GFAPhrGFP-*CB_1*-KO (0.95 ± 0.95%). The number of total mitochondria examined is in parentheses on the top of each column. (b) In the DML, the values of CB_1 receptor immunopositive astrocytic mitochondria in GFAP-*CB_1*-RS (11.48 ± 1.76%) and GFAPhrGFP-*CB_1*-WT (13.74 ± 3.20%) are comparable to the *CB_1*-WT (11.56 ± 2.33%). Background in astroglial mitochondria is: STOP-*CB_1* (5.38 ± 1.22%), GFAP-*CB_1*-KO (3.05 ± 1.04%), *CB_1*-KO (2.49 ± 0.80%), GFAPhrGFP-*CB_1*-KO (1.98 ± 0.91%). The number of total mitochondria examined is in parentheses on the top of each column. Data are expressed as mean ± SEM of three different animals. Data were analyzed by means of Kruskal-Wallis test and the Dunn's multiple comparison post-hoc test. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. As: astrocytic processes; mito: mitochondria.

5.1.3. Distance from the astroglial mtCB1 receptors to the nearest synapse

The distance between the astrocytic $mtCB_1$ receptor particles and the midpoint of the nearest synapse was assessed in *CB_1*-WT, GFAP-*CB_1*-RS and GFAPhrGFP-*CB_1*-WT hippocampi (Fig. 17; table 2).

In the CA1, 10.55 ± 4.01% of the total synapses analyzed were in a range of 0–400 nm from the astrocytic $mtCB_1$ receptor particles in *CB_1*-WT, 2.67 ± 2.67% in GFAP-*CB_1*-RS and 7.41 ± 3.70% in GFAPhrGFP-*CB_1*-WT. 38.54 ± 8.32% of the synapses were located between 400 and 800 nm in *CB_1*-WT, 49.28 ± 2.87% in GFAP-*CB_1*-RS and 51.85 ± 3.70% in GFAPhrGFP-*CB_1*-WT. 29.51 ± 6.85% of the synapses were detected between 800 and 1,200 nm in *CB_1*-WT, 37.26 ± 2.02% in GFAP-*CB_1*-RS and 29.63 ± 7.41% in GFAPhrGFP-*CB_1*-WT. Finally, 21.40

± 5.56% of the synapses were found at more than 1,200 nm from the astrocytic mtCB$_1$ receptor in *CB$_1$*-WT, 10.79 ± 2.94% in GFAP-*CB$_1$*-RS and 14.81 ± 7.41% in GFAPhrGFP-*CB$_1$*-WT (Fig. 17; Table 2).

In the DML, 11.11 ± 6.42% of the total synapses analyzed were at 0–400 nm in *CB$_1$*-WT, 2.82 ± 1.48% in GFAP-*CB$_1$*-RS and 1.52 ± 1.52% in GFAPhrGFP-*CB$_1$*-WT. 50 ± 3.21% of the synapses were located at a distance of between 400 and 800 nm from the astrocytic mtCB$_1$ immunoparticle in *CB$_1$*-WT, 47.57 ± 4.81% in GFAP-*CB$_1$*-RS and 57.37 ± 6.26% in GFAPhrGFP-*CB$_1$*-WT. 23.15 ± 0.93% of them were located between 800 and 1,200 nm in *CB$_1$*-WT, 43.79 ± 3.13% in GFAP-*CB$_1$*-RS and 35.86 ± 2.53% in GFAPhrGFP-*CB$_1$*-WT. Finally, 18.52 ± 3.70% of the synapses in *CB$_1$*-WT, 11.82 ± 3.51% in GFAP-*CB$_1$*-RS and 5.25 ± 2.72% in GFAPhrGFP-*CB$_1$*-WT were observed at more than 1,200 nm from the astrocytic mtCB$_1$ receptor particles (Fig. 17; Table 2).

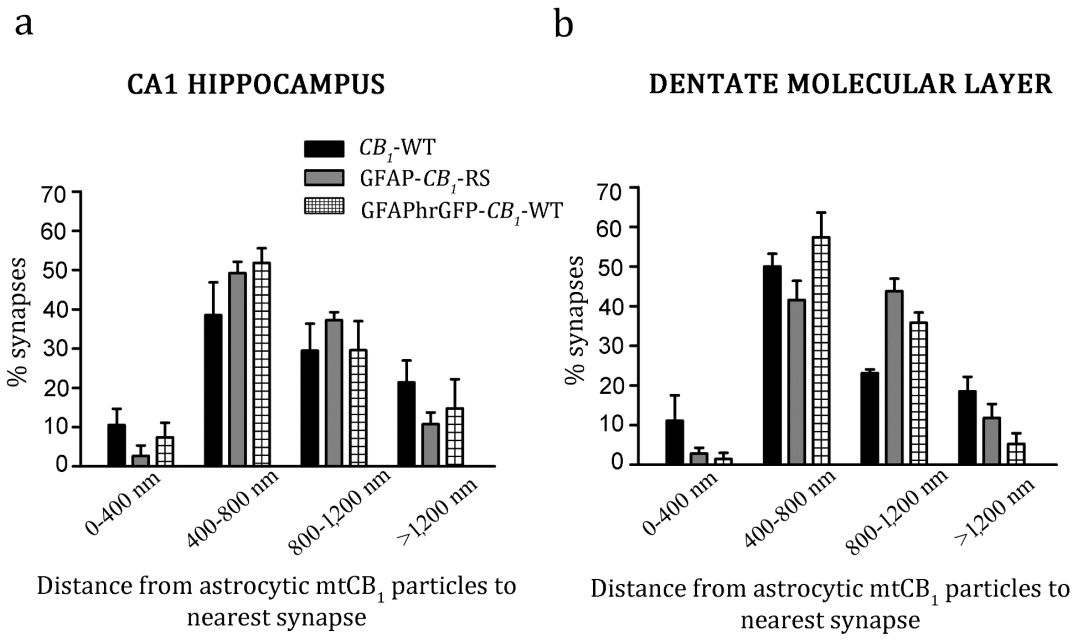


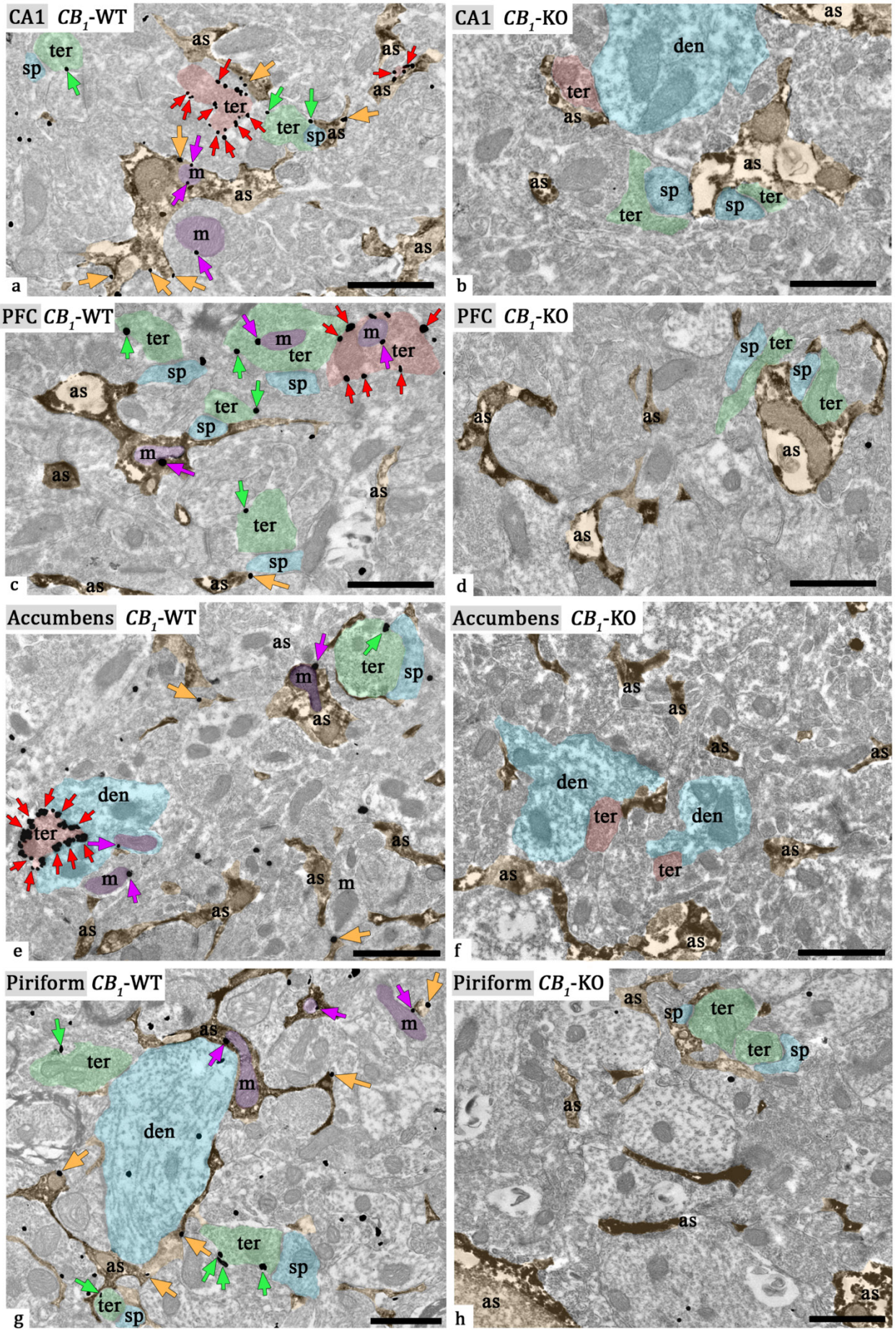
Figure 17. Distance from the mitochondrial CB$_1$ receptor particles in astrocytes to the synapses in the hippocampus. The distance between the CB$_1$ receptor particles on mitocondrial membranes in astrocytic processes and the midpoint of the nearest synapse surrounded by them was assessed in the CA1 (a) and DML (b) of *CB$_1$*-WT, GFAP-*CB$_1$*-RS and GFAPhrGFP-*CB$_1$*-WT (see Table 2 for values).

Table 2. Proportion of synapses visualized in 400 nm bit ranges from the CB_1 receptor labeling in astroglial mitochondria.

CA1	*CB_1*-WT (1,790 µM²)	GFAP-*CB_1*-RS (2,100 µM²)	GFAPhrGFP-*CB_1*-WT (784 µM²)
< 400 nm	10.55 ± 4.01%	2.67 ± 2.67%	7.41 ± 3.70%
400-800 nm	38.54 ± 8.32%	49.28 ± 2.87%	51.85 ± 3.70%
800-1,200 nm	29.51 ± 6.85%	37.26 ± 2.02%	29.63 ± 7.41%
> 1,200 nm	21.40 ± 5.56%	10.79 ± 2.94%	14.81 ± 7.41%
DML	***CB_1*-WT (784 µm²)**	**GFAP-*CB_1*-RS (1,708 µm²)**	**GFAPhrGFP-*CB_1*-WT (1,512 µm²)**
< 400 nm	11.11 ± 6.42%	2.82 ± 1.48%	1.52 ± 1.52%
400-800 nm	50.00 ± 3.21%	41.57 ± 4.81%	57.37 ± 6.26%
800-1,200 nm	23.15 ± 0.93%	43.79 ± 3.13%	35.86 ± 2.53%
> 1,200 nm	18.52 ± 3.70%	11.82 ± 3.51%	5.25 ± 2.72%

5.1.4. Density of CB_1 receptors in mitochondria of astrocytes and neurons

Immunogold electron microscopy revealed that, in addition to the presence of mitochondrial-associated CB_1 receptors in neurons (Benard et al., 2012; Hebert-Chatelain et al., 2014, 2016; Koch et al., 2015), CB_1 protein staining can also be detected in close juxtaposition to astroglial mitochondrial membranes from the hippocampus (Gutiérrez-Rodríguez et al., 2018), prefrontal cortex, piriform cortex and nucleus accumbens (Fig. 18a, c, e and g) (Jimenez-Blasco et al., (submitted to Nature). Negative control tissues from global *CB_1*-KO mice displayed just background staining in both neurons and astrocytes (Fig. 18b, d, f and h) (Gutiérrez-Rodríguez et al., 2018). Interestingly, despite the generally lower absolute levels of CB_1 receptors in astrocytes than in neurons (Metna-Laurent et al., 2015), the proportion of $mtCB_1$ over the total CB_1 receptors density in each cell type was slightly larger in astroglial mitochondria of hippocampus and prefrontal cortex than in neuronal mitochondria (Fig. 19a, b, c and d).


CA1 CB1-WT
ter
sp
as
m
a
CA1 CB1-KO
den
b
PFC CB1-WT
c
PFC CB1-KO
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Accumbens CB1-WT
e
Accumbens CB1-KO
f
Piriform CB1-WT
g
Piriform CB1-KO
h

Figure 18. CB_1 receptor localization in identified astrocytes, neurons and mitochondria in the CA1 stratum radiatum, prefrontal cortex, nucleus accumbens and piriform cortex of wild-type and *CB_1*-KO mice. Pre-embedding immunogold and immunoperoxidase method for electron microscopy. Representative electron immunogold images of the detection of CB_1 receptors on mitochondrial membranes of astrocytes (identified with anti-GLAST immunoperoxidase staining) and neurons in the hippocampus (a, b), prefrontal cortex (c, d), nucleus accumbens (e, f) and piriform cortex (g, h) of WT and *CB_1*-KO mice, respectively. CB_1 receptor labeling (arrows), presynaptic GABAergic terminal (ter, red), presynaptic glutamatergic terminal (ter, green), dendrite (den, blue), spine (sp, blue), mitochondria (m, purple) and astrocyte branch (as, orange) Scale bars: 1 μm.

Note: In this case (Fig. 18), the rabbit polyclonal anti-GLAST antibody was used to detect the astrocytic processes. As cited in the literature, neurotransmitter uptake is one of the important functions of astrocytes (Haydon, 2001). In the vast amount of brain excitatory synapses, astrocytes uptake excess amount of glutamate from synaptic clefts through glutamate transporters such as GLT-1 and GLAST (Chaudhry et al., 1995; Rothstein et al., 1996; Tanaka et al., 1997). Since GFAP immunoreactivity is not uniformly detected in the whole brain (Tatsumi et al., 2018), w*e used the GLAST antibody as a good and reliable astrocytic marker.*

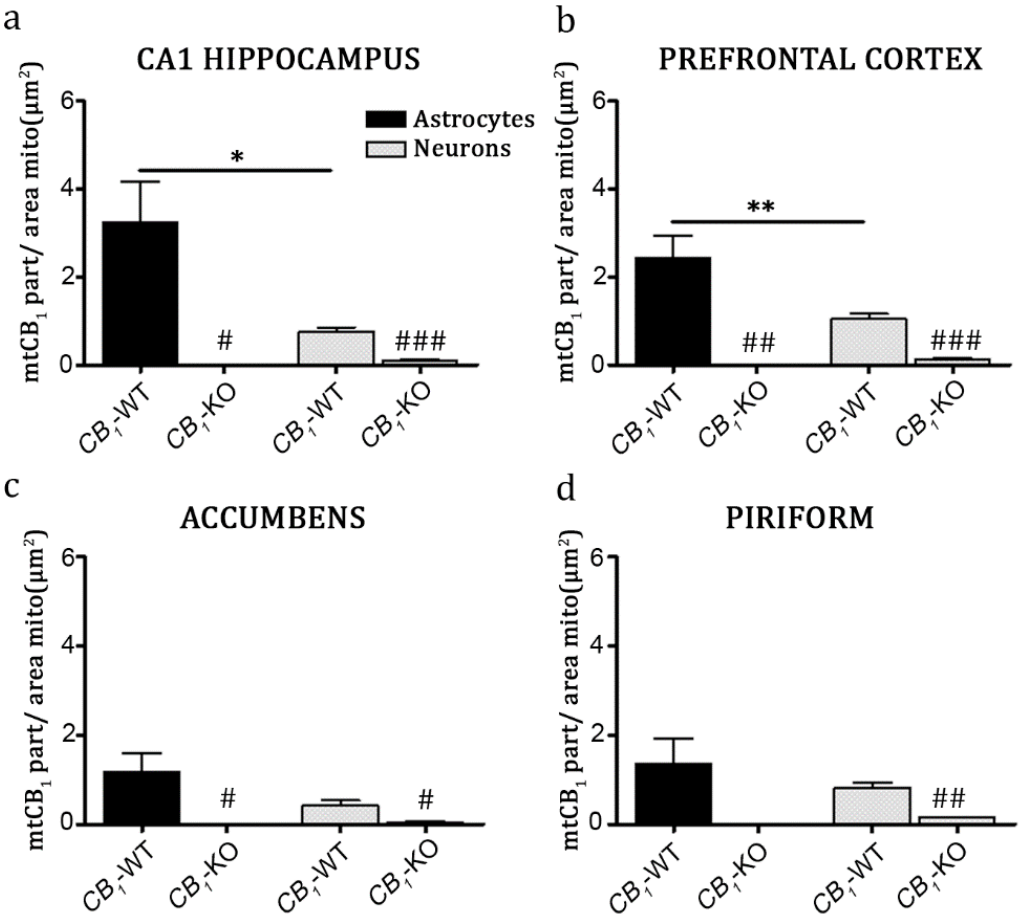

Figure 19. CB_1 density in astrocytic and neuronal mitochondria. The CB_1 density in astrocytic mitochondria of CA1 (3.24 ± 0.94 part/µm²), prefrontal cortex (2.45 ± 0.49 part/µm²), nucleus accumbens (1.16 ± 0.44 part/µm²) and piriform cortex (1.35 ± 0.57 part/µm²), is statistically higher than in neuronal mitochondria of CA1 (0.76 ± 0.09 part/µm²), prefrontal cortex (1.04 ± 0.13 part/µm²), nucleus accumbens (0.42 ± 0.12 part/µm²) and piriform cortex (0.82 ± 0.12 part/µm²). Only residual particles were found in neuronal mitochondria in CA1 (0.09 ± 0.04 part/µm²), prefrontal cortex (0.13 ± 0.02 part/µm²), nucleus accumbens (0.03 ± 0.03 part/µm²) and piriform cortex (0.14 ± 0.02 part/µm²) of *CB_1*-KO. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test, *p<0.05; **p < 0.01 (WT astros vs. WT neurons); #p < 0.05; ##p < 0.01; ###p < 0.001 (WT vs. *CB_1*-KO).

5.2. ADOLESCENT ETHANOL INTAKE

5.2.1. Cellular and subcellular localization of the CB_1 receptor in adult CA1 hippocampus after adolescent EtOH intake

The DAB immunostaining was used to identify GFAP-containing astrocytes and their processes, which then allowed individual immunogold-labeled CB_1 receptors on astrocytes to be counted. To determine whether EtOH intake during adolescence caused a global change in CB_1 receptor expression in the adult CA1 stratum radiatum, the proportion of CB_1 receptor immunoparticles in different cellular compartments was examined. Metal particles were localized on inhibitory and excitatory axon terminals which formed synapses with dendrites and dendritic spines, respectively (Fig. 20). As expected, the highest proportion of the total CB_1 receptor particles counted in 110 μm^2 (control: 74.59 ± 13.72 particles; EtOH: 68.67 ± 6.72 particles) ($p > 0.05$) was found on inhibitory axon terminal membranes making symmetric synapses with dendrites. Overall, there was no significant difference between the CB_1 receptor expression on inhibitory terminals in control (Figs 20a, a´; Fig. 21a: 52.66 ± 3.59% particles) and EtOH (Figs. 20b, b´; Fig. 21a: 53.80 ± 2.89% particles) ($p > 0.05$). Furthermore, 82.69 ± 4.28% of the inhibitory terminals in control and 76.44 ± 4.49% in EtOH-treated mice were CB_1 receptor immunopositive ($p > 0.05$; Fig. 21b). The concentration of immunogold particles was low in the asymmetric (excitatory) synapses in control (11.02 ± 0.75% particles) and even lower in EtOH (8.61 ± 0.46% particles) (*$p < 0.05$; Fig. 21a). In this case, 19.48 ± 2.23% of the excitatory boutons in control and 15.94 ± 1.67% in EtOH animals were CB_1 receptor positive ($p > 0.05$; Fig. 21b). In astrocytes, scattered metal particles were observed on thin and thick processes of GFAP-immunoreactive astrocytes in the CA1 stratum radiatum of control mice (Figs. 20a, a´). Immunoparticles were less frequently observed in astrocytic processes following EtOH exposure (Figs. 20b, b´, c). The proportion of the total CB_1 receptor particles found on astrocyte membranes in control (5.72 ± 0.96% particles) and EtOH treated mice (2.73 ± 0.44% particles) was very low relative to the terminals, mitochondria (control: 13.92 ± 1.57% particles; EtOH: 13.95 ± 1.63% particles) and other membrane compartments (control: 9.95 ± 1.55% particles; EtOH: 12.35 ± 1.70% particles) (Fig. 21a). Remarkably, the decrease in CB_1 receptor labeling in astrocytes was statistically significant after EtOH exposure (*$p < 0.05$; Fig. 21a). Furthermore, the differences between the CB_1 receptor immunopositive astrocytic processes in control (37.22 ± 3.12%) and after EtOH exposure (21.49 ± 2.28%), as well as the density of receptor labeling (particle/μm of astrocytic membrane) in astrocytes of control and EtOH-exposed mice

(control: 0.35 ± 0.02; EtOH: 0.24 ± 0.02), were significantly different (***p < 0.001, **p < 0.01, respectively; Figs. 21c, d). CB_1 receptor density in the other CB_1 receptor-containing profiles remained statistically unchanged after EtOH (p > 0.05; Fig. 21c). Importantly, the CB_1 receptor immunolabeling pattern disappeared in the CA1 stratum radiatum of *CB_1*-KO mice (Fig. 20d) hence demonstrating the specificity of the anti-CB_1 receptor antibody used.

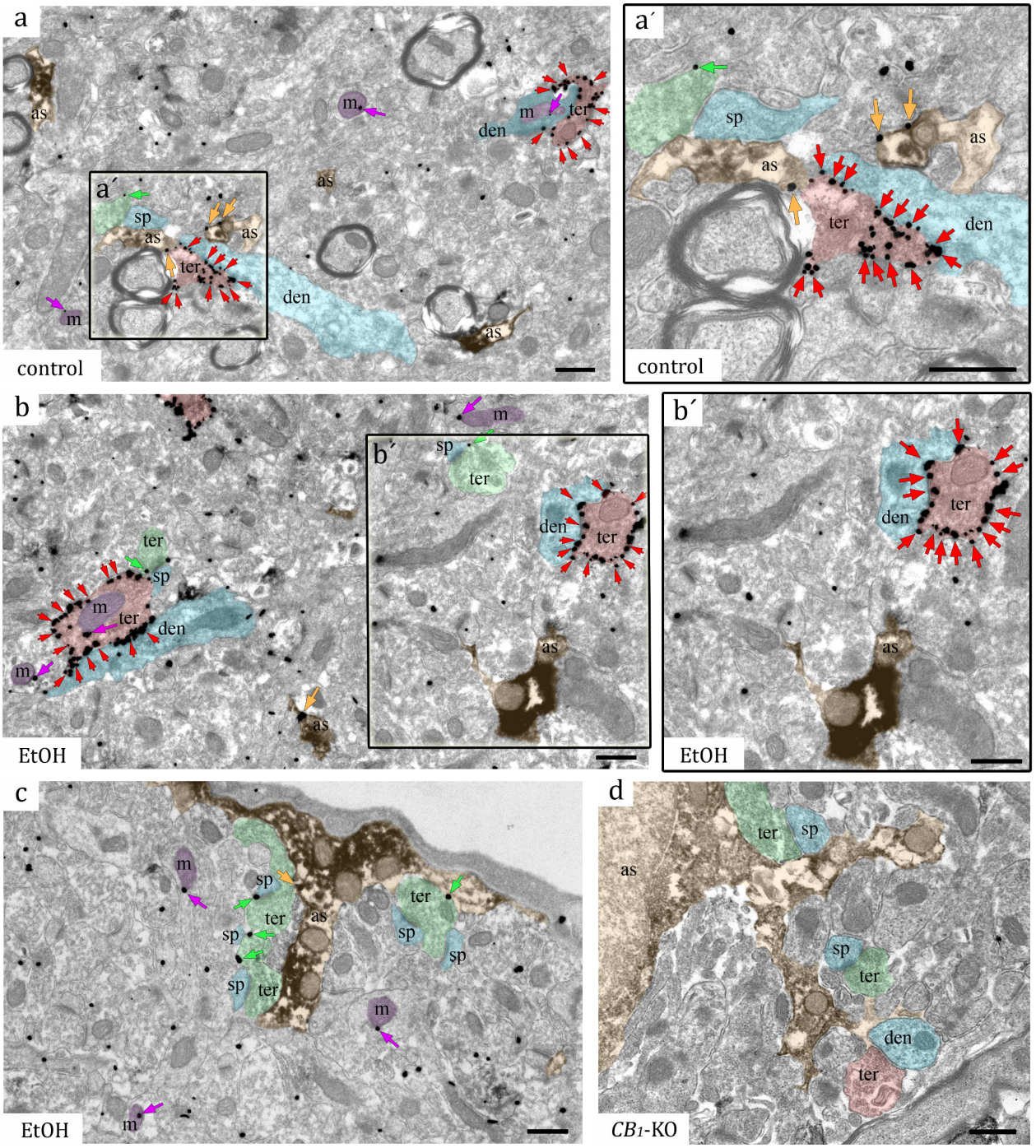

Figure 20. Subcellular CB_1 receptor localization in the adult CA1 stratum radiatum of control and EtOH-treated mice using a combined pre-embedding immunogold/immunoperoxidase labeling methods. (a) In control, CB_1 receptor immunogold labeling is observed on both excitatory (ter, green arrows) and inhibitory

terminals (ter, red arrows), and on astrocytic membranes (as, orange arrows). Note the presence of CB_1 receptor labeling on mitochondria (m, purple arrows). (a´) Higher magnification view showing CB_1 receptor labeling on inhibitory terminals (ter; red arrows) as well as on astrocytic membranes (as, orange arrows) in control. In EtOH mice (b, c), CB_1 receptor particles are also observed on excitatory terminals (ter, green arrows), inhibitory terminals (ter, red arrows), mitochondrial membranes (m, purple arrows) and astrocytes (as, orange arrows). (b´) Enlargement of the enclosed area in b. (c) An astrocytic end-foot (as) around a capillary with CB_1 receptor labeling (orange arrow). Note gold particles on excitatory terminals (ter, green arrows) and mitochondria (m, purple arrows). (d) No CB_1 receptor immunolabeling is detected on terminals (ter), astrocytes (as) and mitochondria in *CB_1*-KO mice, indicating the specificity of the CB_1 receptor antibody used. Scale bars: 1 μm.

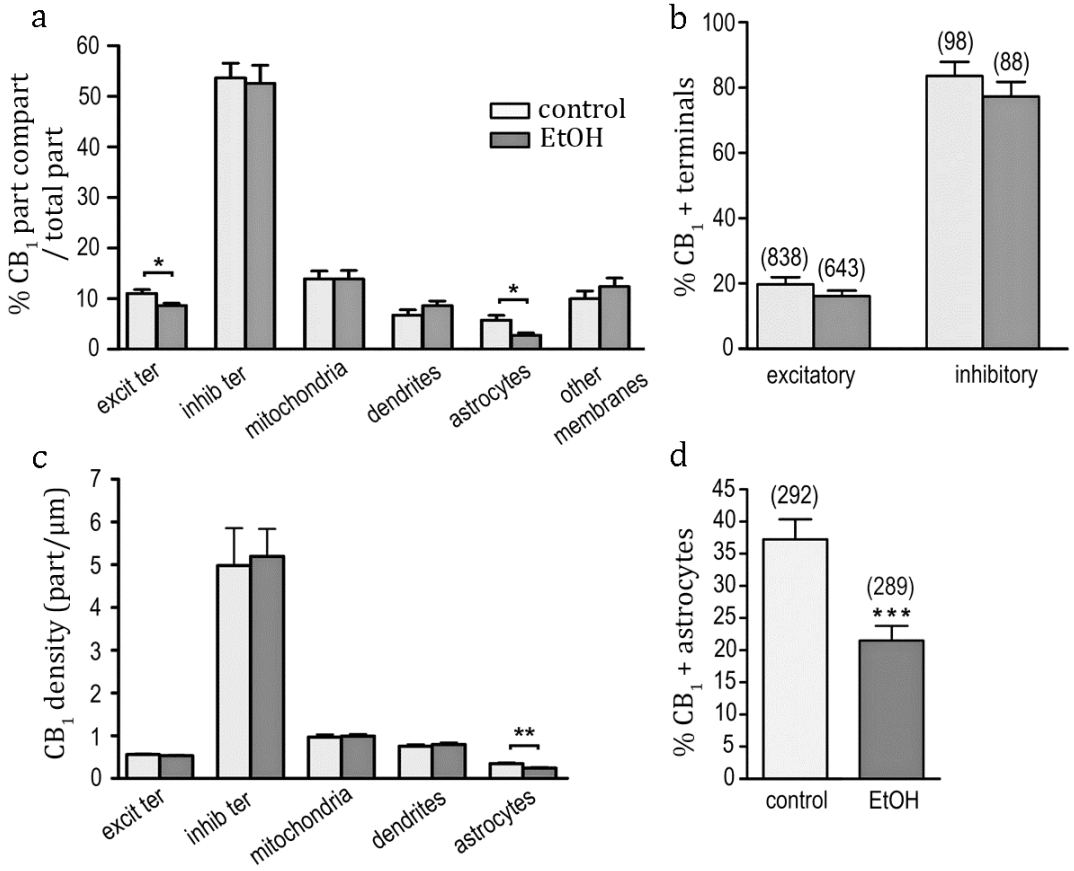

Figure 21. CB_1 receptor distribution in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Proportion of CB_1 receptor labeling in different compartments normalized to the total CB_1 content in control and EtOH mice (analyzed area: ~2,000 μm²). (b) Percentage of CB_1 receptor-immunopositive excitatory and inhibitory synaptic terminals in control and EtOH mice. The number of synaptic terminals studied is in parentheses on the top of each column. (c) CB_1 receptor density (particles/μm) in CB_1 receptor positive profiles in control versus EtOH treated mice. (d) Percentage of labeled astrocytic processes in control and EtOH mice. The number of astrocytic portions studied is in parentheses on the top of the columns. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

5.2.2. Ultrastructural changes in CA1 astrocytes after EtOH exposure during adolescence

First, we assessed the astrocyte morphology. There were fewer processes in astrocytes of EtOH exposed animals (control: 5.80 ± 0.45; EtOH: 3.93 ± 0.23 astrocytic processes/electron micrograph; ***p < 0.001; Fig. 22a), however, their area was significantly larger in EtOH (1.06 ± 0.15 μm²) than in control (0.90 ± 0.15 μm²; **p < 0.01; Fig. 22b). No statistical differences were found in the astrocytic perimeter (control: 4.63 ± 0.26 μm; EtOH: 4.78 ± 0.25 μm; p > 0.05; Fig. 22c). Taken together, these data indicate that the astrocytes in the adult hippocampus have a swollen morphology after EtOH exposure during adolescence.

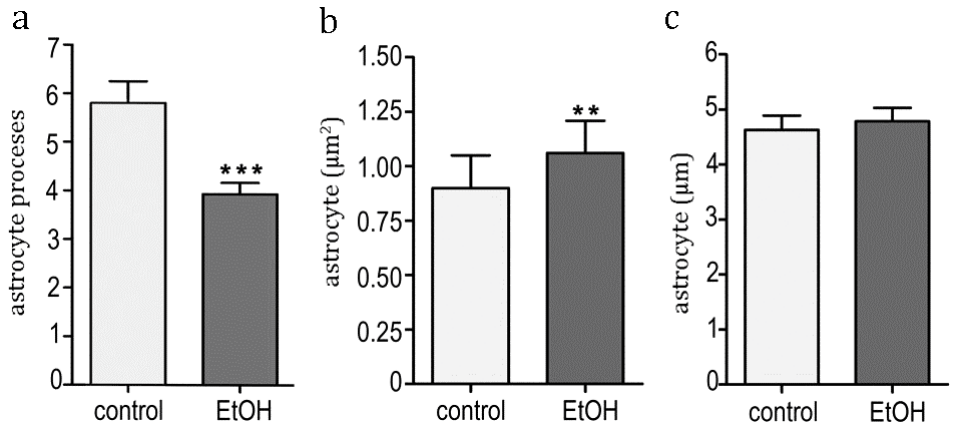

Figure 22. Ultrastructural assessment of the astrocyte morphology in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Number of astrocytes per 110 μm² in control and EtOH mice. (b) Area (in μm²) of the astrocytes in control (n = 292) and EtOH (n = 289) mice. (c) Perimeter (μm) of the astrocytes in control (n = 292) and EtOH (n = 289) mice. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test **p < 0.01; ***p < 0.001).

5.2.3. Distance from the astroglial CB_1 receptors to the synapses in adult CA1 stratum radiatum of control and after EtOH exposure during adolescence

To determine how CB_1 receptor distribution was affected in the swollen astrocytes surrounding the synapses following adolescent EtOH exposure, the distance between the astroglial CB_1 receptors and the midpoint of the nearest synapse was measured in adult CA1 stratum radiatum. In control, while only 12.21 ± 1.41% of the synapses from the total analyzed (n = 123) were localized at a distance 0–400 nm from the closest astrocytic CB_1 receptor particles (Fig. 23a), the majority of them (46.34 ± 0.69%) were at 400–800 nm.

The second most frequent distance occurred between 800 and 1,200 nm (25.27 ± 1.46% of the synapses), while numbers were again reduced (16.18 ± 2.66% of the synapses) at distances more than 1,200 nm from the nearest astrocytic CB_1 receptor labeling. In EtOH-treated mice, 13.57 ± 5.22% of the total synapses (n = 73) was within 400 nm from the astroglial CB_1 receptor particles. The distribution was fairly equivalent between 400–800 nm (33.04 ± 4.61%), and 800–1,200 nm (33.16 ± 6.92%), while 20.23 ± 6.73% of the synapses were localized beyond 1,200 nm (Fig. 23a). No statistical differences were observed between control and EtOH-treated mice in the astrocytic CB_1 receptor distribution with respect to neighboring synapses ($p > 0.05$; Fig. 23a) nor in the proportion of asymmetric/excitatory versus symmetric/inhibitory synapses relative to the CB_1 receptor positive astrocytes (control: excitatory 6.38 ± 0.72%, inhibitory 8.66 ± 1.00%. EtOH: excitatory 4.44 ± 0.61%, inhibitory: 4.50 ± 0.74%) ($p > 0.05$; Fig. 23b). However, the proportion of inhibitory synapses closely related to the astrocytic CB_1 receptor labeling was significantly lower in EtOH- exposed mice than in control (*$p < 0.05$; Fig. 23b) with no differences detected at the excitatory synapses ($p > 0.05$; Fig. 23b).

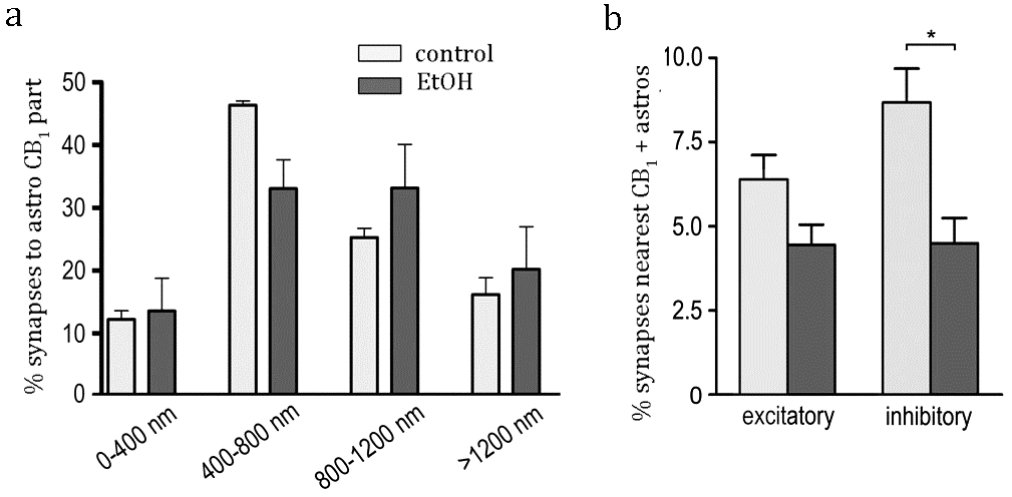

Figure 23. Distance from the astrocytic CB_1 receptors to the nearest synapse in the adult CA1 stratum radiatum of control and EtOH-treated mice. (a) Proportion of synapses in 400 nm bin distances from the astrocytic CB_1 receptor particles to the midpoint of the nearest synapse in control (n = 123 synapses) and EtOH (n = 73 synapses) mice. (b) Percentage of synapses (asymmetric/excitatory versus symmetric/inhibitory synapses) surrounding CB_1 receptor positive astrocytes in control (n = 936 synapses) and EtOH (n = 731 synapses) mice. Data are expressed as mean ± SEM. Data were analyzed by means of non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test; *$p < 0.05$).

5.3. R-mTBI MODEL

5.3.1. Cellular and subcellular localization of the CB_1 receptor in the molecular layer of the rat dentate gyrus

CB_1 receptor immunogold particles in the DML of uninjured and injured rats were mainly localized on inhibitory and excitatory axon terminals forming synapses with dendrites and dendritic spines, respectively, and to a lesser extent, on astrocytic processes and mitochondria (Figure 24, 25 and 27).

To determine whether juvenile concussion (8 hits) caused a global change in CB_1 receptor expression immediately or after the cessation of the repeated ACHI procedures in the mature hippocampus, the proportion of CB_1 positive profiles and CB_1 density was examined in both sexes at post injury day (PID) 1, 10 and 40. Although the CB_1 receptor immunolabeling was not severely impacted on PID 1, the CB_1 receptor expression was significantly reduced in both sexes at PID 10, and even more at PID 40. Thus at PID 10, injured male rats showed a significant reduction in CB_1 positive excitatory terminals (sham: 34.05 ± 3.45%, 8 hits: 21.01 ± 3.74%), astrocytes (sham: 18.01 ± 2.21%, 8 hits: 11.98 ± 1.51%) and mitochondria (sham: 12.05 ± 1.22%, 8 hits: 8.69 ± 0.96%) (**$p < 0.01$; Fig. 26b). Moreover, a remarkable decrease in CB_1 density in inhibitory terminals was observed (sham: 7.69 ± 0.46 part/µm, 8 hits: 4.53 ± 0.56 part/µm) (***$p < 0.001$; Fig. 27b). Interestingly, only the latter change could be detected in injured female rats at PID 10 (CB_1 density in inhibitory terminals in sham: 7.10 ± 0.48 part/µm, 8 hits: 4.56 ± 0.33 part/µm) (**$p < 0.01$; Fig. 27e).

There were significant changes in both sexes at PID 40. In males, the proportion of CB_1 receptor-labeled excitatory terminals (sham: 33.14 ± 3.38%, 8 hits: 24.69 ± 3.36%) and inhibitory terminals (sham: 85.55 ± 1.40%, 8 hits: 76.81 ± 3.04%) decreased significantly, but increased the CB_1 receptor-positive astrocytes (sham: 16.58 ± 1.29%, 8 hits: 22.48 ± 3.39%) (*$p < 0.05$, *$p < 0.05$ and **$p < 0.01$; Fig. 26c). Likewise, significant reductions in CB_1 receptor immunopositive excitatory terminals (sham: 32.86 ± 3.30%, 8 hits: 16.09 ± 1.75%), inhibitory terminals (sham: 84.94 ± 3.46%, 8 hits: 76.06 ± 1.91%) and mitochondria (sham: 13.68 ± 0.68%, 8 hits: 9.41 ± 1.04%) (***$p < 0.001$, *$p < 0.05$ and **$p < 0.01$; Fig. 26f) were seen in females. Finally, the CB_1 immunoparticle density dropped significantly in inhibitory terminals of both males (sham: 7.50 ± 0.73 part/µm, 8 hits: 4.85 ± 0.67 part/µm) (*$p < 0.05$; Fig. 27c) and females (sham: 6.69 ± 0.41 part/µm, 8 hits: 4.84 ± 0.76 part/µm) (**$p < 0.01$; Fig. 27f).

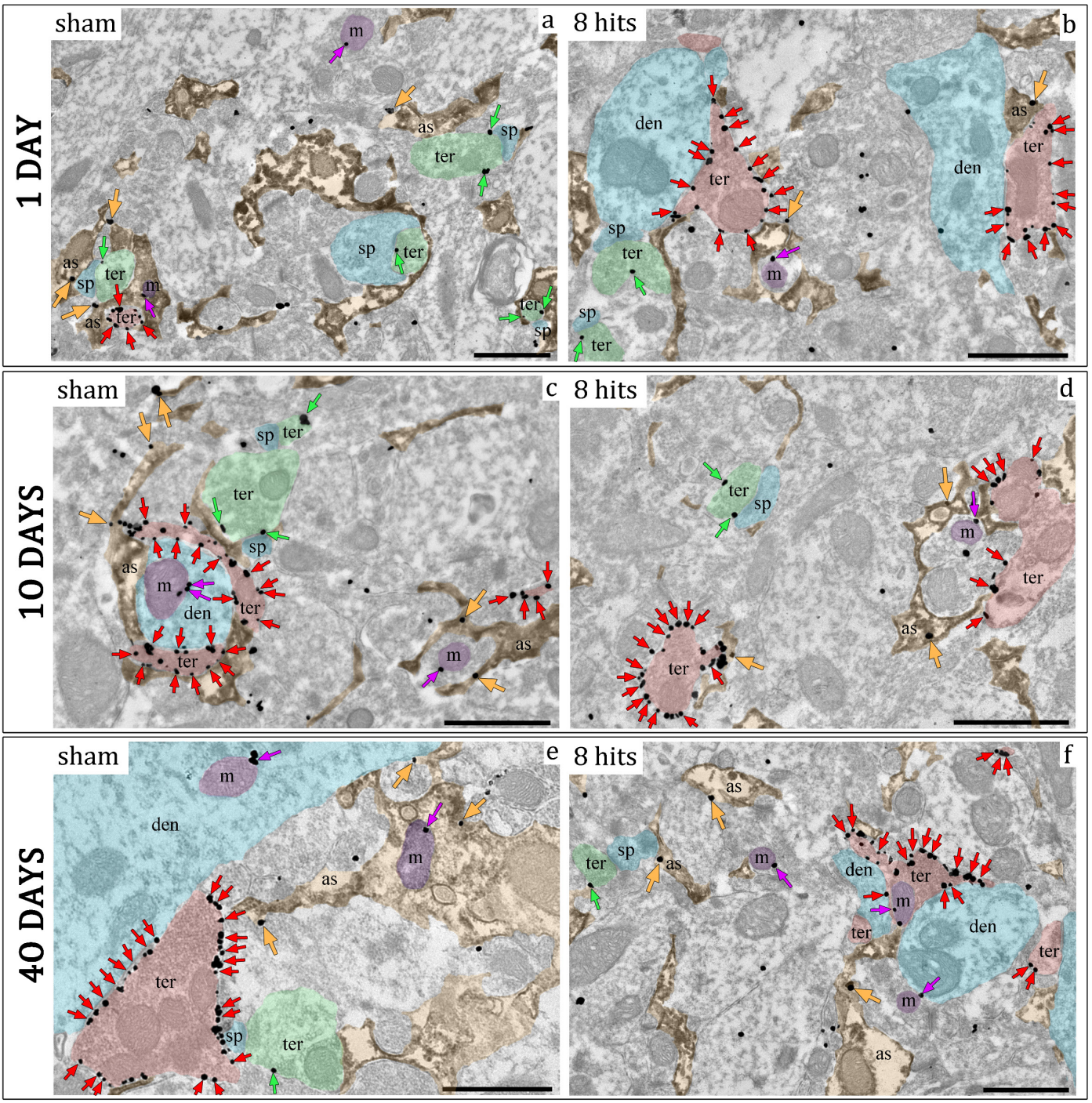

Figure 24. Subcellular localization of CB_1 receptors in DML of injured (8 hits) and uninjured male rats. Combined pre-embedding immunogold/immunoperoxidase method. (a, c and e) In sham at post-injury day 1, 10 and 40 respectively, CB_1 receptor immunolabeling is observed on excitatory terminals (ter, green arrows), inhibitory terminals (ter, red arrows), astrocytic membranes (as, orange arrows) as well as neuronal and astrocytic mitochondria (m, purple arrows). (b) In mTBI at post-injury day 1, CB_1 receptor labeling is also observed on excitatory (ter, green arrows) and inhibitory terminals (ter, red arrows), astrocytic membranes (as, orange arrows) and mitochondria (m, purple arrows). (d) At post-injury day 10, CB_1 receptors are localized to astrocytes (as, orange arrows) and mitochondrial membranes (m, purple arrows) but CB_1 receptor labeling decreases in excitatory (ter, green arrows) and inhibitory terminals. (f) Similarly, at post-injury day 40, CB_1 gold particles are

observed on astrocytes (as, orange arrows) and on mitochondrial membranes (m, purple arrows) but the labeling of excitatory terminals appears to be lower (ter, green arrows) and even more in inhibitory terminals. Scale bars: 1 µm.

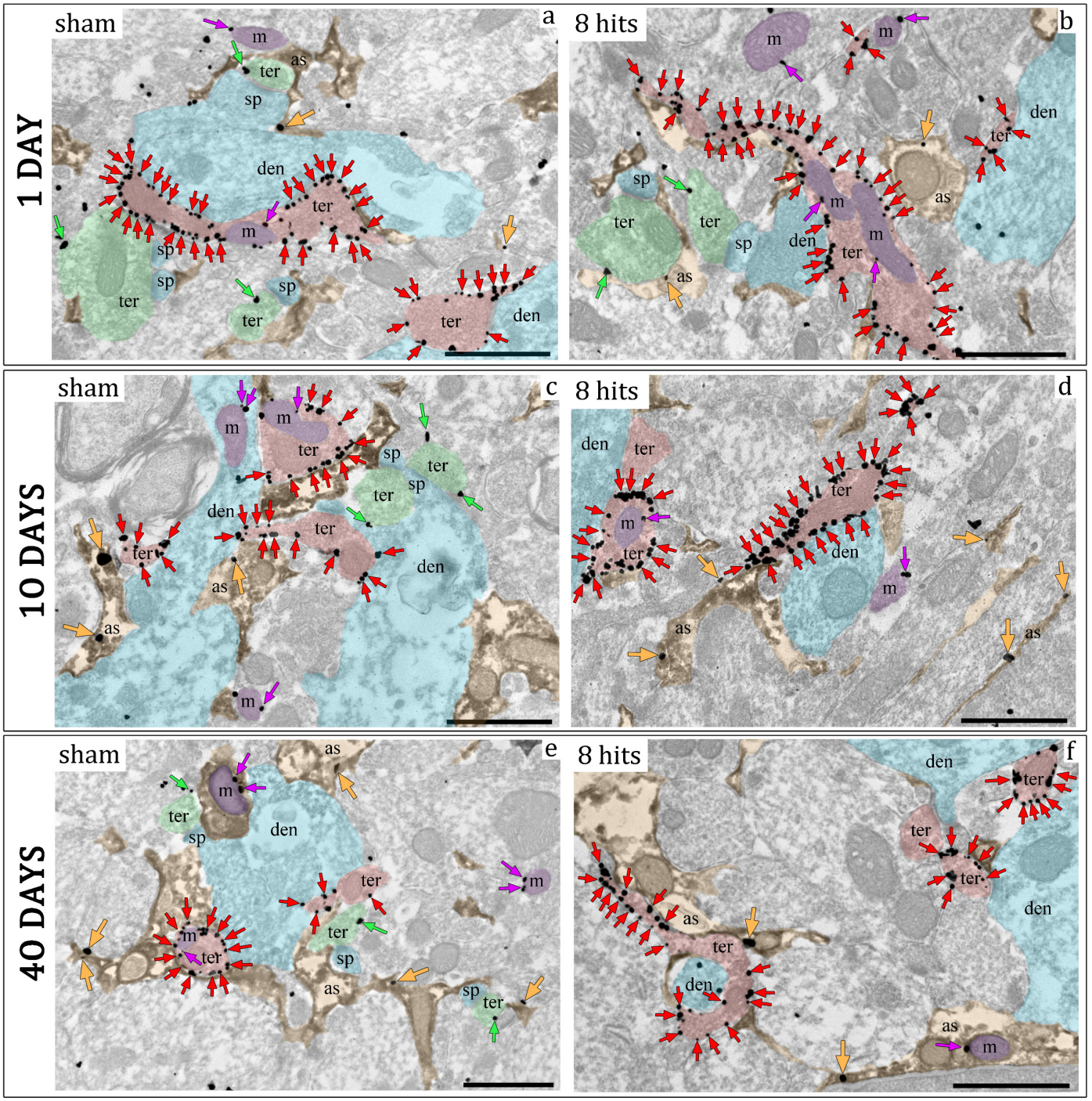

Figure 25. Subcellular localization of CB_1 receptors in DML of injured (8 hits) and uninjured female rats. Combined pre-embedding immunogold/immunoperoxidase labeling method. (a, c and e) CB_1 receptor immunolabeling in sham rats is observed in excitatory (ter, green arrows) and inhibitory terminals (ter, red arrows), astrocytic membranes (as, orange arrows) as well as neuronal and astrocytic mitochondria (m, purple arrows). (b) 1 day after TBI injury, CB_1 receptor immunolabeling is also observed in excitatory (ter, green arrows) and inhibitory terminals (ter, red arrows), astrocytic membranes (as, orange arrows), and mitochondria (m, purple arrows). (d) 10 days after TBI injury, CB_1 receptors are in

astrocytes (as, orange arrows) and mitochondrial membranes (m, purple arrows). Gold particle labeling is reduced in excitatory (ter, green arrows) and inhibitory terminals. (f) Similarly, 40 days post-TBI injury, CB_1 receptors are localized in astrocytes (as, orange arrows) and mitochondrial membranes (m, purple arrows) and appear to be reduced in excitatory terminals (ter, green arrows) and some inhibitory terminals. Scale bars: 1 µm.

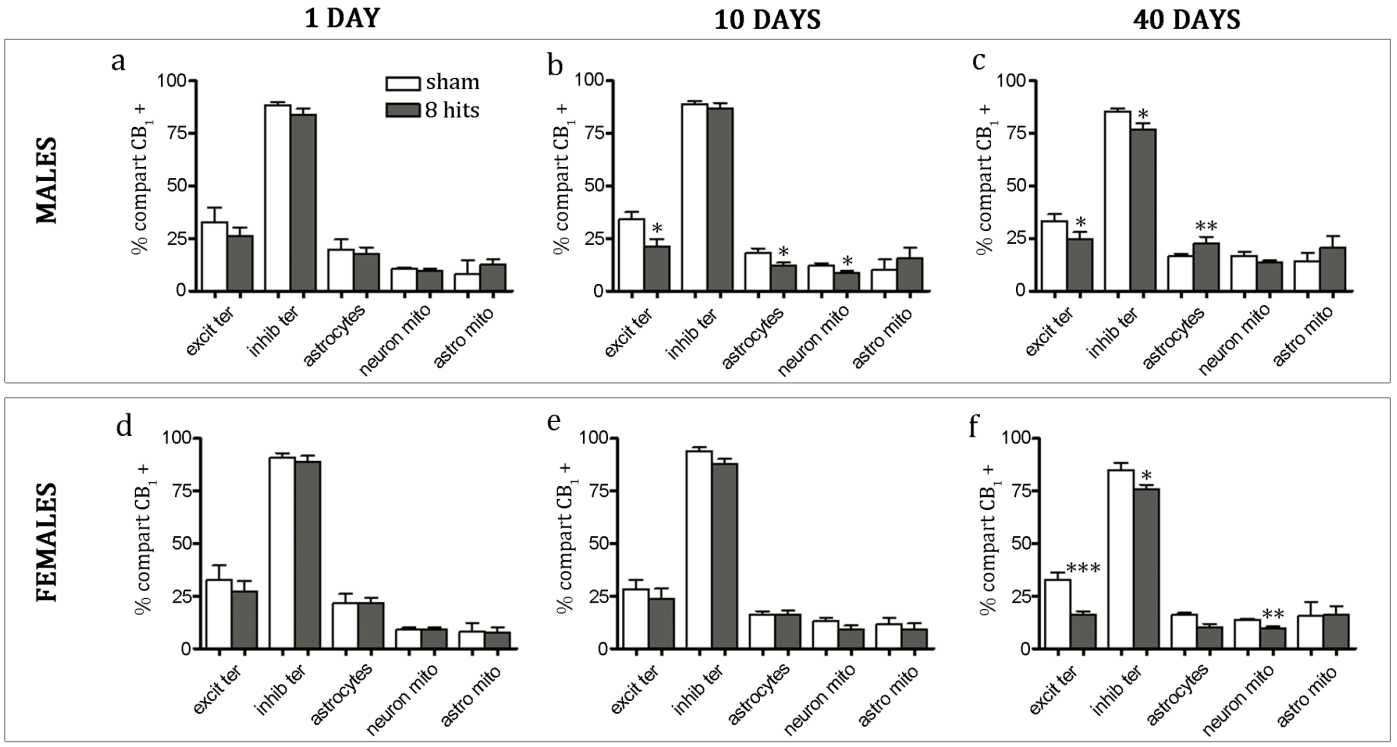

Figure 26. Percentage of CB_1 receptor immunopositive profiles in the DML of injured (8 hits) versus uninjured rats. Males and females at post-injury days 1 (a), 10 (b) and 40 (c). Data are expressed as mean ± SEM and were analyzed by non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test *p < 0.05; **p < 0.01; ***p < 0.001), (see Table 3 for values).

Table 3. CB_1 receptor immunolabeled profiles in injured (8 hits) and uninjured rats.

	MALES	EXCITATORY TERMINALS	INHIBITORY TERMINALS	ASTROCYTES	NEURONAL MITOCHONDRIA	ASTROCYTIC MITOCHONDRIA
1 DAY	sham	32.68 ± 6.97%	88.54 ± 1.45%	19.80 ± 4.83%	10.43 ± 0.89%	8.21 ± 6.45%
	8 hits	26.32 ± 4.01%	83.74 ± 3.17%	17.79 ± 2.97%	9.81 ± 0.82%	12.72 ± 2.59%
10 DAYS	sham	34.05 ± 3.45%	88.95 ± 1.47%	18.01 ± 2.21%	12.05 ± 1.22%	10.32 ± 4.96%
	8 hits	21.01 ± 3.74%	86.91 ± 2.52%	11.98 ± 1.51%	8.69 ± 0.96%	15.82 ± 4.81%
40 DAYS	sham	33.14 ± 3.38%	85.55 ± 1.40%	16.58 ± 1.29%	16.76 ± 1.84%	14.40 ± 3.62%
	8 hits	24.69 ± 3.36%	76.81 ± 3.04%	22.48 ± 3.39%	13.71 ± 0.72%	20.62 ± 5.53%

	FEMALES	EXCITATORY TERMINALS	INHIBITORY TERMINALS	ASTROCYTES	NEURONAL MITOCHONDRIA	ASTROCYTIC MITOCHONDRIA
1 DAY	sham	32.57 ± 6.95%	90.82 ± 2.22%	21.63 ± 4.45%	8.85 ± 1.09%	8.21 ± 4.09%
	8 hits	27.07 ± 5.03%	89.07 ± 2.63%	21.59 ± 2.64%	9.03 ± 1.15%	7.65 ± 2.67%
10 DAYS	sham	28.16 ± 4.67%	93.85 ± 1.98%	16.23 ± 1.55%	13.12 ± 1.47%	11.61 ± 2.91%
	8 hits	23.82 ± 4.67%	88.00 ± 2.33%	16.29 ± 1.83%	9.29 ± 1.57%	9.05 ± 2.82%
40 DAYS	sham	32.86 ± 3.30%	84.94 ± 3.46%	16.01 ± 1.09%	13.68 ± 0.68%	15.50 ± 6.77%
	8 hits	16.09 ± 1.75%	76.06 ± 1.91%	10.05 ± 1.70%	9.41 ± 1.04%	16.17 ± 3.99%

5.3.2. Density of CB_1 receptors in the DML after mTBI

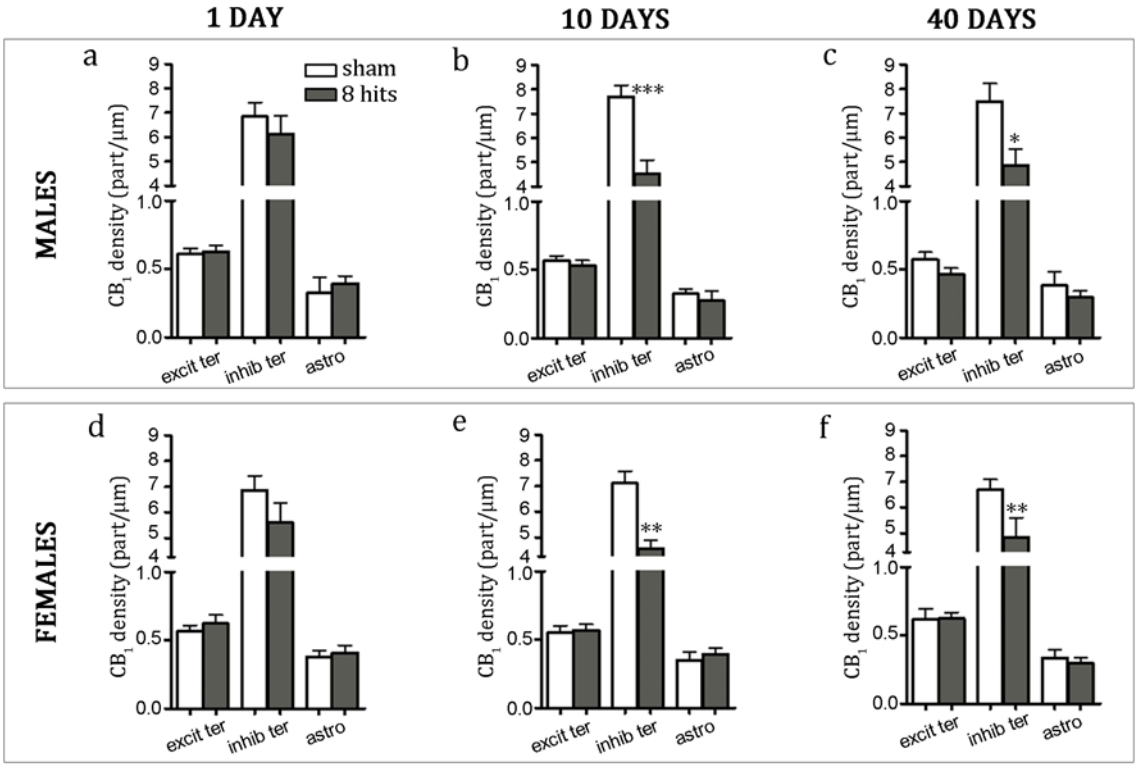

Figure 27. CB_1 receptor density (particles/µm) in the DML of injured (8 hits) and uninjured rats. Males and females at post-injury days 1(a, d) 10 (b, e) and 40 (c, f). Data are expressed as mean ± SEM and were analyzed by non-parametric or parametric tests

(Mann–Whitney U test or unpaired t-test *p < 0.05; **p < 0.01; ***p < 0.001), (see Table 4 for values).

Table 4. CB_1 receptor density (particles/µm) in the DML of injured (8 hits) versus uninjured rats.

	MALES	EXCITATORY TERMINALS	INHIBITORY TERMINALS	ASTROCYTES
1 DAY	sham	0.61 ± 0.04 part/µm	6.84 ± 0.60 part/µm	0.32 ± 0.12 part/µm
	8 hits	0.63 ± 0.05 part/µm	6.12 ± 0.75 part/µm	0.39 ± 0.06 part/µm
10 DAYS	sham	0.56 ± 0.04 part/µm	7.69 ± 0.46 part/µm	0.32 ± 0.03 part/µm
	8 hits	0.53 ± 0.03 part/µm	4.53 ± 0.56 part/µm	0.27 ± 0.07 part/µm
40 DAYS	sham	0.57 ± 0.05 part/µm	7.50 ± 0.73 part/µm	0.38 ± 0.09 part/µm
	8 hits	0.47 ± 0.04 part/µm	4.85 ± 0.67 part/µm	0.29 ± 0.05 part/µm

	FEMALES	EXCITATORY TERMINALS	INHIBITORY TERMINALS	ASTROCYTES
1 DAY	sham	0.57 ± 0.04 part/µm	6.86 ± 0.57 part/µm	0.38 ± 0.05 part/µm
	8 hits	0.62 ± 0.06 part/µm	5.58 ± 0.77 part/µm	0.41 ± 0.05 part/µm
10 DAYS	sham	0.55 ± 0.05 part/µm	7.10 ± 0.48 part/µm	0.35 ± 0.06 part/µm
	8 hits	0.57 ± 0.05 part/µm	4.56 ± 0.33 part/µm	0.39 ± 0.05 part/µm
40 DAYS	sham	0.61 ± 0.08 part/µm	6.69 ± 0.41 part/µm	0.33 ± 0.06 part/µm
	8 hits	0.62 ± 0.04 part/µm	4.84 ± 0.76 part/µm	0.30 ± 0.04 part/µm

Overall, these results show that r-mTBI male and female rats have a significant reduction in CB_1 receptor immunopositive excitatory and inhibitory terminals after 40 days as well as in CB_1 receptor density in inhibitory terminals after 10 days relative to shams. We next wanted to investigate the net effect of more severe mTBI conditions, i.e. 16 hits, on CB_1 receptor expression with no distinction between males and females in order to minimize the number of rats used, as no great differences in CB_1 receptor expression could be detected between both sexes after 8 head impacts.

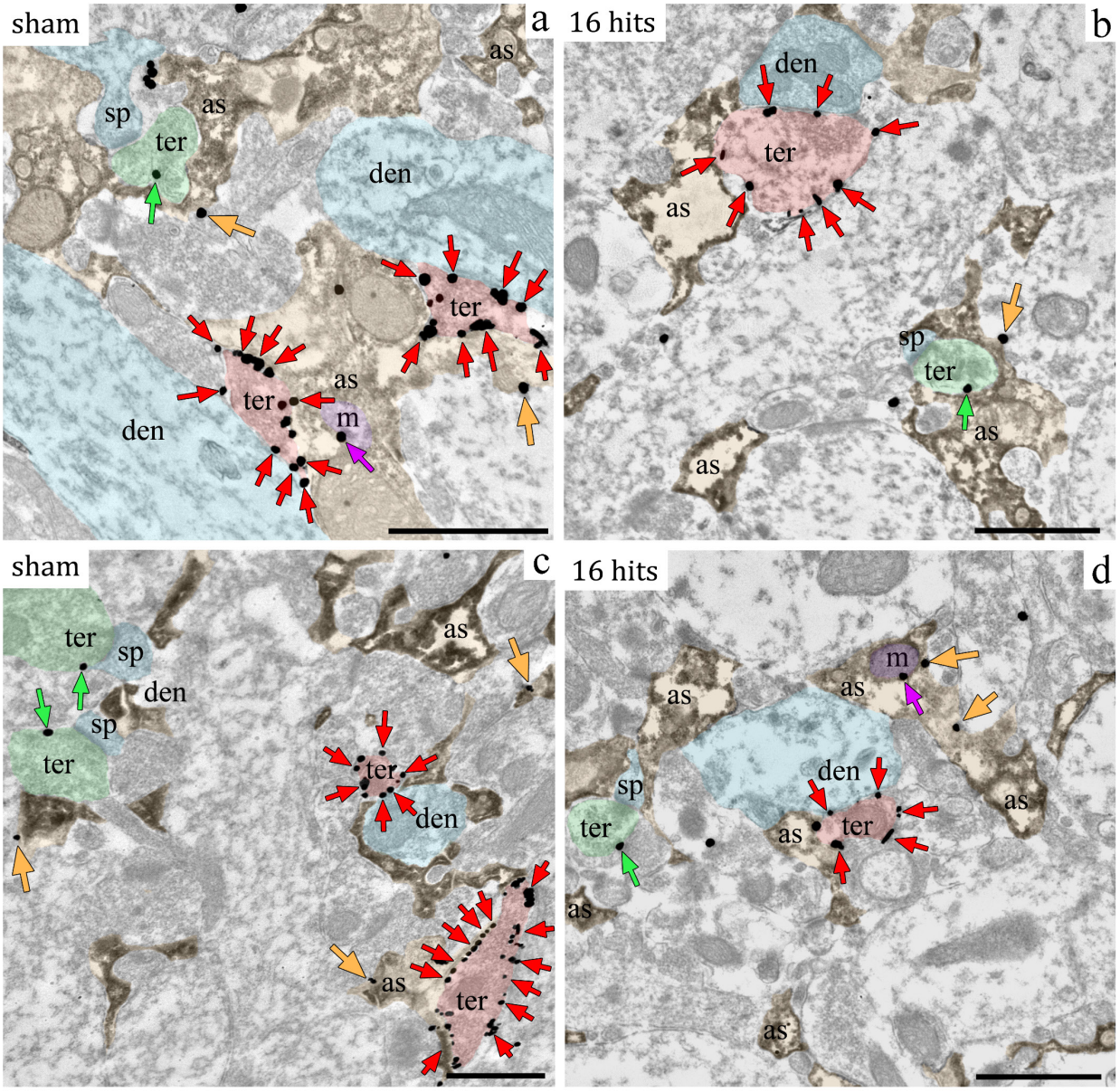

Figure 28. Subcellular CB_1 receptor localization in the DML 40 days after TBI (16 hits) and in sham rats. Combined pre-embedding immunogold/immunoperoxidase method. (a, c) As expected in shams, CB_1 receptor labeling is localized to both excitatory (ter, green arrows) and inhibitory terminals (ter, red arrows), astrocytes (as, orange arrows) and astrocytic mitochondria (m, purple arrows). (b, d) In TBI, CB_1 receptors are also in astrocytes (as, orange arrows) and astrocytic mitochondria (m, purple arrows), but seem to be less abundant in excitatory (ter, green arrows) and inhibitory terminals. Scale bars: 1 µm.

The proportion of CB_1 receptor immunolabeled excitatory terminals (sham: 39.80 ± 5.05%, 16 hits: 15.97 ± 3.93%) and inhibitory terminals (sham: 91.94 ± 3.40%, 16 hits: 64.56 ± 5.79%) (**p < 0.01; Fig. 29a) decreased significantly after 16 hits, as well as the density of CB_1 receptors in inhibitory terminals (sham: 6.58 ± 0.42 part/µm, 16 hits: 4.66 ± 0.32 part/µm) (**p < 0.01; Fig. 29b) (represented in Fig. 28).

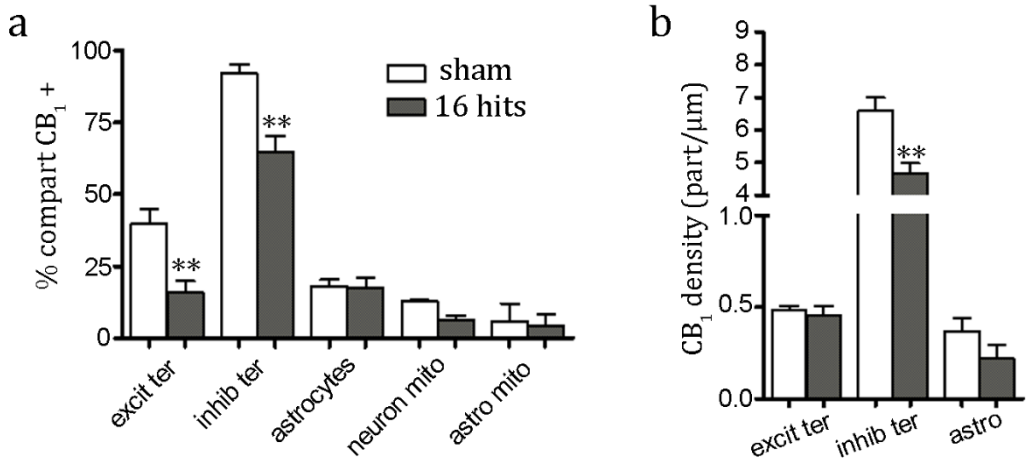

Figure 29. (a) **Percentage of CB_1 receptor positive profiles and** (b) **CB_1 receptor density (particles/µm) in the DML after 40 days of TBI (16 hits)** . Data are expressed as mean ± SEM and were analyzed by non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test **p < 0.01).

5.3.3. Ultrastructural changes in the morphology of astrocytes, neurons and mitochondria in the DML 40 days after TBI (16 hits)

The areas of different cellular compartments and the distance between the astrocytic CB_1 receptor immunoparticles and the midpoint of the nearest synapse surrounded by the immunopositive astrocytic element was measured in the DML to elucidate the possible morphological adaptations following a cumulative effect of 16 head impacts.

First, the size of the excitatory terminals, inhibitory terminals, astrocytic processes and mitochondria did not significantly change after TBI. Second, in sham rats, 15.60 ± 4.26% of the synapses (total analyzed = 70) were localized at 0–400 nm from the closest CB_1 receptor particle in the astrocyte, 42.09 ± 2.23% were at 400–800 nm, 23.72 ± 4.48% at 800-1,200 nm, and 18.59 ± 3.21% were at more than 1,200 nm (Fig. 30b). In injured rats, 21.01 ± 4.20% of the total synapses (n = 70) was within 400 nm from the nearest astroglial CB_1 receptor particle, 40.18 ± 6.50% were at 400–800 nm, 26.81 ± 6.81% at 800–1,200 nm, and 11.99 ± 1.65% were beyond 1,200 nm (Fig. 30b). Furthermore, the statistical analyses of these values revealed that there were not differences in the distribution of CB_1

receptors in astrocytes relative to the synapses of uninjured and TBI rats ($p > 0.05$; Fig. 30b).

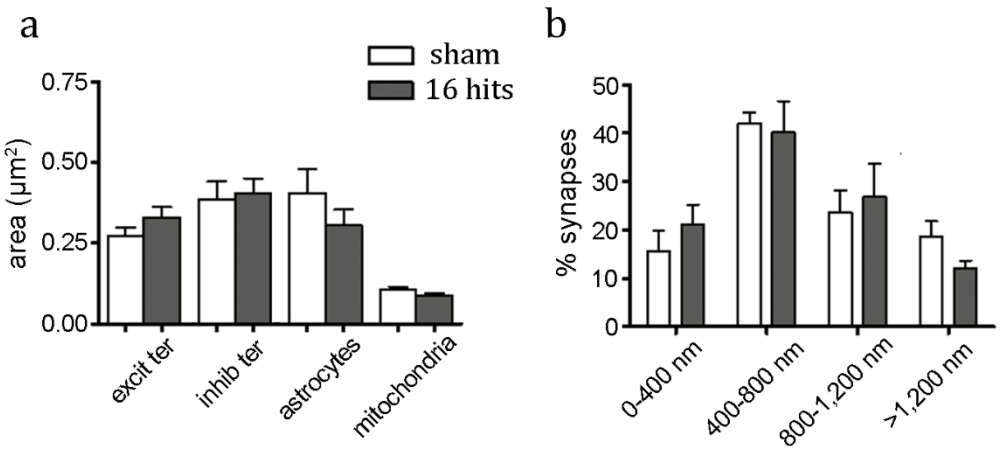

Figure 30. Assessment of the cellular and subcellular architecture in DML 40 days after TBI (16 hits) versus uninjured rats. (a) Area of excitatory terminals (sham: 0.27 ± 0.02 µm², 16 hits: 0.33 ± 0.03 µm²), inhibitory terminals (sham: 0.38 ± 0.05 µm², 16 hits: 0.40 ± 0.04 µm²), astrocytic processes (sham: 0.40 ± 0.07 µm², 16 hits: 0.30 ± 0.05 µm²) and mitochondria (sham: 0.10 ± 0.01 µm², 16 hits: 0.08 ± 0.01 µm²). (b) Proportion of synapses within 400 nm bits from the astrocytic CB_1 particles to the midpoint of the nearest synapse. Data are expressed as mean ± SEM. They were analyzed by non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test).

5.4. EFFECTS OF ACUTE THC EXPOSURE ON CB_1 RECEPTOR EXPRESSION AND HIPPOCAMPAL ULTRASTRUCTURE

5.4.1. Cellular and subcellular localization and density of the CB_1 receptor in CA1 hippocampus after acute THC administration

Hippocampal tissue from sham and THC-treated mice were used to investigate the cellular and subcellular localization of the CB_1 receptor in the CA1 stratum radiatum (Fig. 31). As expected in sham mice, presynaptic inhibitory terminal membranes forming symmetric synapses with postsynaptic dendrites were decorated with a high density of CB_1 receptor immunoparticles, whereas a much lower labeling was observed in excitatory terminals making asymmetric synapses with dendritic spines (Fig. 31a, b, d, e, f and g). Once again, the CB_1 receptor pattern virtually disappeared in *CB_1*-KO meaning that the CB_1 receptor antibody used was highly specific (Fig. 31c).

Notably, the total of CB_1 receptor particles counted in 31.5 μm^2 on plasma membranes decreased significantly after THC exposure versus sham (sham: 34.23 ± 3.24 particles; THC: 21.85 ± 3.39 particles; ***$p < 0.001$). In particular, the highest proportion of the total CB_1 receptor particles found in sham was on inhibitory axon terminal membranes, as expected (17.50 ± 3.09 particles) but was remarkably low in THC treated mice (7.64 ± 2.98 particles) (***$p < 0.001$). Likewise, a great particle decrease was also detected after acute THC exposure in excitatory terminals (sham: 2.77 ± 0.24 particles; THC: 1.71 ± 0.26 particles) (**$p < 0.01$) and mitochondria (sham: 3.38 ± 0.30 particles; THC: 2.63 ± 0.23 particles) (*$p < 0.05$; Fig. 32d). Furthermore, 78.40 ± 0.81% of the inhibitory terminals and 21.91 ± 2.06% of the excitatory terminals in CA1 hippocampus were CB_1 receptor immunopositive in sham mice. In contrast, in acute THC treated mice, the values of CB_1 immunopositive inhibitory terminals decreased significantly (47.74 ± 13.27%; ***$p < 0.001$), but no significant differences were found in the proportion of CB_1 receptor-labeled excitatory terminals (17.55 ± 2.39%; $p > 0.05$; Fig. 32a). Finally, there was a significant decrease in the density of CB_1 receptors in inhibitory terminals (sham: 7.03 ± 0.52 part/µm; THC: 3.68 ± 0.28 part/µm) (***$p < 0.001$) and mitochondria (sham: 0.96 ± 0.02 part/µm; THC: 0.88 ± 0.02 part/µm) (*$p < 0.05$). However, the receptor density in excitatory terminals did not vary after acute THC exposure (sham: 0.55 ± 0.019 part/µm; THC: 0.60 ± 0.028 part/µm) ($p > 0.05$; Fig. 32c). There was also a significant reduction in the CB_1 receptor immunopositive astrocytic processes in acute THC (23.05 ± 3.99%) relative to sham (35.13 ± 4.29%) (**$p < 0.01$; Fig. 32a), associated with a significant increase in CB_1 receptor particle density (sham: 0.20 ± 0.02 part/µm; THC: 0.40 ± 0.06 part/µm) (**$p < 0.01$; Fig. 32c). Additionally, CB_1 receptor immunopositive mitochondria

in neurons (18.66 ± 1.06%) and astrocytes (13.49 ± 2.28%) were significantly reduced after acute THC administration (11.32 ± 0.68% in neurons; ***p < 0.001, and 6.08 ± 1.39% in astrocytes; *p = 0.049; in detail, Fig. 32b).

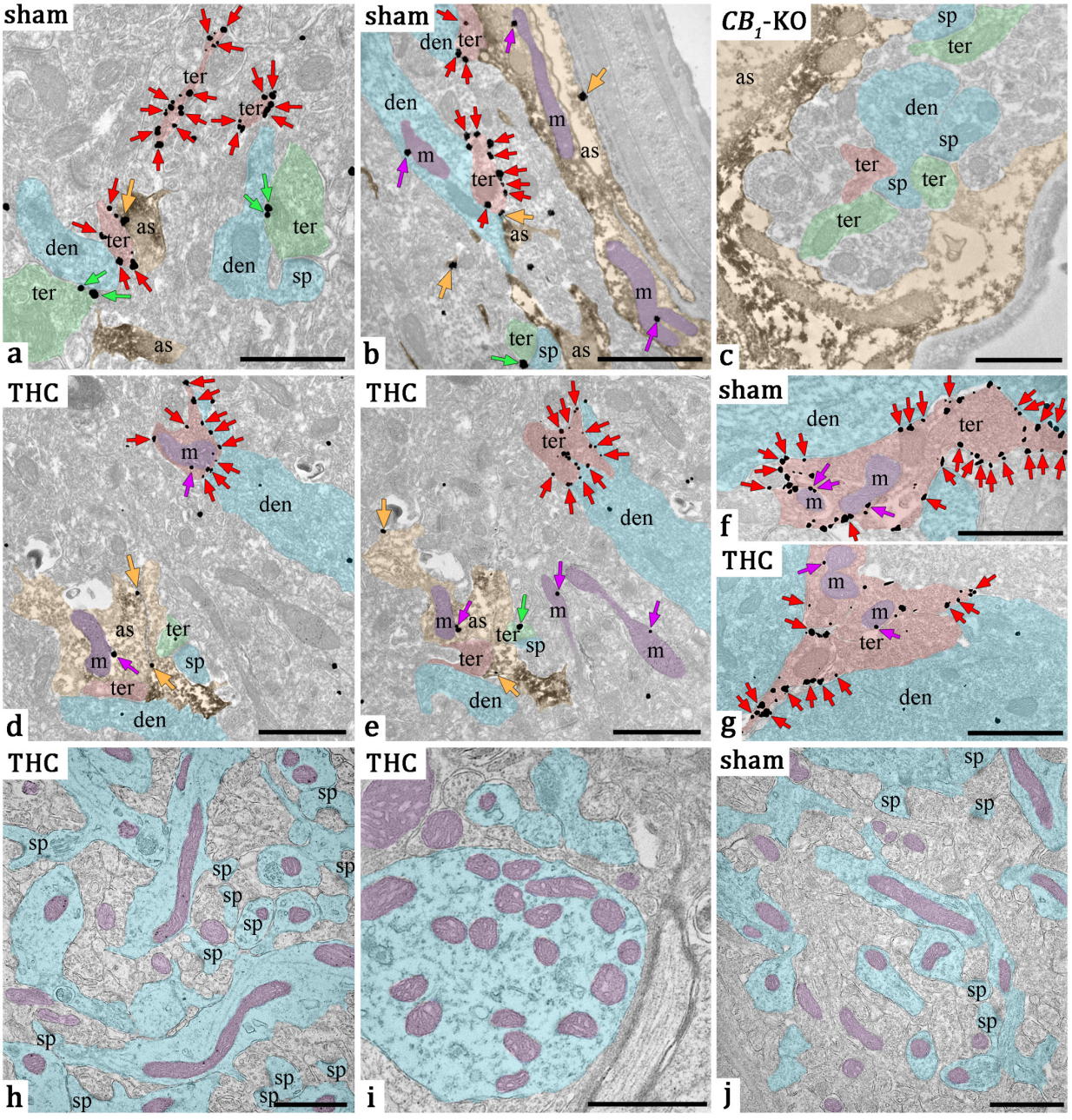

Figure 31. Double pre-embedding immunogold (CB_1 receptor) and immunoperoxidase (GFAP) method for electron microscopy. (a, b) As expected in sham mice, CB_1 receptors are observed in excitatory (ter, green arrows) and inhibitory terminals (ter, red arrows), astrocytic membranes (as, orange arrows), as well as neuronal and astrocytic mitochondria (m, purple arrows). (c) No CB_1 receptor immunolabeling is detected in terminals (ter), astrocytes (as) and mitochondria in *CB_1*-KO mice, indicating the specificity of the CB_1 receptor antibody used. (d, e) Serial ultrathin sections showing a GFAP positive (DAB immunodeposits) astrocytic process with a few CB_1 receptor immunoparticles on the astrocytic membrane (as, orange arrows). A CB_1 receptor positive excitatory terminal (ter, green arrow) and a CB_1 receptor negative inhibitory terminal (ter, red) are also closely associated to this

astrocytic process. In addition, CB_1 receptor labeling is observed in astrocytic mitochondria, another inhibitory terminal (ter, red arrows), and neuronal mitochondria (m, purple arrows). (f) Usual CB_1 receptor metal particles on an inhibitory terminal (ter, red arrows) and mitochondria (m, purple arrows) of sham mice; however, CB_1 receptor labeling decreases in inhibitory terminals (ter, red arrows) after acute THC treated mice (g). (h) Notice that dendritic spines (sp, blue) and (i) and mitochondria (m, purple) seem to be more numerous after acute THC with respect to sham (j). Scale bars: 1µm.

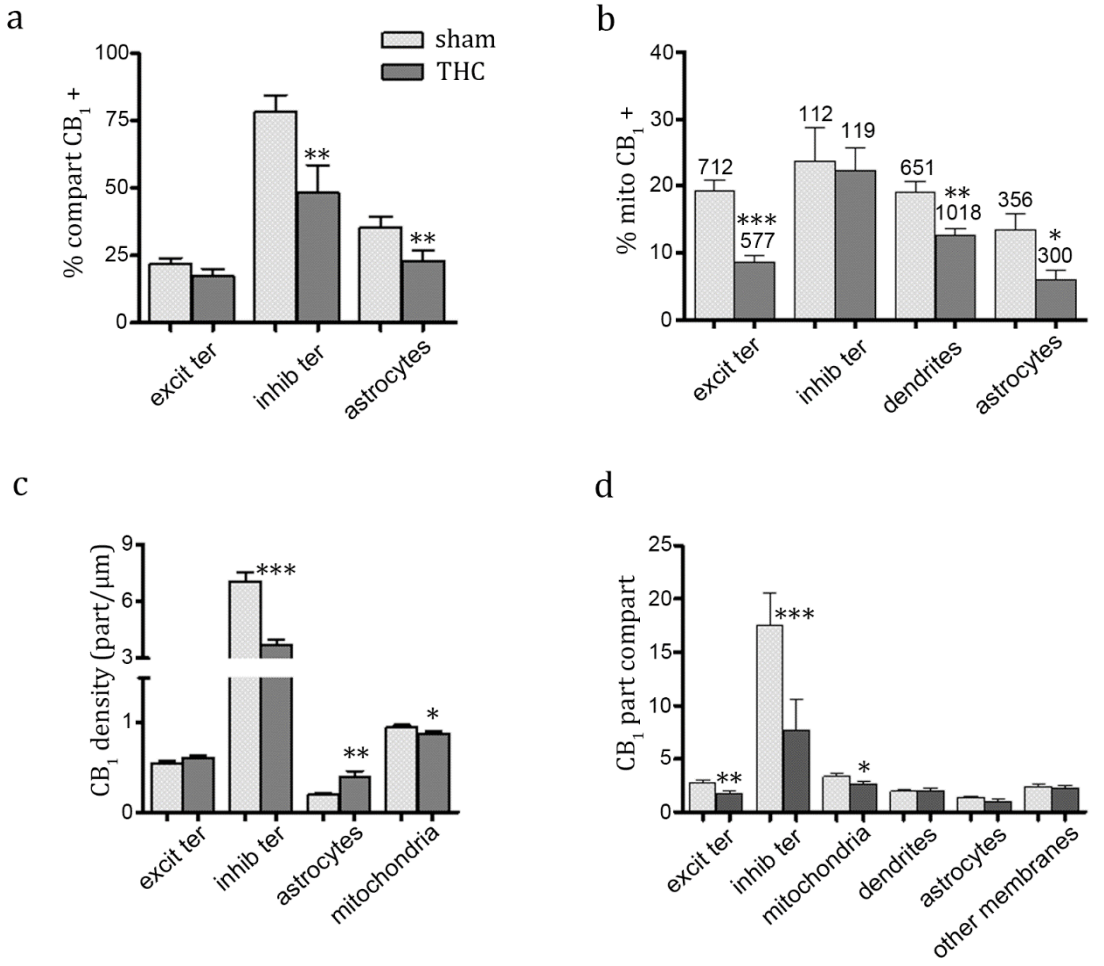

Figure 32. CB_1 receptor distribution in the CA1 stratum radiatum of acute THC treated mice. (a) Percentage of CB_1 receptor immunopositive excitatory and inhibitory synaptic terminals as well as astrocytes in sham and after THC. (b) Percentage of labeled mitochondria in cellular and subcellular compartments in sham and acute THC: in excitatory terminals, sham: 19.27 ± 1.57% vs. THC: 8.69 ± 0.98%; in inhibitory terminals, sham: 23.64 ± 5.02% vs. THC: 22.35 ± 3.39%; in dendrites, sham: 19.05 ± 1.67% vs. THC: 12.66 ± 1.07%; in astrocytes, sham: 13.49 ± 2.28% vs. THC: 6.08 ± 1.39%). The number of mitochondria studied is in parentheses on the top of each column. (c) CB_1 receptor density (particles/µm) in CB_1 receptor positive profiles in sham and acute THC treated mice. (d) Proportion of CB_1 receptor labeling in different compartments per photo (31.5 μm^2) (analyzed area: ~2000 μm^2). (d) Data are expressed as mean ± SEM and were analyzed by non-parametric or parametric tests (Mann–Whitney U test or unpaired t-test *p < 0.05; **p < 0.01; ***p < 0.001).

5.4.2. Ultrastructural changes in CA1 astrocytes, neurons and mitochondria after acute THC exposure

The ultrastructural analysis revealed that 49.57 ± 1.39% of the total area analyzed corresponded to dendritic profiles in sham mice, whereas 67.80 ± 1.13% was occupied by dendrites in THC treated mice (***p < 0.001; Fig. 33a). Furthermore, an increase in the number of dendritic spines was observed in acute THC treated (11.63 ± 0.30 spines/31.5 µm^2) versus sham mice (8.08 ± 0.25 spines/31.5 µm^2;***p < 0.001; Fig. 33e). Alike, a great increase in the number of mitochondria was detected particularly in dendrites (*p < 0.05, **p < 0.01, ***p < 0.001; Fig. 33b). However, not statistically significant differences were found in the number of excitatory terminals in THC and sham mice (p > 0.05; Fig. 33e). Interestingly, the area of the dendritic spines (sham: 0.12 ± 0.00 µm^2; THC: 0.11 ± 0.00 µm^2), synaptic terminals (sham: 0.21 ± 0.01 µm^2; THC: 0.15 ± 0.00 µm^2) (***p < 0.001; Fig. 33d) and mitochondria (sham: 0.13 ± 0.00 µm^2; THC: 0.08 ± 0.00 µm^2) (***p < 0.001; in detail, Fig. 33c) decrease significantly in THC with respect to sham. The area of astrocytes was also significantly smaller in THC (1.51 ± 0.51 µm^2) than in sham (2.19 ± 0.51 µm^2) (***p < 0.001) as well as the astrocytic perimeter (sham: 5.87 ± 0.49 µm; THC: 4.25 ± 0.54 µm) (***p < 0.001; Fig. 33f). These observations suggest that the increase in CB_1 receptor density in astrocytes after acute THC exposure might be due to the reduced astrocytic size induced by this harm condition.

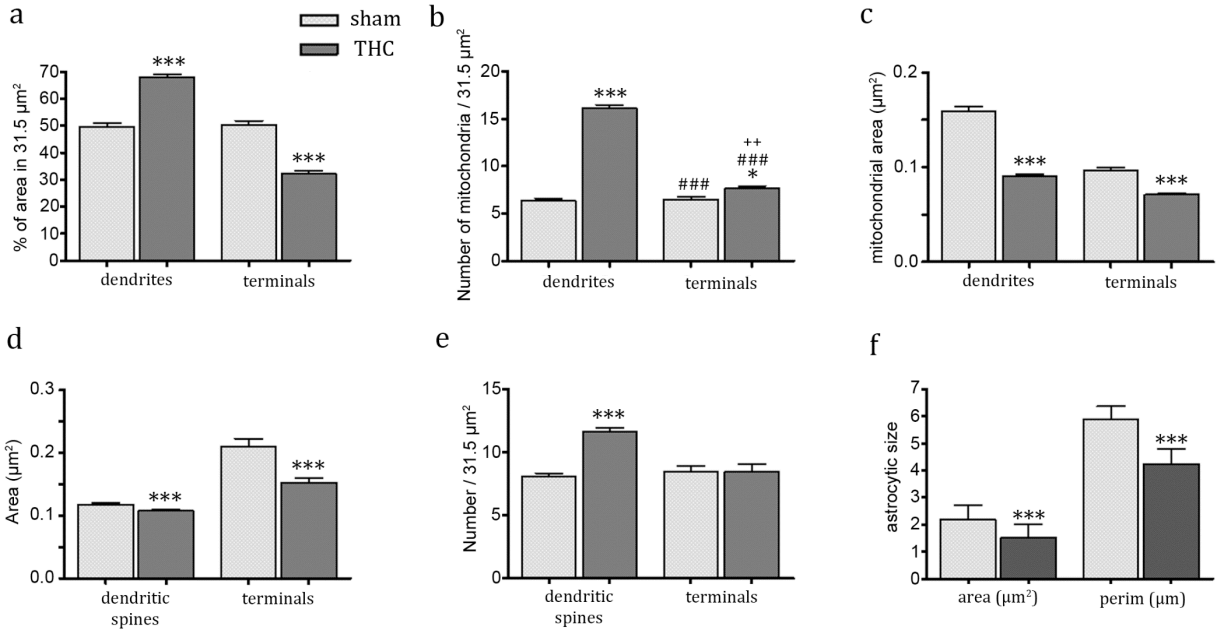

Figure 33. Ultrastructural assessment of the neuronal and astroglial morphology in the CA1 stratum radiatum of sham and THC treated mice. (a) Proportion of the area of dendrites and terminals normalized to the total neuronal area. (b) Number of

mitochondria segments per 31.5 μm^2 (in dendrites, sham: 6.38 ± 0.23, THC: 16.11 ± 0.36; vs. in terminals, sham: 6.52 ± 0.27, THC: 7.68 ± 0.24) (Data, mean ± SEM. *p < 0.05, ***p < 0.001 (sham postsynaptic mitochondria vs. THC mitochondria), $^{\#\#\#}$p < 0.001 (THC postsynaptic mitochondria vs. presynaptic mitochondria), $^{++}$p < 0.01 (THC postsynaptic mitochondria vs. THC presynaptic mitochondria), Dunn's Multiple Comparison Test). (c) Area of mitochondria (in dendrites, sham: 0.16 ± 0.00 μm^2, THC: 0.10 ± 0.00 μm^2; vs. in terminals, sham: 0.09 ± 0.00 μm^2, THC: 0.07 ± 0.00 μm^2) (***p < 0.001, Mann–Whitney test). (d) Area of dendritic spines (sham: 0.12 ± 0.00 μm^2, THC: 0.11 ± 0.00 μm^2), and terminals (sham: 0.21 ± 0.01 μm^2, THC: 0.15 ± 0.00 μm^2) (***p < 0.001, Mann–Whitney test). (e) Number of dendritic spines (sham: 8.08 ± 0.25 spines, THC: 11.63 ± 0.30 spines) (***p < 0.001, Mann–Whitney test) and number of excitatory terminals per 31.5 μm^2. (f) Area of astrocytes (sham: 2.19 ± 0.51 μm^2, THC: 1.51 ± 0.51 μm^2) and astrocytic perimeter (sham: 5.87 ± 0.49 μm, THC: 4.25 ± 0.54 μm) (***p < 0.001, Mann–Whitney test).

6. DISCUSSION

6.1. CB_1 RECEPTORS IN ASTROCYTES OF THE HIPPOCAMPUS

Hippocampal sections of *CB_1*-WT, *CB_1*-KO, conditional CB_1 receptor rescue mice re-expressing CB_1 receptor exclusively in astrocytic GFAP expressing cells (GFAP-*CB_1*-RS), STOP-*CB_1* (carrying a loxP-flanked stop cassette inserted in the 5'UTR upstream of the CB_1 receptor translational start codon) and conditional mutant mice bearing a selective deletion of CB_1 in astrocytes expressing GFAP (GFAP-*CB_1*-KO) were used in this doctoral thesis to investigate the localization of the CB_1 receptor. Besides, *CB_1*-WT and *CB_1*-KO mice expressing the humanized isoform of the Green Fluorescence Protein (hrGFP) from the sea pansy *Renilla* (Ward et al., 1978; Ward and Cormier, 1979; Navarro-Galve et al., 2005; Hadaczek et al., 2009; De Francesco et al., 2015; Kerr et al., 2015) under the control of the promoter of GFAP [GFAPhrGFP-*CB_1*-WT and GFAPhrGFP-*CB_1*-KO, respectively] were also studied. The low CB_1 receptor expression in astrocytes could be accurately detected by high resolution immunocytochemical techniques for electron microscopy (Puente et al., 2019), and a combined pre-embedding immunogold and immunoperoxidase method has previously been shown in our laboratory (Puente et al., 2019) to be an excellent approach for the localization of the CB_1 receptors in astrocytes (Han et al., 2012; Bosier et al., 2013; Gutiérrez-Rodríguez et al., 2018).

My results showed that the CB_1 particles localized on astrocytic profiles versus the total CB_1 receptor particles on plasmalemmal structures of the GFAP-*CB_1*-RS hippocampus were almost all on astrocytic elements. Furthermore, no expression of the CB_1 receptor was observed in astrocytes of STOP-*CB_1*, and only background levels were on astrocyte mitochondria despite previous findings of CB_1 receptor mRNA expression in *CB_1*-STOP mice (Ruehle et al., 2013; de Salas-Quiroga et al., 2015). The CB_1 receptor expression restored in astrocytes of the GFAP-*CB_1*-RS knock-in mice can provide good insights into the sufficiency of the CB_1 receptor in these cells for specific brain functions and behaviours, as it was demonstrated recently for the neuronal CB_1 receptors of rescue mice expressing the gene exclusively in dorsal telencephalic glutamatergic neurons (Glu-*CB_1*-RS) or GABAergic neurons (GABA-*CB_1*-RS) (Ruehle et al., 2013; Soria-Gómez et al., 2014; de Salas-Quiroga et al., 2015).

6.1.1. CB_1 receptor expression in GFAPhrGFP-*CB_1*-WT mutant mice

Additionally, mutant mice target to express hrGFP in astroglial cells were used in my

doctoral thesis. The proportion of the total CB_1 receptor particles (particles/µm) that was localized in astrocytic elements was higher in GFAPhrGFP-*CB_1*-WT than in the WT. These results suggest that the CB_1 receptor expression in astrocytes could actually be higher than previously reported using the astrocytic GFAP marker (Han et al., 2012; Bosier et al., 2013; Gutiérrez-Rodríguez et al., 2018). A plausible explanation could be due to the different molecular nature of the GFAP and the hrGFP proteins. GFAP is a cytoskeletal protein assembled in intermediate filament packet (Inagaki et al., 1994; Eng et al., 2000; Hol & Pekny, 2015) whereas hrGFP is a diffusible protein, which fills all the cytoplasmic regions of the cell. Thus, GFAP immunostaining is mostly restricted to the main radial processes of the astrocyte, while hrGFP extends into the fine processes of the astrocytes that normally lack GFAP (Nolte et al., 2001) therefore accomplishing a better detection of the astrocyte processes.

The CB_1 receptors in astrocytes play a key role in the communication between neurons and astrocytes through an astrocytic calcium rise that triggers a potentiation of the excitatory synaptic transmission at distant synapses (Navarrete & Araque, 2008, 2010; Navarrete et al., 2012, 2014; Gómez-Gonzalo et al., 2015). CB_1 receptor activation in astrocytes contributes to the brain´s energy supply through the control of the leptin receptor expression in these cells (Bosier et al., 2013) and regulates D-aspartate uptake by astrocytes (Shivachar, 2007). Furthermore, the CB_1 receptor expression increases in astrocytes of the sclerotic hippocampus (Meng et al., 2014) and blockade of the astroglial receptor modulates the intracellular calcium signaling reducing hippocampal epileptiform seizures (Coiret et al., 2012). The subcellular compartmentalization of the CB_1 receptors in astrocytes suggests the existence of a molecular architecture that may be crucial for the functional role of the CB_1 receptors at the tripartite synapse (Navarrete & Araque, 2008, 2010; Han et al., 2012; Araque et al., 2014; Pérez-Alvarez et al., 2014; Belluomo et al., 2015; Metna-Laurent & Marsicano, 2015; Da Cruz et al., 2016).

6.1.2. CB_1 receptors in astroglial mitochondria and the nearby synapses

Recent consensus has been reached (Harkany & Horvath, 2017; Mancini & Horvath, 2017) confirming that a low but significant proportion of CB_1 receptors in the brain are functionally associated to mitochondrial membranes, where they mediate cannabinoid effects on cellular mitochondrial activity and on behavioral food intake and memory consolidation (Koch et al., 2015; Hebert-Chatelain et al., 2016). However, whether these effects are due to specific cell-type dependent mechanisms is not known. Immunogold

electron microscopy revealed CB_1 receptors in astroglial mitochondrial membranes from the hippocampus (Gutiérrez-Rodríguez et al., 2018), prefrontal cortex, piriform cortex and nucleus accumbens. Importantly, negative control tissues from global *CB_1*-KO mice displayed just background staining in both neurons and astrocytes. Despite the generally lower absolute levels of CB_1 receptors in astrocytes than in neurons (Metna-Laurent & Marsicano, 2015), the proportion of $mtCB_1$ receptors over the total CB_1 receptors density in each cell type was slightly larger in astroglial mitochondria of the hippocampus and prefrontal cortex than in neuronal mitochondria. Stimulation of astroglial $mtCB_1$ receptors impairs brain glucose metabolism through a molecular signaling cascade involving destabilization of the N-module of mitochondrial complex I, decreased production of mitochondrial reactive oxygen species (ROS) and attenuation of the hypoxia-induced factor-1 (HIF-1) pathway. In turn, these signaling events reduce lactate production in astrocytes and induce bioenergetic stress in neurons, which eventually leads to impairment of social interactions in mice (Jimenez-Blasco et al., Nature, under review).

Furthermore, the findings that astrocytes contain CB_1 receptors in mitochondria and that the cannabinoid-induced reduction of oxygen consumption (Bénard et al., 2012) is absent in mitochondria isolated from the forebrain of GFAP-*CB_1*-KO mice (Jimenez-Blasco et al., Nature, under review) suggest that astroglial $mtCB_1$ receptors play a prominent role in the global effects of cannabinoids on brain mitochondrial respiration. Considering that neurons express much larger absolute levels of CB_1 receptor protein than astrocytes (Busquets-Garcia et al., 2018) and they contain $mtCB_1$ receptors (Bénard et al., 2012), we can speculate that neuronal $mtCB_1$ receptors might mediate other specific effects of cannabinoids, such as reduction of mitochondrial mobility and synaptic transmission (Hebert-Chatelain et al., 2016). In addition, it is also likely that the respiratory effects of cannabinoids on neuronal mitochondria are limited to specific brain regions, subcellular domains and/or neuronal types. Data seem to indicate that the cell types involved in the effects of $mtCB_1$ receptors depend on the functions and the behavioral tasks under scrutiny, as well as on the state of subjects (Busquets-Garcia et al., 2015).
As the CB_1 receptors in astrocytes (Gutiérrez-Rodríguez et al., 2018), the most common distance observed between the astroglial $mtCB_1$ receptors and the nearest synapses was 400-800 nm in the CA1 stratum radiatum and the DML. Thus, it is tempting to suggest that this distance would represent the average distance crossed by the eCBs generated on demand in postsynaptic neurons to the CB_1 receptors localized to the mitochondrial astrocytes.

6.1.3. Conditional CB_1 receptor mutants

Loss of function of mutant mice lacking the CB_1 receptor in specific cell types allowed better identifying the anatomical localization and defining the necessary role of the receptor for several brain functions (Marsicano et al., 2003; Monory et al., 2006, 2015; Han et al., 2012; Bénard et al., 2012; Lutz, 2014; Soria-Gómez et al., 2014; Koch et al., 2015; Martín-García et al., 2015). As to the astrocytes, conditional mutant mice without CB_1 receptors in these cells lack the spatial working memory impairment and *in vivo* hippocampal long-term depression induced by acute cannabinoids (Han et al., 2012). The demonstration that the hippocampus of the GFAP-*CB_1*-RS mutant mice maintain the normal anatomical distribution and expression levels of CB_1 receptors in the astrocytes and their mitochondria with restored receptors (Gutiérrez-Rodríguez et al., 2018), make these mutants ideal for the study of the CB_1 receptor function in astrocytes, as it was recently shown for Glu-*CB_1*-RS (Ruehle et al., 2013; Soria-Gómez et al., 2014; de Salas-Quiroga et al., 2015; Gutiérrez-Rodríguez et al., 2017) and GABA-*CB_1*-RS mice (de Salas-Quiroga et al., 2015; Gutiérrez-Rodríguez et al., 2017). In fact, the rescue strategies have the advantage of the re-establishment and visualization of existing CB_1 receptors levels in cell types with sparse CB_1 receptors (as the astrocytes), allowing a more comprehensive functional characterization of the (endo)cannabinoid system based on the precise cellular and subcellular localization of the CB_1 receptor, and improving the fundamental knowledge for the development of innovative therapeutics against complex brain diseases. Altogether, these observations confirm the high specificity of the CB_1 receptor genetic rescue approach carried out in the astrocytes of the mutant mice, and emerge as excellent models for studying the contribution of the CB_1 receptors in astrocytes to brain function and dysfunction.

6.2. BINGE DRINKING AND CB_1 RECEPTORS IN ADULT CA1 HIPPOCAMPUS

I examined the effect of the adolescent intermittent binge drinking in the dark, a model of chronic alcohol intake, on the CB_1 receptor expression in adult brain astrocytes, for two main reasons: first, the adolescent brain is highly vulnerable to ethanol having a tremendous impact on the brain´s cellular structure and function, including the astrocytes (Pascual et al., 2007; Vetreno & Crews, 2015; Adermark & Bowers, 2016); second, the astroglial CB_1 receptors at the tripartite synapse play a key role in brain functions such as synaptic plasticity, memory and behavior (Navarrete & Araque, 2010; Han et al., 2012; Araque et al., 2014; Navarrete et al., 2014; Gómez-Gonzalo et al., 2015; Metna-Laurent & Marsicano, 2015; Da Cruz et al., 2016) that are altered upon ethanol intake (Lovinger & Roberto, 2013; Lovinger & Alvarez, 2017).

The adolescent EtOH intake has been shown to cause a significant decrease in the relative CB_1 receptor protein and mRNA (Basavarajappa et al., 1998; Mitrirattanakul et al., 2007; Steindel et al., 2013; Peñasco et al., 2020). To determine whether EtOH intake during adolescence elicited a global change in CB_1 receptor expression in the adult CA1 stratum radiatum, the proportion of CB_1 receptor immunoparticles in different cellular compartments was examined. Metal particles were localized on inhibitory and excitatory axon terminals forming synapses with dendrites and dendritic spines, respectively. As expected, the highest proportion of the total CB_1 receptor particles was found on inhibitory axon terminal membranes making symmetric synapses with dendrites. Overall, there was no significant difference between the CB_1 receptor expression on inhibitory terminals in control and EtOH. The concentration of immunogold particles was low in the excitatory synapses in control and even lower in EtOH.

I used the DAB immunostaining to identify GFAP-containing astrocytes and their processes, which then allowed individual CB_1 receptors on astrocytes to be detected using immunogold labeling. My results showed that adolescent mice subjected to a 4-day model of binge drinking have a 40% decrease in astroglial processes expressing CB_1 receptors and a 30% drop in receptor density in adult CA1 stratum radiatum astrocytes relative to control. In addition, the proportion of total CB_1 receptor particles found on astrocytes in EtOH was much lower than in control. Interestingly, the measurements taken (perimeter, area) indicate that astrocytes were swollen in adult CA1 upon cessation of ethanol intake in adolescence. Because of the disrupted cell morphology, the astroglial CB_1 receptor expression was analyzed on a similar number of astroglial processes that were counted

up in about 30% larger area in EtOH than in control. Astrocytic swelling seems to be a phenomenon associated with EtOH consumption that leads to astroglial dysfunction (Adermark & Bowers, 2016) upon disruption of the glial fibrillary acidic protein found in the astrocyte intermediate filaments (Renau-Piqueras et al., 1989). Furthermore, long-term behavioral and cognitive impairments, synaptic plasticity disturbance, late alcohol abuse and addiction related to binge drinking during the adolescence have been associated with neuroinflammatory mechanisms (Nestler, 2001; Montesinos et al., 2016). Astrocytes participate in the inflammatory response through their capacity to release pro-inflammatory molecules (Farina et al., 2007) that can be diminished by anti-inflammatory reactions mediated by eCBs acting on astroglial CB_1 receptors (Metna-Laurent & Marsicano, 2015). Hence, because of the drastic reduction in CB_1 receptors in adult astrocytes it is reasonable to expect an impairment of the astroglial anti-inflammatory reaction in response to the adolescent EtOH intake. Furthermore, the altered astroglial morphology should affect the extracellular matrix components and the perineuronal nets sat between the astrocytes and the synapses, so impairing the homeostasis at the tripartite synapse. The supposedly resulting disturbance of neurotransmitter clearance and gliotransmission may lead to deficits in synaptic plasticity (Dzyubenko et al., 2016) that ought to underlie the brain dysfunction observed after chronic ethanol consumption (Pava & Woodward, 2012; Lovinger & Roberto, 2013; Lovinger & Alvarez, 2017). Interestingly, the astroglial glutamate transporter GLAST (EAAT1) appears to be up-regulated upon ethanol exposure (Rimondini et al., 2002) which should favor glutamate clearance from the synaptic cleft. However, this compensation seems not to be relevant for the ethanol effects, as mice lacking GLAST but equipped with functional presynaptic CB_1 receptors show less alcohol consumption, motivation, and reward (Karlsson et al., 2012). It remains to be determined in our model whether the reduction in CB_1 receptors in astrocytes correlates with changes in GLAST expression.

I observed that adolescent binge drinking does not alter the distribution of the remaining CB_1 receptors in astrocytes relative to neighboring synapses, except the proportion of inhibitory synapses closely related to the astrocytic CB_1 receptors that was significantly lower in EtOH-exposed mice. However, the drastic decrease in astrocytic CB_1 receptors distributed on swollen astrocytes may not be effective in sensing the eCBs produced on demand by neural activity, compromising gliotransmitter availability elicited by cannabinoids at the synapses (Han et al., 2012; Araque et al., 2014).

The CB_1 receptor expression on glutamatergic synaptic terminals was affected too, as a slight but significant decrease in CB_1 receptor immunolabeling (but not the proportion of

CB_1 receptor positive terminals nor the labeling density) was noticed in adult CA1 stratum radiatum upon adolescent binge drinking. This is in line with our recent observations in the DML that ethanol consumption during adolescence negatively impacts on the adult CB_1 receptor-dependent long-term depression of excitatory synaptic transmission, a form of synaptic plasticity (Peñasco et al., 2020). Under normal conditions, CB_1 receptor immunogold particles localized to inhibitory and excitatory axon terminals forming synapses with dendrites and dendritic spines, respectively, and several other cell compartments in the middle one-third of the DML. The proportion of the total CB_1 receptor gold particle distribution was determined in excitatory terminals (12.03 ± 0.91%), inhibitory terminals (54.90 ± 2.18%), mitochondria (8.72 ± 0.22%), dendrites (10.39 ± 1.90%) and other membranes (13.94 ± 1.03%) in this sublayer of the DML (Peñasco et al., 2019). Furthermore, 26.31 ± 1.19% of the excitatory terminals were CB_1 receptor-positive with a density of 0.64 ± 0.04 CB_1 receptor particles/µm (Peñasco et al., 2019). To assess whether adolescent EtOH intake caused a global change in CB_1 receptor expression in the mature hippocampus after the same adolescent binge drinking model applied in my doctoral thesis, the CB_1 receptor immunoparticle distribution (% of CB_1 immunoparticles distributed in different compartments taken from the total CB_1 particles counted in the middle one-third DML) was compared between sham and EtOH-treated mice. The values in sham mice were: excitatory terminals (14.56 ± 2.45%), inhibitory terminals (46.08 ± 4.96%), mitochondria (11.65 ± 1.31%), dendrites (10.69 ± 1.35%), other membranes (17.02 ± 2.26%). In EtOH-treated mice: excitatory terminals (9.52 ± 0.93%), inhibitory terminals (49.70 ± 5.08%), mitochondria (11.80 ± 1.38%), dendrites (12.84 ± 1.54%), other membranes (17.19 ± 2.08%) (Peñasco et al., 2020). Furthermore, the percentage of CB_1 receptor-labeled excitatory terminals was significantly reduced after EtOH exposure (17.78 ± 1.95% in EtOH vs 26.98 ± 3.15% in sham) (Peñasco et al., 2020). Interestingly, no statistical differences were found in CB_1 receptor immunoparticle density (particles/µm) between excitatory boutons of sham (0.63 ± 0.05) and EtOH-treated mice (0.58 ± 0.03) (Peñasco et al., 2020). Hence, the reduction in CB_1 receptors in excitatory terminals could account for at least part of the deficits in the adult CB_1 receptor-dependent synaptic plasticity after adolescent EtOH intake (Peñasco et al., 2020). Thus, field excitatory postsynaptic potentials (fEPSPs) evoked by MPP stimulation in the DML were inhibited upon CB_1 receptor activation in adult sham, but not in EtOH-exposed mice. In addition, MPP but not mossy cell fiber stimulation triggered a novel CB_1 receptor-dependent excitatory long-term depression (CB_1-eLTD) that was absent in adult mice after adolescent EtOH consumption (Peñasco et al., 2020). Furthermore, the CB_1-eLTD was group I metabotropic glutamate receptor (mGluR)-dependent, required intracellular

calcium influx and 2-AG synthesis. Also, adolescent EtOH intake significantly decreased the [35S]guanosine-5*-O-(3-thiotriphosphate) ([35S] GTPγS) basal binding and the guanine nucleotide-binding (G) protein Gαi2 subunit, and significantly increased the monoacylglycerol lipase (MAGL) mRNA and protein in the adult hippocampus. Interestingly, MAGL inhibition recovered the CB_1-eLTD and the significant loss of recognition memory in EtOH-treated mice (Peñasco et al., 2020). However, whether there are also any glial cell-associated changes in CB_1 receptor expression in the medial DML remains unknown.

The expression and localization of CB_1 receptors in GABAergic synaptic terminals was unaffected, meaning that the proportion of the CB_1 receptor-immunopositive inhibitory synaptic terminals in control and EtOH coincided with the CB_1 receptor distribution pattern described for rodent interneurons in *CB_1*-WT (Nyíri et al., 2005; Gutiérrez-Rodríguez et al., 2017). However, the percentage of the CB_1 receptor-immunopositive excitatory terminals in this doctoral thesis and in our preceding study (Gutiérrez-Rodríguez et al., 2017) was to a certain extent lower than the values formerly reported (Katona et al., 2006; Uchigashima et al., 2011), most likely attributable to the different immunocytochemical protocols and antibodies used (Katona et al., 2006; Uchigashima et al., 2011). Furthermore, the proportion of CB_1 receptor particles on mitochondria in control (13.92% ± 1.57%) was similar to our previous reports (Bénard et al., 2012; Hebert-Chatelain et al., 2016) and no changes in the CB_1 receptor expression could be detected in this organelle upon EtOH exposure.

The eCBS participates in ethanol behaviors (Economidou et al., 2006) and, reciprocally, ethanol has effects on the eCB-dependent neural activity and behavior (Pava & Woodward, 2012; Talani & Lovinger, 2015). This system prevents the ethanol-induced potentiation of GABA release (Roberto et al., 2010; Talani & Lovinger, 2015) and supresses the glutamatergic transmission elicited by ethanol (Basavarajappa et al., 2008). Furthermore, CB_1 receptor antagonism reduces ethanol self-administration and seeking while CB_1 receptor activation has opposite effects during the relapse and maintenance phase of alcohol drinking (Getachew et al., 2011). Sardinian alcohol preferring rats, a genetic model of alcoholism, exhibit a higher CB_1 receptor density, coupling to G-proteins and endocannabinoid levels associated with lower expression of FAAH (Vinod et al., 2012). Upon alcohol consumption, however, CB_1 receptor coupling was reduced. This effect was attenuated during alcohol withdrawal and reversed by CB_1 receptor

antagonism that appeared to be associated with reduced alcohol intake (Vinod et al., 2012). In this line, the CB_1 receptor expression tends to normalize after a prolonged withdrawal (Rimondini et al., 2002; Mitrirattanakul et al., 2007; Vinod et al., 2012) probably due to a reduction in endocannabinoid levels (Vinod et al., 2012). However, the rapid CB_1 receptor internalization and lysosomal degradation of CB_1 receptors due to the endocannabinoid increase upon ethanol intake, and the ultimate membrane reinsertion upon alcohol intake cessation (Pava & Woodward, 2012), seem to differently operate in neuronal and astroglial compartments. In our model of adolescent intermittent alcohol intake, a drastic decrease in CB_1 receptor immunoparticles, proportion of CB_1 receptor-expressing profiles and receptor density were only observed in adult CA1 astrocytes, suggesting that some differences should exist between CB_1 receptors located at neuronal and astroglial compartments. Actually, CB_1 receptors signal in neurons through coupling to Gαi/o proteins (Kano et al., 2009) and mitochondrial CB_1 receptors have been shown to signal through Gαi proteins, as pertussis toxin blocks the decrease in mitochondrial cAMP, protein kinase A, complex I activity and respiration induced by cannabinoids (Hebert-Chatelain et al., 2016). Interestingly, no changes in the mitochondrial CB_1 receptors in adult upon adolescent intermittent ethanol intake were observed in my thesis work. In astrocytes, there are pieces of evidence indicating that CB_1 receptors, in addition to Gαi/o proteins, also signal through Gαq proteins enabling astroglial CB_1 receptors to couple to different intracellular signaling pathways (Metna-Laurent & Marsicano, 2015). These biochemical differences might also have consequences on CB_1 receptor-binding proteins, like the G-protein-associated sorting protein 1 (GASP1) responsible for linking CB_1 receptors to degradation, or the cannabinoid receptor associated protein 1a (CRIP1a) involved in the CB_1 receptor function modulated by antagonists (Vinod et al., 2012). Taken together, coupling of the CB_1 receptor to different G-proteins might be behind the distinct effect of adolescent EtOH exposure on the CB_1 receptor expression in astrocytes of adult brain.

The long-lasting effects of adolescent binge drinking on astroglial CB_1 receptors and astroglial morphology suggest the existence of an architectural breakdown of the neuron-astrocyte crosstalk at the tripartite synapse of the adult brain. Yet, the effects of these drastic changes on the adult synaptic function and behavior remain to be elucidated. Lastly, the reciprocal interactions between the eCBS and the acute and chronic effects of ethanol have been taken as targets for treatment of ethanol addiction. Therefore, the changes observed in astroglial CB_1 receptors might represent a novel target of interest to palliate the structural, functional and behavioral consequences of the adolescent binge

drinking at later periods of life.

6.3. REPEATED MILD TRAUMATIC BRAIN INJURY IN JUVENILE RATS AND CB_1 RECEPTORS IN THE DML OF THE ADULT BRAIN

Part of these results was obtained during my research stay in the laboratory of Dr. Brian Christie and Dr. Patrick Nahirney, Division of Medical Sciences, University of Victoria (British Columbia, Canada). As fully described in the Results section, to determine whether juvenile r-mTBI (8 hits) caused in the mature hippocampus a global change in CB_1 receptor expression immediately or after the cessation of the repeated ACHI, I examined in both sexes the proportion of CB_1 receptor positive profiles and CB_1 receptor density in the DML at PID 1, 10 and 40. The CB_1 receptor expression was not affected at PID 1 but was significantly reduced in males and females at PID 10. At this post-injury time, male rats had a significant decrease in CB_1 positive excitatory terminals, astrocytes and mitochondria. Importantly, a remarkable decrease in CB_1 receptor density in inhibitory terminals was also observed. Interestingly, injured female rats showed no significant differences in CB_1 receptor expression at PID 10 except that the CB_1 receptor density in inhibitory terminals was significantly lower than in sham rats. At PID 40, there were significant changes in CB_1 receptors in both sexes: a) in males, the proportion of CB_1 receptor-labeled excitatory and inhibitory terminals was lower after r-mTBI, however, there was a significant increase in CB_1 receptor-positive astrocytes; b) in females, the proportion of CB_1 receptor immunopositive excitatory and inhibitory terminals as well as mitochondria were affected. Also 40 days after injury, the CB_1 immunoparticle density only dropped significantly in inhibitory terminals of both males and females. Also under more severe conditions (16 hits), the proportion of CB_1 receptor immunolabeled excitatory and inhibitory terminals as well as the density of CB_1 receptors in inhibitory terminals decreased significantly 40 days after injury.

The expression of CB_1 receptor mRNA and CB_1 receptor protein have been shown to decrease 24 h and 72 h after a closed-head trauma induced by a 50 g weight dropped from a 36 cm height, with the recovery to normal levels 2 weeks later (López-Rodríguez et al., 2015). Furthermore, these changes in CB_1 receptor expression negatively correlates with edema formation and behavioral impairments (López-Rodríguez et al., 2015). However, this weight-drop model causes a limited contra-coup lesion, functional deficits and mortality (5–15%) within the first 5 min following the impact (Homsi et al., 2009, 2010; Siopi et al., 2013). Many of the models used to study mTBI are associated with disturbing factors. For instance, animal models that feature a non-penetrating mechanical impact, a lack of focal damage, and that incorporate linear and rotational forces are now preferred (Shultz et al., 2017). Indeed, confirming the absence of skull fracture, hemorrhage and

significant cell death following mTBI are becoming common (DeWitt et al., 2013; Shultz et al., 2017; Meconi et al., 2018). Dr. Christie laboratory has recently introduced the ACHI as a model for r-mTBI studies. The ACHI creates linear and rotational forces, and has a demonstrated capacity for reliably producing r-mTBI (Meconi et al., 2018; Wortman et al., 2018; Christie et al., 2019; Pham et al., 2019). My results clearly show no changes in the subcellular expression of CB_1 receptors 24 h after ACHI. However, changes in CB_1 receptors were evident 10 days and even more obvious 40 days after r-mTBI. These changes correlate with small microbleeds throughout the brain which associate with activated microglia, an inflammation mechanism observed in r-mTBI (Triviño Paredes JS, unpublished observations). In my doctoral thesis, CB_1 receptor expression has not been assessed in microglia, but we have some ultrastructural evidences indicating that CB_1 receptors might indeed be expressed in these cells under normal conditions at very low levels. Thus, we have been able to visualize CB_1 receptors in about 9% of the microglial processes (subtracted the background already) in the hippocampus of a transgenic mouse expressing the EGFP in microglia under the C-X3-C motif of the chemokine 1 receptor, by a high resolution immunocytochemical tecnique for electron microscopy (the same used in my thesis work). This percentage might change under pathological conditions. In this sense, we are carrying a colaborative work with Dr. Julián Romero and his laboratory (Faculty of Experimental Sciences, Universidad Francisco de Vitoria, Pozuelo de Alarcón, Spain) using a transgenic mouse model expressing EGFP under the control of the gen cnr2 promoter preceded by the insertion of an IRES (internal ribosomal entry site) sequence in the 3´UTR region of the cnr2 gen. These mice were crossed with mice expressing 5 familial Azheimer´s disease mutations (5xFAD) (López et al., 2018). We used specific antibodies against the ionized calcium binding adaptor molecule 1 (Iba1) as microglial marker, as it labels all types of microglial subpopulations (Ito et al., 1998; Okere & Kaba, 2000; Hirayama et al., 2001; Shapiro et al., 2008). Our preliminary results indicate that CB_1 receptors are localized in 15-20% of the microglial processes in the subiculum of 10-month-old wild-type mice as well as of mice with amyloid plaques. Therefore, it doesn´t seem to happen a significant change in CB_1 receptor expression in microglia in the Alzheimer´s condition. Nevertheless, the number of CB_1 immunoparticles in the microglial processes in Alzheimer´s is higher than in control (Terradillos et al., 2019). Altogether, it is plausible that changes in microglial CB_1 receptor expression could also take place in r-mTBI conditions. Of course, we should also keep in mind that the CB_2 receptor expression increases in activated microglia as a response to certain neuropathological and neuro-inflammatory conditions (Guzmán et al., 2001; Benito et al., 2003, 2005; Zhang et al., 2003; Maresz et al., 2005; Yiangou et al., 2006). The activation of CB_2 receptors in microglia by

cannabinoids regulates immune functions in these cells, stimulating microglial proliferation and migration and reducing neurotoxic factors such as TNFα or free radicals (Walter et al., 2003; Carrier et al., 2004; Ramírez et al., 2005; Eljaschewitsch et al., 2006; Dirikoc et al., 2007), having the microglia lower harmful effects at the lesion sites (Stella, 2010). However, it remains to be discovered the levels of CB_2 receptor expression and localization in microglia under r-mTBI conditions, and how its expression could affect neuro-inflammation, synaptic plasticity and behavior.

Long-term changes in CB_1 receptors in the DML may underlie modifications in synaptic plasticity that is at the base of learning and memory in the hippocampus (Younts et al., 2016; Monday & Castillo, 2017; Monday et al., 2018). Indeed, the laboratory of Dr. Christie have previously shown a significant effect of a single mTBI on LTP in this region (White et al., 2017), and their preliminary data indicate that r-mTBI (8 hits) can impair LTD induction (900 pulses @ 1 Hz) at 7 days post-injury (Pinar, unpublished). Actually, a decrease in CB_1 receptors in the excitatory MPP synapses, as we have observed in the DML 10 and 40 days after r-mTBI (8 hits), suggests that an impairment in eCB-eLTD might also be happening in r-mTBI, as it occurs in adult MPP synapses after binge drinking during adolescence (Peñasco et al., 2020). In this latter study, the disruption of the adult CB_1 receptor-mediated excitatory transmission and eCB-eLTD after adolescent EtOH intake was associated with a defect in recognition memory in adulthood which correlated with a cannabinoid signaling disturbance, as the loss of excitatory synaptic plasticity and the novel object recognition test (NOR) deficits were reversible by the inhibition of MAGL (Peñasco et al., 2020). Moreover, these changes went with 34% decrease in CB_1 receptor immunolabeling in excitatory terminals and 35% reduction in the proportion of CB_1 receptor immunopositive excitatory boutons in the middle one-third of the DML of EtOH-treated versus sham (Peñasco et al., 2020). Hence, the CB_1 receptor reduction in excitatory terminals could account for at least part of the deficits in the adult eCB-eLTD after adolescent EtOH intake.

Another angle to be considered is that CB_1 receptor signaling might also be affected after r-mTBI, as CB_1 receptor coupling to G protein signaling is very efficient in glutamatergic synapses (Steindel et al., 2013). In this sense, the specific reduction in the Gαi2 subunit observed in EtOH-treated mice might be responsible for the reduction in [35S] GTPγS basal binding and the impairment in CB_1 receptor signaling, which may be related to the absence of eCB-eLTD and deficits in the NOR test in the EtOH (Peñasco et al., 2020). In fact, a lack of Gαi2 subunit leads to abnormalities in learning efficiency, sociability and social

recognition (Hamada et al., 2017). Upon agonist (2-AG)-induced stimulation of Gαi/o subunits, inhibition of MAGL could overcome the loss of CB_1 receptors in glutamatergic terminals due to the high coupling efficiency of this CB_1 receptor population (Steindel et al., 2013), leading to functional (eCB-eLTD) and behavioral (recognition memory) recovery in adult mice after EtOH treatment during adolescence.

MAGL inhibition in vivo may primarily act by suppressing $GABA_A$ receptor-mediated inhibition; therefore, CB_1 receptors localized in GABAergic terminals might also be contributing indirectly to the eCB-eLTD recovery in EtOH-treated mice. In our r-mTBI model, drastic reductions in the CB_1 receptor density in inhibitory terminals have been observed in males and females 10 and 40 days after 8 hits. These results suggest that an increase in GABAergic signaling might be contributing to reduce DG output following TBI (Johnson et al., 2014).

6.3.1. Functional context of the eCB-eLTD at MPP-granule cell synapses in r-mTBI

Brain functions regulated by the eCBS rely on its distribution in cerebral tissue (Castillo et al., 2012; Katona & Freund, 2012; Hu & Mackie, 2015; Busquets-Garcia et al., 2018). The hippocampus is required for declarative/episodic memory and is involved in spatial and context-dependent learning (Eichenbaum et al., 2012). Inputs from the postrhinal cortex convey spatial information to the dorsolateral medial entorhinal cortex that projects to the dorsal hippocampus through the MPP (Fyhn et al., 2004; Hargreaves et al., 2005). On the other hand, the perirhinal cortex projects to the lateral entorhinal cortex which gives rise to the LPP (Burwell, 2000). The LPP pathway transmits non-spatial information, and, together with information about spatial clues forwarded by the MPP into the DG, representations for object-place or event-place scenarios are thought to be built (Suzuki et al., 1997; Gaffan, 1998; Hargreaves et al., 2005). At the same time, signal integration by granule cells related to environment or context is under control of hilar mossy cells which are critical in the learning of information sequences (Lisman et al., 2005). The mossy cells receive glutamatergic granule mossy fiber collaterals, and in turn send commissural/associational fibers that travel long distances giving innervation to multiple DG cells forming mossy-granule cell synapses (Amaral & Witter, 1989; Scharfman & Myers, 2013). The glutamatergic synapses of the three excitatory pathways targeting the

dentate granule cells contain CB_1 receptors (Marsicano & Lutz, 1999; Katona et al., 2006; Kawamura et al., 2006; Monory et al., 2006; Uchigashima et al., 2011; Katona & Freund, 2012; Wang et al., 2016; Gutiérrez-Rodríguez et al., 2017) and display different forms of eCB dependent-synaptic plasticity (Chiu & Castillo, 2008; Chávez et al., 2010; Wang et al., 2016, 2018) which correlate with the distinct information processed by each pathway. As previously shown in single BNST neurons (Puente et al., 2011), either the 2-AG and CB_1 receptor-dependent eLTD (Peñasco et al., 2020), or the AEA and TRPV1-dependent eLTD at the MPP synapses (Chávez et al., 2010) might each be switched on by distinct patterns of neural activity conveying spatial information. At the same time, high frequency stimulation of the LPP in the outer one-third of the DML leads to 2-AG production and CB_1 receptor-dependent eLTP at these LPP synapses associated with memories related to odor discrimination, and semantic information and representation (Wang et al., 2016, 2018). Altogether, the spatial and non-spatial information transmitted by granule cells to CA3 pyramidal neurons that provides sequence learning and sequence prediction (Hunt et al., 2013) would involve PP inputs and different forms of cannabinoid-dependent plasticity recruited upon the type of information processed, all being modulated by mossy cell activity. Learning and memory processes that involve the hippocampus can be affected by some pathological conditions. For instance, impaired recognition, spatial, and associative memories can be observed in the adult brain after high ethanol exposure (binge drinking) during adolescence (Rico-Barrio et al., 2019). This also correlates with a decrease in CB_1 receptor expression in astrocytes (Bonilla-Del Río et al., 2019), as well as with changes in CB_1 receptor expression at the PP synapses (Peñasco et al., 2020). Interestingly, the memory impairment observed after adolescent binge drinking is recovered in adults exposed to enriched environmental conditions (Rico-Barrio et al., 2019). It is plausible that changes in different forms of CB_1 receptor-dependent plasticity in the DG underlie the memory deficits observed in adults after r-mTBI.

6.4. ACUTE THC ADMINISTRATION, ULTRASTRUCTURAL ARCHITECTURE AND CB_1 RECEPTORS IN CA1 HIPPOCAMPUS OF THE ADULT MOUSE BRAIN

Although THC acts on CB_1 and CB_2 receptors, the tetrad composed of antinociception, hypothermia, hypolocomotion and catalepsy induced by THC (Pertwee, 1997; Felder & Glass, 1998; Ameri, 1999) is mediated by CB_1 receptors, as these effects are not observed in CB_1 receptor knock-out mice (Ledent et al., 1999; Zimmer et al., 1999), or in animals pretreated with the CB_1 receptor antagonist SR141716A (Lichtman & Martín, 1997; Welch et al., 1998), However, not much is known about changes in CB_1 receptor expression in cellular and subcellular compartments as well as about modifications in brain ultrastructure taking place after acute THC exposure in the young adulthood.

In this study, I have shown that the total CB_1 receptors decrease significantly in acutely THC-exposed young adult mice. In particular, CB_1 receptors were remarkably low in inhibitory terminals and also decreased in excitatory terminals and mitochondria. Furthermore, the percentage of CB_1 receptor immunopositive inhibitory terminals decreased significantly, but not the proportion of CB_1 receptor-labeled excitatory terminals.

CB_1 receptor expression is very high in inhibitory synaptic terminals, mostly in cortical and hippocampal CCK-positive GABAergic interneurons (Kawamura et al., 2006; Ludányi et al., 2008; Marsicano & Kuner, 2008; Katona & Freund, 2012; De-May & Ali, 2013; Steindel et al., 2013; Hu & Mackie, 2015; Lu & Mackie, 2016; Gutiérrez-Rodríguez et al., 2017), low in excitatory glutamatergic synapses (Marsicano et al., 2003; Domenici et al., 2006; Katona et al., 2006; Monory et al., 2006; Takahashi & Castillo, 2006; Kamprath et al., 2009; Bellocchio et al., 2010; Puente et al., 2011; Reguero et al., 2011; Ruehle et al., 2013; Soria-Gómez et al., 2014; Gutiérrez-Rodríguez et al., 2017) and very low in brain astrocytes (Rodríguez et al., 2001; Navarrete & Araque, 2008, 2010; Stella, 2010; Han et al., 2012; Bosier et al., 2013; Metna-Laurent & Marsicano, 2015; Viader et al., 2015; Da Cruz et al., 2016; Kovács et al., 2017; Gutiérrez-Rodríguez et al., 2018). Brain CB_1 receptors are mostly localized in axon terminals and preterminals away from the presynaptic active zones and are also localized at mitochondria in neurons (Bénard et al., 2012; Hebert-Chatelain et al., 2014a,b, 2016; Koch et al., 2015) and in astrocytes (Gutiérrez-Rodríguez et al., 2018). We assessed the CB_1 receptor distribution in subcellular compartments of the CA1 hippocampus as the proportion of CB_1 receptor-dependent silver-intensified gold particles in GABAergic terminals (~56%), glutamatergic terminals (~12%), astrocytes

(~6%) and mitochondria (~15%) (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019). Noticeably, 11% of the immunoparticles were localized to other compartments, and, importantly, the labeling disappeared in the *CB_1*-KO (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019).

Long-term exposure to cannabinoids causes down-regulation of CB_1 receptors in the brain (Romero et al., 1998; Sim-Selley & Martín, 2002; Sim-Selley et al., 2006) which is at the base of receptor desensitization and tolerance (Breivogel et al., 1999; Sim-Selley & Martín, 2002; Martini et al., 2007; Tappe-Theodor et al., 2007). Chronic THC administration uncouples CB_1 receptors from G proteins (Sim et al., 1996) likely through receptor phosphorylation by a G protein-coupled receptor kinase (Jin et al., 1999). CB_1 receptor desensitization is also due to changes in presynaptic membrane dynamics: endocannabinoid signaling may be limited by the rise of receptor immobilization at extrasynaptic sites and a progressive decrease in the number of CB_1 receptors at the synapse (Mikasova et al., 2008). Furthermore, as classical G protein-coupled receptors, prolonged agonist treatment triggers CB_1 receptor internalization via both caveolae/lipid-rafts- and clathrin-coated-pits-mediated pathways in a dynamin-dependent manner (Hsieh et al., 1999; Jin et al., 1999; Wu et al., 2008) with distinct domains of the receptor being involved (Jin et al., 1999). Interestingly, THC and AEA are low CB_1 receptor endocytic agonists but induce a faster desensitization and slower resensitization than the high endocytic agonists WIN and 2-AG (Wu et al., 2008).

Acute activation of CB_1 receptors by a relatively high dose of THC as used in this study (5 mg/Kg) causes a drastic decrease in CB_1 receptors in CA1 hippocampus probably due to receptor internalization. This scenario would lead to an increase in GABA release. At the same time, glutamate release would also be affected, as CB_1 receptor expression is also reduced (but not the density) in excitatory terminals resulting in an excitation/inhibition imbalance. A low dose of THC (0.002 mg/kg) reduces injury-induced cognitive deficits in mice (Assaf et al., 2011) through the biphasic effects of THC which produces analgesia, acute hypothermia, and decreased locomotion at high doses (10 mg/kg), and hyperalgesia, hyperthermia, and increased locomotion at low doses (0.002 mg/kg) (Sarne et al., 2011). Also, a low dose of THC potentiates calcium entry into cells in vitro (Okada et al., 1992) and increases glutamate release (Schurman & Lichtman, 2017).

Both GABAergic and glutamatergic systems have been shown to be altered by cannabis exposure (Skosnik et al., 2014; Zamberletti et al., 2014; Cortes-Briones et al., 2015;

Radhakrishnan et al., 2015; Colizzi et al., 2016; Melis et al., 2017). As to the excitatory transmission, an increase in the NMDA receptor subunit GluN2B, the AMPA subunits GluA1 and GluA2 (Zamberletti et al., 2016), the presynaptic marker synaptophysin and the postsynaptic marker PSD95 have been observed in hippocampal synaptosomes from adult rats after their THC exposure during adolescence (Zamberletti et al., 2016; Melis et al., 2017). However, only an increase in GluN2B and GluA1 subunits in the prefrontal cortex was observed in females after adolescent THC (Rubino et al., 2015), suggesting that there are sex differences in the adult brain regions affected after THC exposure during the adolescence. It has also been postulated that activated astrocytes promoting a pro-inflammatory phenotype might contribute to the alterations in glutamatergic synapses induced by adolescent THC (Melis et al., 2017). The excess of glutamate resulting of the decrease in CB_1 receptors at the glutamatergic terminals might activate microglia and/or astrocytes that trigger inflammatory IL-1b, TNF-α or iNOS which cause learning impairment (Cutando et al., 2013; Zamberletti et al., 2015). On the other and, the drop of CB_1 receptors in GABAergic terminals might also favor glia activation following cannabis use, as the increase in neuro-inflammatory molecules was seen to depend on CB_1 receptors in GABAergic neurons (Albayram et al., 2011).

I also performed an ultrastructural analysis in the CA1 hippocampus to determine the impact of acute THC administration during adolescence on the size of dendrites and the distribution of neuronal mitochondria. The results of my doctoral thesis indicate that CA1 dendrites after acute THC exposure occupy a significantly larger area than in sham mice. Also, an increase in the number of dendritic spines and mitochondrial profiles were observed particularly in dendrites. However, not statistically significant differences were found in the number of excitatory terminals. However, the area of the dendritic spines, synaptic terminals, mitochondria as well as of the astrocytes and their perimeter decrease significantly in THC. Cannabis abuse has been associated with morphological changes in different brain regions of male and females. Interestingly, alterations predominate in the hippocampus in males (Solowij et al., 2013) and in prefrontal cortex and amygdala in females (Medina et al., 2009; McQueeny et al., 2011). The changes in the CA1 ultrastructure induced by acute THC might be related to the CB_1 receptor internalization. It has been shown that mice with the global lack of CB_1 receptors do not show drastic changes neither at the cellular/phenotypic level (Mulder et al., 2008) nor in motor coordination (Bilkei-Gorzo et al., 2005; Kishimoto & Kano, 2006). In cerebellum, CB_1 receptors are expressed at the glutamatergic parallel fiber (PF) terminals of the granule cells that make excitatory synapses with the Purkinje cell (PC) dendritic spines

(Kawamura et al., 2006). The laboratory of Dr. Grandes has demonstrated recently that ultrastructural changes at the PF-PC synapses occur in *CB_1-KO* mice (Buceta et al., 2019). Thus, the spinocerebellar lobule V of the vermis, but not the vestibulocerebellar lobule X, of *CB_1*-KO had significantly less and longer synapses than in *CB_1*-WT. PF terminals were significantly larger in both lobules of *CB_1*-KO with no changes in PC dendritic spines. The PF terminals in lobule V of *CB_1*-KO contained less synaptic vesicles and lower vesicle density; by contrast, vesicle density in lobule X of *CB_1*-KO remained unchangeable relative to *CB_1*-WT. There were as many vesicles in lobule V of *CB_1*-KO as in *CB_1*-WT, but their distribution decreased drastically at 300 nm of the active zone. In lobule X of *CB_1*-KO, less vesicles were found within 150 nm from the presynaptic membrane; however, no vesicles were at 450-600 nm of the active zone. A significant higher amount of synaptic vesicles close to the active zone in lobule V and X of *CB_1*-KO was observed (Buceta et al., 2019). In conclusion, the absence of CB_1 receptors strikingly and distinctively impacts on the ultrastructural architecture of the PF-PC synapses located in cerebellar lobules that differ in vulnerability to damage and motor functions. Thus, a similar phenomenon could account for the ultrastructural changes observed in neuronal and astrocytic compartments in the CA1 hippocampus after the drop of CB_1 receptors induced by the acute administration of THC.

CB_1 receptors are also reduced in CA1 mitochondria after acute THC administration. Both the percentage of CB_1 immunopositive mitochondria in neurons and astrocytes were significantly reduced. Acute cannabinoid intoxication is known to induce amnesia in humans and animals (Marsicano & Lafenêtre, 2009; Broyd et al., 2016) and the $mtCB_1$ activation alters energy production by mitochondria (Bénard et al., 2012; Hebert-Chatelain et al., 2014; Koch et al., 2015). Interestingly, acute cannabinoid-induced memory impairment in mice requires activation of hippocampal $mtCB_1$ receptors, as removal of CB_1 receptors from hippocampal mitochondria prevents cannabinoid-induced reduction of mitochondrial mobility, synaptic transmission and memory formation (Hebert-Chatelain et al., 2016). Signaling of mitochondrial CB_1 receptors through intra-mitochondrial Gαi protein activation inhibits soluble-adenylyl cyclase and PKA-dependent phosphorylation of specific subunits of the mitochondrial electron transport system, which dampens cellular respiration (Hebert-Chatelain et al., 2016). Thus, the drop of CB_1 receptors in mitochondria seen in this doctoral thesis would represent a brain´s compensatory mechanism to palliate the negative consequences of the acute THC intoxication.

There was also a significant reduction in the percentage of CB_1 receptor immunopositive astrocytic processes and a simultaneous increase in CB_1 receptor immunoparticle density in astrocytes after acute THC, probably due to the reduced astrocytic size induced by this harm condition (see below). Some years ago was demonstrated that the impairment of spatial working memory and in vivo LTD at hippocampal CA3-CA1 synapses induced by an acute exposure of exogenous cannabinoids, is fully abolished in conditional mutant mice lacking CB_1 receptors in astrocytes, but is conserved in mice lacking CB_1 receptors in glutamatergic or GABAergic neurons (Han et al., 2012). Blockade of neuronal NMDA receptors and of synaptic trafficking of AMPA receptors also abolishes cannabinoid effects on spatial working memory and LTD. Thus, the impairment of working memory by acute marijuana and cannabinoids in vivo is due to the sequential activation of astroglial CB_1 receptors and postsynaptic NR2B-containing NMDA receptors, which elicits AMPA receptor endocytosis-mediated expression of in vivo LTD at CA3-CA1 synapses, resulting in working memory impairment (Han et al., 2012). Astrocytes produce eCBs (Stella, 2010) and CB_1 receptors in astrocytes control endocannabinoid turnover in the brain (Belluomo et al., 2015). Adolescent THC exposure increases GFAP expression in the DG of male and female rats (López-Rodríguez et al., 2014) and astrocyte activation is associated with an increase in the pro-inflammatory TNF-α and iNOS and reduction in the anti-inflammatory cytokine IL-10 (Melis et al., 2017). To mention that the long-term effects of THC administration on glial cells is sex- and region-dependent, as the hippocampus and cerebellum are the more affected brain regions in males and cerebral cortex in females.

In conclusion, the acute low-dose THC administration affects dendritic morphology and causes an increase in dendritic spines and mitochondria. Also, the CB_1 receptor distribution is drastically reduced in inhibitory synapses. The ultrastructure and receptor modifications observed in CA1 hippocampus after acute THC administration indicate the existence of fast brain adaptations that support morphologically the behavioral alterations provoked by cannabis intoxication.

7. CONCLUSIONS

The conclusions of my doctoral thesis are:

1. The expression levels of CB_1 receptors in hippocampal astrocytes of the mutant GFAPhrGFP-*CB_1*-WT mice are similar to the wild type.
2. Almost all CB_1 receptors in the GFAP-*CB_1*-RS mutant mice that re-express the CB_1 receptors only in GFAP-positive cells localize to astrocytes.
3. The CB_1 receptor expression in astroglial mitochondria of the GFAP-*CB_1*-RS and GFAPhrGFP-*CB_1*-WT hippocampus maintains the same values and localization of the *CB_1*-WT mice.
4. The density of the mitochondrial CB_1 receptors in the hippocampus and prefrontal cortex is higher in astrocytes than in neurons.
5. Adolescent ethanol intake (binge drinking) causes a drastic decrease in CB_1 receptors in astrocytes and changes in the CB_1 receptor distribution in the CA1 hippocampus of the adult mouse. Also, ethanol decreases the number of astroglial processes and alters their morphology.
6. A significant reduction in the CB_1 receptor immunopositive excitatory and inhibitory synaptic terminals takes place in the adult DML after juvenile concussion. These changes start in males 10 days after repeated mild TBI and are well established 40 days after mTBI in males and females.
7. The CB_1 receptor density is severely reduced in inhibitory terminals 10 days after the last concussive impact and is maintained low at least up to 40 days after mTBI in males and females.
8. Acute low-dose of THC affects dendritic morphology and causes an increase in the number of dendritic spines and dendritic mitochondria. However, the size of the dendritic spines, synaptic terminals, astrocytes and mitochondria decreases significantly.
9. The CB_1 receptor expression is drastically reduced in inhibitory synapses, astrocytic processes and in neuronal and astroglial mitochondria after acute THC administration.
10. Altogether, this doctoral thesis has shown the existence of changes in CB_1 receptor expression and structural brain adaptations that support the behavioral alterations caused by pathological conditions such as adolescent binge drinking, concussion and acute THC consumption.

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