Effects of the absence of TRPV1 in the expression of the cannabinoid 1 (CB1) receptor in the brain

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Though cannabinoids functions in the brain are typically associated with the activation of cannabinoid receptor 1 (CB1R), several effects depend on the activation of TRPV1, a receptor from the transient receptor potential channel family involved in synaptic transmission, plasticity and some pathologies. An anatomical arrangement between CB1R and TRPV1 in various regions of the brain has been demonstrated, which allows a complex, but currently largely unidentified, crosstalk that involves activation of both TRPV1 and CB1R by anandamide. The constitutive absence of TRPV1 change the expression and localization of components of the endocannabinoid system (ECS), including CB1R, in the hippocampus, though it is currently unknown whether these changes occur in other regions of the brain. This research aims to analyze the impact of the lack of TRPV1 on the ECS's physical and molecular changes with particular attention to the CB1R in the amygdala, hippocampus, substantia nigra, globus pallidus, retrospenial cortex, cingulate area, motor cortex, olfactory bulb and cerebellum. For this purpose, using avidin-biotin complex immunohistochemistry techniques and light microscopy, the expression and localization of CB1R was studied in a mouse model with the genetic deletion of TRPV1 compared to wild-type mice. A significant increase in CB1R density was observed in the retrosplenial cortex, and a decrease in the cingulate area, motor cortex, and cerebellum upon the constitutive deletion of the TRPV1 gene, supporting the hypothesis of a reciprocal relation between both systems, so the lack of TRPV1 could lead to compensatory processes affecting the expression and localization of the ECS.

Key words: endovanilloid system, endocannabinoid system, immunohistochemistry, cannabinoid (CB) receptor 1

INTRODUCTION

The endocannabinoid system (ECS) is composed by specific cannabinoid receptors, their endogenous ligands and the enzymatic systems of their biosynthesis and degradation (Svíženská et al., 2008). Two main subtypes of cannabinoid receptors, the cannabinoid receptor type 1 (CB1R) and cannabinoid receptor type 2 (CB2R), which are G protein-coupled receptors, have been recognized (Pertwee, 1997) and are responsible for the transduction of different effects of the ECS through both excitatory (glutamatergic) and inhibitory (GABAergic) synapses (Castillo et al., 2012). These receptors are activated in response to different cannabimimetic compounds, including the main cannabis psychotropic component Δ9-tetrahydrocannabinol, and the endogenous cannabinoids arachidonoylethanolamide (anandamide, AEA) and 2-arachydonoyl-glycerol (2-AG) (Di Marzo, 1998). The functions of endocannabinoids in the central nervous system (CNS) are carried out by activation of CB1R, which can also be found in the peripheral nervous system (PNS), while CB2R is mostly found in cells of the immune system and plays a more limited role (Svíženská et al., 2008).

Through these receptors, the ECS exert a wide range of neuromodulatory activities, playing an important role in neurogenesis, memory, emotions, anxiety, behavior, motor coordination, immune response, metabolism and thermoregulation, among others (Chaperon & Thiébot, 1999; Crowe et al., 2014; Gatta-Cherifi & Cota, 2015; Robertson et al., 2017; Soria-Gómez et al., 2014). Moreover, due to its retrograde signaling course of action, the ECS provides a mechanism for inhibitory feedback to regulate neurotransmitter release in the brain, playing a crucial role in the intrinsic response to neuroinflammation. This unique function of endocannabinoids has provided a strong rationale for investigating them as therapeutic targets for autoimmune disease (Moreno-García et al., 2020), brain injury (Schurman & Lichtman, 2017) and some other severe neurodegenerative diseases, including Alzheimer's disease, Huntington's disease and Parkinson's disease (Basavarajappa et al., 2017).

The topographical distribution of CB1 receptors in the brain has been broadly mapped by autoradiography and in situ hybridization methods (Mackie, 2005), whereas immunohistochemistry procedures have provided the necessary information about CB1R expression at the cellular level (Hebert-Chatelain et al., 2014; Melser et al., 2017; Mendizabal-Zubiaga et al., 2016; Puente et al., 2019). Specifically, CB1Rs are highly expressed in brain areas responsible for mood regulation, motor coordination, cognition and pain such as: the hippocampus, including dentate gyrus; the basal ganglia, with prominent density in the globus pallidus and substantia nigra; and the molecular layer of the cerebellum (Cristino et al., 2006; Gutiérrez-Rodríguez et al., 2017; Reguero et al., 2011). Functional CB1Rs are also found in the cortex, hypothalamus, amygdala, olfactory

regions, periaqueductal gray matter, cingulate gyrus and stria terminalis (Cristino et al., 2006; Puente et al., 2010; Svíženská et al., 2008). Only sparse populations of CB1Rs are found in the medulla and thalamus (Cristino et al., 2006).

Though cannabinoid functions in the brain are typically associated with the activation of CB1Rs, endogenous, plant-derived and synthetic cannabinoids can have other molecular targets. In particular, several cannabinoid effects have been proved to depend on the activation of members of the transient receptor potential (TRP) channel family (Muller et al., 2019). As a member of this family, the transient receptor potential vanilloid 1 (TRPV1) is one of the most eminent members of the TRPs. This receptor is a ligand-gated non-selective cation channel known to be activated by capsaicin (Caterina et al., 1997), but also by numerous stimuli including the main endocannabinoids 2-AG and AEA (Muller et al., 2019). Formerly, the main function of TRPV1 was identified in sensory transmission of the nociceptive neurons in the PNS (Martins et al., 2014), where its role has been widely investigated and its potential application as a target for treating neurological disorders has been broadly demonstrated (Shuba, 2021). However, some inconsistencies exist in what concerns the expression of TRPV1 at the CNS. Although a study using a novel approach with high sensitivity/specificity pointed to a sparse distribution and relatively low expression levels of TRPV1 in CNS (Cavanaugh et al., 2011), functional and behavioral studies indicate that this sparse minor concentration of TRPV1 can produce significant effects and should not be discarded (Martins et al., 2014). Therefore, the role of this receptor in CNS remains to be further studied.

Functionally, the TRPV1 is a thermosensitive ion channel that holds the ability to detect alterations in the environmental temperature, maintain normal body temperature (Alawi et al., 2015), and elicit painful and other sensations under conditions of inflammation (Caterina et al., 2000). Accumulating evidence indicate that, in addition to its well-known role in modulating pain transduction, TRPV1 seems to play an important role in regulating brain synaptic transmission and plasticity (Egaña-Huguet et al., 2021b; Marsch et al., 2007; Puente et al., 2015; Saffarzadeh et al., 2015). TRPV1 can be expressed both presynaptically and postsynaptically, and depending on the specific synapse, it can either increase or decrease neurotransmission (Meza et al., 2022). Besides, TRPV1 seem to supports numerous ostensibly unrelated cellular functions, making it an important determinant of diverse physiological processes ranging from nociception to energy metabolism (Shuba, 2021): learning and memory (Li et al., 2008; Marsch et al., 2007), cortical excitability (Mori et al., 2012), fear and anxiety (Marsch et al., 2007) and various pathophysiologic responses both in the CNS and other systems (Kong et al., 2017). Furthermore, TRPV1 intervenes in brain neurogenesis, regulates neural proliferation/differentiation rate (Ramírez-

Barrantes et al., 2016) and contributes to AEA transport into endothelial cells (Hofmann et al., 2014). Thus, TRPV1 expression has been detected in the cortex, hippocampus and dentate gyrus, amygdala, striatum, substantia nigra, hypothalamus, thalamus and cerebellum among others (Canduela et al., 2015; Cristino et al., 2006; Puente et al., 2015).

Current evidence has indicated that TRPV1 contributes to the development and progression of the symptoms of many immune-associated diseases in multiple organs or systems and serves as a therapeutic target that is amenable to blockade by small molecules (Assas et al., 2014). Dysfunction of TRPV1 is involved in the pathogenesis of numerous immune-mediated neurological diseases in the CNS and participates in various pathophysiological responses in tumors (Stock et al., 2012) and other systems, such as respiratory (Ternesten-Hasséus et al., 2015), cardiovascular (Randhawa & Jaggi, 2017), digestive (Liñán-Rico et al., 2016), endocrine (Lee et al., 2015), urogenital (Coelho et al., 2015) and immune system (Parenti et al., 2016). Taken together, these studies suggested that TRPV1 can modulate the release of pro-/anti-inflammatory cytokines and aggravate or alleviate neurological and other system's disorders, representing a potential therapeutic target.

The use of single and double-immunolabeling techniques have shown co-expression of CB1R and TRPV1 in cell bodies, axons or dendrites in various regions of the brain, including the hippocampus, basal ganglia, thalamus, hypothalamus, cerebellar peduncle, pontine nuclei, periaqueductal gray matter and cerebellum (Cristino et al., 2006). The anatomical arrangement between these two receptors can result in enhancement of the biological effects induced by agonists of these receptors (Cristino et al., 2006) and allows a complex, but currently largely unidentified, crosstalk that involves activation of both TRPV1 and CB1 receptor by AEA. Although no consensus has yet been reached on the precise role played by this crosstalk, the literature has witnessed the emergence of extensive proposals that address its role at multiple levels of analysis. The increase of AEA reduces 2-AG effect on presynaptic CB1Rs through postsynaptic TRPV1 resulting in short-term plasticity regulation (Maccarrone et al., 2008). Interestingly, one study showed that TRPV1 can be transiently expressed during embryonic development and its expression may undergo postnatal restriction in some brain regions (Cavanaugh et al., 2011). This allows us to hypothesize that TRPV1 expression may be up- or down-regulated in different brain regions depending on age or even physiological/pathological conditions. Hence, genetic deletion of components of the endocannabinoid system could lead to compensatory changes in TRPV1; indeed, TRPV1 expression has been shown to be decreased in mice lacking CB1R (Cristino et al., 2006). Furthermore, the CB1R sensitizes TRPV1 promoting receptor responsiveness to AEA in the peripheral nervous system (Chen et

al., 2016). These authors described that the AEA-responsiveness was not eliminated by the CB1R deletion, meaning that mechanisms other than TRPV1-CB1 crosstalk could also foster it. Though, TRPV1 changes upon the loss or absence of CB1R in CNS remain to be further investigated.

On the other hand, the constitutive absence of TRPV1 changes the expression and localization of components of the endocannabinoid system, including CB1 receptor (Egaña-Huguet et al., 2021a). In mice with the genetic deletion of TRPV1, the work done in our laboratory proved changes in CB1R in the molecular layer (ML) of the hippocampus, concluding that CB1R might exert a major regulatory effect on the excitatory transmission with important functional consequences in the dentate ML (Egaña-Huguet et al., 2021a; Egaña-Huguet et al., 2021b). Particularly, they observed an increase in the CB1R coupling efficacy and a shift from CB1 receptor-dependent LTD to LTP at the medial perforant path synapses in mice lacking TRPV1, highlighting the importance of the crosstalk between CB1 and TRPV1 signaling in this form of synaptic plasticity (Egaña-Huguet et al., 2021b). So, the change in CB1 receptors in the absence of TRPV1 could be at the base of the shift from MPP-LTD to MPP-LTP. However, whether these changes occur in other brain regions remains unknown.

To date, data prove that the CB1R and TRPV1 exhibit co-expression and complex, functional interactions largely unknown, being suggested that the lack of TRPV1 can trigger compensatory mechanisms in other receptors or channels that replace its function. However, the extent of this relationship and its impact on the nervous system remains mostly unknown, with no studies on its possible use as a therapeutic target.

In order to better understand how the CB1-TRPV1 crosstalk shapes synaptic transmission, we herein investigated the effect that the constitutive deletion of the TRPV1 gene had in the expression and localization of CB1R in several regions throughout the mouse brain. This study aims to describe whether the compensatory mechanisms found in the hippocampal CB1R when TRPV1 is absent, also occur in other brain regions where both receptors are co-expressed. Thus, based on the increase in the total CB1 receptor particles found in the dentate ML in the absence of TRPV1 (Egaña-Huguet et al., 2021a), we hypothesized to find changes in CB1R immunoreactivity in TRPV1-/- compared to WT mice, which could reflect that different TRPV1 expression patterns trigger specific compensatory effects. Therefore, the expression of the CB1 receptor was studied in the amygdala, CA1 hippocampus, substantia nigra, globus pallidus, retrospenial cortex, cingulate area, primary and secondary motor cortex, olfactory bulb and cerebellum of TRPV1-/- mice versus WT littermates. For this purpose, avidin-biotin complex immunohistochemistry was performed and

samples were analyzed and quantified by light microscopy.

MATERIALS AND METHODS

Animal procedures

Guidelines on the ethical use of animals were followed to perform the experiments, which were approved by Committee of Ethics for Animal Welfare of the University of the Basque Country and were in accordance with the European Communities Council Directive and Spanish regulations. All efforts were made to minimize the number and suffering of the animals used. Six male adult C57BL/6J mice were used: three TRPV1-/- and three WT littermates (TRPV1+/+). As described previously in the study by Egaña-Huguet et al., the TRPV1-/- mice were derived from heterozygous breeding pairs generated by backcrossing B6.129X1-Trpv1tm1Jul/J mice (The Jackson Laboratory, Bar Harbor, ME) with C57BL/6 j mice (Janvier Labs) at the General Animal Unit Service of the University of the Basque Country (UPV/EHU) (Egaña-Huguet et al., 2021a). The mice used had been genotyped in the Genomics and Proteomics Unit of the University of the Basque Country (UPV/EHU).

Mice were housed maximum in groups of three littermates in standard Plexiglas cages (17 x 14.3 x 36.3 cm) and they were maintained at standard conditions with food and tap water ad libitum in a room with constant temperature (22 ºC), which was kept in a 12:12 h light/dark cycle with lights off at 9:00 p.m.

Tissue preservation

Brain tissue used for immunohistochemistry was processed in accordance with the routine protocols used in the laboratory (Egaña-Huguet et al., 2021a). After deeply anesthetized with a mixture of ketamine/xilacine (80/10 mg/kg body weight) applied intraperitoneally, animals were transcardially perfused at room temperature (RT) with phosphate buffered saline (0.1M PBS, pH 7.4) for 20 s. Subsequently, the fixative solution made up of 4% formaldehyde, 0.2% picric acid, and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) was applied for 10-15 min. Thereafter, each brain was carefully removed from the skull, post-fixed in the fixative solution for 1 week at 4 ºC and storage in 1:10 fixative solution diluted in 0.1M PB with 0.025% sodium azide at 4 ºC until it was used, two weeks later.

Immunohistochemistry for light microscopy

CB1 immunohistochemistry was performed by using the avidin-biotin-peroxidase complex method, which consists of applying a polyclonal antibody that specifically binds to a CB1R epitope. Serial coronal brain sections were cut through the brain with a vibratome at 50 µm-thickness and collected in 0.1M PB at RT. The cerebellum was cut transversally. Following preincubation in 10% horse normal serum (HNS) in Tris-HCI-buffered saline (TBS, pH 7.4), containing 0.1% sodium azide, and 0.5% Triton X-100 for 30 min at RT, slices were incubated with a primary polyclonal goat anti-CB1R

antibody (2µg/ml; Frontier Institute co., ltd; CB1-Go-Af450; RRID: AB_2571591) on a shaker for 2 days at 4 ºC. After several washes in 1% HNS and 0.5% Triton X-100 in TBS, biotinylated horse anti-goat IgG was used as secondary antibody (1:200, Cat# BA-9500; RRID: AB_2336123; Vector Laboratories, Burlingame, CA) and sections were incubated for 1 h on a shaker at RT, washed in the solution previously described and incubated in the avidin-biotin complex prepared in washing solution (1:50; Elite; Cat#PK-6100, Vector Laboratories, Burlingame, CA; RRID: AB_2336819) for 1 h at RT. The brownish reaction product was obtained by using 0.05% diaminobenzidine tetrahydrochloride (DAB) as chromogen in 0.1M PB containing 0.5% Triton X-100 and 0.01% hydrogen peroxide, for 5 min at RT. Lastly, tissue was mounted on slides, dehydrated using alcohol washes of increasing concentrations (50, 70, 96, 100%) to xylol and coverslipped with DPX.

Observation and study of histological samples

The observations and photography of the samples were performed using a Zeiss Axiophot light microscope equipped with an Axiocam HRc digital camera. Measurements of immunoreactivity intensity were performed with a 10X objective with a grayscale resolution of 8 bits/pixel. All same region's micrographs were taken at the same light intensity and exposure time. The atlas of Keith B.J. Frankling & George Paxinos (Franklin & Paxinos, 1997) was used to classify histological sections and to identify brain structures.

Semi-quantification analysis

Sections were visualized under a light microscope in order to select portions with well-preserved structure. Sampling was accurately carried out in the same way for all the animals studied. Image processing was carried out using the measurement function of the image analysis software Image-J (ImageJ, NIH, Bethesda, MD, U.S.A.; RRID:SCR_00370). Measurements were performed within the regions of interest (ROI) of 150 x 150 µm² and the analysis was done over 162 samples in TRPV1-/- (8 in globus pallidus, 14 in substantia nigra, 18 in amygdala, 26 in CA1 hippocampus, 28 in retrospenial cortex, 12 in olfactory bulb, 30 in cerebellum and 26 in primary and secondary motor cortex and cingulate area) and 167 samples in WT (8 in globus pallidus, 16 in substantia nigra, 18 in amygdala, 21 in CA1 hippocampus, 28 in retrospenial cortex, 18 in olfactory bulb, 30 in cerebellum and 28 in primary and secondary motor cortex and cingulate area). Three measurements were performed for each sample, for a total of 486 values in the TRPV1-/- condition and 501 values in the WT condition. All measurements within the black-white range were calibrated on an arbitrary scale of 0-255. The background staining in each section analyzed was considered as the mean optical density value obtained in the areas of the

blood vessels showing the lowest staining intensity; this value was subtracted from each measurement. Finally, each given optical density data was calculated as the average obtained from all ROIs within that region after correction with the background value. Therefore, all data are expressed as relative optical density. GraphPad Software (GraphPad Prism 4, GraphPad Software Inc., San Diego, U.S.A.) was used to perform the statistical analysis. The Kolmogorov-Smirnov and Shapiro-Wilk normality tests were always applied before running statistical analyses and data were analyzed by parametric and non-parametric tests (Unpaired t-test or Man-Whitney test, depending on its gaussian distribution). Values of $p < 0.05$ were considered statistically significant.

RESULTS

The pattern of CB1 receptor immunostaining was compared between TRPV1-/- and WT brain by light microscopy (**Figures 1, 2**). As expected, strong CB1 receptor staining was observed in the hippocampus, globus pallidus, cerebral cortex, substantia nigra, amygdala, olfactory bulb and cerebellum. Briefly, heavy CB1 receptor immunoreactivity was distributed throughout the Ammon's horn and the dentate gyrus (**Figure 1A**). Thus, CB1 receptor immunoreactivity was conspicuous in the strata oriens and radiatum of the Ammon's horn. Also, a denser CB1 receptor staining was found in the pyramidal cell layer and at the limit between the strata radiatum and the lacunosum-moleculare. Remarkably, a strong band of CB1 receptor immunostaining appeared in the inner one-third of the dentate molecular layer corresponding to the termination zone of the

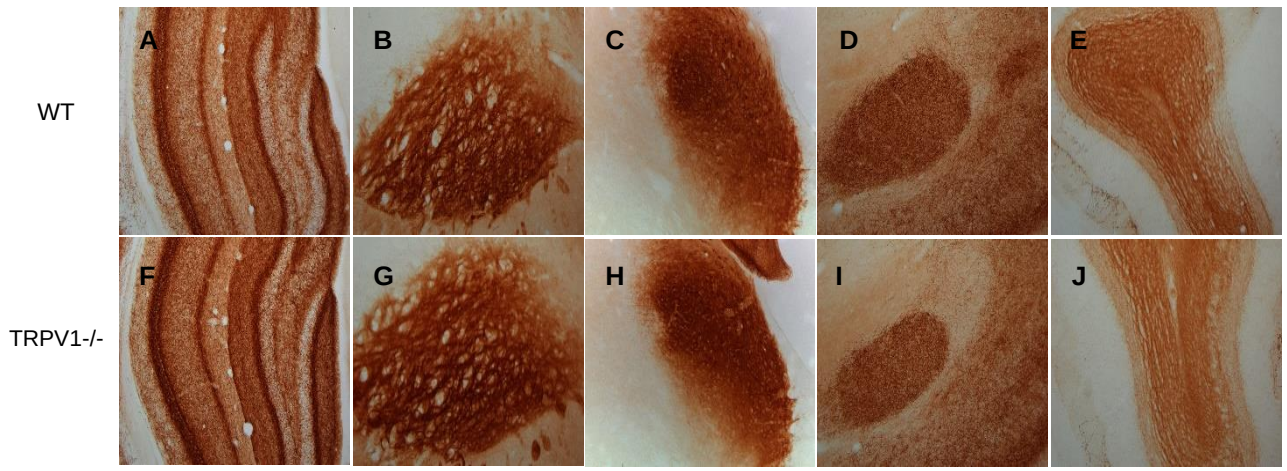


FIGURE 1 | Immunoperoxidase method for light microscopy. Photomicrographs of coronal sections through the brain, showing CB1R immunoreactivity at the levels of the hippocampus (**A**, **F**), globus pallidus (**B**, **G**), substantia nigra (**C**, **H**), amygdala (**D**, **I**) and the olfactory bulb (**E**, **J**). Scale bars: 100 µm.

commissural/associational fibers. Also, heavy labeling in a mesh-like and punctate fashion was seen in the globus pallidus, substantia nigra pars reticulata and basolateral amygdala (**Figure 1B, C, D**). In the olfactory bulb, CB1 receptor staining was almost null in the external plexiform layer and mitral cell layer, was very low in the glomerular layer, moderate in the internal plexiform layer and high in the granule cell layer (**Figure 1E**), as we described previously (Soria-Gómez et al., 2014). The CB1 receptor expression in the cerebral cortex was mostly restricted to layers II/III and V/VI. Thus, a strong CB1 immunopositive mesh made up of dotty elements and thin processes filled the neuropil of the stained layers (**Figure 2A, C**). In the cerebellum (**Figure 2B**), CB1 receptor immunolabeling was very strong in the layer of the GABAergic "pinceaux terminaux" or basket cell axon terminals closely surrounding the Purkinje cell bodies, and strong in the molecular layer where it mostly localizes to the glutamatergic parallel fiber terminals making synapses with Purkinje cell dendritic spines (Buceta et al., 2020).

Importantly, CB1 receptor immunostaining was absent in the CB1-/- mouse brain (not shown), indicating that the CB1 receptor antibody used was specific (Gutiérrez-Rodríguez et al., 2017). The overall CB1 receptor pattern in TRPV1-/- matches the above-described CB1 receptor distribution (**Figures 1, 2**). However, some differences in immunoreactivity intensity in TRPV1-/- vs WT were detected (**Figures 1, 2**). Yet this variation was only significant in three of the ROIs: the retrosplenial cortex (p = 0.0076**), cerebellum (p < 0.0001****) and the cingulate and motor cortex (p = 0.0001****) (**Figure 2**). There were no significant differences in basal ganglia, neither in the globus pallidus (TRPV1-/-: 71.79 ± 5.120; WT: 68.60 ± 6.216; p = 0.5464 ns; **Figure 3A**) nor in the substantia nigra (TRPV1-/-: 75.95 ± 6.209; WT: 74.39 ±

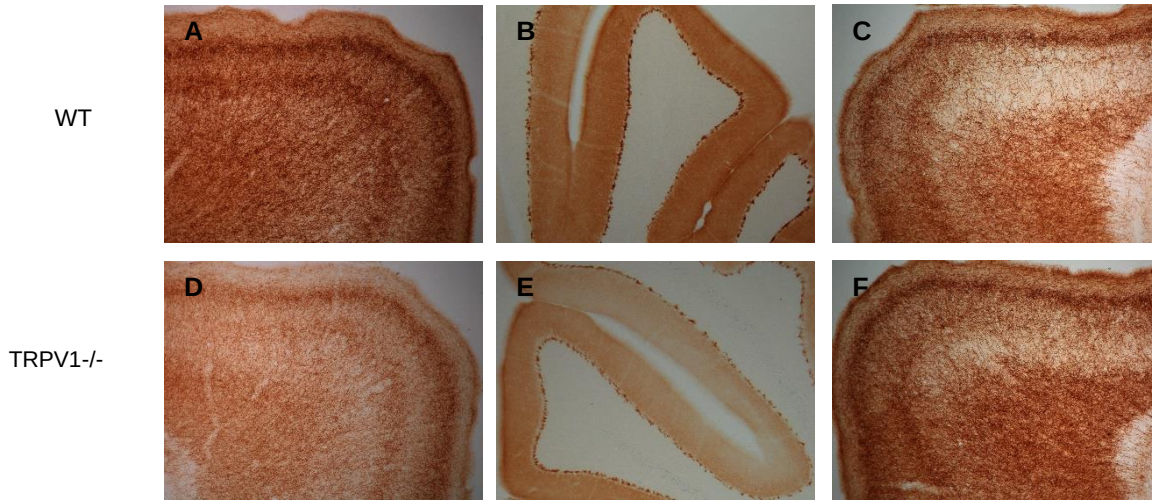
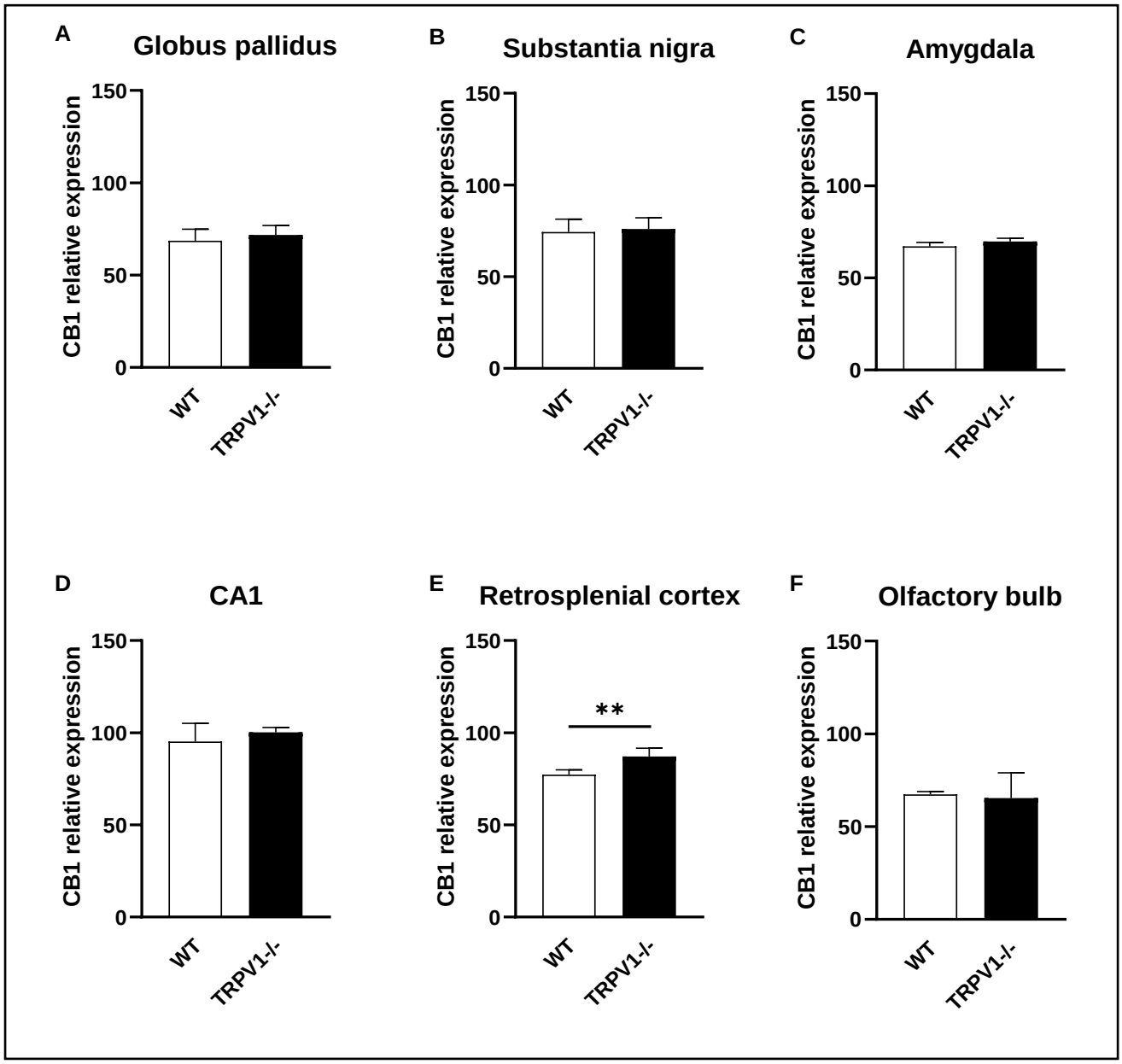


FIGURE 2 | Immunoperoxidase method for light microscopy. Expression patterns of CB1R in WT and TRPV1-/- mice motor cortex (**A**, **D**), cerebellum (**B**, **E**) and retrosplenial cortex (**C**, **F**). CB1R immunoreactivitv decreases in TRPV1-/-. Scale bars: 100 um.

7.019; p = 0.0934 ns; **Figure 3B**). Again, CB1 receptor expression in the amygdala did not vary significantly between TRPV1-/- (69.80 ± 1.641) and WT (67.13 ± 2.116; p = 0.3154 ns; **Figure 3C**) and the reduction was not significant in the CA1 region of the hippocampus (TRPV1-/-: 100.2 ± 2.617; WT: 95.26 ± 1.238; p = 0.1161 ns; **Figure 3D**). However, a significant increase in CB1R was detected in the retrosplenial cortex of WT (TRPV1-/-: 87.14 ± 4.414; WT: 77.31 ± 2.525; p = 0.0076**; **Figure 3E**). In contrast, in the olfactory bulb, there were no differences in CB1R expression between TRPV1-/- (65.43 ± 2.259) and WT (67.41 ± 1.530; p = 0.4527 ns; **Figure 3F**). Lastly, a significant decrease in CB1R immunoreactivity was found in the cerebellum (TRPV1-/-: 35.69 ± 0.9909; WT: 51.47 ± 1.041; p < 0.0001****; **Figure 3G**) as well as in the cingulate and motor cortex of TRPV1-/- mice (TRPV1-/-: 30.10 ± 1.611; WT: 48.13 ± 1.261; p < 0.0001****; **Figure 3H**).



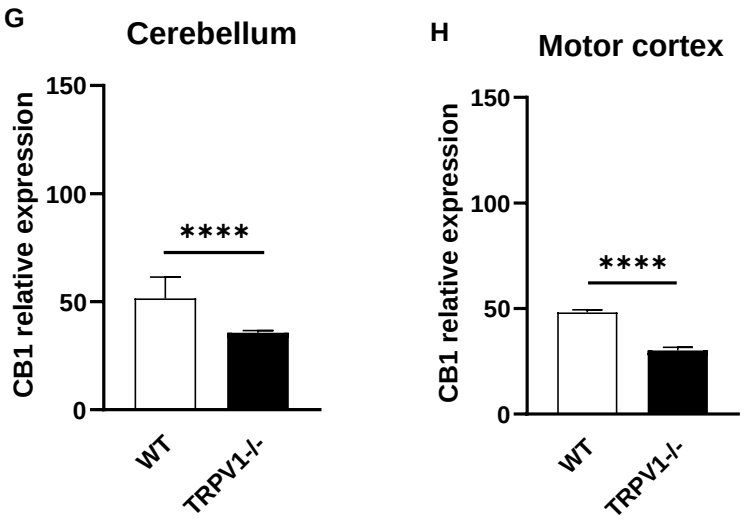


FIGURE 3 | Statistical assessment of CB1R labelling in WT and TRPV1-/- mice in the globus pallidus (**A**), substantia nigra (**B**), amygdala (**C**), CA1 region of the hippocampus (**D**), retrosplenial cortex (**E**), olfactory bulb (**F**), cerebellum (**G**) and the cingulate and motor cortex (**H**). Mann-Whitney *U*-test or Student's *t*-test. $P < 0.01$**; $p < 0.0001$****. All data are represented as mean ± S.E.M.

DISCUSSION

In the present work, the impact of the absence of TRPV1 on the endocannabinoid system was studied, with particular attention to the CB1R in the amygdala, CA1 hippocampus, substantia nigra, globus pallidus, retrospenial cortex, cingulate area, primary and secondary motor cortex, olfactory bulb and cerebellum. Although, to our knowledge, no immunohistochemical study of the expression and localization of CB1R in a mouse model with the genetic deletion of TRPV1 has ever been performed in the brain, our findings are in line with an independent study addressing the biochemical changes taking place in the endocannabinoid system of TRPV1-/- mouse hippocampus, as noted below.

Our results show differences in CB1R expression in some brain regions upon the absence of TRPV1, supporting the hypothesis of a reciprocal relation between both systems. In fact, a significant increase in CB1R density in the retrosplenial cortex and a decrease in the cingulate and motor cortex, as well as in the cerebellum upon the constitutive deletion of the TRPV1 gene. These findings are in consonance with a previous immunohistochemical study where changes in the CB1R density in the hippocampus of TRPV1-/- were reported, though they were not statistically significant (Egaña et al., 2021a). However, the picture could vary using high resolution immunoelectron microscopy to detect the CB1 receptor in TRPV1−/− mice, as CB1 positive excitatory terminals as well as the proportion of CB1 receptor particles in excitatory terminals and astrocytes were observed with this technique to increase, but the CB1R labelling was noticed to decrease in inhibitory terminals in the dentate

molecular layer of TRPV1 mutants (Egaña-Huguet et al., 2021a). This could indicate the existence of a compensatory mechanism between both systems that has also been previously described (Chen et al., 2016; Cristino et al., 2006; Egaña-Huguet et al., 2021a; Egaña-Huguet et al., 2021b). Regarding this system, it has been suggested that the spatial distribution of the CB1R and TRPV1 may contribute to the complexity of their functional interaction and might include also reciprocal influences on gene expression (Chen et al., 2016; Cristino et al., 2006). However, for this cross-talk to occur, the receptors must be expressed in the same cell (Cristino et al., 2006) or at least in close proximity (Chen et al., 2016) and even so, differences can be found depending on the synapse type (excitatory vs. inhibitory) (Egaña-Huguet et al., 2021a), which reflects that distinct TRPV1 expression patterns trigger specific compensatory effects.

In fact, two general patterns of neuronal CB1R-TRPV1 localization have been described: one in which the expression of the two receptors is overlapping in the cytoplasm, and another, where the two receptors co-occur on processes of the same cells (perisomatic and axonal labeling) (Cristino et al., 2006). In the hippocampus, activation of CB1 and TRPV1 receptors by AEA can produce opposing effects on both excitatory and inhibitory neurotransmission in principal neurons and interneurons, respectively (Al-Hayani et al., 2001; Egaña-Huguet et al., 2021a). Conversely, the mechanisms underlying TRPV1 and CB1R co-dependency and their conversion from depressing to potentiating effects remain to be investigated. In the PNS, the CB1R sensitizes TRPV1 promoting receptor responsiveness to AEA only when both receptors were expressed in medium/high density and proximity (Chen et al., 2016), so that the spatial proximity of the two molecules may underlay their effects. In fact, these authors reported that PSN co-expressing CB1 receptor and TRPV1 form two distinct sub-populations based on their pharmacological properties, which could be due to the distribution pattern of the two receptors: neurons respond either only to capsaicin (expressed CB1R and TRPV1 in low density and isolation) or to both capsaicin and AEA (expressed the two receptors in medium/high density and proximity) (Chen et al., 2016).

Given the ability of endocannabinoids like AEA to activate TRPV1, it has been suggested that TRPV1 may act as an ionotropic endocannabinoid receptor with central neuromodulatory effects that either mimic or oppose those exerted by CB1R (Muller et al., 2019; Storozhuk & Zholos, 2018). However, the factors that determine whether endocannabinoids act on TRPV1 or CB1 receptors to regulate synaptic transmission remain unclear. While these results suggest an ability of endocannabinoids to act through both CB1R and TRPV1, the factors that determine directionality of synaptic plasticity and functional consequences in behavior need to be determined.

The absence of significant differences in all the ROIs could be explained by the specific localization of both receptors and the different expression patterns of TRPV1. Therefore, in order to draw firm conclusions, it would be necessary to know the spatial distribution of CB1R and TRPV1 in the studied areas, as well as the place where co-expression occurred. Indeed, based on these findings and in order to know whether the crosstalk between both systems could also be affected by this, it will be interesting to investigate whether the receptors expressed in pre- or post-synaptic localization, which would be conclusively established only with the use of electron microscopy.

On the other hand, the main CB1R staining decrease in TRPV1-/- was found in the motor cortex and cerebellum. The cerebellum plays a major role in the planning, initiation and organization of movement, effects that are mediated, in part, through its influence on the motor cortex. The predominant expression of these receptors, in particular CB1R, and the presence of endocannabinoids in brain areas responsible for the management of movements, together with the conspicuous changes in endocannabinoid transmission in the brains of individuals affected by motor disorders (Fernández-Ruiz & González, 2005), provides a compelling conceptual argument that cannabinoid-based compounds may have the potential to alleviate symptoms of these diseases and provide a novel area of research. Thus, the hypokinetic profile of certain cannabinoid agonists becomes these compounds as promising drugs to attenuate the hyperkinesia that characterizes Huntington's disease (Pazos et al., 2008). Though it was thought that CB1R represents a convincing explanation for the hyperkinesia typical of this disorder and supports the usefulness of enhancing its signaling, further studies revealed that the key property that enables certain cannabinoid agonists to reduce hyperkinesia is their capability to directly activate TRPV1 receptors (Lastres-Becker et al., 2003; Pazos et al., 2008). In a mouse model of Parkinson's disease, the crosstalk between TRPV1 and CB1R suggested a novel neuroprotective mechanism for dopamine neurons by regulating the glial activation and production of proinflammatory mediators (Wi et al., 2020). Therefore, the development of new approaches to understanding the relationship between the endocannabinoid and vanilloid system is critical to advancing our comprehension of the pathophysiology of motor disorders, neurodegenerative diseases and other conditions associated with neuroinflammation, representing a potential therapeutic target.

Future studies are needed to better define the role and exact mechanisms underlying TRPV1- and CB1-mediated changes to synaptic transmission and plasticity in health and disease. For example, it is unknown whether this crosstalk occur at GABAergic synapses and how it influences circuit function. It is also unclear under what conditions AEA acts on TRPV1 or CB1 receptors to modify synaptic function and behavior, as well as whether glial

TRPV1 can be activated by AEA or other vanilloids.

CONCLUSION

The differences in the expression of CB1R upon the constitutive deletion of the TRPV1 gene demonstrated here using immunohistochemistry techniques and light microscopy, support the hypothesis of a reciprocal relation between the endocannabinoid and vanilloid systems in the cingulate and motor cortex, cerebellum and retrosplenial cortex. Furthermore, the presence of compensatory mechanisms opens up the search for new roles for the CB1-TRPV1 crosstalk in brain function and dysfunction, making it a potential therapeutic target.

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